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EDITOR

William E. Paul MD

CONTRIBUTING AUTHORS

Eitan M. Akirav, PhD
Assistant Professor of Research
Department of Medicine
Stony Brook Medical School
Stony Brook, New York
Research Scientist
Biomedical Research Core
Winthrop University Hospital
Mineola, New York

Noelia Alonso Gonzalez, PhD
Postdoctoral Fellow
Department of Epidemiology and Public Health
Yale University
New Haven, Connecticut

Yasmine Belkaid, PhD
Chief, Mucosal Immunology Section
Laboratory of Parasitic Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Albert S. Bendelac, MD, PhD
Professor
Department of Pathology
Howard Hughes Medical Institute
University of Chicago
Chicago, Illinois

Ira Berkower, MD, PhD
Lab Chief, Lab of Immunoregulation
Division of Viral Products, Office of Vaccines
Center for Biologics Evaluation and Research

US Food and Drug Administration
Bethesda, Maryland

Sylvie Bertholet, PhD
Unit Head
Immunology Function
Novartis Vaccines and Diagnostics
Siena, Italy

Jay A. Berzofsky, MD, PhD
Branch Chief
Vaccine Branch, Center for Cancer Research
National Cancer Institute, National Institutes of Health
Bethesda, Maryland

Jean-Laurent Casanova, MD, PhD
Professor and Head of Laboratory
St. Giles Laboratory of Human Genetics of Infectious Diseases
The Rockefeller University
Senior Attending Physician
The Rockefeller University Hospital
New York, New York

Marco A. Cassatella, MD
Professor
Pathology and Diagnostics
Verona University Medical School
Vice Director
General Pathology
Medical School
Verona, Italy

Ameya S. Champhekar, PhD
Postdoctoral Scholar
Division of Biology
California Institute of Technology
Pasadena, California

Akanksha Chaturvedi, PhD
Postdoctoral Fellow
Laboratory of Immunogenetics
NIH, NIAID
Rockville, Maryland

Sangeeta S. Chavan, PhD
Associate Investigator

Center for Biomedical Science
The Feinstein Institute for Medical Research
Manhasset, New York

Yueh-hsiu Chien, PhD
Professor
Department of Microbiology and Immunology
Stanford University
Stanford, California

Mary Ellen Conley, MD
Professor
Department of Pediatrics
University of Tennessee
Le Bonheur Children's Hospital
Memphis, Tennessee

Ken T. Coppieters
La Jolla Institute for Allergy and Immunology
La Jolla, California

Shane Crotty, PhD
Associate Professor
Division of Vaccine Discovery
La Jolla Institute for Allergy and Immunology
La Jolla, California

Angel Davey, PhD
Postdoctoral Fellow
Laboratory of Immunogenetics
NIH, NIAID
Rockville, Maryland

Mark M. Davis, PhD
Investigator, HHMI
Department of Microbiology and Immunology
Stanford University
Stanford, California

Ennio De Gregorio, PhD
Head
Department of Immunology
Novartis Vaccines and Diagnostics
Siena, Italy

Marc K. Jenkins, PhD
Distinguished McKnight Professor
Department of Microbiology and Center for Immunology
University of Minnesota
Minneapolis, Minnesota

Susan M. Kaech, PhD
Associate Professor and HHMI Early Career Scientist
Department of Immunobiology
Yale University
New Haven, Connecticut

Jannet Katz, DDS, PhD
Professor
Department of Pediatric Dentistry
University of Alabama at Birmingham
Birmingham, Alabama

Stefan H.E. Kaufmann, PhD
Director
Department of Immunology
Max Planck Institute for Infection Biology
Professor
Charite University Hospital
Berlin, Germany

Douglas S. Kwon, MD, PhD
Instructor in Medicine
Department of Infectious Disease
Harvard Medical School
Associate Physician
Ragon Institute of MGH, MIT and Harvard
Massachusetts General Hospital
Boston, Massachusetts

Michael J. Lenardo, MD
Senior Investigator
Laboratory of Immunology
National Institutes of Health
Bethesda, Maryland

Warren J. Leonard, MD
Chief, Lab of Molecular Immunology
Director, Immunology Center

National Heart, Lung, and Blood Institute
National Institutes of Health
Bethesda, Maryland

Ian Paul Lewkowich, PhD
Assistant Professor
Department of Cellular and Molecular Immunology
Cincinnati Children's Hospital
Cincinnati, Ohio

Judy Lieberman, MD, PhD
Senior Investigator
Immune Disease Institute
Professor
Department of Pediatrics
Harvard Medical School
Boston, Massachusetts

Kang Liu, PhD
Assistant Professor
Department of Microbiology and Immunology
Columbia University Medical Center
New York, New York

Wanli Liu, PhD
Postdoctoral Fellow
Laboratory of Immunogenetics
NIH, NIAID
Rockville, Maryland

David H. Margulies, MD, PhD
Chief, Molecular Biology Section
Laboratory of Immunology
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Edward E. Max, MD, PhD
Associate Director for Research
Office of Biotechnology Products
Center for Drugs Evaluation and Research
Food and Drug Administration
Bethesda, Maryland

James McCluskey, MD, FRACP, FRCPA, FAA
Professor

Department of Microbiology and Immunology
University of Melbourne
Melbourne, Victoria, Australia

Michael G. McHeyzer-Williams, PhD
Professor
Department of Immunology & Microbial Science
The Scripps Research Institute
La Jolla, California

B. Paul Morgan, MB, PhD, MRCP, FRCPath
Dean and Head of School
School of Medicine
Cardiff University
Honorary Consultant in Medical Biochemistry
Department of Medical Biochemistry & Immunology
University Hospital of Wales
Cardiff, United Kingdom

Philip M. Murphy, MD
Chief
Laboratory of Molecular Immunology
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Moon H. Nahm, MD
Professor
Departments of Pathology and Microbiology
University of Alabama at Birmingham
Birmingham, Alabama

Kannan Natarajan, PhD
Staff Scientist
Molecular Biology Section, Laboratory of Immunology
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Mihai G. Netea, MD
Professor
Department of Medicine
Radboud University Nijmegen Medical Center
Nijmegen, The Netherlands

Chief
Laboratory of Immunogenetics
NIH NIAID
Rockville, Maryland

Carlo Pucillo
Department of Biomedical Science and Technology
Lab of Immunology
University of Udine
Udine, Italy

Rino Rappuoli, PhD
Global Head, Vaccines Research
Novartis Vaccines and Diagnostics
Siena, Italy

Jeffrey V. Ravetch, MD, PhD
Theresa & Eugene Lang Professor
Laboratory of Molecular Genetics & Immunology
The Rockefeller University
New York, New York

Stephen T. Reece
Department of Immunology
Max Planck Institute for Infection Biology
Berlin, Germany

Eleanor M. Riley, PhD
Professor of Immunology
Department of Immunology and Infection
London School of Hygiene and Tropical Medicine
London, United Kingdom

Paul A. Roche, PhD
National Institutes of Health
National Cancer Institute
Experimental Immunology Branch
Bethesda, Maryland

Jamie Rossjohn, PhD
Professor
Department of Biochemistry and Molecular Biology
Monash University
Melbourne, Australia

University of Chicago
Chicago, Illinois

Harry W. Schroeder, Jr. MD, PhD
Professor of Medicine, Microbiology and Genetics
Division of Clinical Immunology and Rheumatology
University of Alabama at Birmingham
Birmingham, Alabama

Alan Sher, PhD
Laboratory of Parasitic Diseases
National Institutes of Allergy and Infectious Disease
National Institutes of Health
Bethesda, Maryland

Ethan M. Shevach, MD
Chief, Cellular Immunology Section
Laboratory of Immunology
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Andrew L. Snow, PhD
Assistant Professor
Department of Pharmacology
Uniformed Services University of the Health Sciences
Bethesda, Maryland

Hae Won Sohn, PhD
Staff Scientist
Laboratory of Immunogenetics
NIH, NIAID
Rockville, Maryland

Megan Sykes, MD
Michael J. Friedlander Professor of Medicine
Professor of Microbiology & Immunology and Surgical Sciences (in Surgery)
Columbia University
Director, Columbia Center for Translational Immunology
Columbia University College of Physicians and Surgeons
New York, New York

Nicola Tamassia
Department of Pathology and Diagnostics
Section of General Pathology
Verona, Italy

Kevin J. Tracey, MD
Investigator
President
Head, Center for Biomedical Science
Feinstein Institute for Medical Research
Manhasset, New York

Lucy A. Truman
Postdoctoral Fellow
Yale University
New Haven, Connecticut

Matthias G. von Herrath, MD
Directory, Type 1 Diabetes Center
Department of Diabetes/Immunology
La Jolla Institute for Allergy & Immunology (LIAI)
La Jolla, California

David Wald, MD, PhD
Assistant Professor
Department of Pathology
Case Western Reserve University
University Hospitals Case Medical Center
Cleveland, Ohio

Bruce D. Walker, MD
Professor
Department of Infectious Disease
Harvard Medical School
Director
Ragon Institute of MGH, MIT, and Harvard
Massachusetts General Hospital
Boston, Massachusetts

Carl F. Ware, PhD
Director and Professor
Department of Infectious & Inflammatory Diseases
Sanford-Burnham Medical Research Institute
La Jolla, California

Marsha Wills-Karp, PhD
Rieveschl Professor
Department of Pediatrics
University of Cincinnati

Director
Division of Immunobiology
Cincinnati Children's Hospital Medical Center
Cincinnati, Ohio

Kathryn J. Wood, DPhil
Professor of Immunology
Nuffield Department of Surgical Sciences
University of Oxford
Oxford, United Kingdom

Thomas A. Wynn, PhD
Laboratory of Parasitic Diseases
National Institutes of Allergy and Infectious Disease
National Institutes of Health
Bethesda, Maryland

Wayne M. Yokoyama, MD
Investigator
Howard Hughes Medical Institute
Sam J. and Audrey Loew Professor of Medicine
Rheumatology Division
Washington University School of Medicine in St. Louis
St. Louis, Missouri

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EDITOR

William E. Paul MD

CONTRIBUTING AUTHORS

Eitan M. Akirav, PhD
Assistant Professor of Research
Department of Medicine
Stony Brook Medical School
Stony Brook, New York
Research Scientist
Biomedical Research Core
Winthrop University Hospital
Mineola, New York

Noelia Alonso Gonzalez, PhD
Postdoctoral Fellow
Department of Epidemiology and Public Health
Yale University
New Haven, Connecticut

Yasmine Belkaid, PhD
Chief, Mucosal Immunology Section
Laboratory of Parasitic Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Albert S. Bendelac, MD, PhD
Professor
Department of Pathology
Howard Hughes Medical Institute
University of Chicago
Chicago, Illinois

Ira Berkower, MD, PhD
Lab Chief, Lab of Immunoregulation
Division of Viral Products, Office of Vaccines
Center for Biologics Evaluation and Research

US Food and Drug Administration
Bethesda, Maryland

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Unit Head
Immunology Function
Novartis Vaccines and Diagnostics
Siena, Italy

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Vaccine Branch, Center for Cancer Research
National Cancer Institute, National Institutes of Health
Bethesda, Maryland

Jean-Laurent Casanova, MD, PhD
Professor and Head of Laboratory
St. Giles Laboratory of Human Genetics of Infectious Diseases
The Rockefeller University
Senior Attending Physician
The Rockefeller University Hospital
New York, New York

Marco A. Cassatella, MD
Professor
Pathology and Diagnostics
Verona University Medical School
Vice Director
General Pathology
Medical School
Verona, Italy

Ameya S. Champhekar, PhD
Postdoctoral Scholar
Division of Biology
California Institute of Technology
Pasadena, California

Akanksha Chaturvedi, PhD
Postdoctoral Fellow
Laboratory of Immunogenetics
NIH, NIAID
Rockville, Maryland

Sangeeta S. Chavan, PhD
Associate Investigator

Center for Biomedical Science
The Feinstein Institute for Medical Research
Manhasset, New York

Yueh-hsiu Chien, PhD
Professor
Department of Microbiology and Immunology
Stanford University
Stanford, California

Mary Ellen Conley, MD
Professor
Department of Pediatrics
University of Tennessee
Le Bonheur Children's Hospital
Memphis, Tennessee

Ken T. Coppieters
La Jolla Institute for Allergy and Immunology
La Jolla, California

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Associate Professor
Division of Vaccine Discovery
La Jolla Institute for Allergy and Immunology
La Jolla, California

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Laboratory of Immunogenetics
NIH, NIAID
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Department of Microbiology and Immunology
Stanford University
Stanford, California

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Head
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Siena, Italy

Marc K. Jenkins, PhD
Distinguished McKnight Professor
Department of Microbiology and Center for Immunology
University of Minnesota
Minneapolis, Minnesota

Susan M. Kaech, PhD
Associate Professor and HHMI Early Career Scientist
Department of Immunobiology
Yale University
New Haven, Connecticut

Jannet Katz, DDS, PhD
Professor
Department of Pediatric Dentistry
University of Alabama at Birmingham
Birmingham, Alabama

Stefan H.E. Kaufmann, PhD
Director
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Max Planck Institute for Infection Biology
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Charite University Hospital
Berlin, Germany

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Ragon Institute of MGH, MIT and Harvard
Massachusetts General Hospital
Boston, Massachusetts

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Senior Investigator
Laboratory of Immunology
National Institutes of Health
Bethesda, Maryland

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Bethesda, Maryland

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New York, New York

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Laboratory of Immunology
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

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Associate Director for Research
Office of Biotechnology Products
Center for Drugs Evaluation and Research
Food and Drug Administration
Bethesda, Maryland

James McCluskey, MD, FRACP, FRCPA, FAA
Professor

Department of Microbiology and Immunology
University of Melbourne
Melbourne, Victoria, Australia

Michael G. McHeyzer-Williams, PhD
Professor
Department of Immunology & Microbial Science
The Scripps Research Institute
La Jolla, California

B. Paul Morgan, MB, PhD, MRCP, FRCPath
Dean and Head of School
School of Medicine
Cardiff University
Honorary Consultant in Medical Biochemistry
Department of Medical Biochemistry & Immunology
University Hospital of Wales
Cardiff, United Kingdom

Philip M. Murphy, MD
Chief
Laboratory of Molecular Immunology
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Moon H. Nahm, MD
Professor
Departments of Pathology and Microbiology
University of Alabama at Birmingham
Birmingham, Alabama

Kannan Natarajan, PhD
Staff Scientist
Molecular Biology Section, Laboratory of Immunology
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Mihai G. Netea, MD
Professor
Department of Medicine
Radboud University Nijmegen Medical Center
Nijmegen, The Netherlands

Chief
Laboratory of Immunogenetics
NIH NIAID
Rockville, Maryland

Carlo Pucillo
Department of Biomedical Science and Technology
Lab of Immunology
University of Udine
Udine, Italy

Rino Rappuoli, PhD
Global Head, Vaccines Research
Novartis Vaccines and Diagnostics
Siena, Italy

Jeffrey V. Ravetch, MD, PhD
Theresa & Eugene Lang Professor
Laboratory of Molecular Genetics & Immunology
The Rockefeller University
New York, New York

Stephen T. Reece
Department of Immunology
Max Planck Institute for Infection Biology
Berlin, Germany

Eleanor M. Riley, PhD
Professor of Immunology
Department of Immunology and Infection
London School of Hygiene and Tropical Medicine
London, United Kingdom

Paul A. Roche, PhD
National Institutes of Health
National Cancer Institute
Experimental Immunology Branch
Bethesda, Maryland

Jamie Rossjohn, PhD
Professor
Department of Biochemistry and Molecular Biology
Monash University
Melbourne, Australia

University of Chicago
Chicago, Illinois

Harry W. Schroeder, Jr. MD, PhD
Professor of Medicine, Microbiology and Genetics
Division of Clinical Immunology and Rheumatology
University of Alabama at Birmingham
Birmingham, Alabama

Alan Sher, PhD
Laboratory of Parasitic Diseases
National Institutes of Allergy and Infectious Disease
National Institutes of Health
Bethesda, Maryland

Ethan M. Shevach, MD
Chief, Cellular Immunology Section
Laboratory of Immunology
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

Andrew L. Snow, PhD
Assistant Professor
Department of Pharmacology
Uniformed Services University of the Health Sciences
Bethesda, Maryland

Hae Won Sohn, PhD
Staff Scientist
Laboratory of Immunogenetics
NIH, NIAID
Rockville, Maryland

Megan Sykes, MD
Michael J. Friedlander Professor of Medicine
Professor of Microbiology & Immunology and Surgical Sciences (in Surgery)
Columbia University
Director, Columbia Center for Translational Immunology
Columbia University College of Physicians and Surgeons
New York, New York

Nicola Tamassia
Department of Pathology and Diagnostics
Section of General Pathology
Verona, Italy

Kevin J. Tracey, MD
Investigator
President
Head, Center for Biomedical Science
Feinstein Institute for Medical Research
Manhasset, New York

Lucy A. Truman
Postdoctoral Fellow
Yale University
New Haven, Connecticut

Matthias G. von Herrath, MD
Directory, Type 1 Diabetes Center
Department of Diabetes/Immunology
La Jolla Institute for Allergy & Immunology (LIAI)
La Jolla, California

David Wald, MD, PhD
Assistant Professor
Department of Pathology
Case Western Reserve University
University Hospitals Case Medical Center
Cleveland, Ohio

Bruce D. Walker, MD
Professor
Department of Infectious Disease
Harvard Medical School
Director
Ragon Institute of MGH, MIT, and Harvard
Massachusetts General Hospital
Boston, Massachusetts

Carl F. Ware, PhD
Director and Professor
Department of Infectious & Inflammatory Diseases
Sanford-Burnham Medical Research Institute
La Jolla, California

Marsha Wills-Karp, PhD
Rieveschl Professor
Department of Pediatrics
University of Cincinnati

Director
Division of Immunobiology
Cincinnati Children's Hospital Medical Center
Cincinnati, Ohio

Kathryn J. Wood, DPhil
Professor of Immunology
Nuffield Department of Surgical Sciences
University of Oxford
Oxford, United Kingdom

Thomas A. Wynn, PhD
Laboratory of Parasitic Diseases
National Institutes of Allergy and Infectious Disease
National Institutes of Health
Bethesda, Maryland

Wayne M. Yokoyama, MD
Investigator
Howard Hughes Medical Institute
Sam J. and Audrey Loew Professor of Medicine
Rheumatology Division
Washington University School of Medicine in St. Louis
St. Louis, Missouri

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Dedication

For Charlotte, Gloria, Lucy, Jenna, Silvie, and Jake—and for Julien

Editors: **Paul, William E.**
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PREFACE

Immunology is the quintessential medical science. Indeed, no branch of the medical sciences has improved the health of people more than the application of immunologic principles to prevention of disease. Smallpox has been eliminated from the planet as a natural infection, as has poliomyelitis from the western hemisphere. Hepatitis B vaccine has prevented more cancers than any intervention other than smoking cessation. The human papilloma virus vaccine promises to cut strikingly the toll of cervical cancer.

The continued need for progress in immunology is clear. The epidemic of human immunodeficiency virus roars on. Glimmers of hope from vaccine trials have led to a redoubling of effort, and the struggle to design effective vaccines for the great infectious scourges goes on, with encouraging results but no breakthroughs yet. Highly effective therapeutic vaccines for cancers still elude us, but some immunologic therapies for cancer have met with encouraging results. We hope for much more as we marshal the tools coming from the study of the innate immune system, regulatory T cells, and lymphocyte differentiation and effector function.

Understanding the basis of inflammation and the cytokine world has given us effective drugs to treat rheumatoid arthritis and a growing number of other autoinflammatory/autoimmune diseases. The value of the interventions based on this knowledge, such as the use of tumor necrosis factor, interleukin-6, and interleukin -1 blockers, is now established. The application of anti-CD20 in the treatment of autoimmune disorders shows great promise. Even more promising strategies are on the horizon.

Fundamental Immunology has the goal of aiding in the education of a new generation of immunologists who can both probe more deeply into the organizing principles of the immune system and can translate this new information into effective treatments and preventatives that will extend and enlarge on the record of immunologic science in bettering the lot of human kind.

Were I beginning the task of preparing a comprehensive text of immunology today, I might have titled it *Immunology, Endless Fascination.* Certainly that describes my own view of this science over the 30 years that I have been working on the seven editions of *Fundamental Immunology.* I had believed that scientific progress was marked by periods of intense creativity, during which new concepts were established, followed by longer periods of consolidation, when work that made important but anticipated advances would dominate. Perhaps that will prove to be true of modern immunology as well when it is looked at by a disinterested observer, but for one in the midst, the pace of discovery seems to speed up with each passing year. *Endless fascination* certainly describes my experience of immunology.

I hope that this seventh edition will convey the dynamism and creativity of modern immunology and provide the reader with a solid introduction to our field and a picture of much

of the very latest that has been achieved. As with each of the previous editions, most of the chapters are entirely new and not simple reworkings of the chapter in the previous edition. In order to contain the seventh edition within one volume, a decision was made to cite references in the printed text but only to provide the detailed citations in the online version. However, the references will be linked to PubMed so cited information can be easily obtained. The electronic version can be accessed at www.fundamentalimmunology.com.

As before, this edition begins with an introductory section consisting of the chapters "The Immune System" and "History of Immunology." These give an overview of modern immunology and of its origins, and provide those new to the field with the basis to go on to the subsequent chapters. This is followed by an "expanded introduction" provided by the sections "Organization and Evolution of the Immune System," "Immunoglobulins and B-Lymphocytes," and "T-Lymphocytes." These are followed by the two core "basic" immunology sections: "The Intersection of Innate and Adaptive Immunity" and "Induction, Regulation, and Effector Functions of the Immune Response." The book concludes with sections devoted to the immune system's role in protection against pathogenic microorganisms, "Immunity to Infectious Agents," and to how the immune system is involved in a variety of human disorders, "Immunologic Mechanisms in Disease."

I repeat a word of caution that has been in the Preface to each edition. Immunology is moving very fast. Each of the chapters is written by an expert in the field, but in some areas there may be differences of opinion expressed by equally accomplished authors. I ask the reader to take note of the differences and to follow developments in the field.

William E. Paul
Washington, DC

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The preparation of the seventh edition required the efforts of many individuals. I particularly wish to thank each of the authors. Their contributions, prepared in the midst of extremely busy schedules, are responsible for the value of this book. Leanne Vandetty of Lippincott Williams & Wilkins saw that the process of receiving, editing, and assembling the chapters went as smoothly as possible. Without her efforts, the completion of this edition would have been immeasurably more difficult. Frances DeStefano's counsel was of utmost importance in planning this edition and when she had to leave the project, Julie Goolsby took over and played a key role in making important publication decisions. I wish to gratefully acknowledge the efforts of each of the members of the editorial and production staffs of Lippincott Williams & Wilkins who participated in the preparation of this edition.

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Front Matter

Nothing is as powerful as an idea whose time has come.

Paraphrased from Victor Hugo

Everything should be made as simple as possible, but not simpler.

ALBERT EINSTEIN

From my teachers I have learned much, from my colleagues still more, but from my students most of all.

The Talmud

Discovery consists of seeing what everybody has seen and thinking what nobody has thought.

ALBERT SZENT-GYORGYI

… the clonal selection hypothesis … assumes that … there exist clones of mesenchymal cells, each carrying immunologically reactive sites … complementary … to one (or possibly a small number) of potential antigenic determinants.

FRANK MACFARLANE BURNET

In the fields of observation, chance favors the prepared mind.

LOUIS PASTEUR

In all things of nature there is something of the marvelous.

ARISTOTLE

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Chapter 1

The Immune System

William E. Paul

The immune system is a remarkable defense mechanism. It makes rapid, specific, and protective responses against the myriad potentially pathogenic microorganisms that inhabit the world in which we live. The tragic examples of acquired immunodeficiency syndrome (AIDS) and the inherited severe combined immunodeficiencies graphically illustrate the consequences of a nonfunctional adaptive immune system. Patients with AIDS and children with severe combined immunodeficiency often fall victim to infections that are of little or no consequence to those with normally functioning immune systems. The immune system also has a role in the rejection of tumors and, when dysregulated, may give rise to a series of autoimmune diseases, including insulin-dependent diabetes mellitus, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, among others.

Fundamental Immunology has as its goal the authoritative presentation of the basic elements of the immune system, of the means through which the mechanisms of immunity act in a wide range of clinical conditions, including recovery from infectious diseases, rejection of tumors, transplantation of tissue and organs, autoimmunity and other immunopathologic conditions, and allergy, and how the mechanisms of immunity can be marshaled by vaccination to provide protection against microbial pathogens.

The purpose of the opening chapter is to provide readers with a general introduction to our current understanding of the immune system. It should be of particular importance for those with a limited background in immunology, providing them with the preparation needed for subsequent chapters of the book. Rather than providing extensive references in this chapter, each of the subject headings will indicate the chapters that deal in detail with the topic under discussion. Those chapters will not only provide an extended treatment of the topic but will also furnish the reader with a comprehensive reference list that can be found in the online version of *Fundamental Immunology*.

KEY CHARACTERISTICS OF THE IMMUNE SYSTEM

Innate Immunity (Chapters 15, 17, 19, and 20)

Powerful nonspecific defenses prevent or limit infections by most potentially pathogenic microorganisms. The epithelium provides both a physical barrier to the entry of microbes and produces a variety of antimicrobial factors. Agents that penetrate the epithelium are met with macrophages and related cells possessing “microbial sensors” that recognize key molecules characteristic of many microbial agents. These “pattern recognition receptors” include

several families of molecules, of which the most intensively studied are the toll-like receptors (TLRs) and the nucleotide oligomerization domain-like receptors. Each TLR recognizes a distinct substance (or set of substances) associated with microbial agents; for example, TLR4 recognizes lipopolysaccharides, TLR3, doublestranded ribonucleic acid, and TLR9, unmethylated CpG-containing DNA. Because the recognized substances are generally indispensable to the infectious agent, microbial sensors provide a highly efficient means to recognize potential pathogens.

The interaction of a TLR with its ligand induces a series of intracellular signaling events, of which activation of the NF-κB system is particularly important. Macrophage activation with enhancement of the cell's phagocytic activity and the induction of antimicrobial systems aid in the destruction of the pathogen. The induction of an inflammatory response as a result of the activation of the innate immune system recruits other cell types, including neutrophils, to the site. The innate system can provide an effective means to control or eliminate pathogens. Indeed, life forms other than vertebrates rely on the innate immune system to allow them to deal with microbial infection.

In vertebrates, the innate immune system also acts to recruit antigen-specific immune responses, not only by attracting cells of the immune system to the site of infection, but also through the uptake of antigen by dendritic cells (DCs) and its transport by these cells to lymphoid tissues where primary immune responses are initiated. Activated DCs express cell surface costimulatory molecules and produce cytokines that can regulate the quality of the immune response so that it is most appropriate to combating the particular infectious agent, be it a virus, bacterium, or parasite.

Primary Responses (Chapters 10 and 14)

Primary immune responses are initiated when a foreign antigenic substance interacts with antigen-specific lymphocytes under appropriate circumstances. The response generally consists of the production of antibody molecules specific for the antigenic determinants of the immunogen and of the expansion and differentiation of antigen-specific helper and effector T-lymphocytes. The latter include cytokine-producing cells and killer T cells, capable of lysing infected cells. Generally, the combination of the innate immune response and the primary adaptive response are sufficient to eradicate or to control the microbe. Indeed, the most effective function of the immune system is to mount a response that eliminates the infectious agent from the body, so-called sterilizing immunity.

Secondary Responses and Immunologic Memory (Chapters 10, 14, 29, and 31)

As a consequence of initial encounter with antigen, the immunized individual develops a state of immunologic memory. If the same (or a closely related) microorganism is encountered again, a secondary response is made. This generally consists of an antibody response that is more rapid, greater in magnitude, and composed of antibodies that bind to the antigen with greater affinity and are more effective in clearing the microbe from the body. A more rapid and more effective T-cell response also ensues. Thus, an initial infection with a microorganism often initiates a state of immunity in which the individual is protected against a second infection. In the majority of situations, protection is provided by high-affinity antibody

molecules that rapidly clear the re-introduced microbe. This is the basis of most licensed vaccines; the great power of vaccines is illustrated by the elimination of smallpox from the world and by the complete control of polio in the western hemisphere.

The Immune Response is Highly Specific and the Antigenic Universe is Vast

The immune response is highly specific. Primary immunization with a given microorganism evokes antibodies and T cells that are specific for the antigenic determinants found on that microorganism but that generally fail to recognize (or recognize only poorly) antigenic determinants expressed by unrelated microbes. Indeed, the range of antigenic specificities that can be discriminated by the immune system is enormous.

The Immune System is Tolerant of Self-Antigens (Chapters 32 and 33)

One of the most important features of the immune system is its ability to discriminate between antigenic determinants expressed on foreign substances, such as pathogenic microbes, and potential antigenic determinants expressed by the tissues of the host. The failure of the system to make full-blown immune responses to self-antigens is referred to as *immunologic tolerance*. Tolerance is a complex process that actually involves several distinct processes. One element, perhaps the most important, is an active process involving the elimination or inactivation of cells that can recognize self-antigens. In addition, there are mechanisms through which cells that encounter antigens (such as selfantigens) in the absence of cues from the innate immune system may fail to make a response, may make a minimal response, or may be inactivated through a process referred to as *anergy*. Finally, a specialized set of T cells exist designated regulatory cells that actively *suppress* responses against self-antigens. Indeed, individuals who have mutations in the key transcription factor *Foxp3* expressed by the regulatory cells develop severe multiorgan autoimmunity (Immunodysregulation polyendocrinopathy, enteropathy X-linked syndrome). The critical necessity to control selfreactivity is clearly shown by this multilayered system that involves elimination, inactivation, and suppression.

Immune Responses Against Self-Antigens can Result in Autoimmune Diseases (Chapter 44)

Failure in establishing immunologic tolerance or unusual presentations of self-antigens can give rise to tissuedamaging immune responses directed against antigenic determinants on host molecules. These can result in autoimmune diseases. As has already been mentioned, a group of extremely important diseases are caused by autoimmune responses or have major autoimmune components, including systemic lupus erythematosus, rheumatoid arthritis, insulin-dependent diabetes mellitus, multiple sclerosis, myasthenia gravis, and inflammatory bowel disease. Efforts to treat these diseases by modulating the autoimmune response are a major theme of contemporary medicine.

Acquired Immunodeficiency Syndrome is an Example of a Disease Caused by a Virus That the Immune System Generally Fails to Eliminate (Chapter 42)

Immune responses against infectious agents do not always lead to elimination of the pathogen. In some instances, a chronic infection ensues in which the immune system adopts a variety of strategies to limit damage caused by the organism or by the immune response. Indeed, herpes viruses, such as human cytomegalovirus, frequently are not eliminated by

immune responses and establish a chronic infection in which the virus is controlled by immune responses. One of the most notable infectious diseases in which the immune response generally fails to eliminate the organism is AIDS, caused by the human immunodeficiency virus (HIV). In this instance, the principal infected cells are those of the immune system

itself, leading to an eventual state in which the individual can no longer mount protective immune responses against other microbial pathogens. Indeed, under the assault of HIV, control of viruses such as cytomegalovirus is lost and they may cause major tissue damage.

Major Principles of Immunity

The major principles of the immune response are:

- Elimination of many microbial agents through the nonspecific protective mechanisms of the innate immune system
- Cues from the innate immune system inform the cells of the adaptive immune system as to whether it is appropriate to make a response and what type of response to make
- Cells of the adaptive immune system display exquisitely specific recognition of foreign antigens and mobilize potent mechanisms for elimination of microbes bearing such antigens
- The immune system displays memory of its previous responses
- Tolerance of self-antigens.

The remainder of this introductory chapter will describe briefly the molecular and cellular basis of the system and how these central characteristics of the immune response may be explained.

CELLS OF THE IMMUNE SYSTEM AND THEIR SPECIFIC RECEPTORS AND PRODUCTS

The immune system consists of several distinct cell types, each with important roles. The lymphocytes occupy central stage because they are the cells that determine the specificity of immunity. It is their response that orchestrates the effector limbs of the immune system. Cells that interact with lymphocytes play critical parts both in the presentation of antigen and in the mediation of immunologic functions. These cells include DCs and the closely related Langerhans cells, monocyte/macrophages, natural killer (NK) cells, neutrophils, mast cells, basophils, and eosinophils. In addition, a series of specialized epithelial and stromal cells provide the anatomic environment in which immunity occurs, often by secreting critical factors that regulate migration, growth and homeostasis, and gene activation in cells of the immune system. Such cells also play direct roles in the induction and effector phases of the response.

The cells of the immune system are found in peripheral organized tissues, such as the spleen, lymph nodes, Peyer's patches of the intestine, and tonsils, where primary immune responses generally occur (see Chapter 3). Many of the lymphocytes comprise a recirculating pool of cells found in the blood and lymph, as well as in the lymph nodes and spleen, providing the means to deliver immunocompetent cells to sites where they are needed and to allow immunity that is initiated locally to become generalized. Activated lymphocytes acquire the capacity to enter nonlymphoid tissues where they can express

effector functions and eradicate local infections. Some memory lymphocytes are “on patrol” in the tissues, scanning for reintroduction of their specific antigens. Lymphocytes are also found in the central lymphoid organs, the thymus, and bone marrow, where they undergo the developmental steps that equip them to mediate the responses of the mature immune system.

Individual lymphocytes are specialized in that they are committed to respond to a limited set of structurally related antigens. This commitment exists before the first contact of the immune system with a given antigen. It is expressed by the presence on the lymphocyte's surface of receptors specific for determinants (epitopes) of the antigen. Each lymphocyte possesses a population of receptors, all of which have identical combining sites (this is a slight oversimplification as occasionally T cells and less frequently B cells may express two populations of receptors). One set, or clone, of lymphocytes differs from another clone in the structure of the combining region of its receptors and thus in the epitopes that it can recognize. The ability of an organism to respond to virtually any non-self-antigen is achieved by the existence of a very large number of different lymphocytes, each bearing receptors specific for a distinct epitope. As a consequence, lymphocytes are an enormously heterogeneous group of cells. Based on reasonable assumptions as to the range of diversity that can be created in the genes encoding antigen-specific receptors, it is virtually certain that the number of distinct combining sites on lymphocyte receptors of an adult human can be measured in the millions.

Lymphocytes differ from each other not only in the specificity of their receptors but also in their functions. There are two broad classes of lymphocytes: the B-lymphocytes, which are precursors of antibody-secreting cells, and the T (thymus-derived)- lymphocytes. T-lymphocytes express important helper functions, such as the ability to aid in the development of specific types of immune responses, including the production of antibody by B cells, the increase in the microbicidal activity of macrophages, and the recruitment of granulocytes to sites of infection. Other T-lymphocytes are involved in direct effector functions, such as the lysis of virus-infected cells or certain neoplastic cells. Regulatory T-lymphocytes have the capacity to suppress immune responses.

B-LYMPHOCYTES AND ANTIBODY

B-Lymphocyte Development (Chapter 8)

B-lymphocytes derive from lymphoid progenitor cells, which in turn are derived from hematopoietic stem cells (Fig. 1.1). A detailed picture has been obtained of the molecular mechanisms through which committed early members of the B lineage develop into mature B-lymphocytes. These events occur in the fetal liver and, in adult life, in the bone marrow. Interaction with specialized stromal cells and their products, including cytokines such as interleukin (IL)-7 and BAFF, are critical to the normal regulation of this process.

The key events in B-cell development involve commitment to the B lineage and repression of the capacity to differentiate to cells of other lineages. In pro-B cells and pre-B cells, the genetic elements that encode the antigen-specific receptors are assembled. These receptors are im

munoglobulin (Ig) molecules specialized for expression on the cell surface. Igs are heterodimeric molecules consisting of heavy (H) and light (L) chains, both of which have

variable (V) regions, which are responsible for the binding of antigen and that differ in sequence from one Ig molecule to another (see Chapters 5, 6, and 7) (Fig. 1.2) and constant (C) regions.

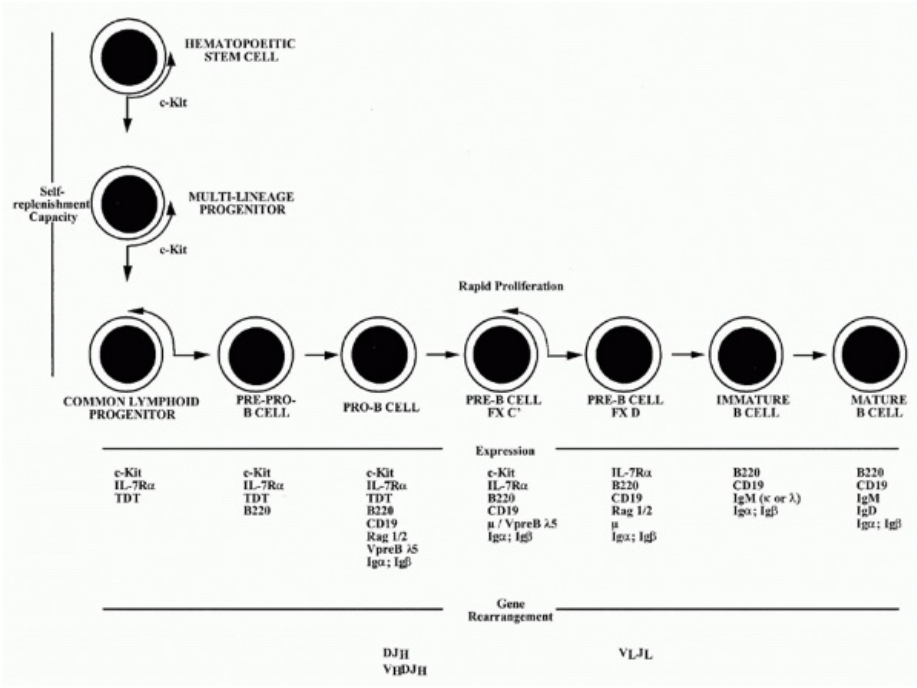

FIG. 1.1. The patterns of gene expression, timing of gene rearrangement events, and capacity for self-replenishment and for rapid proliferation of developing B lymphocytes are indicated. (Adapted from Hardy RR, Hayakawa K. B cell development pathways. *Ann Rev Immunol*. 2001;19:595-621.)

The genetic elements encoding the variable portions of Ig H and L chains are not contiguous in germline DNA or in the DNA of nonlymphoid cells (see Chapter 6) (Fig. 1.3). In pro- and pre-B cells, these genetic elements are translocated to construct an expressible V-region gene. This process involves a choice among a large set of potentially usable V, diversity (D), and joining (J) elements in a combinatorial manner and depends upon the recombinating activating gene (RAG) proteins, RAG1 and RAG2. Such combinatorial translocation, together with the addition of diversity in the course of the joining process, results in the generation of a very large number of distinct H and L chains. The pairing of H and L chains in a quasirandom manner further expands the number of distinct Ig molecules that can be formed.

The H-chain V region is initially expressed in association with the product of the μC-region gene. Together, these elements encode the μ IgH chain, which is used in Igs of the IgM class.

The successful completion of the process of Ig gene rearrangement and the expression of the resultant IgM on the cell surface marks the transition between the pre-B- and B-cell states (see Fig. 1.1). The newly differentiated B cell initially expresses surface Ig solely of the IgM class. The cell completes its maturation process by expressing on its surface a second class of Ig composed of the same L chain and the same H chain V (VDJ) region but of a different H chain C region; this second Ig H chain is designated δ, and the Ig to which it contributes is designated IgD, so that the mature naïve B cells express both IgM and IgD surface molecules

that share the same V region.

The differentiation process is controlled at several steps by a system of checks that determines whether prior steps have been successfully completed. These checks depend on the expression on the surface of the cell of appropriately constructed Ig or Ig-like molecules. For, example,

in the period after a μ chain has been successfully assembled but before an L chain has been assembled, the μ chain is expressed on the cell surface in association with a surrogate L chain, consisting of VpreB and λ5. Pre-B cells that fail to express this μ/VpreB λ5 complex do not move forward to future differentiation states or do so very inefficiently.

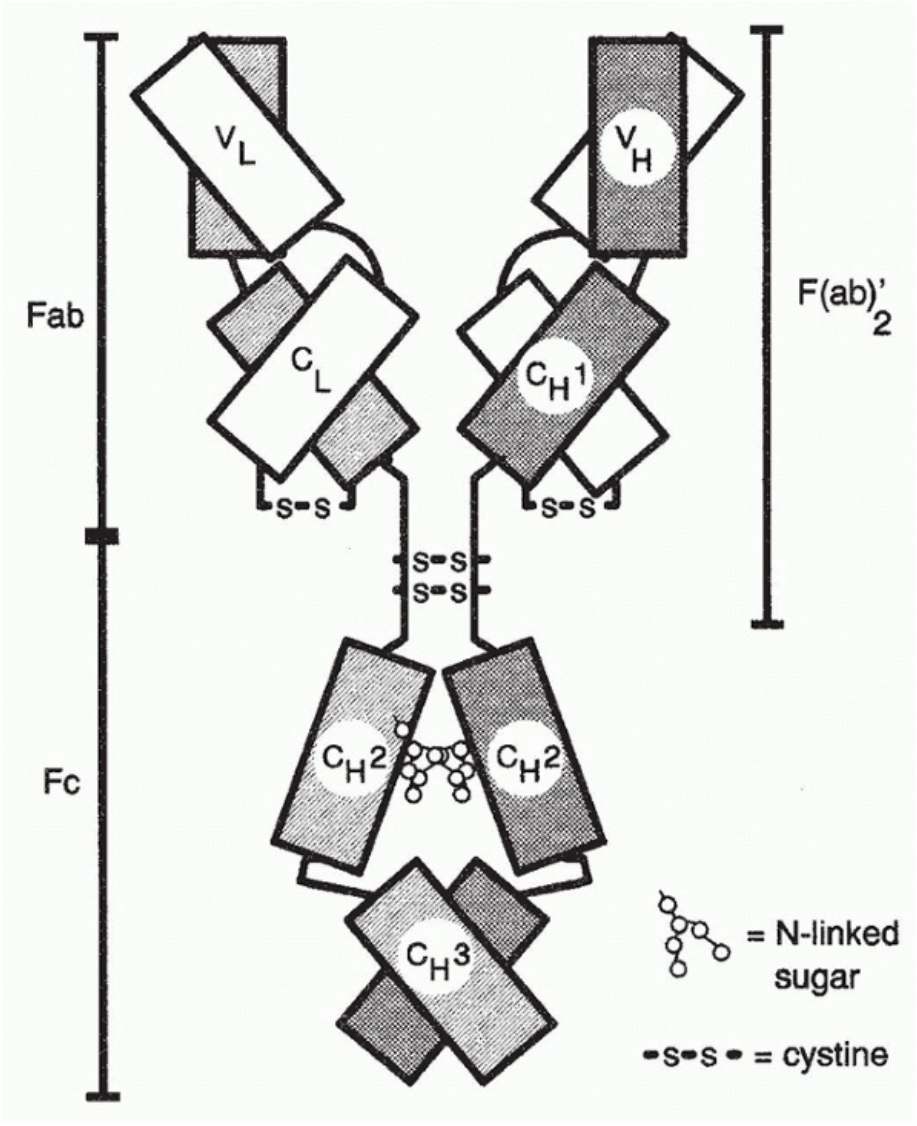

FIG. 1.2. A schematic representation of an immunoglobulin molecule indicating the means through the variable regions and the CH1 and CL regions of heavy and light chains pair with one another and how the CH2 and CH3 regions of the heavy chains pair.

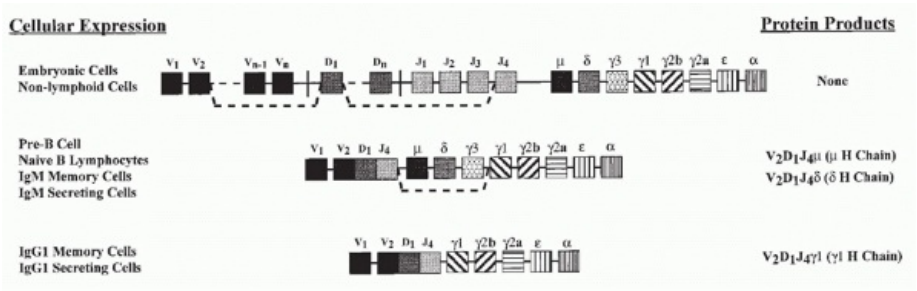

FIG. 1.3. Organization and translocation of mouse immunoglobulin (Ig)H genes. IgH chains are encoded by four distinct genetic elements: Igh-V (V), Igh-D (D), Igh-J (J) and Igh-C. The variable (V), diversity (D), and joining (J) genetic elements together specify the variable region of the heavy chain. The Igh-C element specifies the constant (C) region. The same V region can be expressed in association with each of the C regions (μ, δ, γ3,γ1,γ2b,γ2a, ε, and α). In the germline, the V, D, and J genes are far apart and there are multiple forms of each of these genes. In the course of lymphocyte development, a VDJ gene complex is formed by translocation of individual V and D genes so that they lie next to one of the J genes, with excision of the intervening genes. This VDJ complex is initially expressed with μ and δ C genes but may be subsequently translocated so that it lies near one of the other C genes (e.g., γ1) and in that case leads to the expression of a VDJ γ1 chain.

B-Lymphocyte Activation (Chapter 9)

A mature B cell can be activated by an encounter with antigen-expressing epitopes that are recognized by its cell surface Ig (Fig. 1.4). The activation process may be a direct one, dependent on cross-linkage of membrane Ig molecules by the antigen (*cross-linkage-dependent B-cell activation*), or an indirect one, occurring most efficiently in the context of an intimate interaction with a helper T cell, in a process often referred to as *cognate help*.

Because each B cell bears membrane Ig molecules with identical variable regions, cross-linkage of the cell surface receptors requires that the antigen express more than one copy of an epitope complementary to the binding site of the receptor. This requirement is fulfilled by antigens with repetitive epitopes. Among these antigens are the capsular polysaccharides of many medically important microorganisms such as pneumococci, streptococci, and meningococci. Similar expression of multiple identical epitopes on a single immunogenic particle is a property of many viruses because they express multiple copies of envelope proteins on their surface. Cross-linkage-dependent B-cell activation is a major protective immune response mounted against these microbes. The binding of complement components (see Chapter 36) to antigen or antigen/antibody complexes can increase the magnitude of the cross-linkage-dependent B-cell activation due to the action of a receptor for complement, which, together with other molecules, increases the magnitude of a B-cell response to limiting amounts of antigen.

Cognate help allows B cells to mount responses against antigens that cannot cross-link receptors and, at the same time, provides costimulatory signals that rescue B cells from

inactivation when they are stimulated by weak cross-linkage events. Cognate help is dependent on the binding of antigen by the B cell's membrane Ig, the endocytosis of the antigen, and its fragmentation into peptides within the endosomal/lysosomal compartment of the cell. Some of the resultant peptides are loaded into a groove in a specialized set of cell surface proteins, the class II major histocompatibility complex (MHC) molecules (Fig. 1.5). The resultant class II/

P.6

P.7

peptide complexes are expressed on the cell surface. As will be discussed subsequently, these complexes are the ligands for the antigen-specific receptors of a set of T cells designated CD4 T cells. CD4 T cells that have receptors specific for the class II/peptide complex expressed on the B-cell surface recognize and interact with that B cell. That interaction results in the activation of the B cell through the agency of cell surface molecules expressed by the T cells (e.g., the CD40 ligand [CD154]) and cytokines produced by the T cell (see Fig. 1.4). The role of the B-cell receptor for antigen is to create the T-cell ligand on the surface of antigen-specific B cells; activation of the B cell derives largely from the action of the T cell. However, in many physiologic situations, receptor cross-linkage stimuli and cognate help synergize to yield more vigorous B-cell responses. Recently, it has been shown that the association of ligands for TLRs with antigen will strikingly enhance B cell responses.

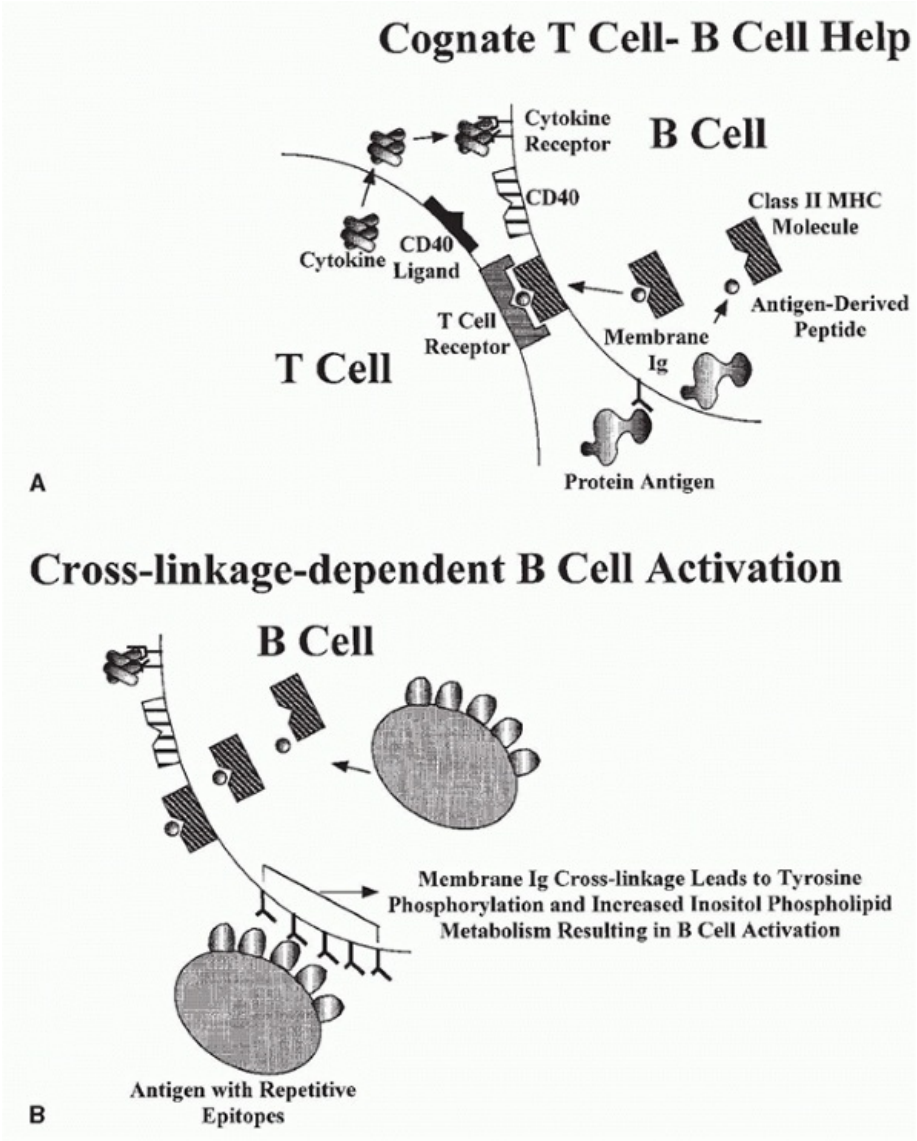

FIG. 1.4. Two Forms of B-Cell Activation. A: Cognate T cell/B cell help. Resting B cells can bind antigens that bear epitopes complementary to their cell surface Ig. Even if the antigen cannot cross-link the receptor, it will be endocytosed and enter late endosomes and lysosomes where it will be degraded to peptides. Some of these peptides will be loaded into class II major histocompatibility complex molecules and brought to the cell surface, where they can be recognized by CD4-positive T cells that bear receptors specific for that peptide/class II complex. This interaction allows an activation ligand on the T cells (CD40 ligand) to bind to its receptor on B cells (CD40) and to signal B-cell activation. In addition, the T cells secrete several cytokines that regulate the growth and differentiation of the stimulated B cell. **B:** Cross-linkage-dependent B cell activation. When B cells encounter antigens that bear multiple copies of an epitope that can bind to their surface immunoglobulin, the resultant crosslinkage stimulates biochemical signals within the cell leading to B-cell activation, growth, and differentiation. In many instances, B-cell activation events may result from both pathways of stimulation.

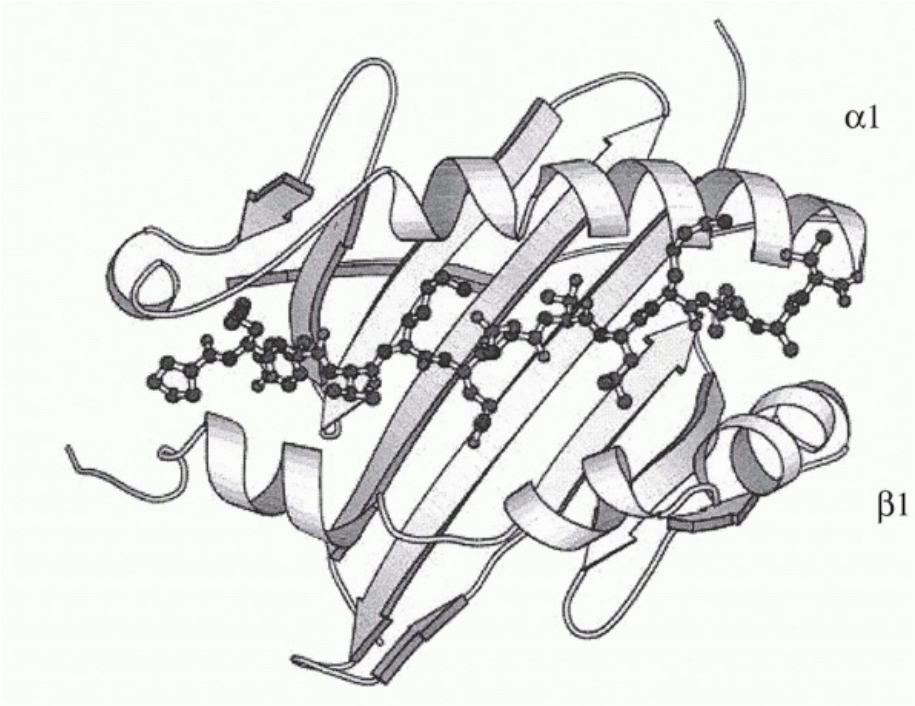

FIG. 1.5. Illustration of the structure of the peptide binding domain (α1 and β1) of a class II major histocompatibility complex molecule bound to an antigenic peptide from influenza hemagglutinin (adapted by D.H. Margulies from Stern LJ, Brown JH, Jardetzky TS, et al. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature*. 1994;68[6468]:215-221.)

B-Lymphocyte Differentiation (Chapters 9 and 10)

Activation of B cells prepares them to divide and to differentiate either into antibody-secreting cells or into memory cells, so that there are more cells specific for the antigen used for immunization. Those cells that differentiate into antibody-secreting cells account for primary antibody responses. Some of these antibody-secreting cells migrate to the bone marrow where they may continue to produce antibody for an extended period of time and may have lifetimes very long.

Memory B cells give rise to antibody-secreting cells upon rechallenge of the individual. The hallmark of the antibody response to rechallenge (a secondary response) is that it is of greater magnitude, occurs more promptly, is composed of antibodies with higher affinity for the antigen, and is dominated by Igs expressing γ, α, or ε C regions (IgG, IgA, or IgE) rather than by IgM, which is the dominant Ig of the primary response.

Division and differentiation of cells into antibody-secreting cells is largely controlled by the interaction of the activated B cells with T cells expressing CD154 and by their stimulation by T-cell-derived cytokines.

The differentiation of activated B cells into memory cells occurs in a specialized microenvironmental structure in the spleen and lymph nodes: the germinal center. The increase in antibody affinity also takes place within the germinal center. This process, designated *affinity maturation*, is dependent on somatic hypermutation. The survival of B

cells within the germinal center depends on their capacity to bind antigen so that as the amount of antigen diminishes, B cells that have higher affinity receptors, either naturally or as a result of the hypermutation process, have a selective survival and growth advantage. Thus, such cells come to dominate the population.

The process through which a single H-chain V region can become expressed with genes encoding C regions other than μ or δ is referred to as Ig class switching. It is dependent on a gene translocation event through which the C-region genes between the genetic elements encoding the V region and the newly expressed C gene are excised, resulting in the switched C gene being located in the position that the Cμ gene formerly occupied (see Fig. 1.3). This process also occurs mainly in germinal centers. Both somatic hypermutation and immunoglobulin class switching depend upon the action of activation-induced cytidine deaminase (AID) that plays an important role in the breakage and repair of DNA, which is essential for recombination events.

B1 and Marginal Zone B-Lymphocytes (Chapters 8 and 10)

B lymphocytes consist of at least three distinct populations: conventional B cells, B1 B cells, and marginal zone B cells. B1 B cells were initially recognized because some express a cell-surface protein, CD5, not generally found on other B cells. In the adult mouse, B1 B cells are found in relatively high frequency in the peritoneal cavity but are present at low frequency in the spleen and lymph nodes. B1 B cells are quite numerous in fetal and perinatal life and appear to be self-renewing, in contrast to conventional B cells, in which division and memory are antigen driven.

Marginal zone B cells are localized in a distinct anatomical region of the spleen (the marginal zone) that represents the major antigen filtering and scavenging area. Like B1 B cells, marginal zone B cells express a repertoire biased toward bacterial cell wall constituents and senescent self-components. Marginal zone and B1 B cells respond very rapidly to antigenic challenge, likely independently of T cells. Uniquely, among all populations of B cells, marginal zone B cells are dependent on Notch-2 signaling for their development.

B1 B cells and marginal zone B cells are responsible for the secretion of the serum IgM that exists in nonimmunized mice, often referred to as natural IgM. Among the antibodies found in such natural IgM are molecules that can combine with phosphatidylcholine (a component of pneumococcal cell walls) and with lipopolysaccharide and influenza virus. B1 B cells also produce autoantibodies, although they are generally of low affinity and in most cases not pathogenic. There is evidence that B1 B cells are important in resistance to several pathogens and may have a significant role in mucosal immunity.

B-Lymphocyte Tolerance (Chapter 32)

One of the central problems facing the immune system is that of being able to mount highly effective immune responses to the antigens of foreign, potentially pathogenic agents while ignoring antigens associated with the host's own tissues. The mechanisms ensuring this failure to respond to self-antigens are complex and involve a series of strategies. Chief among them is elimination of cells capable of self-reactivity or the inactivation of such cells. The encounter of immature, naïve B cells with antigens with repetitive epitopes capable of cross-linking membrane Ig can lead to elimination of the B cells, particularly if no T-cell help is provided at the time of the encounter. This elimination of potentially self-reactive cells is

often referred to as clonal elimination. Many selfreactive cells, rather than dying upon encounter with selfantigens, undergo a further round of Ig gene rearrangement. This *receptor editing* process allows a self-reactive cell to substitute a new receptor and therefore to avoid elimination.

There are many self-antigens that are not encountered by the developing B-cell population or that do not have the capacity to cross-link B-cell receptors to a sufficient degree to elicit the receptor editing /clonal elimination process. Such cells, even when mature, may nonetheless be inactivated through a process that involves cross-linkage of receptors without the receipt of critical costimulatory signals. These inactivated cells may be retained in the body but are unresponsive to antigen and are referred to as anergic. When removed from the presence of the anergy-inducing stimulus, anergic cells may regain responsiveness.

Immunoglobulins

Structure (Chapter 5)

Igs are the antigen-specific membrane receptors and secreted products of B cells. They are members of a large family of proteins designated the Ig supergene family. Members of the Ig supergene family have sequence homology, a common gene organization, and similarities in three-dimensional structure. The latter is characterized by a structural element referred to as the Ig fold, generally consisting of a set of seven β-pleated sheets organized into two opposing layers (Fig. 1.6). Many of the cell surface proteins that participate in immunologic recognition processes, including the T-cell receptor (TCR), the CD3 complex, and signaling molecules associated with the B-cell receptor (Igα and Igβ), are members of the Ig supergene family.

The Igs themselves are constructed of a unit that consists of two H chains and two L chains (see Fig. 1.2). The H and L chains are composed of a series of domains, each consisting of approximately 110 amino acids.

The L chains, of which there are two types (κ and λ), consist of two domains. The carboxy-terminal domain is essentially identical among L chains of a given type and is referred to as the C region. As already discussed, the amino terminal domain varies from L chain to L chain and contributes to the binding site of antibody. Because of its variability, it is referred to as the V region. The variability of this region is largely concentrated in three segments, designated the hypervariable or complementarity-determining regions (CDRs). The CDRs contain the amino acids that are the L chain's contribution to the lining of the antibody's combining site. The three CDRs are interspersed among four regions of much lower degree of variability, designated framework regions.

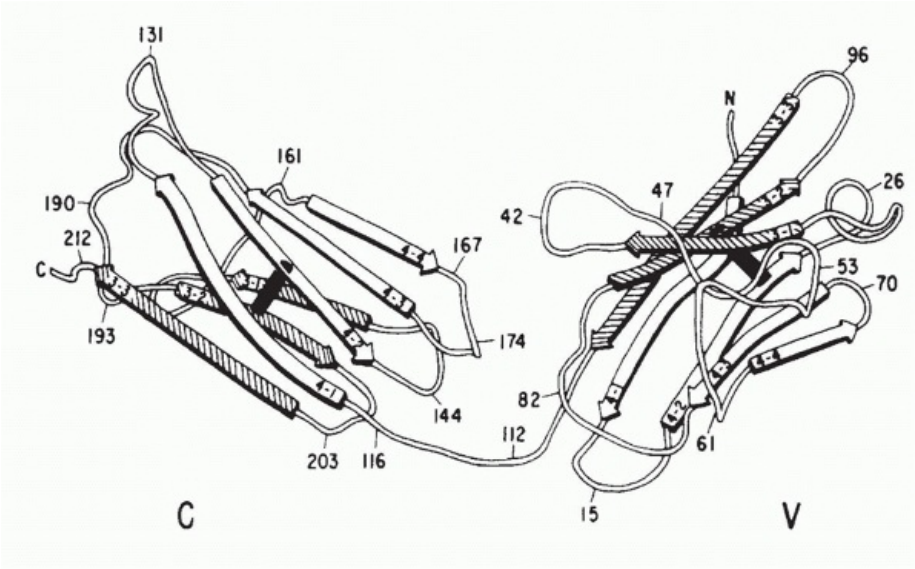

FIG. 1.6. Schematic Drawing of the Variable and Constant Domains of an Immunoglobulin Light Chain Illustrating the "Immunoglobulin Fold." The β strands participating in the antiparallel β-pleated sheets of each domain are represented as *arrows*. The β strands of the three-stranded sheets are *shaded*, whereas those in the four-stranded sheets are *white*. The intradomain disulfide bonds are represented as *black bars*. Selected amino acids are numbered with position 1 as the N terminus. (Reprinted with permission from Edmundson AB, Ely KR, Abola EE, Schiffer M, Panagiotopoulous N. Rotational allomerism and divergent evolution of domains in immunoglobulin light chains. *Biochemistry*. 1975;14:3953-3961).

The H chains of Ig molecules are of several classes determined by their constant regions (μ, δ, γ [of which there are several subclasses], α and ε). An assembled Ig molecule, consisting of one or more units of two identical H and L chains, derives its name from the constant region of the H chain that it possesses. Thus, there are IgM, IgD, IgG, IgA, and IgE antibodies. The H chains each consist of a single amino terminal V region and three or four C regions. In many H chains, a hinge region separates the first and second C regions and conveys flexibility to the molecule, allowing the two combining sites of a single unit to move in relation to one another so as to promote the binding of a single antibody molecule to an antigen that has more than one copy of the same epitope. Such divalent binding to a single antigenic structure results in a great gain in energy of interaction (see Chapter 7). The H-chain V region, like that of the L chain, contains three CDRs lining the combining site of the antibody and four framework regions.

The C region of each H-chain class conveys unique functional attributes to the antibodies that possess it. Among the distinct biologic functions of each class of antibody are the following:

- IgM antibodies are potent activators of the complement system (see Chapter 36).
- IgA antibodies are secreted into a variety of bodily fluids and are principally responsible for immunity at mucosal surfaces (see Chapter 34).
- IgE antibodies are bound by specific receptors (FcεRI) on basophils and mast cells. When

cross-linked by antigen, these IgE/FcεRI complexes cause the cells to release a set of mediators responsible for allergic inflammatory responses (see Chapter 45).

- IgD antibodies act virtually exclusively as membrane receptors for antigen.

- IgG antibodies, made up of four subclasses in both humans and mice, mediate a wide range of functions including transplacental passage and opsonization of antigens through binding of antigen/antibody complexes to specialized Fc receptors on macrophages and other cell types (see Chapters 19, 20, and 24).

IgD, IgG, and IgE antibodies consist of a single unit of two H and L chains. IgM antibodies are constructed of five or six such units, although they consist of a single unit when they act as membrane receptors. IgA antibodies may consist of one or more units. The antibodies that are made up of more than a single unit generally contain an additional polypeptide chain, the J chain, that appears to play a role in the polymerization process. In addition, secreted IgA expresses a chain, a secretory piece, that is derived from the receptor for polymeric IgA, which plays a role in the transport of IgA through the cells lining the lumen of the gut.

Each of the distinct Igs can exist as secreted antibodies and as membrane molecules. Antibodies and cell surface receptors of the same class made by a specific cell have identical structures except for differences in their carboxy-terminal regions. Membrane Igs possess a hydrophobic region, spanning the membrane, and a short intracytoplasmic tail, both of which are lacking in the secretory form.

Immunoglobulin Genetics (Chapter 6)

The components of the Ig H-chain gene have already been alluded to. To reiterate, the IgH chain gene of a mature lymphocyte is derived from a set of genetic elements that are separated from one another in the germline. The V region is composed of three types of genetic elements: V_H, D, and J_H. More than 100 V_H elements exist; there are more than 10 D elements and a small number of J_H elements (4 in the mouse). An H-chain V_HDJ_H gene is created by the translocation of one of the D elements on a given chromosome to one of the J_H elements on that chromosome, generally with the excision of the intervening DNA. This is followed by a second translocation event in which one of the V_H elements is brought into apposition with the assembled DJ_H element to create the V_HDJ_H (V region) gene (see Fig. 1.3). Although it is likely that the choice of the V_H, D, and J_H elements that are assembled is not entirely random, the combinatorial process allows the creation of a very large number of distinct H-chain V-region genes. Additional diversity is created by the imprecision of the joining events and by the deletion of nucleotides and addition of new, untemplated nucleotides between D and J_H and between V_H and D, forming N regions in these areas. This further increases the diversity of distinct IgH chains that can be generated from the relatively modest amount of genetic information present in the germline.

The assembly of L-chain genes follows generally similar rules. However, L chains are assembled from V_L and J_L elements only. Although there is junctional diversity, no N regions exist for L chains. Additional diversity is provided by the existence of two classes of L chains, κ and λ.

An Ig molecule is assembled by the pairing of an IgH-chain polypeptide with an IgL-chain

polypeptide. Although this process is almost certainly not completely random, it allows the formation of an exceedingly large number of distinct Ig molecules, the majority of which will have individual specificities.

The rearrangement events that result in the assembly of expressible IgH and IgL chains occur in the course of B-cell development in pro-B cells and pre-B cells, respectively (see Fig. 1.1). This process is regulated by the Ig products of the rearrangement events. The formation of a μ chain signals the termination of rearrangement of H-chain gene elements and the onset of rearrangement of L-chain gene elements, with κ rearrangements generally preceding λ rearrangements. One important consequence of this is that only a single expressible μ chain will be produced in a given cell, as the first expressible μ chain shuts off the possibility of producing an expressible μ chain on the alternative chromosome. Comparable mechanisms exist to ensure that only one L-chain gene is produced, leading to the phenomenon known as allelic exclusion. Thus, the product of only one of the two alternative allelic regions at both the H- and L-chain loci are expressed. The closely related phenomenon of L-chain isotype exclusion ensures the production of either κ or λ chains in an individual cell, but not both. An obvious but critical consequence of allelic exclusion is that in most cases an individual B cell makes antibodies, all of which have identical H- and L-chain V regions, a central prediction of the clonal selection theory of the immune response. During receptor editing, secondary rearrangements occur. Receptor editing is induced when the initial membrane Ig is capable of self-reactivity. As a consequence of the resultant secondary rearrangement, Ig of a different specificity is expressed, usually no longer self-reactive.

Class Switching (Chapter 6)

An individual B cell continues to express the same IgH-chain V region as it matures but it can switch the IgH-chain C region it uses (see Fig. 1.3). Thus, a cell that expresses receptors of the IgM and IgD classes may differentiate into a cell that expresses IgG, IgA, or IgE receptors and then into a cell secreting antibody of the same class as it expressed on the cell surface. This process allows the production of antibodies capable of mediating distinct biologic functions but that retain the same antigen-combining specificity. When linked with the process of affinity maturation of antibodies, Ig class switching provides antibodies of extremely high efficacy in preventing re-infection with microbial pathogens or in rapidly eliminating such pathogens. The associated phenomena of class switching and affinity maturation account for the high degree of effectiveness of antibodies produced in secondary immune responses.

The process of class switching is known to involve a genetic recombination event between specialized switch (S) regions, containing repetitive sequences, that are located upstream of each C region genetic element (with the exception of the δ C region). Thus, the S region upstream of the μ C_H region gene (Sμ) recombines with an S region upstream of a more 3′ isotype, such as Sγ1, to create a chimeric Sμ/Sγ1

region and in the deletion of the intervening DNA (Fig. 1.7). The genes encoding the C regions of the various γ chains (in the human γ1, γ2, γ3, and γ4; in the mouse γ1, γ2a, γ2b, and γ3), of the α chain, and of the ε chain are located 3′ of the Cμ and Cδ genes.

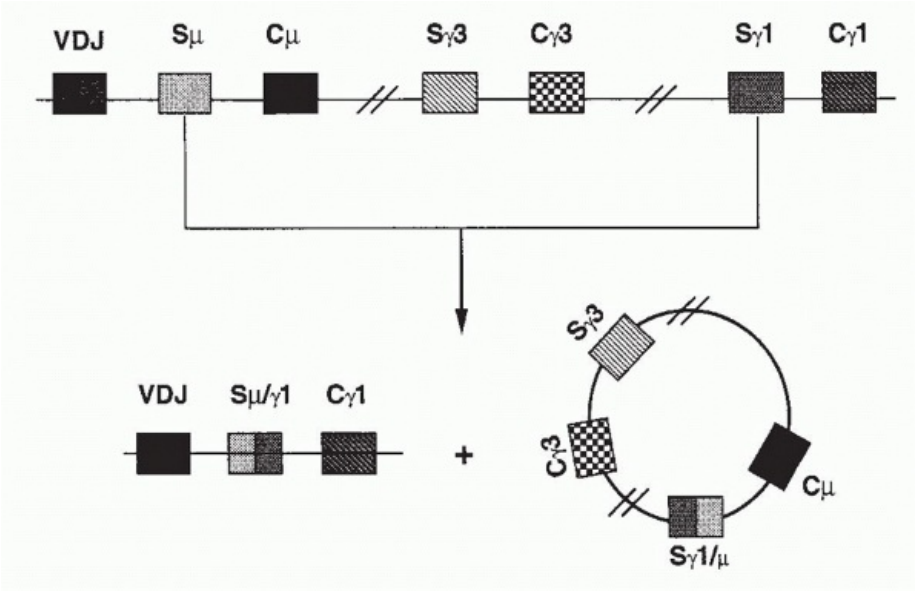

FIG. 1.7. Immunoglobulin Class Switching. The process through which a given VDJ gene in a stimulated B cell may switch the constant region gene with which it is associated from μ to another, such as γ1, is illustrated. A recombination event occurs in which DNA between a cleavage point in Sμ and one in Sγ1 forms a circular episome. This results in Cγ1 being located immediately downstream of the chimeric Sμ/γ1 region, in a position such that transcription initiating upstream of VDJ results in the formation of VDJCγ1 mRNA and γ1 H-chain protein.

The induction of the switching process is dependent on the action of a specialized set of B-cell stimulants. Of these, the most widely studied are CD154, expressed on the surface of activated T cells, and the TLR ligands such as bacterial lipopolysaccharide. The targeting of the C region that will be expressed as a result of switching is largely determined by cytokines. Thus, IL-4 determines that switch events in the human and mouse will be to the ε C region and to the γ4 (human) or γ1 (mouse) C regions. In the mouse, interferon-gamma (IFN-γ) determines switching to γ2a and transforming growth factor-beta (TGF-β) determines switching to α. A major goal is to understand the physiologic determination of the specificity of the switching process. Because cytokines are often the key controllers of which Ig classes will represent the switched isotype, this logically translates into asking what regulates the relative amounts of particular cytokines that are produced by different modes of immunization.

As already noted, both the switching process and somatic hypermutation depend upon the AID. Mice and humans that lack AID fail to undergo both immunoglobulin class switching and somatic hypermutation.

Affinity Maturation and Somatic Hypermutation (Chapters 6 and 10)

The process of generation of diversity embodied in the construction of the H- and L-chain V-region genes and of the pairing of H and L chains creates a large number of distinct antibody molecules, each expressed in an individual B cell. This primary repertoire is sufficiently large so that most epitopes on foreign antigens will encounter B cells with complementary receptors. Thus, if adequate T-cell help can be generated, antibody responses can be made

to a wide array of foreign substances. Nonetheless, the antibody that is initially produced usually has a relatively low affinity for the antigen. This is partially compensated for by the fact that IgM, the antibody initially made, is a pentamer. Through multivalent binding, high avidities can be achieved even if individual combining sites have only modest affinity (see Chapter 7). In the course of T-cell-dependent B-cell stimulation, particularly within the germinal center, a process of somatic hypermutation is initiated that leads to a large number of mutational events, largely confined to the H-chain and L-chain V-region genes and their immediately surrounding introns.

During the process of somatic hypermutation, mutational rates of 1 per 1,000 base pairs per generation may be achieved. This implies that with each cell division close to one mutation will occur in either the H- or L-chain V region of an individual cell. Such a high rate of mutation creates an enormous increase in antibody diversity. Although most of these mutations will either not affect the affinity with which the antibody binds its ligand or will lower that affinity, some will increase it. Thus, some B cells emerge that can bind antigen more avidly than the initial population of responding cells. Because there is an active process of apoptosis in the germinal center from which B cells can be rescued by the binding of antigen to their membrane receptors, cells with the most avid receptors should have an advantage over other antigen-specific B cells and should come to dominate the population of responding cells. Thus, upon rechallenge, the affinity of antibody produced will be greater than that in the initial response. As time after immunization elapses, the affinity of antibody produced will increase. This process leads to the presence in immunized individuals of high-affinity antibodies that are much more effective, on a weight basis, in protecting against microbial agents and other antigenbearing pathogens than was the antibody initially produced. Together with antibody class switching, affinity maturation results in the increased effectiveness of antibody in preventing reinfection with agents with which the individual has had a prior encounter.

T-LYMPHOCYTES

T-lymphocytes constitute the second major class of lymphocytes. They derive from precursors in hematopoietic tissue, undergo differentiation in the thymus (hence the name thymus-derived [T]-lymphocytes), and are then seeded to the peripheral lymphoid tissue and to the recirculating pool of lymphocytes (see Chapters 13 and 14). T cells are subdivided into two distinct classes based on the cell surface receptors they express. The majority of T cells express TCRs consisting of α and β chains. A second group of T cells express receptors made up of γ and δ chains. Among the α/β T cells are two important sublineages: those that express the coreceptor molecule CD4 (CD4 T cells) and those that express CD8 (CD8 T cells). These cells differ in how they recognize

antigen and mediate different types of regulatory and effector functions.

CD4 T cells are the major *helper* cells of the immune system (see Chapter 29). Their helper function depends both on cell surface molecules, such as CD154, induced upon these cells when they are activated and on the wide array of cytokines they secrete upon stimulation. CD4 T cells tend to differentiate, as a consequence of priming, into cells that principally secrete the cytokines IL-4, IL-13, IL-5 and IL-6 (T_{H2} cells) into cells that mainly produce IFN-γ and lymphotoxin (T_{H1} cells), or into cells that produce IL-17 and related cytokines (T_{H17}

cells). T_{H2} cells are very effective in immunity to helminthic parasites, T_{H1} cells are effective inducers of cellular immune responses, involving enhancement in the microbicidal activity of monocytes and macrophages and consequent increased efficiency in lysing microorganisms in intracellular vesicular compartments, while T_{H17} cells are efficient recruiters of granulocytes and other cells of the inflammatory system and play a major role in responses to extracellular bacterial pathogens. CD4 T cells can also acquire the capacity to enter B-cell follicles and help B cells develop into antibody-producing cells and undergo immunoglobulin class switching and affinity maturation; the cells are referred to as T follicular helper (T_{fh}) cells. Another possible fate of naïve CD4 T cells is to differentiate into induced regulatory T cells (iT_{regs}). However, most T_{regs} develop as a independent lineage of CD4 T cells. T_{regs} express the transcription factor Foxp3 and many express large amounts of the α chain of the IL-2 receptor (CD25).

T cells mediate important effector functions. Some of these are determined by the patterns of cytokines they secrete. These powerful molecules can be directly toxic to target cells and can mobilize potent inflammatory mechanisms. In addition, T cells, particularly CD8 T cells, can develop into cytotoxic T-lymphocytes (CTLs) capable of efficiently lysing target cells that express antigens recognized by the CTLs.

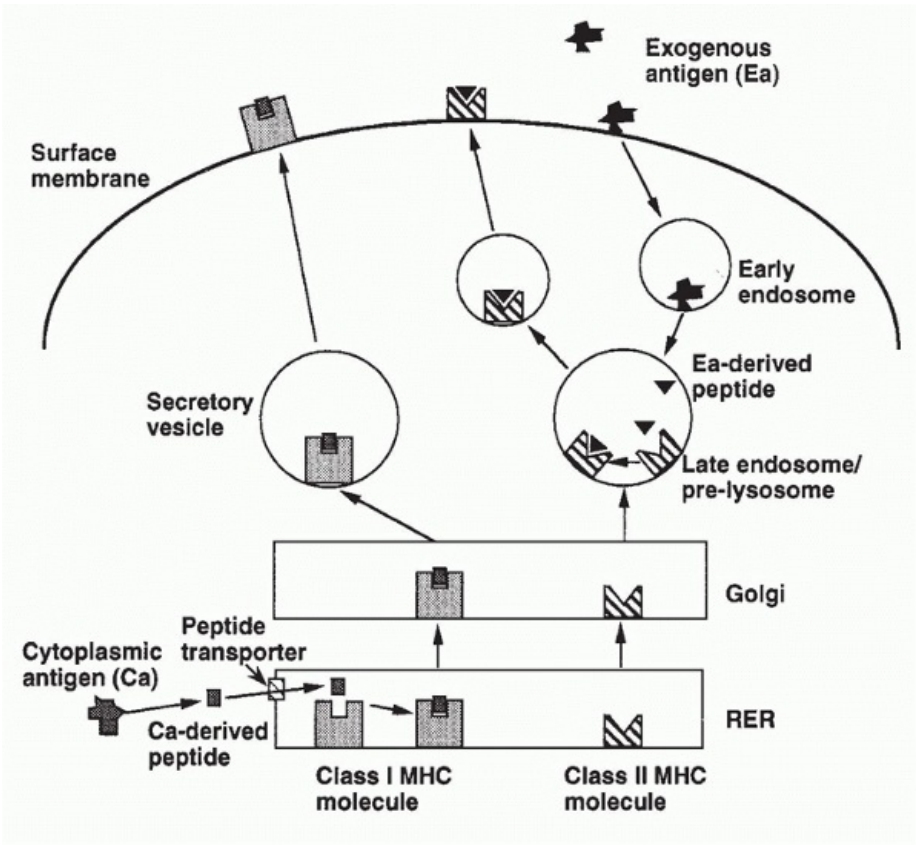

FIG. 1.8. Pathways of Antigen Processing. Exogenous antigen (Ea) enters the cell via endocytosis and is transported from early endosomes into late endosomes or prelysosomes, where it is fragmented and where resulting peptides (Ea-derived peptides) may be loaded into class II major histocompatibility complex (MHC) molecules. The latter have been transported from the rough endoplasmic reticulum (RER) through the Golgi apparatus to the peptide-containing vesicles. Class II MHC molecules/Ea-

derived peptide complexes are then transported to the cell surface, where they may be recognized by T-cell receptor expressed on CD4+ T cells. Cytoplasmic antigens (Ca) are degraded in the cytoplasm and then enter the RER through a peptide transporter. In the RER, Ca-derived peptides are loaded into class I MHC molecules that move through the Golgi apparatus into secretory vesicles and are then expressed on the cell surface where they may be recognized by CD8+ T cells (Reprinted with permission from Paul WE. In: Gallin JI, Goldstein, I, Snyderman, R, ed. *Inflammation*. New York: Raven,;1992:776.)

T-Lymphocyte Antigen Recognition (Chapters 11, 21, and 22)

T cells differ from B cells in their mechanism of antigen recognition. Ig, the B-cell's receptor, binds to individual antigenic epitopes on the surface of native molecules, be they on cell surfaces or in solution. Antibody and B-cell receptors evolved to bind to and to protect against microorganisms in extracellular fluids.

By contrast, T cells invariably recognize cell-associated molecules and mediate their functions by interacting with and altering the behavior of such *antigen-presenting cells* (APCs). Indeed, the TCR does not recognize antigenic determinants on intact, undenatured molecules. Rather, it recognizes a complex consisting of a peptide, derived by intracellular proteolysis of the antigen, bound into a specialized groove of a class II or class I MHC protein. Indeed, what differentiates a CD4 T cell from a CD8 T cell is that the CD4 T cells recognize peptide/class II complexes whereas the CD8 T cells recognize peptide/class I complexes.

The TCR's ligand (i.e., the peptide/MHC protein complex) is created within the APC. In general, class II MHC molecules bind peptides derived from proteins that have been taken up by the APC through an endocytic process (Fig. 1.8). These endocytosed proteins are fragmented by proteolytic enzymes within the endosomal/lysosomal compartment. The resulting peptides are loaded into class II MHC that traffic through this compartment. Peptide-loaded class II molecules are then expressed on the surface of the APC where they are available to be bound by CD4 T cells that have TCRs capable of recognizing

the expressed cell surface peptide/MHC protein complex. Thus, CD4 T cells are specialized to largely react with antigens derived from extracellular sources.

In contrast, class I MHC molecules are mainly loaded with peptides derived from internally synthesized proteins, such as viral gene products. These peptides are produced from cytosolic proteins by proteolysis within the proteasome and are translocated into the rough endoplasmic reticulum. Such peptides, generally nine amino acids in length, are bound by class I MHC molecules. The complex is brought to the cell surface, where it can be recognized by CD8 T cells expressing appropriate receptors. This property gives the T-cell system, particularly CD8 T cells, the ability to detect cells expressing proteins that are different from, or produced in much larger amounts than, those of cells of the remainder of the organism (e.g., viral antigens [whether internal, envelope, or cell surface] or mutant antigens [such as active oncogene products]), even if these proteins, in their intact form, are neither expressed on the cell surface nor secreted.

Although this division of class I-binding peptides being derived from internally synthesized proteins and class II-binding peptides from imported proteins is generally correct, there are important exceptions to this rule that are central for the function of the immune system. The most effective priming of naive CD8 T cells occurs in response to peptide/MHC-I complexes expressed by DCs and yet many viruses do not infect these cells but rather target other cell types. Viral antigens produced by infected cells can be taken up by specialized DCs and loaded into class I molecules in a process referred to as cross-presentation.

T-Lymphocyte Receptors (Chapter 11)

The TCR is a disulfide-linked heterodimer (Fig. 1.9). Its constituent chains (α and β, or γ and δ) are Ig supergene family members. The TCR is associated with a set of transmembrane proteins, collectively designated the CD3 complex, that play a critical role in signal transduction. The CD3 complex consists of γ, δ (note that the CD3 γ and δ chains and the TCR γ and δ chains are distinct polypeptides that, unfortunately, have similar designations) and ε chains and is associated with a homodimer of two ζ chains or a heterodimer of ζ and ε chains. CD3 γ, δ, and ε consist of extracellular domains that are Ig supergene family members. The cytosolic domains of CD3 γ, δ, and ε and of ζ and ε contain one or more copies of the immunoreceptor tyrosine-based activation motif (ITAM) (D/ExxYxxLxxxxxxYxxL/I) that is found in a variety of chains associated with immune recognition receptors. This motif appears to be important in the signal transduction process and provides a site through which protein tyrosine kinases can interact with these chains to propagate signaling events.

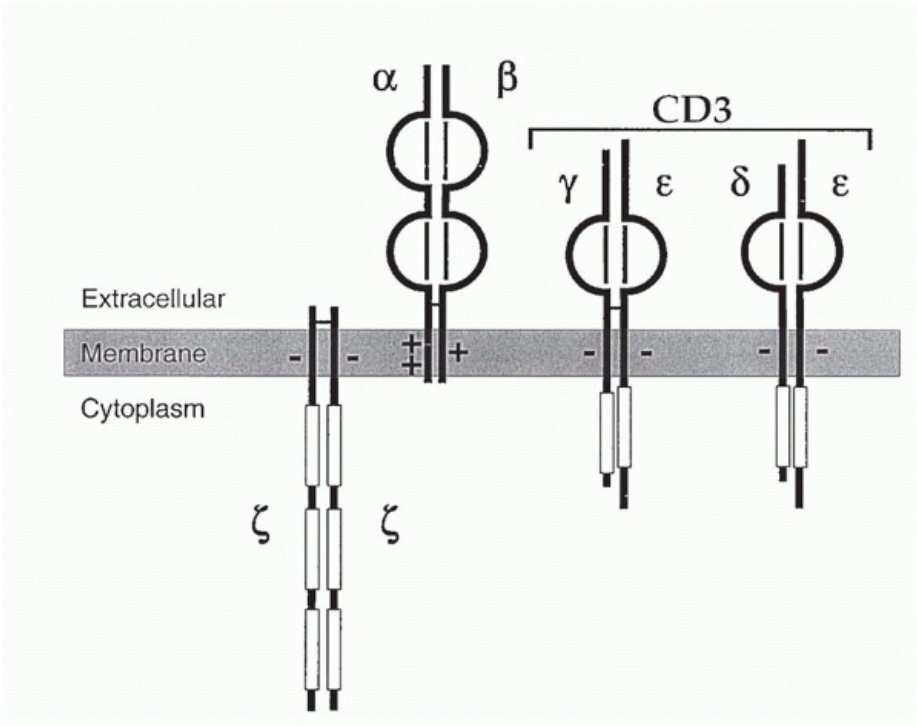

FIG. 1.9. The T-Cell Antigen Receptor. Illustrated schematically is the antigen binding subunit comprised of an αβ heterodimer and the associated invariant CD3 and ζ chains. Acidic (-) and basic (+) residues located within the plasma membrane are indicated. The *open rectangular boxes* indicate motifs within the cytoplasmic domains that interact with

protein tyrosine kinases.

The TCR chains are organized much like Ig chains. Their N-terminal portions are variable and their C-terminal portions are constant. Furthermore, similar recombinational mechanisms are used to assemble the V-region genes of the TCR chains. Thus, the V region of the TCR β chain is encoded by a gene constructed from three distinct genetic elements (*Vβ*, *D*, and *Jβ*) that are separated in the germline. Although the relative numbers of *Vβ*, *D*, and *Jβ* genes differ from that for the comparable Ig H variable region elements, the strategies for creation of a very large number of distinct genes by combinatorial assembly are the same. Both junctional diversity and N-region addition further diversify the genes and their encoded products. TCR β has fewer *V* genes than IgH but much more diversity centered on the *D/J* region, which encodes the equivalent of the third CDR of Igs. The α chain follows similar principles, except that it does not use a *D* gene.

The genes for TCR γ and δ chains are assembled in a similar manner except that they have many fewer V genes from which to choose. Indeed, γ/δ T cells in certain environments, such as the skin and specific mucosal surfaces, are exceptionally homogeneous. It has been suggested that the TCRs encoded by these essentially invariant γ and δ chains may be specific for some antigen that signals microbial invasion and that activation of γ/δ T cells through this mechanism constitutes an initial response that aids the development of the more sophisticated response of α/β T cells.

T-Lymphocyte Activation (Chapter 12)

T-cell activation is dependent on the interaction of the TCR/CD3 complex with its cognate ligand, a peptide bound in the groove of a class I or class II MHC molecule, on the surface of a competent APC. Through the use of chimeric cell surface molecules that possess cytosolic domains largely limited to the ITAM signaling motif alluded to previously, it is clear that cross-linkage of molecules containing such domains can generate some of the signals that result from TCR engagement. Nonetheless, the molecular events set in motion by receptor engagement are complex ones. Among the earliest steps is the activation of tyrosine kinases leading to the tyrosine phosphorylation of a set of substrates that control

several signaling pathways. Current evidence indicates that early events in this process involve the src family tyrosine kinases $p56^{lck}$, associated with the cytosolic domains of the CD4 and CD8 coreceptors, and $p59^{fyn}$, and ZAP-70, a Syk family tyrosine kinase, that binds to the phosphorylated ITAMs of the ζ chain. The protein tyrosine phosphatase CD45, found on the surface of all T cells, also plays a critical role in T-cell activation.

A series of important substrates are tyrosine phosphorylated as a result of the action of the kinases associated with the TCR complex. These include a 1) set of adapter proteins that link the TCR to the Ras pathway; 2) phospholipase Cγ1, the tyrosine phosphorylation of which increases its catalytic activity and engages the inositol phospholipid metabolic pathway, leading to elevation of intracellular free calcium concentration and activation protein kinase C; and 3) a series of other important enzymes that control cellular growth and differentiation. Particularly important is the phosphorylation of LAT, a molecule that acts as an organizing scaffold to which a series of signaling intermediates bind and upon which they

become activated and control downstream signaling.

The recognition and early activation events result in the reorganization of cell surface and cytosolic molecules on the T cell, and correspondingly, on the APC to produce a structure, the *immunological synapse*. The apposition of key interacting molecules involving a small segment of the membranes of the two cells concentrates these interacting molecules in a manner that both strengthens the interaction between the cells and intensifies the signaling events. It also creates a limited space into which cytokines may be secreted to influence the behavior of the interacting cells. The formation of the immunological synapse is one mechanism through which the recognition of relatively small numbers of ligands by TCRs on a specific T cell can be converted into a vigorous stimulatory process.

In general, normal T cells and cloned T-cell lines that are stimulated only by TCR cross-linkage fail to give complete responses. TCR engagement by itself may often lead to a response in which the key T-cell-derived growth factor, IL-2, is not produced and in which the cells enter a state of anergy such that they are unresponsive or poorly responsive to a subsequent competent stimulus (see Chapter 32). Full responsiveness of a T cell requires, in addition to receptor engagement, an accessory cell-delivered costimulatory signal. The engagement of CD28 on the T cell by CD80 and/or CD86 on the APC (or the engagement of comparable ligand/receptor pairs on the two cells) provides potent costimulatory activity. Inhibitors of this interaction markedly diminish antigen-specific T-cell activation in vivo and in vitro, indicating that the CD80/86-CD28 interaction is physiologically important in T-cell activation (see Chapters 12 and 14).

The interaction of CD80/86 with CD28 increases cytokine production by the responding T cells. For the production of IL-2, this increase appears to be mediated both by enhancing the transcription of the IL-2 gene and by stabilizing IL-2 mRNA. These dual consequences of the CD80/86-CD28 interaction cause a striking increase in the production of IL-2 by antigen-stimulated T cells.

CD80/86 has a second receptor on the T cell, CTLA-4, that is expressed later in the course of T-cell activation. The bulk of evidence indicates that the engagement of CTLA-4 by CD80/86 leads to a set of biochemical signals that terminate the T-cell response. Mice that are deficient in CTLA-4 expression develop fulminant autoimmune responses and anti-CTLA-4 antibodies are used as drugs to enhance antitumor immune responses.

T Lymphocyte Development (Chapter 13)

Upon entry into the thymus, T-cell precursors do not express TCR chains, the CD3 complex, or the CD4 or CD8 molecules (Fig. 1.10). Because these cells lack both CD4 and CD8, they are often referred to as double-negative cells. Thymocytes develop from this double-negative pool into cells that are both CD4+ and CD8+ (double-positive cells) and express low levels of TCR and CD3 on their surface. In turn, double-positive cells further differentiate into relatively mature thymocytes that express either CD4 or CD8 (single-positive cells) and high levels of the TCR/CD3 complex.

The expression of the TCR depends on complex rearrangement processes that generate TCR α and β (or γ and

δ) chains. Once TCR chains are expressed, these cells undergo two important selection processes within the thymus. One, termed *negative selection*, is the deletion of cells that

express receptors that bind with high affinity to complexes of self-peptides with self-MHC molecules. This is a major mechanism through which the T-cell compartment develops immunologic unresponsiveness to self antigens (see Chapters 13 and 32). In addition, a second major selection process is *positive selection*, in which T cells with receptors with "intermediate affinity" for self-peptides bound to self-MHC molecules are selected, thus forming the basis of the T-cell repertoire for foreign peptides associated with self-MHC molecules. T cells that are not positively selected are eliminated in the thymic cortex by apoptosis. Similarly, T cells that are negatively selected as a result of high-affinity binding to self-peptide/self-MHC complexes are also deleted through apoptotic death. These two selection processes result in the development of a population of T cells that are biased toward the recognition of peptides in association with self-MHC molecules from which those cells that are potentially autoreactive (capable of high-affinity binding of selfpeptide/self-MHC complexes) have been purged.

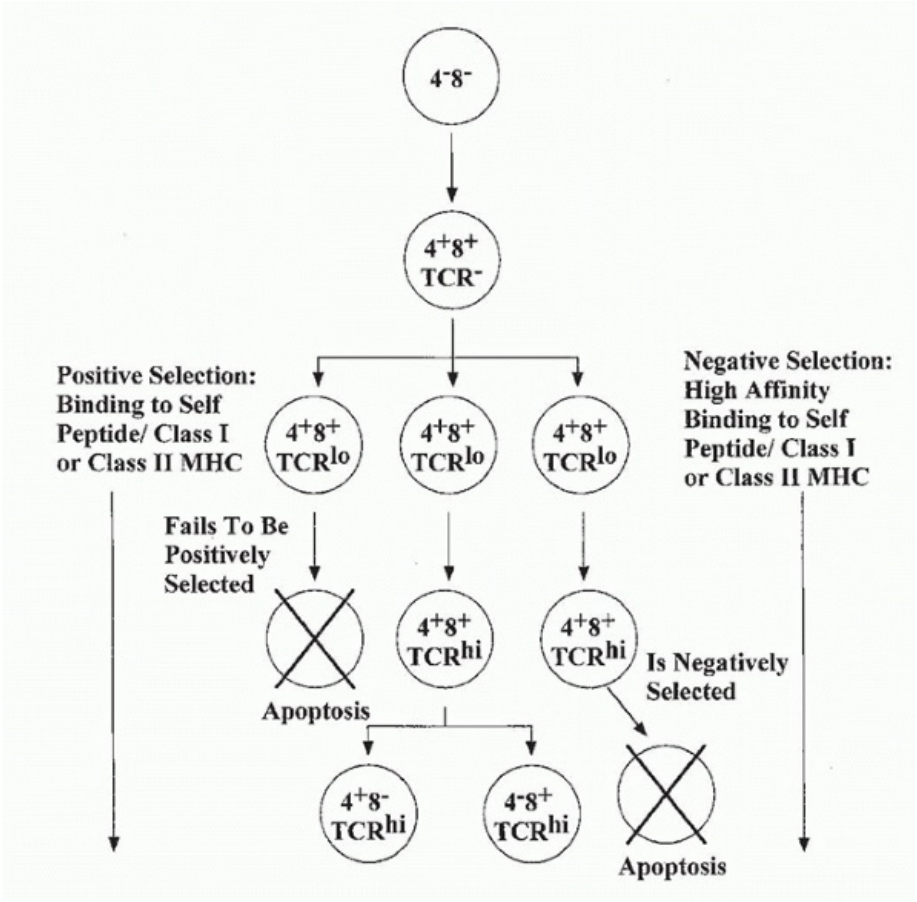

FIG. 1.10. Development of α/β T Cells in the Thymus. Doublenegative T cells (4^-8^-) acquire CD4 and CD8 (4^+8^+) and then express α/β TCRs, initially at low levels. Thereafter, the degree of expression of T-cell receptiors increases and the cells differentiate into CD4 or CD8 cells and are then exported to the periphery. Once the T cells have expressed receptors, their survival depends upon the recognition of peptide/major histocompatibility complex (MHC) class I or class II molecules with an affinity above some given threshold. Cells that fail to do so undergo apoptosis. These cells have failed to be *positively selected*. Positive selection is associated with the

differentiation of 4^+8^+ cells into CD4 or CD8 cells. Positive selection involving peptide/class I MHC molecules leads to the development of CD8 cells whereas positive selection involving peptide/class II MHC molecules leads to the development of CD4 cells. If a T cell recognizes a peptide/MHC complex with high affinity, it is also eliminated via apoptosis (it is *negatively selected*).

One important event in the development of T cells is their differentiation from double-positive cells into CD4+ or CD8+ single-positive cells. This process involves the interaction of double-positive thymocytes with peptide bound to class II or class I MHC molecules on accessory cells. Indeed, CD4 binds to monomorphic sites on class II molecules, whereas CD8 binds to comparable sites on class I molecules. The capacity of the TCR and CD4 (or of the TCR and CD8) to bind to a class II MHC (or a class I MHC) molecule on an accessory cell leads either to the differentiation of doublepositive thymocytes into CD4+ (or CD8+) single-positive T cells or to the selection of cells that have "stochastically" differentiated down the CD4 (or CD8) pathway.

Less is understood about the differentiation of thymocytes that express TCRs composed of γ/δ chains. These cells fail to express either CD4 or CD8. However, γ/δ cells are relatively numerous early in fetal life; this, together with their limited degree of heterogeneity, suggests that they may comprise a relatively primitive T-cell compartment.

T-Lymphocyte Functions (Chapters 14, 29, and 33)

T cells mediate a wide range of immunologic functions. These include the capacity to help B cells develop into antibody-producing cells, the capacity to increase the microbicidal action of monocytes/macrophages, the inhibition of certain types of immune responses, direct killing of target cells, and mobilization of the inflammatory response. In general, these effects depend on their expression of specific cellsurface molecules and the secretion of cytokines.

T Cells that Help Antibody Responses (Chapter 13)

Helper T cells, T_{FH} cells, can stimulate B cells to make antibody responses to proteins and other T-cell-dependent antigens. T-cell-dependent antigens are immunogens in which individual epitopes appear only once or only a limited number of times so that they are unable to cross-link the membrane Ig of B cells or do so inefficiently. B cells bind the antigen through their membrane Ig, and the complex undergoes endocytosis. Within the endosomal and lysosomal compartments, the antigen is fragmented into peptides by proteolytic enzymes and one or more of the generated peptides are loaded into class II MHC molecules, which traffic through this vesicular compartment. The resulting complex of class II MHC molecule and bound peptide is exported to the B-cell surface membrane. T cells with receptors specific for the peptide/class II molecular complex recognize that complex on the B cell.

B-cell activation depends not only on the binding of peptide/class II MHC complexes on the B cell surface by the TCR but also on the interaction of T-cell CD154 with CD40 on the B cell. T cells do not constitutively express CD154; rather, it is induced as a result of an interaction with an activated APC that expresses a cognate antigen recognized by the TCR of the T cell. Furthermore, CD80/86 are generally expressed by activated but not resting B cells so that interactions involving resting B cells and naïve T cells generally do not lead to efficient antibody production. By contrast, a T cell already activated and expressing CD154 can

interact with a resting B cell, leading to its upregulation of CD80/86 and to a more productive T-cell/B-cell interaction with the delivery of cognate help and the development of the B cell into an antibody-producing cell. Similarly, activated B cells expressing large amounts of class II molecules and CD80/86 can act as effective APCs and can participate with T cells in efficient cognate help interactions. Cross-linkage of membrane Ig on the B cell, even if inefficient, may synergize with the CD154/CD40 interaction to yield vigorous B-cell activation.

The subsequent events in the B-cell response program, including proliferation, Ig secretion, and class switching, either depend on or are enhanced by the actions of T-cell-derived cytokines. Thus, B-cell proliferation and Ig secretion are enhanced by the actions of several type I cytokines including IL-2 and IL-4. Ig class switching is dependent both on the initiation of competence for switching, which can be induced by the CD154/CD40 interaction, and on the targeting of particular C regions for switching, which is determined, in many instances, by cytokines. The best studied example of this is the role of IL-4 in determining switching to IgG1 and IgE in the mouse and to IgG4 and IgE in the human. Indeed, the central role of IL-4 in the production of IgE is demonstrated by the fact that mice that lack the IL-4 gene or the gene for the IL-4 receptor α chain, as a result of gene knockouts, have a marked defect in IgE production. Similarly, IFN-γ determines switching to IgG_{2a} in the mouse. The relationship between T_{FH} cells that produce IL-4 and T_{H2} cells and those that produce IFN-γ and T_{H1} cells is still uncertain.

Induction of Cellular Immunity (Chapters 14 and 19)

T cells also may act to enhance the capacity of monocytes and macrophages to destroy intracellular microorganisms. In particular, IFN-γ enhances several mechanisms through which mononuclear phagocytes destroy intracellular bacteria and parasites, including the generation of nitric oxide and induction of TNF. T_{H1} cells are particularly effective in

enhancing microbicidal action because they produce IFN-γ. By contrast, three of the major cytokines produced by T_{H2} cells, IL-4, IL-13, and IL-10, block these activities; IL-4 and IL-13 induce an alternative gene activation program in macrophages resulting in *alternatively activated macrophages*, characterized (in the mouse) by the expression of arginase 1 and chitinase. Thus, T_{H2} cells often oppose the action of T_{H1} cells in inducing cellular immunity and in certain infections with microorganisms that are intracellular pathogens of macrophages; a T_{H2}-dominated response may be associated with failure to control the infection.

Regulatory T Cells (Chapter 33)

There has been a longstanding interest in the capacity of T cells to diminish as well as to help immune responses. Cells that mediate such effects are referred to as T_{regs}. T_{regs} may be identified by their expression of Foxp3 and of CD25, the IL-2 receptor alpha chain. These cells inhibit the capacity of both CD4 and CD8 T cells to respond to their cognate antigens. The mechanisms through which their suppressor function is mediated are still somewhat controversial. In some instances, it appears that cell/cell contact is essential for suppression, whereas in other circumstances production of cytokines by T_{regs} has been implicated in their ability to inhibit responses. Evidence has been presented for both IL-10 and TGF-β as mediators of inhibition.

T_{regs} have been particularly studied in the context of various autoimmune conditions. In the absence of T_{regs}, conventional T cells cause several types of autoimmune responses, including autoimmune gastritis and inflammatory bowel disease. T_{regs} express cell surface receptors allowing them to recognize autoantigens; their responses to such recognition results in the suppression of responses by conventional T cells. Whether the receptor repertoire of T_{regs} and the conventional T cells are the same has not been fully determined, although there is increasing evidence that T_{regs} derive from a thymic CD4 T-cell population with relatively high affinity for selfantigen. As noted previously, iT_{regs} can be derived in the periphery from naive CD4 T-cell populations. This is seen when naive cells are stimulated by their cognate ligands in the presence of TGF-β and IL-2.

Cytotoxic T Cells (Chapter 37)

One of the most striking actions of T cells is the lysis of cells expressing specific antigens. Most cells with such cytotoxic activity are CD8 T cells that recognize peptides derived from proteins produced within the target cell and bound to class I MHC molecules expressed on the surface of the target cell. However, CD4 T cells can express CTL activity, although in such cases the antigen recognized is a peptide associated with a class II MHC molecule; often, such peptides derive from exogenous antigens.

There are two major mechanisms of cytotoxicity. One involves the production by the CTL of perforin, a molecule that can insert into the membrane of target cells and promote the lysis of that cell. Perforin-mediated lysis is mediated a series of enzymes produced by activated CTLs, referred to as granzymes. Many active CTLs also express large amounts of fas ligand on their surface. The interaction of fas ligand on the surface of the CTL with fas on the surface of the target cell initiates apoptosis in the target cell.

CTL-mediated lysis is a major mechanism for the destruction of virus-infected cells. If activated during the period in which the virus is in its eclipse phase, CTLs may be capable of eliminating virus and curing the host with relatively limited cell destruction. On the other hand, vigorous CTL activity after a virus has been widely disseminated may lead to substantial tissue injury because of the large number of cells that are killed by the action of the CTLs. Thus, in many infections, the disease is caused by the destruction of tissue by CTLs rather than by the virus itself. One example is hepatitis B, in which much of the liver damage represents the attack of hepatitis B virus-specific CTLs on infected liver cells.

It is usually observed that CTLs that have been induced as a result of a viral infection or intentional immunization must be reactivated in vitro through the recognition of antigen on the target cell. This is particularly true if some interval has elapsed between the time of infection or immunization and the time of test. This has led to some question being raised as to the importance of CTL immunity in protection against re-infection and how important CTL generation is in the long-term immunity induced by protective vaccines. On the other hand, in active infections, such as seen in individuals with HIV, CTL that can kill their target cells immediately are often seen. There is much evidence to suggest that these cells play an active role in controlling the number of HIV-positive T cells.

CYTOKINES (CHAPTERS 25, 26, 27, 28)

Many of the functions of cells of the immune system are mediated through the production of

a set of small proteins referred to as cytokines. These proteins can now be divided into several families. They include the type I cytokines or hematopoeitins that encompass many of the interleukins (i.e., IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, IL-13, IL-15, IL-21, IL-23, and IL-27), as well as several hematopoietic growth factors; the type II cytokines, including the interferons and IL-10; the TNF-related molecules, including TNF, lymphotoxin, and Fas ligand; Ig superfamily members, including IL-1, IL-18, IL-33, IL-36, and IL-37; and the chemokines, a large family of molecules playing critical roles in a wide variety of immune and inflammatory functions. IL-17 and its congeners, including IL-25, constitute a structurally unique set of cytokines.

Many of the cytokines are T-cell products; their production represents one of the means through which the wide variety of functions of T cells are mediated. Most cytokines are not constitutive products of the T cell. Rather, they are produced in response to T-cell activation, usually resulting from presentation of antigen to T cells by APCs in concert with the action of a costimulatory molecule, such as the interaction of CD80/86 with CD28. Although cytokines are produced in small quantities, they are potent, binding to their receptors with equilibrium constants of approximately 10^{10} M^{-1}. In some instances, cytokines are directionally secreted into the immunological synapse formed between a T cell and an APC. In such cases, the cytokine acts in a paracrine manner. Indeed, many cytokines

have limited action at a distance from the cell that produced them. This appears to be particularly true of many of the type I cytokines. However, other cytokines act by diffusion through extracellular fluids and blood to target cells that are distant from the producers. Among these are cytokines that have proinflammatory effects, such as IL-1, IL-6, and TNF, and the chemokines, that play important roles in regulating the migration of lymphocytes and other cell types.

Chemokines (Chapter 28)

A large family of small proteins that are *chemotactic cytokines* (chemokines) have been described. While members of this family have a variety of functions, perhaps the most dramatic is their capacity to regulate leukocyte migration and thus to act as critical dynamic organizers of cell distribution in the immune and inflammatory responses. The receptors for chemokines are seven transmembrane-spanning, G-protein coupled receptors.

The chemokines are subdivided based on the number and positioning of their highly conserved cysteines. Among chemokines with four conserved cysteines, the cysteines are adjacent in one large group (the CC chemokines), whereas in a second large group they are separated by one amino acid (CXC chemokines). There are also rare chemokines in which the cysteines are separated by three amino acids (CX3C) or in which there are only two conserved cysteines (C chemokines).

Individual chemokines may signal through more than one chemokine receptor, and individual receptors may interact with more than one chemokine, producing a complex set of chemokine/chemokine receptor pairs and providing opportunities for exceedingly fine regulation of cellular functions.

THE MAJOR HISTOCOMPATIBILITY COMPLEX AND ANTIGEN PRESENTATION (CHAPTERS 21 AND 22)

The MHC has already been introduced in this chapter in the discussion of T-cell recognition of antigen-derived peptides bound to specialized grooves in class I and class II MHC proteins. Indeed, the class I and class II MHC molecules are essential to the process of T-cell recognition and response. Nonetheless, they were first recognized not for this reason but because of the dominant role that MHC class I and class II proteins play in transplantation immunity (see Chapter 46).

When the genetic basis of transplantation rejection between mice of distinct inbred strains was sought, it was recognized that although multiple genetic regions contributed to the rejection process, one region played a dominant role. Differences at this region alone would cause prompt graft rejection, whereas any other individual difference usually resulted in a slow rejection of foreign tissue. For this reason, the genetic region responsible for prompt graft rejection was termed the *major* histocompatibility complex.

In all higher vertebrates that have been thoroughly studied, a comparable MHC exists. The defining features of the MHC are the transplantation antigens that it encodes. These are the class I and class II MHC molecules. The genes encoding these molecules show an unprecedented degree of polymorphism. This, together with their critical role in antigen presentation, explains their central role as the target of the immune responses leading to the rejection of organ and tissue allografts.

The MHC also includes other genes, particularly genes for certain complement components. In addition, genes for the cytokines TNF-α and lymphotoxin (also designated TNF-β) are found in the MHC.

Class I MHC Molecules (Chapter 21)

Class I MHC molecules are membrane glycoproteins expressed on most cells. They consist of an α chain of approximately 45,000 daltons noncovalently associated with α2-microglobulin, a 12,000-dalton molecule (Fig. 1.11). The gene for the α chain is encoded in the MHC, whereas that for β2-microglobulin is not. Both the α chain and β2-microglobulin are Ig supergene family members. The α chain is highly polymorphic, with the polymorphisms found mainly in the regions that constitute the binding sites for antigenderived peptides and that are contact sites for the TCR.

The class I α chain consists of three extracellular regions or domains, each of similar length, designated α1, α2, and α3. In addition, α chains have a membrane-spanning domain and a short carboxy-terminal cytoplasmic tail. The

crystal structure of class I molecules indicates that the α1 and α2 domains form a site for the binding of peptides derived from antigens. This site is defined by a floor consisting of β sheets and bounded by α-helical walls. The polymorphisms of the class I molecule are mainly in these areas.

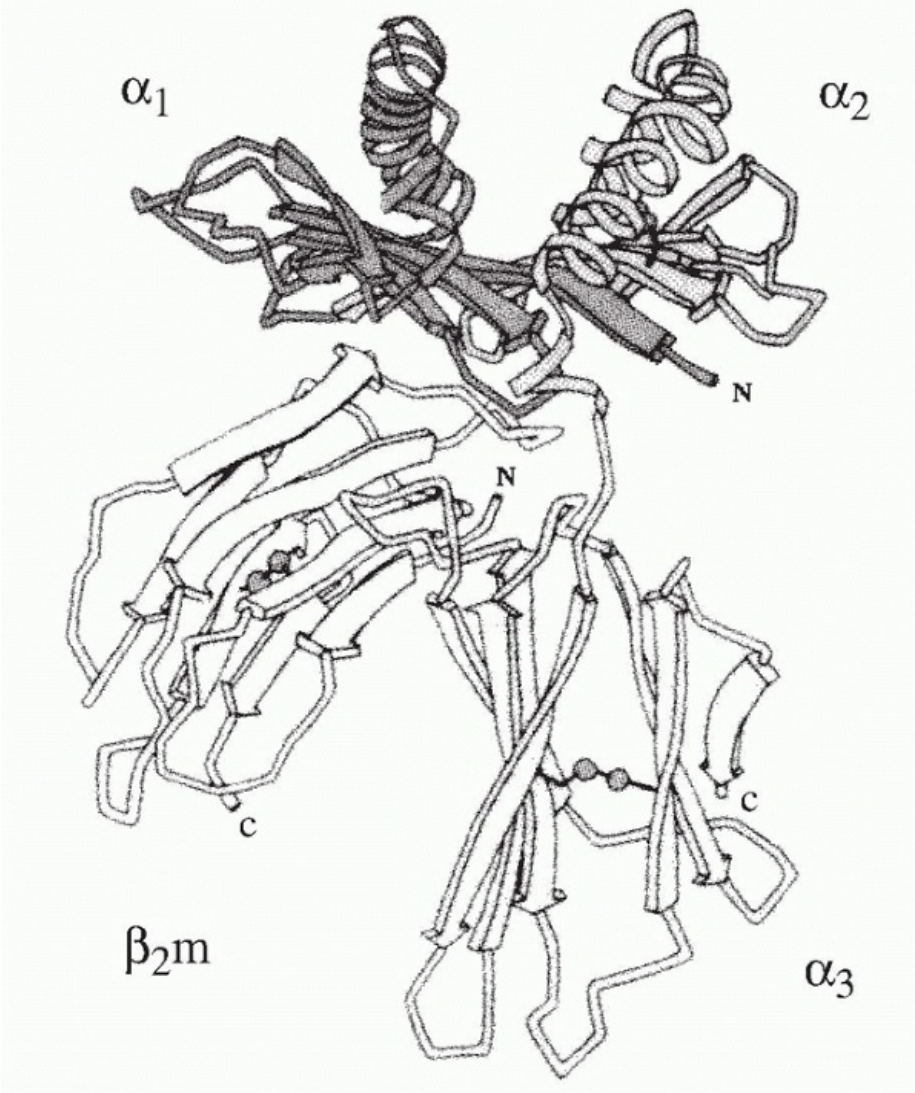

FIG. 1.11. Model of the Class I HLA-A2 Molecule. A schematic representation of the structure of the HLA-A2 class I major histocom-patibility complex (MHC) molecule. The polymorphic α1 and α2 domains are at the top. They form a groove into which antigen-derived peptides fit to form the peptide/MHC class I complex that is recognized by T-cell receptors of CD8 T cells. (Reprinted from Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. Structure of the human class II histocompatibility antigen, HLA-A2. *Nature*. 1987;329:506-512).

In the human, three loci encoding classical class I molecules have been defined; these are designated human leukocyte antigen (HLA)-A, HLA-B, and HLA-C. All display high degrees of polymorphism. A similar situation exists in the mouse. In addition, there are a series of genes that encode class I-like molecules (class Ib molecules). Some of these have been shown to have antigen-presenting activity for formylated peptides, suggesting that they may be specialized to present certain prokaryotic antigens. The class Ib molecule CD1 has been shown to have antigen-presenting function for mycobacterial lipids, providing a mechanism through which T cells specific for such molecules can be generated. CD1d, presenting certain endogenous or exogenous phospholipids, is recognized by a novel class of T cells (NK T cells) that produce large amounts of cytokines upon immediate stimulation.

Class II MHC Molecules (Chapter 21)

Class II MHC molecules are heterodimeric membrane glycoproteins. Their constituent chains are designated α and β; both chains are immunoglobulin supergene family members, and both are encoded within the MHC. Each chain consists of two extracellular domains (α1 and α2; β1 and β2, respectively), a hydrophobic domain, and a short cytoplasmic segment. The overall conformation of class II MHC molecules appears to be quite similar to that of class I molecules. The peptide-binding site of the class II molecules is contributed to by the α1 and β1 domains (see Fig. 1.5); it is within these domains that the majority of the polymorphic residues of class II molecules are found.

A comparison of the three-dimensional structures of class I and class II molecules indicates certain distinctive features that explain differences in the length of peptides that the two types of MHC molecules can bind. Class I molecules generally bind peptides with a mean length of nine amino acids, whereas class II molecules can bind substantially larger peptides.

In the mouse, class II MHC molecules are encoded by genes within the I region of the MHC. These molecules are often referred to as I region-associated (Ia) antigens. Two sets of class II molecules exist, designated I-A and I-E, respectively. The α and β chains of the I-A molecules (Aα and Aβ) pair with one another, as do the α and β chains of I-E (Eα and Eβ).

In the human, there are three major sets of class II molecules, encoded in the DR, DQ, and DP regions of the HLA complex.

Class II molecules have a more restricted tissue distribution than class I molecules. Class II molecules are found on B cells, DCs, epidermal Langerhans cells, macrophages, thymic epithelial cells, and, in the human, activated T cells. Levels of class II molecule expression are regulated in many cell types by interferons and in B cells by IL-4. Indeed, interferons can cause expression of class II molecules on many cell types that normally lack these cell surface molecules. Interferons also can cause striking upregulation in the expression of class I MHC molecules. Thus, immunologically mediated inflammation may result in aberrant expression of class II MHC molecules and heightened expression of class I molecules. Such altered expression of MHC molecules can allow cells that do not normally function as APCs for CD4 T cells to do so and enhances the sensitivity of such cells to CD8 T cells. This has important consequences for immunopathologic responses and for autoimmunity.

Antigen Presentation (Chapter 22)

As already discussed, the function of class I and class II MHC molecules is to bind and present antigen-derived peptides to T cells whose receptors can recognize the peptide/MHC complex that is generated. There are two major types of antigen-processing pathways, specialized to deal with distinct classes of pathogens that the T cell system must confront (see Fig. 1.8).

Extracellular bacteria and extracellular proteins may enter APCs by endocytosis or phagocytosis. Their antigens and the antigens of bacteria that live within endosomes or lysosomes are fragmented in these organelles, and peptides derived from the antigen are loaded into class II MHC molecules as these proteins traverse the vesicular compartments in which the peptides are found. The loading of peptide is important in stabilizing the structure of the class II MHC molecule. The acidic pH of the compartments in which loading occurs facilitates the loading process. Once the peptide-loaded class II molecules reaches neutral

pH, such as at the cell surface, the peptide/MHC complex is stable. Peptide dissociation from such class II molecules is very slow, with a half-time measured in hours. The peptide/class II complex is recognized by those CD4 T cells that have complementary receptors. As already pointed out, the specialization of CD4 T cells to recognize peptide/class II complexes is partly due to the affinity of the CD4 molecule for monomorphic determinants on class II molecules. Obviously, this form of antigen processing can only apply to cells that express class II MHC molecules. Indeed, APCs for CD4 T cells principally include cells that normally express class II MHC molecules, namely DCs, B cells, and macrophages.

T cells also can recognize proteins that are produced within the cell that presents the antigen. The major pathogens recognized by this means are viruses and other obligate intracellular (nonendosomal/nonlysosomal) microbes that have infected cells. In addition, proteins that are unique to tumors, such as mutant oncogenes, or are overexpressed in tumors also can be recognized by T cells. Endogenously produced proteins are fragmented in the cytosol by proteases in the proteasome. The resultant peptides are transported into the rough endoplasmic reticulum through the action of a specialized transport system. These peptides are then available for loading into class I molecules. In contrast to the loading of class II molecules, which is facilitated by the acid pH of the loading environment, the loading of class I molecules is controlled by interaction of the class I α chain

with β2-microglobulin. Thus, the bond between peptide and class I molecule is generally weak in the absence of β2-microglobulin, and the binding of β2-microglobulin strikingly stabilizes the complex. (Similarly, the binding of β2-microglobulin to the α chain is markedly enhanced by the presence of peptide in the α chain groove.) The peptide-loaded class I molecule is then brought to the cell surface. In contrast to peptide-loaded class II molecules that are recognized by CD4 T cells, peptide-loaded class I molecules are recognized by CD8 T cells. This form of antigen processing and presentation can be performed by virtually all cells because, with a few exceptions, class I MHC molecules are universally expressed.

Although the specialization of class I molecules to bind and present endogenously produced peptides and of class II molecules to bind and present peptides derived from exogenous antigens is generally correct, there are exceptions, many of which have physiologic importance. Particularly important is the capacity of some DCs to load peptides from exogenous antigens into class I MHC molecules, allowing sensitization of CD8 T cells to the antigens of pathogens that infect cells other than DCs.

T-Lymphocyte Recognition of Peptide/MHC Complexes Results in MHC-Restricted Recognition (Chapter 11)

Before the biochemical nature of the interaction between antigen-derived peptides and MHC molecules was recognized, it was observed that T-cell responses displayed *MHC-restricted antigen recognition*. Thus, if individual animals were primed to a given antigen, their T cells would be able to recognize and respond to that antigen only if the APCs that presented the antigen shared MHC molecules with the animal that had been immunized. The antigen would not be recognized when presented by APCs of an allogeneic MHC type. This can now be explained by the fact that the TCR recognizes peptide bound to an MHC molecule. MHC molecules display high degrees of polymorphism, and this polymorphism is concentrated in the regions of the class I and class II molecules that interact with the peptide and that can bind to the TCR. Differences in structure of the MHC molecules derived from different

individuals (or different inbred strains of mice) profoundly affect the recognition process. Two obvious explanations exist to account for this. First, the structure of the grooves in different class I or class II MHC molecules may determine that a different range of peptides are bound or, even if the same peptide is bound, may change the conformation of the surface of the peptide presented to the TCR. Second, polymorphic sites on the walls of the α-helices that are exposed to the TCR can either enhance or diminish binding of the whole complex, depending on their structure. Thus, priming an individual with a given antigen on APCs that are syngeneic to the individual will elicit a response by T cells whose TCRs are specific for a complex consisting of a peptide derived from the antigen and the exposed polymorphic residues of the MHC molecule. When the same antigen is used with APCs of different MHC type, it is unlikely that the same peptide/MHC surface can be formed, and thus the primed T cells are not likely to bind and respond to such stimulation.

Indeed, this process also occurs within the thymus in the generation of the T-cell repertoire, as already discussed. T cells developing within the thymus undergo a positive selection event in which those T cells capable of recognizing MHC molecules displayed within the thymus are selected (and the remainder undergo programmed cell death). This leads to the skewing of the population of T cells that emerges from the thymus so that the cells are specialized to respond to peptides on self-MHC molecules. One of the unsolved enigmas of positive selection within the thymus is how the vast array of T cells with receptors capable of reacting with a very large set of foreign peptides associated with self-MHC molecules are chosen by self-MHC molecules that can only display self-peptides. It is believed that a high degree of cross-reactivity may exist so that T cells selected to bind a given class I (or class II) molecule plus a particular self-peptide can also bind a set of other (foreign) peptides bound to the same MHC molecule.

Furthermore, the affinity of an interaction required for positive selection in the thymus appears to be considerably lower that that required for full activation of peripheral T cells. Thus, thymocytes selected by a given self-peptide/self-MHC complex will generally not mount a full response when they encounter the same peptide/MHC complex in the periphery, although they will respond to a set of foreign peptide/MHC complexes to which they bind with higher affinity. Recognition of the self-peptide/self-MHC complex in the periphery nonetheless is important in sustaining the viability of resting T-lymphocytes.

Our modern understanding of T-cell recognition also aids in explaining the phenomenon of immune response gene control of specific responses. In many situations, the capacity to recognize simple antigens can be found in only some members of a species. In most such cases, the genes that determine the capacity to make these responses have been mapped to the MHC. Such immune response gene control of immune responses is based on the capacity of different class II MHC molecules (or class I MHC molecules) to bind different sets of peptides. Thus, for simple molecules, it is likely that peptides can be generated that are only capable of binding to some of the polymorphic MHC molecules of the species. Only individuals that possess those allelic forms of the MHC will be able to respond to those antigens. Based on this, some individuals are nonresponders because of the failure to generate a peptide/MHC molecule complex that can be recognized by the T-cell system.

This mechanism also may explain the linkage of MHC type with susceptibility to various diseases. Many diseases show a greater incidence in individuals of a given MHC type. These include reactive arthritides, gluten-sensitive enteropathy, insulin-dependent diabetes

mellitus, and rheumatoid arthritis (see Chapter 44). One explanation is that the MHC type that is associated with increased incidence may convey altered responsiveness to antigens of agents that cause or exacerbate the disease. Indeed, it appears that many of these diseases are due to enhanced or inappropriate immune responses.

Antigen-Presenting Cells (Chapter 16)

T cells recognize peptide/MHC complexes on the surface of other cells. Such cells are often referred to as APCs. Although

effector cells can mediate their functions by recognizing such complexes on virtually any cell type, naïve cells are most efficiently activated by a set of specialized APC, the DCs. DCs are a multimember family with distinctive locations and functions. Among them are the plasmacytoid DCs that are the principal source of type I interferons in viral infections.

In general, in their immature form, DCs are resident in the tissues where they are efficient at capturing and endocytosing antigen. Their antigen capture activity is dependent upon expression of several surface receptors including Fc receptors, receptors for heat shock proteins, and C-type lectins. If they receive signals, such as various inflammatory stimuli, often mediated by TLRs, they downregulate the expression of these molecules but increase their expression of surface MHC molecules and various costimualtory molecules such as CD80/86. In addition, such stimulation induces expression of chemokine receptors such as CCR2 and CCR7. The latter allow stimulated DCs to follow signals from the chemokines SLC and ELC, and to migrate into the T-cell zone of lymph nodes. As part of the maturation process, they may also acquire the capacity to produce cytokines and express surface molecules that can aid in determining the polarization of T-cell priming. This includes the production of IL-12, IL-23, IL-6, and IL-10, and the expression of inducible costimulator ligand and of Notch ligands. Interaction of naïve T cells with immature DCs may induce a state of peripheral tolerance.

EFFECTOR MECHANISMS OF IMMUNITY

The ultimate purpose of the immune system is to mount responses that protect the individual against infections with pathogenic microorganisms by eliminating these microbes or, where it is not possible to eliminate infection, to control their spread and virulence. In addition, the immune system may play an important role in the control of the development and spread of some malignant tumors. The responses that actually cause the destruction of the agents that initiate these pathogenic states (e.g., bacteria, viruses, parasites, tumor cells) are collectively the effector mechanisms of the immune system. Several have already been alluded to. Among them are the cytotoxic action of CTLs, which leads to the destruction of cells harboring viruses and, in some circumstances, expressing tumor antigens. In some cases, antibody can be directly protective by neutralizing determinants essential to a critical step through which the pathogen establishes or spreads an infectious process. However, in most cases, the immune system mobilizes powerful nonspecific mechanisms to mediate its effector function.

Effector Cells of the Immune Response

Among the cells that mediate important functions in the immune system are cells of the monocyte/macrophage lineage, NK cells, mast cells, basophils, eosinophils, and neutrophils.

It is beyond the scope of this introductory chapter to present an extended discussion of each of these important cell types. However, a brief mention of some of their actions will help in understanding their critical functions in the immune response.

Monocytes and Macrophages (Chapter 19)

Cells of the monocyte/macrophage lineage play a central role in immunity. One of the key goals of cellular immunity is to aid the macrophages in eliminating organisms that have established intracellular infections. In general, non-activated macrophages are inefficient in destroying intracellular microbes. However, the production of IFN-γ and other mediators by T cells can enhance the capacity of macrophages to eliminate such microorganisms. Several mechanisms exist for this purpose, including the development of reactive forms of oxygen, the development of nitric oxide, and the induction of a series of proteolytic enzymes, as well as the induction of cytokine production. Macrophages can act as APCs and thus can enlist the "help" of activated, cytokineproducing CD4+ T cells in regulating their function.

Although macrophages function as APCs for attracting activated T cells, they do not appear to be particularly effective in the activation of naïve CD4 T cells. In instances in which they are the site of infection or have phagocytosed infectious agents or their proteins, antigens from these agents may be transferred to DCs. In such cases, the DCs would be the principal APCs that activate naïve or possibly resting memory CD4 T cells. Such activated T cells would then be available to help infected macrophages.

Natural Killer Cells (Chapter 17)

NK cells play an important role in the immune system. Indeed, in mice that lack mature T and B cells due to the *scid* mutation, the NK system appears to be highly active and to provide these animals a substantial measure of protection against infection. NK cells are closely related to T cells. They lack conventional TCR (or Ig) but express two classes of receptors. They have a set of activating receptors that allow them to recognize features associated with virally infected cells or tumor cells. They also express receptors for MHC molecules that shut off their lytic activity. Thus, virally infected cells or tumor cells that escape the surveillance of cytotoxic T cells by downregulating or shutting off expression of MHC molecules then become targets for efficient killing by NK cells because the cytotoxic activity of the latter cells is no longer shut off by the recognition of particular alleles of MHC class I molecules.

In addition, NK cells express a receptor for the Fc portion of IgG (FcγRIII). Antibody-coated cells can be recognized by NK cells, and such cells can then be lysed. This process is referred to as antibody-dependent cellular cytotoxicity.

NK cells are efficient producers of IFN-γ. A variety of stimuli, including recognition of virally infected cells and tumor cells, cross-linkage of FcγRIII, and stimulation by the cytokines IL-12 and IL-18, cause striking induction of IFN-γ production by NK cells.

Mast Cells and Basophils (Chapters 20 and 45)

Mast cells and basophils play important roles in the induction of allergic inflammatory responses. They express cell surface receptors for the Fc portions of IgE (FcεRI) and for certain classes of IgG (FcγR). This enables them to bind antibody to

their surfaces, and when antigens capable of reacting with that antibody are introduced, the

resultant cross-linkage of FcεRI and/or FcγR results in the prompt release of a series of potent mediators such as histamine, serotonin, and a variety of enzymes that play critical roles in initiating allergic and anaphylactic-type responses. In addition, such stimulation also causes these cells to produce a set of cytokines, including IL-3, IL-4, IL-13, IL-5, IL-6, granulocyte-macrophage colony-stimulating factor, and TNFα, that have important late consequences in allergic inflammatory responses.

Granulocytes (Chapter 20)

Granulocytes have critical roles to play in a wide range of inflammatory situations. Rather than attempting an extended discussion of these potent cells, it may be sufficient to say that in their absence it is exceedingly difficult to clear infections with extracellular bacteria and that the immune response plays an important role in orchestrating the growth, differentiation, and mobilization of these crucial cells. Recent work indicates that T_{H17} cells are particularly important because of their role in recruiting granulocytes to sites of immune responses.

Eosinophils (Chapters 20 and 45)

Eosinophils are bone marrow-derived myeloid cells that complete their late differentiation under the influence of IL-5. They migrate to tissue sites in response to the chemokine eotaxin and as a result of their adhesion receptors. Because T_{H2} cells can produce IL-5 and stimulate the production of eotaxin, eosinophil accumulation is often associated with T_{H2}-mediated inflammation. Eosinophils store a series of proteins in their secondary granules including major basic protein, eosinophil cationic protein, and eosionphil peroxidase. When released, these proteins are responsible for much of the damage that eosinophils mediate both to helminthic parasites and to the epithelium. Eosinophils have been implicated as important in protective responses to helminths and in the tissue damage seen in allergic inflammation in conditions such as asthma.

The Complement System (Chapter 36)

The complement system is a complex system of proteolytic enzymes, regulatory and inflammatory proteins and peptides, cell surface receptors, and proteins capable of causing the lysis of cells. The system can be thought of as consisting of three arrays of proteins. Two of these sets of proteins, when engaged, lead to the activation of the third component of complement (C3) (Fig. 1.12). The activation of C3 releases proteins that are critical for opsonization (preparation for phagocytosis) of bacteria and other particles, and engages the third set of proteins that insert into biologic membranes and produce cell death through osmotic lysis. In addition, fragments generated from some of the complement components (e.g., C3a and C5a) have potent inflammatory activities.

The Classical Pathway of Complement Activation

The two activation systems for C3 are referred to as the classical pathway and the alternative pathway. The classical pathway is initiated by the formation of complexes of antigen with IgM or IgG antibody. This leads to the binding of the first component of complement, C1, and its activation, creating the C1 esterase that can cleave the next two components of the complement system, C4 and C2.

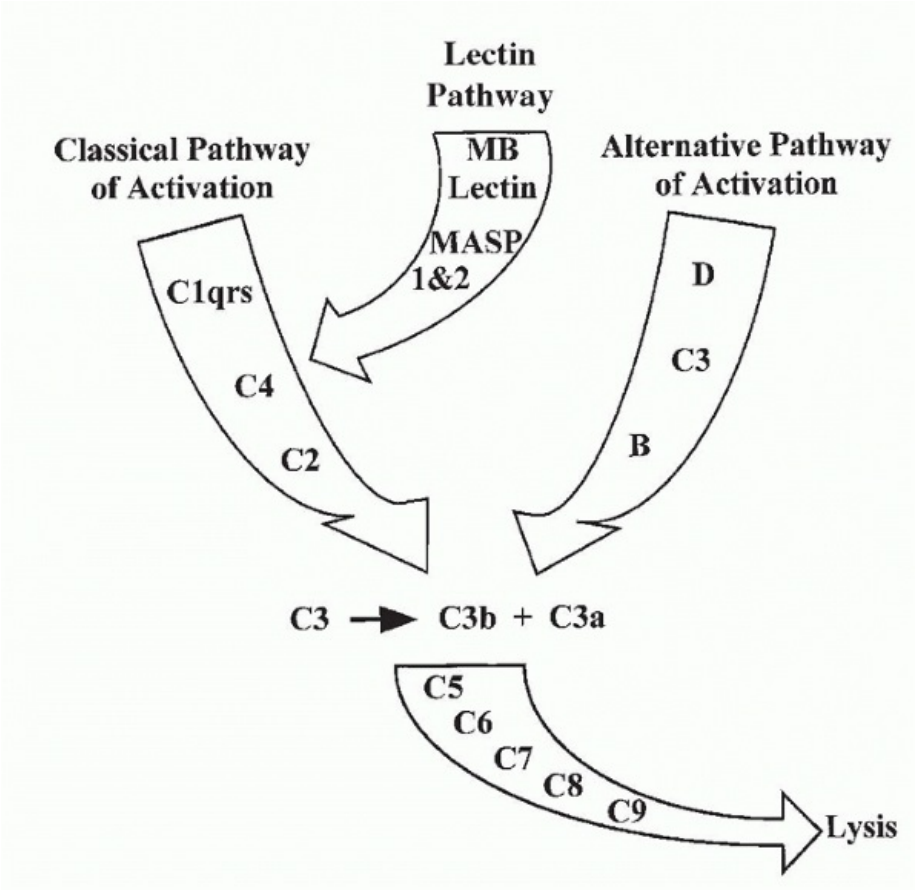

FIG. 1.12. The Complement System. The classical pathway of complement activation, usually initiated by the aggregation of C1 by binding to antigen/antibody complexes, resulting in the formation of an enzyme, a C3 convertase, that cleaves C3 into two fragments, C3b and C3a. The classical pathway can also be initiated by the aggregation of mannan-binding lectin as a result of binding sugars expressed in the capsules of many pathogenic microbes. The components of the lectin pathway appear to mimic the function of C1qrs. The alternative pathway of complement activation provides a potent means of activating complement without requiring antibody recognition of antigen. It results in the formation of a distinct C3 convertase. The fragments formed by cleaving C3 have important biologic activities. In addition, C3b, together with elements of the classical pathway (C4b, C2a) or the alternative pathway (Bb, properdin), form enzymes (C5 convertases) that cleave C5, the initial member of the terminal family of proteins. Cleavage of C5 leads to the formation of the membrane attack complex that can result in the osmotic lysis of cells.

C4 is a trimeric molecule, consisting of α, β, and γ chains. C1 esterase cleaves the α chain, releasing C4b, which binds to surfaces in the immediate vicinity of the antigen/antibody/C1 esterase complex. A single C1 esterase molecule will cause the deposition of multiple C4b molecules.

C2 is a single polypeptide chain that binds to C4b and is then proteolytically cleaved by C1 esterase, releasing C2b. The resulting complex of the residual portion of C2 (C2a) with C4b (C4b2a) is a serine protease whose substrate is C3. Cleavage of C3 by C4b2a (also referred to as the classical pathway C3 convertase) results in the release of C3a and C3b. A single

antigen/antibody complex and its associated C1 esterase can lead to the production of a large number of C3 convertases (i.e., C4b2a complexes) and thus to cleavage of a large number of C3 molecules.

The components of the classical pathway can be activated by a distinct, non-antibody-dependent mechanism, termed the lectin pathway. The mannose-binding lectin (MBL) is

activated by binding to (and being cross-linked by) repetitive sugar residues such as N-acetylglucosamine or mannose. The activation of MBL recruits the MBL-associated serine proteases MASP-1 and MASP-2, which cleave C4 and C2 and lead to the formation of the classical pathway C3 convertase. Because the capsules of several pathogenic microbes can be bound by MBL, the lectin pathway provides an antibody-independent mechanism through which the complement system can be activated by foreign microorganisms.

The Alternative Pathway of Complement Activation

Although discovered more recently, the alternative pathway is the evolutionarily more ancient system of complement activation. Indeed, it, and the MBL activation of the classical pathway, can be regarded as important components of the innate immune system. The alternative pathway can be activated by a variety of agents such as insoluble yeast cell wall preparations and bacterial lipopolysaccharide. Antigen/antibody complexes also can activate the alternative pathway. The C3 convertase of the alternative pathway consists of a complex of C3b (itself a product of cleavage of C3) bound to the b fragment of the molecule factor B. C3bBb is produced by the action of the hydrolytic enzyme, factor D, that cleaves factor B; this cleavage only occurs when factor B has been bound by C3b.

Apart from the importance of the alternative pathway in activating the complement system in response to nonspecific stimulants, it also can act to amplify the activity of the classical pathway because the C3 convertase of the classical system (C4b2a) provides a source of C3b that can strikingly enhance formation of the alternative pathway convertase (C3bBb) in the presence of factor D.

The Terminal Components of the Complement System

C3b, formed from C3 by the action of the C3 convertases, possesses an internal thioester bond that can be cleaved to form a free sulfhydryl group. The latter can form a covalent bond with a variety of surface structures. C3b is recognized by receptors on various types of cells, including macrophages and B cells. The binding of C3b to antibody-coated bacteria is often an essential step for the phagocytosis of these microbes by macrophages.

C3b is also essential to the engagement of the terminal components of the complement system (C5 through C9) to form the membrane attack complex that causes cellular lysis. This process is initiated by the cleavage of C5, a 200,000-dalton two-chain molecule. The C5 convertases that catalyze this reaction are C4b2a3b (the classical pathway C5 convertase) or a complex of C3bBb with a protein designated properdin (the alternative pathway C5 convertase). Cleaved C5, C5b, forms a complex with C6 and then with C7, C8, and C9. This C5b/C9 complex behaves as an integral membrane protein that is responsible for the formation of complement-induced lesions in cell membranes. Such lesions have a donut-like appearance, with C9 molecules forming the ring of the donut.

In addition to the role of the complement system in opsonization and in cell lysis, several of

the fragments of complement components formed during activation are potent mediators of inflammation. C3a, the 9,000-dalton fragment released by the action of the C3 convertases, binds to receptors on mast cells and basophils, resulting in the release of histamine and other mediators of anaphylaxis. C3a is thus termed an anaphylotoxin, as is C5a, the 11,000-dalton fragment released as a result of the action of the C5 convertases. C5a is also a chemoattractant for neutrophils and monocytes.

Finally, it is important to note that the process of activation of the complement cascade is highly regulated. Several regulatory proteins (e.g., C1 esterase inhibitor, decay accelerator factor, membrane cofactor protein) exist that function to prevent uncontrolled complement activation. Abnormalities in these regulatory proteins are often associated with clinical disorders such as hereditary angioedema and paroxysmal nocturnal hemoglobinuria.

CONCLUSION

This introductory chapter should provide the reader with an appreciation of the overall organization of the immune system and of the properties of its key cellular and molecular components. It should be obvious that the immune system is highly complex, that it is capable of a wide range of effector functions, and that its activities are subject to potent, but only partially understood, regulatory processes. As the most versatile and powerful defense of higher organisms, the immune system may provide the key to the development of effective means to treat and prevent a broad range of diseases. Indeed, the last two sections of this book deal with immunity to infectious agents and immunologic mechanisms in disease. The introductory material provided here should be of aid to the uninitiated reader in understanding the immunologic mechanisms brought into play in a wide range of clinical conditions in which immune processes play a major role either in pathogenesis or in recovery.

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Chapter 2

History of Immunology

Steven Greenberg

INTRODUCTION

There comes a time during every argument at which the two opposing parties reach a critical juncture: either resolution or impasse. Variations on this essentially Socratic theme have played out in all spheres of human intellectual activity. The dialectic of science ranges from incremental and relatively harmonious shifts in key, to a few abruptly dissonant ones, taking the form of what Thomas Kuhn would refer to as "paradigm shifts."[1] Such is the case with immunology, a field distinguished by more than its fair share of paradigm shifts. Arguably, its first dialectic, between the "cellularists" and the "humoralists," did not result in an early synthesis, but was characterized by partisan and often entrenched positions. Ultimately, the two parallel paths of cellular and chemical immunology converged, but it was not until the latter half of the 20th century that the two paths became one. How the paths were forged in the first place was an amalgam of the cultural institutions of the time, the creative output of the scientists themselves, and the imperatives of devising effective strategies to combat infection and contagion.

ANTECEDENTS TO THE GERM THEORY OF DISEASE

Ancient Theories of Disease Causation

Religious beliefs in ancient Greece drew contrasts between the sacred or the pure (*katharos*) and the polluted (*miaros*).[2] Pollution, or *miasma*, was blamed for many ancient transgressions, from the petty and personal, to the gravest, most famously embodied in the Oedipus myth. To remove the stain of *miasma*, the transgressor must undergo rites of purification (*catharsis*). To the ancient Greeks in the age of Homer, these were deeply ingrained beliefs that were essentially religious in nature. Because *miasma* was viewed as a source of suffering, it is not surprising that *miasma* was implicated in disease, as described by Hippocrates in his treatise "On Air, Water, and Places" in which *miasma* was associated with "unhealthy vapors."[3,4] Hippocrates is credited with being the first to recognize the potential of disease to arise from the environment and not as a result of religious superstition. *Mal aria*, which is Old Italian for "bad air," was one of many diseases thought to be caused by *miasma*. The concept that *miasma* was the source of disease persisted through the millennia and was a leading theory of how contagious diseases were transmitted up until the time of Pasteur.

Much of what we know about the medicine of ancient Greece is codified in *The Hippocratic*

Corpus, a collection of more than 60 volumes of text. Its authorship is disputed, but it is generally recognized as a compilation of works by Hippocrates himself as well as his students and intellectual heirs. One of his students, as well as son-in-law, Polybus, was credited as the author of *De Natura Hominis* (*On the Nature of Man*), the earliest known text describing the ancient Greek conceptual basis for disease pathogenesis, as embodied in the four humors: black bile, yellow bile, phlegm, and blood.[5]

> The body of man has in itself blood, phlegm, yellow bile and black bile; these make up the nature of his body, although these he feels pain or enjoys health. Now he enjoys the most perfect health when these elements are duly proportioned to one another in respect of compounding, power and bulk, and when they are perfectly mingled. Pain is felt when one of these elements is in defect or excess, or is isolated in the body without being compounded with all the others.

The Greek view that disease arose from an imbalance of the four humors did not supersede the *miasma* theory of disease, but was rather a more general view of disease causation, compared with the subset of apparently communicable diseases best explained by *miasma*.

The Romans developed and refined Greek concepts of disease. Marcus Terentius Varro (116 to 27 bce), referred to as “the most learned of all Romans” by the Roman rhetorician Quintilian,[6] was a prolific Roman scholar, estimated to have written more than 600 volumes. During the civil war of the first century, he served as Pompey's legate in Spain and fought at Pharsalus against Caesar but ultimately reconciled with Caesar, who appointed him director of the public library in 47 bce. Varro is perhaps best known for his only complete extant work, *Rerum Rusticarum Libri Tres* (*On Agricultural Topics*), in which he so presciently anticipated the existence of disease-causing microbes that seemed to Varro to be the immediate cause of diseases[7]:

> Precautions must also be taken in the neighbourhood of swamps, both for the reasons given, and because there are bred certain minute creatures which cannot be seen by the eyes, which float in the air and enter the body through the mouth and nose and there cause serious diseases.

Thus, Varro provided a mechanistic basis for disease that was consistent with the prevailing belief of *miasma* as the source of illness.

The Greek medical tradition was carried on for generations and was ultimately passed on to Claudius Galinus

(Galen), the Greek expatriate who was its greatest explicator. Galen was born in Pergamum in Asia Minor in 130 ce. After beginning his medical training in Pergamum at the behest of his father, he traveled widely in pursuit of “postgraduate” medical training in Smyrna, Corinth, and Alexandria. He returned to Pergamum and practiced surgery on gladiators, which provided a unique opportunity to deepen his knowledge of human anatomy and perfect his surgical technique.[8] Following an outbreak of plague among the Roman troops in Aquileia in

168 ce, he was summoned by the Emperor Marcus Aurelius and was appointed personal physician to his son, Commodus.[8] Galen's view of medicine was based on Hippocrates' *Corpus*. His output was prodigious more than any other ancient author of medical texts. He distinguished symptoms from diseases and offered explanations of the former that were consistent with his interpretations of disease pathogenesis; thus, tertian fever was the result of an "imbalance of yellow bile," quartan was caused by "too much black bile," and quotidian by "an excess of phlegm." Vomiting was viewed as the body's attempt to expel poisons, and the prescription of bleeding was to rid the body of "corrupt humors."[9] Galen's view of medicine remained the dominant one until the 17th century.

Early Concepts of Immunity

The term "immunity" itself is derived from the Latin practice of "exemption" from taxes or public service that normal citizens had to discharge, a favor bestowed by the emperor to meritorious individuals or entire communities.[10] However, the concept of immunity dates back at least as far as Thucydides, who described the plague of Athens of 430 bce that was responsible for the death of more than a quarter of the Athenian population[11]:

> Yet it was with those who had recovered from the disease that the sick and the dying found most compassion. These knew what I had from experience and had now no fear for themselves; the same man was never attacked twice-never at least fatally.

The traditional, religious view of the plague was that it is the work of Apollo who was held responsible for earlier plagues (eg, the plague on the Greek army in Troy because the Greek general Agamemnon abducted the daughter of Apollo's priest, Chryses). The religiously inclined could take some refuge in appealing to Apollo's son, Asklepios, who was revered as the god of healing, as were his daughters Hygeia (Hygiene), Iaso (Remedy), Akæso (Healing), Aglæa (Healthy glow), and Panakeia (Cure-all). In contrast, Thucydides characteristically did not offer facile religious explanations for sickness or recovery. In fact, he described the futility of the Athenians' plight in stark terms[11]:

> Neither the fear of the gods nor laws of men awed any man, not the former because they concluded it was alike to worship or not worship from seeing that alike they all perished, nor the latter because no man expected that lives would last till he received punishment of his crimes by judgment.

It seemed inevitable that in seeking an explanation for why some developed disease and others did not, many others would rely on a moralistic or religious view. In a remarkable passage from Galen's *On the Different Types of Fever* , not only does he set forth an explanation of how disease is transmitted through the air, and using the same term as Fracastoro would, "seeds," some 1300 years later, but he also blames a licentious lifestyle for susceptibility to the plague:

> Suppose, for example, that the circumambient air carries certain seeds

> of plague, and that of the bodies which share [breathe] it, some are full of various residues which are soon to become putrefied in themselves, while others are clean and free of such residues. Assume also that in the former there is a general blockage of their pores, a so-called plethora, and a life of ease devoted to gluttony, drink and sex, with all their necessarily concomitant digestive disorders. The others, which are clean and lack these residues, as well as being fine in themselves, have all a wholesome transpiration through pores that are neither blocked nor constricted; they take appropriate exercise and lead a temperate life. Assuming all this, which of these bodies is most likely to be affected by the rotting air they inspire?

This passage has been analyzed extensively by Nutton, who questioned the extent to which "seed" is used metaphorically[12]; if so, it is particularly apt.

Religious explanations for disease and immunity persisted throughout history. Particularly during the growth of Christianity during the Middle Ages, disease and sin were linked, though not inextricably; the great theologian Thomas Aquinas provided this distinction between sin and other causes of diseases[13]:

> … we need to consider that sin consists of a disorder of the soul, just as physical disease consists of a disorder of the body. And so sin is a disease of the soul, as it were, and pardon is for sin what healing is for disease.

Yet for many, a disease as dire as the plague would continue to be viewed as divine retribution for sin; for others, it was the result of astrological phenomena, while for still others, it was the result of a "conspiracy plotted by Jews to poison wells."[14] With the exception of the latter, which at least offered a proximate physical cause of disease, regardless of the lack of evidence, there was an abstract quality to these explanations that were shrouded in belief and superstition but lacking in substance. Further theories of disease pathogenesis would have to await the 16th century.

Fracastoro's Seeds

Girolamo Fracastoro (1478 to 1553) was an Italian who would have met most definitions of a Renaissance scholar: an accomplished physician, poet, mathematician, botanist, and astronomer. Educated by his father in Verona, and later at the University of Padua, he became an instructor in logic at the University of Padua in 1501 and in anatomy in 1502. He left Padua in 1508 and returned to Verona, where he

dedicated himself to his studies and his medical practice. In 1546, he proposed that disease was caused by *seminaria* ("seeds") that could be transmitted by three ways: direct contact from one person to another; through "fomites," or articles of clothing or dirty linen; and through the air. Although Nutton[12] has pointed out that Galen and Lucretius also used the term "seeds" to describe the transmission of illness through the air (see previous discussion), Fracastoro was the first to make them the focal point for disease transmission and to describe

their predilection for certain organs. Like the 10th century Arab physician Rhazes, who believed smallpox to have an affinity for blood, and specifically for the traces of menstrual blood that were believed to taint everyone in utero, as later suggested by Avicenna, Fracastoro offered the following explanation for immunity to smallpox: Following infection by smallpox seminaria, the menstrual blood would putrefy, rise to the surface, and force its way out via the smallpox pustules.[15] "Hence when this process has taken place, the malady usually does not recur because the infection has already been secreted in the previous attack."

Fracastoro's *seminaria* theory remained influential for nearly three centuries, in many ways serving as an early template for the germ theory of disease.

THE GERM THEORY OF DISEASE AND DEVELOPMENT OF VACCINES

Until the mid-19th century, the Galenic view of disease origins was the dominant one. Not only was the etiology of diseases misunderstood but also was the origin of life itself. The theory of spontaneous generation, which arose from Aristotle, held that life originated spontaneously from inanimate matter. The first experimental evidence against spontaneous generation came from Franceso Redi, the head physician in the Medici court, who in 1668 provided early evidence against the theory.[16*] Nevertheless, no overarching theory was proposed to replace it. Neither the mindset nor the necessary technology were available until the latter part of the 17th century, when an apprentice in a dry goods store, Anton van Leeuwenhoek, began a lifelong obsession with grinding the perfect lens. Leeuwenhoek's lenses were tiny but were ground with high degree of curvature, enabling him to visualize the hitherto undiscovered word of microbes. On September 17, 1683, Leeuwenhoek wrote a letter to the Royal Society, which was the first description of living, motile bacteria obtained from the plaque of his own teeth.[17] Leeuwenhoek was not the first to build a microscope (which was used by, among others, Redi), but his was far superior to existing multilensed or compound microscopes. Other scientists of the time, notably Robert Hooke, also observed microorganisms, and it was Hooke who was the first to publish the first image of a microorganism (the fungus *Mucor*) in 1665.[18] Some 150 years later, Dutrochet and then later Schwann, Schleiden, and Virchow, taking advantage of the microscopes of the their time, advanced the concept that "all living things are composed of cells and cell products."[19] Virchow took this one step further by declaring *omnis cellula e cellula* or "all cells develop only from existing cells." Whether Virchow rejected the germ theory or not is a matter of debate, but it is more likely that Virchow's underlying emphasis was not on external causes, but disease mechanisms, as he wrote in 1885: "First the discovery of the parasite, then the investigation of its etiology, then the question: how does it give rise to the disease."[20] Although it is hard to escape the possibility that a certain degree of professional jealousy may have played a role in Virchow's refusal to embrace the germ theory of disease, his viewpoint is one of but many examples of apparently strict dichotomies in science that would ultimately undergo revision and later synthesis. This is a theme that was to be recapitulated many times in the history of immunology.

The Conceptual Basis for the Germ Theory of Disease

Between Leeuwenhoek's technical breakthroughs in lens design in the late 17th century and the work of Pasteur and Koch in the late 19th century, several individuals endorsed

Fracastoro's “seed” theory, which gained new relevance when bacteria were first visualized. Among these was Jacob Henle, a German pathologist who was later to become Koch's teacher. Henle wrote a treatise that not only laid out the germ theory of disease in great detail but also arguably articulated an early version of what would later be known as “Koch's postulates”[21]: “Before microscopic forms can be regarded as the cause of contagion in man, they must be constantly found in contagious material. They must be isolated from it and their strength tested.” However, Henle's essay was a theoretical one and Henle himself never provided any experimental evidence in support of it. In the same year, decades before Pasteur and Koch would even begin to describe the germ theory of disease, Henry Holland, a Scottish-trained physician to Queen Caroline, who traveled extensively and was acquainted with the scientific luminaries of the time, including Davie, Gay Lussac, Berthollet, and Laplace, wrote a treatise in which he stated,[22]

> The question is, what weight we may attach to the opinion that certain diseases, and especially some of epidemic and contagious kind, are derived from minute forms of animal life, existing in the atmosphere under particular circumstances; and capable, by application to the lining membranes or other parts, of acting as a virus on the human body.

In a footnote, he cited others, including Kircher, and particularly Johannes Nyander, who wrote nearly a century earlier[23]:

> … it may be an easy matter for very minute insects to be the causes of diverse contagious diseases,” of which he included plague, measles, smallpox, and syphilis.

Nyander himself credits Lynceus Leuwenhoekius (“lynx-eyed” or “keen-eyed” Leuwenhoek) as the first to have seen such

“animalcules.” Thus, it is fair to say that the germ theory of disease itself had been “germinating” for some time prior to its scientific proof by Pasteur and Koch.

Experimental Evidence for the Germ Theory of Disease

The honor of the first experimental demonstration for the germ theory of disease may belong to two students of Francesco Redi. In 1687, soon after Redi had offered proof against spontaneous generation, two of his students, Bonomo and Cestoni, went on to observe the causative agent of scabies using the newly developed microscope and were able to transfer disease from person to person.[24] Perhaps the first demonstration of the bacterial pathogenesis of diseases in animals and humans was by Casimir Davaine, a French scientist who provided essentially the same evidence that Pasteur and Koch would years later that anthrax was caused by *bacteridies*. In 1865, Davaine was awarded the Prix Bréant by the Académie des Science for his work.[25]

Davaine's compatriot, Louis Pasteur, was the consummate experimentalist. A chemist by

training, his interest in infectious disease began with his study of fermentation. He made important contributions to the science of fermentation and his work led to many practical benefits to the beer and wine industry of France. His interest in microbes began with his speculation that the same type of microbe required for fermentation was likely responsible for transmitting disease. In 1865, he was asked to investigate a disease called pébrine that affected the silk worm industry. Within a year, Pasteur had established that pébrine was caused by a microbe, which provided further proof for the germ theory of disease. Some 14 years later, his expertise was again sought out of economic interests, in this case by farmers whose poultry stocks were diminished by chicken cholera. In a famous example of scientific serendipity, his assistant, Charles Chamberland, failed to inoculate chickens with cultures of chicken cholera bacilli, as instructed by Pasteur, but instead went on vacation. Upon returning several weeks later, he inoculated chickens with the old bacterial cultures, but the chickens didn't die as expected. Rather than disregard the experiment as a failure, “chance had favored the prepared mind” of Pasteur, who had his assistants inject fresh cholera into the same hens that had previously been injected; now none of the hens became ill. Pasteur had surmised that the bacterial cultures had become weakened by extended culture.[26] Thus began the use of attenuated strains of microbes to immunize against disease,[27] and the birth of the science of vaccination. The term “vaccine,” derived from the Latin *vaccus* for cow, was, coined by Pasteur in honor of Jenner. Attenuation as a strategy of developing vaccines would prove to be enormously valuable, leading to development of the first rabies vaccine by Pasteur himself, a vaccine against the viral causative agent of yellow fever by Theiler,[28] and the Bacillus Calmette-Guérin vaccine at Pasteur's institute.[29]

Although Robert Koch is credited as the originator of “Koch's Postulates,” it may come as a surprise that the essence of the postulates were first formulated by Koch's teacher, Jacob Henle (see previous discussion) and his contemporary, Edwin Klebs.[30] However, neither Henle nor Klebs applied their theories to any practical benefit, which is why Koch received credit for the postulates. Koch was the first to articulate then systematically apply them to prove that *Bacillus anthracis* was the causative agent of anthrax. Koch was a country physician living in Prussia, whose scientific career began unceremoniously with a gift of a microscope from his wife.[31] His first series of investigations began with observing the blood of a dead cow that succumbed to anthrax. Confirming the observation of Davaine and others before him, he observed filamentous bacteria in the blood. Not content merely with the observation, he began a series of technically challenging experiments, necessitating the development of many novel techniques used in microbiology laboratories even today, such as the use of solid medium to grow individual clones or colonies of bacteria. He proved that the filamentous bacteria were present only in infected animals and were capable of reproducing the disease when injected into healthy animals. This was the first systematic application of the eponymous postulates, which has since become the *sine qua non* of disease causation by infectious agents. His work was published in 1876,[32] the first of many groundbreaking publications. Koch's most profoundly important contribution to medicine was the discovery of the causative agent of tuberculosis. The lecture at which he announced his findings, on March 24, 1882, described later by Ehrlich as “the most important experience of his scientific life,” is considered by many to be the single most important lecture in medical history. Koch described the invention of novel staining methods to detect the tubercle bacillus and presented tissue dissections from guinea pigs that were infected with tuberculous material

from the lungs of infected apes and humans who had died from the disease.[33] For “his investigations and discoveries in regard to tuberculosis,” Koch was awarded the Nobel Prize in 1905.

The Unhealthy Rivalry Between Pasteur and Koch, and its Lasting Effects

The Franco-Prusssian war of 1870, the culmination of years of tension between France and Prussia, resulted in a Prussian victory and unity among the German states under King Wilhelm of Prussia. The Treaty of Frankfurt left a unified Germany the city of Strasbourg as well as possession of Alsace and the northern part of Lorraine, which was thought to contribute to further resentment of the Germans by the French and public support for World War I. It is against this backdrop that the relationship between Pasteur and Koch must be viewed. Koch had served in the Prussian army, and Pasteur's son was a conscript fighting for the French. Furthermore, there was intense professional rivalry between the two, especially over their work on anthrax pathogenesis. According to a letter from Charles Ruel, former *privat docent* at the University of Geneva, Koch was in the audience when Pasteur spoke on attenuation and vaccination at the fourth International Congress of Hygiene and Demography held in Geneva in September 1882. Pasteur spoke repeatedly about

German collected works (*recueil Allemand*). According to the letter[34]:

> Koch and his friend Prof. Lichtheim, were sitting side by side; they knew French only imperfectly and both mistook the word pride (orgueil) for collection (recueil). They felt their self-respect profoundly wounded and interpreted the words German pride as a grave insult.

This is but one ironic example of the level of rancor and misunderstanding between the two great men. It is said that Pasteur and Koch underwent some form of reconciliation later in their lives. Regardless, the aftermath of their rivalry, in many ways personifying the bitter relations between France and Germany, had a lasting effect on the evolution of the nascent field of immunology. In the years to come, the intellectual heirs of Pasteur and Koch would reenact the lifelong competitive tensions that characterized their relationship.

The Germ Theory of Disease: A Summation

In many ways, the “germ theory of disease” really did not begin with Pasteur and Koch, but rather by their predecessors, Henle, Klebs, and Davaine, who in turn owed credit to Fracastoro, and ultimately to Galen and Varro, some 1,600 years earlier. What enabled Pasteur and Koch to firmly establish the germ theory of disease began as a thought process that over time became distilled to something tangible: First, the idea that invisible “seeds” might propagate disease; second, the advent of an optically superior microscope, by Leuwenhoek, which enabled scientists their first glimpses at the “minute creatures” postulated by Varro; third, the growing evidence against spontaneous generation that began with Redi, thus opening the door for a new theory of disease; and finally, the inductive genius and careful experimental techniques of Pasteur and Koch.

The Long History of Vaccination: Success and the Unprepared Mind

Vaccination did not originate with Pasteur; its practice had been carried out for centuries without any fundamental understanding of its basis. Probably the earliest recorded example of intentionally inducing immunity to an infectious disease was in the 10th century in China, where smallpox was endemic.[35] The process of "variolation" involved exposing healthy people to material from the lesions caused by the disease, either by putting it under the skin, or, more often, inserting powdered scabs from smallpox pustules into the nose. Variolation was known and practiced frequently in the Ottoman Empire, where it had been introduced by Circassian traders in the 17th century.[35] Unfortunately, because there was no standardization of the inoculum, variolation occasionally resulted in death or disfigurement from smallpox, thus limiting its acceptance. Variolation later became popular in England, mainly due to the efforts of Lady Mary Wortley Montague. Lady Montague was the wife of the British ambassador to the Ottoman court who herself had contracted a severe case of smallpox. While in Istanbul, Lady Montague observed the practice of variolation. Determined not to have her family suffer as she had, she directed the surgeon of the embassy to learn the technique and, in March 1718, to variolate her 5-year-old son. After her return to England, she promoted the technique and had her surgeon variolate her 4-year-old daughter in the presence of the king's physician. The surgeon, Charles Maitland, was given leave to perform what came to be known as the "Royal Experiment," in which he variolated six condemned prisoners who later survived. By these and other experiments, the safety of the procedure was established, and two of the king's grandchildren were variolated on April 17, 1722. After this, the practice of variolation spread rapidly throughout England in the 1740s and then to the American colonies.[35,36]

It is difficult to say what influence the English country physician Edward Jenner (1749 to 1823) had on Pasteur's later discovery of attenuation of bacterial cultures and its application to vaccination. Regardless, it is fair to say that Jenner had a major influence on public health, as he was the first to publish the development and use of a safe alternative to variolation.[37] Although Jenner is rightly celebrated for his development of cowpox as a safe vaccine for smallpox, he was not the first to make use of a relatively nonpathogenic virus to induce immunity. Twenty years earlier, Benjamin Jesty, an English farmer, inoculated his wife and two sons with material taken from the cowpox lesion of the udder of a cow in his neighbor's herd.[36] In 1796, Jenner inoculated James Phipps, an 8-year-old boy, with material obtained from a cowpox lesion that appeared on the hand of a dairymaid (Fig. 2.1). Six weeks later, he inoculated the experimental subject with smallpox without producing disease. Further studies by Jenner established the efficacy of his vaccination procedure. For this feat, Jenner received a cash prize of £30,000 and election to nearly all of the learned societies throughout Europe.[38]

EMERGENCE OF IMMUNOLOGY AS A DISCIPLINE

The Cellularists versus the Humoralists

The international renown of Pasteur and Koch led to the establishment of research institutes that bore their names. Many talented young scientists were drawn to these institutes whose research missions should have been complementary, but were not, and the partisan battle that began with Pasteur and Koch would soon be reenacted on a larger scale.

Metchnikoff and the Birth of Cellular Immunology

Elie Metchnikoff (1845 to 1916), an ambitious student at the University of Kharkoff ("I have zeal and ability, I am naturally talented—I am ambitious to become a distinguished investigator"),[31] borrowed a professor's microscope and began a lifelong quest to understand the cellular basis for immunity. A comparative zoologist by training, his early academic focus was on understanding the development of metazoans. Heavily influenced by Darwin's publication of *The Origin of Species* in 1859,[39] he viewed early embryologic development as a competition among specialized cell

types.[40] Ontogeny was seen as a set of "interactions of cell lineages with each other to limit self-replication by any one component in favour of the interests of the organism as a whole. "[40] He focused his interest on an amoeboid marker cell of the mesoderm, which was dubbed "phagocyte" (devouring cell) by a contemporary of Metchnikoff's, the Viennese zoologist, Carl Claus. In a justly famous passage from Olga Metchnikoff's biography of her husband, she describes the observation that became the defining moment of his scientific career[41]:

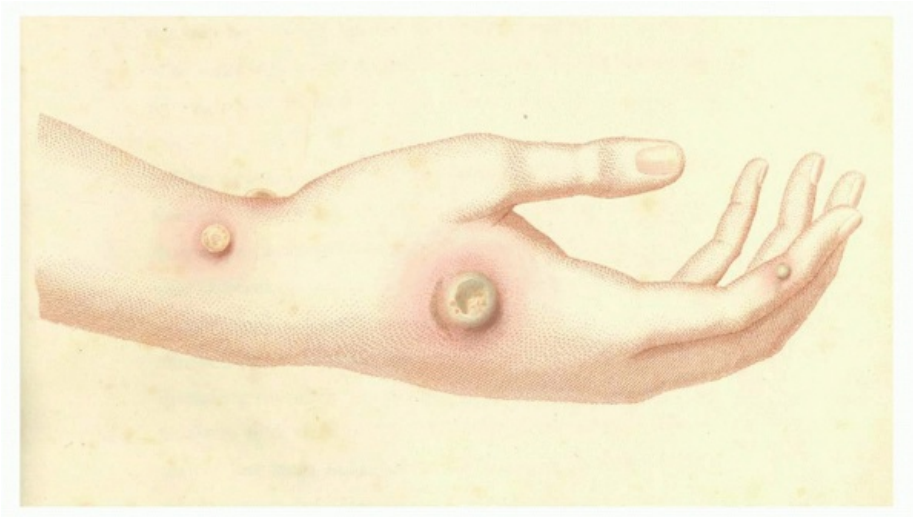

FIG. 2.1. Cowpox Pustule on the Arm of Sarah Nelmes. Reprinted with permission from Jenner37; courtesy of Dr Jenner's House: Birthplace of Vaccination, Gloucestershire, UK.

> One day when the whole family had gone to a circus to see some extraordinary performing apes, I remained alone with my microscope, observing the life in the mobile cells of a transparent star-fish larva, when a new thought suddenly flashed across my brain. It struck me that similar cells might serve in the defense of the organism against intruders. Feeling that there was in this something of surpassing interest, I felt so excited that I began striding up and down the room and even went to the seashore in order to collect my thoughts. I said to myself that, if my supposition was true, a splinter introduced into the

> body of a star-fish larva, devoid of blood-vessels or of a nervous system, should soon be surrounded by mobile cells as is to be observed in a man who runs a splinter into his finger. This was no sooner said than done. There was a small garden to our dwelling, in which we had a few days previously organised a “Christmas tree” for the children on a little tangerine tree; I fetched from it a few rose thorns and introduced them at once under the skin of some beautiful star-fish larvae as transparent as water. I was too excited to sleep that night in the expectation of the result of my experiment, and very early the next morning I ascertained that it had fully succeeded. That experiment formed the basis of the phagocyte theory, to the development of which I devoted the next twenty-five years of my life.

Contrary to popular belief, Metchnikoff was not the first to visualize and describe phagocytosis, nor was he the first to suggest that it played a role in host defense. In a historical recount of the history of phagocytosis,[42] Stossel argues that a Lutheran pastor, Johann August Ephraim Goeze, was the first to describe phagocytosis by microscopic observations of cells derived from hay infusoria in 1777. Much later, German pathologists of the mid-19th century, including Lieberkühn, Henle, and Vogel, drew connections between “pus corpuscles” of wounds and blood corpuscles.[42] Others at the time, particularly the English physicians William Addison and Augustus Waller, observed leukocyte migration through capillaries in response to local injury, and Ernst Haeckel, a German marine biologist who was later to oppose Metchnikoff in an early theory of gastrulation, described molluscan leukocytes ingesting India ink particles in 1862.[43]

A handful of scientists of the time made the conceptual link between phagocytosis and host defense, and Metchnikoff himself cites a few: “When (the phagocytosis theory) is once firmly established, it will be time enough to determine each part taken in its foundation by workers such as Panum, Gaule, Roser, etc…”[44] However, it seems that none of these workers seized upon this concept and appreciated its importance to the degree that Metchnikoff had; certainly, none had further developed and tested what in retrospect was the correct interpretation of the defensive function of phagocytosis.

Following the key experiment in 1882, described previously, Metchnikoff performed many others to test the “phagocytosis theory,” such as the observation of infection in water fleas, which he viewed as a Darwinian struggle between pathogen and host[45]:

> Once they have insinuated themselves into the organism's inmost part, the spores cause an accumulation of the mobile cells round them, which correspond to the white corpuscles in human blood. A battle takes place between the two elements.

Drawing the analogy with host defense in higher organisms, Metchnikoff described the killing of the spores by “mobile cells,” thus ensuring immunity for the organism. His view of phagocytosis expanded to encompass not only host defense but also organismal development, which he viewed in a teleologic context; he cited the dissolution of the tadpole's

tail by the “pervasion of phagocytes.”[46] By the time he moved to Paris to become *Chef de Service* at the Pasteur Institute in

1888, Metchnikoff had already formulated and tested what had become, according to his view, a cornerstone of the science of immune system. Perhaps what he had not appreciated at the time was that his move to Paris would come to signify to the Germans a complicit alliance with the French. He thus had unwittingly entered a battle that not only had been fought in the political arena but in the laboratory as well.

The Ascendance of Humoral Immunity

Several related developments in the new field of immunology occurred at the end of the 19th century that would seal the fate of cellular immunology for at least 50 years: The discovery by Roux and Yersin[47] that toxins alone derived from diphtheria bacilli could reproduce the symptoms of diphtheria; the discovery by von Behring and Kitasato in 1890[48] of humoral immunity to diphtheria and tetanus and the passive transfer of immunity to diphtheria in animals by von Behring and Wernicke in 1892[49]; and the discovery of alexins (Greek for “without a name”) by Hans Buchner in 1899[50] and Jules Bordet at about the same time.[51] Alexin was renamed “complement” by Ehrlich, as it “complemented” the activity of antibodies. Indeed, the ability of humoral components alone to lyse bacteria (the Pfeiffer phenomenon) or erythrocytes in the absence of phagocytosis[51] provided strong independent evidence supporting the humoralists' claims. The discovery of complement also had practical uses, as complement fixation became the basis of a widely used serologic test for the diagnosis of syphilis, the so-called Wasserman test.[52] The collective discoveries of the “humoralists” would lead to the successful treatment of a number of previously intractable diseases, such as diphtheria. Indeed, the first Nobel Prize in physiology of medicine was awarded to von Behring in 1901, “For his work on serum therapy, especially its application against diphtheria, by which he has opened a new road in the domain of medical science and thereby placed in the hands of the physician a victorious weapon against illness and death.” Bordet himself would later be awarded the Nobel Prize “for his studies in regard to immunity.” Among Bordet's contributions was the development of the complement fixation test together with his brother-in-law, Octave Gengou. This formed the basis of what Bordet termed “serodiagnosis.”

Although the lines in the sand had already been drawn by the mostly Prussian humoralists led by Ehrlich on the one hand and the cellularists led by Metchnikoff on the other, it was Metchnikoff who wrote a letter to von Behring, proposing a scientific “truce”[53]:

> I now believe … we can calmly work side by side. We can mutually support one another, just like the phagocytes and antitoxins, since … the phagocytes receive considerable assistance from the antitoxic property, just as the phagocytes … render great assistance to the organism or respectively its antitoxic powers, since they capture and destroy … bacteria.

In fact, von Behring did not seem rigidly against the cellular school, and Metchnikoff viewed

him as supporting the view that "active immunity requires some type of cellular basis."[53,54] It is difficult to know what to make of this passage; it sounds vague and seems to state the obvious, yet it does suggest a degree of flexibility that more intransigent proponents of the humoralist camp seemed to lack at the time. Regardless, scientific reconciliation would not be forthcoming until many years later, although phagocytosis theory was granted a temporary reprieve by the British physician Almoth Wright, who demonstrated the phenomenon of opsonization of bacteria. Wright was the first to describe a mechanism by which humoral and cellular components of immunity cooperate to kill bacteria.[55] Wright is possibly best known in his incarnation as Sir Colenso Ridgeon in Shaw's "The Doctor's Dilemma." Ridgeon defined "opsonin" as "…what you butter the disease germs with to make your white blood corpuscles eat them."[56] In what was viewed as a well-deserved but partly symbolic gesture, nevertheless, Metchnikoff and Ehrlich, two exemplars of the opposing schools of immunity, were awarded the Nobel Prize in 1908 "in recognition for their work on immunity." It would not be until 40 years later that another cell type of the immune system would be first identified as being the source of antibody[57] and 70 years later when dendritic cells (DCs) would be first identified as being the key phagocytic leukocyte responsible for initiating the immune response.[58]

Paul Ehrlich: The Cellularist's Humoralist

Paul Ehrlich embodies a pivotal figure in the history of immunology. Although he would make many practical discoveries in his long research career, his greatest contribution to immunology, like Jerne's over a half century later, was a conceptual breakthrough that served to stimulate the field of immunology for years to come. Although I am tempted to consider his "side chain theory" of antibody formation a paradigm shift, that would presume that there was a preexisting paradigm to shift from, when in fact there was no paradigm of antibody specificity to begin with.

Ehrlich began his research career as a medical student. One of his professors was the pathologist Wilhelm von Waldeyer, who introduced the young Ehrlich, who already showed a strong interest in chemistry, to histologic methods for staining cells and tissues. Following further training in several medical schools, he was influenced by the chemist von Bayer and the pathologists Cohnheim, Haidenhain, and Weigert (his cousin). He presented his thesis on histologic staining in Leipzig at the age of 24, in which he was the first to describe mast cells. As noted by Silverstein, the opening sentence of the thesis gave an inkling of his approach to science[59]:

> While in the modern histological literature, directions on tintorial method are already so numerous, and still increase from day to day, yet their theoretical basis has had only a very negligible consideration.

The same year, he was appointed senior physician in the Department of Internal Medicine at the Charité-Hospital in Berlin. The head of the clinic, Friedrich Frerichs, encouraged Ehrlich to continue his histochemical investigations, which led to the identification of neutrophils, eosinophils,

and basophils as well as the diagnosis of his own case of pulmonary tuberculosis.[60]

It is notable that the term “side-chain” (*seitenketten* in German) was a chemical term in use at Ehrlich's time meaning much the same as it does today. It is inescapable to conclude that Ehrlich's focus and interest in chemistry would be the driving force behind his thinking about how antibody is formed, and “selected for,” by antigen. The essence of Ehrlich's side chain theory, first proposed in 1897 (Fig. 2.2) is well articulated in Ehrlich's Nobel lecture and is paraphrased here[61]:

1. “The relationship between toxin and antitoxin are *strictly specific*—tetanus antitoxin neutralizes exclusively tetanus toxin, diphtheria serum only diphtheria toxin …”
2. “For this reason it must be assumed that the (toxin and antitoxin) enter into a *chemical bond* … fitting each other ‘like lock and key.’”
3. “The group in the protoplasm, the *cell receptor* , must be identical with the *antitoxin* which is contained in solution in the serum of immunized animals.”
4. “As these receptors, which may be regarded as *lateral chains* (“seitenketten”) of the protoplasm…become occupied by the toxin, the relevant normal function of this group is eliminated…the deficiency is not merely *exactly compensated*, but *made up to excess* (hyperregeneration).”

What Ehrlich proposed purely on theoretical grounds is a brilliant argument based on a combination of inductive and deductive reasoning. Characteristic of Ehrlich, it is lucid, logical, and profound. Beginning with a consideration of a simple chemical bond, Ehrlich somehow ends up with an antibody-producing cell; hence, “the cellularist's humoralist.”

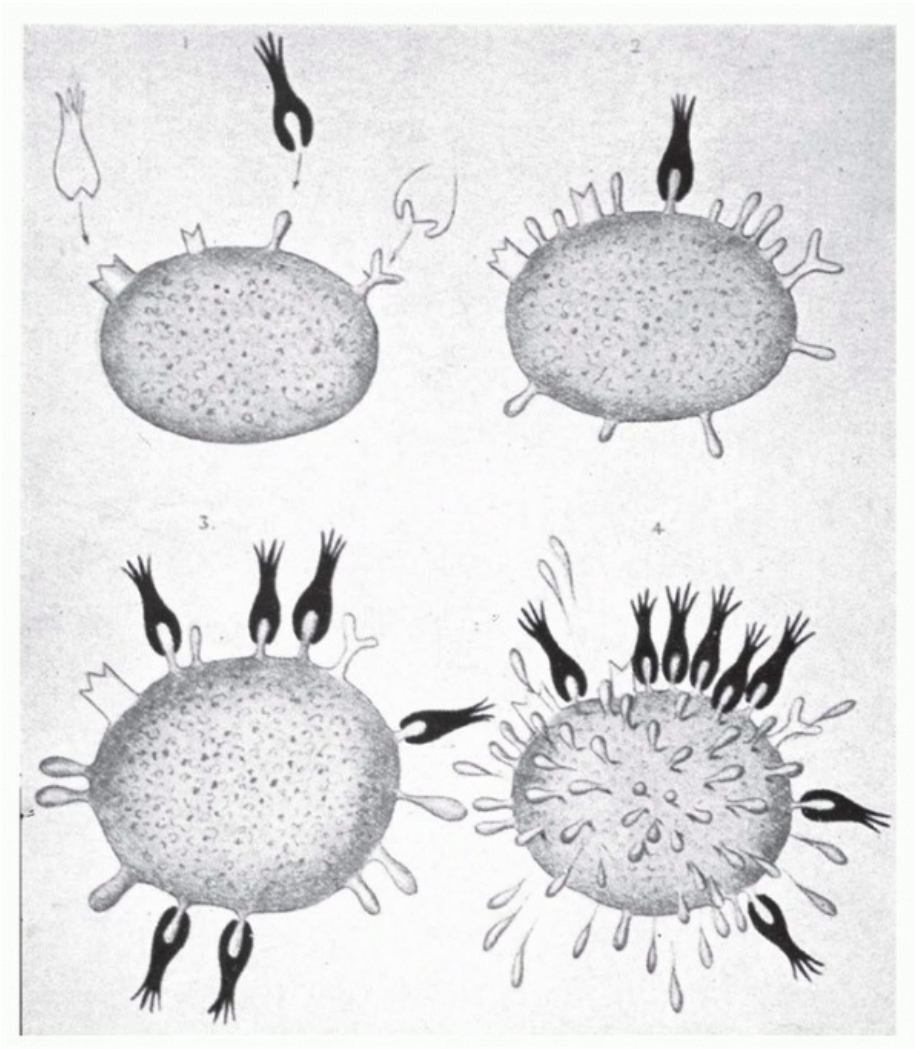

FIG. 2.2. Ehrlich's "Seitenkette" (Side Chains). From Ehrlich.[171]

Not every biologist was enamored of Ehrlich's model, and soon after he proposed it, it came under attack, most notably by Jules Bordet. Bordet objected to Ehrlich's insistence that the specificity of the antigen-antibody reaction required an irreversible bond, whereas Bordet, whose views seemed to be more deductively grounded in his immediate line of investigation (eg, precipitin reactions and complement fixation) thought that adsorptive interactions between antigen and antibody were sufficient to account for specificity.[15,60] Although Ehrlich's side-chain theory provided a logically consistent mechanism to account for antibody specificity, it would later be criticized for failing to account for antibody diversity. That problem would not be conquered for another 60 years in yet another "paradigm shift" when Talmage, Burnet, and Lederberg proposed the clonal selection theory. However, the concept of clonality was not yet conceived of in 1897, and in any case, it is hard to envision how a clonal selection theory could have been developed without the prior description by Ehrlich of antibody selection itself.

IMMUNOLOGY BRANCHES OUT: BENEFICIARIES OF THE EARLY FOCUS ON HUMORAL IMMUNITY

"Man built most nobly when limitations were at their greatest."

Immunology during the early part of the 20th century was heavily influenced by the early victories of the humoralists. Aside from the spectacular practical implications of the work of von Behring, Bordet, and Ehrlich, among others, the immunologist's toolkit of the early 20th century immunologist was very limited. Advanced microscopic techniques were not yet available, and cell fractionation techniques had not yet been invented. Given these limitations, it is hard to envision how the gap between antibody and cell could have been bridged any closer than Ehrlich had managed, at least on a theoretical level. However, the focus on humoral immunity did allow for the rapid development of several fields, most notably immunochemistry as well as hematology and allergy. It would be some time before cellular immunology would catch up.

Karl Landsteiner and the Birth of Transfusion Medicine and Autoimmunity

As described by Silverstein, “No single individual contributed as importantly to so many different areas of immunology as did Karl Landsteiner.”[15] Landsteiner was born in Baden in 1868 and attended Vienna Medical School. He began his training in internal medicine and studied chemistry with Emil Fischer, who would receive the Nobel Prize in chemistry in 1902. He transferred to the Department of Pathological Anatomy, home to Erdheim, Billroth, Escherich, and other accomplished scientists, where he remained until

1907.[62] Landsteiner's first major accomplishment was the discovery of the human ABO blood group antigens at the turn of the century.[63,64] His motivation was succinctly described in his Nobel lecture speech[65]:

> … proteins in individual animal and plant species differ and are characteristic of each species … The problem raised by the discovery of biochemical specificity peculiar to a species … was to establish whether the differentiation extends beyond the species and whether the individuals within a species show similar though smaller differences.

His experiment was a simple one, in which he applied the sera of six heathy men, including himself, to red blood cells of each, and noted that the sera of the men reacted differently with each other—no serum reacted with that same individual's red blood cells. At the end of the paper, Landsteiner noted that the results could account for the variable clinical consequences of human blood transfusion —and thus was borne the discovery of the ABO red blood cell antigens that would later become useful in blood typing prior to transfusions. Many years later, Landsteiner would discover the M, N, P isoantigens in 1927[66] and the Rh system in 1940[67]).

Landsteiner's next major contribution was the codiscovery with Donath of the first autoimmune disease, paroxysmal cold hemoglobinurea,[68] which challenged Ehrlich's dictum that such a situation could not occur.[69]

> The organism possesses certain contrivances by means of which the immunity reaction … is prevented from acting against the organism's own elements and so giving rise to autotoxins … so that one might be justified in speaking of a "horror autotoxicus" of the organism.

The nature of the contrivances was thought to be of "the greatest importance" by Ehrlich, who later stated[70]: "According to our present investigations either the disappearance of receptors or the presence of autoantitoxin is foremost among these contrivances." Depending on how this statement is interpreted, it could be viewed as Ehrlich's formulation of either clonal deletion or anti-idiotypes. Two years after the discovery of paroxysmal cold hemoglobinurea, an Italian ophthalmologist, who observed sympathetic ophthalmia, a disease in which damage to one eye leads to inflammation of the opposite eye, speculated that this disease was due to "autocytotoxins."[71] Autoimmunity research was taken up by a few others, but perhaps owing to either a misinterpretation of Ehrlich, or possibly due to deference to his authority in the field, progress was slow. It would not be years later, until the discovery of the role of sensitization of the newly discovered Rh antigen as an etiology of erythroblastosis fetalis,[72,73] that autoimmunity became an active area of research for immunologists. The first time that autoimmunity was first associated specifically with arthritis was 1957, when Kunkel and colleagues discovered what came to be called "rheumatoid factor," large complexes of immunoglobulin (Ig)M directed against IgG in the sera of some patients with rheumatoid arthritis.[74] This observation fundamentally changed the field of rheumatology.[75]

Landsteiner received the Nobel Prize in 1930 "for his discovery of the human blood groups." Ironically, upon receiving the prize, it is said that he felt the prize should have been awarded for his work on haptens, which would play a great role in the development of the growing field of immunochemistry.

Discovery of Hypersensitivity: The "Other Work"

In 1901 to 1902, Paul Portier and Charles Richet were attempting to raise tolerance in laboratory animals to actinotoxin, an uncharacterized toxin derived from tentacles of sea anemones. Their experiments appeared to be unsuccessful, and, in some cases, it appeared that the animals actually became sensitized to the antigen. They repeated their studies using dogs and obtained completely unanticipated results: All eight dogs collapsed and died within minutes after receiving a relatively small dose. First thinking the results were due to experimental error, they later realized that the sensitized animals had all been exposed to antigen 14 to 23 days previously.[76] They proposed the name "aphylaxis" (against protection), a term later changed to "anaphylaxis."[77] Richet continued his investigations on anaphylaxis and was awarded the Nobel Prize in 1913 for his work. Soon after Portier and Richet made their seminal discovery, Maurice Arthus was able to induce a localized form of anaphylaxis (swelling and ultimately gangrene) by repeated subcutaneous injections of horse serum, considered fairly nontoxic.[76] Yet, a third type of hypersensitivity was described by the pediatricians von Pirquet and Schick,[78] who noted that vaccinated children occasionally developed fever, joint pains, rash, diarrhea, and hypotension. They concluded that the clinical features of what is now called "serum sickness" were not a direct result of the injection of antiserum, but the outcome of "when antigen and antibody meet." As von Pirquet

later explained,[79]

> We are able to observe the effects of the toxic body formed when antigen and antibody meet, that is, the serum disease. We see that at the time when the antibody arises, and therefore the antigen disappears, symptoms of general disease occur. The supposed connection is that these symptoms are due to toxic bodies formed by this digestion of the allergen through the antibody.

Although von Pirquet does not precisely define what he meant by “toxic body,” some have interpreted this to mean antigen-antibody complexes. It is possible that he was reluctant to be more specific as he did not actually have a way of observing or quantifying the complexes; it is also possible that he did not have a ready explanation for how such a complex, if formed, could lead to the symptoms and signs of serum sickness. It would not be until many years later that Frank Dixon and colleagues would precisely delineate the nature of the immune complexes.[80] Nevertheless, it is clear that von Pirquet and Schick viewed this phenomenon as

lying along a continuum with the normal immune response, or rather being a necessary component of it. As they later stated,[81]

> The conception that the antibodies, which should protect against disease, are also responsible for the disease, sounds at first absurd… One forgets too easily that the disease represents only a stage in the development of immunity, and that the organism often attains the advantage of immunity only by means of disease.

It is von Pirquet and Schick who coined the term “ allergy” (from the Greek *allos*, other, and *ergon*, work).[79] By highlighting the role of tissue injury in promoting immunity, they closed the loop that Metchnikoff had begun at the end of the 19th century. This theme would be revisited and expanded upon in the latter part of the 20th century when it was discovered that the inflammation that accompanied the innate immune response was a necessary prequel to the acquired immune response.

THE LONG JOURNEY FROM THE DAWN OF IMMUNOCHEMISTRY TO THE STRUCTURE OF IMMUNOGLOBULINS

Challenges to Ehrlich: The Problem with the “Keys”

One of the difficulties that Ehrlich faced was bridging the gap between the conceptual basis of antibody specificity and the actual basis of antibody specificity. If his side-chain theory was correct, then every cell involved in antibody production would be capable of reacting against every possible antigen it might encounter. Even without considering the cellular origins of antibodies, Ehrlich's critics, such as the Viennese Max von Gruber, raised the question of how the astonishingly large number of different specificities of the antibody molecules themselves could be generated.[15] The size of the repertoire seemed impossibly large if

every “lock” had a unique “key.” Landsteiner became von Gruber's assistant at the University of Vienna in 1896, and he inherited von Gruber's critical view of Ehrlich's theory. Landsteiner's early approach to this problem was to adopt Bordet's “colloid” explanation of the antigen-antibody interaction,[15] which rejected covalent interactions in favor of multiple weaker interactions, a theme that was later taken up by Pauling, with some interesting consequences. Later, stimulated by the work by Obermeyer and Pick, who described chemical modifications of proteins,[82] Landsteiner used structurally related reactive chemicals to derivatize proteins. He showed that antisera raised against one of the chemically modified proteins would react to varying degrees with proteins modified by structurally similar, but not identical, reactive molecules. These results were interpreted as being incompatible with Ehrlich's “lock and key” specificity but called for a more nuanced view of antigen-antibody specificity. It became immediately apparent that the size of the repertoire could be greatly enhanced if one allowed for such graded degrees of binding affinities.

Immunoglobulin Structure: The “Keys” to the Problem

It was clear that further progress on defining the physicochemical nature of the antigen-antibody reaction required the development of specific tools that were unavailable at the turn of the century. Antibodies, whose chemical structures were unknown at the time, were considered “colloids” (from Greek *kolla*, glue), a suspension of particles suspended in a continuous phase of another component. In 1924, the Swedish chemist Svedberg designed a centrifuge based on a modified milk separator. The centrifuge could develop a centrifugal field of up to 5000 g and enabled Svedberg to measure the molecular mass of hemoglobin[83]; he was the first to determine the molecular mass of macroglobulins, derived from a patient with Waldenström macroglobulinemia, which we now know as IgM. A student in Svendberg's laboratory, Arne Tiselius, developed gel electrophoresis,[84] which allowed for the separation of molecules based on charge and size. These tools enabled Michael Heidelberger to establish the field of “immunochemistry.” Heidelberger devoted nearly his entire research career pursuing the implications of a simple but profound discovery he made with Oswald Avery in 1923 that type-specific antigens of pneumococcus bacteria are complex polysaccharides. Over the next three decades, this discovery enabled him to determine, for the first time, the exact weight and chemical composition of antibodies, antigens, and complement. He showed that antibodies are multivalent proteins and used these insights to devise a simple vaccine against pneumonia whose effectiveness was first proven in soldiers who fought in World War II.[85]

The Chemical Nature of Immunoglobulin Molecules

By 1950, it was appreciated that antibodies were proteins of 150,000 molecular mass containing bivalent antigencombining sites. Based on Porter's observation that Igs could be split into smaller products by enzymes such as papain, yielding Fab fragments that bind antigen and Fc (crystallizable) portions that do not, Edelman and Poulik further defined Ig structure by hypothesizing that Bence-Jones proteins, derived from myeloma cells, were free light chains of Ig molecules.[86] As this hypothesis appeared to be correct, this provided a means of obtaining a ready supply of homogeneous Ig subunits. Reduction of disulfide bonds led to still different products, enabling them to propose that the Ig molecule consists of two light chains and two heavy chains, and that the antigen-binding site consisted of

contributions from both heavy and light chains.[86] Further refinement of techniques in protein chemistry allowed Edelman and Porter to work out the primary structure of Ig molecules, for which they received the Nobel Prize in 1972.

CONFRONTING THE SIZE OF THE REPERTOIRE

The value of a large repertoire from which to select is appreciated by any performing musician. Yet in the 1950s, the repertoire problem was unsolved, leading to competing theories of how a large repertoire of diverse antibodies are generated. There were two mutually exclusive leading schools of thought: “instruction” theories and “selection” theories.

Instruction Theories of Antibody Diversity

As noted by Silverstein,[15] the first description of antigen as template was by Bail and Tsuda, who proposed in 1909 that antigen persists after its encounter with antibody, and that by so doing, it leaves an impression on the antibody.[87] This concept was further refined by Breinl and Haurowitz, who suggested that antigen is carried to the site of protein formation, where it would serve as a template to instruct antibody formation.[88] Finally, in 1940, Linus Pauling provided a chemical explanation for antigen-directed instruction: that “antibodies differ from normal serum globulin only the way the ends of the polypeptide chain is coiled” as a result of their amino acid sequence, and that “they have accessible a very great many configurations with nearly the same stability.” Under the “influence” of antigen, they “assume configurations complementary to surface regions of the antigen,” forming two active ends, and that after “freeing one end and the liberation of the central part of the chain, it folds up to form the central part of the antibody molecule, with two oppositely directed ends able to attach themselves to two antigen molecules.” This interaction would be further stabilized by weak interactions between antigen and antibody.[89] Assuming a degree of degeneracy in the initial antigen-antibody interaction, these theories were consistent with the findings of Landsteiner, who showed that antigen-antibody interactions were not absolutely specific, as envisioned by Ehrlich (Fig. 2.3). In retrospect, these theories had great chemical appeal; however, a central weakness is that if the initial interactions between antigen and antibody are degenerate, that implies that the interactions cannot be of high affinity (otherwise they would not be degenerate). Yet, if the initial interactions are weak, how would they occur in the first place? At some level, there has to be a certain degree of preexisting antibody specificity, which implies a preexisting repertoire. Although the instruction theories of antibody selectivity helped explain some of the specificity of the antigen-antibody interactions, they could not account for all of them. It is notable that the underlying basic principles have been since invoked for other encounters in the immune system. For example, a “bar code model” of interactions between the T-cell receptor (TcR) and peptide-major histocompatibility complex (MHC) (pMHC) has recently been proposed, in which the initial encounter between TcR and pMHC is governed by strong interactions, followed by “scanning” the epitope and modest changes in conformation of the TcR, leading to strengthening of the TcR-pMHC association.[90]

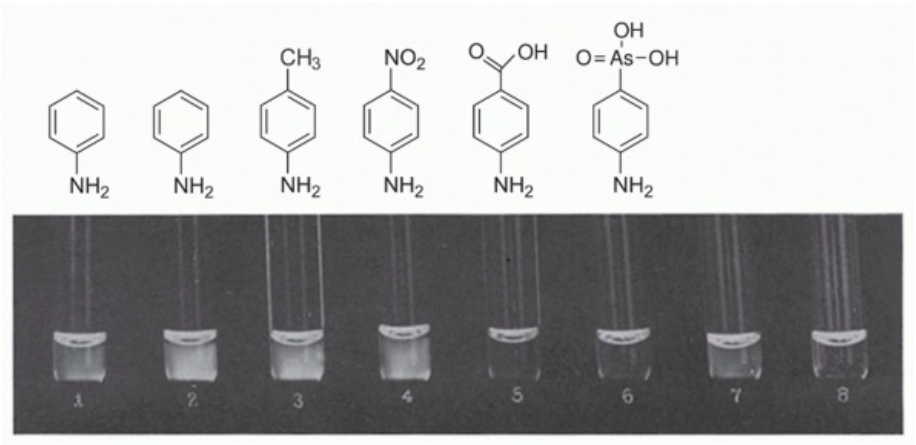

FIG. 2.3. Demonstration of Serologic Specificity by Landsteiner and Scheer. Reactions from immune serum for aniline with various azoproteins and with unchanged horse serum reading after 15 minutes. *(1)* azoprotein from chicken serum and aniline, *(2)* azoprotein from horse serum and aniline, *(3)* azoprotein from horse serum and para-toluidine, *(4)* azoprotein from horse serum and para-nitroaniline, *(5)* azoprotein from horse serum and para-aminobenzoic acid, *(6)* azoprotein from horse serum and para-arsanilic acid, *(7)* unchanged horse serum, *(8)* saline control. Modified from Landsteiner and van der Scheer.[486]

Jerne's Natural Selection Theory of Antibody Formation

Instruction theories of antibody specificity persisted through the 1950s, when they were modified to include participation of enzymes to act as intermediaries between antigen and antibody as well as the newly discovered structure of deoxyribonucleic acid (DNA). In 1955, Niels Jerne published a highly influential paper in which he proposed a new theory of antibody formation that he described as the “natural selection theory of antibody formation.„91

> The antigen is solely a selective carrier of spontaneously circulating antibody to a system of cells which can reproduce this antibody. Globulin molecules are continuously being synthesized in an enormous variety of different configurations…among which… will be fractions possessing affinity toward any antigen to which the animal can respond.

Jerne referred to these preexisting antibodies “natural antibodies,” and went on to state that antigens selectively attach to those globulin molecules that happen to have a complementary configuration. According to Jerne, once the interaction occurs, the antigen-antibody complexes may be engulfed by a “phagocytic cell,” at which point the antigen is eliminated. The antibody within the phagocytic cell can remain or be transferred to another cell, which is the signal for reproduction of the same specific antibodies. More antibody is released into the circulation, resulting in a larger percentage of specific circulating antibody. Jerne states that “the reproduction need not be highly faithful; copying mistakes will be harmless and may

occasionally produce an improved fit."[91] These are remarkable concepts, as Jerne appeared to have invoked Metchnikoff, a Darwinian interpretation of antibody selectivity at the organismic level, as well

as to have anticipated affinity maturation. It is easy to see why Jerne's ideas were so influential. However, the central problem, the lack of an explanation for the huge existing repertoire, which hampered the instruction theories of antibody diversity, as well as Ehrlich's side-chain theory was still unanswered. Jerne admitted this weakness and speculated that the "spontaneous production of globulin molecules of a great variety of random specificities" may reside in a "… specialized lymphoid tissue, such as that of the thymus."[91] Perhaps a more existential problem was that the flow of information was from the existing preformed antibody to more antibody without any genetic intermediary. What was lacking was a specific mechanism to transfer information between a specific antibody and the specific synthesis of that same antibody. Jerne admitted to this problem and nominated ribonucleic acid (RNA) as a key template; he then stated " … a protein molecule may determine the order of the nucleotides in the synthesis of RNA," citing a paper that does not actually make this assertion.[91]

What accounted for this conceptual block that had persisted for more than half a century? On one level, it was ignorance of the "fundamental dogma of molecular biology," which had not yet been articulated.[92] But on another level, it perhaps can be traced back to the decisive victory of the humoralists over the cellularists. So long as the focus was on the antigen-antibody interaction, there was no way to invoke a biologically plausible mechanism of generating a preexisting Ig repertoire and of selectively expanding a specific portion of that repertoire.

Development of the Clonal Selection Theory

In 1957, the American immunologist David Talmage published a review whose focus was allergy; however, in the review, Talmage drew an analogy between natural selection, in which there is "selective multiplication of a few species out of a diverse population" and antibody production. In a very succinct but remarkably insightful passage, he lays out the essence of the clonal selection theory[93]:

> As a working hypothesis it is tempting to consider that one of the multiplying units in the antibody response is the cell itself. According to this hypothesis, only those cells are selected for multiplication whose synthesized product has affinity for the antigen injected.

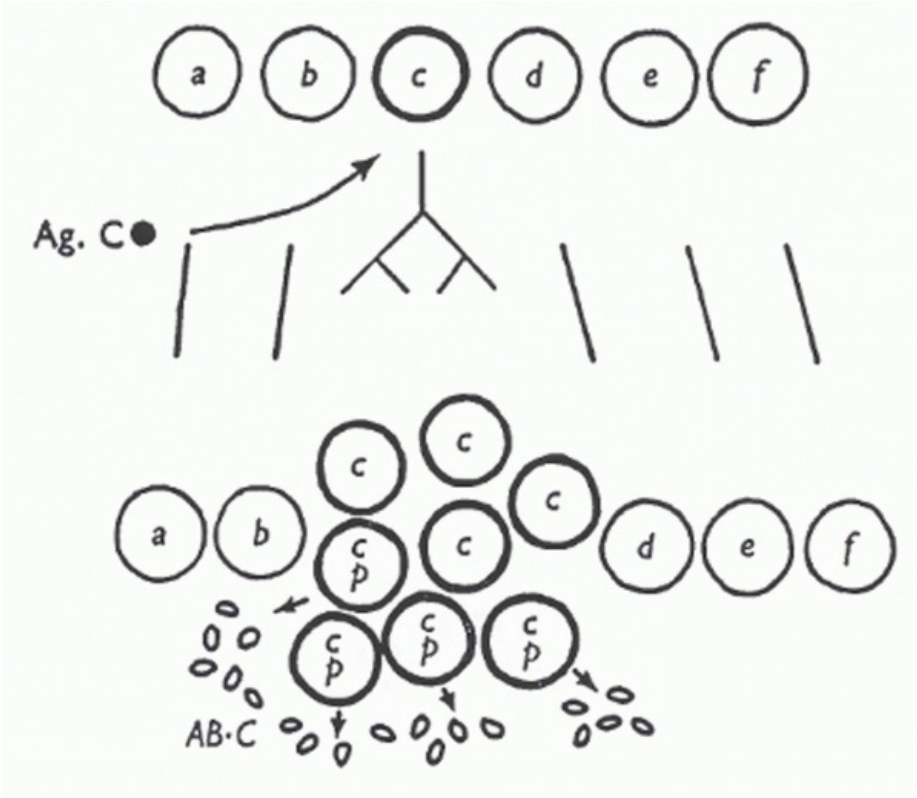

FIG. 2.4. In this diagram, antigen C (Ag.C) is recognized by clone "c," triggering this clone, but not others, to proliferate. Antibody against Ag.C (AB.C) is indicated at the bottom. From Burnet.[246] Copyright ©1959 Sir MacFarlane Burnet. Reprinted with the permission of Cambridge University Press.

At about the same time, Burnet published his own paper outlining the key aspects of the clonal selection theory[94] (Fig. 2.4). In a later interview, Talmage discussed how he provided a preprint of his review to Burnet before he published his landmark paper on the clonal selection theory, but both he and Nossal stated that Burnet developed the theory independently. Talmage thought that the two papers were similar in substance, but that "Burnet had the good fortune to make an analogy of the idea to the clones that grow in bacteria and he gave it a very catchy name, 'clonal selection.'"[95] Among the ideas that led both immunologists to propose the clonal selection theory was the known logarithmic rise in antibody titer during the primary immune response "as if it is a product of some multiplying substance."[95] Other experiments demonstrated that lymphoid cells obtained from previously immunized rabbits that were then transferred to x-radiated recipients were sufficient to induce a recall response in the recipients.[96]

Although we now know that the clonal selection theory is correct, it took more than 10 years for clonal selection to be widely accepted, according to Talmage.[95] Among the key pieces of evidence to prove the theory was the demonstration of antibody production from single cells, by Nossal and Lederberg,[97] and the later finding that cognate antigen was capable of aggregating all surface Ig on individual cells,[98]

which corroborated Nossal and Lederberg's results. Finally, in 1975, Köhler and Milstein demonstrated the production of monoclonal antibodies from single clones of immortalized plasma cells.[99] Along with Jerne, they shared the Nobel Prize in 1984.

The Structural Basis of Antibody Diversity: The Dialectic Revisited

By the mid-1970s, the puzzle of antibody diversity was far from solved—many of the pieces were still missing. The conceptual basis for the clonal selection theory was laid in 1957 by redirecting the focus of investigation from immunochemistry to cell biology. However, what was needed to actually prove the theory was a delineation of the molecular basis for the enormous size of the repertoire. Two lines of investigation converged to provide this evidence: the sequencing of Ig proteins followed by the sequencing of Ig genes. In 1965, Hischmann and Craig sequenced two Bence-Jones proteins and found that there was conservation of the amino acid sequence at the C-termini but considerable diversity at the N-termini.[100] In the ensuing years, sequence data on a number of myeloma proteins became available, and in 1970, Kabat and Wu applied statistical criteria to analyze sequencing data from 77 Ig chains. They identified three regions within the 107 residues comprising the light chain variable region that demonstrated a still further degree of variability (ie, hypervariability). They hypothesized that these regions of extreme diversity represented the complementarity-determining residues and suggested by analogy with prokaryotes that they arose through episomal incorporation into the light chain locus by a recombination event.[101] If this, indeed, was the underlying explanation for antibody diversity, then there would have to be a very large number of episomal elements to account for a diverse repertoire. This was a different view than the one taken by Dreyer and Bennet 5 years earlier, who proposed that variable region genes that had undergone duplication and spontaneous mutation throughout evolution underwent a “genetic scrambling” event, resulting in their juxtaposition to the constant regions of the Ig genes. They even suggested the involvement of enzymes involved in DNA repair as contributing to such an event.[102] Thus, two opposing viewpoints began to emerge to account for the generation of diversity (or GOD, as playfully described by Richard Gershon): somatic mutation of a few genes, as suggested originally by Burnet,[94] and supported by Weigert and Cohn's sequencing data of the mouse λ light chain locus,[103] or somatic recombination among many duplicated genes within the germline, as proposed by Dreyer and Bennet[102] and later refined by Edelman and Gally[104] and Hood and Talmage.[105] In the years that followed, various teleologic arguments were proposed to support one theory over the other. In 1976, at least a partial resolution was provided by Hozumi and Tonegawa, who provided evidence that the V_K and C_K loci from embryonic DNA rearrange to form a contiguous polypeptide in mature lymphocytes.[106] The advent of molecular cloning led to direct proof that the variable and constant regions of the light chain gene had undergone rearrangement at the DNA level.[107,108] This was followed by the demonstration by Weigert et al.[109] that the Ig V_K region in the mouse is encoded by multiple V, J, and C regions joined at the DNA level during differentiation of individual lymphocytes. Finally, in 1980, Early et al.[110] demonstrated how V, D, and J regions of the Ig locus could recombine to generate a virtually unlimited combination of antibody specificities.

In the ensuing years, the molecular mechanisms governing VDJ recombination were

uncovered. These involved recognition of conserved sequences flanking germline V, D, and J segments, introduction of double-strand breaks, potential loss or gain of nucleotides at the coding junctions, and polymerization and ligation to complete the joining process. Many talented scientists contributed to these discoveries, including Alt, Yancopoulos, Blackwell, and Gellert.[111] This culminated in the isolation of the recombinase activating genes (RAG) by Baltimore's group.[112, 113] The dominant view that emerged from these studies largely favored the “germline-ists,” reinforced by Tonegawa's receiving the Nobel Prize in 1984. However, in yet another example of a synthesis of two apparently contradictory approximations of the truth, evidence for somatic hypermutation began to emerge.[114,115] Its importance was established when it was causally linked to the generation of B cells with very high affinity antibodies,[116] a phenomenon termed “affinity maturation.”[117]

SPECIALIZATION WITHIN THE IMMUNE SYSTEM

Division of Labor: Discovery of T and B Cells

The first person to demonstrate delayed type hypersensitivity may have been Robert Koch in 1882. On his quixotic pursuit of developing a vaccination against tuberculosis, he injected himself with spent medium from cultures of human tubercle bacilli and noted a particularly severe reaction, including systemic effects.[118] Although he was not successful in developing a vaccine against tuberculosis, he recognized the diagnostic potential of this procedure. It was not until 1942, and then later in 1945, that Landsteiner and Chase demonstrated that cells from guinea pigs previously immunized with *Mycobacterium tuberculosis* or hapten would transfer reactivity to a naïve recipient when challenged with the immunogen.[119,120] This was the first demonstration that cells, rather than antibody, transmitted specific immunity, a finding that in some ways vindicated Metchnikoff's cellular focus.

The identity of the cells mediating the transferred hypersensitivity was unknown. Based on experiments performed decades earlier, as Silverstein has noted,[121] James Murphy at the Rockefeller Institute argued that lymphocytes were important in the host resistance to tuberculous infection. Murphy used mice exposed to x-rays or splenectomized to manipulate lymphocyte numbers and showed that conditions that would have been predicted to deplete lymphocytes resulted in early death of the mice due to disseminated tuberculosis.[122] In earlier papers, Murphy also showed more directly that lymphocytes were important in graft rejection in transplanted chick embryos. Why were these seemingly important observations ignored? Was it because the

experiments relied to a certain extent on inference, rather than direct proof that lymphocytes were key mediators of tuberculous immunity? Most likely, it was a combination of events: The lingering vestiges of the confrontation between the cellularists and the humoralists and the fact that there was little conceptual basis for understanding how a small innocuous-appearing cell type could participate in immunity.

In a completely independent line of investigation, it was known for some time that certain strains of mice had a high spontaneous rate of developing lymphocytic leukemia. In attempting to explain the finding that thymectomy of 2-month-old mice failed to develop leukemia, Jacques Miller found that neonatally thymectomized mice appeared ill and revealed

a marked deficiency of lymphocytes in blood and lymphoid tissues. Furthermore, these mice failed to reject allografts or xenografts.[123] However, not all areas within lymphoid organs were depleted of lymphocytes, consistent with “thymic-dependent” (paracortical areas of the lymph nodes and periarteriolar sheaths of the spleen) and “thymic-independent” regions (follicles and medullary cords). Miller concluded that the thymus was important for the development of a subset of lymphocytes important in allograft rejection.

The involvement of lymphoid cells in antibody production was considered likely in the 1940s, mainly due to “guilt by association.” For example, Erich and Harris injected antigens, such as typhoid vaccine and sheep erythrocytes, into the feet of rabbits, and then compared the formation of antibody to histologic changes in the draining lymph nodes.[124] Similar experiments were performed using intravenous injection of antigen, and the appearance of antibody and plasma cells in the spleen appeared to be correlated.[125] However, actual proof for the involvement of B cells in antibody production was quite accidental.[126] In 1952, Bruce Glick, a young doctorate student at Ohio State University, was investigating the function of an obscure avian organ, the “bursa of Fabricius.” He removed the organ, which did not result in a discernable phenotype. A fellow graduate student asked to use one of Glick's birds to develop an antibody against *Salmonella* and found that the bursectomized chicken failed to make antibodies. This led to the publication that eventually appeared in *Poultry Science* describing the role of the organ in the generation of bursa-derived or “B” cells.[127] As there was no anatomic equivalent of the bursa in mammals, an exhaustive search finally revealed the bone marrow origin of these cells. It was also found that thymusderived cells, later named “T cells,” were needed to “help” B cells produce antibody.[128,129,130] These distinctions were further clarified when Cooper et al.[131,132] showed that T cells were required for delayed-type hypersensitivity and graft versus host reaction. Thus was borne one of many central dichotomies that Mazumdar[133] has argued drives the field of immunology.

In 1957, Gowans[134] cannulated the thoracic duct of rats and measured the rate of flow of the lymph. He suggested that “the continuous entry of living lymphocytes into the blood may be essential for maintaining the output of lymphocytes from the thoracic duct.” He later showed that intravenous transfusion of radiolabeled lymphocytes resulted in appearance of the radiolabeled cells into the thoracic duct, thus defining the continuous recirculation of lymphocytes from the lymphatics to the blood.[135] In retrospect, these experiments were critically important in understanding how lymphocytes are constantly patrolling the lymphatics, vigilantly on the lookout for antigen.

TRANSPLANTATION BIOLOGY AND THE PURSUIT OF IMMUNOLOGIC TOLERANCE

The history of transplantation began many hundreds of years ago and was vigorously pursued by surgeons and tumor researchers during the early part of the 20th century. These events are well summarized in several texts, notably Brent's *A History of Transplantation Immunology* and Silverstein's *A History of Immunology* . The influence of these early workers, particularly Carrel's, on the surgical aspects of transplantation is clear, but their impact on the field of transplantation immunology was limited because the conceptual framework of immunology was still rudimentary. Analogous to the role that smallpox had in

catalyzing early vaccine development, large-scale bombing campaigns in World War II resulted in many civilian and military burn victims who required skin grafting, compelling surgeons to develop better techniques to avoid homograft rejection. The British zoologist Peter Medawar was assigned to the War Wounds Committee of the Medical Research Council. In 1943, Medawar and Gibson[136] published “The fate of skin homografts in man” based on a single burn victim who received multiple “pinch grafts” of skin. The authors concluded that autografts succeed, whereas allografts fail after an initial take, and that the destruction of the foreign epidermis is brought about by a mechanism of active immunization. Medawar returned to Oxford University to study homograft rejection in laboratory animals and proved that this was an immunologic phenomenon. Medawar[137] concluded that the mechanism by which foreign skin is eliminated belongs to the category of “actively acquired immune reactions.” Shortly after this publication, Ray Owen[138] published the provocative finding that dizygotic twin calves, who share a common circulatory system in utero, exhibit chimaerism with respect to their twin's erythrocytes and fail to produce antibodies against each other's erythrocytes. This led Burnet and Fenner[139] to predict that introduction of a foreign antigen early enough in life would fail to elicit an immune response. Medawar reasoned that the successful exchange of skin grafts between dizygotic calves would verify this hypothesis. Together with his postdoctoral fellow Rupert Billingham, he performed a series of grafting experiments that provided direct support for this model.[140]

> Mammals and birds never develop, or develop to only a limited degree, the power to react immunologically against foreign homologous tissue cells to which they have been exposed sufficiently early in fœtal life … this phenomenon is the exact inverse of “actively acquired immunity”, and we therefore propose to describe it as “actively acquired tolerance.”

At the same time, Milan Hašek[141] in Prague demonstrated successful parabiosis of chick embryos derived from two

different strains. Hašek's stated motivation to perform the experiment was to determine whether exchange of blood in the different chick strains induced a “mutual metabolic assimilation between parabionts,” rather than to induce a state of immune tolerance. It has been suggested that Hašek's real motivation for the experiment was to advance the Lysenkoist genetic doctrine to appease the local communist regime in Czechoslovakia.[142] Regardless, the result has been reinterpreted as an example of the induction of immunologic tolerance. In 1960, Medawar shared the Nobel Prize with Burnet “for the discovery of acquired immunologic tolerance.”

Mechanism versus Metaphor: “Self/Nonself” Discrimination

Medawar's experiments and Burnet's formulation of the clonal selection theory occurred at a critical juncture in the history of the evolving field of immunology. Had their development been dyssynchronous, it is doubtful that much progress would have been made on either front. Indeed, transplantation experiments had been performed earlier in the century with little insight as to why allografts failed. The viewpoint that Burnet espoused, that the function of

the immune system is to distinguish “self” from “nonself,” has proved to have enormous heuristic value ever since its formulation more than 60 years ago. Is this distinction mostly metaphorical, as suggested by Tauber,[143] or does it reflect a more concrete generative reality? To begin to address this question, it is necessary to briefly review a parallel development in immunology, the discovery of the components of the immune system that define molecular self-hood.

Looking Under the Hood: The Discovery of the Major Histocompatibility Complex

The clonal selection theory represented a conceptual breakthrough in the history of immunology, but it did not explain how lymphocytes actually recognize antigen. These insights would eventually come from three sources over the span of 20 years: studies of the genetics of graft rejection in inbred strains of mice by George Snell in the 1940s, studies of the agglutination of white blood cells by sera from transfused patients by Jean Dausset in the 1950s, and studies of the immune response to simple antigens in guinea pigs by Baruj Benacerraf in the 1960s. Snell was interested in identifying genes that controlled the ability of mice to resist tumor transplants. He pioneered the use of congenic mice, which are genetically identical except for a single region or locus. Snell observed that tumor grafts were accepted between inbred mice but not between mice of different strains. The same was true for normal tissues. Snell termed the underlying genes “histocompatibility” genes. In collaboration with Peter Gorer, who had prepared antisera that reacted with cells from one mouse strain but not another, Snell established that the major histocompatibility locus corresponded to a reactivity that Gorer had designated antigen II, renamed locus histocompatibility 2 or H-2.[144] Two loci within this region, designated K and D, carried genes specifying antigens involved in triggering graft rejection. In the 1950s, Dausset observed that patients who had received many blood transfusions produced antibodies that could agglutinate white blood cells from donors but not the patient's own cells. Several of the patients produced antibodies against the same antigen.[145] Subsequent family studies indicated that a genetically determined system, named “human leukocyte antigen” (HLA) system, was found to be the ortholog of H-2 in the mouse. Dissection of the human system would take many years, during which time transplantation surgeons made use of the emerging findings to assist in tissue typing. In the 1960s, Baruj Benacerraf, an immunologist working at New York University, noticed that some outbred guinea pigs responded to simple antigens by developing delayed hypersensitivity reactions upon challenge while others did not, and that this was under genetic control. He termed these genes “immune response genes.” McDevitt and Sela observed similar genes in mice, and McDevitt showed that they are encoded in the MHC.[146] In 1980, Benacerraf, Snell, and Dausset shared the Nobel Prize “for their work on genetically determined structures of the cell surface that regulated immunologic reactions.”

Of course, the identification of the HLA region and the subsequent cloning of the genes encoded in this region proved to be landmarks in the history of immunology. Besides providing a molecular identity to the key orchestrators of antigen presentation to T cells, the identification of specific MHC alleles with autoimmune diseases has led to important insights into their pathogenesis. Ironically, the first recognized HLA association with human disease, HLA-B27, which was associated with the disease ankylosing spondylitis,[144,147] has

generated perhaps more heat than light, as there is no definitive mechanism that explains how HLA-B27 is linked to disease pathogenesis, underscoring that the HLA locus, which took so many years to uncover at the genetic level, still has much to teach us.

The Discovery of Major Histocompatibility Complex Restriction as the Molecular Basis for "Self/Nonself" Discrimination

It was known for several years that cooperation between T and B cells occurred in syngeneic or H-2-compatible animals.[148,149,150] In 1972, Kindred and Shreffler[151] showed that even in nude mice, cooperation between T and B cells required H-2 compatibility; however, the exact role of that H-2 molecules played in this process and the nature of the T- and B-cell interactions remained a mystery. A valuable clue was provided by experiments by Rosenthal and Shevach,[152] who demonstrated that efficient presentation of antigen by antigenpulsed macrophages to T cells also required histocompatibility matching. In 1974, Doherty and Zinkernagel sought to understand the role of T cells in the immune response to viral meningitis. They theorized that it was the strength of the immune response that caused the fatal destruction of brain cells infected with this virus. To test this theory, they mixed virusinfected mouse cells with T lymphocytes from other infected mice. The T lymphocytes did destroy the virus-infected cells, but only if the infected cells and the lymphocytes came from a genetically identical strain of mice. T lymphocytes would

ignore virus-infected cells that had been taken from another strain of mice.[153] Further experiments strongly suggested that the same TcR that recognizes viral antigen also recognizes the MHC molecule.[154] The implications of the Zinkernagel-Doherty experiment were profound. First, it established the principle of MHC restriction: T cells recognize antigen only in the context of MHC molecules. Second, the experiment established that cytotoxic TcR-bearing cells must recognize two separate signals on an infected cell before they can destroy it. One signal is a fragment of the invading virus that the cell displays on its surface and the other is a self-identifying tag from the cell's MHC molecules; it was felt likely that the same TcR probably recognizes both. Thus, the experiment pointed to the identity of the molecular structure that constituted immunologic "self"—it is the MHC molecule—and therefore a virus-infected cell bearing MHC molecules was likely to constitute "altered" or "nonself." Finally, the fact that MHC is highly polymorphic implies that any given allelic product will be capable of forming a different altered self from other MHC allelic products; thus, the specific identity of the MHC molecule itself determines the strength of the immune response.

Although the Zinkernagel-Doherty experiment answered many questions, the steps between encounter of antigen by an antigen-presenting cell (APC) and presentation of that antigen to a T cell was somewhat of a "black box." It had long been thought that intact antigen was presented to T cells, but it was not until 1981 that Ziegler and Unanue[155] showed that an antigen processing event was necessary for I-region (MHC class II)-restricted antigen presentation to T cells. This appeared to require antigen processing in a lysosomal-like compartment.[156] Peptide loading is a complicated affair, involving prior binding of MHC class II by an "invariant chain" to prevent premature loading of incompletely folded proteins in the endoplasmic reticulum.[157,158] Peptide loading requires proteolysis of the invariant chain.[159,160] Mellman and colleagues would go on to show that the actual compartment in

which antigen loading occurred is uniquely specialized for antigen presentation in B cells[161] and DCs.[162] In a landmark paper, Unanue and colleagues purified MHC class II molecules from 10^{11} B cells and showed a 1:1 binding with peptide and MHC class II I-A^d, but not I-A^k,[163] thus demonstrating MHC restriction at the biochemical level.

The processing events required for MHC class I restriction seemed more elusive, as some of the molecular components required for this were not known at the time. It was suspected that an intracellular proteolytic event was needed to process antigen, but that was not proven until 1994 when Rock et al.[164] showed that proteasomal inhibitors blocked degradation of most cell proteins and subsequent generation of peptides presented on MHC class I molecules. The actual mechanism by which peptides generated in the cytosol gained entry into the secretory compartment was provided in 1990, when four groups announced the identification of a member of a family of ABC transporters, called "TAP," which provided this function.[165,166,167,168] Finally, in 1987, Bjorkman et al.[169,170] demonstrated that the antigen in question was a peptide actually bound to the groove of the MHC class I molecule.

SPECIALIZATION WITHIN THE IMMUNE SYSTEM II.

Discovery of B- and T-Cell Antigen Receptors

The discovery of the B-cell receptor (BcR) for antigen began with Ehrlich,[171] when he proposed that cells that produced "amboceptors" expressed them at their surfaces; in fact, Ehrlich's drawings of amboceptor-producing cells emphasized this point (see Fig. 2.2). Evidence for the existence of surface Ig was provided by indirect immunofluorescence and autoradiography, in which immunoreactivity against a single class, IgM, was observed in 1970.[172,173,174,175] The function of the BcR was uncovered by Rock and Lanzavecchia, who showed that MHC class II-restricted antigen presentation by hapten-specific B cells was enhanced 10^3- to 10^4- fold by specific binding, endocytosis, and loading of peptide antigen onto MHC class II molecules.[176,177] In 1952, Colonel Ogden Bruton at the Walter Reed Army Hospital was caring for the 8-year-old son of a general. The boy had had recurrent pneumococcal infections, including bacteremia, but he recovered with antibiotics. Bruton noted that the boy did not mount an antibody response to pneumococcal vaccination and upon testing his serum using Tiselius apparatus, the electrophoretic pattern revealed a complete absence of γ-globulins.[178] Other cases appeared, and it was soon clear that the defect was X-linked. This is often cited as the first description of a primary immunodeficiency. It was not until 1993 that the molecular defect of agammaglobulinemia was uncovered. It was due to deficient expression of a tyrosine kinase, named *Bruton tyrosine kinase*.[179,180] This discovery highlights another function of the BcR, which is to help provide signals for B-cell maturation. Without its expression, B-cell development is blocked beyond the pre-B-cell to immature B-cell stage.[181]

The discovery of the TcR for antigen was likened to the hunt of the apocryphal Snark,[182] according to Mak,[183] except the TcR was finally captured after a hunt that lasted over 20 years or more, depending on one's perspective. The hunt was characterized by extended periods of uncertainty, when it was not clear whether the same receptor bound to antigen and

MHC simultaneously, whether two separate TcR proteins bound MHC and antigen separately (the “intimacy model”), or whether one TcR molecule bound MHC molecules altered by antigen. It was also not known what form the antigen would actually take, and many considered it likely that the binding was strictly analogous to the binding of antibody and antigen, amounting to an “IgT-antigen” interaction.[184] The existence of the TcR was deemed within reach when a clone-specific monoclonal antibody (mAb) against a murine lymphoma was isolated.[185] Finally, in 1984, using differential hybridization approaches, Davis and Mak independently announced the cloning of the β-subunit of the mouse and human TcR, respectively.[186,187] This was soon followed by the cloning of the α-subunits of the mouse and human TcRs,[188,189,190,191] and the identification of other subunits of a different type of TcR, the γδ TcR.[192] Eventually, the crystal structures of pMHC combinations demonstrated that contact between TcR and pMHC was dominated by direct TcR-MHC contacts, rather than TcR-peptide interactions.[193,194,195] Beyond the initial recognition stage, signaling through the

TcR, as in the BcR, is a highly regulated process tuned to recognize cues from pMHC that govern positive and negative selection.[196] There is little doubt that had Medawar survived, he would have appreciated how the clonal selection theory has remained a driving force underlying the molecular details of antigen receptor signaling.

Discovery of Distinct Immunoglobulin Classes

Ehrlich's terminology for antibodies changed depending on the context; he sometimes referred to “amboceptors,” or immune bodies, and at other times he used the more familiar term “antikörper.” Regardless, he did not know the chemical makeup of antibodies, though he recognized that they must contain separate binding sites for antigen and complement. Elucidation of the actual structure of antibodies would have to await the seminal work of Porter and Edelman in the 1960s; however, the actual discovery of specific antibody classes, or isotypes, took place over many years. The first class of antibodies to be discovered was IgG. In 1939, Tiselius and Kabat immunized rabbits with ovalbumin, then absorbed a portion of the resulting antiserum with ovalbumin. When they applied samples of the unabsorbed and antigen-absorbed antisera to electrophoresis, they observed a marked decrease in the amount of protein that migrated in the γ region of electrophoretic mobility, farthest away from the fast-migrating albumin peak. They called the antibody that corresponded to this fraction “γ-globulin.”[197]

The next antibody class to be discovered was a result of an observation by the Swedish oncologist Jan Waldenström. He described two patients with oronasal bleeding, lymphadenopathy, low serum fibrinogen, and increased lymphoid cells in the bone marrow.[198] With the help of a colleague in Svedberg's laboratory, he noted that serum from these patients contained an abnormally large amount of homogeneous globulin with sedimentation coefficients corresponding to a molecular weight of more than one million. Waldenström thought this corresponded to a preformed large molecule, which became known as macroglobulin. Proof that a macroglobulin possessed antibody activity was finally provided in 1967,[199] which ultimately led to its current name, IgM.

IgA was discovered by Gugler et al.[200] who isolated IgA from human milk, and Heremans et al.[201] who isolated IgA from human serum. It was later found in high concentrations in all exocrine secretions[202] and further characterized by Tomasi and Zigelbaum,[203] who suggested that IgA plays an essential role in mucosal immunity. It was not until 1984 that the mechanism of secretion of IgA and IgM across epithelial barriers was uncovered; it was shown to depend on interactions of an Ig-associated polypeptide, the J chain, with a glycoprotein on the surface of epithelial cells, referred to as the "polymeric IgA receptor."[204] This receptor mediated transcytosis of secretory IgA and IgM.

The discovery of IgE had a particularly long gestation period and is well summarized in a review.[205] It began with a report by Prausnitz, in 1921, who injected his forearm with serum from a coworker allergic to fish (and coauthor on the ensuing paper); this was followed by a wheal and flare upon further injection of fish extract.[206] This property was expected for a class of antibodies termed "reagins" (from German *reagieren*, to react). IgE was identified as a specific immunoglobulin class by Teruka and Kimishige Ishizaka in 1966[207,208] and was shown to be increased in sera from asthmatics.[209] In 1993, Kinet and colleagues demonstrated that its high affinity receptor, FcεRI, was required for anaphylaxis.[210]

IgD is the most recent Ig subclass to be recognized. It was discovered from a patient with multiple myeloma.[211] Surface IgD is coexpressed with IgM on mature B cells. The function of IgD is not well understood, although the abundance of IgM- IgD+ B cells in the human upper respiratory mucosa suggests that it is likely involved in mucosal immunity.[212]

Because IgA and IgE were isolated based on their predominant location (IgA) or function (IgE), their role in the immune response was relatively easy to decode, but the relationships between the different Ig classes was unknown. It was discovered in 1963 that the early immune response was initiated by the rapidly sedimenting (19S) antibody, later shown to be IgM, followed by the production of a more slowly sedimenting (7S) antibody, now known to be IgG.[213,214] Although production of the different Ig classes was initially interpreted as being due to the participation of different cells, it was later shown that single clones of B cells were capable of producing multiple isotypes[215,216,217] in a process called class switch recombination (CSR). The enzymology of CSR was worked out much later by Alt, Nussenzweig, Honjo, and others, who showed that it required DNA repair enzymes[218,219] and activation-induced cytidine deaminase (AID). AID is mutated in an autosomal recessive form of the primary immunodeficiency, hyper-IgM type 2, in which afflicted patients are deficient in CSR and somatic hypermutation, consistent with an important role for AID in both processes.[220,221,222]

Discovery of Antibody Effector Functions: Fc Receptors

Almoth Wright was the first to recognize the importance of antibody-mediated effector functions other than neutralization or complement-mediated lysis. His studies were ignored for a long time, and it would take many years to appreciate the importance of the "other end" of the antibody molecule. Since the 1970s, studies on the effector functions of the Fc portion of IgG made extensive use of several in vitro model systems, often involving

phagocytosis.[223,224,225,226,227,228,229,230,231,232] The eventual cloning of receptors for the Fc portion of IgG (FcγRs) by Ravetch and et al.[233] revealed many similarities and some differences, but these early studies did not reveal how the receptors transduced signaling events, such as phagocytosis. It was not until 1989 that Michael Reth[234] noted a short tyrosine-containing sequence in common with several antigen receptors, including subunits of the TcR, the BcR,[235] and Fcγ and Fcε receptors[236] that the signaling function of FcγRs was understood in a larger context. Following receptor engagement, tyrosine residues within this consensus sequence, subsequently named "immunoreceptor tyrosine-based activation motif" (ITAM), become phosphorylated

by Src family tyrosine kinases and serve as docking sites for Syk tyrosine kinase,[237] or ZAP-70, in the case of the TcR. The membrane-associated tyrosine kinases become activated and phosphorylate substrates that further convey downstream signals. Absence of ZAP-70 leads to a severe combined immunodeficiency disease (SCID).[238] These studies uncovered the central role of nonreceptor tyrosine kinases in the immune system, which appeared to explain in large part how antigen and Fc receptors evoke calcium signaling,[239] degranulation,[240] phagocytosis,[229,241,242] and antibody-mediated cellular cytotoxicity.[243] Furthermore, the ITAM-containing γ-chain[244] that is associated with FcγRs is required for immune complex-mediated glomerulonephritis in mice.[245] Thus, it is likely that the hypersensitivity phenomena originally observed by von Pirquet at the beginning of the 20th century, and later attributed to immune complexes by Dixon et al.,[80] has a similar pathophysiology, at least in part.

THE HERMENEUTICS OF LYMPHOCYTE ACTIVATION

Evolution of Early Lymphocyte Activation Models

By the late 1950s, it became clear that lymphocytes were highly adept at interpreting or translating environmental cues and responding by maintaining a state of activation or tolerance. The focus of immunology had shifted from identifying the relevant cell types involved in the immune response to discovering what can be viewed as the "hermeneutics" (a term derived from the Greek ἑρμηνεύω, "translate" or "interpret") of lymphocyte activation. Based on the clonal selection theory, Burnet[246] proposed that activation or tolerance was the result of an antigen stimulating a single cell, depending on the age of the organism. This was in keeping with experiments in which tolerance was induced by exposing antigen during fetal life or shortly thereafter.[138,140,141] In 1959, Lederberg[247] modified this model to describe activation or tolerance as occuring depending on the age of the cell rather than the organism.

> If an antigen is introduced prior to the maturation of any antibody-forming cell, the hypersensitivity of such cells, while still immature, to an antigen-antibody reaction will eliminate specific cell types as the arise by mutation, thereby inducing apparent tolerance to that antigen.

However, the model could not account for several observations, among which were that the

dosage and specific form of the immunogen, such as a hapten, could also influence the outcome of the encounter. This led Talmage and Pearlman[248] to propose an alternative model in which antigen alone would induce tolerance, but aggregated antigen, perhaps associated with complement, could induce an additional nonspecific stimulus to trigger clonal expansion. The suggestion of the need for a second "nonspecific" stimulus, and particularly complement, was remarkably prescient in light of the finding that complement can provide exactly such a stimulus to amplify immunogenicity by 10^3- to 10^4- fold.[249] In 1968, and later revised in 1970, Bretscher and Cohn[250,251] provided a variation on this model, in which a thymus-derived antigen-sensitive cell-bound antigen containing at two separate sites, one for a receptor on the "antiantigen-sensitive" cell and another bound to a "carrier" antibody. If the second site on the antigen was occupied by antibody, then that would deliver a signal to the "antiantigen-sensitive" cell to induce immunity. In contrast, if the site remained unbound, the antigen would induce tolerance. In this model, two cells were involved, but they were separated in time and space. Among the problems with the model was its circularity: What would provide the stimulus for the production of the carrier antibody in the first place? Other theories were proposed to explain the phenomenon of alloreactivity. The most intriguing of these was provided by Lafferty and Cunningham,[252] who proposed the existence of an "antigen bridge," which was now provided by another cell to provide "Signal 2." When MHC restriction was described in 1977, this model was modified somewhat to include the provision that the second signal was triggered by MHC on the stimulator cell[253] (Table 2.1).

Discovery of Costimulation: T Cells as Beneficiaries

By 1975, models for lymphocyte activation had matured, but many details remained sketchy, particularly the identity of the elusive Signal 2. In 1987, Jenkins and Schwartz utilized a system designed to "convert" Ag-specific T-cell clones into suppressor cells. They used a model system in which T-cell clones against a pigeon cytochrome peptide were incubated with ethylene carbodiimide-fixed Ag-pulsed splenocytes as APCs. They obtained the surprising result that the T-cell clones became unresponsive to subsequent restimulation with untreated APCs plus peptide, although they remained viable and proliferated in response to interleukin (IL)-2.[254] This showed that Ag-specific unresponsiveness could be induced in the absence of suppressor cells and that stimulation with cognate antigen alone was insufficient to induce proliferation; the missing Signal 2 was defined as "costimulation" and ultimately identified as cluster of differentiation (CD)28-mediated signaling by Allison and colleagues.[255] Since this seminal discovery, an entire family of CD28-like molecules in T cells and their cognate ligands on APCs has been identified,[256,257] which includes an inhibitory member of the family, cytotoxic T-lymphocyte antigen-4.[258] This has led to the clinical development of cytotoxic T-lymphocyte antigen-4-Ig, an inhibitor of CD28,[259] which was approved for the treatment of rheumatoid arthritis by the U.S. Food and Drug Administration in 2006 and by the European Medicines Agency in 2007.

Discovery of the Mechanism of B-Cell Help

It was known since the 1960s that T cells were needed to "help" B cells produce antibody, but the molecular nature of that help was unknown.[129,260] Experiments had suggested that T-cell help required cell-to-cell contact, implying the existence of the involvement of cell

surface receptor-ligand interactions. Agonistic antibodies against CD40 drove B cells into cell cycle, raising the possibility that a cognate ligand on T helper cells participates in the mechanism of

T-cell help. Using a CD40-Ig fusion protein, murine CD40L was cloned.[261,262] Use of a mAb against the human CD40L confirmed that expression was restricted to mantle and centrocytic zones of lymphoid follicles and the spleen periarteriolar lymphoid sheath in association with CD40+ B cells,[263] which is the distribution that might be expected for a highly localized signal to provide T-cell help to B cells. Indeed, in 2000, the specific type of T cell that provided CD40L-mediated help to B cells, now called a T follicular helper cell, was identified by its unique anatomic location and expression of a homing receptor, CXCR5.[264,265] In 1993, four groups independently announced that defects in the CD40 ligand gene are responsible for X-linked hyper-IgM syndrome in which affected adults express elevated levels of IgM and defects in class switching.[266,267,268,269]

TABLE 2.1 Different Models of Lymphocyte Activation

Basis of Immune Recognition	Signals	Components	Conditions for Tolerance	Authors
Self versus nonself	1	B cell/Ag	Immature organism	Burnet, 1959230
	1	B cell/Ag	Immature Ab-forming cell	Lederberg, 1959231
	2	B cell/Ag-Ab + complement	Absence of bound Ab/complement	Talmage and Pearlman, 1963232
	2	Humoral Ag-sensitive responder cell/bivalent Ag/carrier antigen-sensitive thymusdependent cell	Absence of carrier Ab or lack of	Bretscher and Cohn, 1968,234 1970235
	2	Signal 1: Ab-bound responder cells + Ag	Absence of Signal 2	Lafferty and Cunningham, 1975236

		Signal 2: Stimulator cell + Ag		
Noninfectious self versus infected nonself	2	Signal 1: Lymphocyte + MHC/Ag Signal 2: PAMP+ APC	Absence of Signal 2	Janeway, 1989390
Danger	3+	“Signal 0”: Alarm Signal 1: (Th cell-APC-Ag; Tc-infected cells; B cell/Ag; Signal 2: Help (Th to B) or costimulation (APC to T)	Absence of “Signal 0”	Matzinger, 1994451

Abbreviations: Ag, antigen; Ab, antibody; APC, antigen-presenting cells; MHC, major histocompatibility complex; PAMP, pathogen-associated molecular pattern; Th, T helper.

T-Cell Subsets and T-Cell Signaling Paradigms

The widespread use of mAbs since their discovery by Köhler and Milstein resulted in the production of many antibodies directed against T cells that had nonoverlapping patterns of expression. Much of this early work was done by Schlossman, Reinherz, and colleagues.[270,271,272,273,274,275] In most cases, the antibodies themselves identified T cells with specific functions; and in 1987 and 1988, two groups found that the molecules recognized by two of these mAbs, denoted by their cluster designations, CD8 and CD4, mediated adhesion to MHC class I and II, respectively.[276,277] CD4 and CD8 were shown to bind to nonpolymorphic regions of MHC molecules,[277] suggesting that they might stabilize otherwise weak interactions between TcR and pMHC. Later experiments demonstrated that the cytosolic domains of these coreceptors were able to bind, and recruit, the nonreceptor Src family tyrosine kinase Lck.[278,279] It was not known at the time what the relevant substrates of Lck might be, but in 1989, at about the same time that the ITAM consensus sequence was identified, two groups showed that Lck was capable of phosphorylating subunits of the CD3 complex and the ζ subunits of the TcR.[280,281] Following the discovery of another key tyrosine kinase associated with the ζ subunit of the TcR, ZAP-70, by Chan and Weiss,[238] a complicated cell signaling paradigm began to emerge in which “Signal 1,” delivered by pMHC expressed on either APCs or virus-infected cells, is conveyed by a series of phosphorylation events, leading to phosphorylation of phospholipase C-γ, triggering increases in cytosolic calcium and dephosphorylation of a key transcription factor, NFAT. The elusive machinery of “store-operated calcium channels” required for calcium-based

signaling[282] was finally elucidated in 2006.[283,284,285,286] Defects in this pathway in lymphocytes lead to a form of SCID.[286] Dephosphorylated NFAT, alone or in conjunction with AP-1, translocates to the nucleus where it binds to sites on the promoters of key genes such as IL-2, culminating in T-cell proliferation.[287] The initial interaction between TcR-bearing T cells and pMHC-bearing APCs induces a cooperative series of protein-protein interactions in a spatially delimited fashion (the "immunologic synapse") that facilitates signaling events.[288] We now know that signal transduction by the TcR shares common elements, and in some cases identical kinases and substrates, with signaling by other ITAM-containing receptors, such as the BcR, and Fc receptors or IgG and IgE.[289,290,291] Recent data suggest that similar mechanisms governing "immunologic synapses" in T cells may also be characteristic of "phagocytic synapses,"[292] underscoring a further degree of conservation of ITAM-based pathways. It is ironic that lymphocytes, which had appeared morphologically uninteresting to so many biologists until the latter part of the 20th century, would share so many characteristics in common with their visually more intriguing cousins.

Cell to Cell Communication: Mice Have a Tale to Tell

The first cytokine to be characterized is credited to Isaacs and Lindenmann,[293] who studied an influenza virus-induced factor from chick chorioallantoic membranes that blocked a second viral infection; this factor was called "interferon" (IFN). It was not purified to homogeneity until 20 years later.[294,295] Type I IFNs, which include IFNα and IFN-β, and type II IFN, IFNγ, bind to distinct receptors. A major advance in our understanding of cytokine signaling occurred in 1992, when Schindler et al.[296] demonstrated the tyrosine phosphorylation and nuclear translocation of a complex of four proteins, named IFNα stimulated gene factor 3. IFNα stimulated gene factor proteins were isolated from nuclear extracts derived from over 10^{10} cells, followed by sequencing and cloning of their genes; two of these proteins are members of what are now known as STAT proteins, which were shown to be substrates for members of the Janus-associated kinase family.[297,298,299,300,301,302,303] The most common severe immunodeficiency attributable to defective cytokine signaling is X-linked SCID, which is due to mutation in the common γ_C gene that is shared by receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21.[304] Mutations in Janus-associated kinase 3 were found in two patients with a severe form of SCID indistinguishable from X-linked SCID.[305,306] Dominant-negative mutations in STAT3, which is important in the signal transduction of IL-6 and IL-10, were found to be responsible for hyper-IgE syndrome, a disease associated with highly elevated serum IgE, recurrent staphylococcal skin infections and pneumonia, and skeletal abnormalities.[307,308]

Much of our knowledge of this pathway, as well as many other components of the immune system, is due to the use of gene targeting in mice. In 1985, Smithies et al.[309] introduced a short DNA sequence from the human beta-globin locus into an erythroleukemia cell line and were able to detect a specific exchange of the beta-globin gene with the homologous sequence in about 1 in 10^3, demonstrating the feasibility of gene targeting. At the same time, Capecchi introduced DNA directly into the nucleus of a cell using a microelectrode. Capecchi

noted that multiple copies of the introduced gene were integrated into the host cell's chromosome through homologous recombination. These studies established the potential for homologous recombination in somatic cells. The next major step was based on the ability of using blastocyst-derived embryonic stem cells to introduce genes into the germline of the mouse.[310,311] By injecting blastocysts with cultured embryonic stem cells that were infected with a retrovirus, Evans and colleagues generated chimeric mice in which retroviral DNA was detectable in both somatic and germ-line cells.[311] Eventually, Evans, Smithies, and Capecchi refined these techniques, which led to the first knockout mouse, in which the gene encoding hypoxanthine guanine phosphoribosyl transferase was deleted. The resultant phenotypic resembled that of the Lesch-Nyhan syndrome, which is characterized by mental retardation and self-mutilation.[312,313] Today, gene targeting techniques are used worldwide to study the effects of deletion or overexpression of genes important in immunity and any disease that can be reproduced in the mouse and in other species in which the technique has been used. In 2007, Smithies, Capecchi, and Evans shared the Nobel Prize "for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells."

Chemokines

If cytokines determine what cells do, then chemokines determine where cells go. The first chemokines purified were derived from platelets, although neither protein (platelet factor 4 [CXCL4][314,315] and β-thromboglobulin [CXCL7][316]) was recognized to be chemotactic when first identified. IL-8 (CXCL8) was the first chemokine to be purified and sequenced based on its chemotactic function for neutrophils,[317] and its receptors were cloned in 1991.[318,319] The word "chemotaxis" was coined by Pfeffer in 1884, who observed spermatids migrating toward a pipette containing maleic acid salts.[320] It is not clear who first explicitly observed chemotaxis of leukocytes, although several scientists, notably Schultze, Lieberkühn, Davaine, and Wharton Jones described amoeboid movement in leukocytes in the middle of the 19th century.[321] Since then, chemotaxis has been studied in vitro using various techniques, including specialized chambers that facilitate this process.[322]

The effects of chemokines on cells are complex, but a major function is to orchestrate the process of diapedesis, the process of transendothelial migration first described by Dutrochet in 1824.[323] In 1979, Hayward et al.[324] described six infants from two families whose umbilical cords were still attached at 3 weeks of age. Five of these developed severe local and disseminated infections from which four died. The molecular defect was identified by Springer et al.[325] in 1984, who showed that leukocytes from patients with the disease lacked all known β2 integrin adhesion receptors. This was eventually traced to absent or abnormal β2 subunits (CD18). Marlin and Springer[326] later described the requirement of the β2 integrin, LFA-1, to bind its counter-receptor, ICAM-1, on endothelial surfaces for diapedesis to occur normally. The function of chemokines is to provide chemotactic gradients as well as activate the integrins for tight adhesion prior to the active participation in endothelial cells in diapedesis.[327,328] Interestingly, LFA-1 was shown to be required as an accessory molecule during interaction of T cells with their cognate targets.[329]

Chemokines are critical for initiating the primary immune response as APCs, such as DCs. DCs and naïve T cells both share a common chemokine receptor, CCR7. T cells migrate through high endothelial venules, which secrete the chemokine SLC (CCL21). SLC is needed for integrin-mediated adhesion of the T cells.[330] Mice lacking expression of SLC have defects in lymphocyte and DC localization to the T-cell zone of secondary lymphoid organs.[331] The chemokine-directed "patrolling" of naïve T cells in the lymph nodes is probably the major mechanism by which the very few lymphocytes with the relevant TcR specificity remain there to interact with the antigen-loaded APCs during an immune response. Thus, chemokines are essential components of the "clonal selection" of lymphocytes.

T-Cell Polarization: The Power of Dichotomy

In 1986, Coffman and Mosmann at DNAX Research Institute were interested in determining factors that differentiated different types of T helper cell lines. One such line was capable of inducing a 100-fold increase in IgE secretion from mouse splenocytes, which was potently blocked by IFNγ.[332] When the team characterized other cloned T-cell lines, it became clear that there were very specific patterns of secretion: Some clones, which they called T_H1 cells, produced IL-2 and IFNγ, while other clones, which they called T_H2 cells, produced factors that stimulated B cells (B-cell-stimulating factor-1), mast cells, other T cells, and IgE and IgG1 secretion. All clones produced IL-3.[333] Further work, in collaboration with Paul, who first identified B-cell-stimulating factor-1,[334,335,336] clarified that B-cell-stimulating factor-1 was the same factor that stimulated mast cells and T cells, later renamed IL-4. Coffman and Mosmann[337] defined the now well-established paradigm of polarized T helper cell secretion; they demonstrated that IL-4 and IFNγ reciprocally inhibit the outgrowth of T_H1 and T_H2 cells, respectively. The later identification of transcription factors that are selectively expressed in these cells (GATA-3 and T-bet, respectively) and can even reprogram the cells to transdifferentiate toward the "opposite" phenotype further substantiated this dichotomy.[338,339] These findings had profound implications for immunologists, who began to classify diseases according to their predominant T_H cytokine profiles. Thus, tuberculosis and most bacterial and fungal infections produced a T_H1 pattern of cytokines, whereas asthma and other allergic diseases produced a T_H2 pattern of cytokines. While this reductionist view of cytokine production is no doubt an oversimplification, this simple dichotomy has proved quite robust and has helped guide the development of novel therapeutics for various diseases.

There is, however, a danger to oversimplification[340]—sometimes it is necessary to be a "fox" rather than a "hedgehog."[341] Some diseases, such as Crohn disease, an inflammatory bowel disease that was thought to be driven by the T_H1-polarizing cytokine, IL-12, is more likely driven by a related cytokine, IL-23, with which IL-12 shares a common subunit; indeed, a genome-wide association study in 2006 revealed a highly significant association of the gene encoding a subunit of the IL-23 receptor and early-onset Crohn disease.[342] Ustekinumab, a mAb against the IL-12/IL-23 p40 common subunit was approved by the U.S. Food and Drug Administration for the treatment of moderate-to-severe Crohn disease in 2006 and for moderate-to-severe plaque psoriasis in 2009.

The Language of Immunoregulation: Explaining the "Contrivances"

The concept of immunoregulation is implicit in the term "immunity" (exempt), and the history of how different immunologists have viewed immunoregulation, depending on their perspectives, is worthy of a chapter in itself. Jerne[343] hypothesized that antibodies can act as antigens and elicit an immune response against their idiotypes, which would then serve to regulate the immune response. While appealing, efforts to prove the importance of this concept have achieved limited success. In 1970, Gershon and Kondo[344] found that thymus-derived cells could specifically induce tolerance, and they and others spent many years trying to isolate antigen-specific T suppressor cells but were unable to do so. In the 1990s, the field shifted away from clonotypic suppressor cells toward T cells secreting specific tolerogenic factors, such as IL-10-secreting "Tr1 cells" and transforming growth factor-β-secreting "Th3 cells."[340,345] This was followed by the identification of cell surface markers of a subset of CD4+ cells that were required to suppress autoimmunity in mice. These turned out to be CD25 and CD5 in a CD4+ population of cells in the mouse, cells which were later called regulatory T cells. In 2001, the *FoxP3* gene was identified as the gene that was mutated in Scurfy mice, which develop severe autoimmunity as a result of a single gene mutation.[346] Similarly, mutations in the human homolog of *FoxP3* were found to be associated with the disease called IPEX (*i*mmune dysregulation, *p*olyendocrinophathy *e*nteropathy, *X*-linked syndrome).[347,348,349] Analogous to the capacity for T-bet and GATA3 to drive T_H1 and T_H2 differentiation, respectively, expression of FoxP3 was sufficient to drive the differentiation of T helper cells to a CD4+ CD25+ regulatory T phenotype.[350,351]

Central tolerance, sometimes referred to as "negative selection," is a means of shaping the repertoire that was thought likely to exist following the discovery of the thymus as an immune organ by Miller in 1961; however, proof for this was lacking. How could one prove the existence of a phenomenon that predicted the absence of a cell, rather than its presence? It was not until 1987 that Kappler et al.[352] used a mAb against a specific TcR Vβ segment to show that the mAb recognized immature thymocytes but not mature thymocytes or T cells, thus proving that negative selection occurs. Von Boehmer demonstrated a similar phenomenon using a TcR transgenic mouse model.[353] Negative selection of immature B cells was also demonstrated in the classic experiments of Nossal and Pike,[354] Sidman and Unanue,[355] and Raff et al.[356]

Clearly, the field of immunoregulation is still evolving. Novel mechanisms of immunoregulation will surely be discovered, and novel regulatory interactions will be uncovered. The role of tolerogenic DCs,[357,358] CD8+ T cells,[359,360] and myeloid-derived suppressor cells[361,362,363] are likely to play important roles and some of these will likely find their way to the clinic. Perhaps, however, we have made some progress in support of Ehrlich's contention[364]:

> The organism possesses certain contrivances by means of which the immunity reaction, so easily produced by all kinds of cells, is prevented from working against the organism's own elements.

Immune Subversion: The Case of Tumors

The idea that the immune system is capable of responding to tumors dates back to Ehrlich,[365] who predicted that cancer would occur at a high frequency in the absence of

an immune response. This theme was recapitulated by others, notably Burnet,[366] who argued that T cells would be prominent in what he termed “immune surveillance.” The field enjoyed a resurgence in 1994 when Schreiber and colleagues demonstrated that tumors expressing dominantnegative IFNγ receptors demonstrated enhanced tumorigenicity in syngeneic mice.[367] This was supported by studies using lFNγ receptor-deficient or STAT1-deficient mice.[368] Additional evidence for an active immune response against tumors is the spontaneous development of autoimmunity in some individuals with tumors; for example, paraneoplastic cerebellar degeneration occurs in individuals with a cytotoxic T-cell response to a shared antigen on tumor cells and neuronal cells.[369] The immune response to tumors is complex, with participation from cytotoxic T cells, natural killer (NK) cells, DCs, and myeloid cells.[370] The “counterresponse” by the tumors, which has been termed “escape” or “evasion,” is equally complex.[370] While it is beyond the scope of this chapter to discuss any of these in detail, several relevant points of historical interest should be noted. In 1976, a postdoctoral fellow named Mike Bevan was studying alloreactive cytotoxic T-cell responses and was able to demonstrate that spleen cells from an H-2^b/H-2^d-restricted mouse primed with H-2^b cells that differed in minor histocompatibility loci contained increased numbers of both H-2^b- and H-2^d-restricted cytotoxic CD8 T cells.[371,372] Bevan interpreted this as evidence of “cross-priming,” in which ingestion of cell-associated antigens by phagocytosis led to cytosolic entry of antigens and loading onto MHC class I molecules and then presentation to cytotoxic T cells. It was completely novel and counterintuitive, but subsequent studies validated this interpretation.[373] Recent progress has been made in our understanding mechanisms by which antigens gain entry into the cytosol from phagosomes.[374] These studies were based on key findings by Desjardins and colleagues who first demonstrated recruitment of endoplasmic reticulum membrane to nascent phagosomes.[375,376] Mice that lack CD8α+ DCs, a cell type that is adept at cross-priming, were unable to demonstrate cross-priming and were incapable of generating a cytotoxic response against West Nile virus and a highly immunogenic fibrosarcoma tumor.[377] Thus, crosspriming is important in tumor immunity.

Tumor cells are capable of circumventing the host immune response in various ways, including downregulation of MHC class I molecules, although this would potentially render them susceptible to NK-mediated killing, according to the “missing self” hypothesis.[378] Tumors elaborate a host of cytokines and growth factors that directly inhibit various components of the immune system.[370] An additional means of tumor evasion specifically bears on the meaning of “immunologic self.” Most cells in the body express a cell surface protein, CD47, which is a ligand for a receptor present on macrophages and DCs, SIRPα.[379] SIRPα interacts with tyrosine phosphatases via its cytosolic domain[380]; therefore,

corecruitment of SIRPα to tyrosine kinase-coupled signaling scaffolds is predicted to inhibit kinase-mediated signaling events. Lindberg and colleagues showed in 2000 that CD47 on erythrocytes in mice prevented their phagocytosis until levels fell below a certain level, thus CD47 served as an aging "clock" that signified a "don't eat me" signal.[381] In 2010, in a remarkable study, expression levels of CD47 on non-Hodgkin lymphoma (NHL) cells were negatively correlated with survival. Blocking anti-CD47 antibodies preferentially enabled phagocytosis of NHL cells and synergized with rituximab. Treatment of human NHL-engrafted mice with an anti-CD47 antibody reduced lymphoma burden and improved survival, while combination treatment with rituximab (anti-CD20) led to elimination of lymphoma and cure.[382] In these settings, CD47, rather than MHC proteins, serve as a marker of "self." It is somewhat counterintuitive that, at least in the case of NHL, tumor masquerading as self is one mechanism by which tumors evade the immune system. Ironically, downregulation of MHC class I molecules, the classic marker of self, is yet another mechanism of how tumor evasion of immunity.[370]

Immune Hijacking: The Discovery of Human Immunodeficiency Virus

The first description of what would come to be known as the "acquired immunodeficiency syndrome" appeared in 1981.[383] Initially described in five homosexual men, the disease soon was apparent in Haitians, transfusion recipients, infants, Africans, and female sexual contacts of infected men.[384] In just 2 years, Montagnier's team published the first paper demonstrating the presence of retroviral particles from diseased patients,[385] and a year later, Gallo's group published five papers in *Science* providing convincing evidence that this retrovirus was the cause of acquired immunodeficiency syndrome.[386,387,388,389,390] Barré-Sinoussi and Montagnier won the Nobel Prize for their discovery of human immunodeficiency virus (HIV), along with zur Hausen, for demonstrating that human papillomavirus can cause cervical cancer. Many felt that Gallo should have been awarded the prize as well.

Pneumonia due to *Pneumocystis carinii* (later re-named *P. jerovici*) was common in initial cases of acquired immunodeficiency syndrome, but patients soon presented with an array of opportunistic infectious diseases, malignancies (eg, Kaposi sarcoma and lymphoma), and even autoimmune diseases. In 1986, Maddon et al.[391] demonstrated that CD4 is an essential receptor that mediates HIV-1 entry into lymphocytes; this was followed in 1996 by the demonstration of CXCR4 and CCR5 as coreceptors for HIV-1 entry.[392] Since then, many laboratories worldwide have dedicated their efforts to uncovering HIV pathogenesis.[393,394] One of the major challenges to the immune system as well as the development of an HIV-1 vaccine is the enormous plasticity of the viral sequence due to the high error rate of reverse transcription.[393]

Intensive efforts in academia and the pharmaceutical industry have resulted in highly active antiretroviral therapy based on targeting multiple steps of viral replication, mostly focusing on reverse transcriptase and protease. A disease that was once virtually 100% fatal is now manageable, resulting in an 80% to 90% decrease in mortality rates in the United States and Europe.[393] Among the major

challenges facing immunologists and virologists is eliminating the latent reservoir of virus in resting memory CD4+ lymphocytes, developing an effective HIV-1 vaccine, and providing treatment for the 90% of infected individuals worldwide who reside in developing countries and have poor access to antiretroviral therapy.

METCHNIKOFF'S LEGACY

The Rediscovery of Innate Immunity

Arguably, the most important advance in immunology in the last 15 years has been the "rediscovery" of innate immunity. The closest term to "innate immunity" that was used at the turn of the century was "natural immunity." When the cellularists and the humoralists were debating the relative importance of cells and soluble antikörper at the turn of the century, much of the phenomena that Metchnikoff observed under the microscope represented different aspects of innate immunity. However, Metchnikoff distinguished enhanced immunity due to vaccination as a result of what we would call today an acquired immune response: "an agglutinative substance…in the…fluids of the body becomes much more developed in those of immunised animals."[395] He thought that infections could lead to an acquired immune response, but the nature of that response was to foster an enhanced response in phagocytic cells.[395]

> In certain infective diseases terminating fatally a very marked phagocytosis is observed even in susceptible animals … The acquisition of immunity against microorganisms is, therefore, due not only to the change from negative to positive chemiotaxis, but also to the perfecting of the phagocytic and digestive powers of the leucocytes.

In 1932, Fleming[396] recounted the discovery of lysozyme, which he made in 1921, "because its importance in connection with natural immunity does not seem to be generally appreciated." Although the discovery has been cited to represent another example of scientific serendipity, resulting from accidental dripping of nasal secretions from Fleming himself onto a culture plate,[397] Fleming wrote that "cultures of nasal mucus were made from a person suffering an acute cold" when the bacterolytic phenomenon due to lysozyme was observed.[396] Fleming would go on to discover penicillin, which was a bona fide example of scientific serendipity, and receive the Nobel Prize for his discovery.

Of course, lysozyme is but one of many innate immune molecules important in the early phase of host defense. Other cationic proteins include defensins, which are secreted by leukocytes and epithelial cells, and chemokines, whose tertiary but not primary structures are related to defensins. Some members of both classes have the dual role of recruiting inflammatory cells to sites of infection and killing bacteria.[398,399,400,401] Other examples of components of innate immunity include "innate immune lymphocytes," such as NK cells, NK T cells, and γδ T cells. Although it is beyond the scope of this chapter to describe these cell types except in passing, the discovery of NK cells in 1975[402,403,404] provided a cellular mechanism to account for the observation that some host immune cells recognize and kill

virus-infected or neoplastic cells lacking expression of MHC class I molecules. NK cells also provide an early, immediate source of cytokines, allowing them to rapidly respond to injury prior to maturation of the primary immune response.[405]

The "modern era" of the study of innate immunity began in 1989. In a characteristic example of his remarkable insight, Janeway[406] published an article in which he described a new model for immune recognition. Janeway argued that rather than distinguish "self" from "nonself," the immune system had evolved to distinguish "noninfectious self from infected nonself" (see Table 2.1). Janeway compared the innate and acquired immune systems. He emphasized that the former evolved to respond rapidly to "pathogen associated molecular patterns" by as yet unidentified receptors in a nonclonal fashion. Janeway was acutely aware of the significance of Freund's discovery of adjuvant[407] and the implications this had for the primary immune response. What was remarkable about Janeway's hypothesis is its intuitive appeal and its Darwinian flavor, in some ways analogous to Metchnikoff's view of phagocytosis. In addition, it had great predictive power. In a completely independent line of investigation, Jules Hoffmann became interested in exploring inflammatory pathways in *Drosophila*. Hoffman was aware of Baltimore's discovery of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), a transcription factor, originally identified as a lipopolysaccharide (LPS)-inducible transcription factor in B cells in 1986.[408] Mice rendered deficient in NF-κB demonstrated multifocal defects in immune responses, including those to LPS.[409,410] Based on his observation of similarities between the cytokine-induced activation cascade of NF-κB in mammals and the activation of the morphogen dorsal in *Drosophila* embryos, Hoffmann demonstrated that the dorsoventral signaling pathway and an extracellular toll ligand control expression of antifungal peptide gene expression. Mutations in the toll signaling pathway dramatically reduced survival after fungal infection.[411] Until that time, there was only a limited understanding of pathogen recognition in eukaryotes; the only receptor that was known to participate in the recognition of LPS was CD14, a molecule that is expressed predominantly on macrophages.[412] Shortly after publication of Hoffmann's landmark study, Janeway and Medzhitov published an article in *Nature* that described the cloning of a human homolog of *Drosophila* toll. A constitutively active mutant of human toll transfected into human cell lines induced the activation of NF-κB and the expression of the inflammatory cytokines IL-1, IL-6, and IL-8 as well as the expression of the costimulatory molecule B7.1 "which is required for the activation of naïve T cells."[413] The choice of these proteins was not accidental, and it was clear that Medzhitov and Janeway had Janeway's hypothesis of 1989 in mind. Meanwhile, Beutler et al.[414] were zeroing in on the genetic identification of a mutation in a mouse strain that was incapable of responding to LPS, a component of the cell walls of all gram-negative bacteria. Beutler and Cerami were the first to show that immunization against tumor necrosis factor, the prototypic cytokine

that is produced following administration of LPS, protected mice from lethal shock.[414] Beutler demonstrated that the codominant Lpsd allele of C3H/HeJ mice corresponded to a missense mutation in the toll-like receptor-4 gene.[415] Since these publications, a family of innate immune receptors has been defined, each responding to different ligands encountered by hosts during infection.[416] Initial interactions of innate immune receptors on APCs with

their ligands encounters are necessary for the upregulation of costimulatory molecules, without which tolerance would occur.[417] Janeway's prediction in 1989 was borne out, providing strong empirical evidence in support of his model for immune recognition (see Table. 2.1). In 2012, Beutler and Hoffman, together with Steinman, received the Nobel Prize.

The field of innate immunity has exploded since the key observations of Hoffman, Beutler, Janeway, and Medzhitov. Many other receptors and components of the innate immune system have been discovered. These include components of the complement system,[418] cell surface lectins,[419] collectins such as lung surfactant proteins[420] and mannosebinding proteins,[421] scavenger receptors,[422,423] and pentraxins.[424] Most of these participate in recognition of pathogens, including fungi,[425] bacteria,[426] and viruses such as HIV.[427] In some instances, the phagocytic cells themselves serve as the source of opsonins, such as complement.[428] A variety of innate immune receptors also recognize apoptotic and necrotic cells.[429,430] Secretion of opsonins, such as milk fat globule-EGF factor 8, can enhance uptake of targets such as apoptotic cells,[431] and the absence of this protein has been linked to autoimmunity in mice.[432] Similar results were found in other mouse strains engineered to be deficient in clearance of apoptotic cells.[433,434] The conceptual basis of these experiments was built on earlier work of Savill, Henson, and others who showed that the immunologically “silent” disposal of apoptotic debris is an active process serving to divert self-antigens toward a nonphlogistic mode of phagocytosis.[435,436,437] In the context of resolution of acute infections, a similar function is provided by the production of omega-3 polyunsaturated fatty-acid-derived “anti-inflammatory” lipids (“resolvins”), first identified by Serhan et al. in 2000.[438,439]

Cytosolic Components of Innate Immunity: Discovery of the Nicotinamide Adenine Dinucleotide Phosphate-Oxidase

In 1957, Good and colleagues described an X-linked disease in which children succumbed to chronic suppurative and granulomatous infiltrations and chronic infections.[440] Neutrophils isolated from these children showed decreased bactericidal activity, although they demonstrated normal phagocytosis.[441] However, they showed decreased hydrogen peroxide production and hexose monophosphate shunt activity.[442] In 1974, Curnutte et al.[443] identified defective superoxide anion production in children with this syndrome who also failed to reduce the dye, nitroblue tetrazolium.[444] This simple test has been widely used to diagnose what came to be called “chronic granulomatous disease.” In 1978, Segal et al.[445,446] identified the molecular defect in X-linked chronic granulomatous disease as the absence of cytochrome b. Since then, all the components of this multiprotein enzyme complex have been identified.[447,448,449,450,451,452] Of note is that the oxidase responsible for X-linked chronic granulomatous disease, now referred to as NOX2, is but one of a family of oxidases that are widely expressed and have been implicated in various disease.[453] We now know that there are multiple reactive oxygen species, many of which combine with other reactive molecules, such as nitric oxide, to generate reactive nitrogen species. Nathan and colleagues demonstrated that mice deficient in inducible nitric oxide

synthase proved highly susceptible *Mycobacterium tuberculosis*, resembling wild-type littermates immunosuppressed by high-dose glucocorticoids.[454]

In addition to its role in microbial killing, NOX2 is recruited to early phagosomes in DCs and mediates the sustained production of low levels of reactive oxygen species, causing active and maintained alkalinization of the phagosomal lumen. DCs lacking NOX2 show enhanced phagosomal acidification and increased antigen degradation, resulting in impaired cross-priming.[455]

Cytosolic Components of Innate Immunity Signal Danger

There are many additional cytosolic proteins important in innate immunity. Of particular interest is the discovery of a family of pattern recognition receptors, most commonly referred to as nucleotide oligomerization domain-like receptors.[456] In 2000 and 2001, a number of genetic studies linked defects in *NLR* genes to inflammatory diseases, including Crohn disease,[457] Blau syndrome,[458] Muckle-Wells syndrome,[459] and familial Mediterranean fever.[460] In 2002, Tschopp and colleagues described a multiprotein complex that they called the "inflammasome," which included members of the nucleotide oligomerization domain-like receptors family and caspase-1.[461] They showed that activation of the inflammasome leads to generation of IL-1β, a cytokine that was first cloned by Dinarello and colleagues in 1984,[462] representing one of the key "endogenous pyrogens" first detected in 1953.[463,464] In 2006, Tschopp showed that the NLRP3 inflammasome was activated by monosodium urate crystals, implicating this pathway in gout.[465] From a historical perspective, gout is among the earliest diseases to be described. It was identified by the Egyptians in 2640 bce and was later recognized by Hippocrates in the fifth century bc, who referred to it as "the unwalkable disease." Leeuwenhoek was the first to observe urate crystals from a tophus.[466] Recently, an advisory panel of the U.S. Food and Drug Administration recommended against approval of canakinumab, a humanized mAb against IL-1, for the treatment of gout; although it was effective, it was not deemed safe due to increased risk of serious infections.

In 1994, Matzinger[467] proposed a new model of immune recognition. She proposed that APCs are activated by danger/alarm signals from injured cells (see Table 2.1). Although this was a purely theoretical model, since then, there have been numerous instances in which injured or

damaged components of the host have been recognized to trigger inflammation. As Matzinger explains,[468]

> Although this may seem to be just one more step down the pathway of slowly increasingly complex cellular interaction, this small step drops us off a cliff … in which the "foreignness" of a pathogen is not the important feature that triggers a response, and "selfness" is no guarantee of tolerance.

Thus, release of uric acid crystals can be viewed as an example of a "danger signal." Similarly, release of intracellular stores of adenosine triphosphate from dying cells as a

trigger for signaling cellular injury, predicted in 1988,[469] was shown to be capable of activating the NLRP3 inflammasome.[470] Although Matzinger's model shares features in common with Janeway's, the emphasis on endogenous danger signals rather than direct engagement of pattern recognition receptors by pathogens is novel. The fact that pathogens and “endogenous danger signals” share common receptors and signaling pathways is consistent with the idea that either exogenous or endogenous triggers of innate immune receptors accomplish the same thing: to facilitate the primary immune response.

Cytosolic Components Maintain Safety: The Role of Autophagy

There are numerous examples of microbes that are ingested by phagocytosis but either remain viable within acidic organelles or escape into the host cytoplasm. Macroautophagy is an evolutionarily conserved process in which cytoplasmic components are sequestered by a double membrane sac, eventually acquiring endosomal and lysosomal characteristics.[471] In 2004, Deretic and colleagues showed that IFNγ induced autophagy in macrophages, which contributed to suppression of intracellular survival of mycobacteria.[472] In the same year, Nakagawa et al.[473] demonstrated that autophagy was necessary for killing of group A *Streptococcus*, which had escaped into the host cytoplasm. Since then, many publications have confirmed the importance of autophagy in both innate and acquired immunity.[471]

The Renaissance of Cellular Immunology: Discovery of Dendritic Cells

Although we tend to take for granted the concept that APCs are necessary to process antigen, this was not firmly established until 1967. Mosier used a method pioneered by Mishell and Dutton for measuring in vitro antibody production with sheep erythrocytes as antigen.[474] Mosier separated mouse spleen cells into an adherent fraction and a nonadherent fraction. The adherent cells, which were phagocytic, were deemed “macrophage rich,” and the nonadherent cells were deemed “lymphocyte rich.” Both populations together, but neither alone, were needed for an antibody response, and Mosier[475] concluded that antibody production required “antigen phagocytosis by macrophages and macrophage lymphocyte interactions,” although he did not know the nature of the interactions. Further experiments established that exceedingly few adherent cells were needed for antibody production, perhaps as few as 1 in 10^4 adherent cells.[476] In the mid-1970s, Zanvil Cohn and a postdoctorate in his laboratory at Rockefeller University, Ralph Steinman, were characterizing a novel adherent population of cells from spleen.[477,478,479] The cells had an unusual “tree-like” morphology, prompting the name “dendritic cell.”[477] These cells proved difficult to purify, necessitating Steinman and Cohn to develop a rather laborious density gradient technique for cell purification.[480] In 1978, they used this technique to show that DCs could stimulate a primary mixed leukocyte reaction —an index of lymphocyte proliferation—and showed that DCs are at least 100 times more effective than B cells and macrophages for this function.[58] This landmark paper was the first demonstration of the unique capability of DCs to efficiently present antigen. Since then, many DC subsets have been described with critical functions in shaping the immune response. For example, Banchereau and colleagues showed that one subtype of DC, a “plasmacytoid” DC, is the principal IFNα-secreting cell in systemic lupus erythematosus with the capacity to

induce plasma cell differentiation.[481,482] Other DC subsets can respond to cues from epithelial cells, which secrete the cytokine TSLP, and shape T-cell polarization toward a T_H2 phenotype.[483,484] This may be a critical pathway in promoting allergic inflammation in asthma.[485] Since the initial discovery of DCs in 1973, Steinman and his many colleagues and collaborators have uncovered novel functions of DCs in triggering and shaping immunity as well as inducing tolerance. Many laboratories worldwide are using DCs in both capacities, and Steinman himself was the recipient of a DC-based vaccine against his own pancreatic cancer. Steinman was awarded the Nobel Prize, posthumously, in 2012—tragically, just days after he succumbed to cancer. The Nobel was a fitting tribute to a consummate cellular immunologist whose scientific curiosity and pedigree could be traced to Metchnikoff.

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REFERENCES

1. Kuhn TS. *The Structure of Scientific Revolutions*. 3rd ed. Chicago: University of Chicago Press; 1996.

2. Burkert W. *Ancient Mystery Cults*. Cambridge, MA: Harvard University Press; 1987.

3. Hippocrates. *The Genuine Works of Hippocrates*. London, United Kingdom: Sydenham Society; 1849.

4. Bloom BL. The “medical model,” miasma theory, and community mental health. *Commun Ment Health*. 1965;1:333-338.

5. Hippocrates. *On the Nature of Man*. Cambridge, MA: Harvard University Press; 1959.

6. Quintilian. *Institutio Oratoria*. Loeb Classical Library; 1920.

7. Cato V. *On Agriculture*. Cambridge, MA: Harvard University Press; 1993.

8. Galen. *Three Treatises on the Nature of Science*. India: Hackett Publishing; 1985.

9. Galen. *On Diseases and Symptoms*. Cambridge, MA: Cambridge University Press; 2006.

10. Smith W. Immunitas. In: Murray J, ed. *A Dictionary of Greek and Roman Antiquities*. London, United Kingdom; 1875:628.

11. Thucydides. *History of the Peloponnesian War* . Tufts University.

12. Nutton V. The seeds of disease: an explanation of contagion and infection from the Greeks to the Renaissance. *Med Hist*. 1983;27:1-34.

13. Aquinas T. *De Malo*. New York, NY: Oxford; 2001.

14. Cantor NF. *In the Wake of the Plague: The Black Death and The World it Made* . New York, NY: Simon and Schuster; 2002.

15. Silverstein AM. *A History of Immunology* . Amsterdam: Elsevier; 2009.

16. Redi F. *Experiments on the Generation of Insects, 1688*. Chicago, IL: Open Court; 1909.

17. Gest H. The discovery of microorganisms by Robert Hooke and Antoni Van Leeuwenhoek, fellows of the Royal Society. *Notes Rec R Soc Lond*. 2004;58:187-201.

18. Hooke R. *Micrographia: Or Some Physiological Descriptions of Minute Bodies made by Magnifying Glasses with Observations and Inquiries thereupon*. London, United Kingdom: Jo. Martyn and Ja. Allestry; 1665.

19. Schwann T. *Microscopic Investigations on the Accordance in the Structure and Growth of Plants and Animals*. Sydenham Society; 1839.

20. Otis L. *Membranes: Metaphors of Investion in Nineteenth Century Literature, Science and Politics*. Baltimore, MD: Johns Hopkins; 1999.

21. Henle J. *On Miasmata and Contagia*. Johns Hopkins; 1938.

22. Holland H. *Medical Notes and Reflections*. Philadelphia, PA: Haswell, Barrington, and Haswell; 1839.

23. DeLacy ME. A Linnaean thesis concerning Contagium Vivum: The “Exanthemata Viva” of John Nyander and its placer in contemporary thought. With a new translation by AJ Cain. *Med Hist*. 1995;39:159-185.

24. Redi F. *Osservazioni intorno a pellicelli del corpo umano fatte dal Dottor Gio: Cosimo Bonomo e da lui com altre osservazioni scritte in una lettera all'illustriss.* Florence, Italy: Piero Matini; 1687.

25. Théodoridès J. Casimir Davaine (1812-1882): a precursor of Pasteur. *Med Hist*. 1966;10:155-165.

26. Pasteur L. De l'attenuation du virus du choléra des poules. *Comptes rendus de l'Academie des sciences*. 1880;91:673-680.

27. Bourhy H, Perrot A, Cavaillon JM. Rabies. In: Artenstein AW, ed. *Vaccines: A Biography*. New York, NY: Springer; 2010:73.

28. Theiler M, Smith HH. The effect of prolonged cultivation in vitro upon the pathogenicity of yellow fever virus. *J Exp Med*. 1937;65:767-786.

29. Oettinger T, Jørgensen M, Ladefoged A, et al. Development of the Mycobacterium bovis BCG vaccine: review of the historical and biochemical evidence for a genealogical tree. *Tuber Lung Dis* . 1999;79: 243-250.

30. Carter KC. Koch's postulates in relation to the work of Jacob Henle and Edwin Klebs. *Med Hist*. 1985;29:353-374.

31. de Kruif P. *Microbe Hunters*. San Diego, CA: Harcourt; 1926.

32. Koch R. Untersuchungen über Bakterien: V. Die Ätiologie der Milzbrand-Krankheit, begründet auf die Entwicklungsgeschichte des Bacillus anthracis. *Beitrage zur Biologie der Pflanzen*. 1876;2:277-310.

33. Koch R. Tuberculosis. Nobelprize.org. www.nobelprize.org/educational/medicine/tuberculosis/readmore.html. Accessed February 25, 2012.

34. Mollaret HH. Contribution to the knowledge of relations between Koch and Pasteur. *NTM-Schriftenr Gesch Naturwiss, Technik, Med, Leipzig* ; 1983:S57-65.

35. Artenstein AW. Smallpox. In: Arenstein AW, ed. *Vaccines: A Biography*. New York: Springer; 2010:9-30.

36. Behbehani AM. The smallpox story: life and death of an old disease. *Microbiol Rev*. 1983;47:455-509.

37. Jenner E. *An Inquiry Into the Causes and Effects of the Variolæ Vaccinæ, a Disease Discovered in Some of the Western Counties of England, Partiularly Gloucestershire, and Known by the Name of Cow-Pox*. London, United Kingdom: D.N. Shury; 1798.

38. Morgan AJ, Parker S. Translatonal mini-review series on vaccines: The Edward Jenner Museum and the history of vaccination. *Clin Exp Immunol*. 2007;147:389-394.

39. Darwin C. *On the Origin of Species (by Means of Natural Selection)* . London: John Murray; 1859.

40. Tauber AI. Metchnikoff and the phagocytosis theory. *Nat Rev Mol Cell Biol* . 2003;4:897-901.

41. Metchnikoff O. *Life of Metchnikoff, 1845-1916* . Boston: Houghton and Mifflin; 1921.

42. Stossel TP. The early history of phagocytosis. In: Gordon S, ed. *Phagocytosis: The Host*. Stamford: JAI Press; 1999.

43. Haeckel E. *Die Radiolarien*. Berlin, Germany: George Reimer; 1862.

44. Metchnikoff E. *Lectures on the Comparative Pathology of Inflammation* . London: Kegan Paul, Trench, Truber; 1893.

45. Mechnikov I. Ilya Mechnikov-Nobel Lecture. Nobelprize.org. www.nobelprize.org/nobel_prizes/medicine/laureates/1908/mechnikov-lecture.html. Accessed February 26, 2012.

46. Metchnikoff E. *The Evolutionary Biology Papers of Elie Metchnikoff* . Dordrecht, The Netherlands: Kluwer Academic Publishers; 2000.

47. Roux P, Yersin A. Contribution al'etude de la diphtherie. *Ann Inst Pasteur* . 1888;2:629-661.

48. Behring E, Kitasato S. Ueber das Zustandekekommen de Diphtherie— Immunität und der Tetanus—Immunität bei Tieren. *Deutsche Med Wochenschr* . 1890;16:1113-1114.

49. Behring E, Wernicke E. Ueber immunisirung und Heilung von ver-suchsthieren bei der Diphtherie. *Hyg u Infektionskr* . 1892;12:10-44.

50. Buchner H. Über die nahere Natur der bakterientötenden Substanz in Blutserum. *Zentralbl Bakteriol* . 1889;6:561-572.

51. Bordet J. Mechanisme de l'agglutination. *Ann Inst Pasteur* . 1899;13:225-250.

52. Wassermann A, Neisser A, Bruck C. Eine serodiagnostische Reaktion bei Syphilis. *Deutsche medicinische Wochenschrift*. 1906;32:745-746.

53. Linton DS. *Emil von Behring: Infectious Disease, Immunology, Serum Therapy*. Philadelphia: American Philosophical Society; 2005.

54. Metchnikoff E. L'État actuel de la question de l'immunité (Rapport au Congrès International de Budapest). *Annales de l'Institut Pasteur* . 1894;8:706-721.

55. Wright AE, Douglas SR. An experimental investigation of the role of the blood fluids in

connection with phagocytosis. *Proc Royal Soc London*. 1902;72:357-370.

56. Shaw GB. *The Doctor's Dilemma*. Sioux Falls, SD: NuVision; 2008.

57. Fagraeus A. Antibody production in relation to the development of plasma cells. *Acta Medica (Scand) Supplementum*. 1948;204.

58. Steinman RM, Witmer MD. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc Natl Acad Sci U S A* . 1978;75:5132-5136.

59. Silverstein AM. Paul Ehrlich's passion: the origins of his receptor immunology. *Cell Immunol*. 1999;194:213-221.

60. Silverstein AM. *Paul Ehrlich's Receptor Immunology: The Magnificent Obsession* . San Diego: Academic Press; 2002.

61. www.nobelprize.org/nobel_prizes/medicine/laureates/1908/ehrlich-lecture.html. Accessed December 14, 2011.

62. Schwarz HP, Dorner F. Karl Landsteiner and his major contributions to haematology. *Br J Haematol* . 2003;121:556-565.

63. Landsteiner K. Zur Kenntnis der antifermentativen, lytischen und agglutinierenden wirkungen des blutserums und der lymphe. *Zentbl Bakt Orig* . 1900;27:357-362.

64. Landsteiner K. Ueber agglutinationserscheinungen normalen menschlichen blutes. *Wien Klin Wochenschr*. 1901;14:1132-1134.

65. www.nobelprize.org/nobel_prizes/medicine/laureates/1930/landstein-erlecture.html. Accessed December 14, 2011.

66. Landsteiner K, Levine P. A new agglutinable factor differentiating individual human bloods. *Proc Soc Exp Biol Med*. 1927;24:600-602.

67. Landsteiner K, Wiener AS. An agglutinable factor in human blood recognized by immune sera for Rhesus blood. *Proc Soc Exp Biol Med*. 1940;48:223-224.

68. Donath J, Landsteiner K. Ueber paroxysmale Hämoglobinurie. *Muench med Wochenschr*. 1904;51:1590-1593.

69. Ehrlich P, Morgenroth J. Ueber Haemolysine. *Berlin klin Wchnschr*. 1901;38:599-604.

70. Ehrlich P. *Studies in Immunity*. 2nd ed. London, United Kingdom: John Wiley; 1910.

71. Santucci S. Citotossine. *Rev Ital Ottal Roma*. 1906;2:213.

72. Levine P, Katzin EM, Burnham L. Isoimmunization in pregnancy: its possible bearing on the etiology of erythroblastosis foetalis. *JAMA*. 1941;116:825-827.

73. Coombs RR, Mourant AE, Race RR. A new test for the detection of weak and incomplete Rh agglutinins. *Br J Exp Pathol* . 1945;26:255-266.

74. Franklin EC, Holman HR, Muller-Eberhard HJ, et al. An unusual protein component of high molecular weight in the serum of certain patients with rheumatoid arthritis. *J Exp Med*. 1957;105:425-438.

75. Winchester R. A golden anniversary: recognition that rheumatoid arthritis sera contain autoantibodies specific for determinants on native IgG molecules. *J Immunol* . 2007;178:1227-1228.

76. Cohen SG, Zelaya-Quesada M. Portier, Richet, and the discovery of anaphylaxis: a centennial. *J Allergy Clin Immunol* . 2002;110:331-336.

77. Portier P, Richet C. De l'action anaphylactique de certains venins. *C R Seances Soc Biol*. 1902;54:170.

78. von Pirquet C, Schick B. *Die Serumkrankheit*. Leipzig und Wien: Franz Deuticke; 1905.

79. von Pirquet C. Allergy. *Arch Int Med*. 1911;7:383-440.

80. Dixon FJ, Vazquez JJ, Weigle WO, et al. Pathogenesis of serum sickness. *AMA Arch Pathol*. 1958;65:18-28.

81. Jackson M. “A private line to medicine”: the clinical and laboratory contours of allergy in the early twentieth century. In: Kroker K, Keelan J, Mazumdar PMH, eds. *Crafting Immunity—Working Histories of Clinical Immunology*. Burlington: Ashgate; 2008:60.

82. Obermeyer F, Pick EP. Über die chemischen grundlagen der arteigenschartn der eiweisskörper. Bildung von immiunpracipitinen durch chemische veranderte eiweisskoörper. *Wiener klin Wschr* . 1906;19:327-333.

83. Svedberg T, Rinde H. The untra-centrifuge, a new instrucmnt for the determination of size and distribution of size of partice in amicroscopic colloids. *J Am Chem Soc*. 1924;46:2677-2693.

84. Tiselius A. A new apparatus for teh electrophoretic analysis of colloidal mixtures. *Trans Faraday Soc*. 1937;33:524-531.

85. Van Epps HL. Michael Heidelberger and the demystification of antibodies. *J Exp Med*. 2006;203:5.

86. Edelman GM, Poulik MD. Studies on structural units of the gammaglobulins. *J Exp Med*. 1961;113:861-884.

87. Bail O, Tsuda K. Versuche über bakteriolytische immunokörper mit besonderer berücksichtigung des normalen rinderserums. *Zeitschrift Immunitäts-forschung*. 1909;1:546-612.

88. Breinl L, Haurowitz F. Untersuchung des präzipitates aus hämoglobin und anti-hämoglobin serum und bemerkungen über die natur der antikörper. *Z Physiol Chem*. 1930;192:45-57.

89. Pauling L. A theory of the structure and process of formation of antibodies. *J Am Chem Soc*. 1940;62:2643-2657.

90. Eisen HN, Chakraborty AK. Evolving concepts of specificity in immune reactions. *Proc Natl Acad Sci U S A*. 2010;107:22373-22380.

91. Jerne NK. The natural-selection theory of antibody formation. *Proc Natl Acad Sci U S A*. 1955;41:849-857.

92. Crick FH. On protein synthesis. *Symp Soc Exp Biol*. 1958;12:138-163.

93. Talmage DW. Allergy and Immunology. *Annu Rev Med*. 1957;8:239-256.

94. Burnet FMA. A modification of Jerne's theory of antibody production using the concept of clonal selection. *Aust J Med Sci* . 1957;20:67-69.

95. Cruse JM, Lewis RE Jr, David W. Talmage and the advent of the cell selection theory of antibody synthesis. *J Immunol*. 1994;153:919-929.

96. Roberts JC Jr, Dixon FJ. The transfer of lymph node cells in the study of the immune response to foreign proteins. *J Exp Med*. 1955;102:379-392.

97. Nossal GJ, Lederberg J. Antibody production by single cells. *Nature*. 1958;181:1419-1420.

98. Raff MC, Feldmann M, De Petris S. Monospecificity of bone marrowderived lymphocytes. *J Exp Med*. 1973;137:1024-1030.

99. Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*. 1975;256:495-497.

100. Hilschmann N, Craig LC. Amino acid sequence studies with Bence-Jones proteins. *Proc Natl Acad Sci U S A* . 1965;53:1403-1409.

101. Wu TT, Kabat EA. An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. *J Exp Med*. 1970;132:211-250.

102. Dreyer WJ, Bennett JC. The molecular basis of antibody formation: a paradox. *Proc Natl Acad Sci U S A*. 1965;54:864-869.

103. Weigert MG, Cesari IM, Yonkovich SJ, et al. Variability in the lambda light chain sequences of mouse antibody. *Nature.* 1970;228:1045-1047.

104. Edelman GM, Gally JA. Somatic recombination of duplicated genes: an hypothesis on the origin of antibody diversity. *Proc Natl Acad Sci U S A* . 1967;57:353-358.

105. Hood L, Talmage DW. Mechanism of antibody diversity: germ line basis for variability. *Science.* 1970;168:325-334.

106. Hozumi N, Tonegawa S. Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions. *Proc Natl Acad Sci U S A* . 1976;73:3628-3632.

107. Seidman JG, Leder A, Edgell MH, et al. Multiple related immunoglobulin variable-region genes identified by cloning and sequence analysis. *Proc Natl Acad Sci U S A.* 1978;75:3881-3885.

108. Brack C, Hirama M, Lenhard-Schuller R, et al. A complete immunoglobulin gene is created by somatic recombination. *Cell.* 1978;15:1-14.

109. Weigert M, Gatmaitan L, Loh E, et al. Rearrangement of genetic information may produce immunoglobulin diversity. *Nature*. 1978;276: 785-790.

110. Early P, Huang H, Davis M, et al. An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: VH, D and JH. *Cell*. 1980;19:981-992.

111. Alt FW, Oltz EM, Young F, et al. VDJ recombination. *Immunol Today.* 1992;13:306-314.

112. Schatz DG, Oettinger MA, Baltimore D. The V(D)J recombination activating gene, RAG-1. *Cell*. 1989;59:1035-1048.

113. Oettinger MA, Schatz DG, Gorka C, et al. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science*. 1990;248:1517-1523.

114. Bernard O, Hozumi N, Tonegawa S. Sequences of mouse immunoglobulin light chain genes before and after somatic changes. *Cell.* 1978;15:1133-1144.

115. McKean D, Huppi K, Bell M, et al. Generation of antibody diversity in the immune response of BALB/c mice to influenza virus hemagglutinin. *Proc Natl Acad Sci U S A* . 1984;81:3180-3184.

116. Griffiths GM, Berek C, Kaartinen M, et al. Somatic mutation and the maturation of immune response to 2-phenyl oxazolone. *Nature.* 1984;312:271-275.

117. Siskind GW, Benacerraf B. Cell selection by antigen in the immune response. *Adv Immunol.* 1969;10:1-50.

118. Kaufmann SHE. Robert Koch's highs and lows in the search for a remedy for tuberculosis. *Nature Medicine Special Web Focus: Tuberculosis*. 2000. www.nature.com/nm/focus/tb/historical_perspective.html. Accessed January 18, 2012.

119. Landsteiner K, Chase MW. Experiments on transfer of cutaneous sensitivity to simple compounds. *Proc Soc Exp Biol Med*. 1942;49:688-690.

120. Landsteiner K, Chase MW. The cellular transfer of cutaneous hypersensitivity to tuberculin. *Proc Soc Exp Biol Med*. 1945;59:134-135.

121. Silverstein AM. The lymphocyte in immunology: from James B. Murphy to James L. Gowans. *Nat Immunol*. 2001;2:569-71.

122. Murphy JB, Ellis AW. Experiments on the role of lymphoid tissue in the resistance to experimental tuberculosis in mice. *J Exp Med*. 1914;20: 397-403.

123. Miller JF. Role of the thymus in murine leukaemia. *Nature*. 1959;183:1069.

124. Ehrich WE, Harris TN. The formation of antibodies in the popliteal lymph node in rabbits. *J Exp Med*. 1942;76:335-348.

125. Fagraeus A. The plasma cellular reaction and its relation to the formation of antibodies in vitro. *J Immunol*. 1948;58:1-13.

126. Glick B. This week's citation classic. *Current Contents*. 1979:241.

127. Glick B, Chang TS, Jaap RG. The bursa of Fabricius and antibody production. *Poultry Sci*. 1955;35:224-225.

128. Mitchell GF, Miller JF. Cell to cell interaction in the immune response. II. The source

of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J Exp Med*. 1968;128:821-837.

129. Miller JF, Mitchell GF. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. *J Exp Med*. 1968; 128:801-820.

130. Mitchell GF, Miller JF. Immunological activity of thymus and thoracicduct lymphocytes. *Proc Natl Acad Sci U S A* . 1968;59:296-303.

131. Cooper MD, Raymond DA, Peterson RD, et al. The functions of the thymus system and the bursa system in the chicken. *J Exp Med*. 1966;123: 75-102.

132. Cooper MD, Peterson RD, Good RA. Delineation of the thymic and bursal lymphoid systems in the chicken. *Nature*. 1965;205:143-146.

133. Mazumdar PMH. *Species and Specificity. An Interpretation of the History of Immunology*. New York: Cambridge; 1995.

134. Gowans JL. The effect of the continuous re-infusion of lymph and lymphocytes on the output of lymphocytes from the thoracic duct of unanaesthetized rats. *Br J Exp Pathol*. 1957;38:67-78.

135. Gowans JL. The recirculation of lymphocytes from blood to lymph in the rat. *J Physiol*. 1959;146:54-69.

136. Gibson T, Medawar PB. The fate of skin homografts in man. *J Anat*. 1943;77:299-310.

137. Medawar PB. The behaviour and fate of skin autografts and skin homografts in rabbits: a report to the War Wounds Committee of the Medical Research Council. *J Anat*. 1944;78:176-199.

138. Owen RD. Immunogenetic consequences of vascular anastomoses between bovine twins. *Science*. 1945;102:400-401.

139. Burnet FM, Fenner F. *The Production of Antibodies* . Melbourne: MacMillan; 1949.

140. Billingham RE, Brent L, Medawar PB. “Actively acquired tolerance” of foreign cells. *Nature*. 1953;172:603-606.

141. Hašek M. Parabiosis of birds during embryonic development. *Cesk Biol*. 1953;2:265-277.

142. Ivanyi J. Milan Hasek and the discovery of immunological tolerance. *Nat Rev*

Immunol. 2003;3:591-597.

143. Tauber AI. The immune self: theory or metaphor? *Immunol Today*. 1994;15:134-136.

144. Brewerton DA, Hart FD, Nicholls A, et al. Ankylosing spondylitis and HL-A 27. *Lancet*. 1973;1:904-907.

145. Dausset J. [Iso-leuko-antibodies]. *Acta Haematol*. 1958;20:156-166.

146. Benacerraf B, McDevitt HO. Histocompatibility-linked immune response genes. *Science*. 1972;175:273-279.

147. Schlosstein L, Terasaki PI, Bluestone R, et al. High association of an HL-A antigen, W27, with ankylosing spondylitis. *N Engl J Med*. 1973;288:704-706.

148. Aisenberg AC. Allogeneic thymus grafts and the restoration of immune function in irradiated thymectomized mice. *J Exp Med*. 1970;131:275-286.

149. Stutman O, Yunis EJ, Good RA. Effect of parental strain thymus grafts in neonatally thymectomized F1 hybrids. *Transplantation*. 1969;7:420-423.

150. Leuchars E, Cross AM, Dukor P. The restoration of immunological function by thymus grafting in thymectomized irradiated mice. *Transplantation*. 1965;3:28-38.

151. Kindred B, Shreffler DC. H-2 dependence of co-operation between T and B cells in vivo. *J Immunol*. 1972;109:940-943.

152. Rosenthal AS, Shevach EM. Function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes. *J Exp Med*. 1973;138:1194-1212.

153. Zinkernagel RM, Doherty PC. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature*. 1974;248:701-702.

154. Zinkernagel RM, Doherty PC. Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis. *Nature*. 1974;251:547-548.

155. Ziegler K, Unanue ER. Identification of a macrophage antigen-processing event required for I-region-restricted antigen presentation to T lymphocytes. *J Immunol*. 1981;127:1869-1875.

156. Ziegler HK, Unanue ER. Decrease in macrophage antigen catabolism caused by

ammonia and chloroquine is associated with inhibition of antigen presentation to T cells. *Proc Natl Acad Sci U S A* . 1982;79:175-178.

157. Roche PA, Cresswell P. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature*. 1990;345:615-618.

158. Germain RN. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell*. 1994;76: 287-299.

159. Roche PA, Cresswell P. Proteolysis of the class II-associated invariant chain generates a peptide binding site in intracellular HLA-DR molecules. *Proc Natl Acad Sci U S A*. 1991;88:3150-3154.

160. Nakagawa T, Roth W, Wong P, et al. Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. *Science*. 1998;280: 450-453.

161. Amigorena S, Drake JR, Webster P, et al. Transient accumulation of new class II MHC molecules in a novel endocytic compartment in B lymphocytes. *Nature*. 1994;369:113-120.

162. Turley SJ, Inaba K, Garrett WS, et al. Transport of peptide-MHC class II complexes in developing dendritic cells. *Science*. 2000;288:522-527.

163. Babbitt BP, Allen PM, Matsueda G, et al. Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature*. 1985;317:359-361.

164. Rock KL, Gramm C, Rothstein L, et al. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell*. 1994;78:761-771.

165. Spies T, Bresnahan M, Bahram S, et al. A gene in the human major histocompatibility complex class II region controlling the class I antigen presentation pathway. *Nature*. 1990;348:744-747.

166. Trowsdale J, Hanson I, Mockridge I, et al. Sequences encoded in the class II region of the MHC related to the “ABC” superfamily of transporters. Nature. 1990;348:741-744.

167. Deverson EV, Gow IR, Coadwell WJ, et al. MHC class II region encoding proteins related to the multidrug resistance family of transmembrane transporters. *Nature*. 1990;348:738-741.

168. Monaco JJ, Cho S, Attaya M. Transport protein genes in the murine MHC: possible implications for antigen processing. *Science*. 1990;250:1723-1726.

169. Bjorkman PJ, Saper MA, Samraoui B, et al. Structure of the human class I

histocompatibility antigen, HLA-A2. *Nature*. 1987;329:506-512.

170. Bjorkman PJ, Saper MA, Samraoui B, et al. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature*. 1987;329:512-518.

171. Ehrlich P. Croonian lecture: on immunity with special reference to cell life. *Proc R Soc Lon B*. 1900;66:424-448.

172. Raff MC, Sternberg M, Taylor RB. Immunoglobulin determinants on the surface of mouse lymphoid cells. *Nature*. 1970;225:553-554.

173. Coombs RR, Gurner BW, Janeway CA Jr, et al. Immunoglobulin determinants on the lymphocytes of normal rabbits. I. Demonstration by the mixed antiglobulin reaction of determinants recognized by anti-gamma, anti-mu, anti-Fab and anti-allotype sera, anti-As4 and anti-As6. *Immunology*. 1970;18:417-429.

174. Pernis B, Forni L, Amante L. Immunoglobulin spots on the surface of rabbit lymphocytes. *J Exp Med*. 1970;132:1001-1018.

175. Marcuson EC, Roitt IM. Immunoglobulin allotypic determinants on rabbit lymphocytes. *Nature*. 1970;227:1051-1053.

176. Lanzavecchia A. Antigen-specific interaction between T and B cells. *Nature*. 1985;314:537-539.

177. Rock KL, Benacerraf B, Abbas AK. Antigen presentation by haptenspecific B lymphocytes. I. Role of surface immunoglobulin receptors. *J Exp Med*. 1984;160:1102-1113.

178. Bruton OC. Agammaglobulinemia. *Pediatrics*. 1952;9:722-728.

179. Tsukada S, Saffran DC, Rawlings DJ, et al. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell*. 1993;72:279-290.

180. Vetrie D, Vorechovsky I, Sideras P, et al. The gene involved in X-linked agammaglobulinaemia is a member of the src family of proteintyrosine kinases. *Nature*. 1993;361:226-233.

181. Hendriks RW, de Bruijn MF, Maas A, et al. Inactivation of Btk by insertion of lacZ reveals defects in B cell development only past the pre-B cell stage. *EMBO J*. 1996;15:4862-4872.

182. Carroll L. *The Hunting of the Snark. An Agony, in Elght Fits* . London: Macmillan; 1876.

183. Mak TW. The T cell antigen receptor: “The Hunting of the Snark.” *Eur J Immunol* . 2007;37(Suppl 1):S83-S93.

184. Jensenius JC, Williams AF. The T lymphocyte antigen receptor—paradigm lost. *Nature*. 1982;300:583-588.

185. Allison JP, McIntyre BW, Bloch D. Tumor-specific antigen of murine T-lymphoma defined with monoclonal antibody. *J Immunol* . 1982;129: 2293-2300.

186. Yanagi Y, Yoshikai Y, Leggett K, et al. A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. *Nature*. 1984;308:145-149.

187. Hedrick SM, Cohen DI, Nielsen EA, et al. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature*. 1984;308:149-153.

188. Chien Y, Becker DM, Lindsten T, et al. A third type of murine T-cell receptor gene. *Nature*. 1984;312:31-35.

189. Saito H, Kranz DM, Takagaki Y, et al. A third rearranged and expressed gene in a clone of cytotoxic T lymphocytes. *Nature*. 1984;312:36-40.

190. Sim GK, Yague J, Nelson J, et al. Primary structure of human T-cell receptor alpha-chain. *Nature*. 1984;312:771-775.

191. Yanagi Y, Chan A, Chin B, et al. Analysis of cDNA clones specific for human T cells and the alpha and beta chains of the T-cell receptor heterodimer from a human T-cell line. *Proc Natl Acad Sci U S A* . 1985;82:3430-3434.

192. Brenner MB, McLean J, Dialynas DP, et al. Identification of a putative second T-cell receptor. *Nature*. 1986;322:145-149.

193. Fremont DH, Matsumura M, Stura EA, et al. Crystal structures of two viral peptides in complex with murine MHC class I H-2Kb. *Science*. 1992;257:919-927.

194. Zhang W, Young AC, Imarai M, et al. Crystal structure of the major histocompatibility complex class I H-2Kb molecule containing a single viral peptide: implications for peptide binding and T-cell receptor recognition. *Proc Natl Acad Sci U S A* . 1992;89:8403-8407.

195. Stern LJ, Wiley DC. The human class II MHC protein HLA-DR1 assembles as empty alpha beta heterodimers in the absence of antigenic peptide. *Cell*. 1992;68:465-477.

196. Gascoigne NR, Palmer E. Signaling in thymic selection. *Curr Opin Immunol* . 2011;23:207-212.

197. Tiselius A, Kabat EA. An electrophoretic study of immune sera and purified antibody preparations. *J Exp Med*. 1939;69:119-131.

198. Waldenström J. Incipient myelomatosis or “essential” hyperglobulinemia with fibrinogenopenia—a new syndrome? *Acta Med Scand*. 1944;117:216-247.

199. Metzger H. Characterization of a human macroglobulin. V. A Waldenstrom macroglobulin with antibody activity. *Proc Natl Acad Sci U S A* . 1967;57:1490-1497.

200. Gugler E, Bokelmann G, Datwyler A, Von Moralt G. Uber immunoelektrophoretische Untersuchungen an Frauenmilchproteinen. *Schweiz Med Wochenschr* . 1958;88.

201. Heremans JF, Heremans MT, Schultze HE. Isolation and description of a few properties of the beta 2A-globulin of human serum. *Clin Chim Acta*. 1959;4:96-102.

202. Chodirker WB, Tomasi TB Jr. Gamma-globulins: quantitative relationships in human serum and nonvascular fluids. *Science*. 1963;142: 1080-1081.

203. Tomasi TB Jr, Zigelbaum S. The selective occurence of gamma-1a globulins in certain body fluids. *J Clin Invest*. 1963;42:1552-1560.

204. Brandtzaeg P, Prydz H. Direct evidence for an integrated function of J chain and secretory component in epithelial transport of immunoglobulins. *Nature*. 1984;311:71-73.

205. Hamilton RG. Science behind the discovery of IgE. *J Allergy Clin Immunol* . 2005;115:648-652.

206. Prausnitz C, Küstner H. Studien über die Ueberempfindlichkeit. *Zentralbl Bakteriol* . 1921;86:160-169.

207. Ishizaka K, Ishizaka T, Hornbrook MM. Physico-chemical properties of human reaginic antibody. IV. Presence of a unique immunoglobulin as a carrier of reaginic activity. *J Immunol* . 1966;97:75-85.

208. Ishizaka K, Ishizaka T, Hornbrook MM. Physicochemical properties of reaginic antibody. V. Correlation of reaginic activity wth gamma-Eglobulin antibody. *J Immunol* . 1966;97:840-853.

209. Johansson SG. Raised levels of a new immunoglobulin class (IgND) in asthma. *Lancet*. 1967;2:951-953.

210. Dombrowicz D, Flamand V, Brigman KK, et al. Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor α chain gene. *Cell* . 1993;75:969-976.

211. Rowe DS, Fahey JL. A new class of human immunoglobulins. I. A unique myeloma protein. *J Exp Med*. 1965;121:171-184.

212. Chen K, Cerutti A. The function and regulation of immunoglobulin D. *Curr Opin Immunol*. 2011;23:345-352.

213. Uhr JW, Finkelstein MS. Antibody formation. IV. Formation of rapidly and slowly sedimenting antibodies and immunological memory to bacteriophage phi-X 174. *J Exp Med*. 1963;117:457-477.

214. Bauer DC, Mathies MJ, Stavitsky AB. Sequences of synthesis of gamma-1 macroglobulin and gamma-2 globulin antibodies during primary and secondary responses to proteins, salmonella antigens, and phage. *J Exp Med*. 1963;117:889-907.

215. Pernis B, Forni L, Luzzati AL. Synthesis of multiple immunoglobulin classes by single lymphocytes. *Cold Spring Harb Symp Quant Biol* . 1977;41(pt 1):175-183.

216. Kearney JF, Lawton AR. B lymphocyte differentiation induced by lipopolysaccharide. I. Generation of cells synthesizing four major immunoglobulin classes. *J Immunol*. 1975;115:671-676.

217. Wabl MR, Forni L, Loor F. Switch in immunoglobulin class production observed in single clones of committed lymphocytes. *Science*. 1978;199:1078-1080.

218. Manis JP, Gu Y, Lansford R, et al. Ku70 is required for late B cell development and immunoglobulin heavy chain class switching. *J Exp Med*. 1998;187:2081-2089.

219. Casellas R, Nussenzweig A, Wuerffel R, et al. Ku80 is required for immunoglobulin isotype switching. *EMBO J*. 1998;17:2404-2411.

220. Muramatsu M, Sankaranand VS, Anant S, et al. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. J Biol Chem 1999;274:18470-18476

221. Muramatsu M, Kinoshita K, Fagarasan S, et al. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell*. 2000;102:553-563.

222. Revy P, Muto T, Levy Y, et al. Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell*. 2000;102:565-575.

223. Griffin FM Jr, Silverstein SC. Segmental response of the macrophage plasma membrane to a phagocytic stimulus. *J Exp Med*. 1974;139:323-336.

224. Indik Z, Kelly C, Chien P, et al. Human Fc gamma RII, in the absence of other Fc gamma receptors, mediates a phagocytic signal. *J Clin Invest*. 1991;88:1766-1771.

225. Gresham HD, McGarr JA, Shackelford PG, et al. Studies on the molecular mechanisms of human Fc receptor-mediated phagocytosis. Amplification of ingestion is dependent on the generation of reactive oxygen metabolites and is deficient in polymorphonuclear leukocytes from patients with chronic granulomatous disease. *J Clin Invest*. 1988;82:1192-1201.

226. Brumell JH, Grinstein S. Role of lipid-mediated signal transduction in bacterial internalization. *Cellular microbiology* . 2003;5:287-297.

227. Swanson JA, Hoppe AD. The coordination of signaling during Fc receptor-mediated phagocytosis. *J Leukoc Biol* . 2004;76:1093-1103.

228. Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol*. 1999;17:593-623.

229. Crowley MT, Costello PS, Fitzer-Attas CJ, et al. A critical role for Syk in signal transduction and phagocytosis mediated by Fcγ receptors on macrophages. *J Exp Med*. 1997;186:1027-1039.

230. Caron E, Hall A. Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. *Science*. 1998;282:1717-1721.

231. Van den Herik-Oudijk IE, Capel PJ, van der Bruggen T, et al. Identification of signaling motifs within human Fcγ RIIa and Fcγ RIIb isoforms. *Blood*. 1995;85:2202-2211.

232. Cox D, Greenberg S. Phagocytic signaling strategies: Fcγ receptor-mediated phagocytosis as a model system. *Semin Immunol*. 2001;13:339-345.

233. Ravetch JV, Luster AD, Weinshank R, et al. Structural heterogeneity and functional domains of murine immunoglobulin G Fc receptors. *Science*. 1986;234:718-725.

234. Reth M. Antigen receptor tail clue. *Nature*. 1989;338:383-384.

235. Hombach J, Tsubata T, Leclercq L, et al. Molecular components of the B-cell antigen receptor complex of the IgM class. *Nature*. 1990;343: 760-762.

236. Ra C, Jouvin MH, Blank U, et al. A macrophage Fcγ receptor and the mast cell receptor for IgE share an identical subunit. *Nature*. 1989;341: 752-754.

237. Taniguchi T, Kobayashi T, Kondo J, et al. Molecular cloning of a porcine gene syk that encodes a 72-kDa protein-tyrosine kinase showing high susceptibility to proteolysis.

J Biol Chem. 1991;266:15790-15796.

238. Chan AC, Kadlecek TA, Elder ME, et al. ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency. *Science*. 1994;264:1599-1601.

239. Takata M, Sabe H, Hata A, et al. Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca2+ mobilization through distinct pathways. *EMBO J*. 1994;13:1341-1349.

240. Costello PS, Turner M, Walters AE, et al. Critical role for the tyrosine kinase Syk in signalling through the high affinity IgE receptor of mast cells. *Oncogene*. 1996;13:2595-2605.

241. Cox D, Chang P, Kurosaki T, et al. Syk tyrosine kinase is required for immunoreceptor tyrosine activation motif-dependent actin assembly. *J Biol Chem*. 1996;271:16597-16602.

242. Kiefer F, Brumell J, Al-Alawi N, et al. The Syk protein tyrosine kinase is essential for Fcγ receptor signaling in macrophages and neutrophils. *Mol Cell Biol* . 1998;18:4209-4220.

243. Brumbaugh KM, Binstadt BA, Billadeau DD, et al. Functional role for Syk tyrosine kinase in natural killer cell-mediated natural cytotoxicity. *J Exp Med*. 1997;186:1965-1974.

244. Ra C, Jouvin MH, Blank U, et al. A macrophage Fc gamma receptor and the mast cell receptor for IgE share an identical subunit. *Nature*. 1989;341:752-754.

245. Clynes R, Dumitru C, Ravetch JV. Uncoupling of immune complex formation and kidney damage in autoimmune glomerulonephritis. *Science*. 1998;279:1052-1054.

246. Burnet FM. *The Clonal Selection Theory of Acquired Immunity* . Nashville, TN: Vanderbilt University Press; 1959.

247. Lederberg J. Genes and antibodies. *Science*. 1959;129:1649-1653.

248. Talmage DW, Pearlman DS. The antibody response: a model based on antagonistic actions of antigen. *J Theor Biol* . 1963;5:321-339.

249. Dempsey PW, Allison ME, Akkaraju S, et al. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science*. 1996;271:348-350.

250. Bretscher PA, Cohn M. Minimal model for the mechanism of antibody induction and paralysis by antigen. *Nature*. 1968;220:444-448.

251. Bretscher P, Cohn M. A theory of self-nonself discrimination. *Science*. 1970;169:1042-1049.

252. Lafferty KJ, Cunningham AJ. A new analysis of allogeneic interactions. *Aust J Exp Biol Med Sci*. 1975;53:27-42.

253. Cunningham AJ, Lafferty KJ. A simple, conservative explanation of the H-2 restriction of interactions between lymphocytes. *Scand J Immunol*. 1977;6:1-6.

254. Jenkins MK, Schwartz RH. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J Exp Med*. 1987;165:302-3190

255. Harding FA, McArthur JG, Gross JA, et al. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature*. 1992;356:607-609.

256. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. *Nat Rev Immunol*. 2002;2:116-126.

257. Fife BT, Bluestone JA. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. *Immunol Rev*. 2008;224:166-182.

258. Walunas TL, Lenschow DJ, Bakker CY, et al. CTLA-4 can function as a negative regulator of T cell activation. *Immunity*. 1994;1:405-413.

259. Jani M, Hyrich KL. Abatacept in the long-term treatment of rheumatoid arthritis. *Expert Rev Clin Immunol*. 2012;8:231-234.

260. Claman HN, Chaperon EA, Triplett RF. Thymus-marrow cell combinations. Synergism in antibody production. *Proc Soc Exp Biol Med*. 1966;122:1167-1171.

261. Noelle RJ, Roy M, Shepherd DM, et al. A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proc Natl Acad Sci U S A*. 1992;89:6550-6554.

262. Armitage RJ, Fanslow WC, Strockbine L, et al. Molecular and biological characterization of a murine ligand for CD40. *Nature*. 1992;357:80-82.

263. Lederman S, Yellin MJ, Inghirami G, et al. Molecular interactions mediating T-B lymphocyte collaboration in human lymphoid follicles. Roles of T cell-B-cell-activating molecule (5c8 antigen) and CD40 in contact-dependent help. *J Immunol*. 1992;149:3817-3826.

264. Schaerli P, Willimann K, Lang AB, et al. CXC chemokine receptor 5 expression

defines follicular homing T cells with B cell helper function. *J Exp Med*. 2000;192:1553-1562.

265. Breitfeld D, Ohl L, Kremmer E, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med*. 2000;192:1545-1552.

266. Aruffo A, Farrington M, Hollenbaugh D, et al. The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. *Cell*. 1993;72:291-300.

267. Korthauer U, Graf D, Mages HW, et al. Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. *Nature*. 1993;361:539-541.

268. DiSanto JP, Bonnefoy JY, Gauchat JF, et al. CD40 ligand mutations in x-linked immunodeficiency with hyper-IgM. *Nature*. 1993;361: 541-543.

269. Allen RC, Armitage RJ, Conley ME, et al. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science*. 1993;259:990-993.

270. Kung P, Goldstein G, Reinherz EL, et al. Monoclonal antibodies defining distinctive human T cell surface antigens. *Science*. 1979;206:347-349.

271. Reinherz EL, Kung PC, Goldstein G, et al. Separation of functional subsets of human T cells by a monoclonal antibody. *Proc Natl Acad Sci U S A* . 1979;76:4061-4065.

272. Reinherz EL, Hussey RE, Fitzgerald K, et al. Antibody directed at a surface structure inhibits cytolytic but not suppressor function of human T lymphocytes. *Nature*. 1981;294:168-170.

273. Reinherz EL, Meuer S, Fitzgerald KA, et al. Antigen recognition by human T lymphocytes is linked to surface expression of the T3 molecular complex. *Cell*. 1982;30:735-743.

274. Meuer SC, Acuto O, Hussey RE, et al. Evidence for the T3-associated 90K heterodimer as the T-cell antigen receptor. *Nature*. 1983;303:808-810.

275. Meuer SC, Cooper DA, Hodgdon JC, et al. Identification of the receptor for antigen and major histocompatibility complex on human inducer T lymphocytes. *Science*. 1983;222:1239-1242.

276. Norment AM, Salter RD, Parham P, et al. Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature*. 1988;336:79-81.

277. Doyle C, Strominger JL. Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature*. 1987;330:256-259.

278. Veillette A, Bookman MA, Horak EM, et al. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. *Cell*. 1988;55:301-308.

279. Rudd CE, Trevillyan JM, Dasgupta JD, et al. The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. *Proc Natl Acad Sci U S A*. 1988;85:5190-5194.

280. Nakayama T, Singer A, Hsi ED, et al. Intrathymic signalling in immature CD4+CD8+ thymocytes results in tyrosine phosphorylation of the T-cell receptor zeta chain. *Nature*. 1989;341:651-654.

281. Barber EK, Dasgupta JD, Schlossman SF, et al. The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase (p56lck) that phosphorylates the CD3 complex. *Proc Natl Acad Sci U S A*. 1989;86:3277-3281.

282. Hoth M, Penner R. Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature*. 1992;355:353-356.

283. Vig M, Peinelt C, Beck A, et al. CRACM1 is a plasma membrane protein essential for store-operated Ca2+ entry. *Science*. 2006;312:1220-1223.

284. Prakriya M, Feske S, Gwack Y, et al. Orai1 is an essential pore subunit of the CRAC channel. *Nature*. 2006;443:230-233.

285. Peinelt C, Vig M, Koomoa DL, et al. Amplification of CRAC current by STIM1 and CRACM1 (Orai1). *Nat Cell Biol* . 2006;8:771-773.

286. Feske S, Gwack Y, Prakriya M, et al. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature*. 2006;441: 179-185.

287. Muller MR, Rao A. NFAT, immunity and cancer: a transcription factor comes of age. *Nat Rev Immunol* . 2010;10:645-656.

288. Grakoui A, Bromley SK, Sumen C, et al. The immunological synapse: a molecular machine controlling T cell activation. *Science*. 1999;285: 221-227.

289. Love PE, Hayes SM. ITAM-mediated signaling by the T-cell antigen receptor. *Cold Spring Harb Perspect Biol*. 2010;2:a002485.

290. Underhill DM, Goodridge HS. The many faces of ITAMs. *Trends Immunol*. 2007;28:66-73.

291. Abram CL, Lowell CA. The expanding role for ITAM-based signaling pathways in immune cells. *Sci STKE*. 2007;2007:re2.

292. Goodridge HS, Reyes CN, Becker CA, et al. Activation of the innate immune receptor Dectin-1 upon formation of a “phagocytic synapse.” *Nature*. 2011;472:471-475.

293. Isaacs A, Lindenmann J. Virus interference. I. The interferon. *Proc R Soc Lond B Biol Sci*. 1957;147:258-267.

294. Rubinstein M, Rubinstein S, Familletti PC, et al. Human leukocyte interferon purified to homogeneity. *Science*. 1978;202:1289-1290.

295. Rubinstein M, Rubinstein S, Familletti PC, et al. Human leukocyte interferon: production, purification to homogeneity, and initial characterization. *Proc Natl Acad Sci U S A*. 1979;76:640-644.

296. Schindler C, Shuai K, Prezioso VR, et al. Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science*. 1992;257:809-813.

297. Velazquez L, Fellous M, Stark GR, et al. A protein tyrosine kinase in the interferon alpha/beta signaling pathway. *Cell*. 1992;70:313-322.

298. Muller M, Briscoe J, Laxton C, et al. The protein tyrosine kinase JAK1 complements defects in interferon-alpha/beta and -gamma signal transduction. *Nature*. 1993;366:129-135.

299. Watling D, Guschin D, Muller M, et al. Complementation by the protein tyrosine kinase JAK2 of a mutant cell line defective in the interferon-gamma signal transduction pathway. *Nature*. 1993;366:166-170.

300. Silvennoinen O, Ihle JN, Schlessinger J, et al. Interferon-induced nuclear signalling by Jak protein tyrosine kinases. *Nature*. 1993;366:583-585.

301. Argetsinger LS, Campbell GS, Yang X, et al. Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. *Cell*. 1993;74: 237-244.

302. Shuai K, Ziemiecki A, Wilks AF, et al. Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins. *Nature*. 1993;366:580-583.

303. Witthuhn BA, Quelle FW, Silvennoinen O, et al. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell*. 1993;74: 227-236.

304. Noguchi M, Nakamura Y, Russell SM, et al. Interleukin-2 receptor gamma chain: a functional component of the interleukin-7 receptor. *Science*. 1993;262:1877-1880.

305. Macchi P, Villa A, Giliani S, et al. Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID). *Nature*. 1995;377:65-68.

306. Russell SM, Tayebi N, Nakajima H, et al. Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. *Science*. 1995;270:797-800.

307. Holland SM, DeLeo FR, Elloumi HZ, et al. STAT3 mutations in the hyper-IgE syndrome. *N Engl J Med* . 2007;357:1608-1619.

308. Minegishi Y, Saito M, Tsuchiya S, et al. Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. *Nature*. 2007;448:1058-1062.

309. Smithies O, Gregg RG, Boggs SS, et al. Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. *Nature*. 1985;317:230-234.

310. Gossler A, Doetschman T, Korn R, et al. Transgenesis by means of blastocyst-derived embryonic stem cell lines. *Proc Natl Acad Sci U S A* . 1986;83:9065-9069.

311. Robertson E, Bradley A, Kuehn M, et al. Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. *Nature*. 1986;323:445-448.

312. Kuehn MR, Bradley A, Robertson EJ, et al. A potential animal model for Lesch-Nyhan syndrome through introduction of HPRT mutations into mice. *Nature*. 1987;326:295-298.

313. Hooper M, Hardy K, Handyside A, et al. HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. *Nature*. 1987;326:292-295.

314. Deuel TF, Keim PS, Farmer M, et al. Amino acid sequence of human platelet factor 4. *Proc Natl Acad Sci U S A* . 1977;74:2256-2258.

315. Walz DA, Wu VY, de Lamo R, et al. Primary structure of human platelet factor 4. *Thromb Res*. 1977;11:893-898.

316. Begg GS, Pepper DS, Chesterman CN, et al. Complete covalent structure of human beta-thromboglobulin. *Biochemistry (Mosc)*. 1978;17: 1739-1744.

317. Yoshimura T, Matsushima K, Tanaka S, et al. Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proc Natl Acad Sci U S A* . 1987;84:9233-9237.

318. Murphy PM, Tiffany HL. Cloning of complementary DNA encoding a functional human interleukin-8 receptor. *Science*. 1991;253:1280-1283.

319. Holmes WE, Lee J, Kuang WJ, et al. Structure and functional expression of a human interleukin-8 receptor. *Science*. 1991;253:1278-1280.

320. Pfeffer W. Lokomotorische richtungsbewegungen durch chemische reize. *Untersuch aus d Botan Inst Tübingen*. 1884:363-482.

321. Henderson M. A review of the motility of the leucocytes of the blood, with a few additional observations. *Anatom Rec*. 1928;38:71-95.

322. Zigmond SH, Hirsch JG. Leukocyte locomotion and chemotaxis. New methods for evaluation, and demonstration of a cell-derived chemotactic factor. *J Exp Med*. 1973;137:387-410.

323. Dutrochet JH. *Recherches anatomiques et physiologiques sur la structure intime des animaux et des végétaux, et sur leur motilité*. Paris, France: J.B. Bailliere; 1824.

324. Hayward AR, Harvey BA, Leonard J, et al. Delayed separation of the umbilical cord, widespread infections, and defective neutrophil mobility. *Lancet*. 1979;1:1099-1101.

325. Springer TA, Thompson WS, Miller LJ, et al. Inherited deficiency of the Mac-1, LFA-1, p150,95 glycoprotein family and its molecular basis. *J Exp Med*. 1984;160:1901-1918.

326. Marlin SD, Springer TA. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell*. 1987;51:813-819.

327. Muller WA, Randolph GJ. Migration of leukocytes across endothelium and beyond: molecules involved in the transmigration and fate of monocytes. *J Leukoc Biol*. 1999;66:698-704.

328. Muller WA. Mechanisms of leukocyte transendothelial migration. *Annu Rev Pathol*. 2011;6:323-344.

329. Shaw S, Luce GE, Quinones R, et al. Two antigen-independent adhesion pathways used by human cytotoxic T-cell clones. *Nature*. 1986;323: 262-264.

330. Gunn MD, Tangemann K, Tam C, et al. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proc Natl Acad Sci U S A*. 1998;95: 258-263.

331. Gunn MD, Kyuwa S, Tam C, et al. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J Exp Med*. 1999;189:451-460.

332. Coffman RL, Carty J. A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-gamma. *J Immunol*. 1986;136: 949-954.

333. Mosmann TR, Cherwinski H, Bond MW, et al. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*. 1986;136:2348-2357.

334. Howard M, Farrar J, Hilfiker M, et al. Identification of a T cell-derived b cell growth factor distinct from interleukin 2. *J Exp Med*. 1982;155: 914-923.

335. Rabin EM, Ohara J, Paul WE. B-cell stimulatory factor 1 activates resting B cells. *Proc Natl Acad Sci U S A* . 1985;82:2935-2939.

336. Oliver K, Noelle RJ, Uhr JW, et al. B-cell growth factor (B-cell growth factor I or B-cell-stimulating factor, provisional 1) is a differentiation factor for resting B cells and may not induce cell growth. *Proc Natl Acad Sci U S A* . 1985;82:2465-2467.

337. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol*. 1989;7:145-173.

338. Szabo SJ, Kim ST, Costa GL, et al. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*. 2000;100:655-669.

339. Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell*. 1997;89:587-596.

340. Chen Y, Kuchroo VK, Inobe J, et al. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science*. 1994;265:1237-1240.

341. Berlin I. *The Hedgehog and the Fox: An Essay on Tolstoy's View of History* . London, United Kingdom: Weidenfeld and Nicholson; 1953.

342. Duerr RH, Taylor KD, Brant SR, et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science*. 2006;314:1461-1463.

343. Jerne NK. Towards a network theory of the immune system. *Ann Immunol (Paris)*. 1974;125C:373-389.

344. Gershon RK, Kondo K. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology*. 1970;18:723-737.

345. Groux H, O'Garra A, Bigler M, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature*. 1997;389: 737-742.

346. Brunkow ME, Jeffery EW, Hjerrild KA, et al. Disruption of a new forkhead/winged-

helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet*. 2001;27: 68-73.

347. Chatila TA, Blaeser F, Ho N, et al. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. *J Clin Invest*. 2000;106:R75-81.

348. Wildin RS, Ramsdell F, Peake J, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet*. 2001;27:18-20.

349. Bennett CL, Christie J, Ramsdell F, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet*. 2001;27:20-21.

350. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003;299:1057-1061.

351. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*. 2003;4:330-336.

352. Kappler JW, Roehm N, Marrack P. T cell tolerance by clonal elimination in the thymus. *Cell*. 1987;49:273-280.

353. Kisielow P, Bluthmann H, Staerz UD, et al. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes. *Nature*. 1988;333:742-746.

354. Nossal GJ, Pike BL. Evidence for the clonal abortion theory of B-lymphocyte tolerance. *J Exp Med*. 1975;141:904-917.

355. Sidman CL, Unanue ER. Receptor-mediated inactivation of early B lymphocytes. *Nature*. 1975;257:149-151.

356. Raff MC, Owen JJ, Cooper MD, et al. Differences in susceptibility of mature and immature mouse B lymphocytes to anti-immunoglobulininduced immunoglobulin suppression in vitro. Possible implications for B-cell tolerance to self. *J Exp Med*. 1975;142:1052-1064.

357. Manicassamy S, Pulendran B. Retinoic acid-dependent regulation of immune responses by dendritic cells and macrophages. *Semin Immunol*. 2009;21:22-27.

358. Heitger A. Regulation of expression and function of IDO in human dendritic cells. *Curr Med Chem*. 2011;18:2222-2233.

359. Kim HJ, Verbinnen B, Tang X, et al. Inhibition of follicular T-helper cells by CD8(+)

regulatory T cells is essential for self tolerance. *Nature*. 2010;467:328-332.

360. Jiang H, Canfield SM, Gallagher MP, et al. HLA-E-restricted regulatory CD8(+) T cells are involved in development and control of human autoimmune type 1 diabetes. *J Clin Invest*. 2010;120:3641-3650.

361. Greten TF, Manns MP, Korangy F. Myeloid derived suppressor cells in human diseases. *Int Immunopharmacol* . 2011;11:802-807.

362. Tadmor T, Attias D, Polliack A. Myeloid-derived suppressor cells— their role in haemato-oncological malignancies and other cancers and possible implications for therapy. *Br J Haematol* . 2011;153:557-567.

363. Lees JR, Azimzadeh AM, Bromberg JS. Myeloid derived suppressor cells in transplantation. *Curr Opin Immunol* . 2011;23:692-697.

364. Ehrlich P, Morgenroth J. *On Haemolysins. Fifth Communication*. London: Pergamon; 1957.

365. Ehrlich P. Über den jetzigen stand der karzinomforschung. *Ned Tijdschr Geneeskd* . 1909;5:273-290.

366. Burnet M. Cancer: a biological approach. III. Viruses associated with neoplastic conditions. IV. Practical applications. *Br Med J* . 1957;1: 841-847.

367. Dighe AS, Richards E, Old LJ, et al. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFNγ receptors. *Immunity*. 1994;1:447-456.

368. Kaplan DH, Shankaran V, Dighe AS, et al. Demonstration of an interferon γ-dependent tumor surveillance system in immunocompetent mice. *Proc Natl Acad Sci U S A*. 1998;95:7556-7561.

369. Albert ML, Darnell JC, Bender A, et al. Tumor-specific killer cells in paraneoplastic cerebellar degeneration. *Nat Med*. 1998;4:1321-1324.

370. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science*. 2011;331:1565-1570.

371. Bevan MJ. Minor H antigens introduced on H-2 different stimulating cells cross-react at the cytotoxic T cell level during in vivo priming. *J Immunol* . 1976;117:2233-2238.

372. Bevan MJ. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med*. 1976;143:1283-1288.

373. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature*. 1998;392: 86-89.

374. Amigorena S, Savina A. Intracellular mechanisms of antigen cross presentation in dendritic cells. *Curr Opin Immunol* . 2010;22:109-117.

375. Garin J, Diez R, Kieffer S, et al. The phagosome proteome: insight into phagosome functions. *J Cell Biol* . 2001;152:165-180.

376. Gagnon E, Duclos S, Rondeau C, et al. Endoplasmic reticulummediated phagocytosis is a mechanism of entry into macrophages. *Cell*. 2002;110:119-131.

377. Hildner K, Edelson BT, Purtha WE, et al. *Batf3* deficiency reveals a critical role for CD8α+ dendritic cells in cytotoxic T cell immunity. *Science*. 2008;322:1097-1100.

378. Karre K, Ljunggren HG, Piontek G, et al. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature*. 1986;319:675-678.

379. Seiffert M, Cant C, Chen Z, et al. Human signal-regulatory protein is expressed on normal, but not on subsets of leukemic myeloid cells and mediates cellular adhesion involving its counterreceptor CD47. *Blood*. 1999;94:3633-3643.

380. Fujioka Y, Matozaki T, Noguchi T, et al. A novel membrane glycoprotein, SHPS-1, that binds the SH2-domain-containing protein tyrosine phosphatase SHP-2 in response to mitogens and cell adhesion. *Mol Cell Biol* . 1996;16:6887-6899.

381. Oldenborg PA, Zheleznyak A, Fang YF, et al. Role of CD47 as a marker of self on red blood cells. *Science*. 2000;288:2051-2054.

382. Chao MP, Alizadeh AA, Tang C, et al. Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. *Cell*. 2010;142:699-713.

383. Pnuemocystis pneumonia—Los Angeles. *MMWR*. 1981;30:250-252.

384. Sepkowitz KA. AIDS—the first 20 years. *N Engl J Med* . 2001;344: 1764-1772.

385. Barré-Sinoussi F, Chermann JC, Rey F, et al. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science*. 1983;220:868-871.

386. Gallo RC, Sarin PS, Gelmann EP, et al. Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science*. 1983;220:865-867.

387. Josephs SF, Dalla-Favera R, Gelmann EP, et al. 5′ viral and human cellular sequences corresponding to the transforming gene of simian sarcoma virus. *Science*. 1983;219:503-505.

388. Gelmann EP, Popovic M, Blayney D, et al. Proviral DNA of a retrovirus, human T-cell leukemia virus, in two patients with AIDS. *Science*. 1983;220:862-865.

389. Popovic M, Sarin PS, Robert-Gurroff M, et al. Isolation and transmission of human retrovirus (human t-cell leukemia virus). *Science*. 1983;219:856-859.

390. Dalla-Favera R, Martinotti S, Gallo RC, et al. Translocation and rearrangements of the c-myc oncogene locus in human undifferentiated B-cell lymphomas. *Science*. 1983;219:963-967.

391. Maddon PJ, Dalgleish AG, McDougal JS, et al. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell*. 1986;47:333-348.

392. Alkhatib G, Combadiere C, Broder CC, et al. CC CKR5: a RANTES, MIP-1α, MIP-1β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science*. 1996;272:1955-1958.

393. Ho DD, Bieniasz PD. HIV-1 at 25. *Cell*. 2008;133:561-565.

394. Fauci AS. 25 years of HIV. *Nature*. 2008;453:289-290.

395. Metchnikoff E. *Immunity in Infective Diseases*. London, United Kingdom: Cambridge University Press; 1905.

396. Fleming A. Lysozyme: president's address. *Proc R Soc Med*. 1932;26:71-84.

397. Allison VD. Personal recollections of Sir Almroth Wright and Sir Alexander Fleming. *Ulster Med J* . 1974;43:89-98.

398. Territo MC, Ganz T, Selsted ME, et al. Monocyte-chemotactic activity of defensins from human neutrophils. *J Clin Invest*. 1989;84:2017-2020.

399. Chertov O, Michiel DF, Xu L, et al. Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from interleukin-8-stimulated neutrophils. *J Biol Chem*. 1996;271:2935-2940.

400. Yang D, Chertov O, Bykovskaia SN, et al. β-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science*. 1999;286:525-528.

401. Cole AM, Ganz T, Liese AM, et al. Cutting edge: IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity. *J Immunol* . 2001;167:623-627.

402. Kiessling R, Klein E, Wigzell H. “Natural” killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur J Immunol* . 1975;5:112-117.

403. Kiessling R, Klein E, Pross H, et al. “Natural” killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur J Immunol* . 1975;5:117-121.

404. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. *Int J Cancer* . 1975;16:216-229.

405. Vivier E, Raulet DH, Moretta A, et al. Innate or adaptive immunity? The example of natural killer cells. *Science*. 2011;331:44-49.

406. Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* . 1989;54:1-13.

407. Freund J, McDermott K. Sensitization to horse serum by means of adjuvants. *Proc Soc Exp Biol Med*. 1942;49:548-553.

408. Sen R, Baltimore D. Inducibility of κ immunoglobulin enhancerbinding protein Nf-κB by a posttranslational mechanism. *Cell*. 1986;47: 921-928.

409. Weih F, Carrasco D, Durham SK, et al. Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-κB/Rel family. *Cell*. 1995;80:331-340.

410. Sha WC, Liou HC, Tuomanen EI, et al. Targeted disruption of the p50 subunit of NF-κB leads to multifocal defects in immune responses. *Cell*. 1995;80:321-330.

411. Lemaitre B, Nicolas E, Michaut L, et al. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell*. 1996;86:973-983.

412. Wright SD, Ramos RA, Tobias PS, et al. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science*. 1990;249:1431-1433.

413. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature*. 1997;388:394-397.

414. Beutler B, Milsark IW, Cerami AC. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science*. 1985;229:869-871.

415. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*. 1998;282: 2085-2088.

416. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity*. 2011;34:637-650.

417. Iwasaki A, Medzhitov R. Regulation of adaptive immunity by the innate immune system. *Science*. 2010;327:291-295.

418. Roozendaal R, Carroll MC. Emerging patterns in complementmediated pathogen recognition. *Cell*. 2006;125:29-32.

419. Taylor PR, Tsoni SV, Willment JA, et al. Dectin-1 is required for β-glucan recognition and control of fungal infection. *Nat Immunol*. 2007;8: 31-38.

420. Gardai SJ, Xiao YQ, Dickinson M, et al. By binding SIRPα or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. *Cell*. 2003;115:13-23.

421. Kuhlman M, Joiner K, Ezekowitz RA. The human mannose-binding protein functions as an opsonin. *J Exp Med*. 1989;169:1733-1745.

422. Philips JA, Rubin EJ, Perrimon N. Drosophila RNAi screen reveals CD36 family member required for mycobacterial infection. *Science*. 2005;309:1251-1253.

423. Peiser L, Mukhopadhyay S, Gordon S. Scavenger receptors in innate immunity. *Curr Opin Immunol*. 2002;14:123-128.

424. Bottazzi B, Garlanda C, Salvatori G, et al. Pentraxins as a key component of innate immunity. *Curr Opin Immunol* . 2006;18:10-15.

425. Garlanda C, Hirsch E, Bozza S, et al. Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response. *Nature*. 2002; 420:182-186.

426. Tino MJ, Wright JR. Surfactant protein A stimulates phagocytosis of specific pulmonary pathogens by alveolar macrophages. *Am J Physiol*. 1996;270:L677-688.

427. Geijtenbeek TB, Kwon DS, Torensma R, et al. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell*. 2000;100:587-597.

428. Ezekowitz RA, Sim RB, Hill M, et al. Local opsonization by secreted macrophage complement components. Role of receptors for complement in uptake of zymosan. *J Exp Med*. 1984;159:244-260.

429. Sancho D, Joffre OP, Keller AM, et al. Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature*. 2009; 458:899-903.

430. Devitt A, Moffatt OD, Raykundalia C, et al. Human CD14 mediates recognition and phagocytosis of apoptotic cells. *Nature*. 1998;392: 505-509.

431. Hanayama R, Tanaka M, Miwa K, et al. Identification of a factor that links apoptotic cells to phagocytes. *Nature*. 2002;417:182-187.

432. Hanayama R, Tanaka M, Miyasaka K, et al. Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science*. 2004;304:1147-1150.

433. Scott RS, McMahon EJ, Pop SM, et al. Phagocytosis and clearance of apoptotic cells is mediated by MER. *Nature*. 2001;411:207-211.

434. Rodriguez-Manzanet R, Sanjuan MA, Wu HY, et al. T and B cell hyperactivity and autoimmunity associated with niche-specific defects in apoptotic body clearance in TIM-4-deficient mice. *Proc Natl Acad Sci U S A* . 2010;107:8706-8711.

435. Godson C, Mitchell S, Harvey K, et al. Cutting edge: lipoxins rapidly stimulate nonphlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages. *J Immunol*. 2000;164:1663-1667.

436. Ren Y, Stuart L, Lindberg FP, et al. Nonphlogistic clearance of late apoptotic neutrophils by macrophages: efficient phagocytosis independent of beta 2 integrins. *J Immunol*. 2001;166:4743-4750.

437. Huynh ML, Fadok VA, Henson PM. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-β1 secretion and the resolution of inflammation. *J Clin Invest*. 2002;109:41-50.

438. Schwab JM, Chiang N, Arita M, et al. Resolvin E1 and protectin D1 activate inflammation-resolution programmes. *Nature*. 2007;447: 869-874.

439. Serhan CN, Clish CB, Brannon J, et al. Novel functional sets of lipidderived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2-nonsteroidal antiinflammatory drugs and transcellular processing. *J Exp Med*. 2000;192: 1197-1204.

440. Bridges RA, Berendes H, Good RA. A fatal granulomatous disease of childhood; the clinical, pathological, and laboratory features of a new syndrome. *AMA J Dis Child*. 1959;97:387-408.

441. Quie PG, White JG, Holmes B, et al. In vitro bactericidal capacity of human polymorphonuclear leukocytes: diminished activity in chronic granulomatous disease of

childhood. *J Clin Invest*. 1967;46: 668-679.

442. Holmes B, Page AR, Good RA. Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocytic function. *J Clin Invest*. 1967;46:1422-1432.

443. Curnutte JT, Whitten DM, Babior BM. Defective superoxide production by granulocytes from patients with chronic granulomatous disease. *N Engl J Med*. 1974;290:593-597.

444. Baehner RL, Nathan DG. Quantitative nitroblue tetrazolium test in chronic granulomatous disease. *N Engl J Med*. 1968;278:971-976.

445. Segal AW, Jones OT. Novel cytochrome b system in phagocytic vacuoles of human granulocytes. *Nature*. 1978;276:515-517.

446. Segal AW, Jones OT, Webster D, et al. Absence of a newly described cytochrome b from neutrophils of patients with chronic granulomatous disease. *Lancet*. 1978;2:446-449.

447. Dinauer MC, Orkin SH, Brown R, et al. The glycoprotein encoded by the X-linked chronic granulomatous disease locus is a component of the neutrophil cytochrome b complex. *Nature*. 1987;327:717-720.

448. Volpp BD, Nauseef WM, Clark RA. Two cytosolic neutrophil oxidase components absent in autosomal chronic granulomatous disease. *Science*. 1988;242:1295-1297.

449. Leto TL, Lomax KJ, Volpp BD, et al. Cloning of a 67-kD neutrophil oxidase factor with similarity to a noncatalytic region of p60c-src. *Science*. 1990;248:727-730.

450. Abo A, Pick E, Hall A, et al. Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. *Nature*. 1991;353:668-670.

451. Bokoch GM, Quilliam LA, Bohl BP, et al. Inhibition of Rap1A binding to cytochrome b558 of NADPH oxidase by phosphorylation of Rap1A. *Science*. 1991;254:1794-1796.

452. Diekmann D, Abo A, Johnston C, et al. Interaction of Rac with p67phox and regulation of phagocytic NADPH oxidase activity. *Science*. 1994;265:531-533.

453. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev*. 2007;87:245-313.

454. MacMicking JD, North RJ, LaCourse R, et al. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc Natl Acad Sci U S A*. 1997;94:5243-5248.

455. Savina A, Jancic C, Hugues S, et al. NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. *Cell*. 2006;126:205-218.

456. Dagenais M, Skeldon A, Saleh M. The inflammasome: in memory of Dr. Jurg Tschopp. *Cell Death Differ*. 2012;19:5-12.

457. Ogura Y, Bonen DK, Inohara N, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature*. 2001;411:603-606.

458. Miceli-Richard C, Lesage S, Rybojad M, et al. CARD15 mutations in Blau syndrome. *Nat Genet*. 2001;29:19-20.

459. Hoffman HM, Mueller JL, Broide DH, et al. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nat Genet*. 2001;29: 301-305.

460. Centola M, Wood G, Frucht DM, et al. The gene for familial Mediterranean fever, MEFV, is expressed in early leukocyte development and is regulated in response to inflammatory mediators. *Blood*. 2000;95:3223-3231.

461. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of prolL-beta. *Mol Cell*. 2002;10:417-426.

462. Auron PE, Webb AC, Rosenwasser LJ, et al. Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. *Proc Natl Acad Sci U S A* . 1984;81:7907-7911.

463. Bennett IL Jr, Beeson PB. Studies on the pathogenesis of fever. I. The effect of injection of extracts and suspensions of uninfected rabbit tissues upon the body temperature of normal rabbits. *J Exp Med*. 1953;98:477-492.

464. Bennett IL Jr, Beeson PB. Studies on the pathogenesis of fever. II. Characterization of fever-producing substances from polymorphonuclear leukocytes and from the fluid of sterile exudates. *J Exp Med*. 1953;98:493-508.

465. Martinon F, Petrilli V, Mayor A, et al. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature*. 2006;440:237-241.

466. Nuki G, Simkin PA. A concise history of gout and hyperuricemia and their treatment. *Arthritis Res Ther*. 2006;8(suppl 1):S1.

467. Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol*. 1994;12:991-1045.

468. Matzinger P. The danger model: a renewed sense of self. *Science*. 2002; 296:301-305.

469. Greenberg S, Di Virgilio F, Steinberg TH, et al. Extracellular nucleotides mediate Ca2+ fluxes in J774 macrophages by two distinct mechanisms. *J Biol Chem*. 1988;263:10337-10343.

470. McDonald B, Pittman K, Menezes GB, et al. Intravascular danger signals guide neutrophils to sites of sterile inflammation. *Science*. 2010;330: 362-366.

471. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. *Nature*. 2011;469:323-335.

472. Gutierrez MG, Master SS, Singh SB, et al. Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell*. 2004;119:753-766.

473. Nakagawa I, Amano A, Mizushima N, et al. Autophagy defends cells against invading group A *Streptococcus*. *Science*. 2004;306:1037-1040.

474. Mishell RI, Dutton RW. Immunization of dissociated spleen cell cultures from normal mice. *J Exp Med*. 1967;126:423-442.

475. Mosier DE. A requirement for two cell types for antibody formation in vitro. *Science*. 1967;158:1573-1575.

476. Mosier DE, Coppleson LW. A three-cell interaction required for the induction of the primary immune response in vitro. *Proc Natl Acad Sci U S A* . 1968;61:542-547.

477. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med*. 1973;137:1142-1162.

478. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. *J Exp Med*. 1974;139:380-397.

479. Steinman RM, Lustig DS, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. 3. Functional properties in vivo. *J Exp Med*. 1974;139:1431-1445.

480. Steinman RM, Kaplan G, Witmer MD, et al. Identification of a novel cell type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, new surface markers, and maintenance in vitro. *J Exp Med*. 1979;149:1-16.

481. Blanco P, Palucka AK, Gill M, et al. Induction of dendritic cell differentiation by IFN-α

in systemic lupus erythematosus. *Science*. 2001;294: 1540-1543.

482. Jego G, Palucka AK, Blanck JP, et al. Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. *Immunity*. 2003;19:225-234.

483. Soumelis V, Reche PA, Kanzler H, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol*. 2002;3:673-680.

484. Ito T, Wang YH, Duramad O, et al. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med*. 2005;202:1213-1223.

485. Al-Shami A, Spolski R, Kelly J, et al. A role for TSLP in the development of inflammation in an asthma model. *J Exp Med*. 2005;202: 829-839.

486. Landsteiner K, van der Scheer J. Experiments on the production of Wassermann reagins by means of trypanosomes. *J Exp Med*. 1927;45: 465-482.

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Chapter 3

Lymphoid Tissues and Organs

Eitan M. Akirav

Noelia Alonso-Gonzalez

Lucy A. Truman

Nancy H. Ruddle

INTRODUCTION

The mammalian immune system defends against invading pathogens, by both the innate and adaptive mechanisms. Although cells that can respond to pathogens are scattered in tissues throughout the body, the optimal structures for the response to antigens are organized, compartmentalized cellular aggregates that facilitate antigen concentration and presentation to a large repertoire of antigen-specific lymphocytes. The primary lymphoid organs, the fetal liver, thymus, and bone marrow, are the sites where diverse populations of naïve lymphocytes mature to disperse throughout the body to await foreign invaders. This remarkable differentiation process occurs in a foreign antigen-independent fashion. The secondary lymphoid organs, including the lymph nodes, spleen, Peyer's patches, and other mucosal-associated lymphoid tissues (MALTs), are discrete sites in which naïve, antigen-specific T- and B-lymphocytes encounter invaders to generate an adaptive response. The lymph nodes and spleen have been considered to be somewhat static structures, while, in fact, they are responsive to environmental influences and undergo remarkable changes in the course of antigenic challenge. Precise programs control the development of the spleen, lymph nodes, Peyer's patches, tonsils and adenoids, and (in the mouse and rat) the nasal-associated lymphoid tissue (NALT). Somewhat less anatomically restricted tissues that are even more sensitive to the environment facilitate and include accumulations of lymphoid cells are organized, but less discretely defined: the bronchus-associated lymphoid tissues (BALTs) and inducible lymphoid follicles (ILFs). Tertiary lymphoid organs, or more accurately, tertiary lymphoid tissues, are accumulations of lymphoid cells that arise ectopically in sites that are not anatomically restricted and are not regulated by developmental programs. Tertiary lymphoid tissues respond to environmental stimuli and arise during chronic inflammation subsequent to microbial infection, graft rejection, autoimmunity, or cancer by the process of lymphoid neogenesis.[1,2] In this chapter, the structure, function, trafficking patterns, and developmental signals that regulate the hierarchy of lymphoid organs will be described.

PRIMARY LYMPHOID ORGANS

The primary lymphoid organs are the sites where pre-B- and pre-T-lymphocytes mature into naïve B and T cells in the absence of foreign antigen. Each T cell or B cell expresses a

unique receptor that can recognize and respond to exogenous antigen and, in most cases, discriminate between self-and foreign antigens. Naïve cells leave the primary lymphoid organs having received and responded to developmental cues that result in the rearrangement of their genetic material to generate a repertoire capable of recognizing and responding to a wide variety of foreign antigens. In the course of maturation in the primary lymphoid organs, the naïve lymphocytes express various chemokine receptors and adhesion molecules that direct them to secondary lymphoid organs.

Fetal Liver

The earliest lymphoid cell precursors derive from self-renewing hematopoietic precursors called hematopoietic stem cells (HSCs). During ontogeny, these cells occupy several niches. In the fetal mouse, the first wave of hematopoiesis occurs in the yolk sac and aorta-gonad-mesonephros region at E10.5.[3] The placenta also contains HSC activity.[4,5,6] Cells leave these tissues and migrate to the fetal liver, and then the bone marrow and thymus and spleen under the influence of chemokines and adhesion molecules (Fig. 3.1). The cells in the fetal liver respond to CXCL12 (stromal cell derived factor) and, in contrast to those in the bone marrow, also respond to Steel factor[7] (Table 3.1). The fetal liver is also the source of CD4+CD3- lymphoid tissue inducer cells that express lymphotoxin (LT)α (also called tumor necrosis factor [TNF] β) and LTβ. The requirement of inducer cells for the

development of secondary lymphoid organs is described in the following. Differentiation of HSCs to B cells occurs in the fetal liver, but does not require interleukin (IL)-7. B-cell development is described in detail elsewhere in this volume. Of the several different subsets of B cells, those generated by the fetal liver HSCs are somewhat limited and are of the B-1 subset.[8] They do not give rise to cluster of differentiation (CD)5 B cells, predominately B1-B cells, and do not express terminal deoxynucleotidyl transferase and myosin-like light chain.[8,9] It is not known whether these differences are intrinsic to the cells or are due to differences in the cytokine environment of fetal liver and bone marrow. The ligands for P-selectin, E-selectin, and vascular cell adhesion molecule (VCAM)-1 are required for the cells to leave the fetal liver and home to the bone marrow.[10,11]

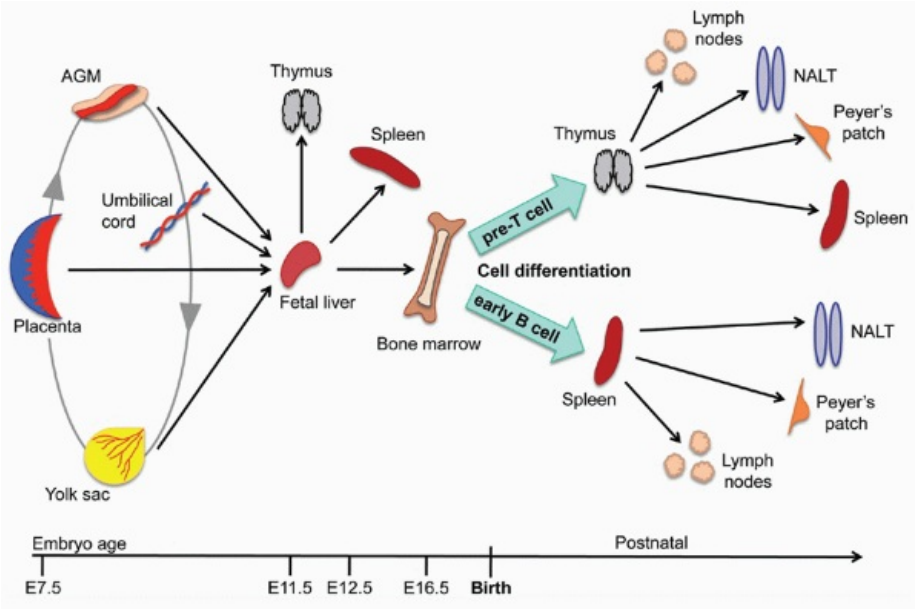

FIG. 3.1. Embryonic and Postnatal Development of T and B Cells in Mouse Primary and Secondary Lymphoid Organs. Embryonic diagram based on information summarized in Medvinsky et al.[6]

TABLE 3.1 Chemokines Implicated in Lymphoid Organ Development and Maintenance

Standard Name	Common Names	Receptor
CXCL12	SDF-1	CXCR4
CXCL13	BCA-1, BLC	CXCR5
CCL17	TARC	CCR4
CCL19	ELC, MIP-3β	CCR7
CCL20	MIP-3α	CCR6
CCL21	SLC, 6Ckine	CCR7
CCL25	TECK	CCR9
CCL28	MEC	CCR10

BCA, B-cell chemoattractant; BLC, B lymphocyte chemoattractant; ELC, EBI-1 ligand

chemokine; MEC, mucosae as-associated epithelial chemokine; MIP, macrophage inflammatory protein; SDF-1, stromal cell derived factor; SLC, secondary lymphoid tissue chemokine; TARC, thymus and activation regulatory chemokine; TECK, thymus-expressed chemokine.

Bone Marrow

Functions

The bone marrow is the source of self-renewing populations of stem cells. These cells include hematopoietic precurscor cells, HSCs, and endothelial progenitor cells, which may derive from a single precursor.[12] The adult bone marrow contains stem cells that can differentiate into adipocytes, chondrocytes, osteocytes, and myoblasts.[13] Collectively, these cells are called hematopoietic/stem progenitor cells. In the adult, cells leave the leave the bone marrow and seed the thymus where they undergo differentiation to naïve T cells. Additional factors enable the differentiation of immature B cells from HSCs.

In addition to serving as a primary lymphoid organ where B-cell differentiation and development occur, the bone marrow is also a home for antibody secreting cells.[14] After B cells

have interacted with antigen in the secondary lymphoid organs, such as the lymph nodes, spleen, and Peyer's patches, they enter the bloodstream and travel to the marrow. Thus, this organ not only serves as a primary lymphoid organ, but also as a reservoir for fully differentiated plasma cells.

Architecture: Cellular and Functional Niches

The microenvironment of the bone marrow, contained in the central cavity of bone, is a complex three-dimensional structure, with cellular niches that influence B cells during their development and later, as plasma cells, as they return to the bone marrow. The bone marrow has a rich blood supply with a nutrient artery that branches into ascending and descending arteries further dividing into cortical capillaries, periosteal capillaries, and endosteal capillaries, finally merging into a sinus.[15] Previously, the prevailing understanding of B-cell differentiation in the bone marrow was that primitive HSCs were located in close contact with the endosteum near osteoblasts (the “endosteal niche”). During the course of differentiation into mature B cells, they moved into the central region of the bone marrow cavity (the “vascular niche”).[16] The former niche was identified as the location of HSC; the latter, as the site of B-cell differentiation.[17] This anatomic concept has been challenged, as it has been reported that HSCs are found throughout the bone marrow. More recently, a reticular niche has been described that includes CXCL12 abundant reticular cells.[18] Growth factors and cytokines produced by different stromal cells influence cells at different stages in their differentiation. Thus, it is more appropriate to consider functional or cellular, rather than anatomical, niches.[15,19] Once the HSCs differentiate into immature B cells expressing cell surface immunoglobulin (Ig) M, they undergo processes of negative selection and receptor editing, leave the bone marrow, and travel through the blood stream to the secondary

lymphoid organs where they complete their differentiation.

Several cytokines and chemokines influence B-cell differentiation in the bone marrow. Flt-3 ligand (also called Flk-2L) signals B-cell differentiation and growth and synergizes with several other hematopoietic growth factors.[20] Its receptor, Flt-3 (also known as Flk-2), expressed by primitive HSCs, is a member of the class II tyrosine kinase family. In contrast to fetal liver, HSCs from adult bone marrow do not respond to Steel factor. Chemokines contribute to B-cell differentiation in the bone marrow and define the functional niches. For excellent reviews of this topic, see Nagasawa,[15] Heissig et al.,[18] and Mazo et al.[21] Many of these factors affect other lymphoid cells, such as dendritic cells (DCs) and T cells. Several of these factors, whose functions have been identified in gene deletion studies in mice, in morphologic analysis, and in cell culture studies, play roles in multiple aspects of lymphoid organ development; their activities, though important in the bone marrow, are not limited to that organ. CXCL12, also known as stromal cell derived factor, is a chemokine that is crucial for recruitment of HSCs to the bone marrow. It is widely expressed by osteoblasts, reticular cells,[19] and endothelial cells.[22] In fact, the interaction of HSCs expressing CXCR4, the receptor for CXCL12, with that chemokine on the endothelial surface is the first step in the HSC's exit from the circulation into the marrow.[23] CXCL12 is also essential for the earliest stage of B-cell development (pre-pro-B cells). Its receptor, CXCR4, is expressed on early B cells and is downregulated in pre-B cells. CXCR4 remains at low levels in immature B cells and mature B cells in secondary lymphoid organs, but is upregulated after B cells interact with antigen and differentiate into plasma cells.[14] This explains the propensity of antibody-secreting cells to return to the bone marrow. Once a pre-pro-B cell has interacted with CXCL12, it moves on to a different cell expressing IL-7. In B-cell development, IL-7 acts later than CXCL12 in a narrow window between pro-B cells and immature B cells in the scheme proposed by Nagasawa.[15]

Traffic In and Out: Chemokines and Adhesion Molecules

The extensive vascularization of the bone marrow allows entrance of hematopoietic precursors and plasma cells and egress of mature cells. Hematopoietic progenitor recruitment to the bone marrow in the mouse is dependent on the interaction of a variety of chemokines, integrins, and selectins, and their receptors, counter receptors, and vascular cell adhesion molecules. These include α_4 integrin (VLA4 or $\alpha_4\beta_1$), VCAM-1,[24] P-selectin glycoprotein ligand-1, E-selectin,[25] $\alpha_4\beta_7$, and mucosal addressin cell adhesion molecule (MAdCAM-1).[26] A small subpopulation of newly formed B cells in the bone marrow that expresses L-selectin has been described,[27] suggesting a mechanism for entrance into lymph nodes (see subsequent discussion).

In addition to the acquisition of Ig expression that occurs in the bone marrow under the control of stromal cells, B cells express various chemokine receptors and adhesion molecules in the process of differentiation. In a study of human bone marrow B cells, it was determined that pro-B and pre-B cells migrate toward CXCL12, but not toward a wide range of other chemokines, including CCL19 and CCL21, though they do express low levels of CCR7, the receptor for those ligands. However, mature bone marrow B cells do respond to CCL 19 and CCL 21,[28] CXCL13 (indicating a functional CXCR5), and CCL20 (MIP3α). On the other hand,

CCR6 expression and responsiveness to CCL20 are only seen in mouse B cells after they have migrated into the periphery during the process of maturation and are in the circulating B cell pool.[29]

Thymus

Functions

The thymus is defined as a primary lymphoid organ due to its inimitable role in T-cell development. Indeed, a mutant known as the “nude” (*nu/nu*) mouse, which lacks a normal thymus, is completely devoid of mature T cells.[30] Although some studies suggest that T cells can develop extrathymically in organs such as the “cervical thymus”[31] and the gut epithelium,[32] the thymus remains the main site for T-cell maturation, education, and selection. T-cell precursors represent more than 95% of total cells in the thymus and give rise to mature T cells. These cells are crucial components of the adaptive immune system in that they are highly specific in their ability to recognize a nearly infinite number of antigens owing to their diverse repertoire.[30,33]

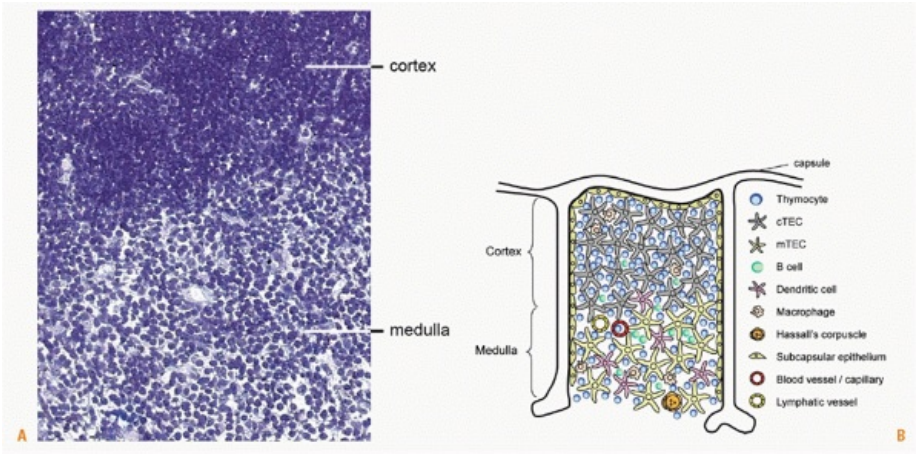

FIG. 3.2. A: Thymic structure. Low power magnification of a hematoxylin-stained frozen section of a mouse thymus. The cortex is well populated with lymphocytes; the medulla is less tightly packed and thus stains less intensely. **B:** Diagram of mouse thymic cellular populations. Precursor T cells enter through blood vessels at the corticomedullary junction. They progress to the medulla where they undergo differentiation from double negative to double positive (DP) cells expressing T-cell receptors. Thymic stromal cells provide growth factors. DP cells undergo positive selection, under the influence of cortical thymic epithelial cells. Single positive (SP) cluster of differentiation (CD)4 or CD8 cells migrate into the medulla where they undergo negative selection, mainly through autoimmune regulator expressing medullary thymic epithelial cells and dendritic cells. SP cells, having undergone differentiation, exit through blood vessels (not shown) to the periphery.

The T-cell repertoire is shaped during development in the thymus by the processes of

positive and negative selection. Negative and positive selection are so stringent that nearly 95% of all T-cell precursors are deleted in the thymus.[34] Negative selection ensures that T cells, which are capable of recognizing the organism's self-antigens presented on major histocompatibility complex (MHC) class I or II molecules with high affinity, are eliminated prior to their export to the periphery. In contrast, positive selection, which requires a higher degree of T-cell receptor (TCR) avidity than negative selection,[35] allows for T cells recognizing self-antigen with low to medium affinity to leave the thymus and eventually protect the organism against invading pathogens.

While negative selection is highly efficient in eliminating the majority of self-reactive T cells, some of these cells do escape the thymus and exit to the periphery. These autoreactive cells impose a threat to various organs as their activation may result in the development of autoimmune diseases. The immune system has evolved several ways to prevent the activation of autoreactive T cells in the periphery. One mechanism that protects against autoimmunity is the generation of a T-cell population capable of suppressing activation of self-reactive T cells. These thymus-derived protective T cells, known as regulatory cells (T_{reg}), recognize self-antigens with relatively high affinity.

Architecture

The thymus in the mouse consists of two symmetric lobes located above the heart, while in humans the thymus is multilobed. Each lobe can be divided into three distinct regions: capsule, cortex, and medulla. The latter two regions harbor thymocytes at various maturation stages. Although maturing T cells constitute the majority of cells in the thymus, other cell types such as macrophages, DCs, B cells, and epithelial cells are also present.[36] Histologic analysis of the thymus reveals a clear distinction between the thymic cortex and medulla, which are separated by a corticomedullary border. The thymic cortex appears darker and more densely populated with T-cell precursors, whereas, the medulla appears considerably lighter and contains smaller numbers of T cells relative to other cell types (Fig. 3.2A). Blood vessels and small blood capillaries are found throughout the thymus. The fact that T-cell progenitors are found in the more highly vascularized corticomedullary border suggests that blood vessels in this region facilitate the entry of T-cell progenitors into the thymic parenchyma.[37] In the thymic medulla, the close association between medullary thymic epithelial cells and thymic blood vessels[38] suggests that these vessels may act as organizers of the medullary thymic compartment. Lymphatic vessel distribution coincides with that of blood vessels and capillaries.[39] The majority of lymphatic vessels are located in the thymic medulla, though some can also be found in the cortex. The role of lymphatic vessels in thymic function is unclear, although it has been proposed that these vessels deliver extrathymic antigens into the thymus or export mature T cells from the thymus into the circulation.

Cellular Composition and Functions

T-cell precursors at various stages of differentiation represent the majority of cells in the thymus. Antigen-presenting cells (APCs) of either hematopoietic or stromal origin mediate the education and selection of T cells in the thymus. The different stages in T-cell selection and maturation that take place in distinct regions of the thymus (Fig. 3.2B) are discussed at length

elsewhere in this volume. Briefly, lymphoid progenitors enter the thymus at the corticomedullary border.[37] Following their entry, these cells, identified as double negative (DN) T cells due to a lack of expression of the cell surface molecules CD4 and CD8, undergo four maturation steps termed DN1 to DN4, which are distinguished by the expression of two additional cell surface molecules: CD25 and CD44.[40,41] DN3 cells migrate to the subcapsular zone while rearranging their TCRβ chain and expressing it in combination with a surrogate α chain. Those cells that have successfully rearranged the genes for α and β chains of the TCR become double positive (DP) cells, and express both CD4 and CD8 surface markers. In the cortex, DP cells undergo negative and positive selection.[42,43] Positively selected DP cells further differentiate into single positive (SP) cells expressing either CD4 or CD8. Following their differentiation, SP cells relocate to the medulla where they mature and undergo further rounds of deletion. SP cells that do survive are then exported out of the thymus.[44] DN precursors can give rise to an additional T-cell population expressing the γδTCR. These T cells are distinct from TCRαβ T cells in their tissue distribution and recognition of antigens. TCRαβ DP cells control the development of TCRγδ cells via the production of LTβ.[45] T_{reg} development in the human thymus occurs at the DP stage allowing for the production of CD4 and CD8 positive T_{reg}s.[46]

The thymic parenchyma consists of a complex threedimensional structure supported by thymic epithelial cells (TECs). TECs in the thymic cortex and thymic medulla are phenotypically and functionally distinct and support different stages of T-cell maturation. Several cell surface markers are used to distinguish medullary TECs (mTECs) from cortical TECs (cTECs) in the mouse. Among these markers are the cytokeratins K5 and K8, the adhesion molecule Ep-CAM, and the glycoprotein Ly-51. K8 and Ly-51 are expressed by cTECs, while K5 and Ep-CAM are expressed by mTECs.[47,48] TECs are unique in that they express MHC II constitutively, similarly to professional APCs. The role of TECs in T-cell selection was recently elucidated by their expression of the transcription factor, autoimmune regulator (AIRE). This transcription factor plays an important role in T-cell selection and prevention of autoimmunity, as illustrated by the fact that humans with a mutated form of the *AIRE* gene exhibit polyendocrine autoimmunity due to inadequate T-cell selection.[49,50] It has become clear that AIRE controls the expression of certain tissue-restricted antigens in the thymus, such as insulin, a protein that is unique to the β cells of the islets of Langerhans in the pancreas.[48,51] The expression of tissue-restricted antigens in the thymus facilitates the negative selection of maturing T cells, which would otherwise be allowed to leave the thymus. The thymic medulla includes distinct structures also known as Hassall corpuscles. Originally described by Arthur Hill Hassall in 1849, this structure consists of concentric stratified keratinizing epithelium. Hassall corpuscles are implicated in several processes of the thymus,[52] including the expression of tissue-restricted antigens, such as insulin (a key antigen in type 1 diabetes),[53] Igs, and filaggrin (key antigens in rheumatoid arthritis),[54,55] and serving as a prominent site for T-cell apoptosis.[56]

DCs are professional APCs that are found in the thymus. Thymic DCs can be divided into two distinct populations. The first population originates from a thymocyte precursor,[57,58] whereas the second population is derived from partially mature peripheral DCs that

continuously enter the thymus from the circulation.[59] DCs do not appear to be involved in positive selection, but do contribute to negative selection.[60,61,62,63] They have also been implicated in the selection of T_{reg}s in humans,[64] and recent reports in the mouse suggest that peripheral DCs can migrate to the thymus and serve as efficient inducers of T_{reg}s.[65] Activation of thymic DCs can be mediated in part by the IL-7-like cytokine, thymic stromal lymphopoietin. In the human thymus, Hassall corpuscles produce thymic stromal lymphopoietin, and thymic stromal lymphopoietin-activated DCs can alter the fate of self-reactive T cells from deletion to positive selection of T_{reg}s.[66] More specifically, thymic stromal lymphopoietin expression in a subset of DCs, plasmacytoid DCs, can induce the generation of particularly potent T_{reg}s from CD4+CD8- CD25- thymocytes.[67] These findings highlight the heterogeneity of DCs and emphasize their ability to fulfill different roles during T-cell development in the thymus.

Macrophages and B cells are additional hematopoieticderived professional APCs in the thymus. In contrast to DCs, thymic macrophages are located throughout the thymus and do not play a significant role in T-cell selection.[68] B cells are detected in human and mouse thymus at relatively low numbers[69,70] and are characterized by the expression of the cell surface molecule CD5. They are capable of producing antibodies of several different isotypes.[71] It has been suggested that thymic B cells induce negative selection in developing thymocytes,[69] although B cell-deficient mice show a limited T-cell repertoire when compared with normal mice, also suggesting a role in positive selection.[72] Recent data consistent with a role for B cells in thymic negative selection have emerged. Mice whose B cells were experimentally manipulated to express a myelin oligodendrocyte glycoprotein peptide show deletion of myelin oligodendrocyte glycoprotein-specific T effector cells.[73] One mechanism for the effect of B cells on T-cell selection is suggested by the observation that B cells play a role in the control of tissue-restricted antigens. Studies done in mice deficient in B cells showed fewer thymic epithelial cells with reduced expression of insulin and myelin oligodendrocyte glycoprotein, native antigens of the pancreas and brain, respectively.[74]

Traffic In and Out: Adhesion Molecules and Chemokines

Adhesion molecules mediate the extravasation of leukocytes from blood and lymphatic vessels into the thymus. These molecules also play an important role in facilitating lymphocyte homing into various regions of the thymus. Indeed,

thymic blood vessels express the adhesion molecules intercellular adhesion molecule (ICAM)-1, VCAM-1, CD34, peripheral node addressin (PNAd) and vascular adhesion protein-1.[75] The expression of high levels of ICAM-1, VCAM-1, and vascular adhesion protein-1 on venules near the corticomedullary border suggests that these molecules may play a role in the recruitment of thymocyte progenitors. More specifically, vascular adhesion protein-1, which is restricted to the venules surrounding the sites of progenitor homing, can mediate the extravasation of leukocytes. The regional distribution of these adhesion molecules further illustrates the importance of a distinct anatomical separation between cortex and medulla and represents their individual functions.

Chemokine-mediated T-cell migration and traffic to and within the thymus is crucial for normal T-cell selection. The compartmentalization of the thymus is orchestrated by a milieu of chemokines. The thymic cortex is involved in the maturation of DN cells to DP cells and by positively selecting DP T cells capable of recognizing MHC:peptide complexes and eliminating the cells that are not. The thymic medulla acts as a site of negative selection of SP T cells based on TCR recognition avidity to self-antigens and possibly positive selection of T_{reg}. Various chemokines that are produced by the thymic cortex and medulla allow T cells expressing different chemokine receptors to home to specific regions of the thymus. This differential expression of chemokines is complemented by the fact that T cells at different maturation states express different chemokine receptors. During development, the entry of lymphoid progenitors into the thymus is highly dependent on CCL21 and CCL25, which bind the chemokine receptors CCR7 and CCR9, respectively. Mice lacking CCR7 or CCL21[76,77] show a transient delay in thymus colonization by lymphocytes (day 14.5), and this delay is further extended (day 17.5) in mice lacking CCR9.[78] Lymphoid progenitors enter the thymus at the corticomedullary border and commence their migration outwards toward the subcapsular region of the cortex as DN3 cells. The expression of the chemokine receptors CXCR4 and CCR7 by DN cells is important in directing cell migration.[79,80] In the subcapsular region, DN thymocytes that have successfully rearranged their TCRαβ chains progress to the DP cell stage. Positively selected DP cells move inwards toward the thymic medulla for further differentiation into SP cells. The ligands for CCR7 are crucial in mediating the migration of positively selected DP cells into the medulla as illustrated by the fact that a deficiency in CCR7 or its ligands, CCL19 or CCL21, prevents DP cell relocation from the cortex to the medulla resulting in abnormal central tolerance.[81,82] This abnormal tolerance is associated with a reduction in thymic B-cell numbers and reduced expression of tissue-restricted antigens.[74] Interestingly, antigen itself can control T-cell migration speed from the cortex to the medulla. In the presence of a negative selection ligand, T cells slow down considerably and are limited to a confined zone 30 μ in diameter allowing for a more prolonged selection and induction of developmental arrest.[83]

The export of positively selected SP T cells out of the thymus is also dependent on chemokines. Chemokines involved in T-cell emigration are CXCL12 and its receptor CXCR4, which repel SP cells out of thymus,[84] and CCL19, which promotes T-cell emigration from the thymus of newborn mice.[85] The chemoattractant, sphingosine 1-phosphate (S1P), is an additional mediator of T-cell emigration. SP T cell express a S1P receptor ($S1P_1$) and are attracted to the high levels of S1P present in the serum promoting their egress.[86,87]

While the role of different thymic compartments and chemokines in the maturation of naïve SP T cells has been extensively studied, the thymic regions and chemokines that control the selection of regulatory T cells remain largely unknown. It may be that chemokines produced by both cTECs and thymic DCs play a role in regulatory T-cell selection, albeit during different stages of maturation.

Development

The initial development of the thymus at midgestation in the mouse is independent of vascularization or bone marrow-derived cells. In the mouse, the thymus rudiment is first

evident on day 11 of gestation as it evolves from the endoderm of the third pharyngeal pouch.[88] This gives rise to the thymic lobes as well as to the parathyroid gland. On day 12.5 of gestation, a separation of the primordium is observed and by day 13.5 a distinct thymus is apparent. Evidence has suggested that not only the pharyngeal pouch endoderm but also the ectoderm may also be involved in thymic development.[89,90] More specifically, it was suggested that the pharyngeal pouch ectoderm contributes to the development of cTECs whereas the endoderm contributes solely to the development of mTECs. This “dual origin” model was mainly supported by histological data as well as data collected from thymi of *nu/nu* mice.[91,92,93] More recently, it was shown that both thymic cortex and medulla are derived solely from the pharyngeal pouch endoderm[94]; although the endoderm and ectoderm are found in close proximity between gestational day 10.5 and 11, only the endoderm actively contributes to thymic development.[95] These findings support the so-called single origin model of thymus development.

The contribution of lymphocytes to the normal development of thymic cortex and medulla is well recognized. In models of T-cell deficiency, cTEC and mTEC development is halted at different stages depending on the stage of T-cell arrest. If T-cell development is arrested at the DN stage, as in the recombination activating gene-deficient mouse, the thymic medulla is greatly reduced while the thymic cortex remains unaffected.[96,97] A more severe phenotype is observed in transgenic mice that overexpress the human CD3 signaling molecule. In these mice, T-cell arrest occurs earlier than in recombination activating gene knockout mice, leading to a loss of both cortex and medulla and to a shift from a three-dimensional to a two-dimensional structure of the thymic epithelia.[97]

Recently, LTα and LTβ have been identified as master regulators of mTEC development and expression of tissue-restricted antigens. LTα and LTβ are members of the TNF superfamily and mediate the processes of secondary lymphoid organ development and inflammation.[1,98] In the absence of LT, tissue-restricted antigen and AIRE expression are reduced and certain mTEC subpopulations fail to develop.[99,100] T cells, owing to their vast numbers, appear

to be the main source for LT production in the thymus[45]; however, additional resident cells also serve as a source of LT. Recently, resident thymic B cells were identified as the highest source of LTα and LTβ on a per cell basis,[74] revealing yet another aspect of thymic B cells in thymus development and function.

The development of the thymus in humans closely follows the model of thymic development in the mouse and bird. Similar to the mouse, human thymic colonization by hematopoietic stem cells occurs relatively early, at week 8.2 of gestation. During this stage, the thymic medulla and cortex are organized, suggesting that thymocytes are required for normal thymic development. Between gestation week 9.5 and 10, the first signs of thymocyte negative selection are evident, and by gestation week 10 to 12.75 the gradual onset of positive selection is detected.[101]

SECONDARY LYMPHOID ORGANS

Naïve cells express their receptors for specific antigen, leave primary lymphoid organs,

circulate through the bloodstream, migrate into the tissues, and lodge in secondary lymphoid organs. The frequency of naïve cells specific for an individual antigen is quite low (estimates range from 1 in 10^5 to 1 in 10^6). Thus, the chance that an individual T or B cell will encounter its specific antigen in the circulation is rather low. Secondary lymphoid organs are strategically located in anatomically distinct sites where foreign antigen and APCs efficiently concentrate and activate rare antigen-specific lymphocytes, thus leading to the initiation of adaptive immune responses and generation of long-lived protective immunity. These organs include highly organized, compartmentalized, and mostly encapsulated tissues such as lymph nodes, spleen, appendix, tonsils, murine NALTs, and Peyer's patches. Naïve cells are also primed in less discrete tissues throughout the body, including the BALTs, cryptopatches, and ILF.

Lymph Nodes

Lymph nodes are bean-shaped structures dispersed along lymphatic vessels. The lymphatic vessel system plays important roles in tissue fluid balance, fat transport, and the immune response. In contradistinction to blood vessels, which form a closed recirculating system, lymphatic vessels comprise a blind-end, unidirectional transportation system. The absorbing lymphatic vessels, or lymphatic capillaries, remove interstitial fluid and macromolecules from extracellular spaces and transport the collected lymph through the primary collector. The collected lymph and its cellular contents are transported into the thoracic duct and returned back to blood circulation. In humans, lymph collected from the entire lower body region, the left head, and left arm region accumulates in the thoracic duct and returns to blood circulation via the left subclavian vein; lymph collected from right head and right arm region returns to blood via the right subclavian vein.

Lymph nodes, usually embedded in fat, are located at vascular junctions, and are served by lymphatic vessels that bring in antigen, and connect them to other lymph nodes. Though most lymph nodes are classified as peripheral lymph nodes, a few (cervical, mesenteric, and sacral), termed mucosal nodes, express a slightly different complement of endothelial adhesion molecules, cooperate with the mucosal system, and are regulated somewhat differently in development (see following discussion). Although all lymph nodes are vascularized, and thus can receive antigens from the bloodstream, they are also served by a rich lymphatic vessel system and are thus particularly effective in mounting responses to antigens that are present in tissues. These antigens may be derived from foreign invaders that are transported by APCs or can be derived from self-antigens. Thus, lymph nodes extend the role of the primary lymphoid organs and discriminate between dangerous foreign antigens and benign self-antigens. This capacity relies on the APCs and their state of activation in the lymph node, and the recognition capacity of the naïve T and B cells.

Lymph nodes can also function as niches for generating peripheral tolerance, an additional mechanism to minimize the effects of those self-reactive T-cells that escape central tolerance in the thymus.[102] DCs constitutively sample selfantigens and migrate to draining lymph nodes even in the steady state.[103,104,105] Because most self-antigen-bearing DCs in lymph nodes are immature[106] and have low levels of costimulatory molecules, they are not effective at activating naïve cells. They regulate self-reactive T cells by inducing anergy, clonal deletion, and/or expanding T_{reg}s.[105,106,107,108,109] Several groups have recently

described populations of cells in secondary lymphoid organs that can present tissue-restricted antigens to CD8 cells.[110,111,112,113,114] These include two different populations of AIRE-expressing stromal cells, DCs, and AIRE-negative lymphatic endothelial cells. These cells may play a role in self-tolerance, or perhaps they can prime for autoimmunity in inflammatory conditions.

Structure and Organization

A collagen capsule surrounds the highly compartmentalized lymph node (Fig. 3.3). The cortical region includes discrete clusters called primary follicles consisting of densely packed naïve B cells and follicular DCs (FDCs). After B cells encounter their cognate antigen, they are activated. They then proliferate secondary follicles and germinal centers develop. T cells and DCs distribute in the paracortex. Macrophages reside in the subcapsular zone and in the medullary area, and those in the subcapsular zone appear to be particularly adept at presenting antigen-antibody complexes to B cells.[115] Follicular DCs, a population of mesenchymal origin, support B-cell follicles or germinal centers under stimulation.[1] Plasma cells are also concentrated in the medulla as they prepare to leave the lymph node and circulate to the bone marrow. A network composed of reticular fibers, fibrous extracellular matrix bundles, and another mesenchymal cell population, the fibroblastic reticular cells, supports the entire lymph node.[116] Compartmentalization of cells in the lymph node is orchestrated by lymphoid chemokines CCL19, CCL21, and CXCL13. Stromal cells in the paracortical region produce CCL19 and the protein is transported to the surface of high endothelial venules (HEVs).[117] CCL21 is encoded by several genes[118]; CCL21-leu is expressed by lymphatic vessels

outside the lymph node[119]; CCL21-ser is made by stromal cells and HEVs in the lymph node. CCL19 and CCL21 recruit CCR7-expressing cells across the HEVs to the paracortical region. CXCL13, produced by stromal cells in the B-cell follicles, attracts CXCR5 expressing B cells.[120] After naïve T and B cells encounter antigen, they undergo extensive changes in expression of chemokine receptors and adhesion molecules that result in their movement to different areas of the lymph node or leaving it all together.[121,122] $S1P_1$ facilitates lymphocyte egress from lymph nodes as they move toward the ligand S1P in the lymph,[123,124] as discussed in more detail subsequently.

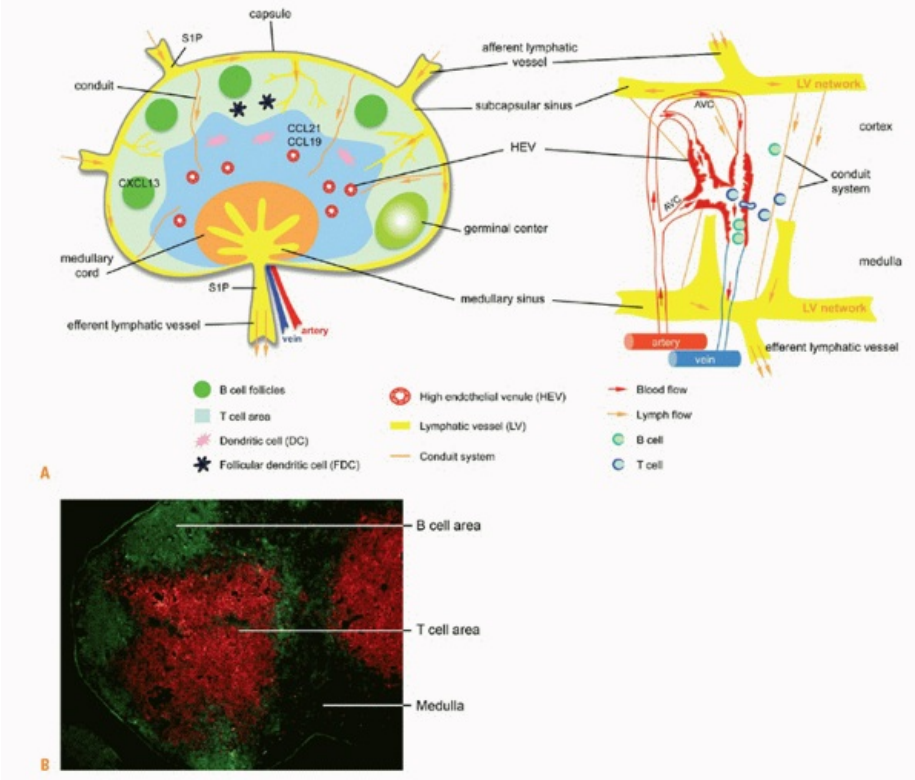

FIG. 3.3. A: Lymph node structure and functional regions. The lymph node is divided into an outer cortex and inner medulla surrounded by a capsule and lymphatic sinus. The cortex includes B cells and follicular dendritic cells. The paracortical region includes T cells and dendritic cells. Macrophages are found in the subcapsular sinus and medullary cord. Lymphocytes enter into the lymph node through an artery at the hilus region, and into the parenchyma through high endothelial venules (HEVs) expressing peripheral node addressin and CCL19 and CCL21. Stromal cells also produce these chemokines. T cells and dendritic cells (DCs) are directed to the paracortical region by CCL19 and CCL21. B cells are directed to the cortex by CXCL13. After interaction with antigen and T cells, germinal centers develop. Antigen and DCs drain into the lymph node from the tissues through afferent lymphatic vessels. Antigen continues to percolate through the node via a conduit system. Activated cells leave through efferent lymphatic vessels. Diagram of the blood network of a rat lymph node (*right*) is adapted from Anderson and Anderson.[125] Note the arteriovenous communications, the venous sphincters, and cells leaving the HEVs into the parenchyma. **B:** Lymph node compartmentalization. Immunofluorescent staining of B cells (anti-B220, *green*) in follicles in the cortex and T cells (anti-cluster of differentiation 3, *red*) in the paracortical area. The medulla is unstained.

Lymph Node Vasculature: Blood Vessels and Lymphatic Vessels

Soluble antigen and APCs enter into lymph nodes via afferent lymphatic vessels. After surveying antigens in the lymph

nodes, lymphocytes leave those organs via efferent lymphatic vessels that can connect to the

next lymph node in the chain and finally return to blood circulation.[125,126,127] In this manner, HEVs and lymphatic vessels maintain lymphocyte homeostasis during the steady state.

Blood endothelial cells play a crucial role in lymphocyte trafficking in the lymph node. One or two arteries enter the lymph node at the hilus. These arteries branch and pass through the medulla area, enter the cortex, and sometimes continue in the subcapsular area. Beneath the subcapsular sinus, the branching capillaries form loops and some of them become arteriovenous communications. Arteriovenous communications become HEVs in the cortex area and occasionally extend from the subcapsular sinus to the medulla. HEVs constitute a specialized postcapillary network in the lymph node, playing a critical role in lymphocyte recirculation. Each main HEV trunk receives three to five branches lined with high endothelial cells and two or three branches lined with flat endothelial cells. The luminal diameters of HEVs progressively increase from cortex to medulla. Finally, HEVs merge into segmental veins in the medulla area and join larger veins in the hilus[125] (see Fig. 3.3A). Intravital microscopy has revealed that the entire venular tree consists of five branching orders with the higher orders in the paracortex and the lower orders located in the medulla and hilus areas. Only the higher order venules, located in the T-cell area, are specialized into HEVs and are recognized by the monoclonal antibody MECA-79.[126,127] Recirculating naive lymphocytes leave the bloodstream via HEVs, specialized vessels with a high cuboidal endothelium, and migrate into the lymph node parenchyma. PNAd, defined by the MECA-79 antigen, is an L-selectin ligand, and is a characteristic HEV adhesion molecule. PNAd is composed of a variety of core glycoproteins, including GlyCAM-1, CD34, Sgp200, and podocalyxin; these proteins must be sialylated, sulfated, and fucosylated to become functional L-selectin ligands. The several enzymes that mediate these posttranslational modification events include FucT-IV, FucT-VII, and GlcNAc6ST2 (also called HEC-6ST, LSST, GST-3, HECGlcNAc6ST, gene name *Chst4*),[128,129,130,131,132] which with the exception of a population of cells in the intestine[133,134] is uniquely expressed in high endothelial cells. Together with another sulfotransferase, GlcNAC6ST1, that is expressed at sites in addition to HEVs, it sulfates glycoproteins in the Golgi apparatus[135] to generate the MECA-79 epitope. PNAd, expressed on the endothelial surface, slows down (tethers) naïve L-selectin^{hi} lymphocytes in their progress through the blood vessels. After this initial interaction, CCL19 and CCL21 on the HEVs are instrumental in activating the lymphocyte integrin lymphocyte function-associated antigen (LFA)-1. This results in tight binding of LFA-1 to ICAM-1 on the HEV, facilitating diapedesis of lymphocytes that migrate between or through the endothelial cells toward the chemokines located in the paracortical region (T cells, DCs) or cortex (B cells). Data suggest that the HEV-lymphocyte interaction is not random in different lymphoid tissues in the mouse.[136,137] T-lymphocytes adhere preferentially to peripheral lymph node HEVs, B cells prefer to adhere to HEVs in Peyer's patches, and T and B cells exhibit an intermediate pattern of adhesion to mesenteric lymph node HEVs.[34,136,138] The selective adhesion of naïve lymphocytes to HEVs is at least partially controlled by the differentially expressed adhesion molecules in different lymphoid organs. PNAd rapidly replaces MAdCAM-1 after birth in mouse peripheral lymph nodes,[139] but is expressed in mucosal lymph nodes together with MAdCAM-1, the ligand for the integrin $\alpha_4\beta_7$.

Lymphatic vessels also play critical roles in the immune response. The collected lymph and cell contents enter the lymph node via several afferent lymphatic vessels and filter through the node where they again are concentrated in the medullary sinus. Lymphatic vessels are concentrated in the subcapsular sinus and medullary area.[125] Factors from afferent lymph can be either transported deep into the lymph node cortex or move via the subcapsular sinus and leave the lymph node through efferent lymphatic vessels.[116] In this manner, soluble antigen and APCs from peripheral tissues are efficiently concentrated in the draining lymph node and initiate an adaptive immune response. Lymphocytes can also enter lymph nodes through afferent lymphatic vessels and leave via efferent vessels and move to the next lymph node in the chain. This is accomplished in part through a gradient of S1P, which is at a high concentration in the lymph and low concentration in the lymph node. Lymphocytes in the lymph node downregulate this receptor ($S1P_1$) and then upregulate it as they prepare to leave and migrate toward the higher concentration in the efferent lymph.[123,124] Human and murine lymphatic vessels express several characteristic markers: lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), Prox-1, podoplanin, CCL21, the vascular endothelial growth factor receptor-3 (VEGFR-3), and neuropilin-2.[140]

Conduit System

A conduit system in the lymph nodes physically connects the lymphatic sinus with the walls of blood vessels and enables the incoming factor(s) from lymph to move rapidly deep into the paracortical area.[116,125] The conduit system consists of four layers: 1) a core of type I and type III collagen bundles; 2) a microfibrillar zone composed largely of fibrillins; 3) a basement membrane abundant with laminins 8 and 10, perlecan, and type IV collagen that provides a supportive structure; and 4) fibroblastic reticular cells that embrace the entire conduit system.[141,142] This conduit system enables incoming lymph to penetrate deep into the T-cell area. A special subset of immature DCs, called conduit-associated DCs, can take up and process antigens moving along the conduit.[142] In this manner, the conduit system probably provides a physical support for rapid initiation of adaptive immune responses after immunization.

The Intimate Relationship Between Lymph and High Endothelial Venules

Incoming lymph is necessary for the maintenance of HEV phenotype and function. After afferent lymphatic vessels are severed, dramatic changes occur in HEVs. These include flattening of the endothelium, a decrease in the uptake of ^{35}S-sulphate[143,144] (a functional marker of GlcNAC6ST-2), a reduction of lymphocyte adherence to the vessels,[145,146,147]

and decreased expression of PNAd and the HEV genes, *Glycam-1* and *Fuc-TVII*.[146,147,148] An increase in MAdCAM-1 expression[146] suggests that these events are not simply due to a general downregulation of blood vessel gene expression and indicates that continual accumulation of afferent lymph factor(s) in lymph nodes is necessary for HEV maintenance. The nature of such lymph factor(s) is unknown.

Topographic relations between HEVs and the lymphatic sinus have been described in the rat mesenteric lymph nodes. Some HEVs are located in the medulla area and positioned closely

in relation to the lymphatic sinus.[125] Occasionally, HEVs are separated from an adjacent lymphatic sinus by only a thin layer of collagen bundles. The closely apposed HEV and lymphatic sinus in the medulla provide the physical support for the intimate relationship between lymph and HEV. However, most HEVs are located in the paracortical area and are separated from the lymphatic sinus by lymphocytes. The conduit system physically connects the subcapsular sinus and HEVs and allows incoming lymph factor(s) to migrate rapidly to the wall of HEVs.[125,149,150] Low-molecular-weight fluorescent tracers (below 70 kD) move rapidly via the conduit system and lead directly to the wall of HEVs. In this manner, low-molecular-weight tracers can migrate with lymph within minutes to HEVs and enter the HEV lumen.[116] Lymph-borne chemokines likely adopt this route to regulate HEV function. IL-8 administration via afferent lymph increases lymphocyte HEV transmigration within minutes.[125,151] In addition, lymph-borne chemokines, such as MIP-1α, also enter the conduit system and move rapidly to HEVs.[116] These data suggest that lymph factor(s) can quickly access and regulate HEVs.

Development

Despite their distinctions in the adult with regard to morphology and expressed genes, a close association of blood vessels and lymphatic vessels is seen during embryogenesis. The generation of embryonic lymphatic vessels from preexisting veins in pig embryos was first described in the early 1900s by Sabin and has recently been molecularly defined.[140] A variety of transcription factors have been identified that contribute to the lymphatic specification and maintenance of the lymphatic vessels phenotype.[152] As early as mouse E9, expression of Sox18 and CoupTFII is apparent in the cardinal vein. These induce Prox1 in the dorsolateral side of the vein resulting in polarization and lymphatic-biased endothelial cells. Prox1 generates a feedback signal for the further maintenance of budding and migration of endothelial cells. Targets of Prox1 include Prox1 itself, podoplanin, VEGFR3, integrin-α, and Nrp-2. Prox1 expression in blood endothelial cells also represses expression of markers of those cells. NfatC1, Foxc2, and Tbx1 are additional transcription factors that are expressed later in lymphatic vessel development and contribute to patterning, pericyte covering of lymphatic vessels, lymphatic vessel valves, and lymphatic vessel maturation. At E11.5-12.0, CCL21 is expressed in these lymphatic-biased endothelial cells, as is VEGFR-3, which is reduced in blood endothelial cells. The endothelial cells expressing LYVE-1, Prox1, VEGFR-3, and CCL21 become irreversibly committed toward a lymphatic pathway.[153,154] The separation of lymphatic endothelial cells from venous endothelium requires a Syk/SLP-76 signal[155] that is provided by platelets.[156,157] Thus, during early lymphangiogenesis, some endothelial cells express both blood vessel and lymphatic vessel markers, indicating the close association of these two vascular systems. Mesenchymal lymphangioblasts may also contribute to early lymphangiogenesis.[158] The lymphatic venous junction remains in adults in only limited regions but plays an essential role in connecting the function of the two vascular systems.

Studies in the mouse have taken advantage of transgenic, knockout, and imaging studies to provide a mechanistic understanding of the process of lymph node development. Although lymph sacs per se do not appear to be essential for the initiation of lymph nodes, a lymphatic vessel system is necessary for the later development and cellular population of lymph

nodes.[159] Furthermore, an interaction between mesenchymal cells and primitive endothelial cells is apparent in the early lymph node anlage.[160]

Cytokines, Chemokines, and Transcription Factors in Lymphoid Organogenesis

The TNF-/LT-receptor family members play key roles in secondary lymphoid organ development. $LT_{\alpha 3}$, signaling through TNFRI and TNFRII, and membrane bound $LT_{\alpha 1\beta 2}$, signaling through the LTβ receptor (LTβR) have been implicated. Mice deficient in LTα lack all lymph nodes and Peyer's patches, and exhibit a disorganized spleen and severely disorganized NALT (see subsequent discussion).[161,162,163] Mice deficient in LTβ lack peripheral lymph nodes but retain mesenteric, sacral, and cervical lymph nodes.[164,165,166] *Ltβr -/-* mice have a phenotype similar to that of Lt^{α}*-/-* mice.[167] LTβR is also recognized by LTβ-related ligand, LIGHT, which also binds to the herpes virus entry mediator. LIGHT-LTβR signaling does not appear to play an essential role during lymphoid organogenesis, as no significant defect is observed in *Light-/-* mice[168]; however, mice doubly deficient in LIGHT and LTβ have fewer mesenteric lymph nodes than mice deficient in LTβ alone, indicating a cooperative effect of the two LTβR ligands. Treatment of pregnant females with an inhibitory soluble protein of LTβR (LTβR and human IgG Fc fusion protein, LTβR-Ig) inhibits most lymph nodes in the developing embryos, depending on the time of administration. Mesenteric lymph nodes are not inhibited by this treatment. These studies indicate that individual lymph nodes differ in the nature and time of cytokine signaling during development.[169] Several additional cytokine and chemokinereceptor pairs are crucial for lymphoid organogenesis. Mice deficient in IL-7 or IL-7R,[170,171] or TRANCE or TRANCER, also called RANKL and RANK,[172,173] exhibit defects in lymph node development. CXCR5- or CXCL13-deficient mice[174,175] lack some lymph nodes and almost all Peyer's patches.[98] The relative importance of the different cytokines in the development of individual lymphoid organs has been recently summarized.[176]

The NF-κB signaling pathways, downstream of the TNF family receptors, play important roles in lymphoid organ development.[177] The alternative pathway, characterized by NF-κB-inducing kinase (NIK) and IKKα is particularly

important. *aly/aly* mice, which have a point mutation in *Nik*, lack all lymph nodes and Peyer's patches.[178,179] In these mice, LTβR, but not TNFR, mediated signaling between NIK and members of the TRAF family appears to be disrupted.[179,180,181] LTβR signaling induces gene expression via both the classical and alternative NF-κB pathways in mouse embryo fibroblasts. The classical pathway mediated by p50:p65 heterodimers induces expression of proinflammatory genes (*Vcam-1*, *Mip1b*, *Mip2*). Intraperitoneal injection of an agonistic LTβR antibody induces splenic chemokines (CCL19, CCL21, and CXCL13) and requires NIK activity and subsequent p100 processing.[182] IKKα is a critical component in alternative NF-κB pathway. Mice with a mutated form of the *Ikka* gene have reduced HEV expression of HEC-6ST (GlcNAc6ST2) and GlyCAM-1, further confirming that the LTβR signal regulates HEVs through the alternative NFκB pathway.[183] Several other signaling pathways that contribute to lymphoid organogenesis include the helix-loop-helix transcription factor inhibitor

(Id2) and retinoid acid-related orphan receptors (RORs) RORγ and RORγt.[184,185,186]

Studies of lymph node anlage formation reveal the mechanisms by which cytokines trigger and coordinate lymphoid organogenesis. There is likely an initiating factor, though this has not been definitively identified. In the case of Peyer's patches, a cell producing the receptor tyrosine kinase has been implicated.[187,188] Recent data indicate that the earliest stages of lymph node development appear to be dependent on retinoic acid that may be produced by nerves in the vicinity of the developing node.[189] Circulating CD4+CD3-CD45+ RORγt+ hematopoietic progenitor cells called lymphoid tissue-inducer cells, derived from fetal liver progenitors,[98,185,190,191,192] provide crucial signals in lymph node organogenesis. These lymphoid tissue-inducer cells accumulate in the developing lymph node, forming clusters with resident stromal organizer cells, to initiate a cascade of intracellular and intercellular events that lead to the maturation of the primordial lymph node.[1,98] During this early step, a positive feedback loop involves several signaling pathways, including LTαβ/LTβR, IL-7R/IL-7, CXCR5/CXCL13, and RANK/RANKL, are expressed on the lymphoid tissue-inducer cells and the stromal organizer cells. The prolonged interaction between lymphoid tissue-inducer cells and stromal organizer cells promotes the development of HEVs, which support the entry of naïve lymphocytes.[193] It is unclear how HEVs differentiate from the flat blood vessels during early lymphoid organogenesis. At birth, HEVs of all lymph nodes express MAdCAM-1, which is replaced in the first few days in peripheral lymph nodes by PNAd.[194] Both MAdCAM-1 and PNAd are expressed in mucosal lymph nodes. LTα alone can induce MAdCAM-1, but PNAd requires LTαβ.[2,195,196] In the remaining mesenteric lymph nodes of *Ltβ-/-* mice, PNAd expression is impaired,[196] indicating that optimal lymph node HEV PNAd expression requires $LT\alpha_1\beta_2$ signaling through the LTβR and the alternative NF-κB pathway.[183] Because the maturation of HEVs is coincident with further development of the lymph node,[139,197] the homing of LT-expressing lymphocytes most likely contributes to HEV maturation. Continual signaling through the LTβR is necessary for maintenance of HEV gene expression.[198,199] Recent data suggest that the actual physical presence of lymphocytes in the close vicinity of HEVs contributes in important ways to the physical cobblestone appearance of these vessels.[200]

Changes in Lymph Nodes after Immunization

Lymph nodes undergo dramatic changes and remodeling after immunization. Early after a variety of immunogenic exposures, such as skin painting with oxazolone, injection of ovalbumin or sheep red blood cells in adjuvant, or bacterial or viral infection, remodeling occurs. This remodeling is apparent as a complex kinetics of changes in lymph flow, lymph cell content, blood flow, HEV gene expression, and lymphatic vessels.[199] Afferent lymph flow and lymph cell content increase soon after initial inflammation, and eventually return to preimmunization levels.[201,202,203,204,205] Lymph node lymphangiogenesis occurs, which eventually resolves.[199,206] Blood flow and lymphocyte migration into lymph nodes peak at 72 to 96 hours,[166,204,207,208] accompanied by an increase in HEV number and dilation,[209,210] accounting for the significant lymph node enlargement apparent at 72 to 96

hours.[207] Efferent lymph flow also increases soon after immunization, but lymph cell content in the efferent lymph drops during the first several hours, indicating the first wave of accumulation of lymphocytes in the draining lymph node. The cell content of the efferent lymph later increases and peaks at 72 to 96 hours.[211] These events, taken together, contribute to the significant enlargement of the draining lymph node at day 4 after immunization. During the early times after immunization, despite the increase in the number of HEVs, the expression of genes that contribute to L-selectin ligand, including *Fuct-vii*, *Glycam-1*, *Sgp200*, and *Chst4*, is initially downregulated followed by a recovery.[148,199,212] However, despite the downregulation of chemokines CCL21 and CXCL12, some genes such as those encoding CXCL9, CCL3, and E-selectin are upregulated,[213] as is MAdCAM-1,[199] suggesting a reversion to an immature phenotype before the eventual recovery of the mature phenotype.

In rodents, HEV maturation is coincident with the continuing development of the lymph node and population of lymphocytes after birth, indicating that the mature HEV phenotype relies on the lymph node microenvironment.[139,197] Plasticity of the mature lymph node is also seen after injection of LTβR-Ig. LTβR-Ig treatment reduces lymph node cellularity, reverts the HEV phenotype to the immature state, inhibits FDC function, and disrupts immune responses to foreign antigens.[198,199,214]

Spleen

Function

The spleen is a large reddish organ located beneath the diaphragm close to the stomach and the pancreas. It is the main filter of the blood and integrates the innate and adaptive responses. Its structure in well-defined different compartments, red pulp and white pulp, determines a variety of functions. The red pulp is a source of hematopoiesis in the embryo that can continue in adult life under stress. Clearance of blood-borne damaged platelets, aged

erythrocytes, and dead cells also occurs in that region. An important function, consequent to its destruction of effete erythrocytes, is its role in iron recycling. In some species, such as horses or dogs, the spleen stores erythrocytes that can be released after stress. The white pulp and marginal zone constitute the highly organized lymphoid compartment of the spleen. Due to this organized lymphoid structure and the special vasculature and circulation, the spleen is a crucial site for blood-borne antigen clearance and presentation to T and B cells. The spleen is crucial in defense against blood-borne pathogens and contributes most significantly to defense against bacterial and fungi infections.[215]

Architecture and Cellular Composition

A fibrous capsule and trabeculae of fibrous connective tissue maintain the structure of the spleen. The general principles of splenic organization are similar across species, though specifics may differ.[216,217,218,219,220] Classically, the spleen can be divided into two areas: red pulp and white pulp. A transit area, the marginal zone (MZ), surrounds the white pulp (Fig. 3.4). These compartments are anatomically and functionally distinct. The red pulp,

in its activities as a hematogenous organ, removes damaged cells and acts as a site for iron storage and turnover. The white pulp is an organized lymphoid structure. The complex structure of the spleen is directly related to the complexity of the vasculature of this organ. The afferent splenic artery branches into central arterioles, surrounded by white pulp areas, and end in cords in the red pulp. Blood then collects in venous sinuses that determine the engulfment of erythrocytes by red pulp macrophages. Finally, the sinuses empty into the efferent splenic vein. Some small arterial branches end in the MZ, which demarcates the red and white pulp. The MZ structure differs somewhat between humans and rodents. A perifollicular zone surrounds the human MZ, which consists of inner and outer marginal zones, whereas the rodent MZ is

a single structure and has no perifollicular zone. The MZ is the transition between the innate and acquired immune systems and is an important transit area for cells reaching the white pulp. It also contains a large number of resident cells. There are two specialized macrophage populations: the MZ macrophages and MZ metallophilic macrophages. MZ macrophages have a high phagocytic activity and are phenotypically different from other macrophage populations in the spleen as exemplified by the expression of SIGNR1 and macrophage-associated receptor (MARCO) with collagenous structure, molecules implicated in the recognition of pathogens. SIGNR1, a C-type lectin in the mouse that is a homologue of human DC-specific intercellular adhesion molecule,[221] is also found in medullary and subcapsular macrophages in the lymph node. MZ macrophages are crucial Marco for the capture of a wide variety of pathogens, including yeast, bacteria, and viruses.[222,223] MARCO, expressed constitutively on MZ macrophages,[224] is a class A scavenger pattern recognition receptor. In addition to the recognition of blood-borne pathogens, MARCO interplays with MZ B cells, modulating their migration to the white pulp.[225] MZ metallophilic macrophages are located at the inner border of the MZ, in contact with marginal sinus lining cells, stromal cells that express MadCAM-1.[226] MZ metallophilic macrophages differ from the MZ macrophages and red pulp macrophages in that they express the sialic acid-binding Ig-like lectin sialoadhesin (Siglec-1), which binds to oligosaccharide ligands present on many cells.[227] Little is known about the specific roles of this population of macrophages during the immune response; however, the phenotype of mice deficient in sialoadhesin implies a role of MZ metallophilic macrophages in T-cell activation.[228,229] Recent studies confirm that MZ metallophilic macrophages are essential for cross-presentation of blood-borne adenovirus antigens to splenic CD8 DCs, activating cytotoxic T-lymphocytes.[230] The MZ also contains a specialized subset of B cells that differ phenotypically and functionally from follicular B cells; they can be considered as a bridge between the innate and adaptive immune systems. They express higher levels of IgM low levels of IgD and the molecule CD1d. These B cells bind antigen in the MZ directly and/or through interactions with MZ macrophages[231] and migrate to the white pulp, where they present blood-borne antigens.[232] The organization of the remainder of the white pulp of the spleen is similar to that of the other secondary lymphoid organs with compartmentalized B- and T-cell areas. The white pulp consists of a central arteriole that is surrounded by T cells, also known as the periarteriolar lymphoid sheath, which is surrounded by B-cell follicles (see Fig. 3.4). T cells interact with DCs and B cells. B cells migrate to the follicles where they interact with FDCs. At the T:B-cell border, T cells,

especially T follicular helper cells,[233] interact with B cells. Germinal centers are the site of somatic hypermutation and Ig class switching. Plasma cells are found mainly in the red pulp.

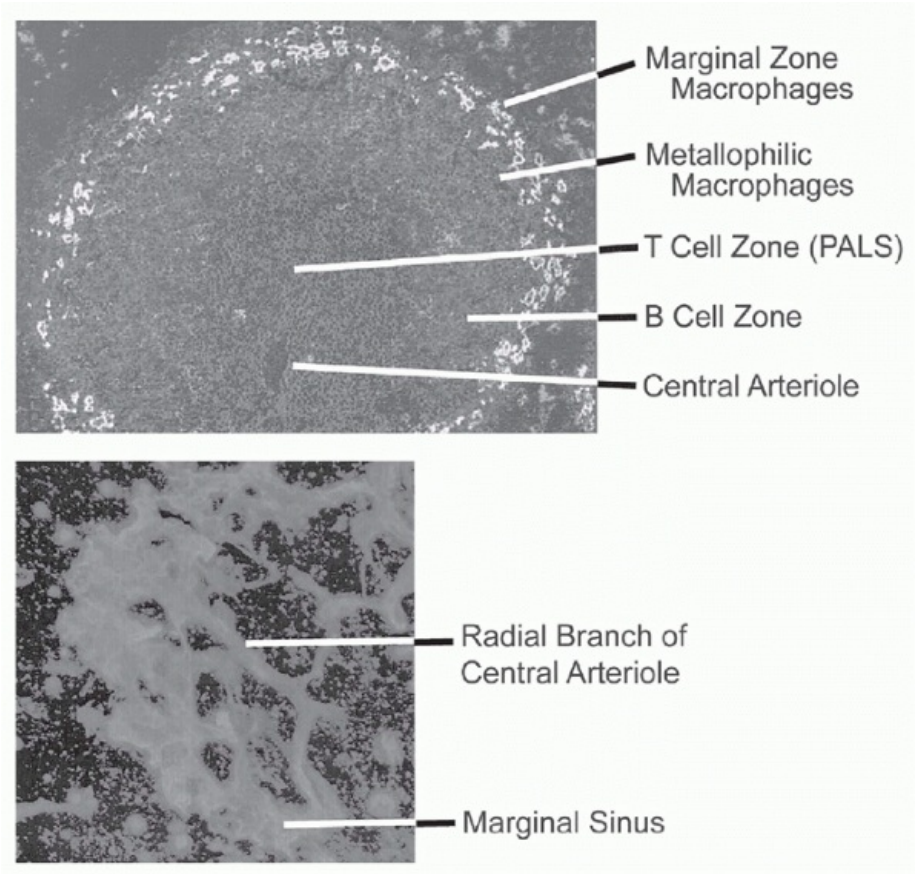

FIG. 3.4. Organization of the Spleen White Pulp. Immunofluorescence staining of a white pulp unit in the mouse spleen. T cells (anti- cluster of differentiation [CD]4+anti-CD8+, *red*) are localized around the central arteriole. B cells (anti-immunoglobulin M, *green*) are localized in follicles around the T-cell area, surrounded by a layer of metalophilic macrophages (labeled with monoclonal antibody-1) and a more peripheral layer of marginal zone macrophages (labeled with monoclonal antibody ERTR9, *orange*). The marginal zone is located between the metalophilic macrophage and the marginal zone macrophage layers (not shown).[357] From Chaplin[358] with permission.

The spleen does not have an afferent lymphatic system, and initial antigen transport must occur through the blood vasculature. A conduit system has been described that allows antigens and chemokines to be transported through the white pulp in a manner similar to that described for the lymph node.[234] This conduit differs from that in the lymph node with regard to the identity of the transported molecules by the fact that it contacts the blood rather than the lymphatic system.

Traffic In and Out: Chemokines and Adhesion Molecules

Cell trafficking in the spleen is similar to that which occurs in the lymph node in some respects, and differs in others. The MZ is the main transit area for blood cells that enter the

white pulp. This process seems to be controlled by marginal sinus lining cells[235] and chemokines, involving signaling through G-couple protein receptors.[236] Stromal cell-produced lymphoid chemokines CXCL13, CCL19, and CCL21 control the migration of lymphocytes and their organization in the various compartments in the white pulp. CXCL13 is essential to positioning of B cells in the follicles, whereas T cells respond to CCL19 and CCL21. After the immune response, germinal center B cells differentiate into plasma cells in the white pulp, then migrate to the red pulp or return the bloodstream to migrate to peripheral tissues. In the migration to the red pulp, plasma cells upregulate CXCR4, a receptor that binds the chemokine CXCL12, which is expressed in the red pulp.

The role of adhesion molecules with regard to lymphocyte traffic in the spleen is not fully well understood. Lymphocytes enter into the white pulp through the marginal sinus and cells lining that structure express MAdCAM-1 and ICAM-1.[226,237] MadCAM-1 expression in these cells seems to be involved in splenic structure development and therefore migration of B and T cells.[235] However, treatment with anti-MAdCAM-1 or anti-α1β7 antibodies does not totally inhibit homing to the spleen, suggesting that this ligand-receptor pair is not the only receptor required for lymphocyte entrance into the white pulp. Early studies indicating that treatment with an antibody that blocks the αL-integrin chain of LFA-1 inhibits homing by only 20%, and that lymphocytes deficient in LFA-1 enter the white pulp,[237] suggested that LFA-1 is not absolutely essential for entry of all cells into the white pulp. However, both $\alpha_4\beta_1$ and LFA-1 integrins are necessary for B-cell retention in the MZ,[238] indicating a role for VCAM-1 and ICAM-1 in cell trafficking in spleen. Once lymphocytes have encountered antigen, they most likely undergo changes in chemokine receptor and adhesion molecule expression similar to those noted in the lymph nodes, leave the white pulp and enter the red pulp (plasma cells) or the circulation. The mechanisms by which lymphocytes leave the white pulp are unclear. The observation of channels that bridge into the MZ[239] suggests one route of egress of lymphocytes. In the lymph nodes, the process of egress of activated lymphocytes through the efferent lymphatic vessels is mediated by upregulation the expression of $S1P_1$. In the spleen, $S1P_1$ is required for B-cell localization in the MZ, and the interplay with CXCR5 regulates the constant trafficking of these cells between the MZ and the white pulp,[232,240] as MZ B cells from $S1P_1$-deficient mice are not found in the MZ but are found in the follicles.

Although it is still unclear which molecules are involved in the egress of lymphocytes from the spleen, and splenic egress cannot be compared to egress in the lymph nodes,

recent studies attribute an important role to the transcription factor Nkx2.3 in the regulation of spleen vasculature and homing of lymphocytes.[241,242]

Development

Because the spleen has characteristics of both a hematopoietic organ and a secondary lymphoid organ, the genes that regulate its development include, in addition to LT, others that are concerned with patterning and hematopoiesis. Several genes that contribute to the development of the spleen are also crucial for normal development of other nonlymphoid organs. The first sign of splenic development in the mouse occurs at E10.5-11. Segregation of red and white pulp starts to be regulated during embryogenesis and continues after birth.

Some structures, such as the MZ, develop during the 3 first weeks after birth. At E10.5-11, progenitor cells begin to condense within the dorsal mesogastrium, adjacent to the stomach and dorsal pancreas. The spleen and pancreas are so intimately associated that it is difficult to distinguish between them at these early stages. In fact, many genes that affect splenic development also contribute to pancreatic development. Several of these are homeobox genes and transcription factors that are expressed in the splenopancreatic mesenchyme at E10.5.[215] The effects of just one of these genes, *Hox11* (now called *Tlx1*[215]), are on the spleen. The others affect multiple organs. *Tlx1* was originally described as an oncogene in T-cell childhood acute leukemias involving the (10;14) translocation breakpoint.[243] *Tlx1* -deficient mice are asplenic,[244] and the product of this gene is a cell survival factor.[245] Several additional genes that are important for development of lymph nodes, NALT, and Peyer's patches also contribute to splenic development. Mice deficient in members of the LT/TNF ligand receptor family and their downstream signaling molecules in the classical and alternative pathways exhibit defects in splenic development. However, none of these molecules is necessary for the early splenic anlagen, as mice deficient in any of the chemokines or cytokines retain a spleen. Recent studies address a role for Glce, an enzyme that modifies heparan sulfate, in early lymphoid tissue morphogenesis, as fetal spleen in Glce deficient mice exhibit a reduced size.[246] The changes in lymphoid tissue organization in mice deficient in LT/TNF ligand receptor family members are due in part to a reduction or near absence of lymphoid chemokines (CXCL13, CCL19, CCL21).[247] Lymphoid chemokine messenger ribonucleic acid (mRNA) expression is reduced in the spleens of mice deficient in TNFR1, TNF, LTα, or LTβ, though CXCL12 mRNA levels are normal. However, treatment with an agonistic LTβR antibody induces expression of the lymphoid chemokines and CXCL12, suggesting that signaling through both the classical and alternative NF-κB pathways are responsible for organization and maintenance of splenic white pulp architecture. Mice deficient in LTα exhibit a disorganized white pulp with loss of T- and B-cell compartmentalization, MZ macrophages, metallophilic macrophages, MZ B cells, MAdCAM-1 sinus lining cells, and germinal centers. *Lt*β-/- mice exhibit similar characteristics except that the disorganization is somewhat less pronounced. However, these lymphoid cytokines seem to be dispensable during fetal spleen development, when segregation between white and red pulp begins, but essential for maintenance of this structure during postnatal development of the white pulp.[248] *Tnf-/-* and *Tnfr1-/-* mice show defects similar to lymphotoxin deficient mice, with the exception of MZ B cells. T cells, B cells, and CD4+CD3- cells produce the cytokines necessary for maintenance of splenic architecture.[249,250]

Plasticity after Virus Infection

Though much is known regarding changes in the lymph node after immunization, the spleen has not been studied as extensively in this regard. However, after infection with cytomegalovirus, white pulp T:B compartmentalization is disrupted.[251] The spleens of *Ltα-/-* deficient mice exhibit a marked reduction in expression of CCL21-ser. This is even further reduced in cytomegalovirus infection, indicating that, in the adult, LT-independent pathways can contribute to maintenance of expression of lymphoid chemokines.

Mucosal-Associated Lymphoid Tissues

General Features

The MALT covers all mucosal surfaces not only in the gut, but also the oropharyngeal and lacrimal mucosae, the nasal and bronchial airways, and the genitourinary tracts. MALT protects a huge surface area and contains approximately half of the lymphocytes of the entire immune system.[252] MALT is considered to be the body's gatekeeper because it is in intimate contact with the commensal flora at the mucosal surfaces.

Although the location of some mucosal lymphoid tissues like the palatine tonsils and appendix are fixed, all MALT are somewhat plastic because of their constant exposure to environmental antigens that induces them to change and remodel. Tonsils, adenoids, and their equivalent in rodents, the NALT, have a fixed location, whereas the location, number, and size of Peyer's patches in the small intestine vary according to antigen exposure. The BALT in the lung and ILFs in the colon are the most plastic MALTs, and their number and location are subject to change by environmental influences.

The mucosal epithelium that surrounds MALT is populated by a dense network of DCs, plasma cells, and intraepithelial lymphocytes that helps to maintain the epithelial barrier. These intraepithelial leukocytes provide retinoic acid and cytokines like IL-10 and TGF-β that condition the gut to become tolerant of antigens produced by the harmless commensal bacteria.[253,254] They are also responsible for inducing and maintaining tolerance to food antigens and commensal bacteria.[255]

The large MALT structures like the tonsils, NALT, and Peyer's patches share many features with lymph nodes. Like lymph nodes, MALT contains HEVs for the entry of naïve lymphocytes, and stromal cells that secrete chemokines that direct lymphocyte traffic in the MALT (CCL19, CCL21 and its receptor CCR7[256]; CCL25 and CCL28 and their receptors CCR9 and CCR10[257]; and CXCL13 and its receptor CXCR5[258,259]). MALT also contains separate T- and B-cell compartments with B-cell follicles, FDCs, germinal centers, and interfollicular T cells and DCs.[260]

MALT differs importantly from other secondary lymphoid organs with regard to its capsules and afferent lymphatic vessels. Unlike the spleen and lymph nodes, which are surrounded by a dense fibrous capsule, MALT often has no capsule, with the exception of the tonsil that has a partial capsule that separates it from the pharyngeal constrictor muscles. Because MALT can sample antigens directly at the epithelial surface, it has no afferent lymphatics. MALT epithelium, especially that of the Peyer's patch, is replaced by specialized lymphoepithelial cells called microfold (M) cells, which because of their high transcytotic capacity, transport antigens to the underlying lymphoid tissue. Tonsils and adenoids are covered by a squamous epithelium and have few M cells. They can sample surface antigens using epithelial DCs that push dendrites through the epithelial surface.[261,262] M cells have not been characterized in human BALT.[263]

The proximity of MALT to the epithelium is an important point, because it helps differentiate MALT from tertiary lymphoid organs (see following discussion). MALT can be defined as organized lymphoid structures in the mucosa that are in direct contact with the epithelium. Using these criteria, a lymphoid aggregate in the submucosa that is not directly in contact with the epithelium (ie, below the lamina propria) is more correctly defined as a tertiary

lymphoid organ.[264]

The Mucosal-Associated Lymphoid Tissue Immune Response: Inductive and Effector Sites

MALT can simultaneously be a site for the induction of an immune response and an effector organ. The MALT surface containing M cells and DCs efficiently samples and transports antigens across the epithelium. Immediately below this single-cell epithelium is a dense network of DCs that can extend dendrites through the epithelium to grasp antigen to present to lymphocytes. Two distinct types of DC have been identified in MALT: CD103+ DC and CX3CR1+ DC.[265] Both types of DC produce tolerogenic responses to commensal bacteria by enhancing the differentiation of Foxp3+ T_{reg}s and inhibiting that of inflammatory Th17 cells. CD103+ DCs express αE integrin, make retinoic acid, induce T_{reg}s, and increase T-cell expression of the two gut homing receptors CCR9 and $\alpha_4\beta_7$. CD103+ DCs exit MALT via the lymphatics and present antigen in the draining lymph nodes. CX3CR1+ DCs that express the chemokine receptor for fractalkine do not migrate to the lymph node. CX3CR1+ DCs are longer-lived mucosal resident DCs that produce less retinoic acid than CD103+ DCs.[265]

MALT has three effector roles: local antibody secretion, systemic antibody secretion, and effector lymphocyte dispersal. MALT plays an important role in defense against pathogens by generating cells that migrate to other sites. MALT can dispatch lymphocytes to lymph nodes, the spleen, and plasma cells to the bone marrow. MALT also sends lymphocytes to other MALT effector sites including the salivary and lacrimal glands, the lactating breast,[266] and the vagina. There are some differences between the effector cells made at different MALT sites. NALT-derived B cells express CCR7 and do not home back to the gut. Instead, cells induced in the NALT home to the salivary glands and the vaginal mucosa lymph nodes,[267] whereas lymphocytes leaving the Peyer's patch express $\alpha_4\beta_7$ and migrate to the gut mucosa. These “preferred pathways” for effector cells coming out of the different MALT has led to the suggestions that MALT is compartmentalized and cells circling in the BALT are in a separate compartment from the GALT. It is more likely that the nature of antigen influences the homing of effector cells. There needs to be flexibility, and there is much overlap and redundancy in the system. For example, after the removal of the tonsils and adenoids in humans, or the NALT in the mouse, the cervical lymph node can act as an inductive site.[268] Likewise, the mouse BALT can mount an immune response in the absence of secondary lymphoid organs.[269,270]

Tonsils and Adenoids

Waldeyer's ring is a group of lymphoid tissues encircling the wall of the throat that are the first defense against pathogens entering through the mouth or nose.[271] Humans have several tonsils with indistinct borders: one pharyngeal tonsil (adenoid), two tubal, two palatine, and one lingual. In addition to T cells, the tonsils contain a large complement of B cells, many of which are positive for IgA.[271] The tonsil epithelium makes the polymeric IgA receptor, or secretory component,[272] crucial for transport of IgA dimers across the epithelium. Secreted IgA provides an early form of defense against pathogens and toxins. Overall, the adenoids produce more secreted IgA than the tonsils.

The common cold virus uses ICAM-1 as receptor to invade the nasal mucosa. ICAM-1 is also an expressed HEV in tonsils.[271] As noted previously, HEVs are specialized vessels that express adhesion molecules that allow naïve lymphocytes enter the tonsil. Naïve L-selectin^{hi} lymphocytes adhere to PNAd on HEVs.[258,259] L-selectin^{hi} cells also bind to MAdCAM-1 predominantly found in the gut HEVs. Considering the "mucosal name" MAdCAM-1, it may be surprising to discover that tonsil HEVs express MAdCAM-1 only weakly or not at all.[273] In mice, the HEVs also manufacture the chemokine CCL21 that can recruit naïve T cells and mature DCs that express CCR7. Human tonsil HEVs do not manufacture CCL21 but are able to display chemokines that have been made and secreted by fibroblasts.[274] During tonsillitis, inflammatory chemokines, cytokines, and adhesion molecules such as CCL19,[256] VCAM-1, and E- and P-selectin are upregulated.[165]

Nasal-Associated Lymphoid Tissue

The NALTs are a pair of lymphoid organs above the soft palate in mice and rats that are considered analogous to Waldeyer's ring.[275] Despite being anatomically separate from the genitourinary tract, NALT has an important "effector" role concerning the generation of immune responses in the genitourinary tract.[276] After nasal immunization with human papillomavirus 16 or ovalbumin, human papillomavirus 16-specific or ovalbumin-specific IgA is detected in vaginal washes,[163,277,278] and cytotoxic T cells are found in vaginal draining lymph nodes.[267] However, the NALT itself is clearly an inductive site for both humoral and cellular immune responses,[279] and supports class switching to IgA.[280]

The stromal cells in murine NALT produce both CCL19 and CXCL13, whereas, in contrast to lymph nodes, only HEVs transcribe CCL21 mRNA.[163] NALT HEVs express high levels of luminal and abluminal PNAd and HEC-6ST.[163] Similar to the tonsils, the major homing receptor-ligand pair in NALT is L-selectin and PNAd, rather than MAdCAM-1 as might have been expected of this mucosal-associated tissue.[281]

Development

Human NALT and tonsils appear early in fetal development, are cellular, and have primary follicles. After birth, secondary follicles and germinal centers appear in response to bacterial antigens.[266] By contrast, the NALT in the mouse and the rat is hypocellular at birth and undergoes dramatic changes after weaning, strongly suggesting that bacterial colonization is important for NALT development in these species. Id2 is required for initiation of the rodent NALT,[282] although RORγT, LTα, or LTβ are not required for this step.[162] The expansion of rodent NALT at weaning includes changes in the expression of LTα and LTβ and lymphoid chemokines, leading to T- and B-cell compartmentalization and HEV maturation.[162,163] The alternative NF-κB pathway is required for the expression of chemokines and the HEV genes *glycam-1* and *chst4* in NALT.[183] LTα, LTβ, IL7R, and the NIK signaling pathways are required for NALT organization and function, and mice that are deficient in these cytokines have a hypocellular NALT.[163,183,283,284]

Bronchus-Associated Lymphoid Tissue

BALT is less organized but more responsive to environmental antigens than the NALT. The number of lymphoid aggregates varies depending on the level of microbial exposure and germ-free pigs have no BALT.[252,285] BALT is found commonly in rabbits and rats, is less frequent in guinea pigs and pigs, and is absent in cats.[264] BALT is not a prominent structure in the laboratory mouse, and its presence varies by strain and age.[286]

An inducible form of the BALT (iBALT) has been described in *Lta-/-* mice after infection with influenza.[287] The term iBALT is misleading because these aggregates have no contact with the bronchial lumen and cannot sample inspired antigens. Therefore, iBALT should be considered as a tertiary lymphoid organ.[288] Splenectomized, lethally irradiated LTα-/- mice reconstituted with normal bone marrow have no lymphoid organs. However, these mice can generate immunological memory in the iBALT.[269,270]

BALT is rare in normal adult human lungs, but tertiary lymphoid organs are common in chronic pulmonary diseases.[283] BALT is frequent in children and is found in fetuses after infections in utero.[289,290] Like NALT, the HEVs in human BALT express PNAd and not MAdCAM-1.[291]

Gut-Associated Lymphoid Tissue

Gut-associated lymphoid tissue is the largest immune system in the body. The gut-associated lymphoid tissue in the small intestine includes the Peyer's patches, smaller isolated lymphoid follicles, and cryptopatches. In the large intestine, there is the appendix, caecal patches, and lymphoglandular complexes.[292]

Peyer's Patches

Peyer's patches are lymphoid aggregates aligned on the antimesenteric border of the small intestine (Fig. 3.5A). They are present in most species, though their number and location vary. They are dome shaped and are covered by a specialized epithelium that lacks surface microvilli and goblet cells; they have numerous M cells. Because Peyer's patches lack afferent lymphatic vessels, these M cells are critical transporters of antigen. Pathogens, including human immunodeficiency virus and salmonella take advantage of the M cells to facilitate their own invasion.[293,294] Below the epithelium is a diffuse area, the subepithelial dome, divided into three to six parts. Peyer's patch organization is similar to that of the lymph node (see Fig. 3.5). Peyer's patches contain 6 to 12 basally located germinal centers, but the B-cell areas are larger with a T:B cell ratio of 0.2; CD4+ cells predominate over CD8+ cells.[295] Although M cells transport antigen across the epithelial barrier, they are not believed to have a crucial role in processing or presenting antigen. Peyer's patches are populated by several subsets of DCs that can carry out these functions.[296,297]

Trafficking In and Out

Cells enter Peyer's patches through HEVs. In contrast to peripheral lymph nodes and the NALT, the HEVs of Peyer's patches express MAdCAM-1,[298] and homing depends on the

interaction of MAdCAM-1 with the integrin $\alpha_4\beta_7$ on lymphocytes. Luminal PNAd is rarely found in Peyer's patch HEVs; only occasional abluminal expression is detected. This pattern is identical to that seen in lymph node HEVs in *chst4-/-* mice.[128] However, lymphocytes from mice that lack both L-selectin ligand and $\alpha_4\beta_7$ home less efficiently to Peyer's patches than do lymphocytes from mice that lack only one or the other of the ligands,[299,300] suggesting that an L-selectin ligand does contribute to homing to Peyer's patches. Even though P-selectin is expressed only weakly on HEVs in Peyer's patches, cells from mice deficient in P-selectin show reduced rolling and adhesion in vivo. This suggests that P-selectin, as well as MAdCAM-1, is important for homing to Peyer's patches.[299]

Lymphoid chemokines CCL19, CCL21, and CXCL13 are found in the T- and B-cell areas[301] (see Fig. 3.5B). Stromal cells make CXCL12 mRNA, but the protein is also found on HEVs, presumably transported in a manner similar to CCL19.[117] The CXCL16/CXCR6 chemokine/receptor pair is important in Peyer's patches but not in lymph nodes. Even under germ-free conditions, CXCL16 is constitutively produced by the dome epithelium.[295] CD4+ and CD8+ cells that express CXCR6 are found in Peyer's patches and require CXCL16 to home to the subepithelial dome. CCL20 and its receptor CCR6 are also important in Peyer's patches. CCL20 is made by the intestinal epithelium and plays a crucial role in dendritic cell trafficking to Peyer's patches.[302] Peyer's patch HEVs express CCL25, the ligand for CCR9, which has been defined as a mucosal homing chemokine receptor for intraepithelial lymphocytes and plasma cells.[78]

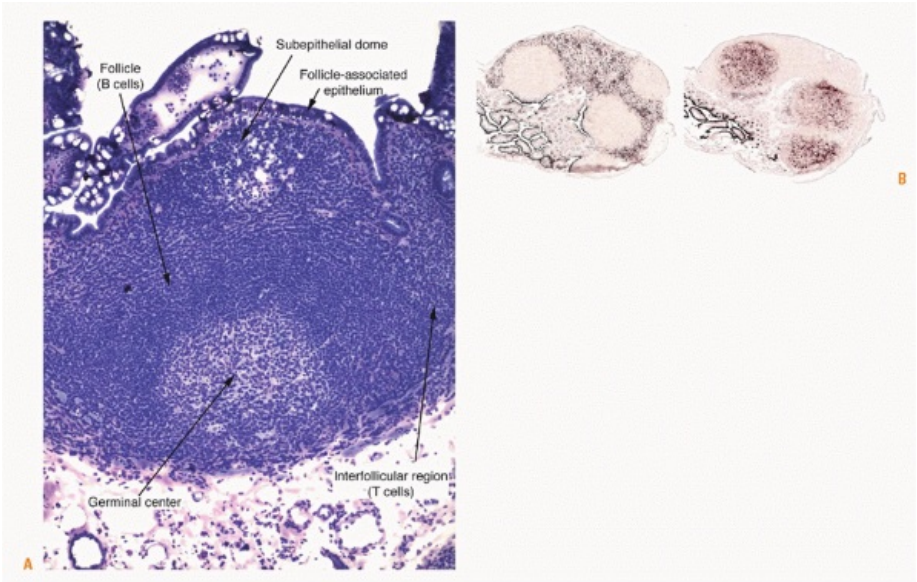

FIG. 3.5. Organization of Peyer's Patches. A: Photomicrograph of Peyer's patch. Peyer's patches are located in the intestine near intestinal villi. The follicle associated epithelium (FAE) is in contact with the gut lumen. M cells (not shown) in the FAE transport antigen into the subepithelial dome, populated by dendritic cells and T cells. The interfollicular T-cell-rich region surrounds the B-cell follicle and germinal center. Courtesy of A. Iwasaki (Yale University School of Medicine, New Haven, CT). **B:** In situ

hybridization of chemokine messenger ribonucleic acids (mRNAs). On the *left*, CCL21 mRNA defines the T-cell zone and high endothelial venules; on the *right*, CXCL13 defines the B-cell zone.

Development

Several cytokines and chemokines have been shown to be crucial for Peyer's patch development. *Lta-/-* and *Lt*β-/- mice completely lack Peyer's patches,[161,303] as do *Cxcr5-/-* mice.[174,175,301] Because mice deficient in the LTβR also lack Peyer's patches,[304] these effects are mediated in large part through $LT\alpha_1\beta_2$. However, some *Tnfr1-/-* mice lack organized Peyer's patches, suggesting that either LTα3 or TNFα also influences generation or later stages in maintenance of Peyer's patches.[305] Mice deficient in IL-7, RORγt, Id2, NIK, and factors in the classical and alternative NFκB pathways[170,177,304] lack Peyer's patches, although RANKL is not required.[172]

A model for the embryonic development of Peyer's patches[306,307,308] provided the framework for studies in that organ. The first sign of Peyer's patch development at E15.5 in the mouse is the appearance of regions on the small intestine that stain positively with antibodies to ICAM-1, VCAM-1, and LTβR. The cells in these aggregates are called the lymphoid tissue organizer cells and express CXCL13, CCL19, CCL21, and IL-7. At E17.5, clusters of IL-7R+CD4+CD3+ inducer cells are found. These express LTα and LTβ, CXCR5 and CCR7, Id2, and RORγ. They also express $\alpha_4\beta1$ integrin activated by CXCR5[309] that allows interaction with the VCAM-1+ organizer cells. At E 18.5, mature T and B cells enter through HEVs, CCL20 is produced by the FAE, and DCs expressing CCR7 and CCR6 are found. By day 4, the typical microarchitecture is apparent, with M cells and T- and B-cell compartmentalization.[310]

Lymphocytes influence the maintenance of Peyer's patches. Mice that lack mature T and B cells have either undetectable or small Peyer's patches that lack follicles and germinal centers.[311,312] B cell-deficient mice retain some M cells, suggesting that T cells may regulate M-cell maintenance. When B cell-deficient mice are reconstituted by a membrane IgM transgene, M cells are found at levels comparable to those of normal mice,[311] indicating that cells in addition to CD4+CD3+ lymphoid tissue-inducer cells are crucial for the maintenance of mature, functioning Peyer's patches.

Cryptopatches and Isolated Lymphoid Follicles

The small intestine of a mouse contains 100 to 200 isolated lymphoid follicles and more numerous cryptopatches. The cryptopatches in the lamina propria containing lymphoid tissue-inducer cells and DCs can be precursors of ILFs. The cryptopatches are composed of lin-c-kit+ cells, DCs, and VCAM-1+ stromal cells with few or no mature T and B cells. The cryptopatch cells express RORγt, IL-7R, and CCR6,[313] and their development is dependent on IL-7, CCR6, and its receptor CCL20. Cryptopatches are quite plastic, and although the LT family is necessary for their development, they can be restored by administration of wild-type bone marrow to adult *Lta-/-* mice.

After mice are exposed to microbes or during some forms of autoimmunity, cryptopatches give rise to ILFs,[314,315] which resemble small Peyer's patches and usually only have a single

dome. ILFs require B cells that express LT, but not T cells, for their formation.[316]. In contrast to lymph nodes, they also require the TNFR1.[317] The location of ILFs is antigen driven, and they resolve completely after mice are treated with antibiotics or cytokine inhibitors.[121,185,314,316,317]

Appendix

Both rabbits and humans have an appendix,[318] a vestigial organ distal to the ileocecal junction that divides the small intestine from the colon. The appendix has epithelial M cells and dense lymphoid accumulations and germinal centers that are similar to Peyer's patches. Similar to the tonsils and adenoids, the appendix involutes and regresses after puberty.

Colon Lymphoglandular Complexes

The colon has the highest bacterial load of any of the MALTs, and the distribution of the MALT is more random than in the small intestine. Lymphoglandular complexes are smaller than Peyer's patches. As their name suggests, the lymphoid cells of the lymphoglandular complexes surround the mucus glands.[292]

TERTIARY LYMPHOID TISSUES

Similarities to Secondary Lymphoid Organs

Tertiary lymphoid tissues, also termed tertiary lymphoid organs, are ectopic accumulations of lymphoid cells that arise in nonlymphoid organs during chronic inflammation through a process termed “lymphoid neogenesis” (also “lymphoid neo-organogenesis”).[2] The iBALT could be considered as either a secondary or tertiary lymphoid tissue. This semantic issue epitomizes the plasticity of all lymphoid organs and the fact that lymphoid organ regulation represents a continuum from ontogeny through chronic inflammation. A notable difference between secondary lymphoid organs and tertiary lymphoid tissues is the fact that the latter can arise in almost any organ in the adult. Nonetheless, tertiary lymphoid tissues exhibit remarkable morphologic, cellular, chemokine, and vasculature similarities to secondary lymphoid organs. They exhibit T- and B-cell compartmentalization; naïve T and B cells; DCs; FDCs; germinal centers; plasma cells; lymphoid chemokines CCL19, CCL21, and CXCL13[1]; HEVs[135,196,319]; and conduits.[320] Lymphatic vessels have also been noted in tertiary lymphoid tissues in chronic graft rejection[321,322] and mouse models of Sjögren syndrome[134] (Fig. 3.6) and Type 1 diabetes.[323] Although it is not completely clear whether they function as afferent and/or efferent vessels, the fact that the tertiary lymphoid tissues respond to S1P inhibitors suggest that their lymphatic vessels function similarly to those in lymph nodes.[323]

Lymphoid neogenesis has been noted in humans in autoimmunity, microbial infection, and chronic allograft rejection. A few examples are shown in Table 3.2 and are described in more detail in Drayton et al.[1] These accumulations also occur in atherosclerotic plaques with

FDCs, organized B-cell follicles, HEVs (HECA-452), and CCL19 and CCL21 in addition to those chemokines more often associated with acute inflammation.[324] Tertiary lymphoid tissues have also been noted in non-small-cell lung cancer[325] and many other solid tumors.[326] The tendency for tertiary lymphoid tissues to develop into lymphomas has also been noted in many studies.[327]

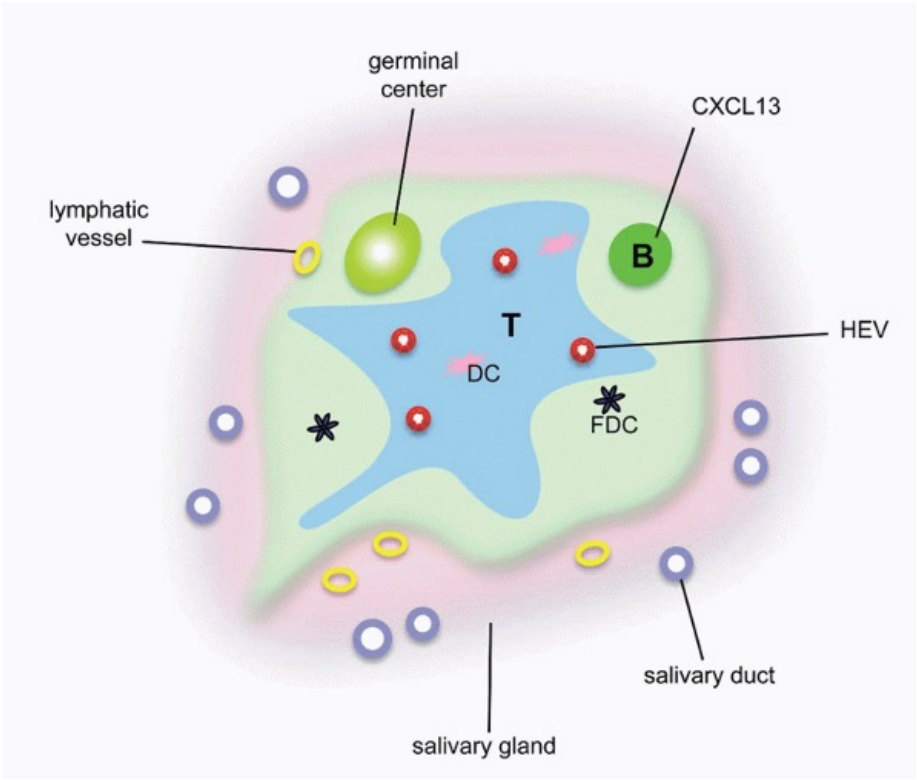

FIG. 3.6. Tertiary Lymphoid Tissue. Schematic diagram of Sjögren syndrome salivary gland. Note similarities with the organization of a lymph node.

Ectopic lymphoid tissues are also apparent in several animal models. The pancreatic infiltrates in early stages of diabetes in the nonobese diabetic mouse express lymphoid chemokines[328] and HEVs expressing MAdCAM-1,[329,330] PNAd, and HEC-6ST.[135] The brain in experimental autoimmune encephalomyelitis, a model of multiple sclerosis, has HEVs, CCL19, CCL21, CXCL13, and FDCs.[331,332,333] The thyroid in the BioBreeding (BB) rat has T- and B-cell compartmentalization and DCs,[334] and the gut in autoimmune gastritis in the mouse has HEVs and CXCL13.[335] Atherosclerotic plaques of apoprotein-E-deficient mice exhibit a marked increase in T and B cells, expression of CCL19 and CCL21, and PNAd+ HEVs.[336,337,338] Lymphoid neogenesis also occurs in chronic mouse heart allograft rejection.[339,340] Tertiary lymphoid tissues have been induced in several transgenic mouse models with the use of tissue-specific promoters driving the expression of inflammatory cytokines or lymphoid chemokines.[1] These mouse models, in addition to serving as examples of human disease, have provided valuable insight into the regulation of secondary lymphoid

organ development.

From Chronic Inflammation to Organized Lymphoid Microenvironments

Data generated from analyzing the cellular and molecular requirements for secondary lymphoid organ development have provided a paradigm for understanding the development of tertiary lymphoid tissues in chronic inflammation. This paradigm proposes that the processes and molecules governing secondary lymphoid organs are also the basis of tertiary lymphoid tissue development,[2] and informs understanding of both secondary and tertiary lymphoid tissues. The physiologic event(s) that precipitate lymphoid neogenesis remain unclear. Data obtained from experiments in knockout and transgenic

mice and clinical observations indicate that cooperative activities of TNF/LT family members and the lymphoid chemokines play central roles in this process.

TABLE 3.2 Lymphoid Neogenesis in Human Autoimmunity, Infectious Diseases, and Graft Rejection

Disease	Affected Tissue	Characteristics
Autoimmunity		
Rheumatoid arthritis	Synovial membrane	T cells and B cells, plasma cells, GCs, FDCs, CXCL13, CCL21, HEVs (PNAd, HEC-6ST)
Sjögren syndrome	Salivary glands	T cells and B cells, plasma cells, GCs, FDCs, CCL21, CXCL12, CXCL13 HEVs (PNAd)
Myasthenia gravis	Thymus	T cells and B cells, GCs, FDCs
Hashimoto thyroiditis	Thyroid	T cells and B cells, GCs, FDCs CCL21, CXCL13, CXCL12, plasma cells, HEVs (PNAd)
Grave disease	Thymus	T cells and B cells, GCs, FDCs, CCL21 CXCL13, CXCL12, HEVs (PNAd)
Multiple sclerosis	Brain	Lymphatic capillaries, B-cell

		follicles and centroblasts, GCs, CCL19, CCL21, CXCL12, CXCL13
Ulcerative colitis	Colon	CXCL13
Inflammatory bowel disease (Crohn disease)	Bowel	T cell-B compartments, LVs, HECA-452+ HEV
Psoriatic arthritis	Joint	T cells and B cells, CXCL13, CCL19, CCL21, HEVs (PNAd)
Infectious Diseases		
Borrelia burgdorferi/Lyme disease	Joints	T and B cells, FDCs, HEVs
Borrelia burgdorferi/neuroborreliosis	Central nervous system/cerebrospinal fluid	CXCL13
Hepatitis C virus	Liver	T-cell and B-cell compartments, MAdCAM-1
Bartonella henselae/cat scratch disease	Granuloma	CXCL13
Graft Rejection		
Organ		
Heart		GCs
Kidney		GCs, LVs

FDC, follicular dendritic cell; GC, germinal center; HEV, high endothelial venule; LV, lymphatic vessel; MAdCAM-1, mucosal addressin cell adhesion molecule; PNAd, peripheral node addressin. Original references are in Drayton et al.1

Inflammation is a localized response to tissue injury, irritation, or infection often marked by tissue damage. Acute inflammation is an early innate immune response that is generally short-lived and self-limiting. However, in some situations, acute inflammation transitions to a

chronic inflammatory response that is long-lived and self-perpetuating. Tertiary lymphoid tissues arise under conditions of constitutive cytokine and/or chemokine expression, but the precise signal(s) that initiates their development is unknown. By integrating studies of lymphoid neogenesis in human pathologies and in animal models, it is becoming clear that at least three critical events promote tertiary lymphoid tissue formation: inflammatory (eg, TNF/LT) cytokine expression, lymphoid chemokine production by stromal cells, and HEV development. It is not known if lymphoid tissue-inducer cells are necessary for lymphoid neogenesis, though such cells have been noted in the mouse models of lymphoid neogenesis,[341] and lymphoid tissue-inducer-like cells have been described in several instances.

Functions of Tertiary Lymphoid Tissues

Ectopic accumulation of lymphoid cells has been considered the hallmark of destructive inflammation. Indeed, some tertiary lymphoid tissues are accompanied by tissue damage. However, it is likely that tertiary lymphoid tissues in chronic inflammation have roles in addition to tissue destruction. In the case of microbial infection, it is likely that lymphoid neogenesis occurs as a way to sequester pathogens and to prevent their access to the other parts of the body. This may represent a primitive form of immunity. Local antigen presentation within the tertiary lymphoid tissue itself likely prevents bacteremia or viremia. Enhanced long-term survival has been noted for those individuals whose lung or breast tumors included tertiary lymphoid tissues.[325,326] The propensity for tertiary lymphoid tissues to develop into lymphomas, as noted previously, the ability of lymphatic vessels to serve as conduits for tumor metastasis, and the ability of tertiary lymphoid tissues to serve as sites of prion accumulation are obvious manifestations of detrimental functions.[342] Furthermore, the development of tertiary lymphoid tissues in autoimmunity may perpetuate clinical disease through epitope spreading.

Data from human and mouse studies provide compelling evidence that tertiary lymphoid tissues are permissive microenvironments for the induction of antigen-specific immune responses. Extensive immunohistochemical analyses of tertiary lymphoid tissues in autoimmunity and other chronic inflammatory states have established the presence

of germinal centers and FDC networks in these tissues; several groups have demonstrated that tertiary lymphoid tissue germinal centers can support B-cell differentiation. Microdissection of discrete lymphocytic foci and subsequent deoxyribonucleic acid sequence analysis of germinal center B cells from the inflamed synovial tissue of patients with rheumatoid arthritis revealed a restricted number of Vκ gene rearrangements, a result consistent with oligoclonal B-cell expansion in the synovial tissue.[343] Somatic hypermutation is apparent in synovial germinal center B cells.[344] Furthermore, synovial B cells exhibit a limited number of heavy and light chain gene rearrangements consistent with local clonal expansion of these cells. The molecular analysis of tertiary lymphoid tissue germinal centers from the salivary glands of patients with primary Sjögren syndrome or the thymus of patients with myasthenia gravis demonstrates oligoclonal B-cell proliferation in these tissues in addition to somatic hypermutation of Ig variable genes.[345,346,347] Together, these studies indicate that tertiary lymphoid tissue germinal centers in several autoimmune pathologies can support antigen-driven clonal expansion and extensive diversification.

Another important hallmark of antigen-driven B-cell responses is the terminal differentiation of activated B cells into Ig-secreting plasma cells. Plasma cells have been detected in tertiary lymphoid tissues associated with germinal centers in rheumatoid arthritis[348] and in Sjögren syndrome. Mice expressing LTα under the control of the rat insulin promoter (RIPLTα) exhibit tertiary lymphoid tissue at the sites of transgene expression (pancreas, kidney, and skin). After immunization with sheep red blood cells, evidence of isotype switching is apparent in these cellular infiltrates.[2] Although the presence of plasma cells in tertiary lymphoid tissues is consistent with local antigen presentation, it is occasionally unclear whether these cells develop in the tertiary lymphoid tissues themselves or migrate from canonical secondary lymphoid tissues. Nonetheless, taken together, these studies indicate that tertiary lymphoid tissues in several human pathologies and animal models support antigen-driven B-cell differentiation marked by somatic hypermutation of Ig variable genes, affinity maturation, isotype switching, and terminal differentiation into antibody-secreting plasma cells.

T-cell priming occurs in tertiary lymphoid tissues as suggested by the presence of isotype switched plasma cells in the RIPLTα tertiary lymphoid tissues,[2] accelerated graft rejection,[349] and T-cell epitope spreading in the central nervous system during experimental allergic encephalomyelitis.[350] The restricted TCR repertoire in a melanoma-associated tertiary lymphoid tissue[351] further supports the concept that tertiary lymphoid tissues can act as priming sites. The demonstration of naïve T-cell proliferation in the islets of nonobese diabetic mice after surgical removal of pancreatic lymph nodes[352] suggests, together with evidence noted previously, that tertiary lymphoid tissues present antigen to naïve cells at the local site and generate an immune response. Determinant or epitope spreading, a phenomenon that arises in several autoimmune diseases, occurs when epitopes other than the inducing antigen become major targets of an ongoing immune response. It is considered to occur subsequent to the tissue damage induced by the initiating autoreactive T cells and therefore is the result of the presentation of new antigens.[353] Data generated in murine models of central nervous system inflammation support the possibility that intermolecular and intramolecular epitope spreading occur in tertiary lymphoid tissues in the central nervous system.[350] More recently, it has become apparent that T_{reg}s can populate tertiary lymphoid tissues,[338] again suggesting that manipulating the immune response at the local site is an avenue of control.

Plasticity and Adaptability of Tertiary Lymphoid Tissues

Tertiary lymphoid tissues are the most plastic and adaptable of the lymphoid tissues. First, lymphoid neogenesis can be induced by a variety of stimuli. Their nimbleness in this regard suggests that they might represent the most primitive tissues in the immune system. Tertiary lymphoid tissues can be “turned off” (ie, resolve) upon removal of the initial stimulus or after therapeutic intervention. The destruction of the islets of Langerhans β cells in type I diabetes mellitus is an example of a situation in which removal of the antigen stimulus is accompanied by tertiary lymphoid tissue resolution. Antibiotic treatment results in the resolution of tertiary lymphoid tissues and even MALT lymphomas.[354] Treatment has been shown to resolve some established tertiary lymphoid tissues, reversing insulitis and protecting against diabetes

in nonobese diabetic mice.[355] Such treatment can also "turn off" established tertiary lymphoid tissues in a mouse model of collagen-induced arthritis.[356] These studies are similar to those described previously regarding the plasticity of lymph nodes after mice are immunized or treated with LTβR-Ig,[198,199] again emphasizing the commonality of these tissues.

CONCLUSION

The immune system depends on a remarkable organization of tissues and cells. The organs have defined functions that include generation of an immune repertoire (primary lymphoid organs) and responding to antigen (secondary lymphoid organs and tertiary lymphoid tissues). Development of primary and secondary lymphoid organs depends on precisely regulated expression of cytokines, chemokines, and adhesion molecules. Similar signals regulate the transition from inflammation to tertiary lymphoid tissues. Chemokines and adhesion molecules regulate trafficking in and out of lymphoid organs. Secondary lymphoid organs are remarkably plastic in their response to antigenic assault and adapt with changes in expression of chemokines and adhesion molecules to maximize encounter of antigen with antigen-specific cells. Tertiary lymphoid tissues, characteristic of many pathologic states, may actually represent the most primitive form of lymphoid tissues in their even greater plasticity and ability to develop directly at the site of antigen exposure.

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REFERENCES

1. Drayton DL, Liao S, Mounzer RH, et al. Lymphoid organ development: from ontogeny to neogenesis. *Nat Immunol*. 2006;7:344-353.

2. Kratz A, Campos-Neto A, Hanson MS, et al. Chronic inflammation caused by lymphotoxin is lymphoid neogenesis. *J Exp Med*. 1996; 183:1461-1472.

3. Medvinsky AL, Samoylina NL, Muller AM, et al. An early pre-liver intraembryonic source of CFU-S in the developing mouse. *Nature*. 1993;364:64-67.

4. Gekas C, Dieterlen-Lievre F, Orkin SH, et al. The placenta is a niche for hematopoietic stem cells. *Dev Cell*. 2005;8:365-375.

5. Ottersbach K, Dzierzak E. The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Dev Cell*. 2005;8: 377-387.

6. Medvinsky A, Rybtsov S, Taoudi S. Embryonic origin of the adult hematopoietic system: advances and questions. *Development*. 2011;138: 1017-1031.

7. Christensen JL, Wright DE, Wagers AJ, et al. Circulation and chemotaxis of fetal hematopoietic stem cells. *PLoS Biol*. 2004;2:E75.

8. Hardy RR, Hayakawa K. B cell development pathways. *Annu Rev Immunol*. 2001;19:595-621.

9. Li YS, Hayakawa K, Hardy RR. The regulated expression of B lineage associated genes during B cell differentiation in bone marrow and fetal liver. *J Exp Med*. 1993;178:951-960.

10. Frenette PS, Subbarao S, Mazo IB, et al. Endothelial selectins and vascular cell adhesion molecule-1 promote hematopoietic progenitor homing to bone marrow. *Proc Natl Acad Sci U S A*. 1998;95:14423-14428.

11. Vermeulen M, Le Pesteur F, Gagnerault MC, et al. Role of adhesion molecules in the homing and mobilization of murine hematopoietic stem and progenitor cells. *Blood*. 1998;92:894-900.

12. Orkin SH, Zon LI. Hematopoiesis and stem cells: plasticity versus developmental heterogeneity. *Nat Immunol*. 2002;3:323-328.

13. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999;284:143-147.

14. Cyster JG. Homing of antibody secreting cells. *Immunol Rev*. 2003; 194:48-60.

15. Nagasawa T. Microenvironmental niches in the bone marrow required for B-cell development. *Nat Rev Immunol*. 2006;6:107-116.

16. Jacobsen K, Osmond DG. Microenvironmental organization and stromal cell associations of B lymphocyte precursor cells in mouse bone marrow. *Eur J Immunol*. 1990;20:2395-2404.

17. Heissig B, Hattori K, Dias S, et al. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell*. 2002;109:625-637.

18. Nagasawa T, Omatsu Y, Sugiyama T. Control of hematopoietic stem cells by the bone marrow stromal niche: the role of reticular cells. *Trends Immunol*. 2011;32:315-320.

19. Tokoyoda K, Egawa T, Sugiyama T, et al. Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity*. 2004;20:707-718.

20. Banu N, Deng B, Lyman SD, et al. Modulation of haematopoietic progenitor development by FLT-3 ligand. *Cytokine*. 1999;11:679-688.

21. Mazo IB, Massberg S, von Andrian UH. Hematopoietic stem and progenitor cell trafficking. *Trends Immunol*. 2011;32:493-503.

22. Heissig B, Ohki Y, Sato Y, et al. A role for niches in hematopoietic cell development. *Hematol*. 2005;10:247-253.

23. Lataillade JJ, Domenech J, Le Bousse-Kerdiles MC. Stromal cellderived factor-1 (SDF-1)\CXCR4 couple plays multiple roles on haematopoietic progenitors at the border between the old cytokine and new chemokine worlds: survival, cell cycling and trafficking. *Eur Cytokine Netw* . 2004;15:177-188.

24. Papayannopoulou T, Craddock C, Nakamoto B, et al. The VLA4/VCAM-1 adhesion pathway defines contrasting mechanisms of lodgement of transplanted murine hemopoietic progenitors between bone marrow and spleen. *Proc Natl Acad Sci U S A* . 1995;92:9647-9651.

25. Katayama Y, Hidalgo A, Furie BC, et al. PSGL-1 participates in E-selectin-mediated progenitor homing to bone marrow: evidence for cooperation between E-selectin ligands and alpha4 integrin. *Blood*. 2003;102:2060-2067.

26. Katayama Y, Hidalgo A, Peired A, et al. Integrin alpha4beta7 and its counterreceptor MAdCAM-1 contribute to hematopoietic progenitor recruitment into bone marrow following transplantation. *Blood*. 2004;104:2020-2026.

27. Lindsley RC, Thomas M, Srivastava B, et al. Generation of peripheral B cells occurs via two spatially and temporally distinct pathways. *Blood*. 2006;109:2521-2528.

28. Honczarenko M, Glodek AM, Swierkowski M, et al. Developmental stage-specific shift in responsiveness to chemokines during human B-cell development. *Exp Hematol*. 2006;34:1093-1100.

29. Bowman EP, Campbell JJ, Soler D, et al. Developmental switches in chemokine response profiles during B cell differentiation and maturation. *J Exp Med*. 2000;191:1303-1318.

30. Pantelouris EM. Absence of thymus in a mouse mutant. *Nature*. 1968;217:370-371.

31. Terszowski G, Muller SM, Bleul CC, et al. Evidence for a functional second thymus in mice. *Science*. 2006;312:284-287.

32. Bubanovic IV. Crossroads of extrathymic lymphocytes maturation pathways. *Med Hypotheses*. 2003;61:235-239.

33. Cunliffe VT, Furley AJ, Keenan D. Complete rescue of the nude mutant phenotype by

a wild-type Foxn1 transgene. *Mamm Genome*. 2002;13:245-252.

34. Scollay RG, Butcher EC, Weissman IL. Thymus cell migration. Quantitative aspects of cellular traffic from the thymus to the periphery in mice. *Eur J Immunol* . 1980;10:210-218.

35. Juang J, Ebert PJ, Feng D, et al. Peptide-MHC heterodimers show that thymic positive selection requires a more restricted set of self-peptides than negative selection. *J Exp Med*. 2010;207:1223-1234.

36. Blackburn CC, Manley NR. Developing a new paradigm for thymus organogenesis. *Nat Rev Immunol* . 2004;4:278-289.

37. Lind EF, Prockop SE, Porritt HE, et al. Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development. *J Exp Med*. 2001;194:127-134.

38. Anderson M, Anderson SK, Farr AG. Thymic vasculature: organizer of the medullary epithelial compartment? *Int Immunol* . 2000;12:1105-1110.

39. Odaka C, Morisada T, Oike Y, et al. Distribution of lymphatic vessels in mouse thymus: immunofluorescence analysis. *Cell Tissue Res*. 2006; 325:13-22.

40. Pearse M, Wu L, Egerton M, et al. A murine early thymocyte developmental sequence is marked by transient expression of the interleukin 2 receptor. *Proc Natl Acad Sci U S A* . 1989;86:1614-1618.

41. Shinkai Y, Rathbun G, Lam KP, et al. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* . 1992;68:855-867.

42. Jameson SC, Hogquist KA, Bevan MJ. Positive selection of thymocytes. *Annu Rev Immunol* . 1995;13:93-126.

43. Kisielow P, Teh HS, Bluthmann H, et al. Positive selection of antigenspecific T cells in thymus by restricting MHC molecules. *Nature*. 1988;335:730-733.

44. Egerton M, Scollay R, Shortman K. Kinetics of mature T-cell development in the thymus. *Proc Natl Acad Sci U S A* . 1990;87:2579-2582.

45. Silva-Santos B, Pennington DJ, Hayday AC. Lymphotoxin-mediated regulation of gammadelta cell differentiation by alphabeta T cell progenitors. *Science*. 2005;307:925-928.

46. Nunes-Cabaco H, Caramalho I, Sepulveda N, et al. Differentiation of human thymic regulatory T-cells at the double positive stage. *Eur J Immunol* . 2011;41(12):3604-3614.

47. Klug DB, Carter C, Crouch E, et al. Interdependence of cortical thymic epithelial cell differentiation and T-lineage commitment. *Proc Natl Acad Sci U S A* . 1998;95:11822-11827.

48. Derbinski J, Gabler J, Brors B, et al. Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J Exp Med*. 2005; 202:33-45.

49. Aaltonen J, Bjorses P, Sandkuijl L, et al. An autosomal locus causing autoimmune disease: autoimmune polyglandular disease type I assigned to chromosome 21. *Nat Genet*. 1994;8:83-87.

50. Akirav EM, Ruddle NH, Herold KC. The role of AIRE in human autoimmune disease. *Nat Rev Endocrinol* . 2011;7:25-33.

51. Anderson MS, Venanzi ES, Klein L, et al. Projection of an immunological self shadow within the thymus by the aire protein. *Science*. 2002;298:1395-1401.

52. Patel DD, Whichard LP, Radcliff G, et al. Characterization of human thymic epithelial cell surface antigens: phenotypic similarity of thymic epithelial cells to epidermal keratinocytes. *J Clin Immunol* . 1995; 15:80-92.

53. Chentoufi AA, Palumbo M, Polychronakos C. Proinsulin expression by Hassall's corpuscles in the mouse thymus. *Diabetes*. 2004;53:354-359.

54. Girbal-Neuhauser E, Durieux JJ, Arnaud M, et al. The epitopes targeted by the rheumatoid arthritis-associated antifilaggrin autoantibodies are posttranslationally generated on various sites of (pro) filaggrin by deimination of arginine residues. *J Immunol* . 1999;162: 585-594.

55. Favre A. Identification of filaggrin in Hassall's corpuscle by histochemical and immunohistochemical methods. *Acta Anat (Basel)* . 1989; 135:71-76.

56. Douek DC, Altmann DM. T-cell apoptosis and differential human leucocyte antigen class II expression in human thymus. *Immunology*. 2000;99:249-256.

57. Ardavin C, Wu L, Li CL, et al. Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. *Nature*. 1993;362:761-763.

58. Foss DL, Donskoy E, Goldschneider I. The importation of hematogenous precursors by the thymus is a gated phenomenon in normal adult mice. *J Exp Med*. 2001;193:365-374.

59. Donskoy E, Goldschneider I. Two developmentally distinct populations of dendritic cells inhabit the adult mouse thymus: demonstration by differential importation of

hematogenous precursors under steady state conditions. *J Immunol*. 2003;170:3514-3521.

60. Anderson G, Owen JJ, Moore NC, et al. Thymic epithelial cells provide unique signals for positive selection of CD4+CD8+ thymocytes in vitro. *J Exp Med*. 1994;179:2027-2031.

61. Anderson G, Partington KM, Jenkinson EJ. Differential effects of peptide diversity and stromal cell type in positive and negative selection in the thymus. *J Immunol*. 1998;161:6599-6603.

62. Brocker T, Riedinger M, Karjalainen K. Targeted expression of major histocompatibility complex (MHC) class II molecules demonstrates that dendritic cells can induce negative but not positive selection of thymocytes in vivo. *J Exp Med*. 1997;185:541-550.

63. Heino M, Peterson P, Sillanpaa N, et al. RNA and protein expression of the murine autoimmune regulator gene (Aire) in normal, RelB-deficient and in NOD mouse. *Eur J Immunol*. 2000;30:1884-1893.

64. Watanabe N, Wang YH, Lee HK, et al. Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. *Nature*. 2005;436:1181-1185.

65. Proietto AI, van Dommelen S, Zhou P, et al. Dendritic cells in the thymus contribute to T-regulatory cell induction. *Proc Natl Acad Sci U S A*. 2008;105:19869-19874.

66. Liu YJ, Soumelis V, Watanabe N, et al. TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. *Annu Rev Immunol*. 2006;25:193-219.

67. Hanabuchi S, Ito T, Park WR, et al. Thymic stromal lymphopoietin-activated plasmacytoid dendritic cells induce the generation of FOXP3+ regulatory T cells in human thymus. *J Immunol*. 2010;184: 2999-3007.

68. Miyazaki T, Suzuki G, Yamamura K. The role of macrophages in antigen presentation and T cell tolerance. *Int Immunol*. 1993;5:1023-1033.

69. Ferrero I, Anjuere F, Martin P, et al. Functional and phenotypic analysis of thymic B cells: role in the induction of T cell negative selection. *Eur J Immunol*. 1999;29:1598-1609.

70. Flores KG, Li J, Hale LP. B cells in epithelial and perivascular compartments of human adult thymus. *Hum Pathol*. 2001;32:926-934.

71. Fukuba Y, Inaba M, Taketani S, et al. Functional analysis of thymic B cells.

Immunobiology. 1994;190:150-163.

72. Joao C, Ogle BM, Gay-Rabinstein C, et al. B cell-dependent TCR diversification. *J Immunol*. 2004;172:4709-4716.

73. Frommer F, Waisman A. B cells participate in thymic negative selection of murine auto-reactive CD4+ T cells. *PLoS One*. 2010;5:e15372.

74. Akirav EM, Xu Y, Ruddle NH. Resident B cells regulate thymic expression of myelin oligodendrocyte glycoprotein. *J Neuroimmunol*. 2011; 235:33-39.

75. Lepique AP, Palencia S, Irjala H, et al. Characterization of vascular adhesion molecules that may facilitate progenitor homing in the postnatal mouse thymus. *Clin Dev Immunol*. 2003;10:27-33.

76. Bleul CC, Boehm T. Chemokines define distinct microenvironments in the developing thymus. *Eur J Immunol* . 2000;30:3371-3379.

77. Liu C, Ueno T, Kuse S, et al. The role of CCL21 in recruitment of T-precursor cells to fetal thymi. *Blood*. 2005;105:31-39.

78. Wurbel MA, Malissen M, Guy-Grand D, et al. Mice lacking the CCR9 CC-chemokine receptor show a mild impairment of early T- and B-cell development and a reduction in T-cell receptor gammadelta(+) gut intraepithelial lymphocytes. *Blood*. 2001;98:2626-2632.

79. Misslitz A, Pabst O, Hintzen G, et al. Thymic T cell development and progenitor localization depend on CCR7. *J Exp Med*. 2004;200:481-491.

80. Plotkin J, Prockop SE, Lepique A, et al. Critical role for CXCR4 signaling in progenitor localization and T cell differentiation in the postnatal thymus. *J Immunol*. 2003;171:4521-4527.

81. Kurobe H, Liu C, Ueno T, et al. CCR7-dependent cortex-to-medulla migration of positively selected thymocytes is essential for establishing central tolerance. *Immunity*. 2006;24:165-177.

82. Ueno T, Saito F, Gray DH, et al. CCR7 signals are essential for cortex-medulla migration of developing thymocytes. *J Exp Med*. 2004;200:493-505.

83. Le Borgne M, Ladi E, Dzhagalov I, et al. The impact of negative selection on thymocyte migration in the medulla. *Nat Immunol*. 2009;10: 823-830.

84. Poznansky MC, Olszak IT, Evans RH, et al. Thymocyte emigration is mediated by active movement away from stroma-derived factors. *J Clin Invest*. 2002;109:1101-1110.

85. Ueno T, Hara K, Willis MS, et al. Role for CCR7 ligands in the emigration of newly generated T lymphocytes from the neonatal thymus. *Immunity*. 2002;16:205-218.

86. Edsall LC, Spiegel S. Enzymatic measurement of sphingosine 1-phosphate. *Anal Biochem*. 1999;272:80-86.

87. Slifka MK, Matloubian M, Ahmed R. Bone marrow is a major site of long-term antibody production after acute viral infection. *J Virol* . 1995;69:1895-1902.

88. Manley NR. Thymus organogenesis and molecular mechanisms of thymic epithelial cell differentiation. *Semin Immunol* . 2000;12:421-428.

89. Gordon J, Bennett AR, Blackburn CC, et al. Gcm2 and Foxn1 mark early parathyroid- and thymus-specific domains in the developing third pharyngeal pouch. *Mech Dev*. 2001;103:141-143.

90. Manley NR, Blackburn CC. A developmental look at thymus organogenesis: where do the non-hematopoietic cells in the thymus come from? *Curr Opin Immunol* . 2003;15:225-232.

91. Kingston R, Jenkinson EJ, Owen JJ. Characterization of stromal cell populations in the developing thymus of normal and nude mice. *Eur J Immunol* . 1984;14:1052-1056.

92. Owen JJ, Jenkinson EJ. Early events in T lymphocyte genesis in the fetal thymus. *Am J Anat*. 1984;170:301-310.

93. Van Vliet E, Jenkinson EJ, Kingston R, et al. Stromal cell types in the developing thymus of the normal and nude mouse embryo. *Eur J Immunol* . 1985;15:675-681.

94. Le Douarin NM, Jotereau FV. Tracing of cells of the avian thymus through embryonic life in interspecific chimeras. *J Exp Med*. 1975;142: 17-40.

95. Gordon J, Wilson VA, Blair NF, et al. Functional evidence for a single endodermal origin for the thymic epithelium. *Nat Immunol* . 2004;5: 546-553.

96. Klug DB, Carter C, Gimenez-Conti IB, et al. Cutting edge: thymocyte-independent and thymocyte-dependent phases of epithelial patterning in the fetal thymus. *J Immunol* . 2002;169:2842-2845.

97. van Ewijk W, Hollander G, Terhorst C, et al. Stepwise development of thymic microenvironments in vivo is regulated by thymocyte subsets. *Development*. 2000;127:1583-1591.

98. Mebius RE. Organogenesis of lymphoid tissues. *Nat Rev Immunol* . 2003;3:292-303.

99. Boehm T, Scheu S, Pfeffer K, et al. Thymic medullary epithelial cell differentiation, thymocyte emigration, and the control of autoimmunity require lympho-epithelial cross talk via LTbetaR. *J Exp Med*. 2003;198:757-769.

100. Chin RK, Lo JC, Kim O, et al. Lymphotoxin pathway directs thymic Aire expression. *Nat Immunol*. 2003;4:1121-1127.

101. Haynes BF, Heinly CS. Early human T cell development: analysis of the human thymus at the time of initial entry of hematopoietic stem cells into the fetal thymic microenvironment. *J Exp Med*. 1995;181:1445-1458.

102. Bouneaud C, Kourilsky P, Bousso P. Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion. *Immunity*. 2000;13:829-840.

103. Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol*. 1991;9:271-296.

104. von Andrian UH, Mempel TR. Homing and cellular traffic in lymph nodes. *Nat Rev Immunol*. 2003;3:867-878.

105. Wilson NS, El-Sukkari D, Belz GT, et al. Most lymphoid organ dendritic cell types are phenotypically and functionally immature. *Blood*. 2003;102:2187-2194.

106. Wilson NS, El-Sukkari D, Villadangos JA. Dendritic cells constitutively present self antigens in their immature state in vivo and regulate antigen presentation by controlling the rates of MHC class II synthesis and endocytosis. *Blood*. 2004;103:2187-2195.

107. Cavanagh LL, Von Andrian UH. Travellers in many guises: the origins and destinations of dendritic cells. *Immunol Cell Biol*. 2002;80:448-462.

108. Steinman RM, Hawiger D, Liu K, et al. Dendritic cell function in vivo during the steady state: a role in peripheral tolerance. *Ann N Y Acad Sci*. 2003;987:15-25.

109. Stoitzner P, Tripp CH, Douillard P, et al. Migratory Langerhans cells in mouse lymph nodes in steady state and inflammation. *J Invest Dermatol*. 2005;125:116-125.

110. Cohen JN, Guidi CJ, Tewalt EF, et al. Lymph node-resident lymphatic endothelial cells mediate peripheral tolerance via Aire-independent direct antigen presentation. *J Exp Med*. 2010;207:681-688.

111. Fletcher AL, Lukacs-Kornek V, Reynoso ED, et al. Lymph node fibroblastic reticular cells directly present peripheral tissue antigen under steady-state and inflammatory conditions. *J Exp Med*. 2010;207:689-697.

112. Gardner JM, Devoss JJ, Friedman RS, et al. Deletional tolerance mediated by extrathymic Aire-expressing cells. *Science*. 2008;321:843-847.

113. Lee JW, Epardaud M, Sun J, et al. Peripheral antigen display by lymph node stroma promotes T cell tolerance to intestinal self. *Nat Immunol*. 2007;8:181-190.

114. Poliani PL, Kisand K, Marrella V, et al. Human peripheral lymphoid tissues contain autoimmune regulator-expressing dendritic cells. *Am J Pathol*. 2010;176:1104-1112.

115. Phan TG, Green JA, Gray EE, et al. Immune complex relay by subcapsular sinus macrophages and noncognate B cells drives antibody affinity maturation. *Nat Immunol*. 2009;10:786-793.

116. Gretz JE, Norbury CC, Anderson AO, et al. Lymph-borne chemokines and other low molecular weight molecules reach high endothelial venules via specialized conduits while a functional barrier limits access to the lymphocyte microenvironments in lymph node cortex. *J Exp Med*. 2000;192:1425-1440.

117. Baekkevold ES, Yamanaka T, Palframan RT, et al. The CCR7 ligand elc (CCL19) is transcytosed in high endothelial venules and mediates T cell recruitment. *J Exp Med*. 2001;193:1105-1112.

118. Nakano H, Gunn MD. Gene duplications at the chemokine locus on mouse chromosome 4: multiple strain-specific haplotypes and the deletion of secondary lymphoid-organ chemokine and EBI-1 ligand chemokine genes in the plt mutation. *J Immunol*. 2001;166:361-369.

119. Vassileva G, Soto H, Zlotnik A, et al. The reduced expression of 6Ckine in the plt mouse results from the deletion of one of two 6Ckine genes. *J Exp Med*. 1999;190:1183-1188.

120. Cyster JG. Chemokines and cell migration in secondary lymphoid organs. *Science*. 1999;286:2098-2102.

121. Ebert LM, Schaerli P, Moser B. Chemokine-mediated control of T cell traffic in lymphoid and peripheral tissues. *Mol Immunol*. 2005;42: 799-809.

122. Rodrigo Mora J, Von Andrian UH. Specificity and plasticity of memory lymphocyte migration. *Curr Top Microbiol Immunol* . 2006;308:83-116.

123. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu Rev Immunol*. 2005;23:127-159.

124. Lo CG, Xu Y, Proia RL, et al. Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to

lymphoid organ transit. *J Exp Med*. 2005;201:291-301.

125. Anderson AO, Anderson ND. Studies on the structure and permeability of the microvasculature in normal rat lymph nodes. *Am J Pathol*. 1975;80:387-418.

126. M'Rini C, Cheng G, Schweitzer C, et al. A novel endothelial L-selectin ligand activity in lymph node medulla that is regulated by alpha(1,3)-fucosyltransferase-IV. *J Exp Med*. 2003;198:1301-1312.

127. von Andrian UH, M'Rini C. In situ analysis of lymphocyte migration to lymph nodes. *Cell Adhes Commun*. 1998;6:85-96.

128. Hemmerich S, Bistrup A, Singer MS, et al. Sulfation of L-selectin ligands by an HEV-restricted sulfotransferase regulates lymphocyte homing to lymph nodes. *Immunity*. 2001;15:237-247.

129. Hemmerich S, Butcher EC, Rosen SD. Sulfation-dependent recognition of HEV-ligands by L-selectin and MECA-79, an adhesion-blocking mAb. *J Exp Med*. 1994;180:2219-2226.

130. Hiraoka N, Kawashima H, Petryniak B, et al. Core 2 branching beta1,6-N-acetylglucosaminyltransferase and high endothelial venule-restricted sulfotransferase collaboratively control lymphocyte homing. *J Biol Chem*. 2004;279:3058-3067.

131. Hiraoka N, Petryniak B, Nakayama J, et al. A novel, high endothelial venule-specific sulfotransferase expresses 6-sulfo sialyl Lewis(x), an L-selectin ligand displayed by CD34. *Immunity*. 1999;11:79-89.

132. Homeister JW, Thall AD, Petryniak B, et al. The alpha(1,3)fucosyltransferases FucT-IV and FucT-VII exert collaborative control over selectin-dependent leukocyte recruitment and lymphocyte homing. *Immunity*. 2001;15:115-126.

133. Kawashima H, Hirakawa J, Tobisawa Y, et al. Conditional gene targeting in mouse high endothelial venules. *J Immunol*. 2009;182:5461-5468.

134. Liao S, Bentley K, Lebrun M, et al. Transgenic LacZ under control of Hec-6st regulatory sequences recapitulates endogenous gene expression on high endothelial venules. *Proc Natl Acad Sci U S A* . 2007;104: 4577-4582.

135. Bistrup A, Tsay D, Shenoy P, et al. Detection of a sulfotransferase (HEC-GlcNAc6ST) in high endothelial venules of lymph nodes and in high endothelial venule-like vessels within ectopic lymphoid aggregates: relationship to the MECA-79 epitope. *Am J Pathol*. 2004;164: 1635-1644.

136. Kraal G, Mebius RE. High endothelial venules: lymphocyte traffic control and

controlled traffic. *Adv Immunol*. 1997;65:347-395.

137. Miyasaka M, Tanaka T. Lymphocyte trafficking across high endothelial venules: dogmas and enigmas. *Nat Rev Immunol*. 2004;4:360-370.

138. Butcher EC, Scollay RG, Weissman IL. Lymphocyte adherence to high endothelial venules: characterization of a modified in vitro assay, and examination of the binding of syngeneic and allogeneic lymphocyte populations. *J Immunol*. 1979;123:1996-2003.

139. Mebius RE, Streeter PR, Michie S, et al. A developmental switch in lymphocyte homing receptor and endothelial vascular addressin expression regulates lymphocyte homing and permits CD4+ CD3- cells to colonize lymph nodes. *Proc Natl Acad Sci U S A*. 1996;93:11019-11024.

140. Oliver G. Lymphatic vasculature development. *Nat Rev Immunol*. 2004; 4:35-45.

141. Anderson AO, Shaw S. Conduit for privileged communications in the lymph node. *Immunity*. 2005;22:3-5.

142. Sixt M, Kanazawa N, Selg M, et al. The conduit system transports soluble antigens from the afferent lymph to resident dendritic cells in the T cell area of the lymph node. *Immunity*. 2005;22:19-29.

143. Drayson MT, Ford WL. Afferent lymph and lymph borne cells: their influence on lymph node function. *Immunobiology*. 1984;168: 362-379.

144. Hendriks HR, Eestermans IL. Disappearance and reappearance of high endothelial venules and immigrating lymphocytes in lymph nodes deprived of afferent lymphatic vessels: a possible regulatory role of macrophages in lymphocyte migration. *Eur J Immunol*. 1983;13: 663-669.

145. Hendriks HR, Duijvestijn AM, Kraal G. Rapid decrease in lymphocyte adherence to high endothelial venules in lymph nodes deprived of afferent lymphatic vessels. *Eur J Immunol*. 1987;17:1691-1695.

146. Mebius RE, Bauer J, Twisk AJ, et al. The functional activity of high endothelial venules: a role for the subcapsular sinus macrophages in the lymph node. *Immunobiology*. 1991;182:277-291.

147. Mebius RE, Breve J, Kraal G, et al. Developmental regulation of vascular addressin expression: a possible role for site-associated environments. *Int Immunol*. 1993;5:443-449.

148. Swarte VV, Joziasse DH, Van den Eijnden DH, et al. Regulation of fucosyltransferase-VII expression in peripheral lymph node high endothelial venules. *Eur*

J Immunol. 1998;28:3040-3047.

149. Gretz JE, Anderson AO, Shaw S. Cords, channels, corridors and conduits: critical architectural elements facilitating cell interactions in the lymph node cortex. *Immunol Rev*. 1997;156:11-24.

150. Gretz JE, Kaldjian EP, Anderson AO, et al. Sophisticated strategies for information encounter in the lymph node: the reticular network as a conduit of soluble information and a highway for cell traffic. *J Immunol*. 1996;157:495-499.

151. Larsen CG, Anderson AO, Appella E, et al. The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. *Science*. 1989;243:1464-1466.

152. Francois M, Harvey NL, Hogan BM. The transcriptional control of lymphatic vascular development. *Physiology (Bethesda)*. 2011;26:146-155.

153. Wigle JT, Harvey N, Detmar M, et al. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J*. 2002;21:1505-1513.

154. Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. *Cell*. 1999;98:769-778.

155. Abtahian F, Guerriero A, Sebzda E, et al. Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk. *Science*. 2003;299:247-251.

156. Bertozzi CC, Schmaier AA, Mericko P, et al. Platelets regulate lymphatic vascular development through CLEC-2-SLP-76 signaling. *Blood*. 2010; 116:661-670.

157. Uhrin P, Zaujec J, Breuss JM, et al. Novel function for blood platelets and podoplanin in developmental separation of blood and lymphatic circulation. *Blood*. 2010;115:3997-4005.

158. Scavelli C, Weber E, Agliano M, et al. Lymphatics at the crossroads of angiogenesis and lymphangiogenesis. *J Anat*. 2004;204:433-449.

159. Vondenhoff MF, van de Pavert SA, Dillard ME, et al. Lymph sacs are not required for the initiation of lymph node formation. *Development*. 2009;136:29-34.

160. Benezech C, White A, Mader E, et al. Ontogeny of stromal organizer cells during lymph node development. *J Immunol*. 2010;184:4521-4530.

161. De Togni P, Goellner J, Ruddle NH, et al. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science*. 1994;264:703-707.

162. Harmsen A, Kusser K, Hartson L, et al. Cutting edge: organogenesis of nasal-

associated lymphoid tissue (NALT) occurs independently of lymphotoxin-alpha (LT alpha) and retinoic acid receptor-related orphan receptor-gamma, but the organization of NALT is LT alpha dependent. *J Immunol*. 2002;168:986-990.

163. Ying X, Chan K, Shenoy P, et al. Lymphotoxin plays a crucial role in the development and function of nasal-associated lymphoid tissue through regulation of chemokines and peripheral node addressin. *Am J Pathol*. 2005;166:135-146.

164. Alimzhanov MB, Kuprash DV, Kosco-Vilbois MH, et al. Abnormal development of secondary lymphoid tissues in lymphotoxin beta-deficient mice. *Proc Natl Acad Sci U S A*. 1997;94:9302-9307.

165. Andoh N, Ohtani H, Kusakari C, et al. Expression of E- and P-selectins by vascular endothelial cells in human tonsils. *Acta Otolaryngol Suppl*. 1996;523:52-54.

166. Soderberg KA, Linehan MM, Ruddle NH, et al. MAdCAM-1 expressing sacral lymph node in the lymphotoxin beta-deficient mouse provides a site for immune generation following vaginal herpes simplex virus-2 infection. *J Immunol*. 2004;173:1908-1913.

167. Futterer A, Mink K, Luz A, et al. The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity*. 1998;9:59-70.

168. Scheu S, Alferink J, Potzel T, et al. Targeted disruption of LIGHT causes defects in costimulatory T cell activation and reveals cooperation with lymphotoxin beta in mesenteric lymph node genesis. *J Exp Med*. 2002;195:1613-1624.

169. Rennert PD, James D, Mackay F, et al. Lymph node genesis is induced by signaling through the lymphotoxin beta receptor. *Immunity*. 1998; 9:71-79.

170. Adachi S, Yoshida H, Honda K, et al. Essential role of IL-7 receptor alpha in the formation of Peyer's patch anlage. *Int Immunol*. 1998;10:1-6.

171. Cao X, Shores EW, Hu-Li J, et al. Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. *Immunity*. 1995;2:223-238.

172. Dougall WC, Glaccum M, Charrier K, et al. RANK is essential for osteoclast and lymph node development. *Genes Dev*. 1999;13:2412-2424.

173. Kim D, Mebius RE, MacMicking JD, et al. Regulation of peripheral lymph node genesis by the tumor necrosis factor family member TRANCE. *J Exp Med*. 2000;192:1467-1478.

174. Ansel KM, Ngo VN, Hyman PL, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature*. 2000;406:309-314.

175. Forster R, Mattis AE, Kremmer E, et al. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell*. 1996;87:1037-1047.

176. Ruddle NH, Akirav EM. Secondary lymphoid organs: responding to genetic and environmental cues in ontogeny and the immune response. *J Immunol*. 2009;183:2205-2212.

177. Weih F, Caamano J. Regulation of secondary lymphoid organ development by the nuclear factor-kappaB signal transduction pathway. *Immunol Rev*. 2003;195:91-105.

178. Miyawaki S, Nakamura Y, Suzuka H, et al. A new mutation, aly, that induces a generalized lack of lymph nodes accompanied by immunodeficiency in mice. *Eur J Immunol*. 1994;24:429-434.

179. Shinkura R, Kitada K, Matsuda F, et al. Alymphoplasia is caused by a point mutation in the mouse gene encoding Nf-kappa b-inducing kinase. *Nat Genet*. 1999;22:74-77.

180. Senftleben U, Cao Y, Xiao G, et al. Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science*. 2001; 293:1495-1499.

181. Yilmaz ZB, Weih DS, Sivakumar V, et al. RelB is required for Peyer's patch development: differential regulation of p52-RelB by lymphotoxin and TNF. *EMBO J*. 2003;22:121-130.

182. Dejardin E, Droin NM, Delhase M, et al. The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways. *Immunity*. 2002;17:525-535.

183. Drayton DL, Bonizzi G, Ying X, et al. I kappa B kinase complex alpha kinase activity controls chemokine and high endothelial venule gene expression in lymph nodes and nasal-associated lymphoid tissue. *J Immunol*. 2004;173:6161-6168.

184. Eberl G, Littman DR. The role of the nuclear hormone receptor RORgamma T in the development of lymph nodes and Peyer's patches. *Immunol Rev*. 2003;195:81-90.

185. Eberl G, Marmon S, Sunshine MJ, et al. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. *Nat Immunol*. 2004;5:64-73.

186. Yokota Y, Mansouri A, Mori S, et al. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature*. 1999;397:702-706.

187. Fukuyama S, Kiyono H. Neuroregulator RET initiates Peyer's-patch tissue genesis.

Immunity. 2007;26:393-395.

188. Veiga-Fernandes H, Coles MC, Foster KE, et al. Tyrosine kinase receptor RET is a key regulator of Peyer's patch organogenesis. *Nature*. 2007;446:547-551.

189. van de Pavert SA, Olivier BJ, Goverse G, et al. Chemokine CXCL13 is essential for lymph node initiation and is induced by retinoic acid and neuronal stimulation. *Nat Immunol*. 2009;10:1193-1199.

190. Mebius RE, Rennert P, Weissman IL. Developing lymph nodes collect CD4+CD3- LTbeta+ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. *Immunity*. 1997;7:493-504.

191. Yoshida H, Honda K, Shinkura R, et al. IL-7 receptor alpha+ CD3(-) cells in the embryonic intestine induces the organizing center of Peyer's patches. *Int Immunol*. 1999;11:643-655.

192. Yoshida H, Kawamoto H, Santee SM, et al. Expression of alpha(4) beta(7) integrin defines a distinct pathway of lymphoid progenitors committed to T cells, fetal intestinal lymphotoxin producer, NK, and dendritic cells. *J Immunol*. 2001;167:2511-2521.

193. Girard JP, Springer TA. High endothelial venules (HEVs): specialized endothelium for lymphocyte migration. *Immunol Today*. 1995;16: 449-457.

194. Mebius RE, Schadee-Eestermans IL, Weissman IL. MAdCAM-1 dependent colonization of developing lymph nodes involves a unique subset of CD4+CD3- hematolymphoid cells. *Cell Adhes Commun*. 1998;6: 97-103.

195. Cuff CA, Schwartz J, Bergman CM, et al. Lymphotoxin alpha3 induces chemokines and adhesion molecules: insight into the role of LT alpha in inflammation and lymphoid organ development. *J Immunol*. 1998; 161:6853-6860.

196. Drayton DL, Ying X, Lee J, et al. Ectopic LT alpha beta directs lymphoid organ neogenesis with concomitant expression of peripheral node addressin and a HEV-restricted sulfotransferase. *J Exp Med*. 2003;197:1153-1163.

197. Eikelenboom P, Nassy JJ, Post J, et al. The histogenesis of lymph nodes in rat and rabbit. *Anat Rec*. 1978;190:201-215.

198. Browning JL, Allaire N, Ngam-Ek A, et al. Lymphotoxin-beta receptor signaling is required for the homeostatic control of HEV differentiation and function. *Immunity*. 2005;23:539-550.

199. Liao S, Ruddle NH. Synchrony of high endothelial venules and lymphatic vessels revealed by immunization. *J Immunol*. 2006;177:3369-3379.

200. Mionnet C, Sanos SL, Mondor I, et al. High endothelial venules as traffic control points maintaining lymphocyte population homeostasis in lymph nodes. *Blood*. 2011;118(23):6115-6122.

201. Hall JG, Hopkins J, Reynolds J. Studies of efferent lymph cells from nodes stimulated with oxazolone. *Immunology*. 1980;39:141-149.

202. Hall JG, Smith ME. Studies on the afferent and efferent lymph of lymph nodes draining the site of application of fluorodinitrobenzene (FDNB). *Immunology*. 1971;21:69-79.

203. Hay JB, Cahill RN, Trnka Z. The kinetics of antigen-reactive cells during lymphocyte recruitment. *Cell Immunol*. 1974;10:145-153.

204. He C, Young AJ, West CA, et al. Stimulation of regional lymphatic and blood flow by epicutaneous oxazolone. *J Appl Physiol*. 2002;93: 966-973.

205. West CA, He C, Su M, et al. Stochastic regulation of cell migration from the efferent lymph to oxazolone-stimulated skin. *J Immunol*. 2001;166:1517-1523.

206. Angeli V, Ginhoux F, Llodra J, et al. B cell-driven lymphangiogenesis in inflamed lymph nodes enhances dendritic cell mobilization. *Immunity*. 2006;24:203-215.

207. Hay JB, Johnston MG, Vadas P, et al. Relationships between changes in blood flow and lymphocyte migration induced by antigen. *Monogr Allergy*. 1980;16:112-125.

208. Ottaway CA, Parrott DM. Regional blood flow and its relationship to lymphocyte and lymphoblast traffic during a primary immune reaction. *J Exp Med*. 1979;150:218-230.

209. Mebius RE, Breve J, Duijvestijn AM, et al. The function of high endothelial venules in mouse lymph nodes stimulated by oxazolone. *Immunology*. 1990;71:423-427.

210. Myking AO. Morphological changes in paracortical high endothelial venules to single and repeated application of oxazolone to mouse skin. *Virchows Arch B Cell Pathol Incl Mol Pathol*. 1980;35:63-71.

211. Cahill RN, Frost H, Trnka Z. The effects of antigen on the migration of recirculating lymphocytes through single lymph nodes. *J Exp Med*. 1976;143:870-888.

212. Hoke D, Mebius RE, Dybdal N, et al. Selective modulation of the expression of L-selectin ligands by an immune response. *Curr Biol*. 1995;5:670-678.

213. Yoneyama H, Matsuno K, Zhang Y, et al. Evidence for recruitment of plasmacytoid dendritic cell precursors to inflamed lymph nodes through high endothelial venules. *Int*

Immunol. 2004;16:915-928.

214. Mackay F, Browning JL. Turning off follicular dendritic cells. *Nature*. 1998;395:26-27.

215. Brendolan A, Rosado MM, Carsetti R, et al. Development and function of the mammalian spleen. *Bioessays*. 2007;29:166-177.

216. Blue J, Weiss L. Electron microscopy of the red pulp of the dog spleen including vascular arrangements, periarterial macrophage sheaths (ellipsoids), and the contractile, innervated reticular meshwork. *Am J Anat*. 1981;161:189-218.

217. Blue J, Weiss L. Periarterial macrophage sheaths (ellipsoids) in cat spleen—an electron microscope study. *Am J Anat*. 1981;161:115-134.

218. Snook T. A comparative study of the vascular arrangements in mammalian spleens. *Am J Anat*. 1950;87:31-77.

219. Waksman BH. *Atlas of Experimental Immunobiology and Immunopathology* . New Haven, CT: Yale University Press; 1970.

220. Mebius RE, Kraal G. Structure and function of the spleen. *Nat Rev Immunol* . 2005;5:606-616.

221. Geijtenbeek TB, Groot PC, Nolte MA, et al. Marginal zone macrophages express a murine homologue of DC-SIGN that captures blood-borne antigens in vivo. *Blood*. 2002;100:2908-2916.

222. Koppel EA, Wieland CW, van den Berg VC, et al. Specific ICAM-3 grabbing nonintegrin-related 1 (SIGNR1) expressed by marginal zone macrophages is essential for defense against pulmonary Streptococcus pneumoniae infection. *Eur J Immunol* . 2005;35:2962-2969.

223. Kraal G, Mebius R. New insights into the cell biology of the marginal zone of the spleen. *Int Rev Cytol* . 2006;250:175-215.

224. Ito S, Naito M, Kobayashi Y, et al. Roles of a macrophage receptor with collagenous structure (MARCO) in host defense and heterogeneity of splenic marginal zone macrophages. *Arch Histol Cytol* . 1999;62:83-95.

225. Karlsson MC, Guinamard R, Bolland S, et al. Macrophages control the retention and trafficking of B lymphocytes in the splenic marginal zone. *J Exp Med*. 2003;198:333-340.

226. Kraal G, Schornagel K, Streeter PR, et al. Expression of the mucosal vascular addressin, MAdCAM-1, on sinus-lining cells in the spleen. *Am J Pathol* . 1995;147:763-771.

227. Crocker PR, Gordon S. Mouse macrophage hemagglutinin (sheep erythrocyte receptor) with specificity for sialylated glycoconjugates characterized by a monoclonal antibody. *J Exp Med*. 1989;169: 1333-1346.

228. Jiang HR, Hwenda L, Makinen K, et al. Sialoadhesin promotes the inflammatory response in experimental autoimmune uveoretinitis. *J Immunol*. 2006;177:2258-2264.

229. Oetke C, Vinson MC, Jones C, et al. Sialoadhesin-deficient mice exhibit subtle changes in B- and T-cell populations and reduced immunoglobulin M levels. *Mol Cell Biol*. 2006;26:1549-1557.

230. Backer R, Schwandt T, Greuter M, et al. Effective collaboration between marginal metallophilic macrophages and CD8+ dendritic cells in the generation of cytotoxic T cells. *Proc Natl Acad Sci U S A* . 2010;107: 216-221.

231. You Y, Myers RC, Freeberg L, et al. Marginal zone B cells regulate antigen capture by marginal zone macrophages. *J Immunol*. 2011;186: 2172-2181.

232. Cinamon G, Zachariah MA, Lam OM, et al. Follicular shuttling of marginal zone B cells facilitates antigen transport. *Nat Immunol*. 2008;9: 54-62.

233. Poholek AC, Craft J. Competing for help: new insights into the function of follicular helper T cells. *Immunol Cell Biol*. 2009;87:438-439.

234. Nolte MA, Belien JA, Schadee-Eestermans I, et al. A conduit system distributes chemokines and small blood-borne molecules through the splenic white pulp. *J Exp Med*. 2003;198:505-512.

235. Pabst O, Forster R, Lipp M, et al. NKX2.3 is required for MAdCAM-1 expression and homing of lymphocytes in spleen and mucosa-associated lymphoid tissue. *EMBO J*. 2000;19:2015-2023.

236. Cyster JG, Goodnow CC. Pertussis toxin inhibits migration of B and T lymphocytes into splenic white pulp cords. *J Exp Med*. 1995;182: 581-586.

237. Nolte MA, Hamann A, Kraal G, et al. The strict regulation of lymphocyte migration to splenic white pulp does not involve common homing receptors. *Immunology*. 2002;106:299-307.

238. Lu TT, Cyster JG. Integrin-mediated long-term B cell retention in the splenic marginal zone. *Science*. 2002;297:409-412.

239. Mitchell J. Lymphocyte circulation in the spleen. Marginal zone bridging channels

and their possible role in cell traffic. *Immunology*. 1973; 24:93-107.

240. Cinamon G, Matloubian M, Lesneski MJ, et al. Sphingosine 1-phosphate receptor 1 promotes B cell localization in the splenic marginal zone. *Nat Immunol*. 2004;5:713-720.

241. Czompoly T, Labadi A, Kellermayer Z, et al. Transcription factor Nkx2-3 controls the vascular identity and lymphocyte homing in the spleen. *J Immunol*. 2011;186:6981-6989.

242. Kellermayer Z, Labadi A, Czompoly T, et al. Absence of Nkx2-3 homeodomain transcription factor induces the formation of LYVE-1-positive endothelial cysts without lymphatic commitment in the spleen. *J Histochem Cytochem*. 2011;59:690-700.

243. Hatano M, Roberts CW, Minden M, et al. Deregulation of a homeobox gene, HOX11, by the t(10;14) in T cell leukemia. *Science*. 1991;253:79-82.

244. Roberts CW, Shutter JR, Korsmeyer SJ. Hox11 controls the genesis of the spleen. *Nature*. 1994;368:747-749.

245. Dear TN, Colledge WH, Carlton MB, et al. The Hox11 gene is essential for cell survival during spleen development. *Development*. 1995;121: 2909-2915.

246. Reijmers RM, Vondenhoff MF, Roozendaal R, et al. Impaired lymphoid organ development in mice lacking the heparan sulfate modifying enzyme glucuronyl C5-epimerase. *J Immunol*. 2010;184:3656-3664.

247. Ngo VN, Korner H, Gunn MD, et al. Lymphotoxin alpha/beta and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. *J Exp Med*. 1999;189:403-412.

248. Vondenhoff MF, Desanti GE, Cupedo T, et al. Separation of splenic red and white pulp occurs before birth in a LTalphabeta-independent manner. *J Leukocyte Biol*. 2008;84:152-161.

249. Ngo VN, Cornall RJ, Cyster JG. Splenic T zone development is B cell dependent. *J Exp Med*. 2001;194:1649-1660.

250. Kim MY, McConnell FM, Gaspal FM, et al. Function of CD4+CD3-cells in relation to B and T zone stroma in spleen. *Blood*. 2006;109(4): 1602-1610.

251. Benedict CA, De Trez C, Schneider K, et al. Specific remodeling of splenic architecture by cytomegalovirus. *PLoS Pathog*. 2006;2:e16.

252. Croitoru K, Bienenstock J. Characteristics and functions of mucosa-associated lymphoid tissue. In: Ogra PL, Mestecky J, Lamm ME, Strober W, McGheee JR, Bienenstock J, eds. *Handbook of Mucosal Immunology* . San Diego, CA: Academic

Press; 1994:141-151.

253. Iwata M, Yokota A. Retinoic acid production by intestinal dendritic cells. *Vitam Horm*. 2011;86:127-152.

254. Weiner HL, da Cunha AP, Quintana F, et al. Oral tolerance. *Immunol Rev*. 2011;241:241-259.

255. Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol*. 2003;3:331-341.

256. Dieu MC, Vanbervliet B, Vicari A, et al. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med*. 1998;188:373-386.

257. Meurens F, Whale J, Brownlie R, et al. Expression of mucosal chemokines TECK/CCL25 and MEC/CCL28 during fetal development of the ovine mucosal immune system. *Immunology*. 2007;120(4):544-555.

258. Puri KD, Finger EB, Gaudernack G, et al. Sialomucin CD34 is the major L-selectin ligand in human tonsil high endothelial venules. *J Cell Biol* . 1995;131:261-270.

259. Berg EL, Mullowney AT, Andrew DP, et al. Complexity and differential expression of carbohydrate epitopes associated with L-selectin recognition of high endothelial venules. *Am J Pathol* . 1998;152:469-477.

260. van Kempen MJ, Rijkers GT, Van Cauwenberge PB. The immune response in adenoids and tonsils. *Int Arch Allergy Immunol* . 2000;122: 8-19.

261. Karchev T, Kabakchiev P. Electron-microscope observations on the tonsillar epithelium in children with recurrent tonsillitis. *Int J Pediatric Otorhinolaryngol* . 1982;4:149-156.

262. Winther B, Innes DJ. The human adenoid. A morphologic study. *Arch Otolaryngol Head Neck Surg*. 1994;120:144-149.

263. Gebert A, Pabst R. M cells at locations outside the gut. *Sem Immunol*. 1999;11:165-170.

264. Pabst R, Gehrke I. Is the bronchus-associated lymphoid tissue (BALT) an integral structure of the lung in normal mammals, including humans? *Am J Resp Cell Mol Biol* . 1990;3:131-135.

265. Schulz O, Jaensson E, Persson EK, et al. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *J Exp*

Med. 2009;206:3101-3114.

266. Brandtzaeg P. Potential of nasopharynx-associated lymphoid tissue for vaccine responses in the airways. *Am J Respir Crit Care Med* . 2011;183: 1595-1604.

267. Dupuy C, Buzoni-Gatel D, Touze A, et al. Nasal immunization of mice with human papillomavirus type 16 (HPV-16) virus-like particles or with the HPV-16 L1 gene elicits specific cytotoxic T lymphocytes in vaginal draining lymph nodes. *J Virol* . 1999;73:9063-9071.

268. Wiley JA, Tighe MP, Harmsen AG. Upper respiratory tract resistance to influenza infection is not prevented by the absence of either nasal-associated lymphoid tissue or cervical lymph nodes. *J Immunol* . 2005;175:3186-3196.

269. Moyron-Quiroz JE, Rangel-Moreno J, Hartson L, et al. Persistence and responsiveness of immunologic memory in the absence of secondary lymphoid organs. *Immunity*. 2006;25:643-654.

270. Constant SL, Brogdon JL, Piggott DA, et al. Resident lung antigen-presenting cells have the capacity to promote Th2 T cell differentiation in situ. *J Clin Invest*. 2002;110:1441-1448.

271. Perry M, Whyte A. Immunology of the tonsils. *Immunol Today*. 1998; 19:414-421.

272. Brandtzaeg P. Regionalized immune function of tonsils and adenoids. *Immunol Today*. 1999;20:383-384.

273. Rebelatto MC, Mead C, HogenEsch H. Lymphocyte populations and adhesion molecule expression in bovine tonsils. *Vet Immunol Immunopathol* . 2000;73:15-29.

274. Carlsen HS, Haraldsen G, Brandtzaeg P, et al. Disparate lymphoid chemokine expression in mice and men: no evidence of CCL21 synthesis by human high endothelial venules. *Blood*. 2005;106:444-446.

275. Goeringer GC, Vidic B. The embryogenesis and anatomy of Waldeyer's ring. *Otolaryngol Clin North Am*. 1987;20:207-217.

276. Wu HY, Russell MW. Induction of mucosal immunity by intranasal application of a streptococcal surface protein antigen with the cholera toxin B subunit. *Infect Immun*. 1993;61:314-322.

277. Balmelli C, Roden R, Potts A, et al. Nasal immunization of mice with human papillomavirus type 16 virus-like particles elicits neutralizing antibodies in mucosal secretions. *J Virol* . 1998;72:8220-8229.

278. Liu XS, Abdul-Jabbar I, Qi YM, et al. Mucosal immunisation with papillomavirus virus-like particles elicits systemic and mucosal immunity in mice. *Virology*. 1998;252:39-45.

279. Zuercher AW, Coffin SE, Thurnheer MC, et al. Nasal-associated lymphoid tissue is a mucosal inductive site for virus-specific humoral and cellular immune responses. *J Immunol*. 2002;168:1796-1803.

280. Shikina T, Hiroi T, Iwatani K, et al. IgA class switch occurs in the organized nasopharynx- and gut-associated lymphoid tissue, but not in the diffuse lamina propria of airways and gut. *J Immunol*. 2004;172:6259-6264.

281. Csencsits KL, Jutila MA, Pascual DW. Mucosal addressin expression and binding-interactions with naive lymphocytes vary among the cranial, oral, and nasal-associated lymphoid tissues. *Eur J Immunol* . 2002;32:3029-3039.

282. Fukuyama S, Nagatake T, Kim DY, et al. Cutting edge: uniqueness of lymphoid chemokine requirement for the initiation and maturation of nasopharynx-associated lymphoid tissue organogenesis. *J Immunol*. 2006;177:4276-4280.

283. Rangel-Moreno J, Hartson L, Navarro C, et al. Inducible bronchusassociated lymphoid tissue (iBALT) in patients with pulmonary complications of rheumatoid arthritis. *J Clin Invest*. 2006;116:3183-3194.

284. Fukuyama S, Hiroi T, Yokota Y, et al. Initiation of NALT organogenesis is independent of the IL-7R, LTbetaR, and NIK signaling pathways but requires the Id2 gene and CD3(-)CD4(+)CD45(+) cells. *Immunity*. 2002;17:31-40.

285. Delventhal S, Hensel A, Petzoldt K, et al. Effects of microbial stimulation on the number, size and activity of bronchus-associated lymphoid tissue (BALT) structures in the pig. *Int J Exp Pathol* . 1992;73:351-357.

286. Kolopp-Sarda MN, Bene MC, Massin N, et al. Immunohistological analysis of macrophages, B-cells, and T-cells in the mouse lung. *Anat Rec*. 1994;239:150-157.

287. Moyron-Quiroz JE, Rangel-Moreno J, Kusser K, et al. Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. *Nat Med*. 2004;10:927-934.

288. Pabst R, Tschernig T. Bronchus-associated lymphoid tissue: an entry site for antigens for successful mucosal vaccinations? *Am J Respir Cell Mol Biol* . 2010;43:137-141.

289. Ersch J, Tschernig T, Stallmach T. Frequency and potential cause of bronchus-associated lymphoid tissue in fetal lungs. *Pediatr Allergy Immunol* . 2005;16:295-298.

290. Tschernig T, Pabst R. Bronchus-associated lymphoid tissue (BALT) is not present in

the normal adult lung but in different diseases. *Pathobiol*. 2000;68:1-8.

291. Csencsits KL, Jutila MA, Pascual DW. Nasal-associated lymphoid tissue: phenotypic and functional evidence for the primary role of peripheral node addressin in naive lymphocyte adhesion to high endothelial venules in a mucosal site. *J Immunol*. 1999;163:1382-1389.

292. Cesta MF. Normal structure, function, and histology of mucosa-associated lymphoid tissue. *Toxicol Pathol*. 2006;34:599-608.

293. Fotopoulos G, Harari A, Michetti P, et al. Transepithelial transport of HIV-1 by M cells is receptor-mediated. *Proc Natl Acad Sci U S A* . 2002; 99:9410-9414.

294. Jones BD, Ghori N, Falkow S. Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *J Exp Med*. 1994;180:15-23.

295. Hase K, Murakami T, Takatsu H, et al. The membrane-bound chemokine CXCL16 expressed on follicle-associated epithelium and M cells mediates lympho-epithelial interaction in GALT. *J Immunol*. 2006;176: 43-51.

296. Iwasaki A, Kelsall BL. Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3alpha, MIP-3beta, and secondary lymphoid organ chemokine. *J Exp Med*. 2000;191:1381-1394.

297. Iwasaki A, Kelsall BL. Unique functions of CD11b+, CD8 alpha+, and double-negative Peyer's patch dendritic cells. *J Immunol*. 2001;166: 4884-4890.

298. Williams MB, Butcher EC. Homing of naive and memory T lymphocyte subsets to Peyer's patches, lymph nodes, and spleen. *J Immunol*. 1997;159:1746-1752.

299. Kunkel EJ, Ramos CL, Steeber DA, et al. The roles of L-selectin, beta 7 integrins, and P-selectin in leukocyte rolling and adhesion in high endothelial venules of Peyer's patches. *J Immunol*. 1998;161:2449-2456.

300. Steeber DA, Tang ML, Zhang XQ, et al. Efficient lymphocyte migration across high endothelial venules of mouse Peyer's patches requires overlapping expression of L-selectin and beta7 integrin. *J Immunol*. 1998;161:6638-6647.

301. Okada T, Ngo VN, Ekland EH, et al. Chemokine requirements for B cell entry to lymph nodes and Peyer's patches. *J Exp Med*. 2002;196:65-75.

302. Cook DN, Prosser DM, Forster R, et al. CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. *Immunity*. 2000;12:495-503.

303. Koni PA, Sacca R, Lawton P, et al. Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta in lymphotoxi-beta deficient mice. *Immunity*. 1997;6:491-500.

304. Kiyono H, Fukuyama S. NALT- versus Peyer's-patch-mediated mucosal immunity. *Nat Rev Immunol* . 2004;4:699-710.

305. Neumann B, Luz A, Pfeffer K, et al. Defective Peyer's patch organogenesis in mice lacking the 55-kD receptor for tumor necrosis factor. *J Exp Med*. 1996;184:259-264.

306. Nishikawa S, Nishikawa S, Honda K, et al. Peyer's patch organogenesis as a programmed inflammation: a hypothetical model. *Cytokine Growth Factor Rev* . 1998;9:213-220.

307. Nishikawa SI, Hashi H, Honda K, et al. Inflammation, a prototype for organogenesis of the lymphopoietic/hematopoietic system. *Curr Opin Immunol* . 2000;12:342-345.

308. Nishikawa S, Honda K, Vieira P, et al. Organogenesis of peripheral lymphoid organs. *Immunol Rev*. 2003;195:72-80.

309. Finke D, Acha-Orbea H, Mattis A, et al. CD4+CD3- cells induce Peyer's patch development: role of alpha4beta1 integrin activation by CXCR5. *Immunity*. 2002;17:363-373.

310. Finke D, Kraehenbuhl JP. Formation of Peyer's patches. *Curr Opin Genet Dev* . 2001;11:561-567.

311. Golovkina TV, Shlomchik M, Hannum L, et al. Organogenic role of B lymphocytes in mucosal immunity. *Science*. 1999;286:1965-1968.

312. Debard N, Sierro F, Browning J, et al. Effect of mature lymphocytes and lymphotoxin on the development of the follicle-associated epithelium and M cells in mouse Peyer's patches. *Gastroenterology*. 2001;120: 1173-1182.

313. Lugering A, Kucharzik T, Soler D, et al. Lymphoid precursors in intestinal cryptopatches express CCR6 and undergo dysregulated development in the absence of CCR6. *J Immunol* . 2003;171:2208-2215.

314. Lorenz RG, Newberry RD. Isolated lymphoid follicles can function as sites for induction of mucosal immune responses. *Ann N Y Acad Sci* . 2004;1029:44-57.

315. Eberl G. Inducible lymphoid tissues in the adult gut: recapitulation of a fetal developmental pathway? *Nat Rev Immunol* . 2005;5:413-420.

316. Newberry RD, Lorenz RG. Organizing a mucosal defense. *Immunol Rev*. 2005;206:6-21.

317. Lorenz RG, Chaplin DD, McDonald KG, et al. Isolated lymphoid follicle formation is inducible and dependent upon lymphotoxin-sufficient B lymphocytes, lymphotoxin beta receptor, and TNF receptor I function. *J Immunol*. 2003;170:5475-5482.

318. Haley PJ. Species differences in the structure and function of the immune system. *Toxicology*. 2003;188:49-71.

319. Pablos JL, Santiago B, Tsay D, et al. A HEV-restricted sulfotransferase is expressed in rheumatoid arthritis synovium and is induced by lymphotoxin-alpha/beta and TNF-alpha in cultured endothelial cells. *BMC Immunol*. 2005;6:6.

320. Link A, Hardie DL, Favre S, et al. Association of T-zone reticular networks and conduits with ectopic lymphoid tissues in mice and humans. *Am J Pathol*. 2011;178:1662-1675.

321. Kerjaschki D. Lymphatic neoangiogenesis in human neoplasia and transplantation as experiments of nature. *Kidney Int*. 2005;68: 1967-1968.

322. Kerjaschki D, Regele HM, Moosberger I, et al. Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. *J Am Soc Nephrol*. 2004;15:603-612.

323. Penaranda C, Tang Q, Ruddle NH, et al. Prevention of diabetes by FTY720-mediated stabilization of peri-islet tertiary lymphoid organs. *Diabetes*. 2010;59:1461-1468.

324. Reape TJ, Rayner K, Manning CD, et al. Expression and cellular localization of the CC chemokines PARC and ELC in human atherosclerotic plaques. *Am J Pathol*. 1999;154:365-374.

325. Dieu-Nosjean MC, Antoine M, Danel C, et al. Long-term survival for patients with non-small-cell lung cancer with intratumoral lymphoid structures. *J Clin Oncol*. 2008;26:4410-4417.

326. Martinet L, Garrido I, Filleron T, et al. Human solid tumors contain high endothelial venules: association with T- and B-lymphocyte infiltration and favorable prognosis in breast cancer. *Cancer Res*. 2011; 71:5678-5687.

327. Hjelmstrom P. Lymphoid neogenesis: de novo formation of lymphoid tissue in chronic inflammation through expression of homing chemokines. *J Leukoc Biol*. 2001;69:331-339.

328. Hjelmstrom P, Fjell J, Nakagawa T, et al. Lymphoid tissue homing chemokines are expressed in chronic inflammation. *Am J Pathol*. 2000; 156:1133-1138.

329. Hanninen A, Jaakkola I, Jalkanen S. Mucosal addressin is required for the development of diabetes in nonobese diabetic mice. *J Immunol*. 1998;160:6018-6025.

330. Yang XD, Sytwu HK, McDevitt HO, et al. Involvement of beta 7 integrin and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) in the development of diabetes in obese diabetic mice. *Diabetes*. 1997;46: 1542-1547.

331. Cannella B, Cross AH, Raine CS. Upregulation and coexpression of adhesion molecules correlate with relapsing autoimmune demyelination in the central nervous system. *J Exp Med*. 1990;172:1521-1524.

332. Columba-Cabezas S, Serafini B, Ambrosini E, et al. Lymphoid chemokines CCL19 and CCL21 are expressed in the central nervous system during experimental autoimmune encephalomyelitis: implications for the maintenance of chronic neuroinflammation. *Brain Pathol*. 2003; 13:38-51.

333. Magliozzi R, Columba-Cabezas S, Serafini B, et al. Intracerebral expression of CXCL13 and BAFF is accompanied by formation of lymphoid follicle-like structures in the meninges of mice with relapsing experimental autoimmune encephalomyelitis. *J Neuroimmunol*. 2004;148:11-23.

334. Mooij P, de Wit HJ, Drexhage HA. An excess of dietary iodine accelerates the development of a thyroid-associated lymphoid tissue in autoimmune prone BB rats. *Clin Immunol Immunopathol*. 1993;69: 189-198.

335. Katakai T, Hara T, Sugai M, et al. Th1-biased tertiary lymphoid tissue supported by CXC chemokine ligand 13-producing stromal network in chronic lesions of autoimmune gastritis. *J Immunol*. 2003;171: 4359-4368.

336. Galkina E, Kadl A, Sanders J, et al. Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. *J Exp Med*. 2006;203:1273-1282.

337. Damas JK, Smith C, Oie E, et al. Enhanced expression of the homeostatic chemokines CCL19 and CCL21 in clinical and experimental atherosclerosis. Possible pathogenic role in plaque destabilization. *Arterioscler Thromb Vasc Biol* . 2006;27(3):614-620.

338. Grabner R, Lotzer K, Dopping S, et al. Lymphotoxin beta receptor signaling promotes tertiary lymphoid organogenesis in the aorta adventitia of aged ApoE-/- mice. *J Exp Med*. 2009;206:233-248.

339. Baddoura FK, Nasr IW, Wrobel B, et al. Lymphoid neogenesis in murine cardiac allografts undergoing chronic rejection. *Am J Transplant*. 2005; 5:510-516.

340. Motallebzadeh R, Rehakova S, Conlon TM, et al. Blocking lymphotoxin signaling abrogates the development of ectopic lymphoid tissue within cardiac allografts and inhibits effector antibody responses. *FASEB J*. 2011;26(1):51-62.

341. Luther SA, Ansel KM, Cyster JG. Overlapping roles of CXCL13, interleukin 7 receptor alpha, and CCR7 ligands in lymph node development. *J Exp Med*. 2003;197:1191-1198.

342. Heikenwalder M, Zeller N, Seeger H, et al. Chronic lymphocytic inflammation specifies the organ tropism of prions. *Science*. 2005;307: 1107-1110.

343. Gause A, Gundlach K, Zdichavsky M, et al. The B lymphocyte in rheumatoid arthritis: analysis of rearranged V kappa genes from B cells infiltrating the synovial membrane. *Eur J Immunol* . 1995;25: 2775-2782.

344. Schroder AE, Greiner A, Seyfert C, et al. Differentiation of B cells in the nonlymphoid tissue of the synovial membrane of patients with rheumatoid arthritis. *Proc Natl Acad Sci U S A*. 1996;93:221-225.

345. Dorner T, Hansen A, Jacobi A, et al. Immunglobulin repertoire analysis provides new insights into the immunopathogenesis of Sjogren's syndrome. *Autoimmun Rev*. 2002;1:119-124.

346. Sims GP, Shiono H, Willcox N, et al. Somatic hypermutation and selection of B cells in thymic germinal centers responding to acetylcholine receptor in myasthenia gravis. *J Immunol*. 2001;167:1935-1944.

347. Stott DI, Hiepe F, Hummel M, et al. Antigen-driven clonal proliferation of B cells within the target tissue of an autoimmune disease. The salivary glands of patients with Sjogren's syndrome. *J Clin Invest*. 1998;102:938-946.

348. Kim HJ, Krenn V, Steinhauser G, et al. Plasma cell development in synovial germinal centers in patients with rheumatoid and reactive arthritis. *J Immunol*. 1999;162:3053-3062.

349. Nasr IW, Reel M, Oberbarnscheidt MH, et al. Tertiary lymphoid tissues generate effector and memory T cells that lead to allograft rejection. *Am J Transplant*. 2007;7:1071-1079.

350. McMahon EJ, Bailey SL, Castenada CV, et al. Epitope spreading initiates in the CNS in two mouse models of multiple sclerosis. *Nat Med*. 2005; 11:335-339.

351. Schrama D, thor Straten P, Fischer WH, et al. Targeting of lymphotoxin-alpha to the tumor elicits an efficient immune response associated with induction of peripheral lymphoid-like tissue. *Immunity*. 2001;14: 111-121.

352. Lee Y, Chin RK, Christiansen P, et al. Recruitment and activation of naive T cells in the islets by lymphotoxin beta receptor-dependent tertiary lymphoid structure. *Immunity*. 2006;25:499-509.

353. Kaufman DL, Clare-Salzler M, Tian J, et al. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature*. 1993;366:69-72.

354. Wotherspoon AC, Doglioni C, Diss TC, et al. Regression of primary lowgrade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of Helicobacter pylori. *Lancet*. 1993;342:575-577.

355. Wu Q, Salomon B, Chen M, et al. Reversal of spontaneous autoimmune insulitis in nonobese diabetic mice by soluble lymphotoxin receptor. *J Exp Med*. 2001;193:1327-1332.

356. Fava RA, Notidis E, Hunt J, et al. A role for the lymphotoxin/LIGHT axis in the pathogenesis of murine collagen-induced arthritis. *J Immunol*. 2003;171:115-126.

357. Martin F, Kearney JF. B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a “natural immune memory.” *Immunol Rev*. 2000;175:70-79.

358. Chaplin DD. Lymphoid tissues and organs. In: Paul WE, ed. *Fundamental Immunology*. 5th ed. Philadelphia, PA: Lippincott Williams and Wilkins; 2003:419-453.

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Chapter 4

Evolution of the Immune System

Martin F. Flajnik

Louis Du Pasquier

INTRODUCTION

Defense mechanisms are found in all living things, even bacteria, where they are surprisingly elaborate. Although new adaptive or adaptive-like (somatically generated) immune systems have been discovered in invertebrates and the jaw-less fish, adaptive immunity based upon immunoglobulin (Ig), T-cell receptors (TCRs), and the major histocompatibility complex (MHC) is only present in jawed vertebrates (gnathostomes); because of clonal selection of lymphocytes, positive and negative selection in the thymus, MHC-regulated initiation of all adaptive responses, etc., the major elements of the adaptive immune system in gnathostomes are locked in a coevolving unit that arose in concert over a short period of evolutionary time.[1,2,3] In addition, a large cast of supporting players, including a large array of cytokines and chemokines, adhesion molecules, costimulatory molecules, and well-defined primary and secondary lymphoid tissues, evolved in the jawed vertebrates as well. This scheme was superimposed onto an innate system inherited from invertebrates, from which many innate molecules and mechanisms were coopted for the initial phase of the adaptive response and others for effector mechanisms at the completion of adaptive responses. Over the last 10 years, we have learned that various components of the innate immune system are also incredibly complex and locked as well in a coevolving unit.[4,5]

In each group of organisms, one can detect a basic set of immune functions, and these are employed in different ways in representative species. For example, we detect that in the jawed vertebrates fine tuning, or adaptations, or even degeneration of molecules/mechanisms in each group (Taxon) are observed, and not a steady progression from fish to mammals as is documented for most other physiologic systems.[6] Given that all the canonical adaptive immune system features are present in cartilaginous fish and apparently none were lost (except in particular groups of organisms that will be discussed), differential utilization of defense molecules rather than sequential installation of new elements is observed. In this chapter, there are only isolated cases of increasing complexity in immune systems, but many examples of contractions/expansions of existing gene families; thus, “more or less of the same” rather than “more and more new features” is the rule. We observe a bush growing from a short stem rather than a tall tree with well-defined branches, and thus deducing the primitive, ancestral traits is not clear cut.

GENERAL PRINCIPLES OF IMMUNE SYSTEM EVOLUTION

The Common Ancestor Hypothesis

Figure 4.1 displays the extant animal phyla ranging from the single-celled protozoa to the metazoan protostome and deuterostome lineages. It is often suggested or assumed by those unfamiliar with thinking in evolutionary terms that molecules or mechanisms found in living protostomes, like the well-studied arthropod *Drosophila*, are ancestral to similar molecules/mechanisms in mouse and human. While this is true in some cases, one must realize that *Drosophila* and humans have taken just as long (over 900 million years) to evolve from a common ancestral triploblastic coelomate (an animal with three germ layers and a mesoderm-lined body cavity, features shared by protostomes and deuterostomes) that looked nothing like a fly or a human, and thus *Drosophila* is not our ancestor (ie, the manner by which flies and humans utilize certain families of defense molecules may be quite different and both may be disparate from the common ancestor). Thus, understanding of how model invertebrates and vertebrates perform certain immune tasks is an important first step in our understanding of a particular mechanism, but we only deduce what is primordial or derived when we have examined similar immune mechanisms/molecules in species from a wide range of phyla. We will touch upon each of the defense molecule families and will emphasize those which have been conserved evolutionarily and those that have evolved rapidly.

Rapid Evolution of Defense Mechanisms

Immune systems are often compared to the Red Queen in *Alice in Wonderland*, (Red Queen's Hypothesis[7,8]) who must continually keep moving just to avoid falling behind. Because of the perpetual conflict with pathogens, the immune system is in constant flux. This is exemplified by great differences in the immune systems of animals that are even within the same phylogenetic group (eg, mosquito [*Anopheles*] and fruitfly [*Drosophila*], both arthropods, or human and mouse, both mammals). In fact, in contrast to what was believed previously, defense mechanisms are extremely diverse throughout the invertebrate phyla, and Kepler et al. have aptly and succinctly described the situation in the title of a recent review: “not homogeneous, not simple, not well understood.”[9] In the jawed vertebrate (or *gnathostomes*), the most rapidly evolving system is an innate system, natural killer (NK) cell recognition, governed by different classes (superfamilies) of receptors in mice and humans, and also extremely plastic even

within the same species, exemplified by the large number of killer immunoglobulin superfamily (KIR) haplotypes found in humans.[10] Studies of Ig superfamily (SF) genes expressed in the nervous system and immune system showed definitively that the immune system molecules evolve at a faster rate.[11] Finally, rapid evolution of immune system molecules and mechanisms is the general rule, but molecules functioning at different levels of immune defense (recognition, signaling, or effector) can evolve at widely varying rates.

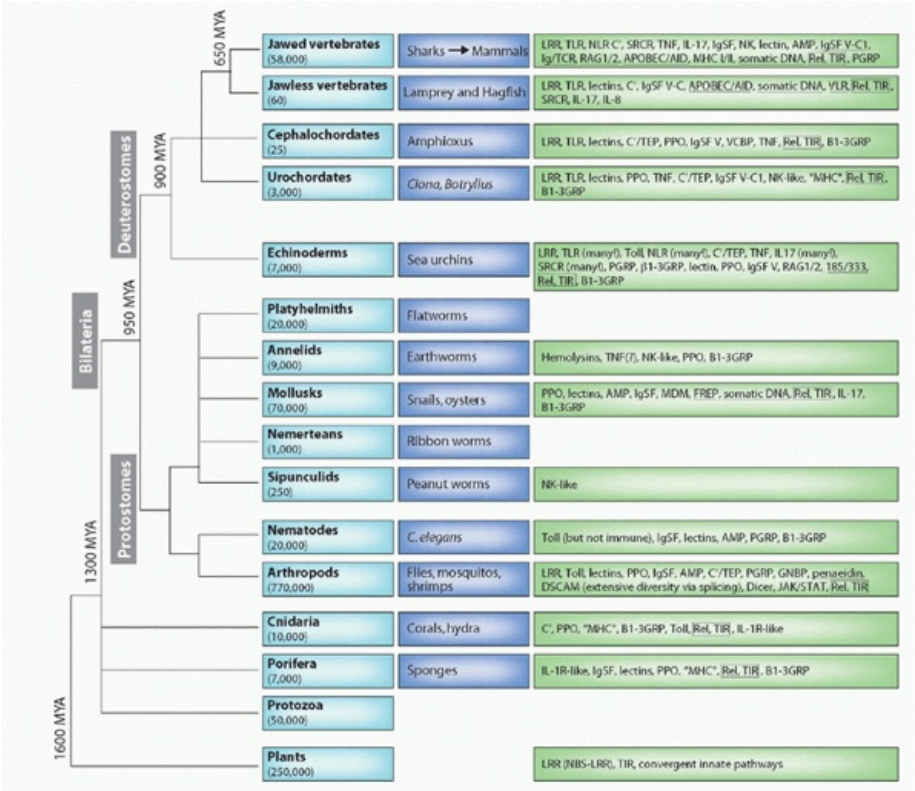

FIG. 4.1. Major Animal Groups and Immune Mechanisms/Molecules Described to Date in Each Group. The first box on the left in each row describes the animal taxon and the approximate number of species in that group. The next box shows specific examples of species or subgroups. The third box lists molecules/mechanisms found in each group: *underlined terms* indicate somatic changes to antigen receptors or secreted molecules. Figure modified from Hibino et al.[48] and Flajnik and Du Pasquier.[61] See Table 4.1 for definition of the acronyms.

Conservation of Defense Mechanisms

While the immune system is the most rapidly evolving physiologic system, nevertheless there is also deep conservation of defense families and mechanisms. Klein[11a] has compared this dichotomy to the two-headed god Janus, the major idea being that certain basic mechanisms/functions are obligatory for immune systems to function, but they still must evolve rapidly to avoid pathogen subterfuge. For example, MHC class I molecules have similar structure/function/features in all gnathostomes, but even within groups of primates class I genes are not orthologous (ie, they can be derived from totally different ancestral class I genes[10]). So, the idea is to preserve vital immune functions but rapidly modify the gene or pathway to outwit the pathogen. Additionally, certain features are conserved (eg, development and function of conventional αβ T cells), but a second, similar system, can be exploited in very different ways in closely related

species (eg, the function[s] of γδ T cells). The “Janus paradigm,” therefore, can be quite useful when examining any pathway in the immune system.

Convergent Evolution

Early on in the comparative study of immunity, it was assumed that the same features appearing in different taxa proved that they were present in the common ancestor as well (ie, they were submitted to divergent evolution). While this dictum still holds true and establishes one of the dogmas of comparative immunology, later we discovered, because of the aforementioned rapid evolution of immune systems, *convergence* of similar functions has occurred in evolution (ie, the same function or even molecular conformation has arisen independently in different organisms, sometimes in species that are relatively closely related). While we will discuss several cases of convergent evolution throughout the chapter, for frame of reference, the NK cells, which use different receptor families in primates and rodents to achieve precisely the same ends of recognizing polymorphic MHC class I molecules, is a striking example of convergence.[12] Additionally, the emergence of a lymphocyte-based somatic generation of two entirely different receptor families for the same function in jawless and jawed vertebrates is another remarkable illustration of convergent evolution.[13] Finally, in innate immunity, the cytosolic nucleotide-binding domain leucine-rich repeat (NLR) proteins, despite their striking similarity in deuterostomes and plants, arose (at least) twice in evolution.[14]

Multigene Families

Genes involved in immunity are often found in clusters, with extensive contraction and expansion via so-called birth and death processes.[15] It is well known that such gene clusters can change rapidly over evolutionary time due to unequal recombination crossovers and gene conversion (and not only in the immune system, but in any *cis* -duplicating gene family). Often, families of related immune genes— especially those involved in recognition events —are found near the telomeres of chromosomes, presumably because this further promotes gene-shuffling events. Nonclassical MHC class I loci, NK receptors, and NLRs are conspicuous examples of this phenomenon, again believed to be a consequence of the race against pathogens. We will discuss many examples of how such multigene families have been exploited in different species throughout the chapter.

Gene duplication, either in the clusters mentioned above or as a consequence of en bloc duplications, certainly has been a major feature of immune system diversity and plasticity. The two types of duplications are not equivalent, the former being more taxon-specific and the latter (en bloc) having a lasting impact on the entire system. It is now universally accepted that two = =genome-wide duplications (the so-called 2R hypothesis[2,16]; see Fig. 4.13) occurred early in vertebrate history, tracking very well with the emergence of the Ig/TCR/MHC-based adaptive immune system.[2,17] This theory forms the basis for much that will be discussed concerning the evolution of the vertebrate adaptive immune response as one can track the emergence of new immune mechanisms, as well as fine tuning of old ones, by examining the paralogous syntenic groups of genes. Our view is that these genomewide duplications were as crucial as the “RAG transposon”[18,19,20] in the development of the Ig/TCR/MHC-based adaptive immunity. In addition, the common ancestor of bony fish (teleosts) underwent a third round of genomewide duplication, which many believe to have played the major role in these fishes' unique outlier status regarding immune system genetics and physiology.[2,21]

Polymorphism

In addition to gene duplications, polymorphism also augments the diversity of immune recognition within a population. It can be generated any time during the history of a gene family of either receptor or effector molecules: MHC, toll-like receptors (TLRs), Ig, TCRs, NK receptors (NKRs) and related molecules, and antimicrobial peptides (AMPs) are just a few examples. Polymorphism, either within the gene itself or in its regulatory elements, provides populations with flexibility in function of the changing pathogenic environment. This subject, central to the studies on MHC, leukocyte receptor complex (LRC), and NK cell complex (NKC), is becoming well documented for immunity-related genes in insects as well. In *Drosophila*, polymorphism in regulatory networks is indeed expected as parasites often target their elements.[22] In humans, there are two major NK cell haplotypes found in all subpopulations, which are under "balancing selection." In such a case, the polymorphisms presumably adopt a division of labor required for the maintenance of the species: one haplotype is believed to be involved in protection from virus and the other perhaps for promoting reproduction.[23]

Somatic Generation of Diversity

Somatic modifications can take place at multiple levels to generate immune system diversity. Long believed to be the sole domain of jawed vertebrates, modifications at the deoxyribonucleic acid (DNA) level via somatic hypermutation, gene conversion, and rearrangement (primary and secondary [eg, receptor editing]) irreversibly modify genes within an individual. The well known V(D)J joining, class switch recombination (CSR), and somatic hypermutation (SHM) are examples of this processes in the IgSF receptors of jawed vertebrates, but modifications to genomic DNA can also occur in the jawless fish and some invertebrates.[7,13] The list of organisms undergoing such diversity of germline immune genes will only grow as more organisms are examined and more genome and expressed sequence tags (EST) sequencing projects are undertaken (see Fig. 4.1).

Alternative splicing can be a source of tremendous diversity in some gene families encoding receptors involved in immunity in insects and crustaceans. The Down syndrome cell adhesion molecule (DSCAM) gene in several arthropods (described in detail in the following) was shown to generate enormous diversity via ribonucleic acid (RNA) processing.[7,24] In the vertebrates, this mechanism is important in

determining the function of different molecules, best known for the Igs (transmembrane [TM] versus secreted forms, as well as inflammatory versus neutralizing forms in non-mammalian vertebrates). Further diversity can be obtained by the assembly of multichain receptors in which different components are combined. The classical example in the jawed vertebrate adaptive immune system is that of Ig light (L) and heavy (H) chains of antibodies but similar combination can occur with insect peptidoglycan-recognizing proteins (PGRP), vertebrate TLRs, and many others.

The study of the evolution of immunity has resulted in a fundamental appreciation of the heart of immunity, both innate and adaptive. Especially now, with studies in many plants and in both invertebrate and vertebrate animals, we can see what features have been conserved and when they arose. "Simple" genetic models such as *Drosophila* and *Candida elegans*

provide a glimpse into these elemental mechanisms and also allow us to remove the clouds that surround studies of mouse and human, with so many interconnected pathways. As mentioned, examination of the well-studied mammalian models in combination with studies of invertebrates allows us to deduce the condition of the common ancestor. Interestingly, major pathways of defense known to all immunologists, such as the ones involving TLR, JAK-signal transducer and activator of transcription (STAT), NOTCH, and tumor necrosis factor (TNF) pathways, clearly arose in an early animal ancestor and have been perpetuated in derived fashions in all major taxa. We shall see how these pathways are manipulated in different animals, always drawing upon the best-known mammalian model as a foundation (whenever possible).

MAJOR GENE FAMILIES INVOLVED IN IMMUNITY

Defense molecules can be composed of a very large number of protein folds, some of which are clearly some used to a large extent.[25,26,27] Some of the most common families (Fig. 4.2) are IgSF, leucine-rich repeats (LRRs), C-type lectins, and the TNF family, and certain other domains in immune recognition (eg, scavenger receptor cysteine-rich [SRCR]). All of the domains discussed in this chapter are found in Table 4.1 and a few are displayed in Figure 4.2. As a means of introduction, two of these families, which constitute the "top two" quantitatively, will be described in the following.

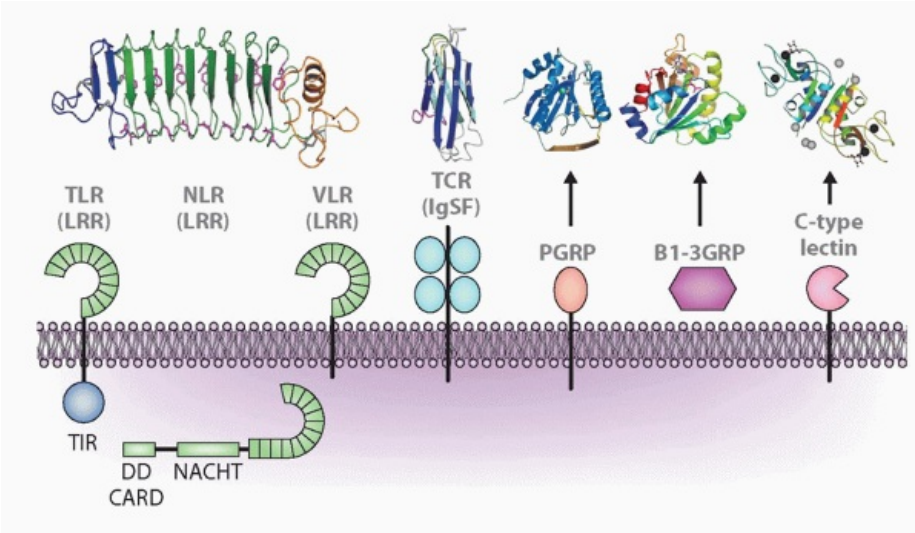

FIG. 4.2. Major Molecular Families Described in the Text and Representatives of Each Family: Leucine-Rich Repeats (LRRs), Immunoglobulin Superfamily (IgSF), Peptidoglycan-Recognition Protein (PGRP), β1-3 Glucan Recognition Protein (β1-3GRP), and C-Type Lectins. Representative structures are shown above for LRR (toll-like receptor, nucleotide-binding domain leucine-rich repeat [NLR], variable lymphocyte receptor), IgSF, PGRP, β1-3GRP, and C-type lectin. Other acronyms are defined in Table 4.1. For the NLR model, the echinoderms have N-terminal death domains, whereas all other animals have caspase-recruitment domains. Figure modified from Hibino et al.[48]

Leucine-Rich Repeats

LRRs consist of 2 to 45 motifs of 20 to 30 amino acids in length (XLXXLXLXXNXHXXHXXXXFXXLX) that fold into an arc shape (see Fig. 4.2).[28] Both the concave and convex parts of the domain have been shown to interact with ligands. Molecular modeling suggests that the conserved pattern LxxLxL is sufficient to impart the characteristic horseshoe curvature to proteins with 20- to 30-residue repeats. LRRs are often flanked by cysteine-rich domains. LRRs occur in proteins ranging from viruses to eukaryotes and are found most famously in the toll/TLRs, as well as tyrosine kinase receptors, cell-adhesion molecules, resistance (R) factors in plants found at the cell surface, and in the cytosol, extracellular matrix (ECM)-binding glycoproteins (eg, peroxidasin), and are involved in a variety of protein-protein interactions: signal transduction, cell adhesion, DNA repair, recombination, transcription, RNA processing, disease resistance, apoptosis, and the immune response. LRR-containing proteins can be associated with a variety of other domains, whether they are extracellular (LRR associated with IgSF or fibronectin [FN] type III) or intracellular (caterpillar family LRR associated with a variety of effector domains; see subsequent discussion). In these chimeric molecules, the LRR moiety is involved in recognition, most likely due to its extraordinarily malleable structure. There are at least six families of LRR proteins, characterized by different lengths and consensus sequences of the repeats.[29] Repeats from different LRR subfamilies never occur simultaneously and have most probably evolved independently in different organisms.

TABLE 4.1 Molecules and Abbreviations Found Throughout the Text

Acronym/Defense Molecule	Full Name	Function
AID	Activation-induced cytidine deaminase	SHM/gene conversion/CSR
APOBEC	Apolipoprotein B mRNA editing enzyme catalytic polypeptide	Innate immunity (antiviral)
AGM	Aorta/gonad/mesonephros	Intraembryonic origin of hematopoietic cells
AMP	Antimicrobial peptide	Innate immunity (eg, defensins)
APAR	Agnathan paired antigen receptor	Similarities to Ig/TCR and NKRs
AVR	Avirulence protein	Pathogen effector recognized by plant NLR

Bf	Factor B	Enzyme of C′ cascade
B1-3GNP	Beta 1-3 glucan-recognizing protein	Binds to gram-negative bacteria
C′	Complement	Innate/adaptive immunity
CARD	Caspase-recruitment domain	Domain in intracellular defense molecules
CATERPILLER or CLR	CARD, transcription enhancer, R(purine)-binding, pyrin, lots of leucine repeats	Apoptosis/immunity/inflammation
CDR	Complementarity-determining region	Portion of Ig/TCR that binds to antigen
CSR	Class switch recombination	Adaptive humoral immunity modification
DD	Death domain	Cytosolic interacting domain
DSCAM	Down syndrome cell adhesion molecule	Insect immune (adaptive?) defense and neuron specification
ECM	Extracellular matrix	
ETI	Effector-triggered immunity	Immunity in plants triggered by NLR
FBA	F box-associated domain	Intracellular domain
FcRN	Fc receptor neonatal	MHC-like FcR
FN3	Fibronectin type III repeat	Domain found in many innate molecules
FREP	Fibrinogen-related protein	Mollusk (adaptive?) defense
FuHC	Fusion histocompatibility	Histocompatibility locus in tunicates

GALT	Gut-associated lymphoid tissue	
GPI Hemolysin	Glycophosphatidylinositol	Lipid linkage to cell membrane (eg, VLR) Cell lysis
ICE	Interleukin-converting enzyme	IL-1β processing
Ig	Immunoglobulin	Adaptive immunity
IgSF	Immunoglobulin superfamily	Innate/adaptive immunity
IFN	Interferon	Innate (type I)/adaptive (type II) immunity
IMD	Immune deficiency	Insect innate defense
IRF	Interferon regulatory factor	Innate (transcription factor)
IRG	Immunity-related GTPases	Innate immunity
ITAM	Immunoreceptor tyrosine-based activation motif	Signaling motif for NK and antigen receptors
ITIM	Immunoreceptor tyrosine-based inhibitory motif	Signaling motif for NK and antigen receptors
JAK	Janus kinase	Signaling molecule associated with cytokine receptors
KIR	Killer IgSF receptor	NK cell receptor
lectins	For example, galectin, C-type, S-type	Many (eg, NKRs, selectins)
LITR	Leukocyte immune-type receptors	Fish NK-like receptors of the IgSF

LMP	Low-molecular-weight protein	Proteasome subunit
LRC	Leukocyte receptor complex	Gene complex containing KIR and many IgSF molecules
LRR	Leucine-rich repeat	Innate/adaptive immunity module
MAC	Membrane-attack complex	C′, pore-forming
MACPF	MAC-perforin domain	Potential pore former
MASP	MBP-associated serine protease	Lectin C′ pathway
MBP (or MBL)	Mannose-binding protein (lectin)	Lectin C′ pathway
MDM	Mollusk defense molecule	IgSF defense molecule
MHC	Major histocompatibility complex	T-cell recognition; innate immunity
MIF	Macrophage inhibitory factor	Innate immunity; inflammation
MyD88 (also dMyD88)	(Drosophila) Myeloid differentiation primary response gene 88	TLR adaptor
NITR	Novel immune-type receptors	Teleost fish NK-like receptors of the IgSF
NK cell	Natural killer cell	Vertebrate innate cellular immunity
NKC	Natural killer cell complex	Gene complex with many C-type lectin genes (especially NK cells)
NKR	Natural killer cell receptor	Receptor on NK cells
NALP	NACHT leucine-rich	Intracellular PRR

	repeat and PYD-containing protein	
NBD-LRR	Nucleotide-binding domain LRR	Motif of intracellular defense molecules
NFκB	Nuclear factor-κB (Rel homology domain)	Evolutionarily conserved transcription factor
NLR	NACHT leucine-rich repeat protein	Intracellular PRR
NOD	Nucleotide oligomerization domain protein	Intracellular PRR
NOS	Nitric oxide synthase	Intracellular killing innate defense molecule
PAMP	Pathogen-associated molecular pattern	Conserved target epitopes on pathogens
PCD	Programmed cell death	Many pathways
Penaedins		Defense molecule in shrimp
PGRP	Peptidoglycan-recognition protein	Gram-positive bacteria defense family; receptor and effector
PPO	Propolyphenol oxidase	Plant/invertebrate defense (melanization)
PRR	Pattern-recognition receptor	Recognize PAMP, innate/adaptive immunity
PMSB	Proteasome subunit beta subunit	Proteolytic member of 20S proteasome
Polμ	DNA polymerase μ	Error-prone polymerase (related to TdT)
PPO	Prophenoloxidase	Invertebrate defense molecule
PYD	Pyrin domain	Domain in intracellular defense molecules

RAG	Recombination-activating gene	Ig/TCR rearrangement
RFP-Y	Restriction fragment polymorphism-Y	Chicken nonclassical MHC gene cluster
RFX	Regulatory factor X	Transcription factor, class I regulation
RIG	Retinoic acid-inducible gene	Intracellular double-stranded RNA recognition
RSS	Recombination signal sequence	DNA element next to Ig/TCR gene segments necessary for RAG-mediated rearrangement
RXR	Retinoid X receptor	Transcription factor encoded in MHC
SHM	Somatic hypermutation	Adaptive humoral immunity
SPE	Spaezle-processing enzyme	Insect defense molecule in toll cascade
SRCR	Scavenger receptor cysteine-rich	Innate immunity recognition molecule
TAK	TGF-β activated kinase	ubiquitin-dependent kinase of innate pathways
TAP (and TAP-L)	Transporter associated with antigen processing	Rransports peptides from cytosol to ER lumen
TAPBP	TAP-binding protein	Tethers TAP to class I
TCR	T-cell receptor	Adaptive defense
TdT	Terminal deoxynucleotidyl transferase	Involved in Ig/TCR rearrangement
TEP	Thioester-containing protein	Opsonization (like C3)

TGF	Transforming growth factor	Immunosuppressive cytokine
TNF	Tumor necrosis factor	
UPD	Unpaired	Protostome cytokine induced by viral infection
TLR	Toll-like receptor	Innate receptor on the cell surface or in endosomes
TM	Transmembrane	
TNF	Tumor necrosis factor	Proinflammatory cytokine (and family)
TRIM	Tripartite motif-containing proteins	Large family of cytosolic innate defense molecules
V-, C1-, C2-, I-	Variable, constant 1 and 2, intermediate IgSF domain	IgSF domain types
VAV	Guanine exchange factor, the “onc F” proto-oncogene	Encoded in MHC, involved in adaptive signaling pathways
VCBP	Variable domain chitin binding	Amphioxus defense molecule
VLR	Variable lymphocyte receptor	Agnathan adaptive defense molecule
WKRY		Plant transcription factor used to upregulate defense genes (analog of NF-κB)
XMIV	*Xenopus* MHC-linked IgSF V region	*Xenopus* MHC-linked NKR-like genes
XNC	*Xenopus* nonclassical	*Xenopus* class Ib cluster
185/333	Sea urchin defense molecule	(Adaptive?) Defense

DNA, deoxyribonucleic acid; ER, endoplasmic reticulim; IL, interleukin; mRNA, messenger ribonucleic acid; RNA, ribonucleic acid.

LRR-containing proteins are involved in immunity from plants to animals. The functions in the immune systems range from control of motility of hemocytes and lymphocytes[30] to specific recognition of antigens via a novel system of gene rearrangement (the variable lymphocyte receptors [VLR] described in the following; see Fig. 4.9). LRRs can occur in soluble forms, the ECM, in the cytosol, or as TM forms, either integral membrane proteins or glycophosphatidylinositol (GPI)-anchored. The bottom line is that because of its basic structure and malleability, the LRR module was locked in early in evolution as an ideal motif for recognition of essentially any ligand.

Immunoglobulin Superfamily

IgSF domains are encountered in a very large number of molecules in the animal kingdom (see Fig. 4.2).[31] They are found intracellularly (eg, connectin) or as cell adhesion molecules, many of which are in the nervous system (eg, the neural cell adhesion molecule, NCAM), coreceptors and costimulatory molecules of the immune system (eg, cluster of differentiation [CD]79, CD80), molecules involved in antigen presentation to lymphocytes (eg, class I molecules), certain classes cytokine receptors (eg, interleukin [IL]-1R), and of course Ig (and TCR), where they were first characterized and were bestowed with their name (Ig). They can be associated with other domains such as FN (eg, titin and FREP) and LRRs,[32] or they can be the sole constitutive elements of the polypeptide chain often associated to a transmembrane segment and a cytoplasmic tail (or GPI-linked). The β barrel IgSF structure was adopted independently in other families such as cadherins, calycins, lipocalin, etc., and the super (or über) family has hundreds of members and has been selected for several different functions. These functions are somehow related, almost all involved in protein-ligand interactions. The vertebrate lymphocyte surface can express 30 different IgSF members simultaneously.

IgSF domains are commonly classified according to different domain constitution in their β strands and loops.[31,33] All conform to the stable shape of a β barrel consisting of two interfacing β sheets, usually linked by a disulfide bridge. There are three types of domains: variable (V), and two types of constant (C1 and C2); the so-called I set domain is intermediate between the C1 and C2. The V domain is most complex with more strands (C′ and C″), which make up complementarity determining region (CDR)[2] in conventional Igs and TCRs. C1 domains lack these strands entirely, and C2/I domains have varying sizes in the C′/C″ region. V domains, either alone (eg, the new antigen receptor [NAR]) or in association with another V domain (eg, Ig H/L), recognize the antigenic epitope and are therefore the most important elements for recognition. Domains with the typical V fold, whether belonging to the true V-set or the I-set, have been found from sponges to insects (eg, amalgam, lachesinm and fascicilin) and even in bacteria. The mollusk fibrinogen-related proteins (FREPs, described in the following) have one or two V-like domains at their distal end, associated with a fibrinogen-like domain. For V domains, the interface between dimers is the

beta strand bearing the C, C′, C″, F, and G strands, so that in Igs the CDR3 are in the center of the binding site; for C domains, the other beta strand bearing the A, B, D, and E strands forms the interface.

The binding capacities of V domains in molecules besides Ig/TCR can reside in different areas of the molecule (interstrand loops, A-A′ strand, F strand), while in Ig/TCR, CD8, and certain NKR, these regions are the targets for variation in shape and charge. The binding capacities can be modulated whether one domain acts as a single receptor unit (eg, IgNAR) or whether it is associated with a contiguous domain (eg, KIR, variable domain chitin-binding protein [VCBP]) or with another polypeptide chain (eg, TCR, Ig). In the case of a dimer, the binding capacity can again be modulated by the presence or the absence in the G strand of a diglycine bulge, which can modify the space between the faces of the Ig domain. In several cases, the sites responsible for binding are known (KIR, Ig, TCR); in many other cases, they are not known but inferred from crystal structures and/or variability plots (leukocyte immune-type receptor [LITR], chicken Ig-like receptor [CHIR], triggering receptor expressed on myeloid cells [TREMs], DSCAM, hemolin).

HEMATOPOIEIS AND CELL TYPES IN THE INVERTEBRATES

Invertebrate Cell Types

Examples of conservation of fundamental mechanisms of genetic control of developmental pathway between protostomes and deuterostomes, even in the absence of homology of the cells or organ considered, are accumulating: the organization and expression of the homeotic gene clusters and eye formation through the function of a complex of proteins including Pax-6.[34] The cell types involved, besides direct interaction with the external layer of cells on the skin, or external teguments, have been specialized cells of mesodermal origin devoted to defense. This is true for all coelomates where effector cells have been identified, but recent data have shown that cnidarian diploblastic organisms that lack mesoderm also have many of the same genetic systems as the coelomates[35] (see Fig. 4.1). The cells can be circulating or sessile, and often are found associated with the gut. Several morphologically distinct hemocyte types in insects cooperate in immune responses: they attach to invading organisms and isolate them, trapping larger organisms in nodules or forming large multi-cellular capsules around them. Indirect evidence for the role of hemocytes in immune responses can be derived by contrasting properties of such cells in healthy and parasitized animals (ie, modifications in adherence and opsonic activity).

All animals show heterogeneity of the free circulating cells, generically called hemocytes (arthropods), coelomocytes, amebocytes (annelids, mollusks, and echinoderms), or leukocytes (sipunculids). However, the repertoire of insect “blood cells” is clearly less heterogeneous than that of vertebrates. Basically, three or four types of cell lineages can be identified in *Drosophila* (Fig. 4.3[36]): plasmatacyte, crystal cells, and lamellocytes, and an equivalent number in *Lepidoptera* (butterflies). The functional roles they play consist of immune defense, disposing of apoptotic and other debris, contributing to the ECM, and modeling of the

nervous system. The immunity role encompasses phagocytosis, encapsulation, and sometimes production of effector molecules (see Fig. 4.3[36]). These roles all require

recognition of pathogen-associated molecular patterns (PAMPs) or self-derived defense molecules (ie, opsonization) at the cell surface.[37,38,39,40]

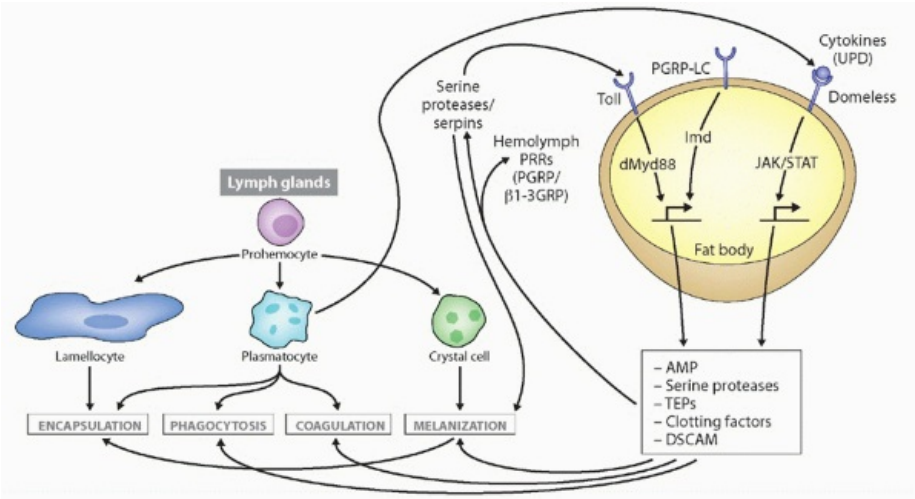

FIG. 4.3. Types of Immune Responses and Cells in Insects, with *Drosophila* as the Prototype. Secreted defense molecules are made by fat body cells in response to pathogens, which either act as direct effector molecules or feed back on hemocytes to stimulate their defense functions. Unpaired (UPD) is produced after virus infection, stimulating defense molecule upregulation via the JAK/signal transducer and activator of transcription pathway. Not shown is the RNAi pathway, also induced upon virus infection. Details on stimulation of the toll and immune deficiency pathways are found in Figure 4.4. This figure was modified from Lemaitre and Hoffmann.[36]

Only in a few organisms has the characterization of hemocyte lineages gone beyond morphologic or basic physiologic functions. Among these free circulating cells are always one or more types that can undergo phagocytosis. Different cells participate in encapsulation, pinocytosis, and nodule formation, and can upon stimulation produce a great variety (within an individual and among species) of soluble effector molecules that may eliminate the pathogen. In an attempt to integrate all of the data available in invertebrates, Hartenstein has proposed a unified nomenclature of four basic types: prohemocytes, hyaline hemocytes (plasmatocytes or monocytes), granular hemocytes (granulocytes), and eleocytes (chloragocytes).[37] These designations will be found in the following description of the blood cell types.

Earthworm (annelid) coelom-tropic coelomocytes are called eleocytes. They contain glycogen and lipid and are considered of the same lineage as the chloragocytes involved in the production of immune effector molecules such as fetidin or lysenin. The phagocytic cells of annelids are apparently granular “leukocytes” derived from the somatopleura and involved in wound healing, whereas the ones derived from the splanchnopleura participate in immunity. Heterogeneity of annelid coelomocytes is not encountered in primitive oligochaetes or in hirudinae (leeches). Phagocytic coelomocytes show an acid phosphatase activity and a beta glucoronidase activity.[41] The large coelomocytes and free chloragocytes (eleocytes) in the typhlosole of *Eisenia foetida* appear to produce the bacteriolytic and cytolytic factor

lysenin.[42] From electron microscopy studies, macrophage-like cells seem to be involved in graft rejection. In the closely related sipunculid phylum, two main cell types can be identified in the blood: erythrocytes (a rare occurrence in invertebrates) and granular leukocytes. The latter are capable of cytotoxicity and even have dense granules reminiscent of vertebrate "NK cells."[43]

Two developmental series have been described in mollusks, the hyaline and granular cells, but cephalopods seem to have only one lineage. They participate in encapsulation, with hemocytes adhering around the foreign body like *Drosophila* lamellocytes. Phagocytosis is carried out by the wandering granular cells, which resemble vertebrate monocytes/macrophages. In oysters, electron microscopy revealed different types of circulating hemocytes, including granular hemocytes resembling the granulocytes of sipunculus mentioned previously.[44,45] In crustaceans, the situation is similar to that in mollusks, with three main populations identified based again on the presence of granules in the cytoplasm.

The hyaline cells are involved in the clotting process and the granular cells in phagocytosis, encapsulation, and the prophenoloxidase (PPO) pathway. The hematopoietic organ is located on the dorsal and dorsolateral regions of the stomach.[39] Crustacean hemocytes can now be cultured and their response to virus can be examined,[46] and markers of the three hematopoietic lineages are available.[47]

In insects, the so-called prohemocytes are believed to be stem cells. They are only found in the embryonic head mesoderm and the larval lymph glands but not in the hemolymph. However, prohemocytes are frequent in both the hemolymph and hematopoietic organs of the lepidopteran *Bombyx* (silkmoth). Plasmatocytes of *Drosophila* have a phagocytic function. This type of hemocyte is equivalent to the granulocytes of *Bombyx*, which play a key role in phagocytosis in larvae. Lamellocytes seem to be unique to *Drosophila*, but they are probably the equivalent of the lepidopteran plasmacytoid cells. Their precursors reside in the larval lymph gland, where they differentiate in response to macroscopic pathogens, following a brief phase of mitosis linked to the presence of the pathogens and under hormonal control via ecdysone. The transcription factors (GATA, Friend-of-GATA, and Runx family proteins) and signal transduction pathways (toll/NF-κB, Serrate/Notch, and JAK/STAT) that are required for specification and proliferation of blood cells during normal hematopoiesis, as well as during hematopoietic proliferation that accompanies immune challenge, have been conserved throughout evolution. The specific differentiation of lamellocytes requires the transcription factor Collier. The mammalian early B-cell factor, an ortholog of Collier, is involved in B-cell differentiation in mice. The *Drosophila* crystal cells are responsible for melanization through the PPO system (see subsequent discussion). In silkworm oenocytoids, crystallike inclusions are also found, but they disappear later after bleeding.[36,37,40]

Echinoderm coelomocytes express a diversity of effector functions, but no studies of lineages have been performed. In echinoderms, the number of different coelomocytes may vary according to the particular family. The sea urchin is endowed with at least four cell types, only one of which only is phagocytic and corresponds to the bladder or filiform forms. Another type is described as the round vibrating cell involved in clotting. Pigment cells (red spherule cells) have been detected ingesting bacteria; the morphology of phagocytic cells can vary

enormously, precluding any easy classification.[48]

In tunicates, amoeboid cells circulate in the blood and are involved in a large number of processes, such as clotting, excretion, nutrition budding, and immunity. Large numbers of blood cells are present (average of 10^7 per mL) in the blood of ascidians such as *Ciona*. Hemoblasts are considered to be undifferentiated cells, perhaps the equivalent of the prohemocytes of arthropods or the neoblasts of annelids. Blood cells in ascidians proliferate in the connective tissue next to the atrium. The pharyngeal hematopoietic nodule of this animal contains a large number of hyaline and granular cells called “leukocytes” with supposed intermediary forms of differentiation between blast and granular mature types. The granular form is likely to be involved in postphagocytic activity, like in earthworms.[49] Adoptive transfer of alloimmunity in the solitary tunicate *Styela* can be achieved via lymphocyte-like cells.

In *Amphioxus*, cells with phagocytic capacity have been identified in the coelom with a morphology resembling more the phagocytic echinoderm cells than urochordate blood cells, a fact that is consistent with the new systematic positions of amphioxus and echinoderms.[50] Both free cells and the lining of the perivisceral coelom are able to phagocytose bacteria. Cells with the morphologic appearance of lymphocytes and expression of lymphocyte-specific genes were detected in this species, the earliest identification of such cells in phylogeny.[51]

Hematopoiesis in the Invertebrates

The history of the hemocytes is associated with that of the mesoderm among triploblastic organisms. The bilaterian ancestor was most likely a small acoelomate or pseudocoelomate worm similar to extant platyhelminths (flatworms) (see Fig. 4.1). A specialized vascular system or respiratory system was probably lacking, although cells specialized for transport and excretions were likely present because they exist in most extant bilaterian phyla. One can further assume that groups of mesoderm cells in the bilaterian ancestor could have formed epithelial structures lining internal tubules or cavities (splanchnopleura). In coelomates, the mesoderm transforms into an epithelial sac, the walls of which attach to the ectoderm (somatopleura) and the inner organs (splanchnopleura). Blood vessels are formed by tubular clefts bounded by the splanchnopleura. Excretory nephrocytes are integrated into those vascular walls, which also gives rise to blood cells circulating within the blood vessels (the pronephros of anurans and head kidney of teleost fish are important hematopoietic organs in vertebrates). Thus, further evolutionary changes separated the three systems, but there was a close original connection between them.

The origin of hemocytes has been investigated mainly in arthropods. When examining principles that govern hematopoietic pathways, similarities have been observed with vertebrates, raising interesting evolutionary issues.[37,40] In jawed vertebrates, the yolk sac or its equivalent gives rise to blood precursors that are primarily erythroid in nature (but see the following: recent data suggest that B1 cells and macrophages are also derived from this embryonic tissue). In succession, definitive hematopoiesis occurs in the aorta/gonad/mesonephros (AGM) region of the embryo, encompassing all of the different cell types and multipotent progenitors (although this is controversial). Like in the vertebrates, hematopoiesis in insects is biphasic. One phase occurs in the embryo and the other during larval development. Additionally, these waves occur in distinct locations of the embryonic

head mesoderm and the larval lymph gland. In the early embryo expression of the GATA factor, serpent (*Srp*) can be detected in the head mesoderm. This GATA family of zinc-finger transcription factors is conserved from yeast to vertebrates where they are involved in various aspects of hematopoiesis. Blood cell formation in the head

follows *Srp* expression, whereas in the lymph gland there is a long delay between *Srp* expression and the appearance of the lymph gland-derived hemocytes.[38] Hematopoiesis in the head mesoderm and yolk sac may be related evolutionarily. A further similarity occurs at the AGM/lymph gland level in *Drosophila*. The lymph gland develops from a part of lateral mesoderm that also gives rise to vascular and excretory cells, much like the vertebrate AGM. The conserved relationship between blood precursors and vascular and excretory systems is intriguing.

Hematopoiesis and Transcription Factors in the Vertebrates

As mentioned previously, transcription factors of the family PAX 2/5/8; GATA 1, 2, 3; ets/erg; and runt domain-containing factors have been cloned in several invertebrates. One plausible model to explain the genesis of true lymphocytes in vertebrates is that closely related members of transcription factor families are the result of a relatively late divergence in lineage pathways followed by specialization of duplicated genes.[52] These duplications could be those that apparently occurred during the history of chordates (see MHC and “Origins” section[2]). Within deuterostomes, the generation of true GATA 2 and 3 probably occurred after echinoderms diverged from the chordate branch and the GATA, ets, early B-cell factor, and Pax5-dependent pathways of T-/B-cell differentiation are thus specific to vertebrates. It is already known that lampreys express a member of the purine box 1/spleen focus-forming virus integration-B gene family that is critically and specifically involved in jawed vertebrate lymphocyte differentiation. Expression has been detected in the gut, which may be related to the fundamental nature of “gut-associated lymphoid tissue (GALT)” as a lymphoid cell-producing organ.

In vertebrates, the generation of T-, B-, and NK lymphocyte lineages from pluripotent hematopoietic stem cells depends on the early and tissue-specific expression of *Ikaros* (and related loci), which by means of alternative splicing produces a variety of zinc-finger DNA-binding transcription factors. The orthologs of *Ikaros*, *Aiolos*, *Helios* , and *Eos* have been identified in the skate *Raja eglanteria*, where two of the four *Ikaros* family members are expressed in their specialized hematopoietic tissues (epigonal and Leydig's organs; see subsequent discussion) like in mammals.[52] In lower deuterostomes, single genes that seem to be related to the ancestor of the Ikaros and Ets family of transcription factors exist, further suggesting that the division of labor between the family members in the jawed vertebrates was a result of en bloc duplications.[52,53] The conservation of *Ikaro*s structure and expression reinforces its role as a master switch of hematopoiesis. We discuss this topic further in the lymphoid tissues section.

Responses of Hemocytes

In this section, we simply touch on classical and specific responses in the invertebrates, responses that are more universal are found in the innate immunity section. Proliferation of hemocytes upon stimulation is an unresolved issue in the invertebrates; clearly, clonal

selection resulting in extensive proliferation is not the rule. The turnover of cell populations has been the object of numerous, often unconvincing experiments. Still, new data have emerged, and it is clear that in several invertebrates, proliferation occurs in certain cell types following encounters with pathogens. Very little cell proliferation occurs in the circulation of crayfish, but cells in the hematopoietic tissue divide after an injection of the PAMP β1-3-glucan. New cells in the circulation developed into functional synthetic germinal centers (GCs) and GCs expressing the proPO transcript. RUNT protein expression was upregulated prior to release of hemocytes. In contrast, proPO was expressed in these cells only after their release into the circulation.[54]

By contrast to the study of transcription factors that regulate hematopoiesis, relatively little is known about cytokines that drive hematopoiesis among invertebrates. It was reported that differentiation and growth of hematopoietic stem cells in vitro from crayfish required the factor astakine, which contains a prokineticin domain[55]; prokineticins are involved in vertebrate hematopoiesis, another case of conservation during the evolution of growth factors and blood cell development.

Parasitization of *Drosophila* by the wasp *Leptopilina boulardi* leads to an increase in the number of both lamellocytes and crystal cells in the *Drosophila* larval lymph gland. This is partially due to a limited burst of mitosis, suggesting that both cell division and differentiation of lymph gland hemocytes are required for encapsulation. In genetic backgrounds where ecdysone levels are low (*ecdysoneless*), the encapsulation response is compromised and mitotic amplification is absent. This ecdysone-dependent regulation of hematopoiesis is similar to the role of mammalian steroid hormones such as glucocorticoids that regulate transcription and influence proliferation and differentiation of hematopoietic cells.[56]

Phagocytosis

To obtain phagocytosis at the site of microorganism invasion implies recruitment of cells via chemoattraction. In vertebrates, this can be done by several categories of molecules such as proinflammatory chemokines/cytokines or the complement fragments C3a and C5a (as mentioned in the following section, C3a fragments as we know from mammals may be found in tunicates but not other nonvertebrates; yet, C3 may be cleaved in different ways in the invertebrates). C3b, mannose-binding lectin (MBL), and many other lectins can function as opsonins, and recent studies of the PGRPs, thioester-containing proteins (TEPs), DSCAMs, and eater have added to this repertoire.[36] Ingestion follows phagocytosis, and then killing occurs by an oxidative mechanism with the production of reactive oxygen radicals and nitric oxide. These mechanisms are conserved in phylogeny, and other basic mechanisms are being examined in more detail now in protozoan models.[57] Signaling pathways in common between vertebrates and the protozoon *Dictyostelium* include involvement of cyclic AMPs, integrins, and perhaps mitogen-activated protein (MAP) kinase cascades. Unique to all jawed vertebrates studied to date, the activation of

phagocytes also leads to upregulation of the antigen processing machinery, costimulatory molecules, and proinflammatory cytokines that can enhance adaptive immunity.

INNATE IMMUNE RESPONSES

Immune responses are often subdivided into recognition, signaling, and effector phases, which are subjected to different pressures, defined by whether orthology is maintained and the relative divergence rates of the genes responsible for the various phases. Recognition molecules are from evolutionarily conserved families, but as described previously, their genes are subjected to rapid duplication/deletion so that orthology is rarely preserved. By contrast, signaling pathways can be conserved (see Fig. 4.5), despite the fact that the genes are often divergent in sequence. Effector molecules can either be extremely conserved (eg, reactive oxygen intermediates) or extremely divergent to the point of being species-specific (eg, AMP). Here, we break the immune response down into these three phases, beginning with the recognition phase.

Initiation of an immune reaction can theoretically involve either the recognition of nonself, altered self, or the absence of self. Nonself-recognition can take place with receptors (pattern recognition receptors [PRRs]) that detect PAMPs, which were originally defined by Janeway and colleagues as evolutionarily conserved epitopes displayed by pathogens but not host cells.[58,59] The second mode, altered self, is typified by molecules that are induced in self-cells during infections and recognized by conserved defense molecules, similar to the SOS systems mentioned in the MHC section, or by peptide presentation on MHC molecules. A third mechanism, “am I still myself,” depends upon recognition of self-tags and their changes in expression[60] (eg, NK recognition of self-MHC molecules through KIR and C-type lectins). These latter two mechanisms have not been described in the invertebrates for immune defense against pathogens, but it would not be surprising if they were revealed in the future, considering the new features of invertebrate immune systems that have been discovered recently and the usage of this mode of recognition in many invertebrate histocompatibility systems.

Whether the invader is related to its host (cells from individuals of the same species or cells from a parasitoid) or are very distant from the host (fungi and bacteria in metazoa), there are different principles of recognition. Yet PAMP determinants have been identified on very different organisms—sugars such as β1-3 glucan of fungi, lipopolysaccharide (LPS) and peptidoglycans of bacteria, phosphoglycan of some parasites, and especially nucleic acids of bacteria and viruses—and they can trigger similar cascades of events. The foreign ligand can be bound by a molecule in solution that initiates an effector proteolytic cascade (eg, clotting or the complement cascade). On the other hand, a proteolytic cascade can be initiated and result in the production of a self-ligand that interacts with a cell surface or endosomic or cytosolic receptor. In this way, there need not be a great diversity of cell surface receptors, especially in the absence of clonal selection.

Of the over 1 million described species of animals (see Fig. 4.1), approximately 95% are invertebrates representing 33 phyla, some with one species (*Placozoa*, *Cycliophora*) and others with over 1 million (*Arthropoda*). Because they have major differences in body plans, development, size, habitat, etc., wildly different types of immune systems in diverse species should be expected. Early studies of invertebrate immunology reached no consensus of how immunity should be examined, but because vertebrate cellular adaptive immunity was often defined (indeed, was discovered for T cells) through transplantation reactions, attempts to reveal specific memory by allograft rejection were often used. After many unsuccessful attempts to demonstrate memory of such responses (see the following) and after extensive molecular studies, a consensus was reached that an invertebrate adaptive immune system

involving somatic generation of antigen receptors and their clonal expression was highly unlikely. However, the term “innate” is rigid and masks the possibility of other somatic alterations of invertebrate immune system molecules, as will be discussed.[61,62] We will categorize the molecules based on their location within the cell.

Intracellular Recognition

Nucleotide-Binding Domain Leucine-Rich Repeat (NLR)

One major group of intracellular sensors in animals and plants is the NLR family (see Figs. 4.2 and 4.4).[14,63] Each of the family members has a central NB/NACHT (nucleotide-binding domain) and C-terminal LRR used for recognition, and a unique N-terminal domain. The subfamilies are defined by their N-terminal domains, coiled-coil and toll-IL-1 receptor (TIR) in plants, and baculoviral inhibitory repeat, caspase-recruitment domain (CARD), pyrin domain (PYD), and activation domain in animals (see Fig. 4.4). Thus far, the NLRs have been found in deuterostomes but not protostomes (see Fig. 4.1), which is surprising considering that plants have intracellular defense proteins with a similar structure, and seem to have been derived via convergent evolution.[14]

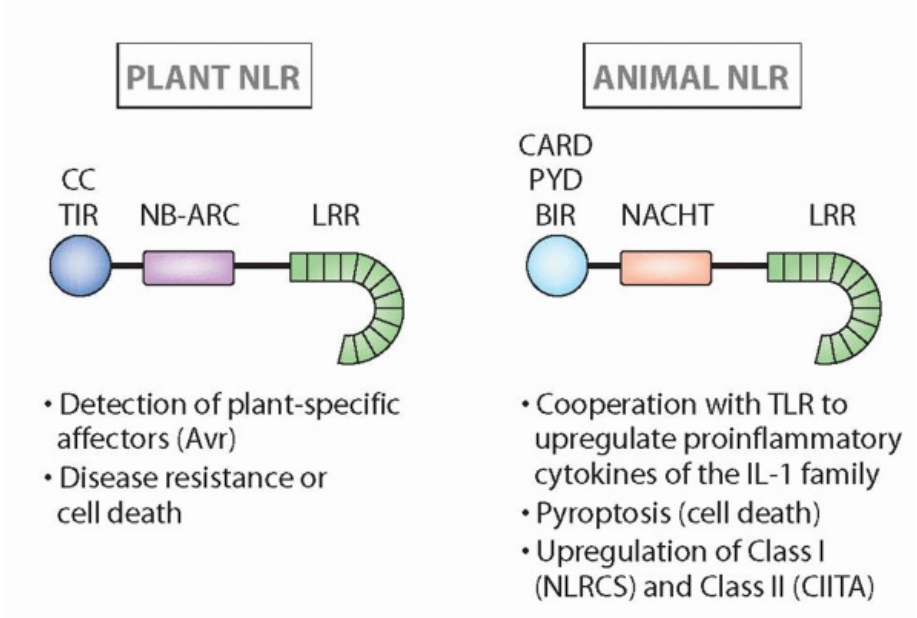

FIG. 4.4. Structure and Major Functions of Nucleotide-Binding Domain Leucine-Rich Repeats in Plants and Animals.[14] Although the structures are quite similar, and recognition can be analogous, these families seem to have arisen via convergent evolution (see text for more details).

The specificity of plant NLR depends principally on the LRRs, and these are targets for diversifying selection, as described previously for multigene families. Plant NLR can recognize pathogen effectors (pathogen-derived avirulence factors), or viral and fungal PAMPs directly via the LRR domains, or via modifications of a host target that interacts with

the N-terminal domains, altered self if you will (see Fig. 4.4). This type of activation in plants is termed effector-triggered immunity, which is specific of the NLRs (see Fig. 4.4). A host of downstream effectors are generated, some involved in defense but others activating cell death pathways.[14,64]

Best described in mammals, the nucleotide oligomerization domain (NOD)/NLR recognizes PAMPs such as peptidoglycan and induces an autophagy-mediated destruction of intracellular pathogens as well as production of proinflammatory cytokines; however, it remains controversial whether there is direct or indirect recognition of the PAMPs (similar to some responses in plants). Polymorphisms in the NOD proteins are associated with inflammatory bowel diseases. The NLRP and NAIP NLRs are activated by in ways that are not well understood by various PAMPs or dangerassociated molecular patterns and form inflammasomes, best known for the activation of caspase 1 and the processing of pro-IL-1beta (or mature IL-18) for release from cells.[14,65] The founding member of the family, CIITA, has long been known to upregulate class II genes (and associated genes, such as cathepsins and invariant chain), and its function is somewhat outside the norm. Another member, NLRC5, has been shown to upregulate MHC class I expression, but the mechanism is unknown.[66] While the shuttling of the vertebrate NLRs CIITA and NLRC5 to the nucleus seems to be a derived characteristic, movement of plant NLRs into the nucleus to activate transcription occurs, either directly or after recruitment of transcription factors like WRKY described in the following.

NLRs are expressed by echinoderm coelomocytes, again representing a highly diversified family[48] (> 200 members, similar to the TLRs and SRCRs). As mentioned, it is surprising that these genes do not seem to be represented in protostomes, and thus the emergence of the family in plants and deuterostomes occurred through convergent evolution.[67] On the contrary, in the vertebrates a search of the *Danio rerio* (and other teleosts) database have yielded a large number of NLR sequences, more similar to the situation in plants.[63] In humans, most NLR genes are encoded in clusters on chromosomes 11p15, 16p12, and 19q13, where six sequences are found in a single telomeric region.

Rig-I-Like Receptors (RLR)

The retinoic acid-inducible gene (RIG)-I is an intracellular defense molecule that is unrelated to the NOD proteins, with N-terminal CARD and C-terminal helicase domains.[68] With the helicase domain, RIG-1 binds to an uncapped 5′ phosphate group, which is diagnostic of viral RNAs. RIG-I also recognizes short double-stranded RNAs, while a second member of this family MDA5 recognizes long double-stranded RNAs. These molecules contain two CARD domains at the N-terminus, a DEXDc domain, a helicase domain, and a regulatory domain. Ligands bind to the regulatory domain, inducing a conformational change leading to interaction with the adaptor protein MAVS (or IPS-1) and ultimately to the induction of type I interferons (IFNs). A third member of the RLR family is LPG2, which lacks the CARD domain; this molecule was originally believed to be a negative regulator of RIG-I/MDA5-induced signaling, but that has been called into question.

This family is found in all of the vertebrates and in lower deuterostomes, such as amphioxus and echinoderms.[63] Somewhat surprisingly, the RLR family is only mildly expanded in sea

urchins (12 members). While there is no report of bonafide RLR family members in protostomes but RLR activity is present,[69] the cnidarian sea anemone has been reported to have a RLR homologue,[70] again showing the importance of studying this taxon for the emergence of immune-related molecules.

Cytosolic Deoxyribonucelic Acid (DNA) Sensors

There are four mechanisms of cytosolic DNA sensing, three of them, the DNA-dependent activator of IFN-regulatory factor, IFI16, and RNA polymerase III (which converts viral DNA into RNA recognized by RIG-I), induce type I IFN production through the intermediate STING, a protein associated with the endoplasmic reticulum (ER).[71] In addition to being an intermediate in IFN upregulation (through IFN regulatory factor-3), STING is also a PRR in its own right, responding to the PAMP cyclic dinucleotides produced by intracellular bacteria like *Listeria*; this suggests that STING was originally a PRR, and then was co-opted by several other PRR sensors to induce effector functions.[72] IFI16 is part of the AIM2-like receptor family; the founding member, AIM2, like the inflammasome, activates caspase-1 to process pro-IL-1beta.

These new molecules/mechanisms have so far only been studied in mammals, but it would be surprising if they were not operative (at least) in other vertebrates as a way to combat DNA viruses. To date, they have not been found in the sea urchin or jawless fish databases.

Tripartite Motifs

Tripartite motif (TRIM) proteins belong to a family induced by type I and II IFNs, with 68 members in the human genome. TRIMs are involved in resistance against pathogens in mammals, especially lentivirus (eg, human Trim 5α is a retroviral restriction factor with activity against human immunodeficiency virus).[73,74] The activity of proteasomes, responsible for cytosolic protein degradation, has been implicated in the TRIM5α-dependent attenuation of retroviral reverse transcription. TRIMs contain an N-terminal moiety composed of three modules: RING (with an E3 ubiquitinase activity)-Bbox-coiled xoil motif followed by different C-terminal domains. TRIMs fit into two major categories by the function of their C-terminal domain: Category 1 with a PHD, MATH, ARF, FNIII, exoII, or NHL domains, and Category 2 with a B 30-2 domain shared with butyrophilins and other proteins and essential for ligand binding.[75] The tertiary structure of TRIM21 revealed two binding pockets in the B30.2 domain formed by six variable loops.[76]

Despite reports to the contrary, the TRIM family is ancient.[77] The family has been greatly diversified in vertebrates and in a taxon-specific manner, as observed for many multigenic immune families.[77] The zebrafish genome harbors a striking diversity of a subset of Category 2 TRIMs not encountered in mammals, called finTRIM, with 84 genes distributed in clusters on different chromosomes. This subset, specific of teleosts, is overexpressed after virus infection in the trout. In the B30.2 domain, residues under positive selection are concentrated within a viral recognition motif first recognized in mammalian Trim 5α.[78]

Finally, *trim* genes encoding Category 2 proteins are preferentially located in the vicinity of MHC or MHC gene paralogs both in fish and human, suggesting that they may have been

part of the ancestral MHC.[79] The B30.2 domains most closely related to finTRIM are found among NLRs, indicating that the evolution of TRIMs and NLRs was intertwined by exon shuffling.[80] Exon shuffling was likely responsible for the presence of the B30.2 domain in butyrophilin and TRIM genes where it was perhaps favored by the proximity of gene in the MHC. It has been argued that during evolution the combination of SPRY and PRY motifs that build up the B30.2 domain were selected and maintained for immune defense.[81]

P47 GTPases

Among IFN-inducible immunity-related genes with an interesting evolutionary history, immunity-related GTPases (IRG/p47 in mouse) function as cell-autonomous resistance factors by disrupting the vacuolar membrane surrounding parasites (eg, toxoplasma).[82] The IRG system studied primarily in mice (absent in humans[83]) is present throughout mammals but the number, type, and diversity of genes differ greatly even between closely related species, one of the common themes in immunity described previously.

Concerning the evolutionary origin of the IRGs, the homologs of zebrafish and pufferfish seem to form two teleost-specific groups, another common theme in this chapter. Their putative promoter regions suggest an expression regulated by an IFN. Homology searches failed to find any convincing ancestral form to the vertebrate IRG proteins in the genomes of invertebrates, but in phylogenetic trees vertebrate IRGs clusters with some families of bacterial GTPases. Thus, IRGs may be derived from a prokaryotic GTPase acquired by a horizontal transfer subsequent to the appearance of eukaryotes.[82]

Integral Membrane (and Sometimes Secreted) Proteins

C-Type Lectins

Lectins were originally defined by their ability to bind carbohydrates in a calcium-dependent manner (how C-type lectins got their name[84]) and some have been described previously (and throughout the chapter). They are found in many phyla in both the deuterostome and protostome lineages in both membrane and/or secreted forms (eg, MBL described in the following). A large number of C-type lectins have been uncovered in the mosquito genome, and some are involved in bacterial defense through direct binding and others through the melanization reaction.[85] Some C-type lectins are encoded in the NKC, including the Ly49 and NKG2 families, as well as CD94 and several other members of the family are central to NK-cell function in mammals. A molecule resembling CD94 but unlikely to be an ortholog (see the following) has been detected on a subset of hemocytes in *Botryllus* and *Ciona*, the functions of which are unknown.[86] Another large gene family that is implicated in the response of the sea urchin to immune challenge includes 100 small C-type lectins,[48] consistent with the enormous expansion of several immune defense families in this animal. We describe other functions of C-type lectins in the NK cell sections.

Scavenger Receptors

The SRCR superfamily is an ancient (from sponges to chordates) and highly conserved group of cell surface and/or secreted proteins, some of which are involved in the development of the immune system as well as the regulation of both innate and adaptive

immune responses; they are especially well known for their function in macrophages.[87] Group B SRCR domains usually contain eight regularly spaced cysteines that allow the formation of a well-defined intradomain disulfide-bond pattern. Scavenger receptors are best known for their housekeeping function of taking up lipids modified by oxidation or acetylation, but they have many other functions as well, such as uptake of apoptotic bodies (eg, croquemort in *Drosophila* of the CD36 subfamily[88]).

SRCRs have been studied mainly in the coelomocytes of echinoderms. Within a few hours after bacterial injection, sea urchin coelomocytes upregulate a variety of genes including an extremely diverse family of SRCRs.[48,89] A very large number of SRCR domains are present (approximately 1,200), but each individual may express different groups of SRCR genes at different levels (and even with differential splicing). To assume that they are all involved in defense is premature, as SRCR genes can be both up- and downregulated after infection with bacteria. As mentioned, this high level of gene duplication is a general rule in the echinoderms.

In mammals, the SRCR family as a whole is also poorly defined but is involved in endocytosis, phagocytosis, and adhesion, and some members acts as PRRs that bind to LPS or other bacterial components. SRCRs are widespread in the human genome and participate as domains in the structure of numerous receptors (eg, S4D-SRCRB, CD6, CD5-L, CD163), but without showing the high level of duplication seen in the echinoderm families.[87]

Down Syndrome Cell Adhesion Molecule

DSCAM in *Drosophila* and other arthropods was described originally by neurobiologists as an axon-guidance protein, dependent upon a large number of isoforms (> 30,000) generated by alternative splicing for the IgSF domains and the transmembrane segment. DSCAM is also involved in insect immunity, expressed in cells of the hematopoietic lineage, and clearly capable of binding to bacteria; like in the nervous system, a large number of splice variants are generated,

clearly different from the ones expressed in neurons.[24] In *Drosophila*, the DSCAM gene is composed of 115 exons, 95 of which encode alternative possibilities for splicing of exons 4, 6, and 9. The molecule consists of 10 IgSF domains and 6 FN domains, and present as either a membrane or soluble form, presumably generated by proteolysis of the membrane form. Each cell expresses only a fraction of the isoform repertoire.

Knock out (RNAi) and anti-DSCAM treatment significantly suppresses phagocytosis, at least in *Drosophila*. Soluble DSCAM constructs with different exon combinations were found to have differential pathogen-binding properties.[90] In addition, suppression of DSCAM in mosquitoes results in an impaired immunity to *Plasmodium*; exposure of hemocytes to different pathogens in culture gives rise to specific modifications and selection of alternative splicing patterns. A similar finding was made in crustaceans, in which particular DSCAM isoforms were induced in response to different pathogens in one species[91] and epitope II was under selection in a study in *Daphnia*.[92] The diversification of DSCAM seems to be specific of arthropods as neither flatworm nor sea urchin nor vertebrate DSCAM are diversified. The vertebrate DSCAM has only two forms, using two alternate TM exons. The

cytoplasmic tail can also be modified by alternative splicing that could change its signaling properties by modulation of tyrosine-based motifs.[93] Human DSCAM is duplicated on chromosomes 21 and 11, but does not appear to be involved in immunity.

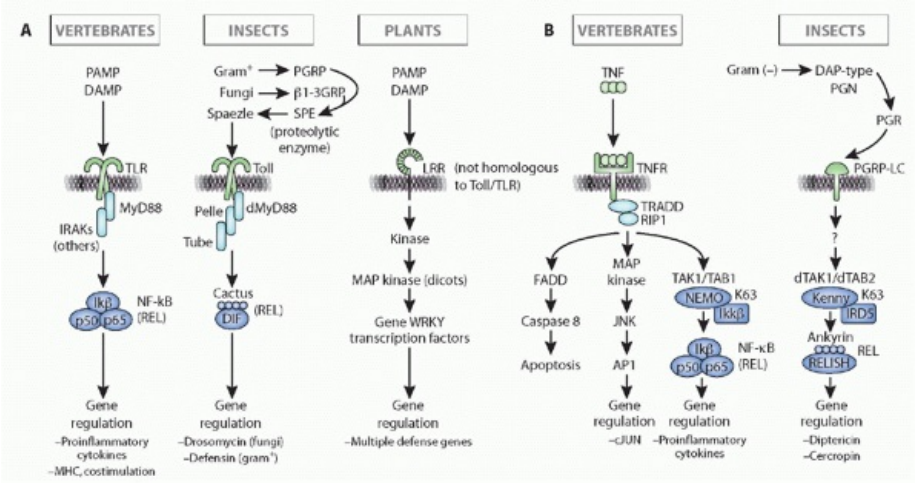

FIG. 4.5. Comparison of Immune Response Induction, Intracellular Pathways, and Immune Outcome in Insect (eg, *Drosophila*), Vertebrates (eg, human), and Plants (a Composite of Pathways in Monocots and Dicots). Note that the initiation of the response and the outcome(s) in insects and vertebrates are quite different for both the toll/toll-like receptor pathway (*left*, **A**) and the immune deficiency/tumor necrosis factor pathways (*right*, **B**), but the intracellular signaling pathways are well conserved evolutionarily in insect and vertebrate (details in the text). Note as well that plants use similar molecules for recognition (leucine-rich repeat-containing molecules) and have similar intracellular pathways with kinase cascades, but *all* of the molecules of recognition, signaling, and effector are derived by convergent evolution as compared to animals. This figure was modified from Beutler et al.[152]

Peptodoglycan-Recognizing Protein and β1-3 Glucan Receptors

PGRPs are found in a wide range of organisms but have been best studied in insects, where they are classified into short (S) and long (L) forms. S forms are soluble and found in the hemolymph, cuticle, and fat-body cells.[94] L forms are mainly expressed in hemocytes as integral membrane proteins where their final structure depends on combinatorial association of different isoforms, modulated by alternative splicing. We provide a short description here, but delve more deeply in the discussion of the insect toll and immune deficiency (IMD) pathways subsequently (Fig. 4.5).[36] The expression of insect PGRPs is often upregulated by exposure to bacteria. PGRPs can activate the toll or IMD signal transduction pathways (see the following) or induce proteolytic cascades that generate AMPs, melanization, or induce phagocytosis. PGRPs directly kill bacteria by inducing a suicide mechanism, first demonstrated to be activated by a type of unfolded protein (stress) response in prokaryotes.[95] Besides their defense functions, insect PGRPs expressed in the gut are believed to promote homeostasis with commensal bacteria (also discussed briefly in the

following). Both soluble and transmembrane forms are present in sea urchins, some with potential catalytic function.[48]

In vertebrates, PGRPs are all secreted and have direct microbicidal activity. Best studied in zebrafish, PGRPs are

expressed in many tissues such as gills, skin, and intestine, providing immune defense. They are expressed before the development of adaptive immunity and likely provide an important protective role.[96] The human PGRP genes are found on the MHC chromosomal paralogs 1q21 and 19q13/p13. All detected splice-variant isoforms bind to bacteria and peptidoglycan. Like the fish molecules, mammalian PGRPs are also positioned at epithelial surfaces and promote intestinal homeostasis by discriminating somewhat between commensal (eg, lactobacilli) and pathogenic bacteria. Knockout mice have increased pathogenic bacteria on mucosal surfaces that induce colitis after injury in the dextran sulphate sodium autoimmune assay.[97]

β1-3 glucan receptor proteins (β1-3GRPs, formerly known as gram-negative binding proteins (GNBPs) are related to bacterial β1-3 glucanases.[98] They are found in insects and other arthropods where they bind bacteria, fungal β-1, 3-glucans, LPS, and/or bacterial lipoteichoic acid (without necessarily showing glucanase activity). An ortholog is present in the sea urchins, but not in vertebrates to date. *Drosophila* GNBP1 together with PGRP-SA are required to activate the toll pathway in response to infection.[36]

Toll and Toll-like Receptors

The toll receptors were originally described in *Drosophila* as genes involved in early development, specifically in dorsoventral patterning. Later, they were also shown to be essential sensors of infection, initiating antimicrobial responses.[36,99] This family was then revealed to be a major force in innate immunity in the vertebrates as well.[100,101] As mentioned previously, across the metazoa structurally closely related members of the toll family range from not being involved in immunity (in *C. elegans* and apparently in the horseshoe crab), to being the equivalent of a cytokine receptor (in *Drosophila*), to being PRR in the vertebrates and invertebrates.[102] Six spaetzle-like and eight toll-like molecules have been identified in *Drosophila*, but only one or two of them are clearly immunity-related.[36,102] In jawed vertebrates, they belong to a multigene family of PRR specific for diverse PAMPs and exhibiting different tissue distributions and subcellular locations.[27] In humans, many are on chromosome 4p and q (TLR 2, 1, 6, 10) but the others are distributed on chromosomes 9, 1, 3, and X.

Ectodomains of TLRs comprise 19 to 25 tandem repeats of LRR motifs made of 20 to 29 aa capped by characteristic N- and C-terminal sequences. All of the toll receptors are homologous and appear similar in domain constitution among all animals. They also share the TIR domain, which is the intracellular segment shared with the IL-1/-18/-33 receptors of vertebrates, as well as other molecules in plants. TIR domains associate with Myd88 to initiate signaling cascades culminating in the activation of NFκB/Rel (see the following) (see Fig. 4.5).

In *Drosophila*, the toll dimer is triggered by an interaction with the unique ligand spaetzle, which is the product of a series of proteolytic cascades, with the most critical enzyme identified (spaetzle-processing enzyme). Activation of the cascades triggers the production of antimicrobial peptides (see Fig. 4.5). The specificity of recognition is not achieved at this receptor level but rather in solution via other intermediates (see the following). *C. elegans* has only one toll receptor, and rather than being antimicrobial responses, it promotes avoidance of a flatworm pathogen upon engagement; the signaling mechanism for the *C. elegans* TOL (toll) is not evolutionarily conserved (it clearly does not induce the NF-κB pathway) and is under investigation.[67] A toll/TLR gene is present in the sea anemone, a cnidarian, but not in other cnidarians such as hydra or coral, which nevertheless have TIR domains associated with other molecules.[35] A TIR domain of the toll-receptor types was detected in sponges, but, like IL-1R in vertebrates, it is associated with a receptor with three IgSF domains.[103] Plants do not have toll/TLR per se, but do have LRR-containing transmembrane sensors that function in a similar fashion[102] (see Fig. 4.5). In summary, toll/TIR arose before the split of protostomes and deuterostomes, but has been lost in some invertebrate groups and has been recruited to perform multiple functions.

The arsenal of TLRs in vertebrates is endowed with specific and diverse capacities. Each vertebrate TLR has its range of specificities and, in addition, combinations of different TLR can create different binding specificities (eg, the association of TLR2 with TLR6 or TLR1 and 2[104] or even TLR2 homodimers in regulatory T cells[105]). This divergence in recognition function is well illustrated by the phylogenetic analysis of the toll and toll-related receptors in different phyla such as arthropods and vertebrates. Toll and related proteins from insects and mammals cluster separately in the analysis, indicating independent generation of the major families in protostomes and vertebrates.[102] Consistent with the expansion of SRCR and NLR genes in sea urchins, hundreds of TLRs also were found in this species.[48] TLRs of the protostome type are in small numbers (3 members) while the vertebrate type has been enormously amplified, all within a single family (222 members) and most without introns. Vertebrate TLRs do not diverge rapidly and evolve at about the same rate, and while there have been some duplications in amphibians and fish, they are not greatly expanded like in the echinoderms (no more than approximately[20] genes in any species).

Signaling Through Innate Surface Recognition Molecules

Four pathways of innate immunity triggering have conserved elements in eukaryotes: the toll/TLRs, the TNF-α/IMD receptors, the intracellular NOD, and the JAK/STAT. Although toll receptors have been found in almost all triploblastic coelomates, most of the work and the elucidation of pathways have been accomplished in *Drosophila* and *Anopheles*.[36] The diversity of AMPs that can be produced via the toll/IMD pathways is substantial, and as described previously is classified in several categories depending upon the type of pathogen recognized (eg, gram (+), drosocin, gram (-), diptericin; fungal, drosomycin) with different effector functions (see Fig. 4.5). Insect antimicrobial molecules were originally discovered by the late Hans G. Boman and colleagues in 1981, a seminal finding that

heralded the molecular analyses of innate immunity in the invertebrates.[106]

Toll and Immune Deficiency Pathways

As described, invertebrate toll receptors are homologous to the vertebrate TLR, in the sense that they are integral membrane LRR-containing proteins (see Fig. 4.5). *Drosophila* toll is activated after it binds spaetzle, the product of a proteolytic cascade activated in solution after the interaction of molecules produced by fungi or gram-positive bacteria with GNBP and PGRP.[107] The TIR cytoplasmic domain of the toll receptor then interacts with MyD88 (itself having a TIR domain) followed by Tube and Pelle, leading to activation of the homologous NF-κB system (Cactus or Diff) that then induces transcription of various defense peptides.[36,99,108] This is remarkably similar to the cascade of events following activation of mammalian TLRs where after their interaction with PAMPs at the cell surface, a cascade is induced through TLR including MyD88, IRAK, TRAF, TAK1, to NF-κB via the IKK signalosome. Thus, infection-induced toll activation in *Drosophila* and TLR-dependent activation in mammals reveal a common ancestry in primitive coelomates (or previous), in which defense genes under the control of a common signaling pathway lead to activation of Rel family transactivators.

The *Drosophila* IMD pathway is employed in responses to gram-negative bacteria[109] (see Fig. 4.5). After interaction with the cell surface receptor PGRP-LC mentioned previously, in a cascade similar to the mammalian TNF-αR signaling pathway, *Drosophila* tak1, an IKK signalosome, and a Relish-mediated (instead of Diff) NF-κB step, results in transcription of antibacterial peptides like diptericin. The *Drosophila* intracellular pathway is similar to the mammalian TNF-α receptor cascade, which also progresses via a death domain Mekk3, the signalosome, and NF-κB resulting in cytokine production. In both cases, a link to pathways leading to programmed cell death is possible; overexpression of *Drosophila* IMD leads to apoptosis. When the activation of either the fly toll or IMD pathway is considered, they are analogous to a mammalian cytokine/cytokine receptor system (eg, TNF-α) in which a soluble self-molecule activates cells via a surface receptor. Fitting with the paradigm put forward on recognition, signaling, and effector phases of the immune response, the diversity of external recognition systems is not matched by an equivalent diversity of intracellular signaling pathways.[22] There are conserved signaling cascades coupled to the receptors, giving the impression of conservation of the innate immunity pathways; yet, these pathways are also used in development, so which is primordial remains an open question.

Plants do not have toll/TLR, but do have transmembrane LRR-containing microbial sensors, of which FLS2 that binds to flagellin, is best characterized[27] (see Fig. 4.5). These molecules do not have an intracellular TIR domain (note that TIR domains exist in plants, but not associated with the TM sensors), but do recruit a kinase of a similar nature to the toll/TLR kinases (the so-called non-RD kinases) to activate downstream mitogen-activated protein kinase cascades. However, as mentioned previously, the NFκB transcriptional system arose early in the animal kingdom[110]; plants employ a different system of transcriptional activators, the WRKY molecules, which are activated directly by the mitogen-activated protein kinase cascades, similar to transcription factor found in animals, AP1.

Extracellular Soluble Receptor with Effector Cascade

Proteolytic cascades are initiated immediately following interaction of foreign material bound by preformed proteins in solution, and this principle is conserved throughout evolution.

Indeed, the proteolytic cascade upstream of production of the toll ligand spaetzle resembles the complement or clotting cascades. The PPO cascade of arthropods leading to melanization and the genesis of antibacterial products described in the following is another example in which peptidoglycans on microbial surfaces initiate the cascade resulting in the degranulation of hemocytes.

The Complement System

The best-studied immune proteolytic cascade that is surprisingly well conserved in the animal kingdom is complement[111,112] (Fig. 4.6). In contrast to the other defense molecules that we have discussed, orthologous complement genes can be detected in all of the deuterostomes without a great deal of expansion/contractions of the gene family. The three major functions of complement in jawed vertebrates are 1) coating of pathogens to promote uptake by phagocytes (opsonization); 2) initiation of inflammatory responses by stimulating smooth muscle contraction, vasodilatation, and chemoattraction of leukocytes; and 3) lysis of pathogens via membrane disruption. Additionally, in the vertebrates, C′ is vital for the removal of immune complexes as well as elicitation of adaptive humoral immunity. The focal point of complement is C3, which lies at the intersection of the alternative, classical, and lectin pathways of complement activation. It is the only known immune recognition molecule (besides its homologue C4) that makes a covalent bond with biologic surfaces via a thioester linkage. C3 has a nonspecific recognition function, and it interacts with many other proteins, including proteases, opsonic receptors, complement activators, and inhibitors. In the alternative pathway, C3 exposes its thioester bond in solution, and in the presence of cell surfaces lacking regulatory proteins that block C3 activation (by cleaving it into iC3b), it associates with the protease factor B (B or Bf). After binding to C3, B becomes susceptible to cleavage by the spontaneously active factor D, resulting in formation of the active protease Bb that in combination with the covalently attached C3 cleaves many molecules of C3 in an amplification step. Another nonadaptive recognition system, the lectin pathway, starts with the MBL (or the lectin ficolin), which is a PRR of the collectin family that binds mannose residues on the surface of pathogens and can act as an opsonin. MBL is analogous to C1q with its high-avidity binding to surfaces by multiple interaction sites through globular C-terminal domains, but apparently it is not homologous to C1q. Like C1q, which associates with the serine proteases C1r and C1s, the MBL-associated serine proteases (MASPs) physically interact with MBL and not only activate

the classical pathway of complement by splitting of C4 and C2 (the same function as C1s; MASP2 appears to be the active protease), but also can activate the alternative pathway in ways that are not understood and thus completely bypass the classical pathway. Indeed, MASP-1 and -2 are homologs of C1r and C1s (see Fig. 4.6). Both C1q and MBL can promote the uptake of apoptotic bodies by phagocytes, via collectin receptors. Another lectin, ficolin, can also initiate the MASP pathway,[113] and it would not be surprising if other activators were discovered in the future (eg, the ancient molecule C-reactive protein is also capable of activating C′). Finally, the classical pathway, which is dependent upon antibody molecules bound to a surface, results in the same potential effector outcomes described previously for the alternative pathway. Novel molecules initiating this pathway are C1q, C1r, C1s, C4, and C2, as well as specific negative regulatory proteins such as C4-binding protein.

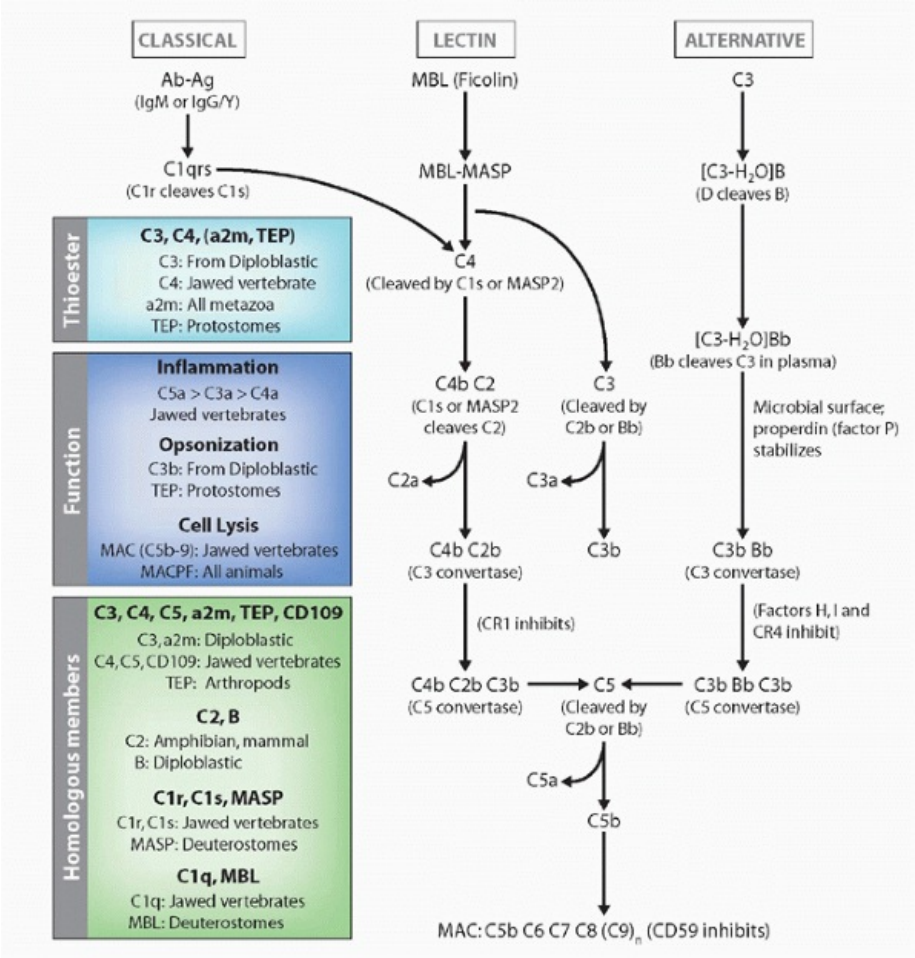

FIG. 4.6. Evolution of the Complement System. The general pathways and appearance of the various components in the phylogenetic tree are emphasized.

C3 and MBL (and ficolin) are vital players in the immediate innate immune response in vertebrates, and both have been described in nonvertebrate deuterostomes.[48,112] Thus far, the best-studied invertebrate systems for investigation of C3 evolution are the sea urchin and the ascidians *Halocynthia* and *Ciona*, in which C3 and B molecules and genes have been analyzed in some detail. In contrast to the very high levels of C3 found in the plasma of jawed vertebrates, sea urchin C3 is not expressed at high levels but is induced in response to infection in coelomocytes.[114] The C3 opsonic function clearly has been identified, but so far initiation of inflammatory or lytic responses (if they exist) has not been obvious. Receptors involved in the opsonization in echinoderms have not been identified, but in the ascidian gene fragments related to the C3 integrin receptor CR3 were identified, and antisera raised to one of the receptors inhibited C3-dependent opsonization.[115]

Hagfish and lamprey C3-like genes were thought to be ancestral C3/C4 genes because the sequence predicts two processing sites (leading to a three-chain molecule), like C4, but a C3-like properdin-binding site is clearly present.[116] However, like C3 in other animals the hagfish protein is composed of only two chains of 115 and 72 kDa, and sea urchin and ascidian C3 sequences predict only two chains (one proteolytic processing site). The

lamprey, but not sea urchin C3, has a recognizable C3a fragment known from gnathostomes to be involved in inflammation, so the role of complement in inflammation may be a vertebrate invention (but see the following).

TEPs have been isolated from *Drosophila* and the mosquito *Anopheles*, as well as several other arthropods.[117,118] While the insect molecules function in a C3-like fashion (opsonization), phylogenetic analysis shows them to be more related to α2-macroglobulin (note that a few insects actually have molecules more related to C3). TEPs in insects function as opsonins, binding to parasites and promoting their phagocytosis or encapsulation. The evolution multimember TEP families in *Drosophila* and mosquito followed independent evolutionary paths, perhaps as a result of specific adaptation to distinct ecological environments as described in the introduction. The *Drosophila* genome encodes six TEPs (whereas there are 15 genes in *Anopheles*, again consistent with the major expansion of many immune gene families in the mosquito), three of which are upregulated after an immune challenge. Mosquito TEPs are involved in killing of parasites, and the reaction is regulated by LRR-containing molecules to avoid destruction of self-tissues; thus, full-blown complement-like systems complete with inhibitors have arisen independently in protostomes and deuterostomes.[119,120]

C3-like genes are present in cnidarians[121,122] and in the horseshoe crab *Limulus*.[123] Good phylogenetic support was obtained for their relationship to C3, as compared to other members of the thioester-containing family like the TEPs. Thus the emergence of C3 as a defense molecule predates the split between protostomes and deuterostomes. A gene resembling the proteolytic enzyme Bf was discovered in these protostomes as well (and in sea anemones), suggesting that the fundamental system was in place a billion years ago (see Fig. 4.1). The lack of C3 in many other protostomes suggests that the ancestral gene was lost and replaced by the TEPs.[117,118]

In jawed vertebrates and some lower deuterostomes, certain species express more than one C3 gene, suggesting that the innate system might compensate in animals that do not optimally make use of their adaptive immune system.[124] Changes in the amino acid composition of the C3-binding site are found that may somehow regulate the types of surfaces bound by the different isotypes.[125] Likewise, in lower chordates such as *Ciona*, C3 and other complement components can be duplicated.[115] Diversification of the carbohydrate recognition domains has been observed also in the *Ciona* MBP family (nine members).

Like Ig/TCR/MHC, the classical pathway and the terminal pathway membrane-attack complex (MAC) appears first in cartilaginous fish.[112] However, as MBL can activate the classical pathway in mammals, it is possible that some portion of this pathway exists in prejawed vertebrates. Nevertheless, C4 and C2 genes have not been detected to date in jawless fish or invertebrates. A bonafide C2 homologue has only been identified to the level of amphibians, although duplicate B genes were isolated from cartilaginous fish and teleost fish that may function both in the classical and alternative pathways. The lytic or MAC pathway, which is initiated by the cleavage of C5 into C5a and C5b, also has not been described in taxa older than cartilaginous fish. Thus, opsonization and perhaps the induction of inflammatory responses were the primordial functions of the lectin/complement pathways. However, a complementary DNA clone for CD59, a molecule that inhibits MAC formation in

self-cells, was identified from a hagfish library, and some of the terminal components of the pathway have been detected in lower deuterostomes with no described functions.[116] Interestingly, proteins with the MAC/perforin domain have been detected throughout the animal kingdom,[48] and some are even involved in cytotoxic reactions; however, it seems that only vertebrates have bonafide terminal C′ components that are highly evolved for targeted destruction of cell membranes. The perforin gene itself also seems to have arisen in gnathostomes, from an ancient MAC/perforin domain-containing gene, macrophage-expressed gene 1 protein, which dates back to sponges; thus, cellular cytotoxic reactions in the invertebrates described in the following must use novel cytotoxic effector molecules.[126]

C3, C4, C5, and α2m (and TEP) are members of the same small family. A cell-surface-expressed (GPI-linked) member of this family, CD109, has been shown to associate with the transforming growth factor (TGF)-β receptor and modulate its expression.[127] The protease inhibitor, α2m, clearly present in invertebrates (protostomes and deuterostomes) and vertebrates, is thought to be the oldest, but obviously this must be viewed with caution considering the data in cnidarians. Along with its ability to bind to and inactivate proteases of all known specificities through a “bait region,” it also has been shown to be opsonic in some situations. α2m, C3, C4, and CD109 (as well as the TEPs) have internal thioester sites, so this feature is primordial; C5 subsequently lost the site. The first divergence probably occurred between α2m and C3, with C5 and then C4 emerging later in the jawed vertebrates.[128] Consistent with Ohno's vertebrate polyploidization scheme is the fact that C3, C4, and C5 genes are located on three of the four previously described paralogous clusters in mammals, and this is also fits with the absence of classical (no antibody) and lytic (no MAC) pathways in phyla older than cartilaginous fish.[2] α2m is encoded at the border of the NKC in mice and human, and there are similarities between these regions and the other MHC paralogs (see Fig. 4.13). The C3a and C5a receptors that promote the inflammatory responses upon complement activation have been identified in several vertebrates and (perhaps) some lower deuterostomes; like the chemokine receptors they are G-protein coupled receptors whose genes may also be found on the ohnologs (C3aR, chr 12p13; C5aR, chr 19q13). If indeed such receptors are found in the prejawed vertebrates

as suggested by recent pioneering experiments in *Ciona* and *Styela*, it will be interesting to determine whether they are involved in some type of inflammation, thought to be the domain of the vertebrates.[129]

Melanization (Prophenoloxidase Cascade)

A major defense system in invertebrates is the melanization of pathogens and damaged tissues,[130] popularized by poor Gregor in Kafka's *Metamorphosis*, when the cockroach Gregor undergoes a melanization reaction from an apple thrown into his thorax by his father. The process is controlled by the circulating enzymes PPO and phenol oxidase. The system is activated by β1-3GRP, PGRP, LPS-binding proteins, and other proteins that can bind to various PAMPs (see Fig. 4.3). The complexes launch a cascade of serine protease activities resulting in cleavage of the pro-form of a prophenoloxidase-activating enzyme into the active form that in turn activates the PPO into phenol oxidase. This leads to the production of quinones and finally melanin. Melanization can completely inhibit parasite growth, whereas

concomitant with PPO activation, many other immune reactions are initiated, such as the generation of factors with antimicrobial-, cytotoxic-, opsonic-, or encapsulation-promoting activities. The presence of specific proteinase inhibitors (of the serpin family) prevents unnecessary activation of the cascade and overproduction of toxic products. Phenoloxidase is the key enzyme responsible for the catalysis of melanization. It is a marker of the PPO activating system, and it can be an immune effector by itself as demonstrated in ascidians. It is therefore interesting to assess its conservation within all metazoa. A survey of the different organisms revealed the presence of phenoloxidase in many deuterostome and protostome phyla, and related molecules are also present in sponges. In arthropods, several PPO genes are present in the genome (nine in *Drosophila* and *Aedes*). Some may have different "immune" functions such as injury repair. Several components that would maintain the role of melanization in immunity may be lacking in different phyla even if many elements are conserved, and so far the best examples of melanization associated with immunity are still found almost exclusively among arthropods and to a lesser extent in annelids. Despite the presence of molecules involved the pathway, the PPO cascade per se does not exist in vertebrates.

Effector Molecules

Peroxidasin

Among molecules containing LRR motifs, peroxidasin occupies a special place because of its involvement in hemocyte biology in insects and because of its homology to the LRR motifs in the agnathan VLR and Ig domains similar to Ig itself. *Drosophila* peroxidasin is an assembly of a cysteinerich motif, six LRR, and four IgSF domains.[131] The molecule is conserved in vertebrates, although a role in immunity has not been reported. Another molecule called peroxinectin, with similarity at the level of the peroxidase region, has been described in crustaceans and shown to be associated with immunity via the PPO cascade.[132] Its involvement in immunity is unlike any other effector so far described but illustrates the utility of LRR in many different types of molecules and processes. Pathogens bound by AMPs can be phagocytosed or walled off by a barrier of flattened hemocytes and ECM. The ECM forms a basement membrane that becomes stabilized partly through peroxidases that generate tyrosine-tyrosine bonds. The combination of LRR and Ig structures suggests that peroxidasin may precisely mediate adhesion of cells to the ECM.

As mentioned, a large number of LRR-Ig-containing proteins has been discovered, most of them playing roles in embryologic development.[32] Many LRR-Ig proteins are encoded in paralogous regions in the vicinity of immune genes, showing an ancient direct connection between the families (see the following).

Fibrinogen-Related Proteins

FREPs are proteins that were first discovered in the hemolymph of snails with an IgSF moiety (one or two V-like domains) and a fibrinogen domain. The fibrinogen domain is found in a large number of defense molecules throughout the animal kingdom (eg, the ficolins). FREP gene expression is upregulated following exposure to the mulluscan parasites such as schistosomes[133]; a snail strain resistant to schistosomes shows an upregulation of the FREP 2 and 4 genes of up to 50-fold. The original discovery of FREPs followed the recovery

of snail proteins that bound to worm antigens, and thus this is one case in which the correlation between an invertebrate receptor and its ligand is clear. However, it is not known whether the IgSF or fibrinogen domain (or both) bind to the antigen or which effector functions are induced after FREP binding.

FREP diversity is remarkable in that there are many polymorphic genes as well as alternate messenger RNA splicing to generate the diversity. In addition, based on the number of genes and alleles in individual snails, there appears to be a somatic diversification mechanism that modifies FREP genes, either via mutation or gene conversion in the region that encodes the IgSF domains.[134] Over 300 unique sequences were found in 22 snails, consistent with a somatic diversification mechanism. Currently, there is no mechanism to account for the mutations, but data accumulate for somatic modifications and the use of FREPs in critical defense against pathogens.[135]

FREPs are also present in arthropods where they lack the Ig domains,[136] once again with an expansion of genes in the mosquito *Anopheles gambiae* as compared to *Drosophila*. RNAi studies have shown that subsets of FREP genes are vital for defense against malarial parasites, and different FREPs bind to bacteria with different affinities.[137] Homo-and heterodimers can form between different FREPs, and multimers can be fashioned that likely increase the avidity of binding. In summary, this ancient family of defense molecules has all of the attributes described in the introduction: rapidly evolving multigene family, conservation in divergent protostomic invertebrate phyla, somatic diversification via alternative splicing, and perhaps an unknown mutational/gene conversion mechanism, as well as heterodimeric (and multimeric) association.

185/333

There are a number of additional expanded gene families in the sea urchin genome that encode proteins with immune-related functions. The *185/333* genes were first noted because they are highly upregulated in coelomocytes after exposure to LPS, constituting up to 7% of the messenger RNAs in such cells.[138] Subsequently, transcripts were shown to be upregulated by many different types of PAMPs. The encoded proteins have no detectable similarity to any other gene family, but they are highly diversified and are produced (at least) by a subset of coelomocytes.

There are estimated to be at least 50 185/333 genes in the sea urchin genome.[139] The gene is composed of two exons, one encoding the leader and the other encoding the mature protein. The second exon is made up of so-called elements and repetitive sequences that are quite different from gene to gene, which can to a large extent explain the diversity of expressed loci. However, there are hints of RNA editing, a unique form of alternative splicing, somatic mutation (perhaps targeting cytosine residues), and other (perhaps) novel mechanisms of diversity to explain the incredible number of different isoforms; additionally, like many other defense molecules, there is evidence of protein multimerization. Whatever the mechanism of diversity generation, it would be surprising if this family were not vital for defense in echinoids. Furthermore, the presence of this unique multigene family in sea urchins is consistent with the great expansion of other immune gene families in this group, including TLRs, SRCRs, and NLRs.[7]

Variable Domain Chitin-Binding Proteins

VCBP, first discovered in *Amphioxus* but present in *Ciona* as well, consist of two Ig domains of the V type but with a different folding motif when compared to Ig or TCR V domains[140] followed by a chitin-binding domain. The chitin-binding domain resembles chitinases found throughout the animal kingdom, and like dedicated chitinases VCBP is usually expressed in the gut. Apparently, there are no cell-surface-expressed forms and thus all VCBPs are likely to be secreted, effector molecules. In *Amphioxus*, their diversity is enormous, apparently entirely because of polymorphism and polygeny, and not somatic alterations. Each individual can carry up to five genes per haplotype, and in limited studies (11 individuals), no identical haplotype has been encountered.[141,142] The general structure of the V domain is like that of the vertebrate rearranging antigen receptors, but with some unusual properties, including packing in a "head-to-tail" dimeric fashion, totally unlike Ig and TCR. VCBP diversity does not reside in the Ig/TCR CDR resides, but rather in the A, A′, and B strands, like in DSCAM.

By contrast to the *Amphioxus* VCBP, there are only a few nonpolymorphic *Ciona* VCBP genes, which are expressed by gut epithelium and amebocytes. Soluble VCBPs bind to bacteria and induce opsonization in amebocytes. It is hypothesized that these molecules may perform a function similar to mucosal IgA in vertebrates, which provides a "firewall" protecting from invasion of intestinal bacteria and promoting homeostasis.[143] If true, this would provide a link to the regulation of commensals in early deuterostomes. It should be noted here, however, that recent data suggest that "tolerance" of commensals occurs in the protostomic invertebrates as well.

Antimicrobial Peptides (Defensins)

Each metazoan taxon produces a variety of molecules with intrinsic antimicrobial activity,[144,145] the majority of which fall into three major categories: defensins, catelicidins, and histatins. Even in species with very small genomes (such as the tunicate *Oikopleura* [60 Mb genome]), selection pressures have been strong enough to lead to expansion of the Phospholipase a2 family, with 128 members.[146] Some families are evolutionarily conserved but generally they diverge rapidly and orthologous relationships are not apparent. The best studied group of AMPs is the *defensins*, which are amphipathic cationic proteins; their positively charged surface allows them to associate with negatively charged membranes (more common in pathogens), and a hydrophobic surface that allows them to disrupt the membranes, either by disordering lipids or actually forming pores. Most of the molecules are proteins, but an antimicrobial lipid called squalamine, which also is modeled to have hydrophobic and positively charged surfaces, is found at very high levels in dogfish and lamprey body fluids.[147] Defensins can either be constitutively expressed (eg, in respiratory epithelia in mammals) or inducible (eg, see the following for *Drosophila* and see Fig. 4.5). Certain responses that seem systemic, like the production of *Drosophila* defensins, can also take place locally in the damaged tissues themselves; otherwise, a systemic response is initiated in organs distant from the site of infection such as the fat body in *Drosophila* where induction of bactericidal peptide expression occurs.[36] Defensins are the focus of great attention in commercially bred species such as oysters, mussels, and crustaceans. Besides their direct defense functions, in mammals defensins play other roles, such as chemotaxis

and immune regulation.

Penaedins. One set of diverse AMPs is the penaedins, present in crustaceans (shrimp). Penaedins are small antimicrobial peptides (5 to 7 kda) that bind to bacteria and fungi, and consist of a conserved leader peptide followed by an N-terminal proline-rich domain and a C-terminal cysteine-rich domain.[7,148] Most of the diversity is found in the proline-rich domain,[149] suggesting that it is most important for recognition, but both domains are required for recognition of bacteria and fungi. Four classes of penaedins, PEN2 to 5, are expressed by shrimp hemocytes. A great diversity of isoforms is generated, with substitutions and deletions within the proline-rich domain, suggesting that this domain recognizes ligand; nevertheless, both domains seem to be required for function. Like the VCBPs, each penaeidin class seems to be encoded by a unique gene and isoform diversity is generated by polymorphism. Multiple copies of penaedin genes are present in different species, and there is rapid expansion and contraction even within closely related organisms.

Responses to Viruses in the Invertebrates

Compared to immunity to extracellular pathogens in the invertebrates, the study of responses to intracellular pathogens like viruses is in its infancy.[150] First discovered in plants and in *C. elegans*, the RNAi pathway of defense against viruses is also operative in *Drosophila* and *Anopheles*.[151] Doublestranded RNAs (viral or otherwise) are recognized by the enzyme Dicer 2, generating small interfering RNAs that can associate with complementary RNAs and induce their degradation.

Viruses also induce an “IFN-like response” through a cytokine receptor (domeless) that is homologous to the IL-6 receptor and signals through the JAK/STAT pathway.[152] After viral infection, unknown signals (RNA, perhaps) induce the production of cytokines of the unpaired family that bind to domeless on neighboring cells and upregulate a large number of genes involved in defense (see Fig. 4.3). Nothing is known about the effector pathways of these responses, but mutants of one of the induced genes results in increased viral load. It should be noted that this pathway is not cell autonomous, inconsistent with the IFN pathway in vertebrates.

In a paradigm-changing paper, foreign antigen was shown to directly interact with *Drosophila* toll7, resulting in the induction of antiviral autophagy and inhibition of viral replication.[153] Toll-7 interacted with the glycoprotein from vesicular stomatitis virus at the cell surface to initiate the response. Thus, the dual paradigm of indirect and direct recognition by toll and TLR, respectively, must now be modified. There are several other tolls without functions in insects that might be involved in immunity, perhaps via such mechanisms.

In summary, arthropods (and presumably other invertebrates) use an RNAi pathway as well as a signaling pathway to combat viruses.[150,152] As opposed to the systemic plant RNAi response, the same pathway in protostomes is rather cell autonomous. This response was lost in the vertebrates, presumably because 1) viruses have been able to effectively counter this response and render it ineffective; 2) there have been remarkable evolutionary innovations in the vertebrate innate and adaptive immune systems to combat viruses; and 3) vertebrates use a Dicer pathway extensively to regulate expression of their own genes. The

discovery of a viral immune response quite like a type I IFN response in vertebrates demonstrates that the three major signaling pathways of defense in *Drosophila*, toll, IMD, and JAK/STAT, are similar to the vertebrate TLR/IL-1R, TNF, and IL-6/IFN pathways, respectively (the first two homologous). Finally, other insect tolls may interact directly with PAMPs to promote antiviral defense such as autophagy or apoptosis. This is a field in which rapid progress will be made in the near future.

Natural Killing Activity Across Metazoa

The word "cytotoxicity" encompasses vastly different protocols of cell killing by of different cell types. It can be an effector function of cells of the adaptive (cytotoxic T-lymphocyte [CTL] or NKT cells) or of the innate arm (bonafide NK, see the following) of the jawed vertebrate immune system. Similarly the term "NK cells" covers different cell types and different functions. NK cells of vertebrates can "recognize" missing self-MHC class I, but also ligands induced on stressed cells following virus infection, transformation or stress. They can also have an immunoregulatory role by interactions with antigen-presenting cells (APCs). Many of these features obviously profit from a comparative approach.

In natural killing, the common denominator is a spontaneous reaction (ie, it does not require any [known] antigenic priming but only cell contact). Some form of natural killing can be observed from the earliest metazoans onwards. Some marine sponge and corals avoid fusion with one another by mechanism of cytotoxic cells or induce apoptosis at the level of the teguments.[154] Phenomena more similar to vertebrate NK killing are observed in sipunculid worms where allorecognition among populations was shown to result in killing of allogeneic erythrocytes by lymphocyte-like cells.[155] Similar cases can be also encountered in annelids and mollusks,[156] The role of IgSF and lectin receptors known to be involved as NK cell receptors in vertebrates has not been examined in these invertebrates, even though some candidate homologs have been identified. Note that the mechanism(s) cannot be perforin-mediated, based on the recent bioinformatics analysis on MAC/perforin domains described previously in the complement section.[126]

When comparative morphology or function is not informative, searching for conservation of transcription factors or cell surface markers may be useful. Survey of IgSF genes across databases have not yielded any promising candidates to date. Despite the presence of polymorphic IgSF members of the receptor tyrosine kinase family in sponges, their role in allorecognition or killing has not been demonstrated.[157] As mentioned, similarities were found among the lectin families, especially in prochordates where cytotoxicity has been reported and associated with a discrete population of hemocytes the granular amoebocytes. The urochordate genome (*Botryllus*, *Halocynthia*, *Ciona*) encodes many lectins with or without typical carbohydrate recognition signatures, among them a putative CD94 homolog has been cloned and its expression followed in *Botryllus*[158]; its predicted sequence does not match well to vertebrate CD94. This gene is differentially regulated during allorecognition in *Botryllus*, and a subpopulation of blood cells has the receptor on their surface in both *Botryllus* and *Ciona*. Phagocytosis is inhibited by an antiserum recognizing the *Ciona* homologue.[159]

Other C-type lection homologs of CD209 and CD69 are linked to the CD94/L gene on *Ciona*

chromosome[1] (L.D.P., personal observation). Could they be part of a “pre-NK complex”? Interestingly, all the human homologs of those lectin genes are present either in the NK complex on 12 p13 (CD94, CD69) or on an MHC paralog 19p13 (CD209). Taken together with studies of the chicken MHC which encodes some C-type lectins (see the following), the data suggest that a conserved MHC-linked region containing several lectin genes was present before the emergence of MHC class I and II genes.[2]

In addition, a number of genes encoding membrane proteins with extracellular C-type lectin or immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and immunoreceptor tyrosine-based activation motif s (ITAMs) (plus their associated signal transduction molecules) were identified in *Ciona*, which suggests that activating and inhibitory receptors have an MHC class I- and II-independent function and an early evolutionary origin.[160] The ligands of these *Ciona* molecules are of great interest to uncover.

Natural Killer and Natural Killer-Like Cells

NK cells express both activating and inhibitory cell surface receptors; in fact, the paradigm for positive and negative signaling via such receptors began with these cells[60,161]; however, activating and inhibitory receptors (often paired) are conserved throughout vertebrates and invertebrate deuterostomes, and are expressed in hematopoietic cells of all types. In NK cells, stimulation of the activating receptors, which associate with proteins having an intracellular ITAM (CD3ζ, DAP12, or DAP10 conserved at least to the level of bony fish[162]), results in killing of target cells. Inhibitory signaling receptors all possess cytoplasmic ITIMs, which recruit phosphatases and generally are dominant over the activating receptors. These receptors fall in two categories IgSF and C-type lectin group V(II). In general, NKRs recognize MHC class I molecules of either the classical or nonclassical type, the latter sometimes encoded by viruses.[163]

General Evolution of Natural Killer Receptor Families . As mentioned, NK cells in mammals can use different types of receptors, even encoded by different gene families, IgSF (KIR) in primates and C-type lectins (Ly49) in rodents. A few receptors are conserved but most others are highly variable. Very few families show conservation of domains throughout the jawed vertebrates.[163] When dealing with the origin of these genes in invertebrates, one has to imagine under what pressures they evolved. The question remains as to whether NK cells, or NK-like cells, preceded the emergence of T- and B-lymphocytes.

As described in the introduction, NKRs are the most rapidly evolving molecular component of the gnathostome immune system. Most ligands for these diverse NKRs are MHC class I molecules, or molecules of host or pathogen origin related to MHC class I. The KIR families are divergent, as very few genes are conserved even between chimpanzees and humans, and there are different numbers of genes in KIR haplotypes within a species. Humans have two KIR haplotypes A and B, one encoding a large number of activating receptors and the other very few.[23] It is speculated that the haplotypes are under balancing selection within the population both for defense against virus and for maternal/fetal interactions. By contrast, CD94 and NKG2 receptors are conserved throughout mammals. So whereas receptors for polymorphic class I molecules are divergent, those for nonpolymorphic or stress-induced

class I molecules are relatively conserved (despite the fact that their ligands Qa1 and HLA-E are not orthologous). NK cells play other important roles in other innate immune responses, for example in antiviral immunity. NK cell recognition of virus-infected cells engages the activating KIR and Ly49 receptors and NKG2D in this process. Thus, viruses are hypothesized to supply the evolutionary pressure on diversification of NKRs. In fact, it has been shown in mice that inhibitory receptors can rapidly mutate into activating receptors when viral "decoy" class I molecules evolve to engage inhibitory receptors.[164] Generally speaking, inhibitory receptors are older and more conserved, whereas activating receptors evolve more rapidly and can be derived from inhibitory receptors via mutations that result in loss of the ITIM.[165]

Comparative Studies of Natural Killer Function . NK cells were detected in *Xenopus* by in vitro ^{51}Cr-release assays. Splenocyte effectors from early thymectomized frogs spontaneously lyse allogeneic thymus tumor cell lines that lack MHC antigen expression.[166] This activity is increased after the injection of tumor cells or after treating the splenocytes in vitro with mitogens, suggesting lymphokine activation of the killers. Splenocytes isolated with an anti-NK monoclonal antibody (mAb) revealed large lymphoid cells with distinct pseudopodia. Immunohistology indicated that each anti-NK mAb routinely labeled cells within the gut epithelium but NK cells were difficult to visualize in spleen sections.[167]

In amphibians, NK cell studies are especially interesting because of natural experiments done by nature (ie, the absence or low levels of MHC classical class I during larval life of some species like *Xenopus*).[168] They are bonafide NK cells, distinct from T cells, as they fail to express TCR Vβ transcripts. NK emerge in late larval life, 7 weeks postfertilization, which is about 2 weeks after the time when cell surface class I can be detected. The proportion of splenic NK cells remains very low until 3 to 4 months of age, but by 1 year there is a sizeable population. Therefore, NK cells fail to develop prior to MHC class I protein normal expression (at least NK cells of the type that can be measured with these assays and with NK cell-specific mAbs) and do not contribute to the larval immune system, whereas they do provide an important backup for T cells in the adult frog by contributing to antitumor immunity.

NK cells have also been described in a number of teleost fish with the most in-depth studies in catfish, in which there are clonal likes of cytotoxic cells,[167] some that clearly lack TCR expression.[169] A subset of the fish NK cell bears a highaffinity FcR that can be utilized for antibody-dependent cellular cytotoxicity.[170] Other subsets of NK cells spontaneously kill allogeneic targets. Further study of these cloned lines may provide much needed information on NK function in phylogeny.

Phylogeny of Natural Killer Lectins

Besides the well-described Ly49 family of receptors in rodents (and horse) and the CD94 and NKG2 families in all mammals, other mammalian NKRs are of interest. Studies in mammals have shown that some NKC-encoded lectin-like receptors in the Nkrp-I family can recognize other lectin-like molecules, termed Clr, also encoded in the NKC.[171] Having linked loci encoding receptor-ligand pairs suggests a genetic

strategy to preserve this interaction; perhaps the CD94 homologs of invertebrates are

genetically linked to genes encoding their ligands. In addition, as described in the following, the close genetic linkage of receptor and ligand genes is a common theme in "histocompatibility reactions" throughout the animal and plant kingdoms described below.

In chickens, a single gene similar to CD94/NKG2 is encoded in a region syntenic with the mammalian NKC.[172] It is linked to CD69, another C-type lectin also encoded in the NKC of mouse/human. Chickens and quail MHC encode two C-type lectin NKR B-lec and B-NK, the latter being most similar to NKPR1.[173] Other C-type lectins are found in the RFP-y locus, one that is also most similar to NKRP1.[174] These linkages give credence to the idea that the NKC and MHC were syntenic in early jawed vertebrates (see the following). While C-type lectin genes with some similarity to mammalian NKR have been detected in ectothermic vertebrates, no convincing orthology or synteny to the NKC has been found to date. In mammals including marsupials, NKG2D is conserved, and CD94/NKG2 is found in mammals and birds (as well as NKPR1).[163] If such C-type lectin NKR are found in the future in cold-blooded vertebrates, they will have to be studied in functional assays.

Given the apparent lack of MHC class I and class II in agnathans and their convergently acquired adaptive immune system (see the following), it is difficult to envisage how NK cells with receptors of any type might function in these animals. It should be mentioned, however, that sequence similarity might be difficult to detect for an MHC peptide-binding region (PBR), given the rapid rate of evolution of this gene family. Furthermore, it would not be shocking if there were NK cells with ligands encoded by other gene families—in mammals, ligands for some activating NKRs have not been identified. It would be of interest to study the non-VLR-expressing lymphocytes in agnathans (if such cells exist) for their killing potential or gene expression.

Phylogeny of Immunoglobulin Superfamily Natural Killer Receptors

IgSF-activating receptors have been recognized from cartilaginous fish onwards with a convincing activating NKp44 homolog first found in carp (called NILT), but with no functional data.[175] Subsequently, this family was found in other bony and cartilaginous fish and definitive orthology to mammalian NKp44 was shown. The activating receptor NKp30 is also conserved, with orthologs found to the level of cartilaginous fish[176]; in addition, as described in the MHC section, there are V(J) genes within the frog MHC called XMIV that are ancient homologues of NKp30 and may be NKRs of both activating and inhibitory types.[177] The ligand for NKp30 has recently been uncovered, the stress-induced molecule B7H6.[178] Interestingly, there is a perfect correlation between the presence or absence of NKp30 and B7H6 in the vertebrate line, with both genes lacking in birds and bony fish but present in all other gnathostome classes.[176] Furthermore, phylogenetic trees suggest a close relationship between NKp30 and NKp44, consistent with their ancient origins within the vertebrate line.

There are other bony fish-specific IgSF NKR families. One family, the novel immune-type receptors (NITRs),[179] have one or two Ig domains with a charged residue in the TM and could therefore be associated (by analogy) to an ITAM DAP12 equivalent, and indeed was shown to interact with mammalian DAP12.[162] Like NKp30, the NITR N-terminal V domain is also of the VJ type. NITRs were originally believed to be part of the LRC, but this was shown to be unlikely upon further analyses. NITRs can be expressed by cells of the hematopoietic

lineage, presumably lymphocytes. NITRs have been found in all bony fish, with rapid contraction and expansion of the gene family, with the majority of proteins predicted to have inhibitory ITIMs in their cytoplasmic tails. In zebrafish, there are many NITR genes that group into 12 distinct families.[180] An extreme level of allelic polymorphism is apparent, along with haplotype variation and family-specific isoform complexity. By contrast, only 11 related genes encoding distinct structural forms have been identified in the channel catfish, and the relatively small number of genes allowed functional studies to be performed. Additionally, taking advantage of the ability to grow clonal lines of catfish hematopoietic cells, one granular cell line lacking all markers of B/T cells was shown to express several NITRs. Expressed NITRs were fused to an ITAM-containing motif and transfected into a T-cell hybridomas line with a nuclear factor of activated T cells (NFAT) promoter, and the specificity for particular catfish MHC alleles was maintained.[181] Subsequent crystal structure analysis showed the NITR V domains to form dimers, much the same as Ig/TCR heterodimeric V regions. Thus, the sequence analysis (ITAM/ITIM), signaling properties, involvement in cytotoxicity, and recognition of MHC molecules identify the NITRs as excellent candidates for NKRs in bony fish. Furthermore, the work serves as a paradigm for study of potential NKRs when homology and/or conserved synteny are lacking or ambiguous.

IgSF inhibitory receptors usually form larger families of molecules in comparison to activating receptors. This function can be devoted to two distinct families of receptors, giving another example of the extremely rapid evolution of these molecules. There are many ITIM-carrying IgSF integral membrane receptors across the classes of vertebrates, and they seem to have had independent histories as it is difficult to convincingly detect orthologous genes between species. This is especially true of multigene families in fish, with members equipped with possible ITIMs, including the teleost NITR and LITR, and bird CHIR and CD300L.[163] Several members of these families can be expressed on NK cells, but expression studies are in their infancy in fish. It is sometimes difficult to distinguish FcR families from NK KIR-like domains, and both FcR and KIR seem to stem from a same lineage. Given the role of the FcR binding to bonafide antibodies and conferring specificities to cells of the innate arms of the immune system, it is likely that these molecules will be restricted to jawed vertebrates. The KIR activity that can incorporate pathogen and virus recognition may be more primitive, but the ancestry of KIR is not well understood, and the bonafide KIR family seems to be restricted to primates.[163]

Genes encoding the classical FcRs on the long arm of human chromosome 1 (1q21-23) are linked to other FcR-like genes. A large multigene family, which includes genes encoding the FcγR and the NK cell Ig-like receptors, is located in the LRC (human chromosome 19q13). This region could in fact be paralogous to 1q23 and may even have been originally associated with the MHC (see the following). These families belong to a larger class of activating or inhibitory receptors. Their phylogenetic conservation in birds, amphibians, and bony fish suggests a biologic importance even though the size of the families, their expression pattern, and the specific nature of the receptors vary greatly among species.[182] In several cases, a commitment to a task in the immune system may not be conserved among homologous members, and the evolutionary fate of the family will be probably affected. Comparison of key residues in the domains may suggest a possible common involvement in

MHC recognition for the two families recently discovered in birds (CHIR)[183] and the teleosts (LITR).[184] Other families were generated within a single class or even within a single order of vertebrates (eg, the KIR described previously). The relationships of KIR with many other multigene families such as IpLITR or NITR remain to be explored. What was the scenario that led to the present mammalian situation? If the fish observation on potential MHC binding holds true, the IgSF type of receptor seems to be the most primitive NKR.[163]

Other Immunoglobulin Superfamily Families to Explore Further

In more primitive vertebrates, the physical or genetic linkage of relatively large IgSF families is well documented in the teleost NITR but not yet elucidated in the case of other interesting families in prochordates like the VCBP. In the sea urchin genome, many IgSF await a complete analysis and will certainly contribute to a better understanding of the evolution and origin of Ig/TCR.[48] Large families of LRR-IgSF in amphioxus[185] could perhaps represent interesting intermediaries in the genesis of either VLR in agnathans or Ig/TCR in gnathostomes (see Fig. 4.10). In hagfish, the discovery of leukocyte expressed receptors agnathan-paired receptors (APARs) revealed what might have been a precursor of Ig or TCR.[186] APARs resemble Ag receptors and are expressed in leukocytes and predicted to encode a group of membrane glycoproteins with organizations characteristic of paired Ig-like receptors. Based on their transmembrane regions, APAR-A molecules are likely to associate with an adaptor molecule with an ITAM and function as activating receptors. In contrast, APAR-B molecules with an ITIM are likely to function as inhibitory receptors Thus, the *APAR* gene family has features characteristic of paired Ig-like receptors. APAR V domains have a J region and are more closely related to those of TCR/B-cell receptor (BCR) than any other V-type domain identified to date outside of jawed vertebrates. Thus, the extracellular domain of APAR may be descended from a VJ-type domain postulated to have acquired recombination signal sequences (RSS) in a jawed vertebrate lineage (see Fig. 4.10).

In jawed vertebrates, three such receptor families with VJ-type domains have been identified: a small family of mammalian proteins known as signal-regulatory proteins, a large family of the previously described teleost NITR, and the MHC-linked XMIV in *Xenopus*. These molecules are examined in more detail in the conclusion. Many IgSF proteins expressed in the immune systems are also expressed in nervous systems where the signaling cacscades may be conserved. This selection of IgSF domains, in two different systems in which homologies are found in molecules with quite different functions, may reveal adaptation capacities and constraints exerted on surface receptors.[187]

VERTEBRATE ADAPTIVE IMMUNITY

Not long ago, it was believed that only jawed vertebrates had a true adaptive immune system. From the previous discussion of invertebrate immune responses, clearly mechanisms exist to generate high levels of immune diversity—even at the somatic level—one of the hallmarks of an adaptive response.[7] As described in the introduction, many in the field agree that the boundary between innate and adaptive immunity is artificial, and it may not be a useful dichotomy when studying immune responses in diverse organisms.[61,62] Despite this reluctance to exclusively classify systems as innate or adaptive, some features clearly fall into the latter category, such as clonal expansion of uncommitted lymphocytes and specific

memory. These conditions are not fulfilled for the DSCAM, FREP, 185/333, or any other invertebrate systems described previously, but of course we must be open to new mechanisms besides the conventional outlook of adaptive immunity. Additionally, such adaptive immunity arose in concert with the emergence of lymphocytes in the lower chordates, as these cells clearly are the major players; we will discuss this in the conclusion.

Immunoglobulins

A typical Ig molecule is composed of four polypeptide chains (two heavy [H] and two light [L]) joined into a macromolecular complex via several disulfide bonds (Fig. 4.7). Each chain is composed of a linear combination of IgSF domains, and almost all molecules studied to date can be expressed in secreted or transmembrane forms.

Immunoglobulin Heavy Chain Isotypes

Like all other building blocks of the adaptive immune system, Ig is present in all jawed vertebrates (see Fig. 4.7). Consistent with studies of most molecules of the immune system, the sequences of IgH chain C region genes are not well conserved in evolution and insertions and deletions in loop segments occur more often in C than in V domains. As a consequence, relationships among non-μ isotypes (and even μ isotypes among divergent taxa) are difficult to establish.[188] Despite these obstacles, the field has developed a working evolutionary tree among all of the isotypes.

Immunoglobulin M

IgM is present in all jawed vertebrates and has been assumed to be the primordial Ig isotype. It is also the isotype expressed earliest in development in all tetrapods; until recently, it was believed to be the case in fish as well, but

this view has changed (see the following). The secretory μ H chain is found in all vertebrates, usually consists of one V and four C1 domains, and is heavily glycosylated. H chains associate with each other and with L chains through disulfide bridges in most species, and IgM subunits form pentamers or hexamers in all vertebrate classes except teleost fish, which form tetramers.[189] The μ C_H4 domain is most evolutionarily conserved, especially in its C-terminal region, whereas the CH2 domain evolves at the fastest rate.[188] There are several μ -specific residues in each of the four C_H domains among vertebrates suggesting a continuous line of evolution, which is supported by phylogenetic analyses. Like TCR TM regions, μ TM regions are also well conserved among sharks, mammals, and amphibians, but the process by which the Ig TM messenger RNA is assembled varies in different species. In all vertebrate classes except teleosts, the μ TM region is encoded by separate exons that are spliced to a site on μ messenger RNA located approximately 30 basepairs from the end of the C_H4-encoding exon. By contrast, splicing of teleost fish μ messenger RNA takes place at the end of C_H3 exon.[190] In holostean fish (gar and sturgeon), cryptic splice donor sites are found in the C_H4 sequence that could lead to conventional splicing, but in the bowfin there is another cryptic splice donor site in C_H3.[191] The TM region itself is interesting as it is the only one that does not contain a residue capable of making an ionic bond with the ITAM-

containing molecule (in this case, Ig-α and Ig-β.) As mentioned, some modifications apparently related to the particular environment were noticed in the Antarctic fish *Trematomus bernacchii*. There are two remarkable insertions, one at the V_H-C_H1 boundary and another at the C_H2-C_H3 boundary; the latter insertion results in a very long CH2-CH3 hinge region. Rates of nonsynonymous substitutions were high in the modified regions, suggesting strong selection for these modifications. These unusual features (also unique glycosylation sites) may permit flexibility of this IgM at very low temperatures.[192]

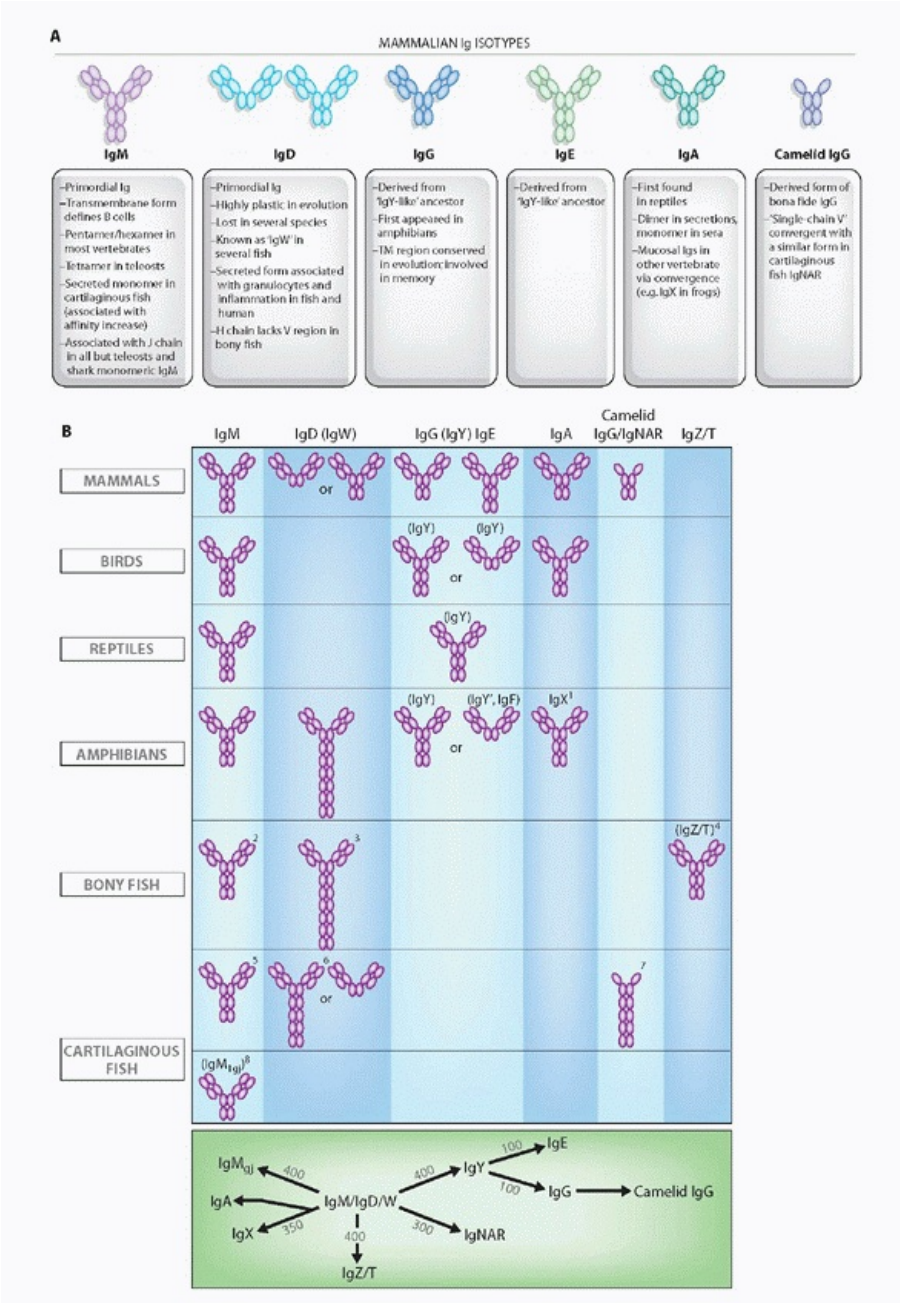

FIG. 4.7. Immunoglobulin (Ig) Isotypes in the Jawed Certebrates. A: Mammalian isotypes and their relationships to Igs in vertebrates from other classes. Each oval represents an Ig superfamily C1 domain. IgD is shown in two forms, mouse (*left*) and human (*right*).[2] **B:** Major Ig isotypes in all vertebrates. The *bottom panel* displays the approximate divergence times of all isotypes. IgM/D/W was found at the inception of

adaptive immunity. 1, IgX is in the IgA column because it is preferentially expressed in the intestine, and IgA seems to have been derived from an IgX ancestor; IgX seems to have been derived from both IgM and IgY ancestors; 2, secreted IgM in teleost fish is a tetramer, and the transmembrane (TM) form only has three C domains; 3, the teleost fish IgD H chains incorporate the μC1 domain via alternative splicing in the TM form, and the secretory form does not have a V region and does not associate with L chains; 4, the new bony fish isotype, IgZ/T, may not be found in all fish species; 5, the secreted form of shark/skate IgM is present as a pentamer and monomer at approximately equal levels; 6, the TM form of IgW has four C domains; 7, a major TM form of IgNAR has three C domains, and IgNAR is related to camelid IgG by convergent evolution; 8, no TM form has been found (to date) for IgM_{1gj}. The *bottom panel* displays the approximate divergence times of all isotypes. IgM/D/W was found at the inception of adaptive immunity.

It has been known for a long time that in all elasmobranchs, IgM is present at very high amounts in the plasma of cartilaginous fish and that it is found in two forms: multimeric (19S) and monomeric (7S).[193] It is unlikely that the two forms are encoded by different gene clusters because 1) peptide maps are identical; 2) early work by Clem found the sequences of the cysteine-containing tail of 19S and 7S H chains to be identical; and 3) all identified germline VH families are represented for the 19S form.[194] Although most studies (but not all) reported that 19S and 7S are not differentially regulated during an immune response, in a recent study, the 19S response wanes over time and a stable 7S titer is maintained for periods of up to 2 years after immunization.[195] In addition, antigen-specific 7S antibodies observed late in the response have a higher binding strength than those found early, suggesting a maturation of the response, also generally at odds with the previous literature. Finally, when specific antibody titers were allowed to drop, a memory response was observed that was exclusively of the 7S class. This work has shown that a “switch” indeed occurs in the course of an immune response; whether the “switch” is due to an induction of the 19S-producing cells to become 7S producers or whether there are lineages of 19S-and 7S-producing B cells is an open question. One working hypothesis is that J chain expression is important for regulating whether a B cell makes 19S or 7S Ig, but of course that could be at the lineage level or the switch level (see the following).

Immunoglobulin M_{1gj}

Nurse shark *Ginglymostoma cirratum* expresses an IgM subclass in neonates.[196] The V_H gene underwent V-D-J rearrangement in germ cells (“germline-joined” or “gj,” see the following). Expression of H_{1gj} is detected in primary and secondary lymphoid tissues early in life, but in adults only in the primary lymphoid tissue, the epigonal organ (see the following). H_{1gj} associates covalently with L chains and is most similar in sequence to IgM H chains, but like mammalian IgG it has three rather than the typical four IgM constant domains; deletion of the ancestral IgM second domain thus defines both IgG and IgM_{1gj}. Because sharks are in the oldest vertebrate class known to possess antibodies, unique or specialized antibodies expressed early in ontogeny in sharks and other vertebrates were likely present at the inception of the adaptive immune system. It is suggested that this isotype interacts either with

a common determinant on pathogens or a self-waste product.

Immunoglobulin New Antigen Receptor and New Antigen Receptor-T-Cell Receptor

A dimer found in the serum of nurse sharks and so far restricted to elasmobranchs, IgNAR is composed of two H chains each containing a V domain generated by rearrangement and five constant C1 domains.[197] IgNAR was originally found in sera, but TM forms exist as ccomplementary DNA and cell-surface staining is detected with specific mAbs. The single V resembles a fraction of camel/llama (camelid) IgG that binds to antigen in a monovalent fashion with a single V region, but it clearly was derived by convergent evolution. In phylogenetic trees, NAR V domains cluster with TCR and L chain V domains rather with that V_H. A molecule with similar characteristics has also been reported in ratfish, although it was independently derived from an ancestral Ig like the camelid molecule emerged from bonafide IgG.[198] IgNAR V region genes accumulate a high frequency of somatic mutations (see the following).

The crystal structure of a Type I IgNAR V regions showed that, in contrast to typical V regions, they lacked CDR2 and had a connection between the two IgSF sheets much like an IgSF C domain.[199] The domain wraps around its antigen (hen egg lysozyme [HEL]), with the CDR3 penetrating into the active site of the enzyme. The structure of a Type II V region has a disulfide bond between CDR1 and CDR3 that forces the most diverse regions of the molecule to form raised loop, similar to what has been described for camelid V domains. In total, the differential placement of disulfide bonds forces major changes in the orientations of CDR1 and CDR3, and provides two major conformations for antigen binding.[200] The structure of a Type II NAR bound to HEL showed that it also interacted with the active site of the enzyme.

While analyzing the TCRVδ repertoire in nurse sharks, an entirely new form of this chain, which encodes three domains, V-V-C, was detected.[201] The C is encoded by the single-copy Cδ gene, and the membrane-proximal V is encoded by a Vδ gene that rearranges to the DJδ elements. The membrane distal V domain is encoded by a gene in the NAR family, found in a rearranging VDJ cluster typical of all cartilaginous fish Ig clusters. The NAR-TCR V genes, unlike IgNAR V genes which have three D segments, only have a single D region in each cluster. The particular Vδ loci linked to each NAR-TCR gene—called NAR-TCR-supporting V δ—encode a cysteine in CDR1 that likely makes a disulfide bridge with the NAR-TCR V domain. The J segment of the rearranged NAR-TCR V gene splices at the RNA level directly to the supporting Vδ segment, which has lost its leader exon (Fig. 4.8). This organization likely arose from an IgNAR V cluster that translocated to the TCRδ locus upstream of a Vδ gene segment. After modifications of the supporting Vδ genes, this entire V-V gene set duplicated and diverged several times in different species of sharks. About 25% of the expressed nurse shark TCR δ repertoire is composed of this TCR (encoded by 15 to 20 V-V genes in this species), and have proposed that the typical γ/δ TCR acts as a scaffold upon which sits the single chain NAR V.

Our interpretation is that, true to the proposal that γ/δ TCRs interact with free antigen, the NAR V is providing a binding site that can interact with antigen in a different way than

conventional heterodimeric Vs. Thus, this is the first case in which a particular V region family has been shown to be associated with a BCR and TCR; in the case of the BCR, the function likely resides within the Fc portion of IgNAR and for the TCR the function (cytokine secretion, killing) lies within the T cell itself. Interestingly, a second TCRδ chain locus has also been described in marsupials and monotremes with properties similar to NAR-TCR.[202] In this case, there are also two V domains, but in marsupials one (proximal to the membrane) is germline-joined, and only the membrane-distal domain undergoes rearrangement. In monotremes, both of the V regions undergo rearrangement, like for the NAR-TCR. This new TCRδ locus is preferentially expressed early in development. This type of TCR is described in more detail in the TCR section.

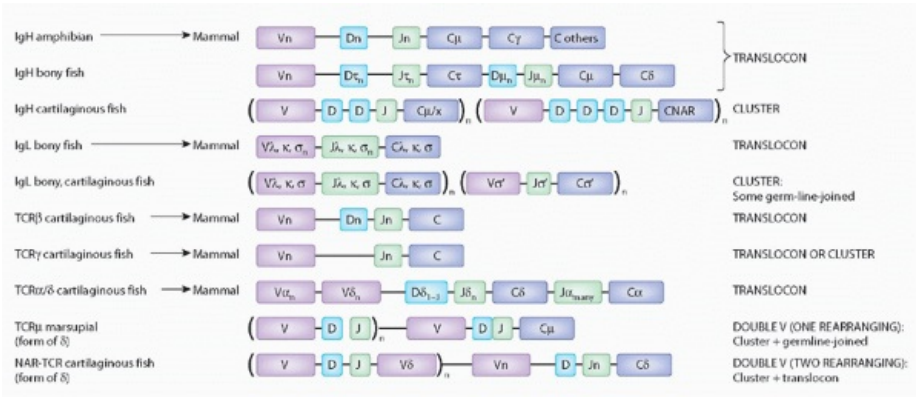

FIG. 4.8. Organization of Immunoglobulin (Ig) Heavy and Light (L) Chain and T-Cell Receptor (TCR) Genes in All Jawed Vertebrates. All coldblooded gnathostomes except bony fish have three L chain isotypes, κ, λ, and σ; mammals have κ and λ; and birds have only λ. RSS are found at the 3′ end of V segments, 5′ end of J segments, and on both sides of D segments. Marsupial μ TCR is related to Ig in its V regions and TCRδ in its C region (and is not related to the μ of IgM).

Immunoglobulin R/Immunoglobulin New Antigen Receptor/Immunoglobulin W/Immunoglobulin X/Immunoglobulin D

All elasmobranchs studied to date have another isotype called IgW. It was probably discovered long ago in skates as a non-IgM secreted isotype called IgR, but no protein sequence of this molecule has been obtained for confirmation.[203] Subsequently, an Ig gene was discovered in skates encoding a three-domain molecule with an unusual secretory tail that was named IgX (not to be confused with another isotype with that name in amphibians).[204,205] A high molecular weight (MW) species detected by northern blotting with an IgW probe suggested that there might be a longer form of this isotype, subsequently shown to be true in the sandbar shark (IgW), nurse shark (IgNARC—it was so-named because the C domains had highest similarity to IgNAR C domains), and skate.[206,207] It was originally believed that sharks only expressed the long (seven-domain) IgW form, but they were later shown to have both secretory forms; the reason for the discrepancy was shark-to-shark variation in expression of the short form, for unknown reasons. The major IgW TM form, like IgM, is composed of five domains, but variants with three domains—like the

secretory forms—were also detected.[208] Very little is known about the function of this isotype, as IgW-specific mAbs have not been generated as they have for IgM and IgNAR.

IgW was thought to be a dead-end isotype in the cartilaginous fish, but a homologue was found in the lungfish.[209] It also is present in two secreted forms, one with eight domains and the other, like in the elasmobranchs, with three domains (unfortunately the secretory tail was not sequenced, and the TM form was not studied.) An Ig isotype was found in *Xenopus tropicalis* most related to the lungfish IgW.[210,211] Computer searches of databases for the *X. tropicalis* genome project uncovered a new isotype flanked by the IgM and IgX genes at the IgH locus. The deduced amino acid sequence obtained from the exons on the genomic scaffold suggests a nine-domain molecule. The N-terminal C domains and the TM regions are most similar to mouse and human IgD regions, and its genomic location also suggest that it is an IgD equivalent. Thus, these new data reveal that like IgM, IgW/D is an isotype that was present at the emergence of all extant vertebrate taxa.

Catfish IgD was the first member of this Ig class found outside of mammals,[212] but this was not well accepted until the isotype was found in all vertebrate classes. It is found attached to FcR on a subset of myeloid cells, and the secreted form does not contain a V region as the leader is spliced to the CH2 domain in plasma cell RNA.[213] This strongly suggests that the IgD is acting as a PRR, perhaps interacting with a conserved region of a pathogen leading to innate immunity. The work on catfish prompted a reappraisal on the role of secreted IgD in humans.[214] Like the fish IgD, the human IgD is bound to a subset of myeloid cells, which are activated upon IgD cross-linking. The receptor on human cells has not been identified, but this new work argues for strong evolutionary conservation of the function of secreted IgD.

An interesting feature of the IgD/W locus is its high plasticity in evolution, both in terms of the number of domains in different fish species and the plethora of splice variants found, at least in cartilaginous fish.[194,215] In sharks, two of the C domains were derived approximately 250 million years ago by a tandem duplication event, and there was a *Xenopus*-specific, two-domain tandem duplication event as well. Within teleost fish, the number of C exons for this isotype is different in various species and the secreted and TM forms are encoded by different loci in the catfish. In addition to the splice variants previously described in the cartilaginous fish and lungfish, in teleosts the IgM C1 domain exon is spliced into the IgD transmembrane transcript.[212] Even in mammals, there are different numbers of C domains in different species, and even exons that have emerged quite recently in evolution (see Fig. 4.7). It is our impression that this is the Ig locus that evolution “plays with,” perhaps using it for different functions in vertebrate taxa. The beginning of understanding the function of the secreted form of IgD is a triumph for comparative immunology; likely the evolutionary approach will reveal the function of the TM form as well.

Immunoglobulin Z/T

A third, novel bony fish isotype was uncovered in screens of the EST and genomic databases called IgZ in zebrafish[216] and IgT in trout.[217] Its genomic organization parallels the TCR α/δ locus in that the IgZ/T D, J, and C elements are found between the VH and Cζ/Cμ exons (see Fig. 4.8). IgZ/T is a five-domain H chain that associates with L chains (see

Fig. 4.7). The authors of the zebrafish paper proposed that lymphocytes bearing IgZ may be B1-cell equivalents, but a preliminary VH repertoire analysis did not suggest that unique sets of V regions are expressed on IgZ compared to IgM. IgT is not preferentially expressed over IgM early in trout development.

Recent work has shown that IgT is the mucosal Ig of trout, with preferential expression in the intestine and skin.[218] Mucosal immunization with the parasite *Toxoplasma gondii* showed a preferential induction of specific IgT. While all teleosts have lost the J chain in evolution,[219] biochemical analysis of IgT showed that it contained a secretory piece derived from the poly-Ig receptor (pIgR). Furthermore, like mammalian dimeric IgA, IgT coats trout commensal bacteria, presumably acting as a "firewall" preventing commensals from breeching the blood-brain barrier. In sum, IgT, while displaying unique evolutionary features, has attained almost all of the characteristics of IgA via convergent evolution.

Other Isotypes Related to Immunoglobulin G, Immunoglobulin E, Immunoglobulin A, and the Class Switch

Other isotypes consist of four C domains in nonmammalian vertebrates including *Xenopus* IgY and IgX, non-μ isotypes of *Rana*, IgY of axolotl, and IgA and IgY of birds. [194,220] In *Xenopus*, IgY is thymus-dependent; IgM and IgX are not, although thymectomy impacts specific IgM antibody production (ie, antigen-specific IgM can be produced, but there is neither an increase in affinity after immunization nor elicitation of plaque-forming cells). IgM and IgX plasma cells are abundant in the gut,[221] whereas IgY is expressed primarily in spleen. Axolotl IgM is present in the serum early during development and represents the bulk of specific antibody synthesis after antigenic challenge.[222] In contrast to *Xenopus* IgY, the axolotl ortholog appears late in development and is relatively insensitive to immunization. From 1 to 7 months posthatching, axolotl IgY is present in the gut epithelium associated with a secretory component.[223] IgY progressively disappears from the gut and is undetectable in the serum of 9-month-old animals. Thus, axolotl IgY, like *Xenopus* IgX and trout IgT, may be analogous to mammalian IgA. *Xenopus* IgX seemed to be most similar to IgM, but as more sequences have become available it does seem to be orthologous to IgA, obviously consistent with its assumed function (Criscitiello, personal communication). In addition, IgX was formerly believed to be derived from IgM, but recent data suggest that the N-terminal C-domains were derived from an IgY ancestor and the C-terminal domains from IgM.[224]

The TM and cytoplasmic domains of *Xenopus* TM IgY share residues with avian IgY and mammalian IgG and IgE, suggesting that mammalian/avian isotypes share a common ancestry with amphibian IgY.[225] This homology is especially interesting because studies in mice suggested that the IgG cytoplasmic tail is the central molecular element promoting rapid memory responses of plasmablasts.[226,227] The motif in

the IgY/G tail is also found in other receptors and seems to recruit PI3kinase, which amplifies the signal derived from Ig-α and -β. Finally, another *Xenopus* isotype was discovered from the databases, called IgF or IgY′ (because it is closely related to IgY, probably duplicating within the *Xenopus* lineage). Like other isotypes described in this section, it only has two C domains, but rather than alternative splicing, the gene is organized in such a manner.[211]

There has been no biochemical identification of IgF.

CSR results from fusing switch regions upstream of the μ gene and another 3′ isotype gene, accompanied by the deletion of the intervening sequences. Among vertebrates, mammalian and bird class switch regions are GC-rich and contain tandem repeats in which certain motifs, such as TGGGG, GGGGT, AGCT, and GGCT, are abundant. Because of the GC richness of these regions, transcription generates stable R loops, which provide single-stranded substrates for activation-induced deaminase (AID) activity. The first comparative studies on switch were done in the amphibian *Xenopus* where the switch regions are not GC-rich but adenine-thymine (AT)-rich and cannot form R loops.[228] Replacement in mouse of the switch region with a *Xenopus* switch region showed that it mediated efficient CSR and that the junctions were associated with the short palindromic AGCT motifs, already recognized as the main component in *Xenopus* switch.[229] As predicted from the absence of R loops, the *Xenopus* switch region supported recombination in both orientations. The breakpoints were located in the AGCT palindrome-rich region of the switch box. Other motifs have been identified in the other switch regions, such as in IgX and chicken isotypes; all of these correspond to the DGYW hotspot consensus. AID-mediated deamination in the context of these motifs may be the conserved major event in the initiation of CSR. As described in the following, until recently it was believed that CSR first appeared in amphibians, but recent data have uncovered a precursor to switch in cartilaginous fish (but not teleosts). Thus, we should add another conserved feature of adaptive immunity that appears to have arisen in the early gnathostomes.[230]

As mentioned, a single gene can encode different Ig forms, like for duck IgY, cartilaginous fish IgW, and camelid IgG loci. It has been suggested that the two avian IgY short and long forms could be the functional equivalents of both IgE and IgG, respectively; the same may be true of the cartilaginous fish IgW short and long forms with two and six domains, respectively. Also as mentioned, IgF or IgY′ also falls into this category but not through alternative splicing.[194,211]

Immunoglobulin Light Chains

L chains can be classified phylogenetically not only by their sequence similarity, but also by the orientation of their V and J RSS, which differ for mammalian λ and κ. There has been much debate regarding the affiliations of L chains in various vertebrates, but as sequences have accumulated, we have a grasp on their phylogenetic emergence. Contrary to what was believed, κ and λ L chains emerged early in vertebrate evolution, in the cartilaginous fish or placoderms. In addition, a third L chain, σ, originally described in *Xenopus* as a dead-end isotype, is in the ancestral clade and is present in all cold-blooded vertebrates.[231]

Elasmobranchs have four L chain isotypes (Type I, II, III, and IV), and the combined data suggest that they are present in all chodricthyans.[231] Type IV is the ortholog of the unusual *Xenopus* σ L chain, and Type I is a dead-end variant of this isotype called σ′ or σ-cart (for cartilaginous fish). Type II L chains are of the λ isotype and have been cloned from all groups of cartilaginous fish; all genes of this isotype are “germline-joined.” The type III is clearly κ-like, at least in the V region and RSS orientation (κ is the only antigen receptor gene in which the V is associated with a 12mer RSS). These relationships are more noticeable in the V sequences, which have defining characteristics such as CDR length; although, the C

sequences also fall into the same clusters, they do so with much less phylogenetic support. Different elasmobranch species express the L chain isotypes preferentially (eg, κ in nurse sharks, σ in horned sharks, and λ in sandbar sharks); this pattern of expression may be due to expansions/contractions of the different isotype genes in various elasmobranch lineages.

Almost all of the L chains in bony fish can be categorized as either κ or σ, despite the large number of gene expansions and contractions in this group.[232] As expected, recent data have shown that λ exists in some species, consistent with the work in sharks.[233] Genes in different species can be found either in the cluster type organization, translocon, or some intermediate type, and this organization is especially well suited to allow for receptor editing, either to generate the repertoire or as a consequence of binding to self-ligand.[234,235]

mAb studies suggested the existence of three *Xenopus* L chain isotypes of 25, 27, and 29 kD with heterogeneous two-dimensional gel patterns and preferential association of some L chain isotypes with IgY H chains.[236] Indeed, three *Xenopus* L chains genes have been isolated: ρ (now κ), σ, and λ. Only one C gene is present in the ρ locus, and it encodes the most abundant L chain. The V and J RSS are of the κ-type and the five identified J segments are nearly identical. The locus is deleted, like mammalian κ, when the other isotype genes are rearranged. Southern hybridizations with genomic DNA from different animals showed V_L sequences to be both diverse and polymorphic. The λ *Xenopus laevis* L chain isotype predicted from the biochemical studies consists of six distinct V_L families. In the σ locus, the J segment has an unusual replacement of the diglycine bulge by two serines. The *Rana* major L chain type has an unusual intrachain disulfide bridge that is seemingly precludes covalent association of its H and L chains.[237]

Two L chain types were identified in reptiles. Chickens and turkeys only express one L chain (λ) with a single functional V and J gene, and the manner by which diversity is generated is likely responsible for this unusual evolutionarily derived arrangement (see the following). Nonproductive rearrangements are not detected on the unexpressed L chain allele, and thus there is a strong pressure to generate functional joints (see the following). Such a system probably rendered a second (or third) L chain locus

superfluous.[236] Within mammals (marsupial *Monodelphis domestica*), the Vλ repertoire is comprised of at least three diverse families related to distinct placental families, suggesting the divergence of these genes before the separation of metatherians and eutherians more than 100 million years ago. Opossum λJC sequences are phylogenetically clustered, as if these gene duplications were recent and the complexity of the λ locus seems greater than that found at the H chain locus.[238]

In summary, all vertebrate groups except the birds have two to four L chain isotypes, all of which can be categorized as κ, λ, or σ (and in cartilaginous fish σ-cart).[231] However, we are still at a loss to understand the significance of possessing multiple isotypes as there is scant evidence that L chains have any effector functions. It has been suggested that different isotypes may provide distinct CDR conformations in association with H chains, or there may be L chain/H chain preferences that provide some advantage that is not obvious.[239,240] At least the differential CDR sizes for σ as compared to κ/λ suggest that the former rationale is plausible. The preferential association of certain L chains with particular IgH isotypes is

suggestive of different functions as well, but there is no evidence for such functions to date.

V_H Evolution

Diversity of the immune repertoire depends on the variety of V segments inherited in the germline and upon the further diversification by rearrangement (CDR3 only) and SHM (all CDR). Early in life, the repertoire depends chiefly on the inheritable genes as one finds little N-region diversity and somatic mutation (with exceptions; see the following). A central question is how antibody germline V genes diversify CDR during evolution while they are subject to homogenizing forces operating in most multigene families. Perhaps environmental antigens have played a major role in shaping the germline repertoire and have selected some V_H/V_L germline sequences used by neonates. V_H families arose in a bony fish lineage and have been conserved for hundreds of millions of years.[241] Conserved regions defining families are found on solvent-exposed faces of the V_H, at some distance from the antibody-combining site. Phylogenetic analyses show clustering of V_H into groups A, B, C, D, and E. All cartilaginous fish V_H belong to the monophyletic group E; bony fish V_H genes to cluster into all Groups (one group [D] unique only to them). By contrast, group C includes bony fish sequences as well as V_H from all other classes except cartilaginous fish. Another phylogenetic analysis classifies mammalian V_H genes in three "clans" (I, II, and III), which have coexisted in the genome for > 400 million years. Only in cartilaginous fish does it appear that V_H gene families have been subjected to concerted evolution that homogenized member genes (except for the $IgM1_{gj}$ V region described previously). It has been debated whether Ig V genes could be under direct positive selection or not because these genes hypermutate somatically. However, several features (eg, codon bias) and discovery of high replacement/silent ratios in germline gene CDR codons indeed argue for positive selection during evolution.[242,243]

In summary, much of the V_H germline repertoire has been conserved over extremely long periods of vertebrate evolution. The birds and some mammalian species that rely on GALT to generate Ig diversity (see the following) are exceptions with a reduced germline repertoire (at least expressed repertoire), but as will be seen in the following, gene conversion and SHM compensate for this situation in formation of the primary repertoire. Even in the cartilaginous fish where there is a single V_H family, there is nevertheless great heterogeneity in CDR1 and CDR2 sequences (as well as hypermutation) that must boost diversity in the expressed repertoire.

The J Chain

The joining (J) chain is a small polypeptide, expressed by plasma cells, that regulates polymer formation of IgA and IgM.[244] J-chain incorporation into dimeric IgA and pentameric IgM endows these antibodies with the ability to be transported across epithelial cell barriers. J chain facilitates creation of the binding site for pIgR (secretory component in the Ig polymers), not only by regulating the polymeric structure but apparently also by interacting directly with the receptor. Therefore, both the J chain and the pIgR/secretory component are key proteins in secretory immunity. J chain complementary DNAs have been reported in all jawed vertebrates except the teleost fish, which have lost it.[245] The existence of *Xenopus* J chain

suggests that, unlike mouse IgM, *Xenopus* IgM forms hexamers with J chain; alternatively, the previous electron microscopy studies identified IgX as the hexameric isotype (the ξ chain has a stop codon before the Cys of C_{H4} domain and thus cannot make a covalent attachment to J chain). The highest level of J chain expression was detected in frog and bird intestine, correlating well with a role for J chain in mucosal immunity (although obviously not for IgX secretion). Elasmobranch J chain shows high similarity to the N-terminal half of J chains from other vertebrates, but is divergent or even absent in the other regions. This result suggests that the function of J chain may be solely for IgM polymerization in elasmobranchs, and the transporting function arose later in evolution; consistent with this idea, *Xenopus*, but not shark, J chain is capable if interacting with human IgA and pIgR.[245] As mentioned previously, the loss of J chain in bony fish does not preclude the interaction of IgT with pIgR and transport across epithelia, implying a strong pressure to maintain a mucosalspecific Ig.[218] There was a claim for the presence of J chain in many protostomes because a homologue was cloned in earthworms, but no J chain sequences have appeared in the numerous protostomes or deuterostome invertebrate databases.[246]

T-Cell Receptors

Ig, TCRs, and MHC class I and class II are all composed of IgSF domains. The membrane-proximal domains of each Ig/TCR/MHC chain are IgSF C1-set domains, while the N-terminal domains of Ig and TCR proteins are V-set domains encoded by genes generated via rearrangement of two or three gene segments during ontogeny (the membrane-distal domains of

MHC are a special case; see the following). In all vertebrates studied to date, TCRs are membrane-bound and never secreted, while almost all Ig proteins have transmembrane and secreted forms.[247]

α/β Constant Domains

Genes encoding the two types of TCR, α/β (which accounts for all known MHC-restricted regulatory and effector functions) and γ/δ (which to the best of our knowledge recognizes antigens in an Ig-like manner and may play immunoregulatory or homeostatic roles during certain infections), existed in the earliest jawed vertebrates. The α/β TCR is considered rather “boring” evolutionarily, as all gnathostomes from the basal cartilaginous fish appear to have this MHC restricted form.[248] As mentioned, although many IgSF members exist in the invertebrates, thus far no bonafide Ig/TCR sequences (ie, IgSF genes generated by somatic rearrangements) have been isolated from jawless vertebrates or invertebrates, although a renewed search in jawless vertebrates may be fruitful (see the following).

While TCR genes have been cloned from representatives of most vertebrate classes, few biochemical data are available, except in birds where α/β and γ/δ TCR have been identified with mAbs.[249] In amphibians, the *Xenopus* α/β TCR was coimmunoprecipitated with cross-reactive antibodies raised against human CD3ε chains[250] (note that this same antibody indentifies CD3 epsilon from many divergent species, which is quite unusual,[251] in turtles). α chains from diverse vertebrates are poorly conserved and the structure of the Cα IgSF domain itself is unusual: only strands A, B, C, E, and F can be identified, although strands E

and F are shorter than those of mammals and strand D is absent; this modification has an important role in TCR dimerization and subsequent signaling.[252] The lack of conservation in this extracellular domain as well as deletions found in bird and teleost fish TCR (especially in the connecting peptide) suggest that the coreceptor may be structurally distinct from mammalian CD3 complex components.

Pre-Tα, which associates with TCR β chains during thymocyte development, has been identified only in warmblooded vertebrates.[253] The pre-Tα protein has no V domain, and its interaction with the TCRβ chain is unique, based on recent structural studies.[254] Unlike all of the other TCR chains, pre-Tα has a long cytoplasmic tail, which seems to be important for T-cell differentiation. Interestingly, the pre-Tα gene is linked to the MHC in mammals, and more phylogenetic studies should be performed to determine whether this linkage group is ancient (see the following).

The TM region and cytoplasmic tail of Cα are the most conserved parts of the molecule. Cα and Cβ TM segments in all species have the so-called conserved antigen receptor transmembrane motif (CART) motif, in which conserved amino acids form an interacting surface with the CD3 complex.[255] Besides CART, the opposite TM face with conserved residues Ile-Lys-Leu interacts with other CD3 components. The cytoplasmic region is remarkably conserved among all vertebrates. TCR β genes have been sequenced from several species of cartilaginous and bony fish and two species of amphibians (axolotl and *Xenopus*). In addition to the typical IgSF domain features, there are several conserved regions among vertebrate TCRβ chains, especially at positions 81 to 86, probably involved in TCR dimerization. There are also remarkable differences: the solvent-exposed segment 98 to 120 in mammals is absent in all nonmammalian vertebrates. This loop has been shown in mouse TCR to be important for negative selection events in the thymus; perhaps the absence of this region in nonmammalian vertebrates results in subtle differences in tolerance induction as compared to mouse/human.[256] The number of Cβ genes varies in different species.

Like Cα, Cβ sequences are not well conserved in evolution (eg, the *X. laevis* Cβ gene does not cross-hybridize with *X. tropicalis* genomic DNA, and catfish Cβ has only 41% to 42% identity with other teleost Cβ and 26% identity with horned shark Cβ). Two different catfish Cβ complementary DNA sequences were identified, suggesting the existence of either two loci or allotypes, as is found in mammals.[257] Indeed, the damselfish Cβ was shown to be encoded by two polymorphic genes, and this feature seems to extend to other teleosts. As the polymorphic sites are believed to interact with the associated CD3-signaling molecules, the authors suggested that signals might be transduced to T cells in different ways depending on the particular expressed Cβ allele. The damselfish Cα gene seems to be encoded by polymorphic alleles as well.[258]

α/β T-Cell Receptor Variable Domains

Because T-cell recognition is MHC-restricted, TCR V regions have been evolutionarily selected for different properties as compared to Ig; indeed, TCR V regions are much less similar to each other than are Ig V regions from other antigen receptor loci.[257] Furthermore, TCR Vs, unlike IgV_H, have conserved CDR3 lengths, suggesting that there is a restricted size for recognition of MHC-peptide complexes.[259] α loci in all vertebrates examined have

many J segments, and consistent with the mammalian paradigm, the absence of D and the large numbers of J segments favors the potential for receptor editing during thymic positive selection.[260] A number of Vβ gene families are another evolutionarily conserved feature. There are 12 families in nurse sharks and 19 in *Xenopus*. In axolotls Vβs are classified into nine categories each with 75% or more nucleotide identity; as only 35 genes were cloned, there are probably more families, and several are related to mammalian Vβ genes (human Vβ13 and Vβ20[257]).

There is evidence that the TCR has coevolved with MHC, displayed by the canonical types of interactions seen in crystal structures.[261] Vβ sequences from many different vertebrates share residues in CDR2 that interact with class II and CD1 MHC molecules (Y-46 and Y-48, especially).[262] It will be of interest to search for other conserved interaction sites as crystal structures become available.

Studies of several teleost α/δ loci suggests an organization similar to mouse/human, but with more rearrangement by inversion and more gene segments than are found in mammals.[263] Pulsed field gel analysis suggested that the horned shark α and δ TCR loci are closely linked.[264] There

has been a recent, major modification in our thinking of the α/δ locus that will be described in the following.

The γ/δ TCR

Complementary DNA sequences from the skate have significant identity with prototypic mammalian γ and δ TCR genes with extensive V region diversity, putative D segments in δ, and varying degrees of junctional diversity.[248] In the nurse shark, there is a great diversity of the δ V regions, the highest among the TCR chains; consistent with this diversity is the presence of the NAR-TCRδ, which is found in approximately 25% of the δ Vs.[257] The NAR-TCR has been found in all cartilaginous fish to date, including holocephalins, whereas NAR and IgW have only been detected in elasmobranchs. Thus, it is possible that NAR (especially the V region) arose as a TCR and was transferred to an Ig cluster sometime in evolution.

In axolotls, Vδ diversity was diminished in thymectomized animals and TCR δ chains are expressed by cells in lymphoid organs, skin, and intestine.[265] Chicken γ/δ T cells were identified long ago. Expression is found in thymus, spleen, and a γ/δ T-cell line, but not in B cells or α/β T-cell lines. Three V subfamilies, three J gene segments, and one C gene were identified at the TCR γ locus. All Vγ subfamilies participate in rearrangement during the first wave of thymocyte development, and the γ repertoire diversifies from embryonic day 10 onwards with random V-J recombination, nuclease activity, and P- and N-nucleotide addition.[249]

In ruminants and chickens (so-called GALT species; see the following), the γ/δ repertoires are quite diverse, and there seems to be ligand-mediated selection of γ/δ cells during ontogeny. In sheep, where γ/δ TCR diversity is thymus-dependent and follows a developmentally regulated progression, no invariant γ/δ TCRs are found.[266] The degree of γ/δ expression is correlated with the evolution of the TCR V families in warm-blooded vertebrates. Indeed,

mammals can be classified into “γ/δ low” (humans and mice, in which γ/δ T cells constitute limited portion of the T-cell population) and “γ/δ high” (chicken, sheep, cattle, and rabbits, in which such γ/δ cells comprise up to 60% of T cells). TCR V genes form subgroups in phylogenetic analyses, and humans and mice have representative loci in most subgroups whereas the other species appear to have lost some.[267] Thus, γ/δ-low species have a high degree of TCR-V gene diversity, while γ/δ-high species have limited diversity. Interestingly, this pattern is similar to that found for IgV$_H$ genes.

Recent work in *Xenopus* has shown that the presence of prototypic V$_H$ at the TCRδ locus, in addition to typical α and δ V domains.[268] Furthermore, consistent with the presence of VH, the αδ locus is closely linked to IgH and IgL λ in the frog genome. These data suggest that these heterodimeric loci arose via a *cis* duplication early in vertebrate history.[263] The TCRμ locus in monotremes and marsupials[202,269] can now be extended to the chicken as well,[270] although like *Xenopus* there are chicken V$_H$ with a three-domain molecule as is found in monotremes, marsupials, and sharks. In total, all of the nonplacental mammals except bony fish show some sort of chimeric Ig/TCR locus, with TCR δ V domains that are more Iglike than TCR-like. Generally speaking, comparative studies have revealed that the γ/δ TCR can either be used for innate recognition (eg, the cells in the skin of mice or the blood of humans) or adaptively with an enormous repertoire in ways we do not understand—this will be discussed in more detail in the conclusion. Consistent with the potential of γδ TCR to interact with nominal antigen, SHM has been detected in the γ genes of the sandbar shark, although it is not known whether the mutations generate the repertoire or appear in mature T cells after immunization.[271,272]

Immunoglobulin Gene Organization

V$_H$ Regions

A rearranged V$_H$ gene consists of a leader segment, encoded by a canonical split exon, followed by four framework regions and three CDRs (see Fig. 4.8). Canonical V$_H$ CDR1 nucleotide sequences are conserved in all jawed vertebrates and serve as targets for SHM.[242,243] A major germline difference is the lack of conserved octamers and TATA box in the 5′ region of shark Vs. In all species, functional V genes are assembled by rearrangement and joining of germline V, D, and J elements. Cartilaginous fish H chains are encoded by large numbers of clusters (> 100 in horned shark and dogfish; approximately 15 in the nurse shark). For IgNAR, there are only four V genes/haploid genome and only a few IgW V genes are detected in nurse sharks, but a large number in skates and dogfish.[273] There are widely varying numbers of V genes in different species; importantly, the V$_H$ complexity does not seem to limit diversity of the antibody repertoire in any ectothermic vertebrate studied to date. There are actually fewer functional human V$_H$ (44 functional, 79 pseudogenes that fall into seven families) than in many ectotherms. Dynamic reorganization of the H chain V regions seems to have occurred at least eight times between 133 and 10 million years ago.[241] Perhaps species that utilize somatic mutation/selection “optimally” rely less on germline diversity and therefore fewer functional genes are required. Only approximately 10% of *Xenopus* V$_H$ are pseudogenes in the three families (V$_{H1-3}$) that have been exhaustively

studied; thus, *Xenopus* with fewer lymphocytes has a greater number of functional V_H genes than humans.

Chondrichthyan Germline-Joined Genes

In all vertebrate species, functional Ig genes are assembled by rearranging DNA segments scattered on the chromosome. However, in cartilaginous fish some V genes are the products of V(D)J rearrangement in eggs/sperm.[196,274,275] Type I L chain (σ) genes are all germline-joined in skates but split in horned sharks, and the piecemeal germline joins (eg, VD, VDD, VDDJ) found in many horned shark H chain gene clusters and in nurse shark L chain Type I (σ) clusters strongly suggest that the germline-joining is a derived feature. Definitive proof came from a study of a germline-joined nurse shark Type III (κ) L chain gene, shown by phylogenetic analysis to have been joined within the last 10 million years[236]; this was followed by the identification of the nurse shark germline-joined $IgM1_{gj}$ described previously.[196] When there is a *mixture* of joined and conventional genes, the split genes are expressed in adults, while the joined genes are

expressed at significant levels only early in ontogeny. When *all* of the genes in a particular family are joined (eg, skate Type I L chain genes and Type II [λ] L chains in all elasmobranchs), they continue to be expressed into adult life at high levels. In mammals, what may appear like germlinerearranged V genes are in most cases processed pseudogenes (eg, pseudo Vκ on chromosome[22] in human or in mouse). However, it is possible that the surrogate L chain gene VpreB is the product of a germline-joining event in the line leading to mammals.[276] Additionally, as described previously, there is a marsupial germline-joined V that serves as the membrane-proximal domain of TCRμ.[202]

Organization of Rearranging Genes

As mentioned, shark IgH-chain genes are structured into tens to hundreds of clusters, each consisting of V, D, J, and C elements[277]; all evidence from studies in horned shark, nurse shark, skate, and sandbar shark (and holocephalin rat-fish) suggest that V, D, and J genes rearrange only within one cluster (but, as detailed in the following, switch may occur between clusters, most likely as a consequence of antigen stimulation). While there is extensive N-region diversity and sometimes usage of two D segments (three Ds in IgNAR), and there are V_H subfamilies having substantial CDR1/2 heterogeneity, diversity of the primary repertoire is lower than in other vertebrates as there is no (or infrequent) rearrangement between clusters. The special constitution of the shark H and L chain loci suggests an exclusion mechanism similar to that of mouse TCR γ loci, also found in clusters. It appears that only one V_H transcript is expressed in each lymphocyte, consistent with isotypic exclusion, despite the many clusters (see the following).[278,279] Bony fish (teleosts and chondrosteans like the sturgeon), frogs, reptiles, and mammals have very similar architectures of their H chain locus —the so-called translocon configuration. As described previously, multiple families of V_H genes, each consisting of many apparently functional elements (1 to 30 per family), are separated from a smaller number of genomic D and J elements. The possibility of combinatorial rearrangement enables more diversification than is possible with the cartilaginous fish clusters for a given number of segments. In birds, the organization is similar

but all V genes except those most 3′ to the D elements are pseudogenes (see the following). One exception is the coelacanth (*Latimeria*), in which V genes are immediately followed by D segments, and then multiple J segments as are found in all tetrapods.[280]

L chain gene organization is variable. In elasmobranchs, the organization is the same (ie, in clusters) as the H-chain locus without the D segments. The prototypic horned shark Type I (σ) L chain has a cluster organization in which V, J, and C segments are closely linked. As mentioned previously, bony fish L-chain genes have the shark cluster-type organization, but some species have multiple V genes in some clusters, demonstrating that there is rapid evolution of not only sequence but gene organization as well in this taxon.[232] In *Xenopus*, there are multiple Vκ(ρ) presumably derived from one family: five J and a single C gene segment. In cartilaginous fish and birds there has been coevolution of Ig gene architecture for H and L loci, but the teleosts have shown that this is not a rule. As mentioned previously, two groups have suggested that the teleost gene organization seems to promote receptor editing.[234,281]

D Segments

D segments are always present in one of the two loci encoding an Ig/TCR heterodimer (IgH and TCR β,δ), and the pressure maintaining this asymmetry is unknown. Cartilaginous fish usually have two D genes/H chain cluster, and there are only minor variations among the clusters; one RSS follows the 12-12 paradigm seen in tetrapods and the other is like the TCR 12-23. In teleosts, amphibians, and reptiles where the organization of the H chain locus is similar to humans, the number of Ds deduced from complementary DNAs ranges from 10 to 16. Two germline D segments have been identified in *Xenopus*, and their RSS follow the rules defined in mammals. In birds, there are 15 very similar D_H.

There are several reasons why D segments may have been preserved throughout evolution. Incorporation of D segments augments CDR3 diversity and size, obviously directly influencing the combining site.[259] Three different Ds contribute to IgNAR CDR3, and besides generating great diversity, CDR3 length and amino acid composition fulfills special tertiary structure requirements: D-encoded cysteine residues bond with cysteine(s) in the body of the V domain, thereby stabilizing a loop involved in the antigen-binding of this unusual monomeric receptor.[197,200] A similar situation has been reached by convergence in the monomeric variant of camel IgG.[198] Finally, rearrangement of one locus “locks it in” and allows the second locus to undergo receptor editing, as is the case for negative selection of the B-cell repertoire and positive selection of the T-cell repertoire. Finally, in mice, one of the TCRδ D segments encodes a section that interacts with the ligand, the nonclassical class I molecule T lymphocyte antigen (TLA).[282]

Agnathan Variable Lymphocyte Receptor Structure and Function

In the 1960s, jawless vertebrates (hagfish and lampreys; see Fig. 4.1) were reported to mount humoral responses to foreign antigens. However, for anti-group A streptococcal antigens, the hagfish “antibodies” (at least a proportion of them) were actually the complement component C3[283]! Lamprey and hagfish were long known, however, to possess cells resembling lymphocytes and plasma cells,[284] with expression of lymphocyte- or at least

leukocyte-specific genes,[285] but the quest for RAG or bonafide Ig/TCR/MHC genes was a complete failure. Reports of specific memory in allograft rejection and other specific humoral responses were difficult to reconcile with absence of the RAG-based rearranging machinery and the possibility of generating specific lymphocyte clones.

Our view of the jawless fish immune system was radically transformed when Pancer et al. prepared complementary DNA libraries of naïve lymphocyte RNA subtracted from lymphocyte RNA derived from lamprey larvae (ammocoetes) that had been immunized to a bacterial/PAMP mixture, and found a highly diverse set of LRR sequences enriched in the

immunized animals.[286] The clones were not only diverse in sequence, but also in the number of LRR "cassettes" found between invariant 5′ (LRR NT) and 3′ (LRR CT) cassettes. Unlike what has been discussed previously for immune genes many species (especially sea urchins), there were not a great number of the germline NT and CT cassettes, suggesting that a somatic recombination process, convergent with Ig/TCR rearrangement, occurred in the developing lamprey lymphocytes (Figs. 4.9 and 4.10). The genomic organization of germline VLR (the original gene discovered was VLRB) have 5′ and 3′ LRR cassettes separated by an intron. Upstream and downstream of the invariant exons are a large number of LRRs, which become inserted between the 5′ and 3′ cassettes during lymphocyte differentiation. Individual lymphocytes apparently express a uniquely rearranged VLR gene in monoallelic fashion. The potential VLR repertoire can be as great as 10^{14}, vastly outnumbering the lymphocytes within an individual.[287,288]

Fragments of homology between the LRR cassettes allow for joining and then priming of synthesis of a copy of the particular transferred cassette.[289] Because these small regions of homology are found throughout the cassettes, "hybrid" LRR can also be formed, further enhancing the diversity over a simple insertion of cassettes. The insertion of LRRs can occur at either end of the somatic recombinant, but it is always 5′-to-3′ on the growing strand. This type of genomic modification resembles the initial stage of gene conversion, but because there is not a complete transfer of genetic material between two homologous gene segments, but actually an addition of sequence, the assembly of the mature VLR is more similar to a recombinatiorial mechanism described in yeast called "copy choice." The enzymology of "copy choice" has not been examined; because a gene conversion-like process occurs, it is likely that an APOBEC family member is involved. Indeed, APOBEC family members were detected in the lamprey genome project and are expressed in a lymphocyte-specific fashion[288]; in fact, the two members that have been discovered, CDA1 and CDA2, are differentially expressed in agnathan lymphocyte subsets (see the following). Because APOBEC family members are involved in repertoire building in jawless and jawed vertebrates, mutation/gene conversion may have predated RAG-mediated repertoire building of the repertoire (see the following).

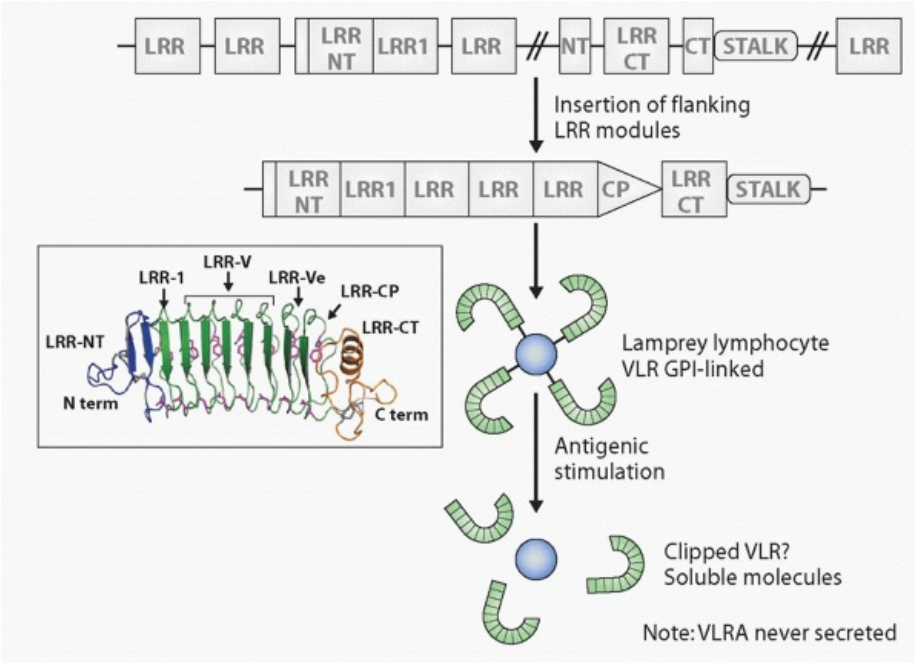

FIG. 4.9. Genetics, Generation of Diversity, and Speculative Cell Biology of the Hagfish Variable Lymphocyte Receptor (VLR) System.[13] The *top line* shows an incomplete VLR gene (NT and CT, N-terminal and C-terminal cassettes, respectively). During lymphocyte ontogeny, upstream and downstream LRR cassettes are inserted between the NT and CT gene segments, resulting in an intronless, mature VLR gene (*second line*). VLR proteins are attached to the lymphocyte surface via a glycophosphatidylinositol linkage, and may be released into the blood upon antigenic stimulation (also see Fig. 4.10). *Boxed* is a hagfish VLR protein, the first structure to be elucidated by Kasahara and colleagues.[499] Note the loop at the C-terminal leucinerich repeat, which is mentioned in the text as a prominent region inserting into antigens, like single-domain antibodies.[293,294]

VLR homologs were found in two additional lamprey species and in hagfish, the only other order of living jawless vertebrates reviewed in Boehm et al.[13] As in the sea lamprey, the incomplete hagfish germline VLR generate somatically highly diverse repertoires. Interestingly, the *Amphioxus* genome harbors a large number of intronless VLR-like sequences that could represent an alternative germline VLR diversity akin to the echinoderm gene families (TLR, SRCR) described previously; one could even speculate that they are related to the ancestral VLR before invasion of its analogous “RAG transposon” (see the following).

There are three VLR types: A, B, and C. VLR-B cell-surface receptors and secreted molecules are analogous to the jawed vertebrate membrane and secreted BCR, and it was proposed that cell activation was similar to T-independent pathways in jawed vertebrates (ie, either direct stimulation through the surface VLR or surface VLR stimulation in combination with a PRR/PAMP interaction). It was suggested that the jawless vertebrate adaptive system might be dedicated exclusively to humoral immunity, with the PAMPs providing the second signal to activate lymphocytes.[290] Thus, it was proposed that the lamprey system was “B

cell-centric" and therefore focused on humoral immunity.

This model has been disproved as subsequent work demonstrated that the VLRA and VLRB were differentially expressed (see Fig. 4.10). The former receptor could not be found in plasma and was expressed exclusively as

a cell-surface receptor, while, as described, the latter was present as both a lymphocyte receptor and a secreted molecule.[291] When microarray expression analysis was performed on VLRA- and VLRB-positive cells, the patterns were consistent with expression profiles in gnathostome T cells and B cells, respectively. The lamprey adaptive scheme, therefore, parallels the situation in jawed vertebrates where Ig is found both as a cell-surface receptor on B cells and as a secreted effector molecule in the serum, whereas the TCR is present only as a cell-surface receptor on T cells; effector functions in T cells, such as cytokine secretion or production of cytotoxic mediators, are properties of the T cells themselves. It is not clear how VLRC fits into this scheme at the moment.

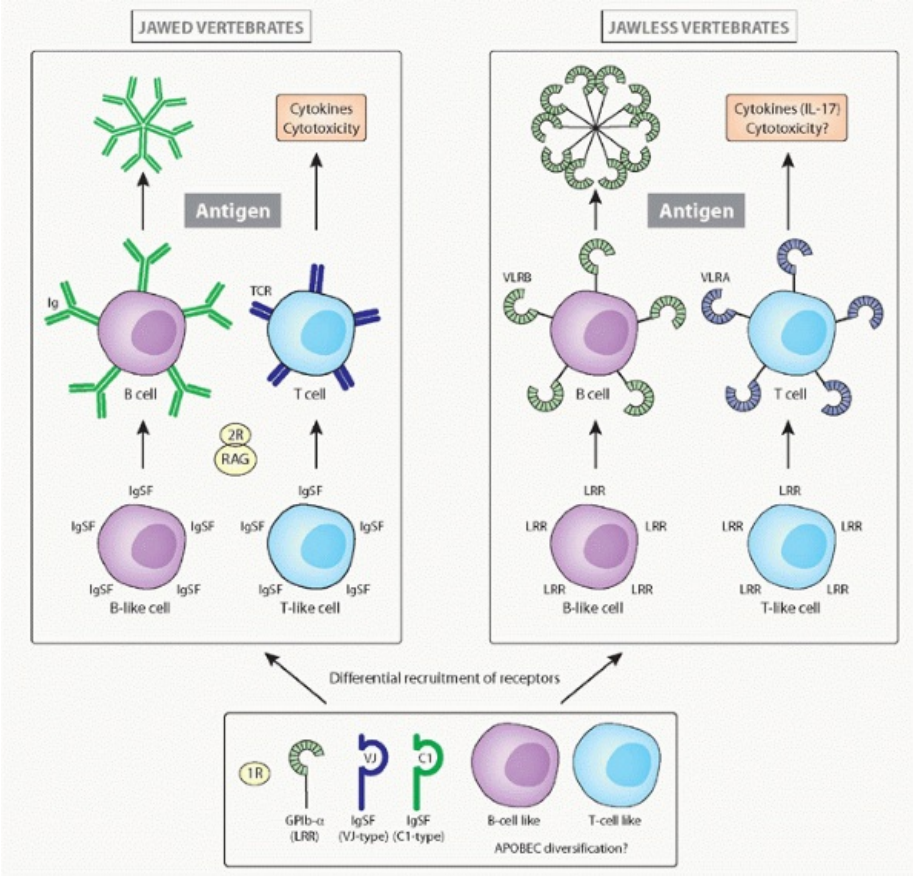

FIG. 4.10. Emergence of Variable Lymphocyte Receptor (VLR) and Immunoglobulin (Ig) Early in Vertebrate Evolution. T and B cells likely preceded the divergence of the leucine-rich repeat and Ig superfamily antigen receptors. The VLR is most similar to a molecule called GPIb-α[288] present in *Amphioxus*, and Ig superfamily antigen receptors are derived from molecules with so-called VJ and C1 domains, probably in the proto-major histocompatibility complex (see text for candidates). Both precursor genes were present in basal chordates. The RAG transposon and 2R likely ignited the appearance of the Ig superfamily receptors (Ig/T-cell receptor) (see text for

full explanation). This figure also shows B and T cells today in jawless and jawed vertebrates, with secretion of IgM from gnathostome B cells and a VLR tetramer of dimers from VLRB-secreting lamprey B cells. Figure modified from Flajnik and Kasahara.[2]

As expected for members of this family, the framework, or backbone, of the LRR is very similar between the cassettes. Diversity between cassettes is concentrated in the concave surface, presumably the region coming in contact with antigen. The crystal structures of two VLR-B molecules have been determined, one specific for HEL[292] and the other for H-trisaccharide.[293] In both cases, the concave surface makes contacts as well as a loop in the LRR-CT, which in the case of HEL inserts into the active site, similar to

what has been found for single-domain camelid and shark Vs.[198] One VLR-A crystal structure was determined, also specific for HEL.[294] This VLRA was derived from a HEL-hyperimmunized adult lamprey, which apparently underwent affinity maturation with induced molecules having affinities in the picomolar range. The antigen is bound in the region of the molecule that is most diverse among all VLRAs; surprisingly, the LLRNT among all VLRA are not diverse, suggesting that this region might bind to a self-"restricting element" and the rest of the binding site to the true antigen.

These are early days, but the comparisons to date suggest that VLRA and VLRB recognize antigen in different ways. Like gnathostome BCR, VLRB is expressed at the cell surface and is secreted as a pentamer of dimers, much like IgM (see Fig. 4.10). The affinities detected to date from immunized animals are in the micromolar range, again similar to low-affinity pentameric IgM in gnathostomes. By contrast, VLRA has never been detected in the sera or secreted in culture, fitting with its similarity to TCR. The affinity of VLRA for soluble antigen was high (in the nanomolar range) for the HEL-immunized animal, which appeared to undergo affinity maturation.[294] Combining the information with the conserved and diverse regions of the VLRA biding site, perhaps VLRA recognizes antigen (budding virus?) associated with a self-protein (MHC analogue?) at the cell surface. It remains to be seen whether VLRA clones will be found from other immunized animals, and the HEL experiment was an artifact of the immunization schedule and not physiologic.

The independent development of two different strategies for receptor somatic diversification at the dawn of vertebrate evolution approximately[500] million years ago reveals the magnitude of the selective pressure applied on to the immune system and the emergence of individualized adaptive responses (see Fig. 4.10). Are VLR responsible for the graft rejection results seen in the old experiments? If so, this is most likely a "convergent MHC" coopted for the VLR system. Skin grafting experiments (or other tests of alloreactivity) should be repeated to study potential VLR involvement. The VLR system is discussed below as well in the lymphoid tissues section and the conclusion.

Adaptive Immune Responses in Gnathostomes

The quality of T-cell and B-cell responses depends on the heterogeneity and the diversity of the antigen receptor repertoires, and the ability to select cells in secondary lymphoid tissues.

Because of the indefinite number of potential Ig/TCR V region sequences in most taxa, potential diversity exceeds the number of available lymphocytes; furthermore, all jawed vertebrates are capable of AID-dependent SHM or gene conversion after antigen stimulation. Yet, while potential repertoires are diverse in all vertebrate classes, and polymorphic MHC class I and II and TCR genes have been isolated from all classes, antibody diversity in nonmammalian vertebrates is relatively low.[295,296,297] The expressed repertoire has been studied via structural studies, affinity measurements during the maturation of the immune response, enumeration of antigen-binding Igs by isoelectrofocusing (IEF), and idiotypic analysis. Sequences of Ig and TCR genes expressed over the course of a response help to estimate diversity at another level, allowing studies of V genes diversified by gene conversion and/or somatic mutation during a response in a precise way. In the following survey, we describe studies of specific antibody synthesis, T-cell responsiveness (T-B collaboration, MHC restriction).

Cartilaginous Fish

Natural antibodies binding many antigens have been detected at surprisingly high levels in chondrichthyans and in some teleosts. In older experiments, after immunization the horned shark mounted a low-affinity 19S (pentameric) IgM antibody response, which varied little among individuals and did not increase in affinity after prolonged immunization.[298]

The relative homogeneity and large number of V genes hindered SHM studies until a single unique reference horned shark IgM V_H gene was found. Mutations in this gene were slightly more frequent than those in *Xenopus* (see the following). This first study proved that SHM preceded diversity obtained by combinatorial association of gene segments in evolution.[299] In contrast to mutations in the horned shark IgM V_H genes, unusual patterns of somatic mutation were detected in nurse shark IgNAR (see the following) and Type II (lambda) germline-joined L chains. Half of the mutations (338/631) occur in tandem without the GC bias seen in *Xenopus* or horned shark H chain V genes. Tandem mutations and point mutations that take place simultaneously were not generated by gene conversion as there are no repeated patterns or potential donor genes.[300,301] The germline-joined L chain genes can only diversify through SHM, perhaps like the hypothetical prototypic V region gene prior to RAG-mediated rearrangement (ie, SHM may have preceded gene rearrangement as the primordial somatic diversification mechanism)[300] (see the following). Lastly, a reappraisal of mutation at H chain and other L chain loci in the nurse shark showed that the mutations were not so different from the L chains; the differences from the previous work were the different shark species and the analysis of all H chain loci in the species rather than only one unusual locus.[302]

As mentioned, the small number of IgNAR genes also made it possible to analyze SHM, and in the first experiments, random complementary DNAs were examined.[197,296] The mutation frequency was about 10 times that of *Xenopus* and horned shark IgM, and even higher than in most studies in mammals. It was difficult to establish a pattern for the mutations due to their high frequency and because they are often contiguous, like in the L chain gene study described previously. Mutations even in randomly isolated clones appeared to be under positive selection in IgNAR secretory but not TM clones, strongly suggesting that mutations do not generate the primary repertoire like in sheep but arise only after antigenic

stimulation.[303,304] In total, the shark mutations seem quite mammalian-like but with unusual features. Analysis of the mutations in noncoding DNA suggests

an AID-dependent process coupled with an error-prone polymerase.[305]

Affinity maturation and memory generation can be detected in sharks.[195] Soon after immunization with HEL, an IgM response can be detected, primarily of the pentameric class. Over time, 7S IgM and IgNAR responses develop, and the 7S antibodies have a higher binding strength than those of the 19S class. When titers were permitted to drop to baseline (or close to baseline), a memory response was induced by immunization of antigen without adjuvant. However, unlike responses in higher vertebrates, the titers do not increase over those in the primary response, suggesting a unique type of regulation of antigen-specific IgM and IgNAR. Nevertheless, these data strongly suggest that the hallmarks of an adaptive response occur in sharks. This type of response is reminiscent of IgA responses of intestinal lymphocytes in mice.[306] In another study, a family of HEL-specific IgNAR clones was followed over time after immunization, and a 10-fold increase in the affinity of an already high-affinity germline clone (10^{-9} M) was observed.[307] These results suggest that affinity maturation, memory, and “switch” to the monomeric IgM isotype occurs, but it takes much longer to attain these adaptive hallmarks compared to mammals, perhaps a paradigm for ectotherms (see the following).

Recent work showed that isotype switch can occur in sharks, despite many reports to the contrary.[230] Previously, studies of nurse and horned sharks complementary DNAs showed that the V and C regions were derived from the same gene clusters. By contrast, in the new work, immunized animals showed switching between IgM clusters and even between IgM and IgW clusters. It is not known whether there is any functional significance to the switch, but one might predict the switch could dictate a change from 19S to 7S antibodies (see the following), or a modification of effector class in IgM and IgW. None of the classic hotspots (RGYW) upstream of constant region genes in tetrapods vital to switch appear to be targeted to initiate the switch in sharks, but other repetitive elements were detected that might play a role.

The role of T cells in shark immune responses has not been studied in detail. No thymectomy experiments have been performed, and T cells have not been monitored during an immune response. Shark mixed lymphocyte responses (MLR) and graft rejection have been attempted, MLR with little success (probably for technical reasons) and grafts with the demonstration of a chronic type of rejection for which the genetics has not been analyzed. However, from the MHC and TCR studies, it is clear that all of the molecular components are available for proper antigen presentation in sharks and skates, and studies of splenic architecture suggestive class II+ dendritic cells in the white pulps argue for a prominent T-cell regulatory role in adaptive immunity.[308] Furthermore, an increase in binding strength and memory response, as well as mutation and now switch, also strongly suggests a T-cell involvement in humoral immunity. Finally, recent studies of the thymus have shown that the architecture and expression of well-known markers are wholly consistent with a typical T-cell regulation of adaptive immunity.[257] In summary, it appears that all of the components of adaptive immunity in mammals occur in the cartilaginous fish.

Bony Fish

There are high levels of low-affinity natural antibody (up to 11% of total Ig) to nitrophenylacetate in some bony fish. Natural antibodies in catfish have been correlated with resistance to virus infection or furonculosis. As a rule, and similar to cartilaginous fish, little affinity maturation has been detected in fish, although some changes in fine specificities were noticed in the trout with a sensitive enzyme-linked immunosorbant assay-based test.[309] The mild increase in trout antibody affinity (similar to that found in *Xenopus* and shark IgNAR) is attributed to selection of either minor preexisting B-cell populations or somatic mutants. In partially-inbred self-fertilized or gynogenetic trout, variability of specific responses is even more restricted. Affinity measured by equilibrium dialysis was of the order of 2.0×10^{-6} M for trinitrophenol (TNP)-specific antibodies. A large literature deals with vaccination attempts in teleost fish, due to their economic importance. The availability of catfish B-cell, macrophage, and T-cell lines have been instrumental in analyses of antibody production.[310] There are puzzling differences in responses from different teleost groups, much like differences between urodeles and anurans (amphibians). Cod, for example, do not respond well to specific antigen and have very high levels of “natural antibodies”; recent studies showing that cod have lost class II genes provides an explanation for the poor humoral responses (see the following).

Like the sharks, isolation of TCR genes and the existence of a polymorphic class I and class II molecules suggest that antigen presentation is operative teleosts, but unlike sharks, functional experiments examining mammalian-like T-APC interactions have been performed. TCR messenger RNAs are selectively expressed, and specific TCR rearrangements have been detected in catfish clonal cell lines, which produce factor(s) with leukocyte growth-promoting activity reviewed in Miller et al.[310] Modifications of the trout T-cell repertoire during an acute viral infection (rhabdovirus) have also been followed.[311,312] In nonintentionally immunized trout, adaptation of the spectratyping technique for TCRβ CDR3 length revealed a polyclonal naïve T-cell repertoire. After an acute infection with viral hemorrhagic septicemia virus, CDR3 size profiles were skewed for several Vβ/Jβ combinations, corresponding to T-cell clonal expansions. Both “public” and “private” T-cell expansions were detected in the infected genetically identical individuals. The “public” response resulted in expansion of Vβ4/Jβ1-positive T cells that appeared first in the primary response and were boosted during the secondary response. Further work examined fine specificity of the viral T-cell response, which is a model for studies in cold-blooded animals.

Recent results suggest that, despite the fact that IgM is the major Ig expressed in an immune response, high- and lowaffinity IgM appears to be a function of the degree of polymerization of the tetramer.[309] It is suggested that high-affinity interactions of TM IgM on B cells modifies the enzymes

involved in disulfide-bond formation, resulting in modifications of secreted IgM.[313] In addition, expression of transcription factors in different subpopulations of trout B cells suggests that the typical populations of naïve, memory, and long- and short-lived plasma cells exist in bony fish; these populations have been followed in blood, spleen, and head kidney by measuring proliferation and B cell-specific transcription factors.[314,315,316] Such studies

should prove very useful for vaccination of large populations of teleosts.

Studies of SHM in teleost fish have been hampered by the lack of good reference genes, but it has been clear that AID-targeting motifs are found in Ig V genes.[317,318] Furthermore, AID is expressed in the spleen during immune responses, again consistent with its role in SHM.[319] A recent study examined mutations in a reference zebrafish L chain gene and detected the typical mutational pattern described previously (without the tandems seen in sharks).[320] AID from several bony fish species is capable of inducing CSR in mammalian B cells, despite the fact that teleosts do not undergo CSR[318]; perhaps the new results that demonstrate switching in sharks may shed light on this conundrum.[230]

As mentioned, species living in extreme cold develop adaptive structural differences in their Igs.[192] At the level of global immune response, temperature exerts a great influence in ectothermic vertebrates in general, low temperature generally being immunosuppressive. Lowering the water temperature from 23° to 11°C over a 24-hour period suppresses both B- and T-cell functions of catfish for 3 to 5 weeks as assessed by in vitro responses.[321] Virgin T cells are most sensitive to this cold-induced suppression, a property shared with mammals when tested appropriately. Fish have developed ways to adapt to the lack of fluidity of their B-cell membranes by altering the composition of fatty acid by using more oleic acid at low temperatures. After appropriate in vivo acclimation, catfish T cells are better able to cap cell surface molecules at low assay temperatures than are B cells, suggesting that capping is not the low temperature-sensitive step involved in T-cell immunosuppression in catfish.

In the NK section, we briefly discussed fish cytotoxic cells.[169] In vitro studies have now shown that leukocytes from immunized fish specifically kill a variety of target cells (allogeneic erythrocytes and lymphocytes, hapten-coupled autologous cells); fish CTL of the αβ (and perhaps γδ) lineages as well as NK cells were found (see previous discussion). Naïve catfish leukocytes spontaneously kill allogeneic cells and virally infected autologous cells without sensitization, and allogeneic cytotoxic responses were greatly enhanced *by in vitro* alloantigen stimulation.[322] Cloned cytotoxic cells contain granules and likely induce apoptosis in sensitive targets via a putative perforin/granzyme or Fas/FasL-like interactions. All catfish cytotoxic cell lines express a signaltransduction molecule with homology to the Fcγ chain of mammals; this chain with an ITAM is an accessory molecule for several activating receptors on mammalian NK cells.[161] Importantly, these cytotoxic cells do not express a marker for catfish nonspecific cytotoxic cells. As described previously, nonspecific cytotoxic cells have been found in other fish species, including trout, carp, damselfish, and tilapia, and they spontaneously kill a variety of xenogeneic targets, including certain fish parasites and traditional mammalian NK cell targets.

Amphibians

Differences in immune system features between urodele (axolotl) and anuran (*Xenopus*) amphibians, already discussed for MHC and Ig complexity, are also seen in immune responses. Rarely is such divergence seen within one vertebrate class (although the two groups diverged over 250 million years ago!). Urodeles express a very restricted antibody repertoire in response to specific antigen that peaks at 40 days postimmunization, and is entirely of the IgM class, even though the serum also contains IgY.[323] They do not respond

well to thymus-dependent antigens, which may be due to lack of T-cell help, yet their expressed TCR diversity looks normal. A population of axolotl B cells proliferates specifically in response to LPS and also secretes both IgM and IgY. Moreover, a distinct lymphocyte subpopulation proliferates significantly in response to the T cell mitogens Con A. T cells from young axolotls (before 10 months) do not have this functional ability. Axolotl T cells also can be stimulated with to SEA/SEB, known from mammalian studies to be superantigens.[324]

Anuran larvae can respond specifically (with only 10^6 lymphocytes) to many antigens, with a modest affinity maturation of the IgM anti-dinitrophenol (DNP) response.[325] In adults, the number of different anti-DNP antibodies does not exceed 40, versus 500 in mammals. In secondary responses, the peak of the response is about 10-fold higher and is reached in 2 weeks; there are no major changes in affinity over this initial rise. Isogeneic *Xenopus* produce homogenous antibodies to DNP, xenogeneic red blood cells, or phosphorylcholine with identical or similar IEF spectrotypes and idiotypes, while outbred individuals differ.[326] Both IEF spectrotypes and idiotypes are inheritable, suggesting that diversity is a reflection of the germline repertoire without a major contribution from somatic mutations. Thus, somatic mutations were followed during the course of an antigenspecific immune response at the peak of the modest affinity maturation.[327] The V_H genes, like their mammalian homologues, contain the sequence motifs that target hypermutation, as described previously. Of the[32] members of the V_H1 family involved in the anti-DNP response, expression of only[5] was detected, indicating that immunization was being monitored. Few mutations were detected (average: 1.6 mutations per gene; range: 1 to 5), and there was not a strong preference for mutations in CDR1 and 2 and virtually none in CDR3. Like in the horn shark IgM study noted previously (but not IgNAR or Type II L chains), the mutations were targeted to GC bases, and such a pattern has been suggested to be the first phase of the SHM phase in mouse/human; perhaps *Xenopus* has lost the second phase of the process that results in an evening of mutation frequency for all bases. While the mutation frequency was lower than in mammalian B cells, the rates were only four- to sevenfold less in *Xenopus*. Thus, there is no shortage of variants, and the reasons for the low heterogeneity and poor affinity maturation

may be due to less than optimal selection of the mutants. Indeed, because of a relatively low ratio of replacement to silent mutations in the CDRs, it was argued that there is no effective mechanism for selecting mutants, which in turn might be related to the absence of GC in *Xenopus*. In summary, the data from hypermutation, complementary DNA heterogeneity, and spectratype dominance suggests that in the absence of refined modes of selection in late-developing clones, B cells producing somatic mutants may be outcompeted by antibodies generated earlier in the response.

Essential T-cell functions in anurans have been demonstrated with in vitro assays for T-B collaboration and MHC restriction, demonstrating the similarity of the role of MHC in *Xenopus* and mammals.[328] Regulatory T cells have been shown indirectly in hematopoeitic/thymic chimaeras for control of CTL generation and in antibody responses. Ig synthesis can be enhanced following late thymectomy in axolotl or *Xenopus*, again implying a role for thymic-dependent regulatory cells. Thymectomy early in life totally prevents CSR from IgM to IgY, but not IgX synthesis; thus, T cells are absolutely required for the switch to the “IgG-like isotype”

and for high-affinity IgM responses, but switch to the mucosal Ig can be T-independent. Switching can also be induced in tadpoles, although one must hyperimmunize animals for this response, due to a paucity of T cells in larvae. The switch is also temperature-dependent, and as described previously for channel catfish, ectotherm T cells are quite temperature-sensitive. AID is expressed in lymphocytes in the spleen as well as in secretory cells.[329] However, consistent with many previous studies, there is no evidence for a typical GC response to date in amphibians or any other ectothermic species. The continued expression of AID in plasma cells (and, presumably, early in embryogenesis) is of interest and deserves further study.

Similar to studies in mammals, the chaperone gp96 has been shown to shuttle peptides into cells making them targets for MHC-restricted CTL lysis.[330] Immunization of frogs with gp96 from a thymic tumor results in the elicitation of CTL that display antitumor activity. Elegant experiments with gp96 vaccination have also shown that CTL activity against minor histocompatibility antigens is MHC-restricted. As mentioned previously, NK cells have been characterized in *Xenopus* with mAbs that recognize non-B/T cells. Those cells kill MHC class I-negative target tumor cells but not class I-positive lymphocytes, and after thymectomy these cells are enriched in the spleen.[331] CD8+ cells expressing TCR were isolated with the same mAb, suggesting the existence of amphibian NKT cells; expression of the mAb epitope on cells is induced by phorbol 12-myristate 13-acetate (PMA)/ionomycin, and is also detected in CTL when MHC-dependent cytotoxicity is reduced.[332] Robert and colleagues have developed one of the best-defined model of innate and adaptive immunity to viral infection in cold-blooded vertebrates, the ranavirus FV3 in *Xenopus*.[70,333] Involvement of CD8 cytotoxic cells and humoral responses have been studied over the course of primary and secondary infections with this virus.[334,335] The system is now primed for the study of other cell type involvement, ontogeny, repertoire, etc.

Reptiles

Lack of an increase in affinity and homogeneity of IEF spectrotypes suggest low-antibody heterogeneity in reptiles. In the turtle *Pseudemys scripta*, a number of genomic VH sequences, representing possibly four families, were isolated, as was a genomic Cμ, all shown to be encoded at a single locus. In northern hybridizations, the Cμ4 probe detected two transcripts; of the four VH groups, only one was expressed, and multiple bands indicated the presence of at least two non-μ transcripts. Among 32 unique VDJ rearrangements from one animal, there were 22 sequence variants in FR4, suggesting either a large number of J segments or somatic modification.[336] The latter interpretation is supported by point mutations found in FR3 and CDR3. For T cells, there are no data on T effector function, but there are studies on the behavior of T-cell population changes due to seasonal and hormonal variations. Thymocytes from the turtle *Mauremys caspica* proliferate in response to phytohemagglutinin (PHA) and ConA, and can kill tumor target cells by both antibody-dependent cellular cytotoxicity-mediated and NK-mediated cytotoxicity. Proliferative responses to PHA and Con A were higher for both sexes in spring and for females in winter than in the other seasons.[337]

Birds and Mammals

The poor increase in affinity of chicken anti-DNP and anti-fluorescein antibodies again indicates lower heterogeneity in chickens. Few changes occur after immunization, even if one waits 1 year after several injections. Perhaps similar to the trout study described previously, a restricted population of high-affinity antibodies was found only after immunization in complete Freund's adjuvant (CFA).[338] Hyperconversion and somatic mutation in Ig genes have been found in splenic GC B cells after immunization.[339] The relatively poor affinity maturation of the chicken response may be due to a balance between gene conversion and somatic mutation. Indeed, modification of V genes with segments of DNA is not an optimal strategy for fine-tuning antibody responses.[340] In the rabbit, there is also conversion/mutation by B cells in GCs after immunization. Within mammals, large variations are found from marsupials with no obvious secondary response, to mouse with 1,000-fold increases in affinity, but the basis for the relatively poor responses has not been established.

In conclusion, although all vertebrates have a very large potential for generating diverse antibodies after immunization, only some mammals studied to date make the most of this potential. Perhaps pressures on the immune system of cold-blooded vertebrates have been less intense due to a stronger innate immunity, and architecture of their lymphoid system is not optimal for selecting somatic mutants, or the great rises in affinity detected in antihapten responses are not physiologically relevant.[297] An immune system using somatic diversification at its “best” is well adapted to species where the value of single individuals is important (ie, species with small progenies); has that been the condition for the creation and selection of somatic rearrangement and of the optimal usage of somatic mutations? If this explanation provides a rationale for the utilization of somatic mechanisms

in generating a repertoire and improving it, it does not tell us why it works so well in certain species and not in others. Perhaps one key is the organization of secondary lymphoid organs. Likely a combination of factors (eg, quantitative and qualitative effects such as endothermy, secondary lymphoid tissues, mutation versus conversion, the hypermutation mechanism itself, rates of proliferation thogen and lymphocyte], etc.) are at work in the regulation of antibody responses.[296,297] Finally, significant differences have been detected in the mutational mechanism and targeting in ectotherms (eg, GC-richness in amphibians, tandem mutations in sharks, TCR loci mutation in sharks, etc.) that may enrich our understanding of the general mutational mechanism, including the enzymes involved and the targeting to V genes.[318]

Lymphoid Tissues

In addition to the molecules and functions characteristic of adaptive immunity, primary (lymphocyte-generating) and secondary (immune response-generating) lymphoid tissues also define the specific immune system[341] (Fig. 4.11). The thymus is present in all jawed vertebrates, and recent evidence suggests its origin at the dawn of vertebrate emergence.[342] *All* animals have hematopoietic cell-generating tissues, and outside of the so-called GALT species, B cells develop in such bone marrow equivalents in all jawed vertebrates. With the advent of clonal selection, the accumulation and segregation of T and B cells in specialized organs for antigen presentation became necessary and indeed the spleen as such an organ is found in all jawed vertebrates, but not in agnathans or

invertebrates.

All jawed vertebrate species rearrange their antigen receptor genes somatically (except the case mentioned previously for some shark germline-joined genes[276]). Besides rearrangement, with combinatorial joining of gene segments and imprecision of the joins, there are two other sources of diversity to generate the repertoires: the terminal deoxynucleotidyl transferase (TdT) enzyme that modifies boundaries of rearranging gene segments, and somatic mutations, found exclusively in B cells usually introduced during immune responses. However, progression of rearrangement during B- and T-cell development and diversification follow different rules in different vertebrates.[220] It is conceivable that species hatching early with just a few lymphocytes are under pressures to develop a rapid response and may not use the same mechanisms as species protected by the mother's uterine environment. It is also possible that immune systems of species with few offspring are under stronger pressures than species that have many offspring, and this could be reflected in the manner diversity is generated. Studies of B- and T-cell differentiation have been performed in many vertebrates. RAG and TdT genes have been cloned in representatives of all vertebrate classes, probes that allow the monitoring of lymphocyte development (see the following). Reagents have become available permitting a monitoring of T-cell appearance in the lymphoid organs of ectotherms (crossreactive anti-CD3 sera mentioned previously or TCR probes), as well as mAbs and gene probes specific for Ig H/L chains that allow examination of B cells. As a rule, the thymus is the first organ to become lymphoid during development. Another emerging rule is that development of the thymus-dependent MHC-restricted T-cell repertoire is similar in all species, and this is reflected in the evolution of TCR gene organization described previously as well as a core set of molecules that are required for thymocyte migration and differentiation[343]; in contrast, B-cell repertoire generation differs dramatically among different species, at times even within the same class of vertebrates.[220]

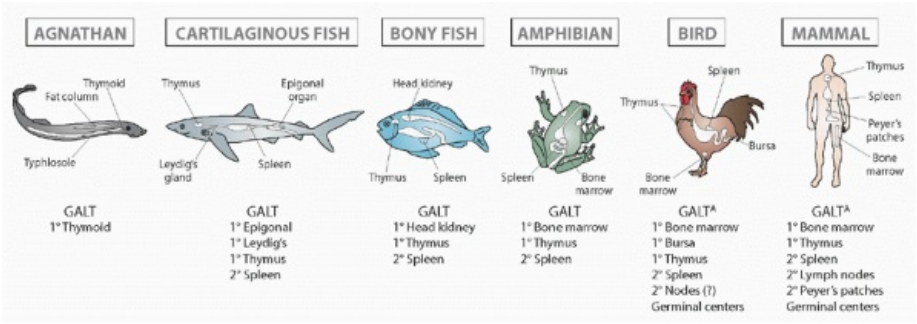

FIG. 4.11. Evolution of Lymphoid Tissues in the Vertebrates. All jawed vertebrates have a thymus and a spleen with demarcated T- and B-cell zones. Fish have different bone marrow equivalents (epigonal, Leydig's gland, head kidney), and amphibians are the oldest group with lymphopoietic bone marrow (also the first to have a typical immunoglobulin [Ig]H chain class switch, although sharks seem to have switch as well, despite the IgH cluster organization). Germinal centers are found only in warm-blooded vertebrates. A thymus equivalent (thymoid) has recently been discovered in

agnathans.[375]

Cartilaginous Fish

Like all other major adaptive immune system components, cartilaginous fish are the first in evolution to possess a prototypic thymus originating from pharyngeal pouches. As

in mammals, it has a distinct cortex/medulla structure, and TdT expression was detected in thymocytes with crossreactive antisera and more recently by northern blotting and in situ hybridization, where it is found throughout the cortex.[257] Interestingly, unlike most other vertebrates, age is not an indicator as to the size of the cartilaginous fish thymus; it can be small or large at any stage of development. GALT is also found in elasmobranchs, but lymphoid tissue in the spiral valve (intestine) clearly does not have typical secondary lymphoid tissue structure; the spleen is the only tissue with compartmentalization of cells into discrete T-cell and B-cell zones.[308] The Leydig's and epigonal organs (associated with the gonads) are lymphopoietic and erythropoietic, producing mainly granulocytes and lymphocytes, and there is high RAG expression in these tissues (see the following). Lymphocytes form nodules in the epigonal organ, probably indicative of differentiative events. In addition, many plasma cells are found peppered throughout the epigonal, fitting with the bone marrow connection.

At hatching, when dogfish embryos are exposed to waterborne antigens, structural development of the lymphomyeloid tissues is well advanced.[344] In the nurse shark, neonatal spleen white pulp consists entirely of class II-negative B cells; by 5 months after birth, T-cell zones appear adjacent to the B-cell zones. Both the B-cell and T-cell zones are vascularized, and no detectable marginal zone separates red pulp from white pulp. Class II-positive dendritic-like cells are found throughout the white pulp.[345]

In the skate *Raja eglanteria*, Ig and TCR expression is sharply upregulated relatively late in development (8 weeks) by quantitative polymerase chain reaction. IgM expression is first detected in the spleen of young skates but IgW is expressed first in gonad, liver, Leydig's organ, and thymus.[346] In adults, Leydig's organ and spleen are sites of the highest IgM and IgW expression. In nurse sharks, IgM1gj and 19S IgM appears in the serum before 7S IgM and IgNAR, and this profile is reflected in the lack of IgNAR+ cells in the spleen until 2 months after birth. RAG and TdT expression in the thymus and epigonal organ of the nurse shark suggests that lymphopoiesis is ongoing in adult life. In contrast to most other vertebrates, N-region diversity is detected (albeit reduced by approximately 50%) in skate and nurse shark IgM and IgNAR CDR3 from the earliest stages analyzed, suggesting that a diverse repertoire is important for young elasmobranchs.[308] As mentioned previously, a subset of Ig genes is prerearranged in the germline of chondrichthyans, and many of those germline-joined genes are transcribed in the embryo and hatchling, but not in the adult. This pattern fits with the expression of the nurse shark IgM_{1gj} with its germline-joined V region, and suggests that some germline-joined genes “take advantage” of their early transcriptional edge and thus some clusters can be selected for specialized tasks in early development. With many gene clusters, it is not known how “clusteric exclusion” is achieved at the molecular

level (and why the germline-joined gene expression is extinguished in adult life), but as mentioned previously, studies in two cartilaginous fish species suggest that only one H chain cluster is expressed in each lymphocyte.[278,347] It was suggested, based on the paucity of rearranged gene segments at the nonexpressed IgH loci, that there are limiting factors regulating accessibility to RAG proteins and a short time window for rearrangement of accessible loci in B cells.[347]

The architecture of cartilaginous fish Ig loci allows greatest diversity only in CDR3 because the CDR2 and CDR1 are always encoded in the germline and V segments do not combine with (D)J segments from other clusters. Yet the number of possible CDR3 is essentially limitless, and the number of germline clusters is also high (at least 15 genes in each species and as many as 100 genes; and usually three rearrangement events take place because two D segments are in each cluster). Thus, the potential diversity is greater than the number of lymphocytes, the general rule for generation of diversity in the vertebrates.

Bony Fish

In all teleosts examined, the thymus is the primary organ for T-lymphocyte generation and head kidney the primary organ for B-cell development. The teleost thymus gland originates from the pharyngeal pouches and can be uni-, bi-, or trilobed, depending on the species,[348] and it is the first organ to become lymphoid. The cortex/medulla architecture is not as precise in other vertebrate species, but the duality of the compartment is apparent and varies from species to species.[349] The spleen contains the basic elements seen in other vertebrates —blood vessels, red pulp, and white pulp—but the distinction between red and white pulp is less obvious (the white pulp being poorly developed). In spleen, the ellipsoids, which are actually terminal capillaries, have a thin endothelial layer surrounded by fibrous reticulum and an accumulation of cells, mainly macrophages. Lymphocyte accumulations are often seen in their vicinity, especially during immune responses, which have been suggested to be primitive GCs, but they are not homologous; as mentioned, AID expression is found in cells[319] during immune responses. Red pulp is rich in melanomacrophage centers, groups of pigment-containing cells at bifurcations of large blood vessels, which may regulate immune responses. The other main lymphoid organ is head kidney, believed to function as mammalian bone marrow. The transparent zebrafish is being developed as a new model to study T-cell differentiation.[343]

In the sea bass *Dicentrarchus labrax*, a mAb detects differentiating T cells (perhaps pre-T cells) as well as mature T cells as evidenced by the presence of TCR messenger RNA in the sorted populations. Cells seem to migrate from surrounding mesenchyme and subsequently mature in the thymus like in all vertebrates studied so far. T cells appear earlier in ontogeny (between 5 to 12 days after hatching) than cytoplasmic Ig+ pre-B cells, which are detected only at 52 days posthatching. Adult levels of T and B cells are reached between 137 to 145 days after hatching, which is quite a long time compared to young amphibians.[350,351]

Teleost RAG1 differs from mammalian RAG1 genes by the presence of an intron of 666 base pairs (an intron is also found in the sea urchin RAG1 gene in a similar position[352]).

Compared with other RAG1 sequences, trout RAG1 has a minimum of 78% similarity for the

complete sequence and 89% similarity in the conserved region (amino acid 417-1042). RAG1 transcripts are detected starting at day 20 after fertilization. Trout TdT is highly expressed within the thymus and to a lesser extent in the pronephros beginning at 20 days postfertilization, which correlates with the appearance of lymphocytes in these two tissues.[353] Because the H chain cluster is in the translocon configuration and there are many V_H families, it is assumed that diversity is generated in the mouse/human mode.

As described previously, studies of mature B-cell activation and homing have been done in trout. Immunization of animals results in the production of short-term Ig-secreting cells in the blood and spleen and long-lived plasma cells in the head kidney. Further analysis with B cell-specific transcription factors like PAX5 and BLIMP1 reinforced the functional studies and showed that the blood contains primarily “resting” B cells and the head kidney both plasma cells and B-cell precursors.[314,354] These last findings appear to be true of the cartilaginous fish as well, with the epigonal organ as the head kidney primary lymphoid tissue equivalent. Interestingly, recent studies have shown trout B cells to be quite efficient at phagocytosis, raising questions about myeloid/lymphoid lineage commitment in the vertebrates.[355] It is predicted that B cells from all ectothermic vertebrates are capable of phagocytosis (shown for *Xenopus*), and the work was recently extended to B1 cells in mice.[356] It certainly will be of interest to study these innate characteristics of B-cell subsets in the future, considering the early appearance of B1 cells and macrophages differentiating from the yolk sac of mouse embryos.[328,357]

Amphibians

In anurans, the thymus develops from the dorsal epithelium of the visceral pouches (the number of pouches varies with species) and is the first tissue to become lymphopoietic. It is colonized from days 6/7 onward by precursors derived from lateral plate and ventral mesoderm through the head mesenchyme. Precursors proliferate in situ as the epithelium begins to express MHC class II molecules but not classical class I molecules. By day 8, thymic cortex/medulla architecture resembles that of other vertebrates.[328] Amphibians possess a spleen with red and white pulp, GALT with no organized secondary lymphoid tissue, and many nodules (but no lymph nodes), with lymphopoietic activity in the kidney, liver, mesentery, and gills. The general morphology of lymphoid organs varies greatly according to species and changes with the season. In *Xenopus*, splenic white pulp is delineated by a boundary layer, and the central arteriole of the white pulp follicle terminates in the red pulp perifollicular area, a T-dependent zone. Anurans, like all ectothermic vertebrates, lack GCs. In *Bufo calamita*, colloidal carbon particles injected via the lymph sac are trapped by red-pulp macrophages, which then move through the marginal zone to the white pulp.[358] Giant, ramified, nonphagocytic cells found in both white and red pulp have been proposed to be dendritic cells. *Xenopus* bone marrow does not appear to be a major lymphoid organ from histologic observation, but high RAG expression in this tissue suggests lymphopoietic activity.[359] The maintenance of RAG expression throughout adult life suggests that lymphocytes are continually produced.

Thymectomy decreases or abolishes allograft rejection capacity, MLR and PHA responsiveness, IgY antibody synthesis, and all antibody responses that increase in affinity to

classic thymus-dependent antigens.[331,360] MLR reactivity matures before the ability to mount IgY responses in primary responses. Thymectomy at 7 days of age delays allograft rejection and abrogates specific IgY responses, whereas later in life it only abrogates antibody responses. Thymectomy performed later greatly affects the pool of peripheral T cells, as monitored with mAbs specific for molecules such as CD8. Early thymectomy results in the complete absence of T cells, but lymphocytes with T-cell markers, perhaps corresponding to NKT cells, can still be detected. In *Xenopus*, thymocytes induce weak graft-versus-host reactions, whereas splenic T cells are good helpers and strong graft-versus-host inducers. The thymus contains some IgM-producing B cells and memory cells poised to switch to IgY synthesis, and in vitro responses are downregulated by naïve thymus cells. *Xenopus* B cells respond in vitro to low doses of LPS not by proliferation, but rather by Ig synthesis, and also respond to PMA. Old reports of B-cell proliferation can be attributed to contaminants in LPS preparations.[328]

Urodele embryos initially produce five pairs of thymic buds, the first two of which disappear.[284] This results in a three-lobe thymus in *Ambystoma*, but in *Pleurodeles* and *Triturus* it forms one lobe. No cortex-medulla boundary is present, and the thymus generally resembles a canonical cortex. There are at least three types of stromal epithelial cells. There is no lymphopoietic activity in axolotl bone marrow, and hematopoiesis takes place in the spleen and in the peripheral layer of the liver. The spleen is not clearly divided into white and red pulp.

About 40% of TCR-β VDJ junctions in 2.5-month-old *Ambystoma* larvae have N-additions, compared to about 73% in 10- to 25-month-old animals.[247] These VDJ junctions had approximately 30% defective rearrangements at all stages of development, which could be due to the slow rate of cell division in the axolotl lymphoid organs and the large genome in this urodele. As mentioned previously, many axolotl CDRβ3 sequences, deduced from in-frame VDJ rearrangements, are the same in animals of different origins. In contrast, in *Xenopus*, rearrangement starts on day 5 after fertilization for the V_H locus, and within 9 days all V_H families are used. V_H1 rearranges first followed by V_H3, and by day 9/10 V_H 2, 6, 9, and 10 begin being rearranged and then V_H 5, 7, 8, 11 on day 13. For VL, the κ locus is the first to rearrange on day[7] (2 days after V_H), a situation similar to that found in mammals. During this early phase, B cells are present in the liver, where their number increases to approximately 500 cells.[325] Later in larval life, rearrangement resumes at metamorphosis, as suggested by the low incidence of pre-B cells and by the reexpression of RAG during the second histogenesis of the lymphoid system. T cells show a similar type of RAG expression/cell renewal during

ontogeny as the B cells, and the larval and adult Vβ T-cell repertoires differ significantly. Even early in development, tadpoles express a highly variable TCR-β repertoire despite the small number of lymphocytes (8,000 to 10,000 splenic T cells); little redundancy in TCR complementary DNA recovered from young larvae implies that clone sizes must be extremely small, unlike in axolotls.

In *Xenopus*, no lymphoid organ apart from the thymus is detectable until day 12 when the spleen appears and with it the ability to respond to antigen. For B cells, until this time no

selection occurs as suggested by the random ratio of productive/non-productive VDJ rearrangements (2:1). After day 12, this ratio becomes 1:1 (ie, the rearrangements have been selected). Complementary DNA sequences on days 10 to 12 (when the number of B cells increases from 80 to 500) are not redundant as if each sequence was represented by one cell.[325] RAG expression together with the detection of DNA rearrangement circles in the bone marrow suggests that rearrangement is ongoing throughout life and is not restricted to an early period, like in birds and rabbits. Tadpole rearrangements are characterized by a lack of N-region diversity, like in mammals but not axolotls or shark/skate (see previous discussion), and thus very short CDR3.[361] During ontogeny, TdT appears in significant amounts in thymus of tadpoles at metamorphic climax, but little expression is detected at earlier stages, which correlates well with the paucity of N-region addition in larval IgH chain sequences.[362] Studies of the ontogeny of the *Xenopus* immune system have revealed a less efficient tadpole immune response (skin graft rejection and Ig heterogeneity and affinity); the absence of TdT expression during tadpole life fits well with the findings of lower larval Ig (and perhaps TCR) diversity.

Reptiles

In all reptiles studied, the thymic cortex and medulla are clearly separated. The spleen has well-defined white and red pulp regions, but T- and B-cell zones have not been delineated with precision.[348] In *Chrysemys scripta*, white pulp is composed of two lymphoid compartments: lymphoid tissue surrounds both central arterioles and thick layers of reticular tissue called ellipsoids. Even after paratyphoid vaccine injection, splenic GCs are not formed, as in fish and amphibians. Splenic red pulp is composed of a system of venous sinuses and cords. In *Python reticulatus*, dendritic cells involved in immune complex trapping have been identified and may be related to mammalian follicular dendritic cells. GALT develops later than spleen during development, and it appears to be a secondary lymphoid organ (but does not seem to contain the equivalent of the bursa of Fabricius). Lymph node-like structures, especially in snakes (*Elaphe*) and lizards (*Gehyra*), have been reported.

Reptiles, the evolutionary precursors of both birds and mammals, are a pivotal group, but unfortunately the functional heterogeneity of reptile lymphocytes is poorly documented. There seems to be T-/B-cell heterogeneity because an antithymocyte antiserum altered some T cell-dependent functions in the viviparous lizard *Chalcides ocellatus*. Embryonic thymocytes responded in MLR at all stages, but ConA responsiveness increased gradually during successive stages and declined at birth. In the alligator (*Alligator mississippiensis*), like in mammals after glass-wool filtration, nonadherent peripheral blood leukocytes (PBL) responded to PHA and not to LPS, whereas adherent cells were stimulated by LPS.

Birds

The thymus, which develops in chickens from the third and fourth pharyngeal pouches, consists of two sets of seven lobes each with definitive cortex/medulla. The thymus becomes lymphoid around day 11 of incubation. Splenic architecture is less differentiated than in mammals. It is not lymphopoietic during embryogenesis as RAG-positive cells are found mainly in yolk sac and blood. Birds are the first vertebrate group where follicular GCs and T-dependent areas comprising the periarteriolar lymphatic sheath are encountered. Plasma cells are located in the red pulp. γ/δ TCR+ T-lymphocytes are chiefly concentrated in

sinusoids, whereas α/β T cells fill the periarteriolar lymphatic sheath.[249] Lymph nodes seem to be present in water and shore birds but not in chickens and related fowl.

The bursa of Fabricius is a primary lymphoid organ unique to birds in which B cells are produced.[363] It arises at day 5 of development and involutes 4 weeks later. T-B heterogeneity is obviously well defined in birds (indeed, the "B" in B cell stands for bursa.) The effects of thymectomy—T- and B-cell collaboration and generation of MHC-restricted helper and killer cells—are very similar to mammals, the other class of warm-blooded vertebrates.

During the embryonic period, chicken stem cells found in yolk sac and blood rearrange their IgH and L V genes simultaneously over a very restricted period of time, and very few cells colonize each bursal follicle (about 10^4 follicles).[364] Three weeks after hatching, these cells have differentiated in the bursa and then seed the secondary lymphoid tissues, after which time B cells are no longer be generated from multipotent stem cells; thus, only approximately 2×10^4 productive Ig rearrangements occur in the life of the chicken. When an antiserum to chicken IgM was administered in ovo to block this early bursal immigration, there were no stem cells arising later in development that can colonize the bursa, and these chickens lacked B cells for their entire lives.[365] Although the general Ig locus architecture is similar to that of frogs and mammals, only one rearrangement is possible as there is only one functional V_L or V_H on each allele.[366,367] Diversity is created during bursal ontogeny by a hyperconversion mechanism in which a pool of pseudogenes (25 ψL and approximately 80 ψH) act as donors and the unique rearranged gene acts as an acceptor during a proliferative phase in bursal follicles. For H chains, the situation is more complex as there are multiple D elements. During ontogeny, selection of productive rearrangements parallels the selection of a single D reading frame, suggesting that the many D segments favor D-D joins to provide junctions that are diversified by gene conversion; the hyperconversion mechanism can also modify Ds because most donor pseudogenes are fused VD segments. The gene conversion process requires AID, which is also required for SHM and CSR.[368]

Because diversification by gene conversion occurs after Ig rearrangement and cellular entry into bursal follicles, and there is only a single germline V_H and V_L expressed on all developing B cells, it was tempting to implicate a bursal ligand binding to cell surface IgM to initiate and sustain cellular proliferation and gene conversion. However, surface expression of IgM devoid of V regions permitted the typical B-cell developmental progression, demonstrating that such receptor/ligand interactions are not required.[369] Thus, currently we know little of how cells enter the bursa, which signals induce them to proliferate/convert, and how cells arrest their development and seed the periphery.[364]

Generation of Diversity in Mammals

Mechanisms leading to the generation of repertoire diversity vary among mammalian species.[220] Categories can be made depending upon the mode of B-cell development: rabbits, cattle, swine, and chickens, unlike fish, amphibians, reptiles, and primates/rodents, use a single V_H family, of which only a few members (sometimes only one) are functional. To

diversify their antibody repertoire, this group uses gene conversion or hypermutation in hindgut follicles of GALT early in life (rather than bone marrow throughout life) and is therefore known as the “GALT group.” At the rabbit H locus, as in the chicken, a single V_H is expressed in most peripheral B cells. During development, B cells that have rearranged this particular V in the bone marrow (and other sites) migrate to the appendix where this rearranged gene is diversified by gene conversion using upstream donor V segments.[370,371] This development of B cells in rabbits is dependent on the intestinal microflora, and efforts are being made to define the potential bacterial superantigens involved, as well as binding sites on IgM necessary for the differentiation.[372] In ruminants, the ileal Peyer patches are the bursa-like primary B cell-generating tissues.[373] Although bursa, appendix, and sheep ileal Peyer patches show morphologic similarities, the mechanisms generating diversity are different: conversion in the chicken and hypermutation in sheep, and both in the rabbit. As described previously, most of the “GALT group” also appears to lack IgD; thus, IgD might serve some purpose in repertoire development in some groups of mammals and not others.

In summary, the organization of the lymphoid tissues is perhaps the only element of the immune system that shows increasing complexity that can be superimposed on the vertebrate phylogenetic tree. The absence of primary and secondary lymphoid tissues (thymus and spleen) is correlated with the absence of a rearranging and/or hypermutating receptor family in other animals, with the exception of the agnathan VLR in which the relevant lymphoid tissues are being defined (see the following). While all jawed vertebrates have a true secondary lymphoid tissue (spleen), ectotherms lack lymph nodes and organized GALT. In addition, while ectotherms clearly have B-cell zones resembling follicles, and despite the clear ability for ectothermic B cells to undergo SMH and at least some degree of affinity maturation, GCs with follicular dendritic cells are not formed after immunization; clearly, this was a major advance in the evolution of the vertebrate immune system.

The potential repertoire of Ig and TCR as well as VLR-combining sites is enormous in all vertebrates. The potential antigen receptor repertoire in all species for both T and B cells is far greater than could ever be expressed in an animal because of cell number limitations. Not all species or all gene families use combinatorial joining for repertoire building, but all species assemble V, (D), and J gene segments to generate their functional Ig genes during B-cell ontogeny, and the imprecision of this assembly creates great somatic diversity. Thus, from this survey in various species, one could not predict that there would be major differences in immune responses in representatives of different vertebrate classes, and yet as mentioned previously the mouse/human antibody responses are superior to those in many taxa.

More on Evolution of the Thymus

The discovery of a second (cervical) functional thymus in mice has raised ontogenetic and immunologic questions.[342,374] Is this second thymus the result of an atavism, or does it correspond to what is seen in human when a cervical thymus can form under certain pathogenic conditions during the migration of the thymus to its final mediastinal location? There are examples of cervical thymi in primitive mammals such as marsupials but also in some prosimians. An “extra” thymus also is reminiscent of the multiple thymi encountered quite frequently in cold-blooded vertebrates.

As described, the thymus is promiscuous with regard to its precise developmental origin.[284] The thymus arises from pouches two to six in cartilaginous fish, from the second pouch in frogs, the second and third in reptiles, and from the third and/or fourth in bony fish, birds, and mammals. The final number of thymi can also be variable. It ranges from five pairs of organs in sharks, to four in caecilian amphibians, to three in urodeles, and finally to one in many teleost fish species, anurans, and many mammals. The thymus of reptiles varies in term of location and number of lobes, reflecting variation in embryonic origin. The adult thymus may be found anywhere from the base of the heart to the neck. For example, in lizards and snakes, there are two lobes on each side of the neck with no subdivision in lobules. The crocodilian thymus is an elongated chain-like structure, not unlike that of birds. In turtles, the thymus is a pair of lobes divided in lobules at the bifurcation of the common carotid associated with the parathyroids. The multilobed thymus in birds is not the equivalent of the multiple thymi found in sharks because the subdivision is secondary to primary organogenesis. Regardless of the underlying ontogenetic mechanism leading to the development the cervical thymus in mice, the result is suggestive of the secondary “thymus spreading” in birds, but it differs with regard to an uneven final size distribution. All marsupials, except koalas and some species of wombats that only have a cervical thymus, have a thoracic thymus similar to that of placentals. Some marsupials (kangaroos and possums) also have a cervical thymus; their thoracic thymus derives from the third and fourth pharyngeal pouches, whereas the cervical thymus arises mainly from the ectoderm of the cervical sinus with some participation from the second and third pouch (like in reptiles).

The second mouse thymus seems to show primitive characteristics such as the existence of a single lobe as is found in amphibians, a superficial location, and a position compatible with an origin involving another pharyngeal pouch (presumably the second) that would be a marsupial and therefore perhaps a reptilian character. Embryology helps in determining the possible scenarios: During mouse ontogeny, the canonical thymus anlage can be recognized, beginning on day 11.5 in development, as a group of Foxn1-expressing cells located ventrally in the third pharyngeal pouch. If the cervical thymus were derived from an independent anlage, one would rather expect to detect Foxn1+ cells in the endoderm outside the third pouch. This argues for a common origin of thoracic and cervical thymi and against a second thymus anlage outside the third pouch, and, hence, against the above hypothesis that the cervical thymus represents an atavistic organ.

A Thymus in Jawless Fish?. Until recently, it was believed that the thymus appeared in evolution with the emergence of adaptive immunity in the extinct placoderm lineage approximately 500 million years ago. There has never been any controversy concerning the presence of a thymus in all living jawed vertebrates, and as described, its requirement for T-cell differentiation is universal. The lack of a thymus in the jawless fish was consistent with the non-Ig/TCR adaptive immunity in these animals, but this has changed in a new study.[375]

The finding that lampreys have two types of lymphocytes heralded a renewed search for a thymus equivalent in these animals.[291] Indeed, there was an extensive literature on this topic over the past century identifying accumulations of cells in various cranial regions, suggesting that lymphocytes could be differentiating in these areas. However, it has always been clear that there is no specialized tissue with a defined cortex and medulla in lampreys or hagfish, as is seen in all gnathostomes. This conclusion was not well supported because there were

no molecular markers for either lymphocytes or thymic epithelium in jawless fish. Indeed, consistent with almost all other accumulated data, a recent study concluded that there was "no evidence for a thymus in lampreys."[343(p189)] In that study, the major transcription factor involved in the development of T cells described previously, *foxn1*, was expressed by the pharyngeal epithelium, but the lack of expression of any known lymphocyte markers made it unlikely that this region was truly the thymus equivalent; a similar transcription profile was seen in the gill epithelium of the model basal chordate *Amphioxus*, which truly seems to lack an adaptive immune system.

The discovery of "T cells" in lampreys opened a new panorama with the expression of T cell-specific genes besides the antigen receptors. As mentioned, Pancer and colleagues discovered that lamprey lymphocytes express two APOBEC family member genes, CDA1 and CDA2, and suggested that they were involved in the rearrangement (and perhaps mutational) events in the VLR genes.[288] In the T/B split paper described previously, these APOBEC family members seemed to be expressed specifically in either VLRA (CDA1) or VLRB (CDA2) cells. In the new study, CDA1 was shown to be expressed by lymphocytes in close proximity to the *foxn1* -positive pharyngeal epithelial cells.[375] Furthermore, only in these "developing" lymphocytes, but not in mature VLRA-positive cells, could a high percentage (approximately 25%) of out-of-frame VLRA genes be detected, implying that cells were differentiating in this region. In summary, this tissue in lampreys, which was christened the "thymoid," 1) is derived from the pharyngeal epithelium, 2) expresses classical thymic epithelial markers such as *foxn1* and notch ligands, and 3) is associated with developing VLRA cells, based on expression of the APOBEC family member CDA1, out-of-frame VLRA gene sequences, and failure to respond to activation signals (such as the T cell mitogen PHA) that stimulate mature lymphocytes. In addition, consistent with the high percentage of cells with a nonfunctional receptor, many lymphocytes undergo apoptosis in the thymoid, which is also comparable to the situation in jawed vertebrates. Much more work is necessary to understand this system, but the basic finding is extraordinary.

Because the thymoid is expressed at the tips of all of the gill filaments,[375] thymectomy will not be possible in jawless fish. Perhaps procedures will be developed to block the interactions between the VLRA cells and the pharyngeal epithelium, or the development of the thymoid itself can be disrupted. Assuming that this tissue indeed is the thymic equivalent in lampreys, what is the significance of having the VLRA cells develop in a unique organ? In gnathostomes, T cells recognize antigen in the form of peptides in association with MHC class I or class II molecules. Because of the high levels of MHC polymorphism, as mentioned previously, T cells are positively selected in the thymus for cells that recognize antigen in association with the thymic MHC. Despite major effort, neither MHC molecules nor the specialized proteins associated with antigen processing have been detected in the jawless fish, and thus if there is positive selection it must be orchestrated by a convergent system of antigen processing/presentation. In the same vein, perhaps there is an AIRE equivalent expressed by the thymoid that ensures deletion of self-reactive clones[376]; if so, it would also imply that a convergent antigen presentation system will be discovered in lampreys. It will be of interest to reexamine differentiation of lymphocytes in the pharyngeal epithelium of basal chordates; perhaps this will lead us to an understanding of the origins of adaptive immunity in the vertebrates. Finally, what is the significance of T cells developing in association with the pharyngeal epithelium? Is it because this area in the gill region is evolutionarily plastic or is

there some relevance to exposure of the thymoid to the external environment?

Discovery of a New Proteasome Element Expressed in Thymus . Recently, a new form of the proteasome only expressed in the thymus was discovered in mammals, called β5t. The β5t gene was generated via a cis duplication from LMPX (β5), and in its absence cytotoxic T cells are impaired.[377] Like many other components of adaptive immunity we have discussed, β5t is found in all gnathostomes, beginning with the cartilaginous fish. As we will see in the following, the LMPX equivalent of the immunoproteasome, β5i or LMP7, is polymorphic in amphibians and bony fish,

suggesting that it is a lynchpin in formation of the immunoproteasome.[378] It is interesting as well that both β5t and β5i (and all of the immunoproteasome elements) have been lost in birds (see the following).

The Major Histocompatibility Complex

T cells distinguish self from nonself through the presentation of small peptides bound to MHC class I and class II molecules (ie, MHC restriction). The genetic restriction of T cell-APC collaboration, processing of antigen by professional APCs, and T-cell education in the thymus described in mice hold true (or is assumed) for most jawed vertebrate classes. For technical reasons, no MHC-regulated T-cell responses have been documented in cartilaginous fish, but the identification of polymorphic class I and II and rearranging TCRα/β genes and segregated T- and B-cell zones in spleens (see previous discussion) strongly suggest that functional analyses will reveal MHC restriction of adaptive responses.[257] By contrast, urodele amphibians and teleost cod are notorious for their poor immune responses (see the following), but biochemical and molecular evidence suggests that class II polymorphism is low in the axolotl and cod have lost the entire class II-based immune system. Recent results in agnathans and other vertebrates have opened our eyes to new functions in MHC.

Class I/II Structure Through Evolution

The three-dimensional organizations of class I and class II are remarkably similar: the two membrane-distal domains of both molecules form a PBR composed of two antiparallel α helices resting on a floor of eight β strands, and the two membrane-proximal domains are IgSF C1. Although sequence identity among class I and class II genes in vertebrates is low (like most other immune genes), the four extracellular domain organization and other conserved features are likely to be found in the ancestral class I/II gene.[379] An intrachain disulfide bridge exists within the class I PBR α2 and class IIβ1 domains, but not the class I/II PBR α1 domains, and phylogenetic trees show that these respective domains are most similar. Bony fish class II α1 domains, like class II DMα molecules, do have a disulfide bridge. The exon/intron structure of class I and class II extracellular domains is also well conserved, but some teleosts have acquired an intron in the exon encoding the IgSF β2 domain. Other conserved features of class I genes include a glycosylation site on the loop between the α1 and α2 domains important in biosynthesis (shared with class II β chains), a Tyr and one to three Ser in the cytoplasmic regions that can be phosphorylated in mammals, as well as several stabilizing ionic bonds. Class II with its two TM regions differs from class I with only one; conserved residues in the class II α and β TM/cytoplasmic regions facilitate dimerization. In summary, because sequence similarity is very low among MHC genes in different taxa,

these conserved features are important for function, biosynthesis, and maintenance of structure.

β2m was the second IgSF molecule (C1 type) ever to be identified, originally found at high levels in the urine of patients with kidney disease. It associates with most class I molecules (see the following). Besides mammals, β2m genes have been cloned from representatives of all jawed vertebrate classes. The β2m gene is outside the MHC in all tetrapods and bony fish tested, and is a single copy gene in all species except cod and trout, in which it has undergone multiple duplications. Based on the levels of similarity between the various domains of class I and class II, it was predicted that β2m was originally encoded in the MHC; indeed, recent work has shown that it is linked to the MHC in the nurse shark, adjacent to the ring3 gene.[380] Like TCR/Ig/class I/II, β2m has not been found in jawless vertebrates, so its origin remains mysterious.

Classical and Neoclassical Class I and Class II

Class Ia (classical) and class Ib (nonclassical) genes are found in all of the major groups of jawed vertebrates. Class Ia genes are defined by their ubiquitous expression, their presence in the MHC proper, and by high polymorphism (in most species). In addition, class Ia proteins almost always have eight conserved residues at both ends of the PBR that interact with "mainchain" atoms of bound peptides and constrain their size to eight or nine residues; this feature often distinguishes class Ia from class Ib (see the following). Thus, tight binding of peptides, a likely source of conformational changes in class I allowing transport through the endoplasmic reticulum and cell surface expression, is an evolutionarily conserved trait.[381] In nonmammalian vertebrates, one of these residues at the C-terminus is lysine rather than tyrosine, the functional significance of which is unclear.[379]

The class Ia/Ib distinction holds in most taxa: one to three polymorphic class Ia genes are expressed ubiquitously in most species, whereas other minimally polymorphic or monomorphic class Ib genes are expressed in a tissue-specific fashion. The class Ib genes can be split into two major groups: one set that is most related to the class Ia genes within a taxon and thus recently derived, and one group that is ancient and emerged early in evolution.[381] In mouse and human, the set most closely related to class Ia genes are closely linked within the MHC. In nonmammalian vertebrates, however, this first set is found in gene clusters on the same chromosome as the MHC proper but far enough away to segregate independently from MHC (eg, chicken [*Rfpy*] and *Xenopus* [*XNC*]). One *Xenopus* class Ib gene is expressed specifically in the lung and thus likely has a specialized function, and another gene (XNC10) is expressed by T cells and may serve as a ligand for unconventional subsets of T cells.[382,383] Chicken Rfpy is associated with resistance to pathogens, and the recent structure of one of these class Ib genes has shown an unusual hydrophobic structure in the groove.[384] Class Ib genes related to but unlinked to the classical class I have also been found in bony and cartilaginous fish, and a lineage of class II-linked class I genes was discovered in bony fish.[385] Thus, class Ib genes that arise in each taxon seem to have true class I-like functions, but perhaps have become specialized (sometimes the distinction between class Ia and class Ib is blurry; see the following). The second set of older class Ib genes that predates divergence of taxa can have very different functions.[381] For example, the neonatal Fc receptor is involved in binding

and transport of IgG molecules across epithelia as well as protecting them from degradation (the Brambell receptor). Furthermore, molecules only described so far in mammals are composed only of a PBR without IgSF domains; these unusual class I molecules do not bind peptides but rather are important for the regulation of NK- and T-cell function during infection. The paradigm for these SOS responses is the MHC class I-related protein (MIC) and UL16-binding protein (ULBP) class Ib molecules, which clearly do not bind peptides. Some teleost class Ib genes that fall outside the major cluster of fish class I genes may fit into this category.[385,386] Finally, molecules like CD1 bind nonpeptidic antigens for presentation to innate-like NKT cells. The phylogenetic analysis predicts that CD1 and FcRN are old class I genes (see the following on CD1); the age revealed by the phylogenetic tree also correlates well with the hypothesis that ancient duplication events predating the emergence of jawed vertebrates resulted in the appearance of CD1, FcRN, and MHC-linked class I genes (see the following). Was the original function of class I linked to antigen presentation (peptidic or otherwise), induction of an SOS response, or to housekeeping functions? We do not have the answer because class Ia and class Ib molecules are present in the oldest living gnathostomes. The discovery of class I-like genes in animals derived from ancestors predating adaptive immunity or in the jawless fish would help resolve this question, but to date no recognizable MHC molecules or their kin have been detected in prejawed vertebrates; this topic is especially of interest since the dichotomy of lymphocytes as well as a thymus candidate in jawless fish were discovered.[291,375]

Class II molecules also have nearly invariant and evolutionarily conserved residues that bind to main-chain atoms of peptides, but these are in the center of the groove.[379] Thus, tight binding to main-chain peptide atoms occurs in the center of the class II PBR, and peptides are free to protrude from both ends. The only nonclassical class II molecules so far identified are the previously mentioned DM molecules that lack these residues and DO proteins. DM molecules so far have been cloned only from tetrapods[177] and have not been detected in any fish species despite the large genomic and EST databases for the bony fish. Thus, they either had not emerged at the time fish arose or were lost in the bony fish lineage; phylogenetic trees suggest the latter, and would be consistent with the rapid rate of genome evolution in the teleosts. DO class II molecules, believed to modulate DM function, have only been detected in placental mammals.[387] The invariant chain is another protein found only in gnathostomes[388]; its recent role in class I cross-presentation suggests that it may have been important for biosynthesis of both class I and class II.[389]

Class I/II Expression

In *Xenopus* species, immunocompetent larvae express high levels of class II on APCs such as B cells, but express only very low levels of class Ia molecules on all hematopoietic cells until metamorphosis.[328] Expression of the immunoproteasome element *Imp7* and all identified class Ib isotypes is also very low.[390] Larval skin and gut, organs with epithelia in contact with the environment, appear to coexpress class I (transcripts) and class II. Such expression may provide immune protection during larval life; perhaps expression of class Ia is limited to organs that undergo massive destruction and remodeling at metamorphosis. Class II molecules also change their distribution after metamorphosis and are highly expressed by

unstimulated T cells.[391] Axolotl class II molecules are also regulated differentially during ontogeny, expressed in young animals on B cells, and then expanded to all hematopoietic cells, including erythrocytes, later in life.[392] Changes in MHC expression are not correlated with cryptic metamorphosis in axolotls, but class II expression by erythrocytes is correlated to the switch from larval to adult globins. Unlike *Xenopus*, class I transcripts isolated so far are expressed early in ontogeny, from hatching onwards.

Carp class I and class II transcripts are detected in embryos 1 day after fertilization and reach a plateau at day 14. However, the suspected class Ia protein does not appear until week 13, whereas β2m can be detected several weeks earlier.[393] It was suggested that another class I molecule is expressed during early development of the carp hematopoietic system, perhaps one of the unusual nonclassical molecules that groups outside the teleost cluster. Interestingly, class I is expressed in the brain of young, but not adult fish, suggesting that class I molecules may play a role in neurogenesis.[394]

As mentioned previously, the cod was recently shown to have lost class II genes, as well as the invariant chain and CD4 genes.[395] The apparent lack of the entire class II system of antigen recognition correlates well with the inability of cod to make antigen-specific antibody responses.[396] The authors speculate that the large numbers of class I molecules in cod may somehow compensate for the loss of a class II system. Axolotls, which have very low class II polymorphism,[397] also have a highly expanded class I system.[398] It is possible that in both cases there has been a “use it or lose it” scenario, in which a major arm of the immune system was lost due to a low pathogen load.

Major Histocompatibility Complex Gene Organization

As class I and class II proteins are structurally similar, it is no surprise that their genes are linked, a primordial trait subsequently lost only in bony fish (Fig. 4.12).[2] But why are structurally unrelated class I processing genes, including the immune proteasome components *Imp2* and *Imp7* and the *TAP* and *TAPASIN* genes, also found in the MHC? There are two possible scenarios: primordial linkage of ancestral processing and presenting genes in the MHC, or later recruitment of either the processing/presenting genes into a primordial MHC. Based on the presence of similar clusters of MHC genes on paralogous chromosomal regions in humans and mice, Kasahara et al.[17,399] proposed that ancestors of class I, class II, proteasome, transporter, and class III genes were already linked before the emergence of the adaptive immune system. Genomewide duplications around the time of the origin of vertebrates (the so-called 2-Round or 2R hypothesis), as proposed by Ohno et al.,[16] may have provided the raw material from which the immune system genes was assembled (see the following; Fig. 4.13). As for all other adaptive immune genes described so far, neither class I/II nor immunoproteasome/transporter associated with antigen processing (TAP) have been isolated from hagfish nor

lampreys, and all of these genes as well as other genes involved in immunity could have emerged as a consequence of the duplications. Because class I genes are found on two or three of the clusters, class I-like molecules may have predated class II in evolution. Indeed,

NK-like recognition of a class I or class I-like molecule encoded in an ancestral linkage group may have been at the origin of the adaptive immune system (see the following).

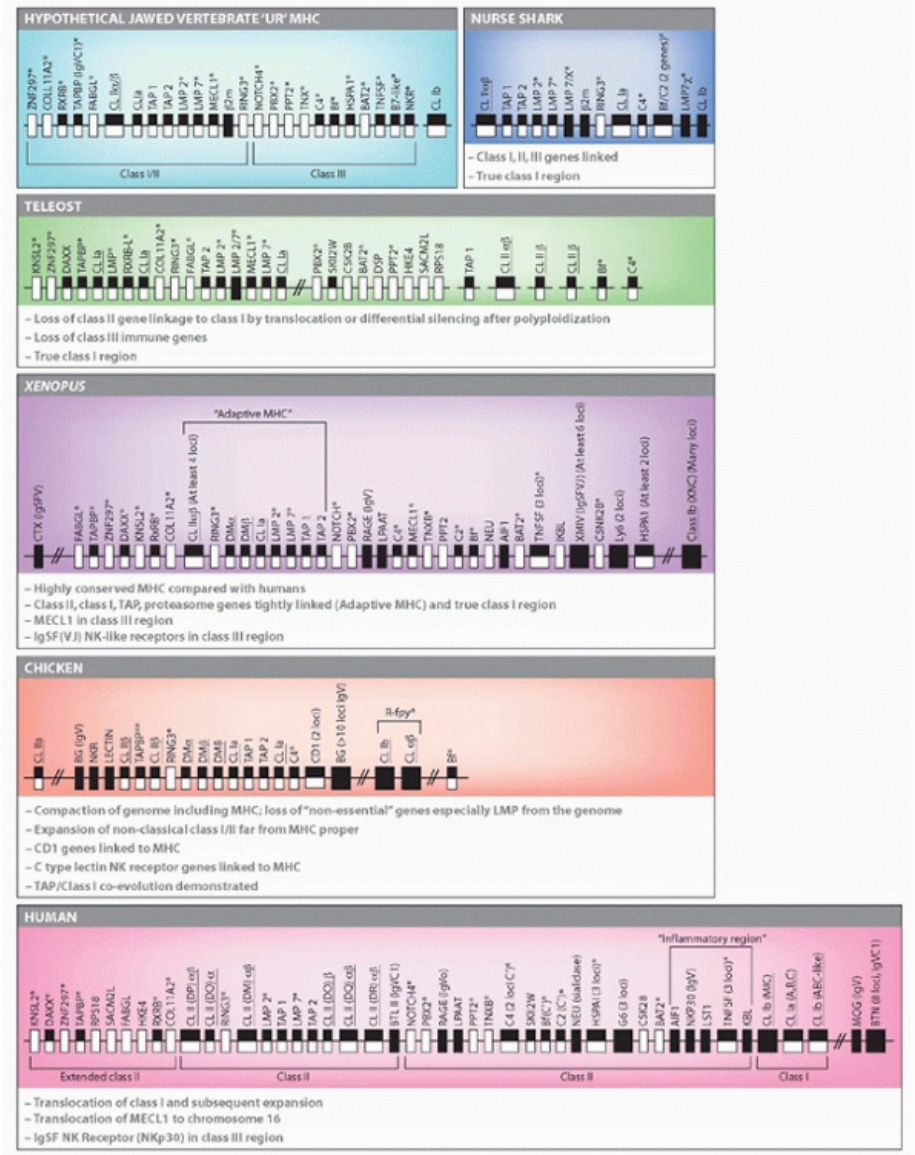

FIG. 4.12. Major Histocompatibility Complex (MHC) Evolution. *White* indicates that the gene is found in at least one other species besides human; *black* designates a gene with known or inferred immune function; *asterisk* indicates an ancestral gene found on at least two paralogous MHC regions in mammals; *large box* indicates at least two genes in the particular region; *double slash* indicates linkage far away on the same chromosome; *space between lines* indicates that the genes are on different linkage groups. The teleost MHC is a composite of the zebrafish, trout, *Fugu*, and medakafish maps. Extensively modified from Flajnik and Kasahara.[498]

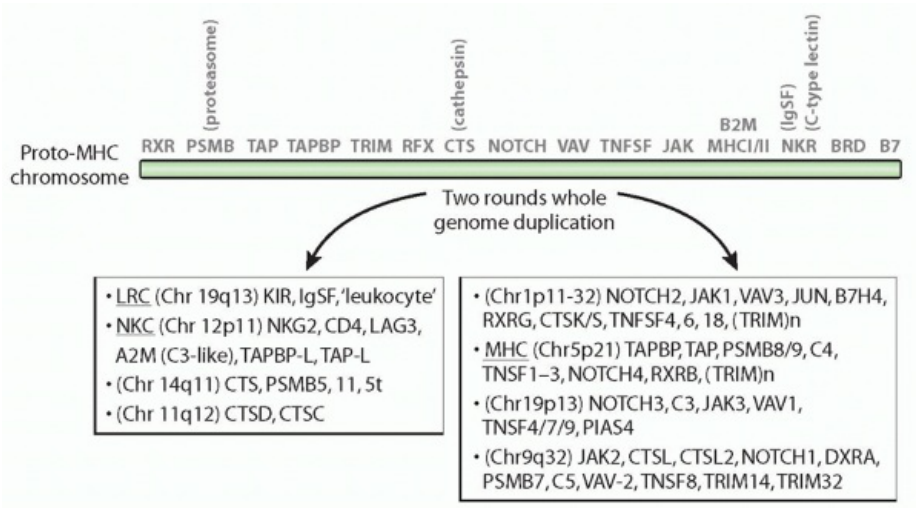

FIG. 4.13. Genomewide Duplication (2R) Model of Major Histocompatibility Complex (MHC) Evolution. Displayed are the MHC paralogous regions and their human chromosomal designations, first identified by Kasahara and modified over the years.[2,17] Note that such paralogous regions have also been studied for chemokine receptors,[477] tumor necrosis factor and tumor necrosis factor receptors,[459] immunoglobulin superfamily molecules involved in cell-cell interactions,[442] and others. Note that the natural killer cell complex and leukocyte receptor complex, respectively, are likely to have been in the Ur MHC based upon linkages seen today in various taxa. The *box on the right* indicates the original MHC paralogous regions identified by Kasahara on chromosomes 1, 6, 9, and 19.[17] The *box on the left* shows paralogous regions uncovered in further analyses.[500] Modified from Flajnik and Kasahara.[2]

TAP is an interesting case in that the MHC-linked TAP 1/2 genes were clearly not part of the Ur MHC, but rather they were "recruited" to the MHC early in evolution. TAPs are members of the very large ABC transporter family, and are most closely related to bacterial ABC transporters; this suggests that TAP1/2 were derived from a bacterial, or more likely a mitochondrial gene, via horizontal transfer.[400] Furthermore, a close homologue of TAP1/2, TAP-L (TAP-like), unlike all of the other Ig/TCR/MHC genes we have discussed, is present in jawless fish.[401] The function of TAP-L is not known, but it may be involved in cross-presentation[402] or "typical" presentation in jawless fish. In summary, the TAP genes are not following any of the "rules" that seem to hold true for the other genes involved in adaptive immunity, and this story will be exciting to monitor, especially in the context of the agnathan VLR system.

In all nonmammalian vertebrates, and even in marsupials, the immunoproteasome and TAP genes are closely linked to class I genes, not to class II, in a true "class I region."[2] This result is most striking in bony fish (*Fugu*, zebrafish, medaka, trout) because class I/Imp/TAP/TAPBP and class II are found on different chromosomes.[403,404] The class III region, historically defined by the innate immune genes such as Bf/C2, and C4 are also present in the *Xenopus* and elasmobranch MHC, showing that the class III association of class I/II with such genes is

ancient.[177] If Kasahara's interpretation is correct (ie, MHC syntenic groups found on different mammalian chromosomes resulted from ancient block duplications), it is expected that the physical association of ancestral class I, II, and III genes predated the emergence of jawed vertebrates, and such syntenies in ectothermic vertebrates are not surprising. Indeed, linkage studies in nonvertebrates *Amphioxus* and *Ciona* do support an ancient linkage of class I, II, and III genes.[405] Taken together, the data reveal that lack of synteny of class I, class II, and class III genes in teleosts is a derived character. Independent assortment of class I and class II may allow these genes to evolve at different rates: in some teleosts, class Ia alleles form ancient, slowly evolving lineages, whereas class II genes evolve at similar rates as mammalian MHC alleles.[406,407]

The chicken MHC, the B complex, is on a microchromosome, and intron sizes and intergenic distances are both quite small so that the entire complex is only a few hundred kb as compared to over 4,000 kb in humans and *Xenopus*.[408] Class Ia (BF), class IIβ (BL and DM), and TAP genes are in the MHC, but there is no evidence for immunoproteasome genes, and almost all class III genes have been deleted except for C4. The quail MHC is similar, although is somewhat expanded, especially in genes related to the C-type lectin NK receptors.[409] Although most class III genes are found on other chromosomes, *Imp2/7* and *MECL1* genes are actually absent from the genome; indeed, peptides bound to chicken class I molecules sometimes have C-terminal glutamic acid or aspartic acid, which are rare after proteolysis by mammalian proteasomes containing Imp2 and Imp7. Indeed, the recent crystal structure of a chicken class I molecule shows that the alleles can interact with a broader array of peptides as compared to mammalian class I alleles; the PBR is “broader” and one of the conserved peptide-binding residues is noncanonical.[410] To explain the correlation of

diseases with particular haplotypes, Kaufman proposed that the chicken has a minimal essential MHC composed of only those genes absolutely required to remain in the complex. This concept has been reinforced recently by an analysis of resistance to viral infection (Rous sarcoma virus) governed by classical class I molecules.[411,412] Additionally, the presence of polymorphic TAP alleles closely linked to particular class I alleles has demonstrated coevolution of the transporters and peptide-binding molecules.[413]

Surprisingly, CD1 genes are closely linked to the chicken MHC.[414,415] As mentioned previously, CD1 genes in mammals seem to be on one of the MHC paralogous regions and it was suggested that it arose as a consequence of the en bloc duplication. While this is still possible (ie, there was differential silencing of CD1 and other class I genes on the two paralogous chromosomes), the more likely scenario is that CD1 arose by gene duplication within the MHC itself in an ancestor of warm-blooded vertebrates; no bonafide CD1 genes have been detected to date in any fish or amphibians, so the entire NKT system may be specific of warm-blooded vertebrates.

In summary, in all animals except placental mammals, classical class I genes map closely to the *TAP*, *Imp*, and tapasin genes, suggesting that the processing, transport, and presenting genes were in an original “class I region.”[416] The tight linkage of the functionally, but not structurally, related genes strongly suggests that such genes coevolve within particular MHC haplotypes. Indeed, in *Xenopus* there are biallelic lineages of class Ia, LMP7, and TAP, which

are always found as a set in wild-caught animals.[417] Although teleosts underwent an explosive adaptive radiation 100 million years ago and primordial syntenies have been lost in many cases, there are deep lineages of class Ia genes in many species, also found for *Xenopus* and cartilaginous fish class Ia genes. A study in medaka suggests that divergent noncoding regions between the class I-processing and -presenting genes do not permit recombination between lineages, hence preserving the linkage disequilibrium.[418] Nonaka et al. proposed that these deep proteasome/class I lineages emerged at the dawn of vertebrate immunity and may even have been maintained perhaps by convergent evolution in certain groups.[419] In (most) eutherian mammals, the class I region is not closely linked to Imp/TAP and is very unstable, with rapid duplications/deletions expected in a multigene complex (see Fig. 4.12); the same class I instability extends to the non-MHC-linked class Ib genes in *Xenopus* species.[420]

EVOLUTION OF ALLORECOGNITION

Histocompatibility Reactions in Invertebrates

Scrutinizing graft rejection within a species (allograft) across the animal kingdom demonstrated that allorecognition was almost universal among metazoa. Scrutinizing specific memory in the same systems was *the* major tool to establish the universality or not of adaptive immunity. Moreover, it fueled speculations the origins of vertebrate MHC. Most of these studies led to inconclusive results because of the poor immunogenetic aspects of the reactions. However, the genetic regions and mechanisms responsible and molecular underpinnings of such alloreactions are now becoming known, and no (or little) resemblance to the vertebrate MHC has been found even though regions homologous of the extended vertebrate MHC have been identified in some organisms but without functional correlation.[405,421]

Colonies of *Porifera*, *Cnidaria*, *Bryozoa*, and *Tunicata* (see Fig. 4.1) often compete for space and may develop histocompatibility reactions in the zone of contact. In addition, cell-lineage parasitism, in which the somatic and/or germ cell lineage of one partner replaces that of the other, may ensue if colonies fuse into a chimera. Thus, effector functions following allorecognition also protect the genetic integrity of the individual. In some species, fusion is apparently restricted to tissues of the same individual (complete matching), in other species such as bryozoans, fusion also occurs between genetically distinct individuals if they share kinship (partial matching). Two divergent invertebrate phyla have been studied in detail: *Cnidaria* and *Tunicata*.

Porifera

Sponges (*Porifera*) and placoza are the phylogenetically oldest extant metazoan phyla. Sponges, whether marine or freshwater species, possess a sophisticated histocompatibility system.[422] Elements of the sponge immune system involved in these reactions have been analyzed at the molecular level. Sponge cells associate in a species-specific process through multivalent calcium-dependent interactions of carbohydrate structures on a 200 kd extracellular membrane-bound proteoglycan called “aggregation factor,” well studied in *Microciona* and *Geodia*.[422] The glycan moiety is involved in cell adhesion and exhibits differences in size and epitope content among individuals, suggesting the existence of allelic

variants. Therefore, strong carbohydrate-based cell adhesion evolved at the very start of Metazoan history. Other genes involved in these reactions include one that is similar to the vertebrate MHC-linked allograft inflammatory factor and another to the T-cell transcription factor. AIF-1 and T-cell transcription factor genes are upregulated in vivo after tissue transplantation, and in vitro in mixed sponge cell reaction.[423] Polymorphic IgSF molecules are found on the surface of sponge cells, but their relationship to allorecognition events (if one exists) is not clear.[424]

Allogeneic recognition in vitro led to apoptotic cell death in one partner and survival in the other.[425] The process is controlled by a differential expression of the proapoptotic and prosurvival proteins that are characteristic for the initiation of apoptosis (caspase, MA3, ALG-2 protein) and the prevention of programmed cell death (2 Bcl-2 homology proteins, FAIM-related polypeptide, and DAD-1-related protein). In an apoptotic mixed cell combination, characteristic apoptotic genes were expressed, while in the nonapoptotic aggregates the cell-survival genes are upregulated.[423] In another species, *Microciona*, allogeneic interactions also

induce cellular reactions involving gray cells (sponge immunocytes) and finally apoptosis. Analogous (but most likely not homologous) to T-cell responses, the response is inhibited by cyclosporin A.[426]

From observation of 50 pairs of larval grafts within one F1 progeny of the marine sponge *Crambe*, 75% could fuse, a proportion suggesting that the genetic control depends on one locus and sharing of one haplotype results in fusion; a 100% fusion between mother and offspring is consistent with this interpretation. Unfortunately, the issue is complicated as individuals from a given mother may not have the same father. Still, the few data available are consistent with a single or at least major histocompatibility locus (or region), the “rule” in other invertebrate phyla described in the following.

Cnidaria

The existence of highly polymorphic histocompatibility loci was demonstrated long ago in various corals where apoptosis induction was responsible for the death of partners in incompatible combinations,[154] whereas in sea anemone nematocyte discharge was induced between incompatible individuals.[427] The colonial cnidarian *Hydractinia* was the only species to provide a model to analyze genetic control of such reactions. In 1950, Hauenschild noticed that allorecognition seemed to be under the control of a single genetic region with multiple alleles.[428] Colonies of *Hydractinia* encrust the shells of hermit crabs, where they grow by elongation and branching of stolons. Embryos and larvae fuse indiscriminately. However, when two or more larvae are recruited to the same substratum, colonial forms may come into contact through their stolons. If the two colonies are histocompatible, stolon tips adhere and fuse, establishing gastrovascular connections and a permanent genetic chimera. If tips of incompatible colonies fail to adhere, they swell (hyperplastic stolons) with the migration of nematocysts, which discharge and damage the tissues. In addition, transitory fusions can also occur in a few cases. Similar to the genetics of the sponge alloreactions previously mentioned (and in other invertebrates and plants), colonies fuse if they share one

or two haplotypes, reject if they share no haplotypes, and display transitory fusion if they share only one allele at one haplotype and no alleles at the other. Examination of the polymorphic locus governing this reaction (*alr*) revealed the involvement of two closely-linked polymorphic loci 1.7 cm apart (likely encoding receptor and ligand), *alr1* and *alr2*.[429] *Alr1* encodes a three IgSF domain surface molecule, not unlike other metazoan nectin-like molecules, with a cytoplasmic tail equipped with multiple signaling motifs (including a ITAM-like motif). In the first domain, the molecule shows a high level of variability at particular residues, suggesting positive selection. Alr1 is embedded in a genetic region consisting of multiple IgSF members. Alr2 encodes another highly polymorphic gene with three IgSF domain IgSF with the distal V-like domain being the most variable, and an ITIM-like motif in the cytoplasmic tail adjacent to various phosphorylation motifs. Like the corals studied by Theodor,[430] a high level of allele diversity was found at the alr2 level as nearly all sampled alleles encoded unique gene products.[431]

Urochordates

Compared to *Cnidaria*, tunicates shared a recent common ancestor with the vertebrates (see Fig. 4.1), and thus might be expected to have a histocompatibility system more related to MHC. Allorecognition has been studied in both colonial and solitary ascidians. In *Botryllus*, a colonial ascidian, extra attention was afforded this system because the locus controlling histocompatibility was linked to (or was the same locus as) the locus controlling fertilization by preventing self-fertilization. For fusion, at least one histocompatibility locus must be shared between the colonies and for fertilization the sperm must be mismatched from the egg.[432]

Metamorphosis in *Botryllus* is followed by budding that eventually gives rise to a large colony of asexually derived genetically identical individuals (zooids), united through a vascular network. At the periphery of the colony, the vasculature ends in ampullae, which are the sites of interaction when two colonies meet during their expansion. The interaction results in either fusion of the two ampullae to form a single chimeric colony sharing a common blood supply or a rejection reaction during which the interacting ampullae are destroyed, thus preventing vascular fusion. Hemocytes (morula cells) are involved in the reaction.[433] Fusion or rejection is governed by a single highly polymorphic (tens to hundreds of alleles) locus called the FuHC (for fusion/histocompatibility; 10 wild-type individuals collected from around the Monterey Bay area yielded 18 cFuHC alleles). As mentioned, when two colonies share one or both FuHC alleles, they will fuse; rejection occurs if no alleles are in common. However, like in *Hydractinia*, intermediary pathways have been reported in the past and have not been entirely elucidated.

The C-terminal region of the FuHC molecule consists of a nectin-like segment with three Ig domains, showing most similarity to chicken Igsf4 and related members conserved in all vertebrates as well as to one of the *Ciona* nectins.[434] Those members are found in a tetrad of paralogs in vertebrates but not linked to the tetrad of MHC paralogs (see the following). The N-terminal region contains an epidermal growth factor (EGF) domain and some other unrecognizable regions not conserved among ascidians. Any two FuHC alleles differ by an average of 4% at the nucleotide level. Unlike MHC class I and class II, polymorphic residues in FuHC alleles are not concentrated in particular regions, and alternative splicing can generate a fragment devoid of the IgSF moiety.

Some[200] kb away from FuHC is a second polymorphic (and polygenic) locus, *fester*, which is inherited in distinct haplotypes.[435] Diversified through extensive alternative splicing, with each individual expressing a unique repertoire of splice forms (each individual expressing up to three splice variants in addition to the regular full-length product), it potentially exists as both membrane-bound and secreted forms, all expressed in tissues intimately associated with histocompatibility. After *fester* knockdown, the histocompatibility reaction is blocked at the stage of initiation as

if the colonies ignored each other. By contrast, when Fester was blocked with specific mAbs, fusion reactions were unaffected, but rejection reactions were turned into fusions in an allele-dependent manner. These data combined with its genetic location suggest that Fester encodes the FuHC ligand. Fester contains a short consensus repeat (or sushi domain) often found in vertebrate complement receptors. Beside these two polymorphic products, another *Botryllus* cell surface-expressed nonpolymorphic molecule related to fester, uncle fester, is required for incompatibility reactions, while fester seems to be required for allele discrimination and inhibition of killing.[436] So, several independent pathways seem to control the final outcome of the interaction between individuals.[437] It should be mentioned that a recent study, based essentially on the incongruence between histocompatibiity profiles and FuHC polymorphism, questions the validity of these reports.[438]

Solitary Ascidians

In *Halocynthia roretzi*, a "polymorphism of color" has been observed and histocompatibility reset by a mixed hemocyte technique in vitro resulting in a melanization reaction likely to involve the PPO cascade.[439] Depending on the strains, the percentage of positive reaction varied from approximately 55% to 70%, indicative of polymorphism. Grafting experiments had already shown the existence of allorecognition in solitary ascidians, and investigation at the cellular level had demonstrated the occurrence of cytotoxic cells in such organisms.[440] In order to shed light on allorecognition in urochordates and on the molecules involved in preventing self-fertilization, gonadal complementary DNAs of three genetically unrelated *Ciona intestinalis* individuals were compared by suppression subtractive hybridization. This led to the discovery of the highly polymorphic variable complement receptor-like 1 gene coding for a transmembrane protein with several short consensus repeat domains (short consensus repeat/complement control protein [CCP]), a motif shared with the variable fester receptor of *Botryllus* described previously.[441] Genes encoding variable complement receptor-like are in the same linkage group as a set of IgSF domains with homology to nectin (CD155/poliovirus receptor [PVR], cortical thymocyte protein [CTX]/junctional-adhesion molecule [JAM] family members, etc.) and other adhesion molecules of which related members can be found also on one genetic region in vertebrate, the 19q 34 human chromosome segment with the extended LRC (see the following).[442]

The Meaning of Histocompatibility Reactions in the Invertebrates

The association between allorecognition in *Botryllus* and fertilization led to the proposal that histocompatibility systems were selected during evolution to avoid inbreeding. The hypothesis made sense in the case of sessile colonial invertebrates that might have difficulty

dispersing their gametes and therefore are susceptible to inbreeding depression. Indeed, the partial matching mentioned previously is a general characteristic of fusion compatibility in colonial invertebrates, perhaps driven by “selection operating on an error-prone genetic system for self-recognition that is perhaps constrained by derivation from a gametic function selected to reduce inbreeding.[443] Furthermore, even in mammals there is a large literature suggesting a selection both at the mate-choice and pregnancy levels for preserving heterozygocity at the MHC.[443a] However, the possibility of a common genetic system, or linked systems, governing fusion and gametic compatibility awaits confirmation. Because animals that are neither sessile nor unable to disperse their gametes can possess alloimmune responses is inconsistent with the general hypothesis. Moreover, inbreeding avoidance can only explain the selection of histocompatibility alleles if the histocompatibility loci are genetically linked to a large fraction of its genome. This is inconsistent with the tight linkage of histocompatibility genes to a single major locus, especially in invertebrates. So inbreeding avoidance is unlikely to contribute significantly to the selection of histocompatibility alleles, although in jawed vertebrates, selection for heterozygocity at the MHC itself has obvious advantages.

Another hypothesis is that alloimmunity was selected because it avoided intraspecific parasitism and/or competition for attachment sites.[444] Indeed, after fusion of compatible colonies, bloodborne germline or totipotent stem cells are transferred between colonies, and can expand and differentiate in the newly arising, asexually derived individuals of the vascular partner.[445] This can result in a situation where only one genotype is represented in the gametic output of the fused individuals. The FuHC polymorphism in *Botryllus* could function to restrict to compatible individuals the vascular fusion and the germline parasitism. The high allelic polymorphism characteristic of all invertebrate recognition systems may have evolved in response to selection for fusion with self rather than kin. Fusion with self will allow the development of a colony that benefits from fusion while eliminating the possible cost of somatic cell parasitism, and thus would be the raison d'etre of the allorecognition mechanisms.[446]

However, most animals are not sessile and thus are neither prone to intraspecific competition nor to compete for limited substrate attachment sites. Tunicates besides *Botryllus* have evolved differently (eg, in *Diplosoma*, large numbers of chimaeras are encountered in nature). In addition, the intraspecific competition hypothesis demands that individuals maintain expression of histocompatibility alleles, even when the expression of these alleles enables their own destruction during intraspecific competition. These considerations suggest that intraspecific competition might affect histocompatibility allele frequencies in some organisms under certain conditions.

Both previous hypotheses are essentially based on observation made in colonial tunicates, but colonial living has evolved several times independently. Urochordates are most related to the vertebrates, but the data argue against any of the genes involved in their histocompatibility reactions being ancestral to MHC class I and class II. It is clear in all of these studies that a strong pressure for polymorphism is the rule, but the evidence from all of these fascinating

reactions suggests that such “pressures” select for similar systems via convergence, at least

at the level of the receptor. The selecting molecular environment can perhaps show more conservation (see the folllowing). On the other hand, as described previously, genetic regions with homology to the vertebrate MHC have been detected in the invertebrates, yet are (most likely) unrelated to the reactions detailed here (see conclusion).

As described, in all of these allorecognition systems, convergent mechanisms have been encountered to reach an analogous end, and thus it is unlikely that a unique "cause" arose for selecting them in diverse organisms. However, in an attempt to find an ultimate and general explanation to the selection during evolution of highly polymorphic allorecognition systems, it has been suggested that pathogen and retroviruses are the force behind the selection of allopolymorphism.[447] This does not imply intimate phylogenetic relationships between the systems observed but rather emphasizes analogous solutions when facing similar pressures. We discuss "connections" between vertebrate immunity and these histocompatibility reactions in the conclusion.

CYTOKINES AND CHEMOKINES

Many cytokines/chemokines and their receptors, like most molecules of the immune system, evolve rapidly. However, consistent with the "Big Bang" theory, it is an emerging picture that the majority of cytokines and chemokines found in mouse and human are also found in the genome and EST projects of nonmammalian jawed vertebrates, best studied in chickens and certain bony fish, but now extending to the cartilaginous fish as well. This suggests that whatever the initiating pressures in the evolution of the Ig/TCR adaptive immune system, the network of cytokines and chemokines emerged (practically) full blown early in evolution. A picture starts to emerge, but the gestalt is far from clear; this is, in part, because the situation in mouse human is clouded by the plasticity of T helper cell lineages, as well as an entirely new subset of immune cells, the innate lymphoid cells (ILCs), that produce cytokines previously thought to be within the domain of adaptive lymphocytes.

The Proinflammatory Cytokines, Interleukin-1 Family Members, Interleukin-6, Interleukin-8, and Tumor Necrosis Factor α

IL-1 and related family members IL-18 and IL-33, IL-6, TNF-α, and IL-8 (CXCL8) are the prototypic cytokines associated with inflammatory responses, which are defined by induction of vasodilatation and vascular permeability, and upregulation of innate immune system-specific molecules that have direct functions or that costimulate/attract T and B cells. Classically, many of these activities can be assayed in supernatants from PAMP (eg, LPS)-stimulated phagocytes by determining whether thymocytes are induced to proliferate when one also adds suboptimal concentrations of T-cell mitogens. It was reasonable to hypothesize that such cytokines, which act both at a distance as well as in a cognate fashion, might be found in the invertebrates. Indeed, IL*-1-like *activities* have been described for echinoderm coelomocytes (either IL-1-like production by such cells, or the ability of the cells to respond to mammalian IL-1), but unfortunately no molecular data revealing the structures of the active invertebrate cytokine/cytokine receptor have been reported. In fact, no ortholog has been detected in the genome projects from protostomic invertebrates (see Fig. 4.1), and only IL-17 and TNF homologues have been detected in nonvertebrate deuterostomes and protostomes,[48,448,449] and IL-8 in agnathans; thus, we may consider these as primordial cytokines related to the vertebrate versions. A molecule from earthworms capable of activating the prophenoloxidase defense pathway cross-reacted with a mAb directed to

mammalian TNF-α.[450] However, this molecule had no homology to TNF-α upon sequencing.

IL-1 activity as measured by costimulation assays or as a consequence of PAMP stimulation has been detected in all nonmammalian vertebrates. IL-1β upregulation has been detected after treatment of macrophages with LPS, consistent with its inflammatory function in mammals. In addition, injection of gram-negative bacteria into trout induced IL-1β expression in many tissues.[451] Identity with the mammalian IL-1β gene in all other species ranges from 28% to 40% (identity between mammalian IL-1α and IL-1β is about 25%). In nonmammalian species, IL-1β lacks the so-called ICE cleavage site, important for function in mouse/human.[452] IL-18 is an IL-1-related cytokine, and in contrast to IL-1 seems more focused in its function of potentiating TH1 responses. IL-18 has been detected in birds and fish, but the tissue distribution in fish seems to be expanded as compared to mammals. Additionally, IL-18 in nonmammalian vertebrates contains the ICE cleavage site, unlike its cousin IL-1β. Other IL-1-related cytokines, including IL-33 and the IL-1F series, have also been found in several jawed vertebrates, but with little study of functional activity. This will be of great interest to uncover, considering the IL-1 family members' roles in regulating Th1, Th2, and Th17 (and others?) differentiation.[453]

Both chicken IL-1β and the IL-1R were identified and have been expressed as recombinant proteins.[454] The IL-1R homology to mammalian orthologs is quite high (61% identity), but the highest similarity is found in the cytoplasmic domains. In addition, there are four blocks of high similarity to the cytoplasmic tail of toll/TLR proteins, and IL-1R and TLR use similar signal transduction cascades (see previous discussion).

As mentioned, IL-8 (actually, the CXC chemokine CXCL8) has been identified in the jawless lamprey and seems to be expressed specifically in B cells, whereas the IL-8 receptor is found in T cells; this has been one of the mechanisms proposed to permit the cells to interact in a cognate fashion.[13,291] IL-8 has also been found in various gnathostomes such as trout, flounder, and perhaps chicken; a chicken CXC chemokine called K60 clusters with IL-8 in phylogenetic trees and is upregulated in macrophages stimulated with LPS, IL-1β, and IFN. Interestingly, Marek disease virus expresses an IL-8 homologue (v-IL-8), which may be

involved in inducing immune deviation.[455] A molecule related to the IL-R was detected in the sea urchin genome, but the ligand has not been found[48]; becuase there are so many members of the IL-1 family, the search should continue.

The TNF family in mammals includes the canonical TNF-α, lymphotoxin (LT)α, and LTβ, all encoded at the distal end of the MHC class III region,[403] as well as a large number of other members with diverse immune functions. TNF-α is the best studied of these cytokines, and it is one of the key regulators of innate and adaptive immunity. The other two cytokines have a more limited tissue distribution and function, and are noted in their roles in lymphoid tissue development, especially in the formation of splenic white pulp and segregation of T and B cells in lymph nodes. In contrast to IL-1, TNF homologues have been detected in cnidarians, protostomes (see previous discussion), sea urchin,[48] *Ciona*,[448] and *Amphioxus*,[456] consistent with the idea that such a multifunctional cytokine would predate the jawed vertebrate adaptive immune system. Homologues have also been cloned from several teleost

species, and TNF-α expression in leukocytes is upregulated within 4 hours after treatment with LPS, IL-1β, and PMA. While there is good phylogenetic support for orthology of fish TNF-α to that of mammals, the other TNF genes seem to be teleost-specific duplicates rather than the LT genes.[457] Conversely, in *Xenopus*, the three TNF family members described previously in mammals are closely linked in the class III region of the MHC.[177] This is a bit of a surprising result as all of these family members seem to be lacking in chickens, consistent (according to the authors) with a lack of lymph nodes in these animals; however, the unusual nature of the MHC in birds (eg, the immunoproteasome genes are missing) rather is consistent with a loss of TNF genes in these animals, which is indeed surprising, especially for TNF-α.[458]

An excellent review detailing the evolution of all TNF and TNF receptor family members demonstrated that all of the genes are found in paralogs on four chromosomes, consistent with the 2R hypothesis. This work demonstrated that the earliest member was linked to the proto MHC, and thus it is not surprising that TNF is an ancient gene family.[459]

IL-6 and its related members IL-11 and IL-31 are also found in most gnathostomes examined, with few functional data.

Interleukin-2 and the γC Family of Cytokines

The family of cytokines that signals through the γC receptor includes IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Similar to what was described for most molecules involved in adaptive immunity, all of these cytokines (except perhaps IL-9) are present in all gnathostomes studied.[460] Costimulation assays of thymocytes, as described previously for IL-1, and perpetuation of T-cell lines with stimulated T-cell supernatants are performed to detect IL-2 or "T-cell growth factor" activities. Unlike IL-1, IL-2-like factors generally stimulate cells only from the same species, and it is a "cognate" cytokine, meant for release only between closely opposed cells, or as an autocrine factor. From teleost fish to mammals, stimulated T-cell supernatants costimulate thymocyte proliferation or can maintain the growth of T-cell blasts, and the ortholog has been detected in bony fish and birds. The chicken IL-2 protein is only 24% identical to human IL-2 and only 70% identical to a near cousin, the turkey.[454] IL-15, a relative of IL-2, has also been cloned in the chicken. A candidate IL-2R in chicken was identified by a mAb recognizing a 50-kDa molecule only on stimulated T-cells (thus an IL-2Rα homologue). This mAb blocks costimulation by IL-2-like molecules in chicken T-cell supernatants and also reduces the capacity of T-cell blasts to absorb IL-2-like activity from supernatants. IL-2 has been studied in the bony fish *Fugu*. In both chicken and the deduced *Fugu* IL-2 protein, there is a second set of cysteine residues, which are found in IL-15 and thus is a primordial feature.[461]

As mentioned, γC is the signaling subunit of the IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptors; absence of this chain in mammals leads to major defects in lymphocyte development ("boy in the bubble"). In fish, a γC homologue was cloned in rainbow trout with unusually high identity (44% to 46%) to mouse/human genes.[462] IL-1β, but not LPS, upregulated the trout gene in macrophage cultures and a fibroblast cell line. Since then, many other orthologs have been uncovered in fish, often duplicated in various species.[460]

IL-21, which is encoded adjacent to IL-2 in mammals, has been found in ectothermic

vertebrates.[460] Because one of its major functions is the differentiation of T-follicular cells and the GC response, it will be of interest to study it role in those animals.

IL-7 is involved in lymphocyte differentiation as well as homeostasis of mature lymphocytes. Recently, mutations in Il-7R and downstream signaling molecules in the zebrafish have shown a block in T-cell development.[463] These mutants will permit future study of lymphocyte production in this crucial developmental model.

TH2 Cytokines: Interleukin-4, Interleukin-5, Interleukin-9, and Interleukin-13

Reponses to extracellular pathogens, especially filarial worms, are largely regulated by TH2 cytokines in mammals. IL-4 is the most pleiotropic cytokine in this regard, stimulating the production of neutralizing antibodies, stimulation of eosinophils and mast cells, and an antagonism of TH1 responses. IL-4, IL-5, and IL-13 (and granulocyte macrophage colony-stimulating factor) are encoded in the so-called TH2 cytokine complex in mammals, and all of the genes are coordinately upregulated after stimulation of a nearby locus-control region. This same "TH2 complex" is found in chickens but with a psuedogene for IL-5, but in *Xenopus* only the IL-4 gene has been identified to date. Fish have genes in which the ancestor of IL-4 and IL-13 seems to be in a preduplicated state, syntenic with genes in the TH2 complex.[464] Again, duplicates of IL-4/-13 and their receptor have been found in various fish.[460,465]

At this stage of study, it may be that lower vertebrates do not have "full-blown" TH2 responses, consistent with the lack of canonical allergic responses in ectothermic vertebrates. On the other hand, we must be careful because cytokine genes evolve rapidly and relying on synteny is not always

dependable, especially in the teleost fish. It will be of interest to see whether IL-4 in nonmammalian vertebrates will promote switching to isotypes that are incapable of promoting inflammatory responses. In addition, further studies of this family and its receptors in teleost and cartilaginous fish will reveal whether this facet of the adaptive immune response is indeed a relative newcomer.

Interferons

IFNs, classically known for their antiviral properties, are divided into three groups: type I, type II, and type III. Perhaps surprisingly, this entire group has only been uncovered in jawed vertebrates to date. However, as described previously, viral immunity in insects is partially regulated via a JAK/STAT response.[152] Types I (α and β) and III (λ) IFN are widely expressed in cells of many types and induces inhibition of viral replication in neighboring cells, as well as molecules of the innate immune system such as inducible nitric oxide synthase (iNOS) and IFN regulatory factor-1.[466] In contrast, type II IFN (IFNγ or immune IFN) is synthesized by activated T cells, activates macrophages, and upregulates class I, class II, immune proteasome subunits, and TAP, and a large number of other genes.

Antiviral activity is detected in supernatants from virally infected fish fibroblasts, epithelial cell lines, and leukocytes. All of the biochemical properties of mammalian type I IFN (eg, acid-stable, temperature-resistant) are present in these fish supernatants, and the putative IFN

reduces viral cytopathic effects in homologous cell lines infected with virus.[466] In vivo, passive transfer of serum from virally infected fish protects naïve fish from acute viral pathogenesis. There appears to be two lineages of type I IFNs in fish that are specific to this group. In chickens there are up to 10 closely related, intronless type I IFN genes.[455] Sequence identity to human type I IFN ranges from 25% to 80%, with the apparent functional gene having highest similarity. Interestingly, bats have greatly expanded their type I and III IFN genes and receptors (as well as many other genes associated with viral immunity).[467] These animals are highly infected with (and susceptible to) virus, and some IFN forms are constitutively expressed at low levels.

While type I/III IFNs can be highly duplicated in some vertebrates, this is not true of IFN receptors, and in some cases many cytokines will used two to four receptors within a species. How can such a system work and result in different readouts? It appears that even single amino acid changes in the cytokine can induce differential conformational changes in the receptor that can modify downstream signaling.[468] This paradigm will be exciting to follow in a phylogenetic context.

Type II or IFNγ has been cloned in most jawed vertebrates. The chicken gene is 35% identical to human type II IFN and only 15% identical to chicken type I IFN.[454,469] Recombinant chicken IFN stimulates nitric oxide production and class II expression by macrophages. The gene has been cloned in many fish, and has been studied mostly in the trout where it has been shown to upregulate CXCL10 and activate protein kinase C. Thus, at least in chickens and teleost fish, type II IFN seems to have the same function as in mammals, suggesting that the TH1-type responses emerged early in vertebrate evolution.[466]

Heterodimeric Cytokines

The IL-12 family are composed of heterodimers that share chains (p19, p40, p35, and EB13) and include IL-12, IL-23, IL-27, and IL-35. IL-12 is generally considered to be a proinflammatory cytokine produced by APCs that promotes a TH1 response after exposure to intracellular pathogens. Consistent with the ancient derivation of TH1 responses, the two subunits of IL-12 p70 (p35 and p40) have been found in chickens and several teleost species.[454,470] The p40 subunit of IL-12 can also associate with p19 to form the cytokine IL23, which is involved in the perpetuation of TH17 cells. IL-27 generally inhibits all TH subsets but expands regulatory T cells and is composed of p28 and EB13; IL-35 is a relatively new regulatory T-produced cytokine composed of p35 and EB13. All of these chains have been detected in gnathostomes and several studies have been done to examine their expression at the RNA level; however, to date no reagents have been prepared to the heterodimers for functional analysis.

Transforming Growth Factor-β and Interleukin-10

TGF forms a large family with pleiotropic effects in many developmental systems. For the immune system, TGF-β isoforms are best known for their capacity to suppress adaptive immune responses (even across species barriers), although they can also stimulate lymphocytes under certain conditions, especially in mucosal surfaces. TGF-β inhibits macrophage activation in trout and growth of T-cell lines in *Xenopus* species. Recombinant *Xenopus* TGF-β, like the mammalian form, also can inhibit IL-2-like dependent growth of

splenic lymphoblasts.[471] Four TGF-β isoforms were isolated from chickens, as opposed to three major forms in mammals.[455] The three major forms of the cytokine have been isolated in several teleost species.[470]

IL-10 is often considered along with TGF-β because it is mostly an immunosuppressive cytokine with multiple effects; both cytokines are expressed in subsets of "regulatory T" cells in mammals. This cytokine was originally discovered by its ability to suppress TH1 responses, but now is known to have a much expanded role in immunity. IL-10 has been found in chickens and a large number of teleosts.[455,470] As usual, functional experiments have lagged behind the molecular work, but recombinant chicken IL-10 can block IFNγ production by splenocytes. IL-10 is a class II cytokine, related to IFNγ, IL-19, IL-20, IL-24, and IL-26. IL-22, a cytokine released by TH17 cells and ILCs in mammals, is upregulated in fish vaccinated with pathogens and is correlated with immune protection.[472]

Note that there have been very few "regulatory T" experiments reported in the comparative literature, but many older experiments that suggest that such cells exist. Over 25 years ago, experiments in *Xenopus* showed that graft rejection could be delayed when lymphocytes from metamorphosing animals were adoptively transferred into adult frogs.[473] This

result suggested that a "wave" of suppressor cells emerged near the time of metamorphosis that evolved to protect animals from autoimmunity when adult-specific molecules were expressed. It is time to reexamine such experiments with modern tools. In a recent experiment in zebrafish, immunization of animals with central nervous system (CNS) antigens induced an autoimmune reaction. Overproduction of foxp3 inhibited the production of IL-17 and IFNγ in these animals, ameliorating the disease.[474]

Interleukin-17

TH17 cells in mammals are important for inflammatory responses to extracellular bacteria via indirect stimulation of neutrophils. Despite its late appearance in the immunologic literature, IL-17 appears to be one of the most ancient cytokines, with homologues in protostomes and lower deuterostomes, and extensively amplified in sea urchins. Even the different isoforms seem to be conserved among the jawed vertebrates, which should initiate many new avenues of study.[470] The family is composed of IL-17A/F, with the A and F forms being the best studied to date. IL-17A/F genes have been found in all mammals studied, as well as IL-17C/E. The most ancient IL-17 form is IL-17D, present in both oysters and agnathans.[291,449] This form is expressed in lamprey T cells but not B cells, and has been proposed as a helper factor for cognate interactions.[13] Finally, like many other immune genes, as many as 20 IL-17 genes have been found in sea urchins, again suggesting expanded innate immunity in this species.[475]

Cytokine Summary

The isolation of nonmammalian cytokines and cytokine receptor genes has lagged behind molecular characterization of antigen receptors and MHC. However, with the advent of the genome and EST projects, we are rapidly acquiring a comparative view of this field, at least at the genetic and molecular level. Teleost fish and chickens have paved the way in this field,

but cartilaginous fish and agnathan databases will soon be complete and provide the big picture. Already, it seems that cytokines like TNF and IL-17 seem to be most primordial, with homologues in *Ciona*, *Amphioxus*, and sea urchins, as well as deeper homologues in certain protostomes. Conversely, *none* of the other so-called adaptive cytokines (or even type I/III IFNs) seem to be present in the lower deuterostomes, consistent with the Big Bang theory of adaptive immunity.[1,2] If it is true that the jawless fish lack these genes as well, despite their convergent adaptive immune system, one can point to the evolution of lymphoid organs and the segregation of lymphocyte subsets into discrete areas to help to explain the explosion and recruitment of these genes. We discuss this concept in more detail in the conclusion section, especially regarding the relationship of adaptive lymphocytes with ILCs.

Despite our "big picture" knowledge of the emergence of cytokines, obviously functional experiments have lagged behind. Furthermore, is it true that TH2 responses are evolutionary latecomers, or are our early attempts at finding the genes in ectotherms because of some "missing pieces" in the databases (or our ability to find some of the genes)? Some genes may have been lost, as described previously for the chicken—again, further studies of amphibians and cartilaginous fish should help in our understanding. In mammals, the LT TNF family members are important in the development of lymph nodes and splenic white pulp, and yet the three genes are present in lymph node-less *Xenopus* —what are their functions in ectotherms? If we can uncover these other roles in nonmammalian vertebrates, it could be quite informative to reinvestigate such functions in mammals. Finally, chickens and fish have evolved their own paralogs of some of the well-known cytokine genes; their study should reveal selection pressures on particular species that could also be quite informative in our understanding of the gestalt of the cytokine network.

General Evolution of Chemokines and Their Receptors

Chemokines and chemokine receptors are essential for many aspects of the immune system, including inflammation, the differentiation of lymphoid tissues, trafficking of hematopoietic cells during ontogeny and immune responses, and even stimulation of cells under various conditions.[476] With the advent of the genome and EST projects, a seminal paper showed that, consistent with the Big Bang theory of adaptive immune system evolution, all of the major classes of chemokines and their receptors were present in the bony fish lineage, and probably will be found in cartilaginous fish as well when the genomes are completed.[477] Recently, the analysis was updated to include all of the vertebrates in the databases.[476] Similar to most of the immune-related genes discussed throughout the chapter, bony fish have more chemokines/receptors than any other vertebrate, including amphibians and mammals.

Interestingly, no CC (so-called homeostatic) or CXC (so-called inflammatory) chemokines/receptors were detected in lower deuterostomes like *Ciona* and sea urchin. The chemokine receptors are member so the G protein-coupled receptor family, and of course such molecules are found in all animals. However, no members of the specialized family (G protein-coupled receptor γ) to which chemokine receptors belong were found in lower deuterostomes. The chemokine receptor genes are found on four chromosomes in mammals, four of which contain the HOX genes, one of the gene families that provided evidence for the 2R hypothesis early in the reawakening of this theory. The distribution of chemokine receptor genes on these chromosomes correlates very well with 2R, and helps to account for the

large-scale gene expansion of this family in the jawed vertebrates. Thus far, very few chemokines/receptors (eg, IL-8 [CXCL8] and CXCR4) have been detected in agnathans, but as mentioned previously, agnathan T cells express IL-8R and B cells IL-8, believed to be a factor in promoting the cognate interactions between the cells.[291] CXCL12, a chemokine vital for thymic differentiation, is also present in lamprey, consistent with new work on the agnathan thymus discussed previously.[375] In summary, it appears that a few chemokines/receptors arose at the dawn of vertebrate adaptive immunity, and they

were greatly expanded by both en bloc and *cis* duplications and became "full-blown" shortly after the 2R Big Bang.[476] Currently, this is our most complete story of the evolution of a family of immune genes in deuterostomes.

ORIGINS OF ADAPTIVE IMMUNITY

The immune system of vertebrates is unique because the antigen-specific receptor expressed by lymphocytes, which initiates cascades leading to activation of the adaptive immune system, is not the product of a complete germline-inherited gene. Rather, receptors are generated somatically during lymphocyte ontogeny from gene segments scattered at a particular locus. As described previously, the receptors in gnathostomes are IgSF members composed of V and C domains, with the C domains being of the rare "C1" type, which is shared by MHC class II and class I molecules. There are many specific questions: 1) Did MHC class I or class II come first?; 2) What is the origin of the MHC PBR?; 3) Was the MHC involved with innate immunity before the emergence of adaptive immunity?; 4) Did somatic rearrangement or somatic mutation come first to diversify antigen receptors?; 5) Which of the extant antigen receptors in gnathostomes, α/β TCR, γ/δ TCR, or IgH/L (if any) resembles the primordial receptor?; and 6) How did two different antigen receptor gene families emerge in evolution, *after* the appearance of separate lymphocyte populations? The answers to these questions are speculative, but deductions can be made based upon the wealth of molecular and emerging functional data. We base many of our arguments on the large-scale duplications that were revealed for MHC by Kasahara[17] in 1996. The remarkable syntenies of paucicopy genes on the paralogous regions, and the recent finding that an animal that predated the duplications (*Amphioxus* and *Ciona*) has only single copy genes in the same syntenic group orthologous to the four mammalian copies, make it clear the en bloc duplications were involved.[405,421] Further analysis of this region in model lower deuterostomes, as described in the histocompatibility section, will continue to be vital in our understanding of early immunity. Genetic analyses in protostome lineages have not been very informative, but we must reevaluate once we piece together data from more deuterostome species.

Major Histocompatibility Complex Origins

Class I and class II molecules have been found only on the jawed vertebrates—so far, painstaking screenings of the lower deuterostome or lamprey/hagfish databases have yielded no indication of these proteins. Based upon phylogenetic analyses and thermodynamic arguments, most investigators believe that class II preceded class I in evolution.[478] However, as stated previously, class I is much more plastic than class II as there are many different types of class I molecules, some that do not even bind to peptides. Because class I genes

may be on two or three MHC paralogs, and they can have functions outside the immune system; this is evidence that the primordial "PBR" may not have even bound to anything. If this is true, and because class I and class II do bind peptides, it would suggest class I arose first. Again, genome scans of jawless fish and lower deuterostomes should be informative on this point, but to date no luck! As described previously, we should be diligent because with the discovery of T cells and a thymus-like structure in lamprey, one expects some sort of "MHC" in the agnathans[291,375]; to date, the only homologue we have uncovered to date is TAP-L, which may hold the key to antigen presentation in this group.[401]

From the paralog data, genes encoding the complement components C3 and Bf, TNF superfamily members, the signaling molecule Vav, B7 family members, and proteasome subunits among other genes (such as tripartite motifcontaining) should have been present in the proto-MHC, before emergence of the adaptive immune system (see Fig. 4.13). Some of these genes were found in the *Amphioxus* and *Ciona* "MHC" linkage groups, and C3 and Bf genes are linked in the sea urchin. A fifth paralogous region on human chr 12p13 contains the α2-macroglobulin gene (recall the C3/4/5 homologue), a tapasin homologue, the C3areceptor, and this "complex" is linked to the NKC. Taken together, the data suggest that the proto-MHC included vital nonhomologous genes of the innate immune system, which perhaps were linked to allow coordinate regulation of expression. After the en bloc duplications, Pontarotti et al. has suggested that "functional restraints upon the complex were relaxed" and hence the duplicated members could evolve new functions, including features indispensable to the adaptive system.[405] If indeed innate immunity genes were already linked to allow upregulation at times of infection, it is no surprise that the adaptive immune system piggybacked on such a gene complex. A final point: why did the duplicate genes survive rather well over hundreds of millions of years; *cis* duplicates have been shown to degenerate rapidly over evolutionary time. Evidence suggests that duplicates arising from polyploidy (and by inference large en bloc duplications) survive better than *cis* duplicates, most likely because they cannot be inactivated by unequal crossovers; the ability of the genes to survive over very long periods, perhaps combined with strong selection pressures, would allow for subspecialization.[479]

Origins of Rearranging Receptors

The Rearranging Machinery

Most models propose that the generation of somatically rearranging receptors occurred abruptly in evolution via the generation of the RAG machinery made of two lymphocytespecific proteins, RAG1 and RAG2. *RAG* genes have so far been isolated in all classes of jawed vertebrates and have been quite conserved. In every case examined, *RAG1* and *RAG2* genes are closely linked and in opposite transcriptional orientation. Some regions of RAG1 and RAG2 are similar to bacterial recombinases or to molecules involved in DNA repair (eg, RAD16) or the regulation of gene expression (such as rpt-1r). Similarities to prokaryotic proteins and the gene structure suggest that vertebrates acquired the RAG machinery by horizontal transfer and transposition from bacteria.[19,480] Indeed, *RAG* genetic organization has

some transposon characteristics: the RSS are reminiscent of sequences involved in targeting

excision of transposons. A class of transposons detected in several protostome and deuterostome invertebrate species that shows similarity to the catalytic domain of RAG1.[481] It is believed that RSS were derived from the terminal inverted repeats of this transposon, called *transib*. In invertebrate genomes there are many of such "RAG1 core regions," but only one has an open reading frame throughout the core and shows similarity to vertebrate RAG1 in other regions as well. Furthermore, a *RAG2* homologue is adjacent to the urchin *RAG1* gene, in a similar orientation as is found in gnathostomes.[352] This finding was a big surprise and suggests that both *RAG* genes were in place approximaltey 100 million years before the origin of the Ig/TCR system. Their tissue distribution and expression during ontogeny are not known; nor have any candidate genes been recognized to date with RSS that might be recognized by the echinoderm homologues. It must be admitted that it is unclear how this new result fits into the puzzle of the origins of adaptive immunity. Because transib is so often found in the animal kingdom, it is certainly part of the transposon. By contrast, RAG2 is now believed to have been present in the genome, and recent data have shown it to interact with active chromatin.[482,483] Thus, the new idea is that RAG2 recruits RAG1 to open chromatin, and rag1 is the active enzyme in the major rearrangement events, which is wholly consistent with the transposon hypothesis with RAG1 (ie, transib) being the genome "invader."

Another source of somatic antigen receptor diversity shared by all gnathostomes characterized to date is a unique DNA polymerase, TdT, which diversifies CDR3 during Ig and TCR gene rearrangement through the addition of nucleotides in a template-independent fashion.[484] Furthermore, as detailed previously, its expression serves as an unambiguous developmental marker for the sites of lymphopoiesis. TdT has been highly conserved in both sequence (> 70% amino acid similarity, > 50 amino acid identity) and overall structure during vertebrate evolution. An amino acid alignment of all known TdT sequences reveals that some, but not all, structural motifs believed to be critical for TdT activity are particularly well conserved in all vertebrates studied. TdT protein alignments, and the crystal structure for rat β-polymerase, support the hypothesis that both evolved from a common ancestral DNA repair gene. In addition, four protein kinase C phosphorylation sites are conserved, and hence may be involved in TdT regulation. Homologues related to the ancestor of polymerase β and TdT have been found in sea urchin and other lower deuterostomes. Thus, unlike RAG, TdT has evolved by gradual evolution from a polymerase family and was recruited for immune system function.

Rearrangement or Somatic Hypermutation First?

Because all antigen receptor genes use somatic rearrangement of V genes to generate diversity in CDR3 regions as well as to promote combinatorial diversity, there is no doubt that this mechanism is at the heart of adaptive immunity. Indeed, as described, most investigators believe that the introduction of the tranposable element into a V gene was *the* driving force in the abrupt appearance of vertebrate adaptive immunity; the finding of the RAG genes in sea urchins challenges this notion at some level. However, it cannot be overemphasized that SHM is also at the origins of the immune system; furthermore, all evidence to date suggests a gradual evolution of the SHM machinery (the AID/APOBEC family and associated polymerases/mismatch repair proteins) rather than the "hopeful monster" generated by the famous RAG transposon. Thus, diversity generated via SHM or gene conversion may have existed in an adaptive immune system prior to rearrangement, and V gene rearrangement

was superimposed onto this already existing system.[296,300] Indeed, the presence of APOBEC family members in the jawless fish (CDA1 and CDA2, as described previously) suggest that diversity generated via mutation/conversion preceded rearrangement. The RAG-induced rearrangement break and subsequent repair provided something new and novel in gnathostomes, not only diversity in sequence but heterogeneity in size[300]; this was a remarkable innovation and likely indeed heralded the sophistication of jawed vertebrate adaptive immunity.

Which Antigen Receptor First? Phylogenetic analyses have suggested that among IgSF receptors γ/δ TCR-like ancestor may have predated α/β TCR and Ig H/L.[485] This would suggest that direct antigen recognition, perhaps by a cell surface receptor, arose first in evolution followed by a secreted molecule and an MHC-restricted one. Hood and colleagues argue that phylogenetic analyses over such large evolutionary distances obscure true relationships among the antigen receptor genes (eg, the relationships of the molecules in the phylogenetic trees has to impose multiple loss/gain of D segments in the different antigen receptor families) and suggest a model based upon genomic organization, not so different from the Kasahara model.[263] They propose an alternative phylogeny in which an ancestral chromosomal region with linked genes encoding both chains of an ancestral antigen receptor heterodimer, one having D segments the other not (eg, IgH/IgL). The α and δ TCR loci are still closely linked in all vertebrates analyzed (human chromosome 14), and a pericentric inversion is suggested to have separated the TCR β and γ loci (linked on human chromosome 7). This model predicts that D segments only emerged once, and also explains the existence of inverted V elements in both the TCR β and δ loci. This model does not predict which antigen receptor is oldest, but does provide a "simple view" of receptor evolution, consistent with the Kasahara/Ohno model. Recent data have added to this scenario. First, as mentioned previously, VH elements have been found at the TCR-δ locus in many vertebrates, including sharks, amphibians, birds, and marsupial mammals.[269] In part, this is due to the close proximity of the IgH/L (lambda) and TCR alpha/delta loci in many living vertebrates (especially *Xenopus*[268]), consistent with the Hood hypothesis of ancient en bloc duplications of antigen receptor loci.[268] Additionally, the idea that most TCR-δ receptors do not recognize MHC-restricted antigen suggests that they can continue to "borrow" elements from Ig loci over evolutionary time.

Thus, an alternative scenario is that MHC-restricted antigen recognition arose first, perhaps derived from a NKR with a "VJ" IgSF domain that recognized SOS proteins, like the ULBPs or MIC today. The rearrangement break induced by

RAG occurs in CDR3, which is in the center of the antigen receptor-combining site, in perfect position to interact with peptide bound to MHC, which could have been another innovation to detect foreign antigens within cells.[486] Thus, such cells would be most like extant αβ T cells with an MHC-restricted antigen receptor encoded by linked genes. Once rearrangement was introduced, the combining site may have been "relaxed" so that it was no longer forced to be MHC-restricted (see the section on TCR above). The genomewide duplication then would provide two types of TCR, one MHC-restricted and the other not, like γδ TCR today. One locus, according to the Hood model, then underwent an en bloc cis duplication to give rise to the IgH/L loci. Ig, then, developed a new differentiation pathway with an alternative splicing

mechanism for the transmembrane and secreted forms of BCRs.

For the origin of the rearranging receptors, IgSF lineages have to be traced back through phylogeny as such receptors generated by somatic rearrangement do not exist outside the jawed vertebrates. In a quest for molecules related to elusive ancestors, without focusing on genes expressed in the immune systems of various phyla (ie, structure is more important than function in this case), the most homologous sequences and gene architectures in the various metazoan phyla must be scrutinized.

V(J) and C1 Domains

C1 domains are found in the antigen receptors, MHC class I and class II, and very few other molecules (see Fig. 4.10). This IgSF domain is so far most prevalent in gnathostomes, as if C1 domains arose concurrently with the adaptive immune system and coevolved with it. What was the value of the C1 domain, and why is it found almost exclusively in adaptive immune system-related molecules? All of these molecules interact with coreceptors such as CD3 (TCR), Igα and β chains (Ig on B cells), CD4, and CD8 (with MHC on opposing cells), and it is conceivable that in sections of IgSF domain in which C1 differs from the C2 there is a specific region favoring interaction with other molecules.

The G strand of Ig/TCR V domains is encoded by the J gene segment, separated from the V region-encoded A-F strands, and rearrangement is necessary in order to assemble a complete V gene. The primary structure of each Ig/TCR chain bears hallmarks of the dimeric nature of the receptor in which they participate. A diglycine bulge (Gly-X-Gly), present in all V domains, is thought either to be a beneficial adaptation, or to promote dimer formation by inducing a twist in the G strand that results in V domain pairing that appropriately orients the CDR. Monitoring this feature, therefore, might reveal genes that had the ability to form dimers similar to that of modern antigen-specific receptors. In V genes that do not somatically rearrange, the G stand is an integral part of the V exon. In other remotely related IgSF genes, introns have invaded the V domain exon creating a variety of V gene families. Many examples of such events can be found in the history of the Ig superfamily, for example in the genes encoding CD8 and CTX.[33,487]

As described, no Ig/TCR genes have been isolated from hagfish or lampreys, although there are some tantalizing molecules potentially related to their ancestors (eg, APAR, see previous discussion). Were Ig and TCR “invented” in a class of vertebrates now extinct (eg, the placoderms, which are more primitive than cartilaginous fish but more advanced than agnathans)? The discovery of the three tapasin paralogs all with C1 domains suggests an origin prior to the full establishment of the vertebrate genome.[33]

V domains, either alone (eg, IgNAR) or in association with another V domain (eg, Ig H/L), recognize the antigenic epitope and are therefore the most important elements for recognition. For this reason, they will be the first to be traced back in metazoan evolution by asking whether V domains exist in invertebrates. Domains with the typical V fold, whether belonging to the true V-set or the I-set, have been found from sponges to insects (although not necessarily involved in immune reactions; the first ones were discovered by nonimmunologists among molecules involved in nervous system differentiation in invertebrates [eg, amalgam, lachesin, and fascicilin]). Invertebrates also use IgSF members in immunity, but so far they are not V domains, but more I- or C2-set (eg, mollusk defense molecule, hemolin, DSCAM). However, the mollusk FREPs have a V domain at their distal

end, associated with a fibrinogen-like domain. As described, they are involved in antiparasitic reactions and form a multigenic family with polymorphism.[135] Similarly, as mentioned previously, prochordate VCBP has a VJ domain, but it mediates innate immunity.[143]

Besides searching for VC1-encoding genes in nonverte-brates, surveying the human genome for such genes has been fruitful. Indeed, nonrearranging V-containing molecules, either VJ alone, VC1, or C1 alone, have been found in the human genome. Interestingly, many of them are present in the MHC class III region (human chromosome 6p21) or its paralogs (see Fig. 4.12 and 4.13). Two MHC-linked gene segments stand out: a single VJ, NKP30, and a gene containing a VJC1 core, tapasin (TAP-binding protein), involved in antigen processing. NKP30, made of a single Ig domain of the VJ type, is an NK cell-activating receptor, and it may offer a link to cell types in invertebrates. It could be a relative of an ancient receptor whose history is linked to the emergence of MHC class II and class I; unlike most NKRs, NKp30 is evolutionarily conserved, present from sharks to mammals.[176] In order to resemble an ancestor, the NKP30 V domain need only be associated with a C1 domain. In fact, a C1 single-domain gene, pre-TCR-α, is also encoded in the MHC.[253] Besides Ig and TCR, tapasin is one of the rare cases, if not the only other case, of a gene segment with a VJC1 structure existing on several paralogous linkage groups (6, 9q33, 19q13). In other words, while this gene is related to the rearranging receptor structure, it is undoubtedly very old, and probably predated the ancient block (genomewide) duplications. It could have acted as a donor of C1 to a V domain-containing gene in the MHC class III region (like the XMIV), which then could have been the first substrate of the rearrangement. Another set of molecules with distal VJC1 segments, the signal-regulatory proteins (VJ C1 C1), and the poliovirus receptor (VJ C1 C2) could represent another group linked to the history of the Ig and TCR (see the following).

TREM1 and TREM2, receptors on monocytes/neutrophils involved in inflammatory responses, are composed of single VJ domains whose genes are MHC-linked. Myelin oligodendrocyte protein (MOG) and P0, two single V domains

involved in the synthesis of myelin sheath, are encoded in the MHC paralogous region on chromosome 1. Chicken BG, which is related to MOG but probably has a different function, is encoded in the chicken MHC. Butyrophilin, CD83, and tapasin all have VJ domains, and butyrophilin also has a C1-type domain. More distant relatives with VJC2-based architectures are also found in MHC (receptor for advanced glycosylation end products, CTX, lectin-related genes; see Fig. 4.12), and some of these genes related to the rearranging receptor ancestors are found on several paralogs (whether the MHC paralogs or other), suggesting that the V-C1 core was generated early in vertebrate evolution subsequent to the emergence of the chordate superphylum (see Fig. 4.13). Among all these molecules, butyrophilin is perhaps not on the direct track to antigen-specific receptors. Its C domain, although proven to be C1 through its crystal structure, is more like a C2 at the primary sequence level, and belongs to the CD80/86 family, rather than the TCR. Finally, tracing the VJ NITR gene family previously described in evolution may lead us to an understanding of the original NKC/LRC/MHC, as well as identifying more candidate genes related to the ancestral gene invaded by the RAG transposon.

Many invertebrate molecules not involved in immune responses are present as a distal V

domain associated with one or more C2-type domains. In the vertebrates many molecules have retained this feature such as CD2 and CTX. Some members resemble “primitive” antigen receptors and several of them map, for instance in humans, to at least two tetrads of paralogous genes, the MHC on chromosomes 1, 6. 9 (12), 19 and the LRC on chromosomes 1, 3, 11, 19 (21) (see Fig. 4.13).[160,442] Many form dimers and are expressed in lymphocytes, where they form a family of adhesion molecules. The crystal structure analysis of a CTX-related molecule (JAM) revealed a unique form of dimerization, suggesting that the diversity of ligand binding and domain-interactions used by different IgSF domain is extensive. Two JAM molecules form a U-shaped dimer with highly complementary interactions between the N-terminal domains. Two salt bridges are formed in a complementary manner by a novel dimerization motif, R (V, I, L) E. The receptor for advanced glycosylation end products gene has a rather “generic” receptor function as it recognizes aged cells exposing particular carbohydrate motifs. CD47, another conserved IgSF member with a CTX-like V domain, suggests an ancient function.[488] Perhaps such nonrearranging VJC1 genes that regulated cytotoxicity/phagocytosis were predecessors of the antigen receptors. Some molecules with VJ/C1 cores involved in cell-cell interactions often serve as ports of entry for viruses. In a move from “property to function,” an arms race consequence could result in a virus receptor developing into an immune receptor. The best examples of such molecules are in the CTX JAM family, in which receptor interactions with viruses may trigger an apoptosis, a primitive form of antiviral immunity.[33,489]

Emergence of T and B Cells

As described previously, agnathans have now been shown to have cells in the T and B lineages, similar to what is found in gnathostomes.[291] Because the jawless and jawed vertebrates have antigen receptors derived from different gene families, at first glance it seemed that the T-B split occurred twice in evolution. However, the gene expression profiles for the lamprey T and B cells are too similar to the patterns in gnathostome lymphocyte populations to be derived by convergent evolution; furthermore, the development of lamprey T cells in a thymus-like structure also suggests evolution from a common ancestor. Thus, it seems most likely that both the LRR and IgSF receptor families were present in the common ancestor,[2,13] with the VLR system predominating early in vertebrate evolution (see Fig. 4.10). Such a scenario exists today with NKRs in mammals, with different gene families (C-type lectin or IgSF) predominating in different species.[12]

Because APOBEC family members are most likely involved in VLR assembly and perhaps in mutation, there may have been a primordial IgSF receptor that was also modified somatically by an AID-like molecule, which functioned side-by-side with VLRs. The rearrangement break caused by the RAG transposon in a primordial IgSF gene offered a great advantage, as was detailed previously, and the IgSF receptors then could have supplanted VLRs in gnathostomes, while maintaining the AID-dependent machinery to diversify it further. It is difficult to imagine how a rearrangement break in an antigen receptor gene generated by LRR insertion could be advantageous; conversely, as mentioned previously, a break in the CDR3 loop in the center of the binding site is in perfect position to interact with peptide bound to MHC. While lampreys have a thymoid for development of their “T cells,” as described previously, there was a great leap in complexity of thymus structure in the gnathostomes, consistent with a new TCR/MHC system in which positive and negative

selection became a requirement for a functioning adaptive immune system. In addition, the general transcriptional and cytokine/chemokine networks for T-cell (and B-cell) development would have also been in place in the jawless vertebrates; in gnathostomes, this network became more complex in the Big Bang (eg, see the chemokine section in which the leap in sophistication can be examined with precision).

In summary, a working hypothesis proposes that the divergence of lymphocytes into T and B cells occurred early in vertebrate evolution (see Fig. 4.10). LRR- and IgSF-based antigen receptors existed simultaneously as well at this time (in the same organism or organisms in the same epoch), with VLR perhaps predominating in early vertebrates. The RAG transposon insertion into an IgSF receptor (perhaps one already diversifying by mutation or conversion), along with a genomewide duplication, resulted in the Ig/TCR/MHC-based system of gnathostomes, with all of its accompanying sophistication in all aspects of adaptive immunity, including development of the thymus and spleen, complexity of cytokine and chemokine networks, and intimate interactions of cells with primary and secondary lymphoid tissues.

CONCLUSION

Since the last edition of *Fundamental Immunology* was published[5] years ago, there have been several astonishing findings, as well as other interesting discoveries and integrations, regarding the evolution of the structure and function of the

immune system. Furthermore, there have been many genome and EST projects that have allowed us to determine whether or not particular genes or gene families are present in different taxa. Here, we briefly describe these findings and discuss their broad relevance and relationship to future studies.

A new system of defense in bacteria and archaebacteria against bacteriophage, CRISPR, has been elucidated.[490] Short phage DNA sequences have been acquired by prokaryotic genomes to direct sequence-specific protection against phages. These elements are transcribed into small RNAs that recruit endonucleases to cleave the phage nucleic acid. This system is widespread and its study is in its infancy. The discovery of this system fits well with studies of plants and animals over the last decade uncovering the plethora of mechanisms used to detect foreign nucleic acids (eg, TLR, NLR, RIG, APOBEC, etc.), as described previously. It should be clear to all immunologists by now that *all* living things must distinguish self from nonself, and have developed intricate mechanisms to achieve this end.

Cytokines such as IFNγ, IL-2, and IL-4, and other adaptive cytokines (and type I/III IFNs) and their receptors are not present outside of jawed vertebrates, nor is the vast array of chemokines seen in gnathostomes. However, at the moment it appears that (almost) all of the cytokines/chemokines discovered in mammals are also present in basal jawed vertebrates: Big Bang indeed! IL-17, a cytokine garnering great attention in basic immunology, and TNF, a central cytokine in both innate and adaptive immunity, seem to be the first cytokines to have emerged in evolution based on studies of lower deuterostomes. If, as suggested from studies in mammals, IL-17 is important for responses to extracellular bacteria, nonvertebrate deuterostomes may hold the key to understanding the physiology of the entire system (ie, in the absence of the competing TH1, TH2, and regulatory T cells), and one may be able to study the dynamics of IL-17 development and function in lower deuterostomes and even protostomes.

Additionally, over the past few years a new type of cell, the ILC, has come to prominence.[491] These cells are predominantly found in epithelia and mucosa and are involved in homeostasis and a first line of defense. As they do not bear antigen receptors, ILCs respond via cytokine receptors or PRRs. ILCs express cytokines consistent with T-cell subsets, and it is tempting to propose that they preceded adaptive T cells in evolution, and the cytokine-production profiles were coopted by the antigen receptor-bearing cells. However, as mentioned, it appears that "adaptive" cytokines arose late in evolution, coincident with adaptive immunity. Thus, it seems more like that ILCs arose simultaneously with the adaptive cells in the immunologic Big Bang, and there has been adaptation over time. However, as IL-17 and TGF-β are old cytokines, it has been proposed that their opposing functions arose before adaptive immunity.[492] TGF-β belongs to an ancient family of cytokines, and at least one of its functions in mammals is to antagonize proinflammatory responses (regulatory T differentiation, regulation of IgA synthesis, etc.). One model suggests that sophisticated innate immunity arose in mucosal tissues, regulated by the pro- and anti-inflammatory properties of IL-17 and TGF-β, respectively, by their expression in gut epithelia or by infiltrating hematopoietic cells; later, this was superimposed onto adaptive lymphocytes in the vertebrate lineage. Consistent with this idea, sea urchins have an expanded family of IL-17 genes,[48] suggestive of a very important function for this cytokine. So, such a system may have been the forerunner of both the ILCs and adaptive lymphocytes, perhaps found in tissues directly exposed to the environment.

Additionally, we are poised for more seminal studies of mucosal immunity. Mammals have dedicated secondary lymphoid tissues in their mucosa, and thus it is often difficult to tease apart the innate from adaptive responses in these tissues, except in contrived knockout or transgenic models. By contrast, ectothermic vertebrates have no Peyer patches or mesenteric lymph nodes, and thus are excellent models to examine responses in the lamina propria, which are predominantly T-independent.[493]

Lastly, studies of how the microbiota interact with the immune system have filled journals to the gills over the past 5 years. McFall-Ngai suggested that the driving force for adaptive immunity was not so much to fight off invaders, but rather to tolerate commensals,[494] perhaps not so different from Weaver's proposal.[492] This framework of interactions between leukocytes, and between leukocytes and commensals/pathogens, may be the key to understanding the genesis of adaptive immunity.

A new defense system in *Drosophila*, important for viral defense, has been uncovered. Previously, the recognition of pathogens by TLRs in the invertebrates was believed to be indirect, interacting with self-molecules similar to a cytokine interaction. However, *Drosophila* Tol7 has now been shown to interact directly with viruses, similar to TLRs in vertebrates,[153] but, as expected, the effector phase is different from the canonical (historical) toll pathway. There are several other tolls in the protostomes without a described function that can be studied in a new light.

One of the great triumphs in the field of comparative immunology was the discovery that similar mechanisms are used to recognize extracellular pathogens and initiate immune responses. It has been approximately 15 years since the *Drosophila* toll/IMD systems and their relationship to vertebrate TLRs and TNF receptors has been uncovered. Over the past few years, the intracellular defense mechanism such as NOD/NLR, RIG, helicase, etc., have

been elucidated in vertebrates, and we are beginning to piece together their evolutionary histories. The amazing similarities between the deuterostome and plant NOD/NLR systems will challenge us to understand their recognition events for years to come.[14]

The VLR system continues to amaze us.[13] Most comparative immunologists never expected a unique antigen receptor system to be uncovered in jawless fish. To then realize that the divergence of lymphocytes into two lineages occurred in the ancestor of jawed and jawless fish was astonishing. Now to have uncovered a primitive type of thymus candidate in jawless fish is a total shock. All of these discoveries force us to acknowledge that we should not be surprised by future discoveries in the evolution of immunity, such as a "convergent MHC," or an antigen presentation system not based on peptide presentation, or even a primordial type of adaptive immune system in lower deuterostomes or protostomes.

P.128

Furthermore, from comparative studies, Boehm and colleagues have proposed a simple model for the most fundamental elements required for thymocyte differentiation, which has been superimposed onto the lamprey thymus-like structures.[375,495] Further study of this minimal network of transcription factors, chemokines, and cytokines could uncover the origins of thymus differentiation in lower deuterostomes that clearly have no T/B cells.

Certain groups of organisms are interesting to study for different reasons. The genome projects in cnidarians demonstrated that certain gene families were more ancient than previously believed (from studies of the classical models *Drosophila* and *C. elegans*).[35] Thus, this taxonomic group has become the new rock stars of comparative immunology, not only for their immune complexity in the absence of a mesodermal germ layer, but also for elucidation of a histocompatibility reaction that has fascinated us for decades.[496]

Teleost fish have been shown again and again to have rapidly evolving immune systems, often expanding gene families in unprecedented ways (eg, NLR, tripartite motifcontaining), generating specific paralogs and losing others (eg, cytokines, chemokines, etc.), losing canonical syntenies found in all other vertebrates (eg, MHC class I and class II), and having unique forms of ancient molecules (eg, tetrameric IgM with no J chain). The third genomewide duplication (3R) is believed to have been a major force in this instability and rapid evolution.[20] This group provides an opportunity to study the plasticity of immune systems in related organisms over a short period of evolutionary time. With the cod story, they even offer a natural class II knockout system[395]!

Amphibians with metamorphosis offer models to study two modalities of self-tolerance, metamorphosis, and polyploidy. The larval immune system must somehow be suppressed with the appearance of adult self-determinants during metamorphosis and the refurbishing of the immune system. The amphibian *Xenopus* speciates by polyploidy, allowing an experimental system to examine the effects of whole-genome duplications on immune system loci (silencing, deletion, gene conversion, conservation), a key issue given the importance attributed to the genome-wide duplications in the evolution of immune systems in vertebrates.[328,399]

Birds offer another case of rapid evolution, probably because of attack by viruses. For the MHC, birds have lost immunoproteasomes and the thymic-specific proteasome, and have modified their class I groove to be more promiscuous in peptide binding. Chickens (but not

ducks) have lost the RIG-1 gene, apparently making them susceptible to influenza.[497] Clearly, birds have something fundamental and unique to teach us about how pathogens (most likely viruses in this case) can force a remodeling of innate and adaptive immunity.

The genomewide duplications early in the evolution of the vertebrates (the 2R hypothesis) have been confirmed in a variety of studies, and they were indeed important in the Big Bang emergence of the adaptive immune system. The 2R paradigm is useful for studying *any* gene family found on multiple chromosomes in the vertebrates; good examples of how it has aided in our understanding of immunity were the study of IgSF members involved in cell-cell interaction/costimulation, and the evolution/emergence of chemokines and TNF family members. An emerging paradigm is the possibility that genes encoding NKC, LRC, MHC, as well as the antigen receptors and costimulatory molecules (and other immune genes) were all linked at an early point in evolution.[2,498] In addition, while the agnathan adaptive system is similar to the Ig/TCR/MHC system of gnathostomes, the great sophistication that we see in the latter is not present in lampreys and hagfish. Our best guess at the moment is that the RAG transposon, in combination with the genomewide duplications, both superimposed onto an already existing adaptive framework, resulted in the Big Bang of adaptive immunity (see Fig. 4.10).

With the genome projects and advances in molecular biology, we have made strides in understanding old problems in vertebrate adaptive immunity, such as 1) when the L chains emerged and what the significance of more than one isotype is; 2) which antigen receptor (if any of the extant ones) came first, and all of them came to evolve to their present state; 3) when IgD emerged and what its function is in different vertebrate phyla, especially the transmembrane form; and 4) how γ/δ T cells recognize antigen. We propose that are two arms of the γ/δ T-cell lineage, one innate and the other adaptive, similar to B cells and α/β T cells.

The long-awaited molecular mechanisms of histocompatibility in nonvertebrates have been at least partially uncovered in plants, *Botryllus*, and *Hydractinia*. The common themes thus far are that the genes encoding the polymorphic receptors/ligands are closely linked and that the genes all appear to be unrelated to the vertebrate MHC. However, there are tantalizing links to immune gene clusters found throughout the animal kingdom that suggest there may be an underlying fundamental similarity in recognition, perhaps related to 1) the paucity of surface receptors available because of multiple constraints resulting in sharing of receptor/ligands, and 2) the signaling cascades.

Despite the great variation in mechanisms of allorecognition across phyla, are there commonalities? Whether in cnidarians or in tunicates, the polymorphic genetic regions involved contains from one to many IgSF members resembling members of the nectin PVR/CD155 family. In *Ciona*, these genes are not only closely linked in a single region but also are homologs found in the LRC. Could this region be the conserved and link aspects of immunity between vertebrates and invertebrates? The frequent presence of ITIM and ITAM indeed suggest the conservation of receptors interacting among these molecules.[160] It is clearly of ancient origin. With paralogs on four different chromosome regions in gnathostomes as well as an ancient connection to MHC and NKC, this region was present before 2R. It is not surprising now to detect very different molecular families involved in immune recognition events: LRR, lectins, IgSF. The comparison of allorecogniton in invertebrates has perhaps helped to focus our attention on genetic regions that may have

provided the “context” for leukocyte interactions.

REFERENCES

1. Bernstein RM, Schluter SF, Bernstein H, Marchalonis JJ. Primordial emergence of the recombination activating gene 1 (RAG1): sequence of the complete shark gene indicates homology to microbial integrases. *Proc Natl Acad Sci U S A* . 1996;93(18):9454-9459.

2. Flajnik MF, Kasahara M. Origin and evolution of the adaptive immune system: genetic events and selective pressures. *Nat Rev Genet* . 2010;11(1):47-59.

3. Dzik JM. The ancestry and cumulative evolution of immune reactions. *Acta Biochim Pol* . 2010;57(4):443-466.

4. Elinav E, Strowig T, Henao-Mejia J, Flavell RA. Regulation of the antimicrobial response by NLR proteins. *Immunity* . 2011;34(5):665-679.

5. Fontana MF, Vance RE. Two signal models in innate immunity. *Immunol Rev* . 2011;243(1):26-39.

6. Smith LC, Davidson EH. The echinoid immune system and the phylogenetic occurrence of immune mechanisms in deuterostomes. *Immunol Today* . 1992;13(9):356-362.

7. Ghosh J, Lun CM, Majeske AJ, Sacchi S, Schrankel CS, Smith LC. Invertebrate immune diversity. *Dev Comp Immunol* . 2011;35(9):959-974.

8. Danilova N. The evolution of immune mechanisms. *J Exp Zoolog B Mol Dev Evol* . 2006;306(6):496-520.

9. Loker ES, Adema CM, Zhang SM, Kepler TB. Invertebrate immune systems—not homogeneous, not simple, not well understood. *Immunol Rev* . 2004;198:10-24.

10. Trowsdale J, Parham P. Mini-review: defense strategies and immunity-related genes. *Eur J Immunol* . 2004;34(1):7-17.

11. Hughes AL. Rapid evolution of immunoglobulin superfamily C2 domains expressed in immune system cells. *Mol Biol Evol* . 1997;14(1):1-5.

11a. Klein J, Ono H, Klein D, et al. *The Accordion Model of Mhc Evolution, 1992, Progress in Immunology,* Vol. VIII, 137-143. Springer-Verlag.

12. Howard JC, Jack RS. Evolution of immunity and pathogens. *Eur J Immunol* . 2007;37(7):1721-1723.

13. Boehm T, McCurley N, Sutoh Y, Schorpp M, Kasahara M, Cooper MD. VLR-based adaptive immunity. *Annu Rev Immunol*. 2012;30:203-220.

14. Maekawa T, Kufer TA, Schulze-Lefert P. NLR functions in plant and animal immune systems: so far and yet so close. *Nat Immunol*. 2011;12(9):817-826.

15. Nei M, Gu X, Sitnikova T. Evolution by the birth-and-death process in multigene families of the vertebrate immune system. *Proc Natl Acad Sci U S A* . 1997;94(15):7799-7806.

16. Ohno S, Wolf U, Atkin NB. Evolution from fish to mammals by gene duplication. *Hereditas*. 1968;59(1):169-187.

17. Kasahara M, Hayashi M, Tanaka K, et al. Chromosomal localization of the proteasome Z subunit gene reveals an ancient chromosomal duplication involving the major histocompatibility complex. *Proc Natl Acad Sci U S A* . 1996;93(17):9096-9101.

18. Sakano H, Huppi K, Heinrich G, Tonegawa S. Sequences at the somatic recombination sites of immunoglobulin light-chain genes. *Nature*. 1979;280(5720):288-294.

19. Agrawal A, Eastman QM, Schatz DG. Transposition mediated by RAG1 and RAG2 and its implications for the evolution of the immune system. *Nature*. 1998;394(6695):744-751.

20. Postlethwait JH. The zebrafish genome in context: ohnologs gone missing. *J Exp Zool B Mol Dev Evol*. 2007;308(5):563-577.

21. Catchen JM, Braasch I, Postlethwait JH. Conserved synteny and the zebrafish genome. *Methods Cell Biol*. 2011;104:259-285.

22. Waterhouse RM, Kriventseva EV, Meister S, et al. Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes. *Science*. 2007;316(5832):1738-1743.

23. Parham P. The genetic and evolutionary balances in human NK cell receptor diversity. *Semin Immunol*. 2008;20(6):311-316.

24. Watson FL, Puttmann-Holgado R, Thomas F, et al. Extensive diversity of Ig-superfamily proteins in the immune system of insects. *Science*. 2005;309(5742):1874-1878.

25. Ortutay C, Siermala M, Vihinen M. Molecular characterization of the immune system: emergence of proteins, processes, and domains. *Immunogenetics*. 2007;59(5):333-348.

26. Zhang Q, Zmasek CM, Godzik A. Domain architecture evolution of pattern-recognition receptors. *Immunogenetics*. 2010;62(5):263-272.

27. Ronald PC, Beutler B. Plant and animal sensors of conserved microbial signatures. *Science*. 2010;330(6007):1061-1064.

28. Enkhbayar P, Kamiya M, Osaki M, Matsumoto T, Matsushima N. Structural principles of leucine-rich repeat (LRR) proteins. *Proteins*. 2004;54(3):394-403.

29. Kajava AV, Kobe B. Assessment of the ability to model proteins with leucine-rich repeats in light of the latest structural information. *Protein Sci*. 2002;11(5):1082-1090.

30. Wong K, Park HT, Wu JY, Rao Y. Slit proteins: molecular guidance cues for cells ranging from neurons to leukocytes. *Curr Opin Genet Dev* . 2002;12(5):583-591.

31. Barclay AN. Membrane proteins with immunoglobulin-like domains— a master superfamily of interaction molecules. *Semin Immunol*. 2003; 15(4):215-23.

32. Homma S, Shimada T, Hikake T, Yaginuma H. Expression pattern of LRR and Ig domain-containing protein (LRRIG protein) in the early mouse embryo. *Gene Expr Patterns*. 2009;9(1):1-26.

33. Du Pasquier L, Zucchetti I, De Santis R. Immunoglobulin superfamily receptors in protochordates: before RAG time. *Immunol Rev*. 2004;198:233-248.

34. Gehring WJ. New perspectives on eye development and the evolution of eyes and photoreceptors. *J Hered*. 2005;96(3):171-184.

35. Miller DJ, Hemmrich G, Ball EE, et al. The innate immune repertoire in cnidaria —ancestral complexity and stochastic gene loss. *Genome Biol*. 2007;8(4):R59.

36. Lemaitre B, Hoffmann J. The host defense of Drosophila melanogaster. *Annu Rev Immunol*. 2007;25:697-743.

37. Hartenstein V. Blood cells and blood cell development in the animal kingdom. *Annu Rev Cell Dev Biol*. 2006;22:677-712.

38. Evans CJ, Hartenstein V, Banerjee U. Thicker than blood: conserved mechanisms in Drosophila and vertebrate hematopoiesis. *Dev Cell*. 2003;5(5):673-690.

39. Jiravanichpaisal P, Lee BL, Soderhall K. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. *Immunobiology*. 2006;211(4):213-236.

40. Crozatier M, Meister M. Drosophila haematopoiesis. *Cell Microbiol* . 2007;9(5):1117-

1126.

41. Engelmann P, Molnar L, Palinkas L, Cooper EL, Nemeth P. Earthworm leukocyte populations specifically harbor lysosomal enzymes that may respond to bacterial challenge. *Cell Tissue Res*. 2004;316(3):391-401.

42. Prochazkova P, Silerova M, Felsberg J, et al. Relationship between hemolytic molecules in Eisenia fetida earthworms. *Dev Comp Immunol* . 2006;30(4):381-392.

43. Valembois P. Aims and methods in comparative immunology. *Dev Comp Immunol* . 1982;6(2):195-198.

44. Kuchel RP, Raftos DA, Birch D, Vella N. Haemocyte morphology and function in the Akoya pearl oyster, Pinctada imbricata. *J Invertebr Pathol* . 2010;105(1):36-48.

45. Bachere E, Gueguen Y, Gonzalez M, Garnier J, Romestand B. Insights into the anti-microbial defense of marine invertebrates: the penaeid shrimps and the oyster Crassostrea gigas. *Immunol Rev*. 2004;198: 149-168.

46. Li C, Shields JD. Primary culture of hemocytes from the Caribbean spiny lobster, Panulirus argus, and their susceptibility to Panulirus argus Virus 1 (PaV1). *J Invertebr Pathol*. 2007;94(1):48-55.

47. Wu C, Soderhall I, Kim YA, Liu H, Soderhall K. Hemocyte-lineage marker proteins in a crustacean, the freshwater crayfish, Pacifastacus leniusculus. *Proteomics*. 2008;8(20):4226-4235.

48. Hibino T, Loza-Coll M, Messier C, et al. The immune gene repertoire encoded in the purple sea urchin genome. *Dev Biol* . 2006;300(1):349-365.

49. Wright RK. Protochordate immunity. I. Primary immune response of the tunicate Ciona intestinalis to vertebrate erythrocytes. *J Invertebr Pathol* . 1974;24(1):29-36.

50. Delsuc F, Brinkmann H, Chourrout D, Philippe H. Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature*. 2006;439(7079):965-968.

51. Huang G, Xie X, Han Y, et al. The identification of lymphocyte-like cells and lymphoid-related genes in amphioxus indicates the twilight for the emergency of adaptive immune system. *PLoS One*. 2007;2(2):e206.

52. Anderson MK, Pant R, Miracle AL, et al. Evolutionary origins of lymphocytes: ensembles of T cell and B cell transcriptional regulators in a cartilaginous fish. *J Immunol*. 2004;172(10):5851-5860.

53. Schorpp M, Bialecki M, Diekhoff D, et al. Conserved functions of Ikaros in vertebrate

lymphocyte development: genetic evidence for distinct larval and adult phases of T cell development and two lineages of B cells in zebrafish. *J Immunol*. 2006;177(4):2463-2476.

54. Soderhall I, Bangyeekhun E, Mayo S, Soderhall K. Hemocyte production and maturation in an invertebrate animal; proliferation and gene expression in hematopoietic stem cells of Pacifastacus leniusculus. *Dev Comp Immunol*. 2003;27(8):661-672.

55. Soderhall I, Kim YA, Jiravanichpaisal P, Lee SY, Soderhall K. An ancient role for a prokineticin domain in invertebrate hematopoiesis. *J Immunol*. 2005;174(10):6153-6160.

56. Sorrentino RP, Carton Y, Govind S. Cellular immune response to parasite infection in the Drosophila lymph gland is developmentally regulated. *Dev Biol*. 2002;243(1):65-80.

57. Bozzaro S, Bucci C, Steinert M. Phagocytosis and host-pathogen interactions in Dictyostelium with a look at macrophages. *Int Rev Cell Mol Biol* . 2008;271:253-300.

58. Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RA. Phylogenetic perspectives in innate immunity. *Science*. 1999;284(5418):1313-1318.

59. Medzhitov R, Janeway CA Jr. Innate immunity: the virtues of a nonclonal system of recognition. *Cell*. 1997;91(3):295-298.

60. Karre K. NK cells, MHC class I molecules and the missing self. *Scand J Immunol*. 2002;55(3):221-228.

61. Flajnik MF, Du Pasquier L. Evolution of innate and adaptive immunity: can we draw a line? *Trends Immunol*. 2004;25(12):640-644.

62. Vivier E, Raulet DH, Moretta A, et al. Innate or adaptive immunity? The example of natural killer cells. *Science*. 2011;331(6013):44-49.

63. Hansen JD, Vojtech LN, Laing KJ. Sensing disease and danger: a survey of vertebrate PRRs and their origins. *Dev Comp Immunol*. 2011;35(9):886-897.

64. Bonardi V, Cherkis K, Nishimura MT, Dangl JL. A new eye on NLR proteins: focused on clarity or diffused by complexity? *Curr Opin Immunol* . 2012;24(1):41-50.

65. Franchi L, Munoz-Planillo R, Nunez G. Sensing and reacting to microbes through the inflammasomes. *Nat Immunol*. 2012;13(4):325-332.

66. Robbins GR, Truax AD, Davis BK, Zhang L, Brickey WJ, Ting JP. Regulation of class I major histocompatibility complex (MHC). by nucleotide-binding domain, leucine-rich repeat-containing (NLR) proteins. *J Biol Chem*. 2012;287:24294-24303.

67. Irazoqui JE, Urbach JM, Ausubel FM. Evolution of host innate defence: insights from Caenorhabditis elegans and primitive invertebrates. *Nat Rev Immunol* . 2010;10(1):47-58.

68. Rehwinkel J, Reis e Sousa C. RIGorous detection: exposing virus through RNA sensing. *Science*. 2010;327(5963):284-286.

69. Takeuchi O, Akira S. RIG-I-like antiviral protein in flies. *Nat Immunol* . 2008;9(12):1327-1328.

70. Augustin R, Fraune S, Bosch TC. How Hydra senses and destroys microbes. *Semin Immunol* . 2010;22(1):54-58.

71. Sharma S, Fitzgerald KA. Innate immune sensing of DNA. *PLoS Pathog* . 2011;7(4):e1001310.

72. Burdette DL, Monroe KM, Sotelo-Troha K, et al. STING is a direct innate immune sensor of cyclic di-GMP. *Nature*. 2011;478(7370):515-518.

73. Ozato K, Shin DM, Chang TH, Morse HC III. TRIM family proteins and their emerging roles in innate immunity. *Nat Rev Immunol* . 2008;8(11):849-860.

74. McNab FW, Rajsbaum R, Stoye JP, O'Garra A. Tripartite-motif proteins and innate immune regulation. *Curr Opin Immunol* . 2011;23(1):46-56.

75. Henry J, Mather IH, McDermott MF, Pontarotti P. B30.2-like domain proteins: update and new insights into a rapidly expanding family of proteins. *Mol Biol Evol* . 1998;15(12):1696-1705.

76. James LC, Keeble AH, Khan Z, Rhodes DA, Trowsdale J. Structural basis for PRYSPRY-mediated tripartite motif (TRIM) protein function. *Proc Natl Acad Sci U S A* . 2007;104(15):6200-6205.

77. Du Pasquier L. Fish 'n' TRIMs. *J Biol* . 2009;8(5):50.

78. van der Aa LM, Levraud JP, Yahmi M, et al. A large new subset of TRIM genes highly diversified by duplication and positive selection in teleost fish. *BMC Biol* . 2009;7:7.

79. Boudinot P, van der Aa LM, Jouneau L, et al. Origin and evolution of TRIM proteins: new insights from the complete TRIM repertoire of zebrafish and pufferfish. *PLoS One*. 2011;6(7):e22022.

80. Laing KJ, Purcell MK, Winton JR, Hansen JD. A genomic view of the NOD-like receptor family in teleost fish: identification of a novel NLR subfamily in zebrafish. *BMC Evol Biol* . 2008;8:42.

81. Rhodes DA, de BB, Trowsdale J. Relationship between SPRY and B30.2 protein domains. Evolution of a component of immune defence? *Immunology*. 2005;116(4):411-417.

82. Martens S, Howard J. The interferon-inducible GTPases. *Annu Rev Cell Dev Biol* . 2006;22:559-589.

83. Bekpen C, Hunn JP, Rohde C, et al. The interferon-inducible p47 (IRG) GTPases in vertebrates: loss of the cell autonomous resistance mechanism in the human lineage. *Genome Biol*. 2005;6(11):R92.

84. Osorio F, Reis e Sousa C. Myeloid C-type lectin receptors in pathogen recognition and host defense. *Immunity*. 2011;34(5):651-664.

85. Cirimotich CM, Dong Y, Garver LS, Sim S, Dimopoulos G. Mosquito immune defenses against Plasmodium infection. *Dev Comp Immunol*. 2010;34(4):387-395.

86. Zucchetti I, Marino R, Pinto MR, Lambris JD, Du Pasquier L, De Santis R. ciCD94-1, an ascidian multipurpose C-type lectin-like receptor expressed in Ciona intestinalis hemocytes and larval neural structures. *Differentiation*. 2008;76(3):267-282.

87. Taylor PR, Martinez-Pomares L, Stacey M, Lin HH, Brown GD, Gordon S. Macrophage receptors and immune recognition. *Annu Rev Immunol*. 2005;23:901-944.

88. Franc NC, Dimarcq JL, Lagueux M, Hoffmann J, Ezekowitz RA. Croquemort, a novel Drosophila hemocyte/macrophage receptor that recognizes apoptotic cells. *Immunity*. 1996;4(5):431-443.

89. Pancer Z. Dynamic expression of multiple scavenger receptor cysteinerich genes in coelomocytes of the purple sea urchin. *Proc Natl Acad Sci U S A* . 2000;97(24):13156-13161.

90. Dong Y, Taylor HE, Dimopoulos G. AgDscam, a hypervariable immunoglobulin domain-containing receptor of the Anopheles gambiae innate immune system. *PLoS Biol*. 2006;4(7):e229.

91. Watthanasurorot A, Jiravanichpaisal P, Liu H, Soderhall I, Soderhall K. Bacteria-induced Dscam isoforms of the crustacean, Pacifastacus leniusculus. *PLoS Pathog*. 2011;7(6):e1002062.

92. Brites D, Encinas-Viso F, Ebert D, Du Pasquier L, Haag CR. Population genetics of duplicated alternatively spliced exons of the Dscam gene in Daphnia and Drosophila. *PLoS One*. 2011;6(12):e27947.

93. Brites D, McTaggart S, Morris K, et al. The Dscam homologue of the crustacean Daphnia is diversified by alternative splicing like in insects. *Mol Biol Evol* . 2008;25(7):1429-1439.

94. Royet J, Gupta D, Dziarski R. Peptidoglycan recognition proteins: modulators of the microbiome and inflammation. *Nat Rev Immunol* . 2011;11(12):837-851.

95. Dziarski R, Kashyap DR, Gupta D. Mammalian peptidoglycan recognition proteins kill bacteria by activating two-component systems and modulate microbiome and inflammation. *Microb Drug Resist* . 2012;18(3):280-285.

96. Li X, Wang S, Qi J, et al. Zebrafish peptidoglycan recognition proteins are bactericidal amidases essential for defense against bacterial infections. *Immunity*. 2007;27(3):518-529.

97. Saha S, Jing X, Park SY, et al. Peptidoglycan recognition proteins protect mice from experimental colitis by promoting normal gut flora and preventing induction of interferon-gamma. *Cell Host Microbe*. 2010;8(2):147-162.

98. Hughes AL. Evolution of the betaGRP/GNBP/beta-1,3-glucanase family of insects. *Immunogenetics*. 2012;64(7):549-558.

99. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell*. 1996;86(6):973-983.

100. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*. 1998; 282(5396):2085-2088.

101. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature*. 1997;388(6640):394-397.

102. Leulier F, Lemaitre B. Toll-like receptors—taking an evolutionary approach. *Nat Rev Genet*. 2008;9(3):165-178.

103. Gauthier ME, Du Pasquier L, Degnan BM. The genome of the sponge Amphimedon queenslandica provides new perspectives into the origin of Toll-like and interleukin 1 receptor pathways. *Evol Dev*. 2010; 12(5):519-533.

104. Triantafilou M, Gamper FG, Haston RM, et al. Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and intracellular targeting. *J Biol Chem*. 2006;281(41):31002-31011.

105. Round JL, Lee SM, Li J, et al. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science*. 2011;332(6032):974-977.

106. Steiner H, Hultmark D, Engstrom A, Bennich H, Boman HG. Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature*. 1981;292(5820):246-248.

107. Jang IH, Chosa N, Kim SH, et al. A Spatzle-processing enzyme required for toll signaling activation in Drosophila innate immunity. *Dev Cell*. 2006;10(1):45-55.

108. Sun SC, Faye I. Cecropia immunoresponsive factor, an insect immunoresponsive factor with DNA-binding properties similar to nuclear-factor kappa B. *Eur J Biochem*. 1992;204(2):885-892.

109. Lemaitre B, Kromer-Metzger E, Michaut L, et al. A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the Drosophila host defense. *Proc Natl Acad Sci U S A* . 1995;92(21): 9465-9469.

110. Gilmore TD, Wolenski FS. NF-kappaB: where did it come from and why? *Immunol Rev*. 2012;246(1):14-35.

111. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol*. 2010;11(9):785-797.

112. Nonaka M, Kimura A. Genomic view of the evolution of the complement system. *Immunogenetics*. 2006;58(9):701-713.

113. Endo Y, Matsushita M, Fujita T. Role of ficolin in innate immunity and its molecular basis. *Immunobiology*. 2007;212(4-5):371-379.

114. Smith LC, Clow LA, Terwilliger DP. The ancestral complement system in sea urchins. *Immunol Rev*. 2001;180:16-34.

115. Nonaka M, Satake H. Urochordate immunity. *Adv Exp Med Biol*. 2010;708:302-310.

116. Kimura A, Ikeo K, Nonaka M. Evolutionary origin of the vertebrate blood complement and coagulation systems inferred from liver EST analysis of lamprey. *Dev Comp Immunol*. 2009;33(1):77-87.

117. Blandin S, Levashina EA. Thioester-containing proteins and insect immunity. *Mol Immunol*. 2004;40(12):903-908.

118. Sekiguchi R, Fujito NT, Nonaka M. Evolution of the thioester-containing proteins (TEPs) of the arthropoda, revealed by molecular cloning of TEP genes from a spider, Hasarius adansoni. *Dev Comp Immunol*. 2012;36(2):483-489.

119. Fraiture M, Baxter RH, Steinert S, et al. Two mosquito LRR proteins function as complement control factors in the TEP1-mediated killing of Plasmodium. *Cell Host Microbe*. 2009;5(3):273-284.

120. Volohonsky G, Steinert S, Levashina EA. Focusing on complement in the antiparasitic defense of mosquitoes. *Trends Parasitol*. 2010;26(1):1-3.

121. Dishaw LJ, Smith SL, Bigger CH. Characterization of a C3-like cDNA in a coral: phylogenetic implications. *Immunogenetics*. 2005;57(7):535-548.

122. Kimura A, Sakaguchi E, Nonaka M. Multi-component complement system of Cnidaria: C3, Bf, and MASP genes expressed in the endodermal tissues of a sea anemone, Nematostella vectensis. *Immunobiology*. 2009;214(3):165-178.

123. Zhu Y, Thangamani S, Ho B, Ding JL. The ancient origin of the complement system. *EMBO J*. 2005;24(2):382-394.

124. Nakao M, Tsujikura M, Ichiki S, Vo TK, Somamoto T. The complement system in teleost fish: progress of post-homolog-hunting researches. *Dev Comp Immunol*. 2011;35(12):1296-1308.

125. Ichiki S, Kato-Unoki Y, Somamoto T, Nakao M. The binding spectra of carp C3 isotypes against natural targets independent of the binding specificity of their thioester. *Dev Comp Immunol*. 2012. Epub ahead of print.

126. D'Angelo ME, Dunstone MA, Whisstock JC, Trapani JA, Bird PI. Perforin evolved from a gene duplication of MPEG1, followed by a complex pattern of gene gain and loss within Euteleostomi. *BMC Evol Biol*. 2012;12(1):59.

127. Solomon KR, Sharma P, Chan M, Morrison PT, Finberg RW. CD109 represents a novel branch of the alpha2-macroglobulin/complement gene family. *Gene*. 2004;327(2):171-183.

128. Hughes AL. Phylogeny of the C3/C4/C5 complement-component gene family indicates that C5 diverged first. *Mol Biol Evol*. 1994;11(3):417-425.

129. Yano K, Gale D, Massberg S, et al. Phenotypic heterogeneity is an evolutionarily conserved feature of the endothelium. *Blood*. 2007;109(2): 613-615.

130. Cerenius L, Soderhall K. The prophenoloxidase-activating system in invertebrates. *Immunol Rev*. 2004;198:116-126.

131. Nelson RE, Fessler LI, Takagi Y, et al. Peroxidasin: a novel enzyme-matrix protein of Drosophila development. *EMBO J*. 1994;13(15):3438-3447.

132. Johansson MW, Lind MI, Holmblad T, Thornqvist PO, Soderhall K. Peroxinectin, a novel cell adhesion protein from crayfish blood. *Biochem Biophys Res Commun*. 1995;216(3):1079-1087.

133. Adema CM, Hertel LA, Miller RD, Loker ES. A family of fibrinogen-related proteins that precipitates parasite-derived molecules is produced by an invertebrate after infection. *Proc Natl Acad Sci U S A* . 1997;94(16): 8691-8696.

134. Zhang SM, Adema CM, Kepler TB, Loker ES. Diversification of Ig superfamily genes in an invertebrate. *Science*. 2004;305(5681):251-254.

135. Hanington PC, Forys MA, Dragoo JW, Zhang SM, Adema CM, Loker ES. Role for a somatically diversified lectin in resistance of an invertebrate to parasite infection. *Proc Natl Acad Sci U S A*. 2010;107(49):21087-21092.

136. Middha S, Wang X. Evolution and potential function of fibrinogen-like domains across twelve Drosophila species. *BMC Genomics*. 2008; 9:260.

137. Dong Y, Dimopoulos G. Anopheles fibrinogen-related proteins provide expanded pattern recognition capacity against bacteria and malaria parasites. *J Biol Chem*. 2009;284(15):9835-9844.

138. Buckley KM, Smith LC. Extraordinary diversity among members of the large gene family, 185/333, from the purple sea urchin, Strongylocentrotus purpuratus. *BMC Mol Biol*. 2007;8:68.

139. Ghosh J, Buckley KM, Nair SV, et al. Sp185/333: a novel family of genes and proteins involved in the purple sea urchin immune response. *Dev Comp Immunol* . 2010;34(3):235-245.

140. Hernandez Prada JA, Haire RN, Allaire M, et al. Ancient evolutionary origin of diversified variable regions demonstrated by crystal structures of an immune-type receptor in amphioxus. *Nat Immunol* . 2006;7(8): 875-882.

141. Cannon JP, Haire RN, Litman GW. Identification of diversified genes that contain immunoglobulin-like variable regions in a protochordate. *Nat Immunol* . 2002;3(12):1200-1207.

142. Dishaw LJ, Ota T, Mueller MG, et al. The basis for haplotype complexity in VCBPs, an immune-type receptor in amphioxus. *Immunogenetics*. 2010;62(9):623-631.

143. Dishaw LJ, Giacomelli S, Melillo D, et al. A role for variable region-containing chitin-binding proteins (VCBPs) in host gut-bacteria interactions. *Proc Natl Acad Sci U S A* . 2011;108(40):16747-16752.

144. Selsted ME, Ouellette AJ. Mammalian defensins in the antimicrobial immune response. *Nat Immunol*. 2005;6(6):551-557.

145. Bulet P, Stocklin R, Menin L. Anti-microbial peptides: from invertebrates to vertebrates. *Immunol Rev*. 2004;198:169-184.

146. Denoeud F, Henriet S, Mungpakdee S, et al. Plasticity of animal genome architecture unmasked by rapid evolution of a pelagic tunicate. *Science*. 2010;330(6009):1381-1385.

147. Zasloff M, Adams AP, Beckerman B, et al. Squalamine as a broad-spectrum systemic antiviral agent with therapeutic potential. *Proc Natl Acad Sci U S A* . 2011;108(38):15978-15983.

148. Cuthbertson BJ, Deterding LJ, Williams JG, et al. Diversity in penaeidin antimicrobial peptide form and function. *Dev Comp Immunol*. 2008; 32(3):167-181.

149. Cuthbertson BJ, Deterding LJ, Williams JG, et al. Diversity in penaeidin antimicrobial peptide form and function. *Dev Comp Immunol*. 2008; 32(3):167-181.

150. Sabin LR, Hanna SL, Cherry S. Innate antiviral immunity in Drosophila. *Curr Opin Immunol*. 2010;22(1):4-9.

151. Montgomery MK, Xu S, Fire A. RNA as a target of double-stranded RNA-mediated genetic interference in Caenorhabditis elegans. *Proc Natl Acad Sci U S A* . 1998;95(26):15502-15507.

152. Beutler B, Eidenschenk C, Crozat K, et al. Genetic analysis of resistance to viral infection. *Nat Rev Immunol*. 2007;7(10):753-766.

153. Nakamoto M, Moy RH, Xu J, et al. Virus recognition by Toll-7 activates antiviral autophagy in Drosophila. *Immunity*. 2012;36(4):658-667.

154. Theodor JL, Senelar R. Cytotoxic interaction between gorgonian explants: mode of action. *Cell Immunol*. 1975;19(2):194-200.

155. Boiledieu D, Valembois P. [In vitro study of the cytotoxic activity of sipunculid leukocytes towards allogeneic and xenogenic erythrocytes]. *C R Acad Sci Hebd Seances Acad Sci D*. 1976;283(3):247-249.

156. Nappi AJ, Ottaviani E. Cytotoxicity and cytotoxic molecules in invertebrates. *Bioessays*. 2000;22(5):469-480.

157. Pancer Z, Skorokhod A, Blumbach B, Muller WE. Multiple Ig-like featuring genes

divergent within and among individuals of the marine sponge Geodia cydonium. *Gene*. 1998;207(2):227-233.

158. Khalturin K, Becker M, Rinkevich B, Bosch TC. Urochordates and the origin of natural killer cells: identification of a CD94/NKR-P1-related receptor in blood cells of Botryllus. *Proc Natl Acad Sci U S A* . 2003;100(2): 622-627.

159. Zucchetti I, Marino R, Pinto MR, Lambris JD, Du Pasquier L, De Santis R. ciCD94-1, an ascidian multipurpose C-type lectin-like receptor expressed in Ciona intestinalis hemocytes and larval neural structures. *Differentiation*. 2008;76(3):267-282.

160. Daeron M, Jaeger S, Du Pasquier L, Vivier E. Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future. *Immunol Rev*. 2008;224:11-43.

161. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol*. 2008;9(5):495-502.

162. Wei S, Zhou JM, Chen X, et al. The zebrafish activating immune receptor Nitr9 signals via Dap12. *Immunogenetics*. 2007;59(10):813-821.

163. Yoder JA, Litman GW. The phylogenetic origins of natural killer receptors and recognition: relationships, possibilities, and realities. *Immunogenetics*. 2011;63(3):123-141.

164. Voigt V, Forbes CA, Tonkin JN, et al. Murine cytomegalovirus m157 mutation and variation leads to immune evasion of natural killer cells. *Proc Natl Acad Sci U S A* . 2003;100(23):13483-13488.

165. Abi-Rached L, Parham P. Natural selection drives recurrent formation of activating killer cell immunoglobulin-like receptor and Ly49 from inhibitory homologues. *J Exp Med*. 2005;201(8):1319-1332.

166. Horton TL, Ritchie P, Watson MD, Horton JD. NK-like activity against allogeneic tumour cells demonstrated in the spleen of control and thymectomized Xenopus. *Immunol Cell Biol*. 1996;74(4):365-373.

167. Horton JD, Horton TL, Dzialo R, et al. T-cell and natural killer cell development in thymectomized Xenopus. *Immunol Rev*. 1998;166:245-258.

168. Horton TL, Stewart R, Cohen N, et al. Ontogeny of Xenopus NK cells in the absence of MHC class I antigens. *Dev Comp Immunol*. 2003;27(8): 715-726.

169. Shen L, Stuge TB, Zhou H, et al. Channel catfish cytotoxic cells: a minireview. *Dev Comp Immunol*. 2002;26(2):141-149.

170. Shen L, Stuge TB, Evenhuis JP, et al. Channel catfish NK-like cells are armed with IgM via a putative FcmicroR. *Dev Comp Immunol*. 2003; 27(8):699-714.

171. Plougastel BF, Yokoyama WM. Extending missing-self? Functional interactions between lectin-like NKrp1 receptors on NK cells with lectin-like ligands. *Curr Top Microbiol Immunol*. 2006;298:77-89.

172. Rogers SL, Gobel TW, Viertlboeck BC, Milne S, Beck S, Kaufman J. Characterization of the chicken C-type lectin-like receptors B-NK and B-lec suggests that the NK complex and the MHC share a common ancestral region. *J Immunol*. 2005;174(6):3475-3483.

173. Shiina T, Briles WE, Goto RM, et al. Extended gene map reveals tripartite motif, C-type lectin, and Ig superfamily type genes within a subregion of the chicken MHC-B affecting infectious disease. *J Immunol*. 2007;178(11):7162-7172.

174. Delany ME, Robinson CM, Goto RM, Miller MM. Architecture and organization of chicken microchromosome 16: order of the NOR, MHC-Y, and MHC-B subregions. *J Hered*. 2009;100(5):507-514.

175. Stet RJ, Hermsen T, Westphal AH, et al. Novel immunoglobulin-like transcripts in teleost fish encode polymorphic receptors with cytoplasmic ITAM or ITIM and a new structural Ig domain similar to the natural cytotoxicity receptor NKp44. *Immunogenetics*. 2005;57(1-2):77-89.

176. Flajnik MF, Tlapakova T, Criscitiello MF, Krylov V, Ohta Y. Evolution of the B7 family: co-evolution of B7H6 and NKp30, identification of a new B7 family member, B7H7, and of B7's historical relationship with the MHC. *Immunogenetics* 2012;64(8):571-590.

177. Ohta Y, Goetz W, Hossain MZ, Nonaka M, Flajnik MF. Ancestral organization of the MHC revealed in the amphibian Xenopus. *J Immunol*. 2006;176(6):3674-3685.

178. Brandt CS, Baratin M, Yi EC, et al. The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. *J Exp Med*. 2009;206(7):1495-1503.

179. Litman GW, Hawke NA, Yoder JA. Novel immune-type receptor genes. *Immunol Rev*. 2001;181:250-259.

180. Yoder JA, Litman GW. The zebrafish fth1, slc3a2, men1, pc, fgf3 and cycd1 genes define two regions of conserved synteny between linkage group 7 and human chromosome 11q13. *Gene*. 2000;261(2):235-242.

181. Cannon JP, Haire RN, Magis AT, et al. A bony fish immunological receptor of the NITR multigene family mediates allogeneic recognition. *Immunity*. 2008;29(2):228-237.

182. Davis RS, Ehrhardt GR, Leu CM, Hirano M, Cooper MD. An extended family of Fc receptor relatives. *Eur J Immunol* . 2005;35(3):674-680.

183. Viertlboeck BC, Habermann FA, Schmitt R, Groenen MA, Du Pasquier L, Gobel TW. The chicken leukocyte receptor complex: a highly diverse multigene family encoding at least six structurally distinct receptor types. *J Immunol* . 2005;175(1):385-393.

184. Stafford JL, Bengten E, Du Pasquier L, et al. A novel family of diversified immunoregulatory receptors in teleosts is homologous to both mammalian Fc receptors and molecules encoded within the leukocyte receptor complex. *Immunogenetics*. 2006;58(9):758-773.

185. Pancer Z, Cooper MD. The evolution of adaptive immunity. *Annu Rev Immunol* . 2006;24:497-518.

186. Suzuki T, Shin I, Fujiyama A, Kohara Y, Kasahara M. Hagfish leukocytes express a paired receptor family with a variable domain resembling those of antigen receptors. *J Immunol* . 2005;174(5):2885-2891.

187. Boulanger LM. Immune proteins in brain development and synaptic plasticity. *Neuron*. 2009;64(1):93-109.

188. Hsu E. The variation in immunoglobulin heavy chain constant regions in evolution. *Semin Immunol* . 1994;6(6):383-391.

189. Kaattari S, Evans D, Klemer J. Varied redox forms of teleost IgM: an alternative to isotypic diversity? *Immunol Rev* . 1998;166:133-142.

190. Wilson MR, Marcuz A, van GF, et al. The immunoglobulin M heavy chain constant region gene of the channel catfish, Ictalurus punctatus: an unusual mRNA splice pattern produces the membrane form of the molecule. *Nucleic Acids Res* . 1990;18(17):5227-5233.

191. Lundqvist M, Stromberg S, Bouchenot C, Pilstrom L, Boudinot P. Diverse splicing pathways of the membrane IgHM pre-mRNA in a Chondrostean, the Siberian sturgeon. *Dev Comp Immunol* . 2009;33(4):507-515.

192. Coscia MR, Varriale S, Giacomelli S, Oreste U. Antarctic teleost immunoglobulins: more extreme, more interesting. *Fish Shellfish Immunol* . 2011;31(5):688-696.

193. Marchalonis J, Edelman GM. Polypeptide chains of immunoglobulins from the smooth dogfish (Mustelus canis). *Science*. 1966;154(756): 1567-1568.

194. Hsu E, Pulham N, Rumfelt LL, Flajnik MF. The plasticity of immunoglobulin gene

systems in evolution. *Immunol Rev*. 2006;210:8-26.

195. Dooley H, Flajnik MF. Shark immunity bites back: affinity maturation and memory response in the nurse shark, Ginglymostoma cirratum. *Eur J Immunol* . 2005;35(3):936-945.

196. Rumfelt LL, Avila D, Diaz M, Bartl S, McKinney EC, Flajnik MF. A shark antibody heavy chain encoded by a nonsomatically rearranged VDJ is preferentially expressed in early development and is convergent with mammalian IgG. *Proc Natl Acad Sci U S A* . 2001;98(4):1775-1780.

197. Greenberg AS, Avila D, Hughes M, Hughes A, McKinney EC, Flajnik MF. A new antigen receptor gene family that undergoes rearrangement and extensive somatic diversification in sharks. *Nature*. 1995;374(6518):168-173.

198. Flajnik MF, Deschacht N, Muyldermans S. A case of convergence: why did a simple alternative to canonical antibodies arise in sharks and camels? *PLoS Biol*. 2011;9(8):e1001120.

199. Stanfield RL, Dooley H, Flajnik MF, Wilson IA. Crystal structure of a shark single-domain antibody V region in complex with lysozyme. *Science*. 2004;305(5691):1770-1773.

200. Stanfield RL, Dooley H, Verdino P, Flajnik MF, Wilson IA. Maturation of shark single-domain (IgNAR) antibodies: evidence for induced-fit binding. *J Mol Biol* . 2007;367(2):358-372.

201. Criscitiello MF, Saltis M, Flajnik MF. An evolutionarily mobile antigen receptor variable region gene: doubly rearranging NAR-TcR genes in sharks. *Proc Natl Acad Sci U S A*. 2006;103(13):5036-5041.

202. Parra ZE, Baker ML, Schwarz RS, Deakin JE, Lindblad-Toh K, Miller RD. A unique T cell receptor discovered in marsupials. *Proc Natl Acad Sci U S A* . 2007;104(23):9776-9781.

203. Kobayashi K, Tomonaga S. The second immunoglobulin class is commonly present in cartilaginous fish belonging to the order Rajiformes. *Mol Immunol*. 1988;25(2):115-120.

204. Hsu E, Flajnik MF, Du Pasquier L. A third immunoglobulin class in amphibians. *J Immunol*. 1985;135(3):1998-2004.

205. Harding FA, Amemiya CT, Litman RT, Cohen N, Litman GW. Two distinct immunoglobulin heavy chain isotypes in a primitive, cartilaginous fish, Raja erinacea. *Nucleic Acids Res*. 1990;18(21):6369-6376.

206. Greenberg AS, Hughes AL, Guo J, Avila D, McKinney EC, Flajnik MF. A novel "chimeric" antibody class in cartilaginous fish: IgM may not be the primordial immunoglobulin. *Eur J Immunol* . 1996;26(5):1123-1129.

207. Berstein RM, Schluter SF, Shen S, Marchalonis JJ. A new high molecular weight immunoglobulin class from the carcharhine shark: implications for the properties of the primordial immunoglobulin. *Proc Natl Acad Sci U S A* . 1996;93(8):3289-3293.

208. Rumfelt LL, Diaz M, Lohr RL, Mochon E, Flajnik MF. Unprecedented multiplicity of Ig transmembrane and secretory mRNA forms in the cartilaginous fish. *J Immunol* . 2004;173(2):1129-1139.

209. Ota T, Rast JP, Litman GW, Amemiya CT. Lineage-restricted retention of a primitive immunoglobulin heavy chain isotype within the Dipnoi reveals an evolutionary paradox. *Proc Natl Acad Sci U S A* . 2003;100(5):2501-2506.

210. Ohta Y, Flajnik M. IgD, like IgM, is a primordial immunoglobulin class perpetuated in most jawed vertebrates. *Proc Natl Acad Sci U S A* . 2006;103(28):10723-10728.

211. Zhao Y, Pan-Hammarstrom Q, Yu S, et al. Identification of IgF, a hinge-region-containing Ig class, and IgD in Xenopus tropicalis. *Proc Natl Acad Sci U S A* . 2006;103(32):12087-12092.

212. Wilson M, Bengten E, Miller NW, Clem LW, Du Pasquier L, Warr GW. A novel chimeric Ig heavy chain from a teleost fish shares similarities to IgD. *Proc Natl Acad Sci U S A*. 1997;94(9):4593-4597.

213. Edholm ES, Bengten E, Stafford JL, et al. Identification of two IgD+ B cell populations in channel catfish, Ictalurus punctatus. *J Immunol* . 2010;185(7):4082-4094.

214. Chen K, Xu W, Wilson M, et al. Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cell-stimulating programs in basophils. *Nat Immunol* . 2009;10(8):889-898.

215. Edholm ES, Bengten E, Wilson M. Insights into the function of IgD. *Dev Comp Immunol* . 2011;35(12):1309-1316.

216. Danilova N, Bussmann J, Jekosch K, Steiner LA. The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. *Nat Immunol* . 2005; 6(3):295-302.

217. Hansen JD, Landis ED, Phillips RB. Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: implications for a distinctive B cell developmental pathway in teleost fish. *Proc Natl Acad Sci U S A* . 2005;102(19):6919-6924.

218. Zhang YA, Salinas I, Li J, et al. IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nat Immunol*. 2010;11(9):827-835.

219. Hohman VS, Stewart SE, Rumfelt LL, et al. J chain in the nurse shark: implications for function in a lower vertebrate. *J Immunol*. 2003;170(12): 6016-6023.

220. Flajnik MF. Comparative analyses of immunoglobulin genes: surprises and portents. *Nat Rev Immunol*. 2002;2(9):688-698.

221. Mussmann R, Du Pasquier L, Hsu E. Is Xenopus IgX an analog of IgA? *Eur J Immunol*. 1996;26(12):2823-2830.

222. Fellah JS, Vaulot D, Tournefier A, Charlemagne J. Ontogeny of immunoglobulin expression in the Mexican axolotl. *Development*. 1989;107(2): 253-263.

223. Fellah JS, Iscaki S, Vaerman JP, Charlemagne J. Transient developmental expression of IgY and secretory component like protein in the gut of the axolotl (Ambystoma mexicanum). *Dev Immunol*. 1992;2(3):181-190.

224. Wei Z, Wu Q, Ren L, et al. Expression of IgM, IgD, and IgY in a reptile, Anolis carolinensis. *J Immunol*. 2009;183(6):3858-3864.

225. Mussmann R, Wilson M, Marcuz A, Courtet M, Du Pasquier L. Membrane exon sequences of the three Xenopus Ig classes explain the evolutionary origin of mammalian isotypes. *Eur J Immunol* . 1996;26(2): 409-414.

226. Martin SW, Goodnow CC. Burst-enhancing role of the IgG membrane tail as a molecular determinant of memory. *Nat Immunol*. 2002;3(2): 182-188.

227. Liu W, Meckel T, Tolar P, Sohn HW, Pierce SK. Intrinsic properties of immunoglobulin IgG1 isotype-switched B cell receptors promote microclustering and the initiation of signaling. *Immunity*. 2010;32(6): 778-789.

228. Stavnezer J, Amemiya CT. Evolution of isotype switching. *Semin Immunol*. 2004;16(4):257-275.

229. Zarrin AA, Alt FW, Chaudhuri J, et al. An evolutionarily conserved target motif for immunoglobulin class-switch recombination. *Nat Immunol*. 2004;5(12):1275-1281.

230. Zhu C, Lee V, Finn A, et al. Origin of immunoglobulin isotype switching. *Curr Biol* . 2012;22(10):872-880.

231. Criscitiello MF, Flajnik MF. Four primordial immunoglobulin light chain isotypes, including lambda and kappa, identified in the most primitive living jawed vertebrates. *Eur J Immunol*. 2007;37(10): 2683-2694.

232. Edholm ES, Wilson M, Bengten E. Immunoglobulin light (IgL) chains in ectothermic vertebrates. *Dev Comp Immunol* . 2011;35(9):906-915.

233. Edholm ES, Wilson M, Sahoo M, et al. Identification of Igsigma and Iglambda in channel catfish, Ictalurus punctatus, and Iglambda in Atlantic cod, Gadus morhua. *Immunogenetics*. 2009;61(5):353-370.

234. Zimmerman AM, Yeo G, Howe K, Maddox BJ, Steiner LA. Immunoglobulin light chain (IgL) genes in zebrafish: Genomic configurations and inversional rearrangements between (V(L)-J(L)-C(L)) gene clusters. *Dev Comp Immunol* . 2008;32(4):421-434.

235. Hsu E, Criscitiello MF. Diverse immunoglobulin light chain organizations in fish retain potential to revise B cell receptor specificities. *J Immunol* . 2006;177(4):2452-2462.

236. Lee SS, Greenberg A, Hsu E. Evolution and somatic diversification of immunoglobulin light chains. *Curr Top Microbiol Immunol* . 2000; 248:285-300.

237. Mikoryak CA, Steiner LA. Noncovalent association of heavy and light chains in Rana catesbeiana immunoglobulins. *J Immunol* . 1984;133(1): 376-383.

238. Miller RD, Belov K. Immunoglobulin genetics of marsupials. *Dev Comp Immunol* . 2000;24(5):485-490.

239. Rast JP, Anderson MK, Ota T, et al. Immunoglobulin light chain class multiplicity and alternative organizational forms in early vertebrate phylogeny. *Immunogenetics*. 1994;40(2):83-99.

240. Greenberg AS, Steiner L, Kasahara M, Flajnik MF. Isolation of a shark immunoglobulin light chain cDNA clone encoding a protein resembling mammalian kappa light chains: implications for the evolution of light chains. *Proc Natl Acad Sci U S A* . 1993;90(22):10603-10607.

241. Ota T, Sitnikova T, Nei M. Evolution of vertebrate immunoglobulin variable gene segments. *Curr Top Microbiol Immunol* . 2000;248: 221-245.

242. Chang B, Casali P. The CDR1 sequences of a major proportion of human germline Ig VH genes are inherently susceptible to amino acid replacement. *Immunol Today* . 1994;15(8):367-373.

243. Hsu E. Canonical VH CDR1 nucleotide sequences are conserved in all jawed vertebrates. *Int Immunol* . 1996;8(6):847-854.

244. Braathen R, Hohman VS, Brandtzaeg P, Johansen FE. Secretory antibody formation: conserved binding interactions between J chain and polymeric Ig receptor from humans

and amphibians. *J Immunol*. 2007;178(3):1589-1597.

245. Hohman VS, Stewart SE, Rumfelt LL, et al. J chain in the nurse shark: implications for function in a lower vertebrate. *J Immunol*. 2003; 170(12):6016-6023.

246. Takahashi T, Iwase T, Takenouchi N, et al. The joining (J) chain is present in invertebrates that do not express immunoglobulins. *Proc Natl Acad Sci U S A* . 1996;93(5):1886-1891.

247. Charlemagne J, Fellah JS, De Guerra A, Kerfourn F, Partula S. T-cell receptors in ectothermic vertebrates. *Immunol Rev*. 1998;166:87-102.

248. Rast JP, Anderson MK, Strong SJ, Luer C, Litman RT, Litman GW. Alpha, beta, gamma, and delta T cell antigen receptor genes arose early in vertebrate phylogeny. *Immunity*. 1997;6(1):1-11.

249. Chen CL, Bucy RP, Cooper MD. T cell differentiation in birds. *Semin Immunol*. 1990;2(1):79-86.

250. Gobel TW, Meier EL, Du Pasquier L. Biochemical analysis of the Xenopus laevis TCR/CD3 complex supports the “stepwise evolution” model. *Eur J Immunol* . 2000;30(10):2775-2781.

251. Munoz FA, Estrada-Parra S, Romero-Rojas A, Work TM, Gonzalez-Ballesteros E, Estrada-Garcia I. Identification of CD3+ T lymphocytes in the green turtle Chelonia mydas. *Vet Immunol Immunopathol*. 2009;131(3-4):211-217.

252. Kuhns MS, Girvin AT, Klein LO, et al. Evidence for a functional sidedness to the alphabetaTCR. *Proc Natl Acad Sci U S A* . 2010;107(11): 5094-5099.

253. Smelty P, Marchal C, Renard R, et al. Identification of the pre-T-cell receptor alpha chain in nonmammalian vertebrates challenges the structure-function of the molecule. *Proc Natl Acad Sci U S A* . 2010;107(46):19991-19996.

254. Pang SS, Berry R, Chen Z, et al. The structural basis for autonomous dimerization of the pre-T-cell antigen receptor. *Nature*. 2010; 467(7317):844-848.

255. Campbell KS, Backstrom BT, Tiefenthaler G, Palmer E. CART: a conserved antigen receptor transmembrane motif. *Semin Immunol*. 1994; 6(6):393-410.

256. Sasada T, Touma M, Chang HC, Clayton LK, Wang JH, Reinherz EL. Involvement of the TCR Cbeta FG loop in thymic selection and T cell function. *J Exp Med*. 2002;195(11):1419-1431.

257. Criscitiello MF, Ohta Y, Saltis M, McKinney EC, Flajnik MF. Evolutionarily conserved

TCR binding sites, identification of T cells in primary lymphoid tissues, and surprising trans-rearrangements in nurse shark. *J Immunol*. 2010;184(12):6950-6960.

258. Criscitiello MF, Wermenstam NE, Pilstrom L, McKinney EC. Allelic polymorphism of T-cell receptor constant domains is widespread in fishes. *Immunogenetics*. 2004;55(12):818-824.

259. Rock EP, Sibbald PR, Davis MM, Chien YH. CDR3 length in antigenspecific immune receptors. *J Exp Med*. 1994;179(1):323-328.

260. Petrie HT, Livak F, Schatz DG, Strasser A, Crispe IN, Shortman K. Multiple rearrangements in T cell receptor alpha chain genes maximize the production of useful thymocytes. *J Exp Med*. 1993;178(2):615-622.

261. Garcia KC, Adams JJ, Feng D, Ely LK. The molecular basis of TCR germline bias for MHC is surprisingly simple. *Nat Immunol*. 2009; 10(2):143-147.

262. Scott-Browne JP, White J, Kappler JW, Gapin L, Marrack P. Germline-encoded amino acids in the alphabeta T-cell receptor control thymic selection. *Nature*. 2009;458(7241):1043-1046.

263. Glusman G, Rowen L, Lee I, et al. Comparative genomics of the human and mouse T cell receptor loci. *Immunity*. 2001;15(3):337-349.

264. Rast JP, Haire RN, Litman RT, Pross S, Litman GW. Identification and characterization of T-cell antigen receptor-related genes in phylogenetically diverse vertebrate species. *Immunogenetics*. 1995;42(3):204-212.

265. Andre S, Kerfourn F, Affaticati P, Guerci A, Ravassard P, Fellah JS. Highly restricted diversity of TCR delta chains of the amphibian Mexican axolotl (Ambystoma mexicanum) in peripheral tissues. *Eur J Immunol* . 2007;37(6):1621-1633.

266. Cahill RN, Kimpton WG, Washington EA, Walker ID. Origin and development of the gamma delta T-cell system in sheep: a critical role for the thymus in the generation of TcR diversity and tissue tropism. *Semin Immunol*. 1996;8(6):351-360.

267. Su C, Jakobsen I, Gu X, Nei M. Diversity and evolution of T-cell receptor variable region genes in mammals and birds. *Immunogenetics*. 1999;50(5-6):301-308.

268. Parra ZE, Ohta Y, Criscitiello MF, Flajnik MF, Miller RD. The dynamic TCRdelta: TCRdelta chains in the amphibian Xenopus tropicalis utilize antibody-like V genes. *Eur J Immunol*. 2010;40(8):2319-2329.

269. Wang X, Parra ZE, Miller RD. Platypus TCRmu provides insight into the origins and evolution of a uniquely mammalian TCR locus. *J Immunol*. 2011;187(10):5246-5254.

270. Parra ZE, Mitchell K, Dalloul RA, Miller RD. A second TCRdelta locus in Galliformes uses antibody-like V domains: insight into the evolution of TCRdelta and TCRmu genes in tetrapods. *J Immunol*. 2012;188(8):3912-3919.

271. Chen H, Kshirsagar S, Jensen I, et al. Characterization of arrangement and expression of the T cell receptor gamma locus in the sandbar shark. *Proc Natl Acad Sci U S A*. 2009;106(21):8591-8596.

272. Chen H, Bernstein H, Ranganathan P, Schluter SF. Somatic hypermutation of TCR gamma V genes in the sandbar shark. *Dev Comp Immunol*. 2012;37(1):176-183.

273. Smith LE, Crouch K, Cao W, et al. Characterization of the immunoglobulin repertoire of the spiny dogfish (Squalus acanthias). *Dev Comp Immunol*. 2012;36(4):665-679.

274. Kokubu F, Litman R, Shamblott MJ, Hinds K, Litman GW. Diverse organization of immunoglobulin VH gene loci in a primitive vertebrate. *EMBO J*. 1988;7(11):3413-3422.

275. Lee SS, Fitch D, Flajnik MF, Hsu E. Rearrangement of immunoglobulin genes in shark germ cells. *J Exp Med*. 2000;191(10):1637-1648.

276. Lewis SM, Wu GE. The old and the restless. *J Exp Med*. 2000;191(10): 1631-1636.

277. Hinds KR, Litman GW. Major reorganization of immunoglobulin VH segmental elements during vertebrate evolution. *Nature*. 1986; 320(6062):546-549.

278. Eason DD, Litman RT, Luer CA, Kerr W, Litman GW. Expression of individual immunoglobulin genes occurs in an unusual system consisting of multiple independent loci. *Eur J Immunol* . 2004;34(9):2551-2558.

279. Malecek K, Lee V, Feng W, et al. Immunoglobulin heavy chain exclusion in the shark. *PLoS Biol*. 2008;6(6):e157.

280. Amemiya CT, Ohta Y, Litman RT, Rast JP, Haire RN, Litman GW. VH gene organization in a relict species, the coelacanth Latimeria chalumnae: evolutionary implications. *Proc Natl Acad Sci U S A* . 1993; 90(14):6661-6665.

281. Hsu E, Criscitiello MF. Diverse immunoglobulin light chain organizations in fish retain potential to revise B cell receptor specificities. *J Immunol*. 2006;177(4):2452-2462.

282. Adams EJ, Strop P, Shin S, Chien YH, Garcia KC. An autonomous CDR3delta is sufficient for recognition of the nonclassical MHC class I molecules T10 and T22 by gammadelta T cells. *Nat Immunol*. 2008;9(7):777-784.

283. Hanley PJ, Hook JW, Raftos DA, Gooley AA, Trent R, Raison RL. Hagfish humoral

defense protein exhibits structural and functional homology with mammalian complement components. *Proc Natl Acad Sci U S A* . 1992;89(17):7910-7914.

284. Zapata A, Amemiya CT. Phylogeny of lower vertebrates and their immunological structures. *Curr Top Microbiol Immunol* . 2000;248:67-107.

285. Uinuk-Ool T, Mayer WE, Sato A, Dongak R, Cooper MD, Klein J. Lamprey lymphocyte-like cells express homologs of genes involved in immunologically relevant activities of mammalian lymphocytes. *Proc Natl Acad Sci U S A* . 2002;99(22):14356-14361.

286. Pancer Z, Amemiya CT, Ehrhardt GR, Ceitlin J, Gartland GL, Cooper MD. Somatic diversification of variable lymphocyte receptors in the agnathan sea lamprey. *Nature*. 2004;430(6996):174-180.

287. Alder MN, Rogozin IB, Iyer LM, Glazko GV, Cooper MD, Pancer Z. Diversity and function of adaptive immune receptors in a jawless vertebrate. *Science*. 2005;310(5756):1970-1973.

288. Rogozin IB, Iyer LM, Liang L, et al. Evolution and diversification of lamprey antigen receptors: evidence for involvement of an AID-APOBEC family cytosine deaminase. *Nat Immunol*. 2007;8(6):647-656.

289. Nagawa F, Kishishita N, Shimizu K, et al. Antigen-receptor genes of the agnathan lamprey are assembled by a process involving copy choice. *Nat Immunol*. 2007;8(2):206-213.

290. Alder MN, Herrin BR, Sadlonova A, et al. Antibody responses of variable lymphocyte receptors in the lamprey. *Nat Immunol*. 2008;9(3): 319-327.

291. Guo P, Hirano M, Herrin BR, et al. Dual nature of the adaptive immune system in lampreys. *Nature*. 2009;459(7248):796-801.

292. Velikovsky CA, Deng L, Tasumi S, et al. Structure of a lamprey variable lymphocyte receptor in complex with a protein antigen. *Nat Struct Mol Biol* . 2009;16(7):725-730.

293. Han BW, Herrin BR, Cooper MD, Wilson IA. Antigen recognition by variable lymphocyte receptors. *Science*. 2008;321(5897):1834-1837.

294. Deng L, Velikovsky CA, Xu G, et al. A structural basis for antigen recognition by the T cell-like lymphocytes of sea lamprey. *Proc Natl Acad Sci U S A* . 2010;107(30):13408-13413.

295. Diaz M, Flajnik MF. Evolution of somatic hypermutation and gene conversion in adaptive immunity. *Immunol Rev*. 1998;162:13-24.

296. Du Pasquier L, Wilson M, Greenberg AS, Flajnik MF. Somatic mutation in ectothermic vertebrates: musings on selection and origins. *Curr Top Microbiol Immunol* . 1998;229:199-216.

297. Hsu E. Mutation, selection, and memory in B lymphocytes of exothermic vertebrates. *Immunol Rev*. 1998;162:25-36.

298. Makela O, Litman GW. Lack of heterogeneity in antihapten antibodies of a phylogenetically primitive shark. *Nature*. 1980;287(5783):639-640.

299. Hinds-Frey KR, Nishikata H, Litman RT, Litman GW. Somatic variation precedes extensive diversification of germline sequences and combinatorial joining in the evolution of immunoglobulin heavy chain diversity. *J Exp Med*. 1993;178(3):815-824.

300. Lee SS, Tranchina D, Ohta Y, Flajnik MF, Hsu E. Hypermutation in shark immunoglobulin light chain genes results in contiguous substitutions. *Immunity*. 2002;16(4):571-582.

301. Diaz M, Velez J, Singh M, Cerny J, Flajnik MF. Mutational pattern of the nurse shark antigen receptor gene (NAR) is similar to that of mammalian Ig genes and to spontaneous mutations in evolution: the translesion synthesis model of somatic hypermutation. *Int Immunol*. 1999;11(5):825-833.

302. Malecek K, Brandman J, Brodsky JE, Ohta Y, Flajnik MF, Hsu E. Somatic hypermutation and junctional diversification at Ig heavy chain loci in the nurse shark. *J Immunol*. 2005;175(12):8105-8115.

303. Diaz M, Greenberg AS, Flajnik MF. Somatic hypermutation of the new antigen receptor gene (NAR) in the nurse shark does not generate the repertoire: possible role in antigen-driven reactions in the absence of germinal centers. *Proc Natl Acad Sci U S A* . 1998;95(24):14343-14348.

304. Zhu C, Hsu E. Error-prone DNA repair activity during somatic hypermutation in shark B lymphocytes. *J Immunol*. 2010;185(9):5336-5347.

305. Zhu C, Feng W, Weedon J, et al. The multiple shark Ig H chain genes rearrange and hypermutate autonomously. *J Immunol*. 2011;187(5): 2492-2501.

306. Lindner C, Wahl B, Fohse L, et al. Age, microbiota, and T cells shape diverse individual IgA repertoires in the intestine. *J Exp Med*. 2012; 209(2):365-377.

307. Dooley H, Stanfield RL, Brady RA, Flajnik MF. First molecular and biochemical analysis of in vivo affinity maturation in an ectothermic vertebrate. *Proc Natl Acad Sci U S A*. 2006;103(6):1846-1851.

308. Rumfelt LL, McKinney EC, Taylor E, Flajnik MF. The development of primary and secondary lymphoid tissues in the nurse shark Ginglymostoma cirratum: B-cell zones precede dendritic cell immigration and T-cell zone formation during ontogeny of the spleen. *Scand J Immunol*. 2002;56(2):130-148.

309. Ye J, Bromage ES, Kaattari SL. The strength of B cell interaction with antigen determines the degree of IgM polymerization. *J Immunol*. 2010;184(2):844-850.

310. Miller N, Wilson M, Bengten E, Stuge T, Warr G, Clem W. Functional and molecular characterization of teleost leukocytes. *Immunol Rev*. 1998;166:187-197.

311. Boudinot P, Boubekeur S, Benmansour A. Rhabdovirus infection induces public and private T cell responses in teleost fish. *J Immunol*. 2001;167(11):6202-6209.

312. Castro R, Bernard D, Lefranc MP, Six A, Benmansour A, Boudinot P. T cell diversity and TcR repertoires in teleost fish. *Fish Shellfish Immunol*. 2011;31(5):644-654.

313. Ye J, Bromage E, Kaattari I, Kaattari S. Transduction of binding affinity by B lymphocytes: a new dimension in immunological regulation. *Dev Comp Immunol*. 2011;35(9):982-990.

314. Bromage ES, Kaattari IM, Zwollo P, Kaattari SL. Plasmablast and plasma cell production and distribution in trout immune tissues. *J Immunol*. 2004;173(12):7317-7323.

315. Zwollo P, Mott K, Barr M. Comparative analyses of B cell populations in trout kidney and mouse bone marrow: establishing “B cell signatures.” *Dev Comp Immunol*. 2010;34(12):1291-1299.

316. Zwollo P. Dissecting teleost B cell differentiation using transcription factors. *Dev Comp Immunol*. 2011;35(9):898-905.

317. Yang F, Waldbieser GC, Lobb CJ. The nucleotide targets of somatic mutation and the role of selection in immunoglobulin heavy chains of a teleost fish. *J Immunol*. 2006;176(3):1655-1667.

318. Barreto VM, Magor BG. Activation-induced cytidine deaminase structure and functions: a species comparative view. *Dev Comp Immunol*. 2011;35(9):991-1007.

319. Saunders HL, Oko AL, Scott AN, Fan CW, Magor BG. The cellular context of AID expressing cells in fish lymphoid tissues. *Dev Comp Immunol*. 2010;34(6):669-676.

320. Marianes AE, Zimmerman AM. Targets of somatic hypermutation within immunoglobulin light chain genes in zebrafish. *Immunology*. 2011;132(2):240-255.

321. Bly JE, Quiniou SM, Clem LW. Environmental effects on fish immune mechanisms. *Dev Biol Stand*. 1997;90:33-43.

322. Zhou H, Stuge TB, Miller NW, et al. Heterogeneity of channel catfish CTL with respect to target recognition and cytotoxic mechanisms employed. *J Immunol*. 2001;167(3):1325-1332.

323. Charlemagne J. Antibody diversity in amphibians. Noninbred axolotls used the same unique heavy chain and a limited number of light chains for their anti-2,4-dinitrophenyl antibody responses. *Eur J Immunol* . 1987;17(3):421-424.

324. Salvadori F, Tournefier A. Activation by mitogens and superantigens of axolotl lymphocytes: functional characterization and ontogenic study. *Immunology*. 1996;88(4):586-592.

325. Du Pasquier L, Robert J, Courtet M, Mussmann R. B-cell development in the amphibian Xenopus. *Immunol Rev*. 2000;175:201-213.

326. Wabl MR, Du Pasquier L. Antibody patterns in genetically identical frogs. *Nature*. 1976;264(5587):642-644.

327. Wilson M, Hsu E, Marcuz A, Courtet M, Du Pasquier L, Steinberg C. What limits affinity maturation of antibodies in Xenopus—the rate of somatic mutation or the ability to select mutants? *EMBO J*. 1992;11(12): 4337-4347.

328. Du Pasquier L, Schwager J, Flajnik MF. The immune system of Xenopus. *Annu Rev Immunol*. 1989;7:251-275.

329. Marr S, Morales H, Bottaro A, Cooper M, Flajnik M, Robert J. Localization and differential expression of activation-induced cytidine deaminase in the amphibian Xenopus upon antigen stimulation and during early development. *J Immunol*. 2007;179(10):6783-6789.

330. Robert J, Goyos A, Nedelkovska H. Xenopus, a unique comparative model to explore the role of certain heat shock proteins and non-classical MHC class Ib gene products in immune surveillance. *Immunol Res*. 2009. Epub ahead of print.

331. Horton JD, Horton TL, Dzialo R, et al. T-cell and natural killer cell development in thymectomized Xenopus. *Immunol Rev*. 1998;166: 245-258.

332. Rau L, Gantress J, Bell A, et al. Identification and characterization of Xenopus CD8+ T cells expressing an NK cell-associated molecule. *Eur J Immunol* . 2002;32(6):1574-1583.

333. Chen G, Robert J. Antiviral immunity in amphibians. *Viruses*. 2011; 3(11):2065-2086.

334. Morales HD, Robert J. Characterization of primary and memory CD8 T-cell responses against ranavirus (FV3) in Xenopus laevis. *J Virol*. 2007; 81(5):2240-2248.

335. Morales H, Robert J. In vivo and in vitro techniques for comparative study of antiviral T-cell responses in the amphibian Xenopus. *Biol Proced Online*. 2008;10:1-8.

336. Turchin A, Hsu E. The generation of antibody diversity in the turtle. *J Immunol*. 1996;156(10):3797-3805.

337. Munoz FJ, De la Fuente M. The immune response of thymic cells from the turtle Mauremys caspica. *J Comp Physiol B*. 2001;171(3): 195-200.

338. Hoffmeister MJ, Voss EW Jr. Hapten-binding capacity and population heterogeneity of chicken anti-Dnp IgG antibody. *Immunochemistry*. 1975;12(9):745-749.

339. Arakawa H, Furusawa S, Ekino S, Yamagishi H. Immunoglobulin gene hyperconversion ongoing in chicken splenic germinal centers. *EMBO J*. 1996;15(10):2540-2546.

340. Schiaffella E, Sehgal D, Anderson AO, Mage RG. Gene conversion and hypermutation during diversification of VH sequences in developing splenic germinal centers of immunized rabbits. *J Immunol*. 1999;162(7):3984-3995.

341. Boehm T, Bleul CC. The evolutionary history of lymphoid organs. *Nat Immunol*. 2007;8(2):131-135.

342. Ge Q, Zhao Y. Evolution of thymus organogenesis. *Dev Comp Immunol*. 2012. Epub ahead of print.

343. Bajoghli B, Aghaallaei N, Hess I, et al. Evolution of genetic networks underlying the emergence of thymopoiesis in vertebrates. *Cell*. 2009;138(1):186-197.

344. Hart S, Wrathmell AB, Harris JE. Ontogeny of gut-associated lymphoid tissue (GALT) in the dogfish Scyliorhinus canicula L. *Vet Immunol Immunopathol*. 1986;12(1-4):107-116.

345. Rumfelt LL, McKinney EC, Taylor E, Flajnik MF. The development of primary and secondary lymphoid tissues in the nurse shark Ginglymostoma cirratum: B-cell zones precede dendritic cell immigration and T-cell zone formation during ontogeny of the spleen. *Scand J Immunol*. 2002;56(2):130-148.

346. Miracle AL, Anderson MK, Litman RT, et al. Complex expression patterns of lymphocyte-specific genes during the development of cartilaginous fish implicate unique lymphoid tissues in generating an immune repertoire. *Int Immunol*. 2001;13(4):567-580.

347. Malecek K, Lee V, Feng W, et al. Immunoglobulin heavy chain exclusion in the shark. *PLoS Biol*. 2008;6(6):e157.

348. Zapata A, Amemiya CT. Phylogeny of lower vertebrates and their immunological structures. *Curr Top Microbiol Immunol* . 2000;248:67-107.

349. Koppang EO, Fischer U, Moore L, et al. Salmonid T cells assemble in the thymus, spleen and in novel interbranchial lymphoid tissue. *J Anat*. 2010;217(6):728-739.

350. dos Santos NM, Romano N, de SM, Ellis AE, Rombout JH. Ontogeny of B and T cells in sea bass (Dicentrarchus labrax, L.). *Fish Shellfish Immunol*. 2000;10(7):583-596.

351. Zapata A, Diez B, Cejalvo T, Gutierrez-de FC, Cortes A. Ontogeny of the immune system of fish. *Fish Shellfish Immunol*. 2006;20(2):126-136.

352. Fugmann SD, Messier C, Novack LA, Cameron RA, Rast JP. An ancient evolutionary origin of the Rag1/2 gene locus. *Proc Natl Acad Sci U S A* . 2006;103(10):3728-3733.

353. Hansen JD. Characterization of rainbow trout terminal deoxynucleotidyl transferase structure and expression. TdT and RAG1 co-expression define the trout primary lymphoid tissues. *Immunogenetics*. 1997;46(5): 367-375.

354. Barr M, Mott K, Zwollo P. Defining terminally differentiating B cell populations in rainbow trout immune tissues using the transcription factor Xbp1. *Fish Shellfish Immunol*. 2011;31(6):727-735.

355. Li J, Barreda DR, Zhang YA, et al. B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. *Nat Immunol*. 2006;7(10):1116-1124.

356. Parra D, Rieger AM, Li J, et al. Pivotal advance: peritoneal cavity B-1 B cells have phagocytic and microbicidal capacities and present phagocytosed antigen to CD4 + T cells. *J Leukoc Biol*. 2012;91(4):525-536.

357. Montecino-Rodriguez E, Dorshkind K. B-1 B cell development in the fetus and adult. *Immunity*. 2012;36(1):13-21.

358. Garcia Barrutia MS, Villena A, Gomariz RP, Razquin B, Zapata A. Ultrastructural changes in the spleen of the natterjack, Bufo calamita, after antigenic stimulation. *Cell Tissue Res*. 1985;239(2):435-441.

359. Greenhalgh P, Olesen CE, Steiner LA. Characterization and expression of recombination activating genes (RAG-1 and RAG-2) in Xenopus laevis. *J Immunol*. 1993;151(6):3100-3110.

360. Horton JD, Manning MJ. Response to skin allografts in Xenopus laevis following thymectomy at early stages of lymphoid organ maturation. *Transplantation*. 1972;14(2):141-154.

361. Schwager J, Burckert N, Courtet M, Du Pasquier L. The ontogeny of diversification at the immunoglobulin heavy chain locus in Xenopus. *EMBO J*. 1991;10(9):2461-2470.

362. Lee A, Hsu E. Isolation and characterization of the Xenopus terminal deoxynucleotidyl transferase. *J Immunol*. 1994;152(9):4500-4507.

363. Cooper MD, Raymond DA, Peterson RD, South MA, Good RA. The functions of the thymus system and the bursa system in the chicken. *J Exp Med*. 1966;123(1):75-102.

364. Ratcliffe MJ. Antibodies, immunoglobulin genes and the bursa of Fabricius in chicken B cell development. *Dev Comp Immunol*. 2006; 30(1-2):101-118.

365. Ratcliffe MJ, Ivanyi J. Allotype suppression in the chicken. IV. Deletion of B cells and lack of suppressor cells during chronic suppression. *Eur J Immunol* . 1981;11(4):306-310.

366. Reynaud CA, Anquez V, Grimal H, Weill JC. A hyperconversion mechanism generates the chicken light chain preimmune repertoire. *Cell*. 1987;48(3):379-388.

367. Reynaud CA, Bertocci B, Dahan A, Weill JC. Formation of the chicken B-cell repertoire: ontogenesis, regulation of Ig gene rearrangement, and diversification by gene conversion. *Adv Immunol*. 1994;57:353-378.

368. Arakawa H, Hauschild J, Buerstedde JM. Requirement of the activation-induced deaminase (AID) gene for immunoglobulin gene conversion. *Science*. 2002;295(5558):1301-1306.

369. Sayegh CE, Drury G, Ratcliffe MJ. Efficient antibody diversification by gene conversion in vivo in the absence of selection for V(D)J-encoded determinants. *EMBO J*. 1999;18(22):6319-6328.

370. Knight KL, Becker RS. Molecular basis of the allelic inheritance of rabbit immunoglobulin VH allotypes: implications for the generation of antibody diversity. *Cell*. 1990;60(6):963-970.

371. Knight KL, Crane MA. Generating the antibody repertoire in rabbit. *Adv Immunol*. 1994;56:179-218.

372. Rhee KJ, Jasper PJ, Sethupathi P, Shanmugam M, Lanning D, Knight KL. Positive selection of the peripheral B cell repertoire in gut-associated lymphoid tissues. *J Exp Med*. 2005;201(1):55-62.

373. Reynaud CA, Mackay CR, Muller RG, Weill JC. Somatic generation of diversity in a mammalian primary lymphoid organ: the sheep ileal Peyer's patches. *Cell*. 1991;64(5):995-1005.

374. Terszowski G, Muller SM, Bleul CC, et al. Evidence for a functional second thymus in mice. *Science*. 2006;312(5771):284-287.

375. Bajoghli B, Guo P, Aghaallaei N, et al. A thymus candidate in lampreys. *Nature*. 2011;470(7332):90-94.

376. Saltis M, Criscitiello MF, Ohta Y, et al. Evolutionarily conserved and divergent regions of the autoimmune regulator (Aire) gene: a comparative analysis. *Immunogenetics*. 2008;60(2):105-114.

377. Murata S, Sasaki K, Kishimoto T, et al. Regulation of CD8 + T cell development by thymus-specific proteasomes. *Science*. 2007;316(5829): 1349-1353.

378. Sutoh Y, Kondo M, Ohta Y, et al. Comparative genomic analysis of the proteasome beta5t subunit gene: implications for the origin and evolution of thymoproteasomes. *Immunogenetics*. 2012;64(1):49-58.

379. Kaufman J, Salomonsen J, Flajnik M. Evolutionary conservation of MHC class I and class II molecules—different yet the same. *Semin Immunol*. 1994;6(6):411-424.

380. Ohta Y, Shiina T, Lohr RL, et al. Primordial linkage of beta2-microglobulin to the MHC. *J Immunol*. 2011;186(6):3563-3571.

381. Hansen TH, Huang S, Arnold PL, Fremont DH. Patterns of nonclassical MHC antigen presentation. *Nat Immunol*. 2007;8(6):563-568.

382. Goyos A, Guselnikov S, Chida AS, et al. Involvement of nonclassical MHC class Ib molecules in heat shock protein-mediated anti-tumor responses. *Eur J Immunol* . 2007;37(6):1494-1501.

383. Goyos A, Sowa J, Ohta Y, Robert J. Remarkable conservation of distinct nonclassical MHC class I lineages in divergent amphibian species. *J Immunol*. 2011;186(1):372-381.

384. Hee CS, Gao S, Loll B, et al. Structure of a classical MHC class I molecule that binds “non-classical” ligands. *PLoS Biol*. 2010;8(12):e1000557.

385. Dijkstra JM, Katagiri T, Hosomichi K, et al. A third broad lineage of major histocompatibility complex (MHC) class I in teleost fish; MHC class II linkage and processed genes. *Immunogenetics*. 2007;59(4):305-321.

386. Phillips RB, Zimmerman A, Noakes MA, et al. Physical and genetic mapping of the rainbow trout major histocompatibility regions: evidence for duplication of the class I region. *Immunogenetics*. 2003;55(8):561-569.

387. Liljedahl M, Kuwana T, Fung-Leung WP, Jackson MR, Peterson PA, Karlsson L. HLA-DO is a lysosomal resident which requires association with HLA-DM for efficient intracellular transport. *EMBO J*. 1996;15(18):4817-4824.

388. Criscitiello MF, Ohta Y, Graham MD, Eubanks JO, Chen PL, Flajnik MF. Shark class II invariant chain reveals ancient conserved relationships with cathepsins and MHC class II. *Dev Comp Immunol*. 2012;36(3): 521-533.

389. Basha G, Omilusik K, Chavez-Steenbock A, et al. A CD74-dependent MHC class I endolysosomal cross-presentation pathway. *Nat Immunol*. 2012;13(3):237-245.

390. Salter-Cid L, Nonaka M, Flajnik MF. Expression of MHC class Ia and class Ib during ontogeny: high expression in epithelia and coregulation of class Ia and lmp7 genes. *J Immunol*. 1998;160(6):2853-2861.

391. Du Pasquier L, Flajnik MF. Expression of MHC class II antigens during Xenopus development. *Dev Immunol*. 1990;1(2):85-95.

392. Volk H, Charlemagne J, Tournefier A, et al. Wide tissue distribution of axolotl class II molecules occurs independently of thyroxin. *Immunogenetics*. 1998;47(5):339-349.

393. Rodrigues PN, Hermsen TT, van MA, et al. Expression of MhcCyca class I and class II molecules in the early life history of the common carp (Cyprinus carpio L.). *Dev Comp Immunol*. 1998;22(5-6): 493-506.

394. Fischer U, Dijkstra JM, Kollner B, et al. The ontogeny of MHC class I expression in rainbow trout (Oncorhynchus mykiss). *Fish Shellfish Immunol*. 2005;18(1):49-60.

395. Star B, Nederbragt AJ, Jentoft S, et al. The genome sequence of Atlantic cod reveals a unique immune system. *Nature*. 2011;477(7363):207-210.

396. Magnadottir B, Jonsdottir H, Helgason S, Bjornsson B, Solem ST, Pilstrom L. Immune parameters of immunised cod (Gadus morhua L.). *Fish Shellfish Immunol*. 2001;11(1):75-89.

397. Kaufman J, Volk H, Wallny HJ. A "minimal essential Mhc" and an "unrecognized Mhc": two extremes in selection for polymorphism. *Immunol Rev*. 1995;143:63-88.

398. Sammut B, Du Pasquier L, Ducoroy P, Laurens V, Marcuz A, Tournefier A. Axolotl MHC architecture and polymorphism. *Eur J Immunol* . 1999; 29(9):2897-2907.

399. Kasahara M. The 2R hypothesis: an update. *Curr Opin Immunol* . 2007;19(5):547-552.

400. Hughes AL. Evolution of the ATP-binding-cassette transmembrane transporters of vertebrates. *Mol Biol Evol* . 1994;11(6):899-910.

401. Uinuk-ool TS, Mayer WE, Sato A, et al. Identification and characterization of a TAP-family gene in the lamprey. *Immunogenetics*. 2003; 55(1):38-48.

402. Zhao C, Tampe R, Abele R. TAP and TAP-like—brothers in arms? *Naunyn Schmiedebergs Arch Pharmacol* . 2006;372(6):444-450.

403. Kelley J, Walter L, Trowsdale J. Comparative genomics of major histocompatibility complexes. *Immunogenetics*. 2005;56(10):683-695.

404. Sambrook JG, Figueroa F, Beck S. A genome-wide survey of major histocompatibility complex (MHC) genes and their paralogues in zebrafish. *BMC Genomics*. 2005;6:152.

405. Vienne A, Shiina T, Abi-Rached L, et al. Evolution of the proto-MHC ancestral region: more evidence for the plesiomorphic organisation of human chromosome 9q34 region. *Immunogenetics*. 2003;55(7):429-436.

406. Shum BP, Guethlein L, Flodin LR, et al. Modes of salmonid MHC class I and II evolution differ from the primate paradigm. *J Immunol* . 2001;166(5):3297-3308.

407. Consuegra S, Megens HJ, Leon K, Stet RJ, Jordan WC. Patterns of variability at the major histocompatibility class II alpha locus in Atlantic salmon contrast with those at the class I locus. *Immunogenetics*. 2005; 57(1-2):16-24.

408. Kaufman J, Milne S, Gobel TW, et al. The chicken B locus is a minimal essential major histocompatibility complex. *Nature*. 1999;401(6756): 923-925.

409. Shiina T, Shimizu S, Hosomichi K, et al. Comparative genomic analysis of two avian (quail and chicken) MHC regions. *J Immunol* . 2004; 172(11):6751-6763.

410. Koch M, Camp S, Collen T, et al. Structures of an MHC class I molecule from B21 chickens illustrate promiscuous peptide binding. *Immunity*. 2007;27(6):885-899.

411. Wallny HJ, Avila D, Hunt LG, et al. Peptide motifs of the single dominantly expressed class I molecule explain the striking MHC-determined response to Rous sarcoma virus in chickens. *Proc Natl Acad Sci U S A* . 2006;103(5):1434-1439.

412. Suzuki K, Matsumoto T, Kobayashi E, et al. Genotypes of chicken major histocompatibility complex B locus associated with regression of Rous sarcoma virus J-

strain tumors. *Poult Sci* . 2010;89(4):651-657.

413. Walker BA, Hunt LG, Sowa AK, et al. The dominantly expressed class I molecule of the chicken MHC is explained by coevolution with the polymorphic peptide transporter (TAP) genes. *Proc Natl Acad Sci U S A* . 2011; 108(20):8396-8401.

414. Salomonsen J, Sorensen MR, Marston DA, et al. Two CD1 genes map to the chicken MHC, indicating that CD1 genes are ancient and likely to have been present in the primordial MHC. *Proc Natl Acad Sci U S A* . 2005;102(24):8668-8673.

415. Miller MM, Wang C, Parisini E, et al. Characterization of two avian MHC-like genes reveals an ancient origin of the CD1 family. *Proc Natl Acad Sci U S A* . 2005;102(24):8674-8679.

416. Nonaka M, Namikawa C, Kato Y, Sasaki M, Salter-Cid L, Flajnik MF. Major histocompatibility complex gene mapping in the amphibian Xenopus implies a primordial organization. *Proc Natl Acad Sci U S A* . 1997;94(11):5789-5791.

417. Ohta Y, Powis SJ, Lohr RL, Nonaka M, Du Pasquier L, Flajnik MF. Two highly divergent ancient allelic lineages of the transporter associated with antigen processing (TAP) gene in Xenopus: further evidence for co-evolution among MHC class I region genes. *Eur J Immunol* . 2003;33(11):3017-3027.

418. Tsukamoto K, Hayashi S, Matsuo MY, et al. Unprecedented intraspecific diversity of the MHC class I region of a teleost medaka, Oryzias latipes. *Immunogenetics*. 2005;57(6):420-431.

419. Tsukamoto K, Miura F, Fujito NT, Yoshizaki G, Nonaka M. Long-lived dichotomous lineages of the proteasome subunit beta type 8 (PSMB8) gene surviving more than 500 million years as alleles or paralogs. *Mol Biol Evol* . 2012. Epub ahead of print.

420. Flajnik MF, Kasahara M, Shum BP, Salter-Cid L, Taylor E, Du Pasquier L. A novel type of class I gene organization in vertebrates: a large family of non-MHC-linked class I genes is expressed at the RNA level in the amphibian Xenopus. *EMBO J*. 1993;12(11):4385-4396.

421. Danchin E, Vitiello V, Vienne A, et al. The major histocompatibility complex origin. *Immunol Rev*. 2004;198:216-232.

422. Fernandez-Busquets X, Burger MM. Cell adhesion and histocompatibility in sponges. *Microsc Res Tech*. 1999;44(4):204-218.

423. Wiens M, Perovic-Ottstadt S, Muller IM, Muller WE. Allograft rejection in the mixed cell reaction system of the demosponge Suberites domuncula is controlled by differential expression of apoptotic genes. *Immunogenetics*. 2004;56(8):597-610.

424. Muller WE, Krasko A, Skorokhod A, et al. Histocompatibility reaction in tissue and cells of the marine sponge Suberites domuncula in vitro and in vivo: central role of the allograft inflammatory factor 1. *Immunogenetics*. 2002;54(1):48-58.

425. Tepsuporn S, Kaltenbach JC, Kuhns WJ, Burger MM, Fernandez-Busquets X. Apoptosis in Microciona prolifera allografts. *Biol Bull*. 2003;205(2):199-201.

426. Sabella C, Faszewski E, Himic L, et al. Cyclosporin A suspends transplantation reactions in the marine sponge Microciona prolifera. *J Immunol*. 2007;179(9):5927-5935.

427. Lubbock R. Clone-specific cellular recognition in a sea anemone. *Proc Natl Acad Sci U S A*. 1980;77(11):6667-6669.

428. Hauenschild C. Wilhelm Roux's Archives. 1954;147:1-114.

429. Rosa SF, Powell AE, Rosengarten RD, et al. Hydractinia allodeterminant alr1 resides in an immunoglobulin superfamily-like gene complex. *Curr Biol*. 2010;20(12):1122-1127.

430. Theodor JL. Distinction between “self” and “not-self” in lower invertebrates. *Nature*. 1970;227(5259):690-692.

431. Rosengarten RD, Moreno MA, Lakkis FG, Buss LW, Dellaporta SL. Genetic diversity of the allodeterminant alr2 in Hydractinia symbiolongicarpus. *Mol Biol Evol*. 2011;28(2):933-947.

432. Oka H, Watanabe H. Colony specificity in compound ascidians as tested by fusion experiments (a preliminary report). *Proc Japan Acad Sci*. 1957;33:657-658.

433. Cima F, Sabbadin A, Ballarin L. Cellular aspects of allorecognition in the compound ascidian Botryllus schlosseri. *Dev Comp Immunol*. 2004;28(9):881-889.

434. De Tomaso AW, Nyholm SV, Palmeri KJ, et al. Isolation and characterization of a protochordate histocompatibility locus. *Nature*. 2005;438(7067):454-459.

435. Nyholm SV, Passegue E, Ludington WB, et al. fester, A candidate allorecognition receptor from a primitive chordate. *Immunity*. 2006;25(1):163-173.

436. McKitrick TR, Muscat CC, Pierce JD, Bhattacharya D, De Tomaso AW. Allorecognition in a basal chordate consists of independent activating and inhibitory pathways. *Immunity*. 2011;34(4):616-626.

437. McKitrick TR, De Tomaso AW. Molecular mechanisms of allorecognition in a basal chordate. *Semin Immunol*. 2010;22(1):34-38.

438. Rinkevich B, Douek J, Rabinowitz C, Paz G. The candidate Fu/HC gene in Botryllusschlosseri (Urochordata) and ascidians' historecognition— an oxymoron? *Dev Comp Immunol*. 2012;36(4):718-727.

439. Ishii T, Sawada T, Sasaki K, Ohtake S. Study of color variation in the solitary ascidian Halocynthia roretzi, collected in the Inland Sea of Japan. *Zoolog Sci*. 2004;21(8):891-898.

440. Raftos DA, Briscoe DA, Tait NN. The mode of recognition of allogeneic tissue in the solitary urochordate Styela plicata. *Transplantation*. 1988;45(6):1123-1126.

441. Kurn U, Sommer F, Hemmrich G, Bosch TC, Khalturin K. Allorecognition in urochordates: identification of a highly variable complement receptor-like protein expressed in follicle cells of Ciona. *Dev Comp Immunol*. 2007;31(4):360-371.

442. Zucchetti I, De SR, Grusea S, Pontarotti P, Du Pasquier L. Origin and evolution of the vertebrate leukocyte receptors: the lesson from tunicates. *Immunogenetics*. 2009;61(6):463-481.

443. Burnet FM. “Self-recognition” in colonial marine forms and flowering plants in relation to the evolution of immunity. *Nature*. 1971; 232(5308):230-235.

443a. Flajnik MF, Du Pasquier L. Evolution of the immune system. In: Paul W, ed. *Fundamental Immunology*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2008:56-124.

444. Buss LW. Somatic cell parasitism and the evolution of somatic tissue compatibility. *Proc Natl Acad Sci U S A*. 1982;79(17):5337-5341.

445. Laird DJ, De Tomaso AW, Weissman IL. Stem cells are units of natural selection in a colonial ascidian. *Cell*. 2005;123(7):1351-1360.

446. Hughes RN, Manriquez PH, Morley S, Craig SF, Bishop JD. Kin or self-recognition? Colonial fusibility of the bryozoan Celleporella hyalina. *Evol Dev*. 2004;6(6):431-437.

447. Gould SJ, Hildreth JE, Booth AM. The evolution of alloimmunity and the genesis of adaptive immunity. *Q Rev Biol*. 2004;79(4):359-382.

448. Azumi K, De SR, De TA, et al. Genomic analysis of immunity in a Urochordate and the emergence of the vertebrate immune system: “waiting for Godot.” *Immunogenetics*. 2003;55(8):570-5781.

449. Roberts S, Gueguen Y, de LJ, Goetz F. Rapid accumulation of an interleukin 17 homolog transcript in Crassostrea gigas hemocytes following bacterial exposure. *Dev Comp Immunol*. 2008;32(9):1099-1104.

450. Bilej M, Joskova R, Van den BR, et al. An invertebrate TNF functional analogue activates macrophages via lectin-saccharide interaction with ion channels. *Int Immunol*. 2006;18(12):1663-1670.

451. Hong S, Peddie S, Campos-Perez JJ, Zou J, Secombes CJ. The effect of intraperitoneally administered recombinant IL-1beta on immune parameters and resistance to Aeromonas salmonicida in the rainbow trout (Oncorhynchus mykiss). *Dev Comp Immunol*. 2003;27(9):801-812.

452. Hong S, Zou J, Collet B, Bols NC, Secombes CJ. Analysis and characterisation of IL-1beta processing in rainbow trout, Oncorhynchus mykiss. *Fish Shellfish Immunol*. 2004;16(3):453-459.

453. Paul WE, Zhu J. How are T(H)2-type immune responses initiated and amplified? *Nat Rev Immunol*. 2010;10(4):225-235.

454. Kaiser P, Poh TY, Rothwell L, et al. A genomic analysis of chicken cytokines and chemokines. *J Interferon Cytokine Res*. 2005;25(8):467-484.

455. Kaiser P, Poh TY, Rothwell L, et al. A genomic analysis of chicken cytokines and chemokines. *J Interferon Cytokine Res*. 2005;25(8):467-484.

456. Jin P, Hu J, Qian J, Chen L, Xu X, Ma F. Identification and characterization of a putative lipopolysaccharide-induced TNF-alpha factor (LITAF) gene from Amphioxus (Branchiostoma belcheri): an insight into the innate immunity of Amphioxus and the evolution of LITAF. *Fish Shellfish Immunol*. 2012;32(6):1223-1228.

457. Wiens GD, Glenney GW. Origin and evolution of TNF and TNF receptor superfamilies. *Dev Comp Immunol*. 2011;35(12):1324-1335.

458. Awasthi G, Singh S, Dash AP, Das A. Genetic characterization and evolutionary inference of TNF-alpha through computational analysis. *Braz J Infect Dis*. 2008;12(5):374-379.

459. Collette Y, Gilles A, Pontarotti P, Olive D. A co-evolution perspective of the TNFSF and TNFRSF families in the immune system. *Trends Immunol*. 2003;24(7):387-394.

460. Wang T, Huang W, Costa MM, Secombes CJ. The gamma-chain cytokine/receptor system in fish: more ligands and receptors. *Fish Shellfish Immunol*. 2011;31(5):673-687.

461. Bird S, Zou J, Kono T, Sakai M, Dijkstra JM, Secombes C. Characterisation and expression analysis of interleukin 2 (IL-2) and IL-21 homologues in the Japanese pufferfish, Fugu rubripes, following their discovery by synteny. *Immunogenetics*. 2005;56(12):909-923.

462. Wang T, Secombes CJ. Cloning and expression of a putative common cytokine receptor gamma chain (gammaC) gene in rainbow trout (Oncorhynchus mykiss). *Fish Shellfish Immunol*. 2001;11(3):233-244.

463. Iwanami N, Mateos F, Hess I, et al. Genetic evidence for an evolutionarily conserved role of IL-7 signaling in T cell development of zebrafish. *J Immunol*. 2011;186(12):7060-7066.

464. Li JH, Shao JZ, Xiang LX, Wen Y. Cloning, characterization and expression analysis of pufferfish interleukin-4 cDNA: the first evidence of Th2-type cytokine in fish. *Mol Immunol*. 2007;44(8):2078-2086.

465. Wang T, Huang W, Costa MM, Martin SA, Secombes CJ. Two copies of the genes encoding the subunits of putative interleukin (IL)-4/IL-13 receptors, IL-4Ralpha, IL-13Ralpha1 and IL-13Ralpha2, have been identified in rainbow trout (Oncorhynchus mykiss) and have complex patterns of expression and modulation. *Immunogenetics*. 2011;63(4):235-253.

466. Zou J, Secombes CJ. Teleost fish interferons and their role in immunity. *Dev Comp Immunol*. 2011;35(12):1376-1387.

467. Papenfuss AT, Baker ML, Feng ZP, et al. The immune gene repertoire of an important viral reservoir, the Australian black flying fox. *BMC Genomics*. 2012;13(1):261.

468. Thomas C, Moraga I, Levin D, et al. Structural linkage between ligand discrimination and receptor activation by type I interferons. *Cell*. 2011;146(4):621-632.

469. Kaiser P, Sonnemans D, Smith LM. Avian IFN-gamma genes: sequence analysis suggests probable cross-species reactivity among galliforms. *J Interferon Cytokine Res*. 1998;18(9):711-719.

470. Secombes CJ, Wang T, Bird S. The interleukins of fish. *Dev Comp Immunol*. 2011;35(12):1336-1345.

471. Haynes L, Cohen N. Transforming growth factor beta (TGF beta) is produced by and influences the proliferative response of Xenopus laevis lymphocytes. *Dev Immunol*. 1993;3(3):223-230.

472. Corripio-Miyar Y, Zou J, Richmond H, Secombes CJ. Identification of interleukin-22 in gadoids and examination of its expression level in vaccinated fish. *Mol Immunol*. 2009;46(10):2098-2106.

473. Du Pasquier L, Bernard CC. Active suppression of the allogeneic histocompatibility reactions during the metamorphosis of the clawed toad Xenopus. *Differentiation*.

1980;16(1):1-7.

474. Quintana FJ, Iglesias AH, Farez MF, et al. Adaptive autoimmunity and Foxp3-based immunoregulation in zebrafish. *PLoS One*. 2010;5(3): e9478.

475. Hibino T, Loza-Coll M, Messier C, et al. The immune gene repertoire encoded in the purple sea urchin genome. *Dev Biol*. 2006;300(1):349-365.

476. Nomiyama H, Osada N, Yoshie O. A family tree of vertebrate chemokine receptors for a unified nomenclature. *Dev Comp Immunol*. 2011;35(7): 705-715.

477. DeVries ME, Kelvin AA, Xu L, Ran L, Robinson J, Kelvin DJ. Defining the origins and evolution of the chemokine/chemokine receptor system. *J Immunol*. 2006;176(1):401-415.

478. Klein J, O'Huigin C. Composite origin of major histocompatibility complex genes. *Curr Opin Genet Dev*. 1993;3(6):923-930.

479. Lynch M, O'Hely M, Walsh B, Force A. The probability of preservation of a newly arisen gene duplicate. *Genetics*. 2001;159(4):1789-1804.

480. Sakano H, Huppi K, Heinrich G, Tonegawa S. Sequences at the somatic recombination sites of immunoglobulin light-chain genes. *Nature*. 1979;280(5720):288-294.

481. Kapitonov VV, Jurka J. RAG1 core and V(D)J recombination signal sequences were derived from Transib transposons. *PLoS Biol*. 2005; 3(6):e181.

482. Ramon-Maiques S, Kuo AJ, Carney D, et al. The plant homeodomain finger of RAG2 recognizes histone H3 methylated at both lysine-4 and arginine-2. *Proc Natl Acad Sci U S A*. 2007;104(48):18993-18998.

483. Liu Y, Subrahmanyam R, Chakraborty T, Sen R, Desiderio S. A plant homeodomain in RAG-2 that binds Hypermethylated lysine 4 of histone H3 is necessary for efficient antigen-receptor-gene rearrangement. *Immunity*. 2007;27(4):561-571.

484. Bartl S, Miracle AL, Rumfelt LL, et al. Terminal deoxynucleotidyl transferases from elasmobranchs reveal structural conservation within vertebrates. *Immunogenetics*. 2003;55(9):594-604.

485. Richards MH, Nelson JL. The evolution of vertebrate antigen receptors: a phylogenetic approach. *Mol Biol Evol*. 2000;17(1):146-155.

486. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. *Nature*. 1988;334(6181):395-402.

487. Chrétien I, Robert J, Marcuz A, Garcia-Sanz JA, Courtet M, Du Pasquier L. CTX, a novel molecule specifically expressed on the surface of cortical thymocytes in Xenopus. *Eur J Immunol* . 1996;26(4):780-791.

488. Compte E, Pontarotti P, Collette Y, Lopez M, Olive D. Frontline: characterization of BT3 molecules belonging to the B7 family expressed on immune cells. *Eur J Immunol* . 2004;34(8):2089-2099.

489. Barton ES, Forrest JC, Connolly JL, et al. Junction adhesion molecule is a receptor for reovirus. *Cell*. 2001;104(3):441-451.

490. Bikard D, Marraffini LA. Innate and adaptive immunity in bacteria: mechanisms of programmed genetic variation to fight bacteriophages. *Curr Opin Immunol* . 2012;24(1):15-20.

491. Spits H, Cupedo T. Innate lymphoid cells: emerging insights in development, lineage relationships, and function. *Annu Rev Immunol*. 2012; 30:647-675.

492. Weaver CT, Hatton RD. Interplay between the TH17 and TReg cell lineages: a (co-)evolutionary perspective. *Nat Rev Immunol* . 2009;9(12): 883-889.

493. Flajnik MF. All GOD's creatures got dedicated mucosal immunity. *Nat Immunol*. 2010;11(9):777-779.

494. McFall-Ngai M. Adaptive immunity: care for the community. *Nature*. 2007;445(7124):153.

495. Calderon L, Boehm T. Synergistic, context-dependent, and hierarchical functions of epithelial components in thymic microenvironments. *Cell*. 2012;149(1):159-172.

496. Lakkis FG, Dellaporta SL, Buss LW. Allorecognition and chimerism in an invertebrate model organism. *Organogenesis*. 2008;4(4): 236-240.

497. Barber MR, Aldridge JR Jr, Webster RG, Magor KE. Association of RIG-I with innate immunity of ducks to influenza. *Proc Natl Acad Sci U S A* . 2010;107(13):5913-5918.

498. Flajnik MF, Kasahara M. Comparative genomics of the MHC: glimpses into the evolution of the adaptive immune system. *Immunity*. 2001; 15(3):351-362.

499. Kim HM, Oh SC, Lim KJ, et al. Structural diversity of the hagfish variable lymphocyte receptors. *J Biol Chem*. 2007;282(9):6726-6732.

500. Hallbook F, Wilson K, Thorndyke M, Olinski RP. Formation and evolution of the chordate neurotrophin and Trk receptor genes. *Brain Behav Evol*. 2006;68(3):133-144.

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Chapter 5

Immunoglobulins: Structure and Function

Harry W. Schroeder Jr
David Wald
Neil S. Greenspan

INTRODUCTION

Immunoglobulins (Igs) are marked by a duality of structure and function.[1] In common with other members of the Ig superfamily,[2] they provide the immune system with a conserved set of effector molecules. These effectors can activate and fix complement and they can bind to Fc receptors on the surfaces of granulocytes, monocytes, platelets, and other components of the immune response. Both activation of complement and binding to Fc receptors can contribute to the induction or maintenance of inflammation. They also provide the immune system with a polyclonal set of diverse ligand binding sites, which allow Igs, as a population, to recognize an almost unlimited array of self- and non-self-antigens, which may range from compounds as fundamental to life as deoxyribonucleic acid to manmade molecules that could not have played a role in the evolution of the immune system.

Differential splicing allows individual Ig molecules to serve as either membrane-bound receptors for the B cell that allow antigen-specific activation or as soluble effectors, which act at a distance. In vivo, proper effector function requires more than just antigen-specific binding; it requires successful neutralization of the offending antigen while avoiding potentially pathogenic self-reactivity.

The receptor and effector functions of each individual Ig can be localized to a separate region or domain of the molecule. Each variable (V) or constant (C) domain consists of approximately 110 to 130 amino acids, averaging 12,000 to 13,000 kD. A typical light (L) chain will thus mass approximately 25 kD, and a three C domain Cγ H chain with its hinge will mass approximately 55 kD.

Immunoglobulins are Heterodimers

Igs are heterodimeric proteins, consisting of two H and two L chains (Figs. 5.1 and 5.2). The eponymous Ig domain serves as the basic building block for both chains. Each of the chains contains a single amino-terminal V Ig domain and one, three, or four carboxy-terminal C Ig domains. H chains contain three or four C domains, whereas L chains contain only one. H chains with three C domains tend to include a spacer hinge region between the first (C_H1) and second (C_H2).[3]

At the primary sequence level, Igs are marked by the interspersion of regions of impressive sequence variability with regions of equally impressive sequence conservation. The V domains demonstrate the greatest molecular heterogeneity, with some regions including non-germline-encoded variability and others exhibiting extensive germline conservation across 500 million years of evolution.[4] The molecular heterogeneity of the V domains permits the creation of binding sites, or *paratopes*, which can discriminate between antigens that may differ by as little as one atom. Thus it is the V domains that encode the receptor function and define the monovalent specificity of the antibody. The H chain C_H1 domain, which is immediately adjacent to the V, associates with the single L chain C domain. Together, the C_H1 and C_L domains provide a stable platform for the paired set of V_H and V_L domains,

which create the antigen binding site. The distal C_H2 and C_H3 domains, for those antibodies with a hinge, or the C_H3 and C_H4 domains, for those with an extra (C_H2) domain, typically encode the effector functions of soluble antibody. Each of these Ig C_H domains is encoded by a separate exon. Although the sequences of the individual C_H domains are constant within the individual (and nearly constant within a species), they can vary greatly across species boundaries. The carboxy-terminal C_H domain encodes a secretory tail, which permits the antibody to exit the cell.

Also encoded within the germline sequence of each C_H gene are two membrane/cytoplasmic tail domain exons, termed M1 and M2. Alternative splicing removes the secretory sequence typically encoded by the terminal C_H3 or C_H4 domain and replaces it with the peptides encoded by the M1 and M2 exons, converting a secretory antibody to a

membrane-embedded receptor.[5] Between species, the membrane/cytoplasmic tail region is the most highly conserved portion of the C_H domains, which befits its role as a link to the intracellular signal transduction pathways that ultimately regulate B-cell function.

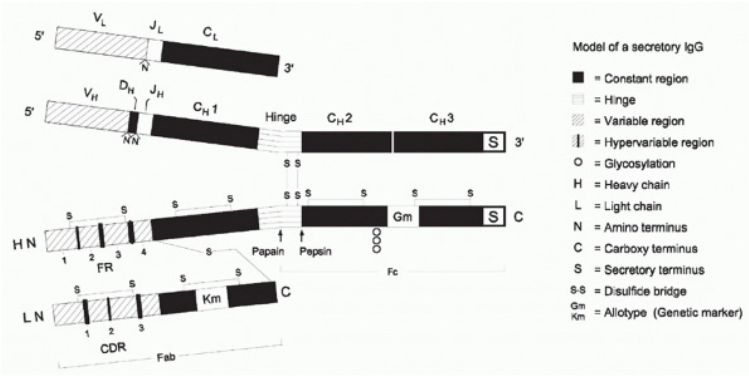

FIG. 5.1. Model of a Secreted Immunoglobulin (Ig)G. The germline exonic derivation of the sequence is shown at the *top*, and the protein structure is shown at the *bottom*. The location of the various cysteine residues that help hold both the individual domains and the various Ig subunits together are illustrated. Papain digests IgG molecules above the cysteine residues in the hinge that holds the two H chains together yielding two Fab molecules and an Fc, whereas pepsin digests below releasing an (Fab)'2 fragment and two individual Fcs (which are typically degraded to smaller peptide fragments). The location of some allotypic variants is illustrated. C_H2 domains can be variably glycosylated, which can also affect Ig structure and effector function.

Paratopes and Epitopes

The immunoglobulin-antigen interaction takes place between the paratope, the site on the Ig at which the antigen binds, and the *epitope*, which is the site on the antigen that is bound. It is important to appreciate that antibodies do not recognize antigens; they recognize epitopes borne on antigens.[6] This makes it possible for Igs to discriminate between two closely related antigens, each of which can be viewed as a collection of epitopes. It also is one scenario that permits the same antibody to bind divergent antigens that share equivalent epitopes, a phenomenon referred to as *cross-reactivity*.

It has been estimated that triggering of effector functions in solution typically requires aggregation of three or more effector domains, and thus tends to involve the binding of three or more epitopes.[6] For antigens encoding repeating epitope structures, such as polysaccharides or antigen aggregates, binding of a single polymeric Ig molecule carrying multiple effector domains, such as pentameric IgM, can be sufficient to induce effector function. For antigens encoding diverse epitopes, which is more typical of monodisperse

single-domain molecules in solution, triggering of inflammatory effector functions may require the binding of a diverse set of Ig molecules, all binding the same antigen, but at different epitopes.[7]

Membrane and Secretory Immunoglobulin

Alternative splicing allows Igs to serve either as soluble antibodies or as membrane-bound antigen receptors. In their role as antibodies, Igs are released into the circulation from where they may traffic into the tissues and across mucosal surfaces. In their role as the B-cell antigen receptor, they are anchored to the membrane by means of their M1:M2 transmembrane domain. Soluble antibodies can also be pressed into service as heterologous cell surface antigen receptors by means of their attachment to membrane-bound Fc receptors.[8] This permits the power of antibody recognition to be extended to nonlymphoid cells, such as Fc-expressing granulocytes, macrophages, and mast cells. The major difference between these two forms of cell surface receptors is that Igs as B-cell antigen receptors provide a monoclonal receptor for each B cell, whereas antibodies bound to Fc receptors endow the cell with a polyclonal set of antigen recognition molecules. This gives greater flexibility and increases the power of the effector cells to recognize antigens with multiple non-self-epitopes.

Isotypes and Idiotypes

Igs can also serve as antigens for other Igs. Immunization of heterologous species with monoclonal antibodies (or a restricted set of Igs) has shown that Igs contain both common

and individual antigenic determinants. Epitopes recognized within the V portion of the antibodies used for immunization that identify individual determinants are termed *idiotypes* (Fig. 5.3), whereas epitopes specific for the constant portion are termed *isotypes*. Recognition of these isotypes first allowed grouping of Igs into recognized classes. Each class of Ig defines an individual set of C domains that corresponds to a single H chain constant region gene. For example, IgM utilizes μ H chain C domains and IgE utilizes ε C domains.

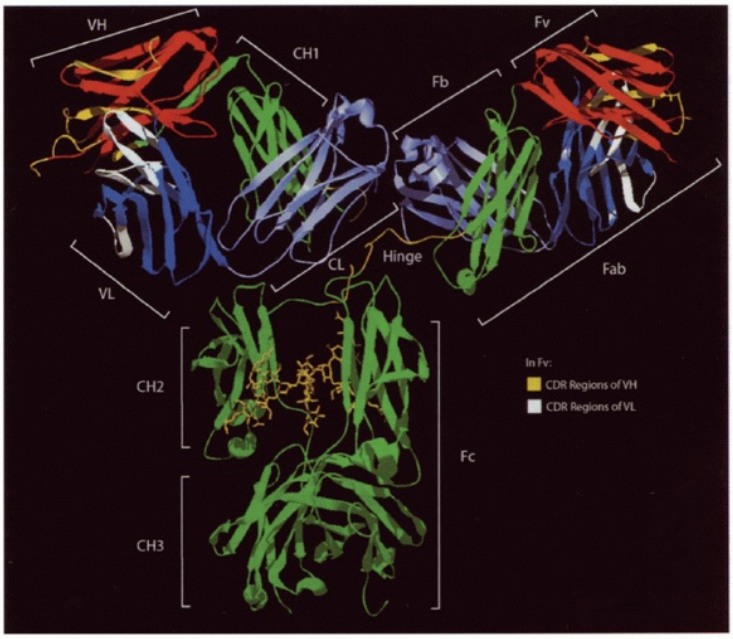

FIG. 5.2. Ribbon Diagram of a Complete Immunoglobulin (Ig)G1 Crystal (1 hzh in the protein data bank [PDB] from Data of Harris et al.[202]). The major regions of the Ig are illustrated. The heavy-chain constant regions (*green*) also include the hinge (*yellow*) between the first two domains. Cγ2 is glycosylated (also seen in *yellow*). The

heavy- and light-chain variable regions (*red* and *dark blue*, respectively) are N terminal to the heavy- (*green*) and light-chain (*light blue*) constant regions. Complementarity determining region loops in the heavy- and light-chain variable regions (*yellow* and *white*) are illustrated as well.

Some V domain epitopes derive from the germline sequence of V gene exons. These shared epitopes, commonly referred to as *public idiotopes* or *cross-reactive idiotypes* (see Fig. 5.3), are, from a genetic perspective, isotypic because they can be found on many Igs of different antigen-binding specificities that derive from the same germline V region. Examples include the cross-reactive idiotypes found on monoclonal IgM rheumatoid factors derived from individuals with mixed cryoglobulinemia, each of which can be linked to the use of individual V gene segments.[9]

Classes and Allotypes

Each of the various classes and subclasses of Igs has its own unique role to play in the immunologic defense of the individual. For example, IgA is the major class of Ig present in all external secretions. It is primarily responsible for protecting mucosal surfaces. IgG subclasses bind Fc receptors differently, and thus vary in effector function.[10] Determinants common to subsets of individuals within a species, yet differing between other members of that species, are termed *allotypes* and define inherited polymorphisms that result from allelic forms of immunoglobulin C (less commonly, V) genes.[11]

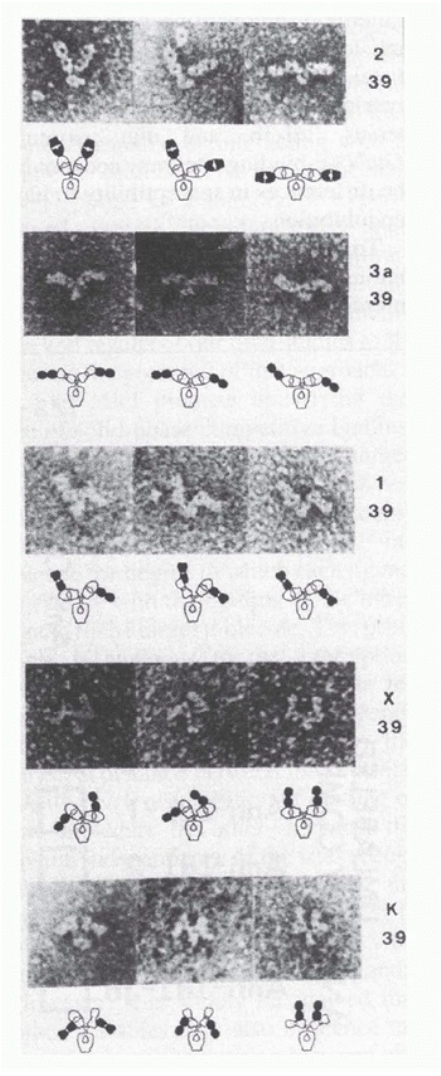

FIG. 5.3. Electron micrographs (*top*; ×350,000) and interpretive diagrams (*below*) of murine mAb hybridoma group A carbohydrate (HGAC) 39 (specific for the cell wall polysaccharide of *Streptococcus pyogenes*) in complex with anti-idiotypic mAb Fab fragments. HGAC 39 is represented in the diagrams as an open figure, and the Fab anti-Id probes are represented as solid figures. The Fab arms of the antibody targets and probes are drawn to indicate their rotational orientation as planar (oval with open center), intermediate (bone shape with or without central opening), or perpendicular ("dumbbell shaped"). Different complexes illustrate the range of Fab-Fab angles made possible by segmental flexibility. 1, anti-Id1-1 Fab; 2, anti-Id1-2 Fab; 39, HGAC 39; 3a, anti-Id1-3a Fab; K, anti-Cκ Fab; X, anti-IdX Fab. Id1 designates an individual idiotope, and IdX, a crossreactive idiotope. Antibody complexes were stained with 2% uranyl formate as described by Roux et al.[108] Reproduced from Proceedings of the National Academy of Sciences (from Roux et al.[108] with permission).

Glycosylation

N-linked carbohydrates can be found in all constant domains as well as in some variable domains.[12] The structure of the attached N-linked carbohydrate can vary greatly, depending on the degree of processing. These carbohydrates can play a major role in Ig function.[13] For example, human IgG molecules contain a conserved glycosylation site at Asn 297, which is buried between the C_H2 domains.[14] This oligosaccharide structure is almost as large as the C_H2 domain itself. O-linked sugars are also present in some Igs.[12] Human IgA1, but not IgA2, possesses a 13 amino acid hinge region that contains three to five O-linked carbohydrate moieties.[15] A deficiency in proper processing of these O-glycans can contribute to IgA nephropathy, which is a disease that is characterized by the presence of IgA1-containing immune complexes in the glomerular mesangium.[16]

A HISTORICAL PERSPECTIVE

The identification of Ig as a key component of the immune response began in the 19th century. This section describes the history of the identification of Ig and introduces fundamental terminology.

Antibodies and Antigens

Aristotle and his contemporaries attributed disease to an imbalance of the four vital humors: the blood, the phlegm, and the yellow and black biles.[17] In 1890, Behring (later, von Behring) and Kitasato reported the existence of an activity in the blood that could neutralize diphtheria toxin.[18] They showed that sera containing this humoral antitoxin activity would protect other animals exposed to the same toxin. Ehrlich, who was the first to describe how diphtheria toxin and antitoxin interact,[19] made glancing reference to "Antikörper" in a 1891 paper describing discrimination between two immune bodies, or substances.[20] The term antigen was first introduced by Deutsch in 1899. He later explained that antigen is a contraction of "Antisomatogen+- Immunkörperbildner," or that which induces the production of immune bodies (antibodies). Thus, the operational definition of antibody and antigen is a classic tautology.

Gamma Globulins

In 1939, Tiselius and Kabat immunized rabbits with ovalbumin and fractionated the immune serum by electrophoresis into albumin, alpha-goblulin, beta-globulin, and gamma-globulin fractions.[21] Absorption of the serum against ovalbumin depleted the gamma-globulin fraction, hence the terms immunoglobulin and IgG. "Sizing" columns were used to separate Igs into those that were "heavy" (IgM), "regular" (IgA, IgE, IgD, IgG), and "light" (light chain dimers). Immunoelectrophoresis subsequently permitted identification of the various Ig classes and

subclasses.

Fab and Fc

In 1949, Porter first used papain to digest IgG molecules into two types of fragments, termed Fab and Fc (Table 5.1).[22]

Papain digested IgG into two Fab fragments, each of which could bind antigen, and a single Fc fragment. Nisonoff developed the use of pepsin to split IgG into an Fc fragment and a single dimeric $F(ab)_2$ that could cross-link antigens.[23] Edelman broke disulfide bonds in IgG and was the first to show that IgG consisted of two H and two L chains.[24]

TABLE 5.1 Definitions of Key Immunoglobulin Structure Nomenclature

Fc	A constant region dimer lacking C_H1
Fab	A light chain dimerized to V_H-C_H1 resulting from papain cleavage; this is monomeric because papain cuts above the hinge disulfide bond(s)
F(ab)′2	A dimer of Fab′ resulting from pepsin cleavage below the hinge disulfides; this is bivalent and can precipitate antigen
Fab′	A monomer resulting from mild reduction of F(ab)′2: an Fab with part of the hinge
Fd	The heavy chain portion of Fab (V_H-C_H1) obtained following reductive denaturation of Fab
Fv	The variable part of Fab: a V_H-V_L dimer
Fb	The constant part of Fab: a C_H1-C_L dimer
pFc′	A C_H3 dimer

From Carayannopoulos and Capra206 with permission.

Two Genes, One Polypeptide

The portion of the constant domain encoded by the Fc fragment was the first to be sequenced and then analyzed at the structural level. It could be readily crystallized when chilled. The heterogeneity of the V domain precluded sequence and crystallographic analysis of an intact Ig chain until Bence-Jones myeloma proteins were identified as clonal, isolated Ig light chains. These intact chains could be purified and obtained in large quantities, which finally permitted rational analysis of antibody structure and function.[25] Recognition of the unique nature of a molecule consisting of one extremely variable V domain and one highly conserved C domain led to the then-heretical Dreyer-Bennett proposal of “two genes, one polypeptide,”[26] which was subsequently and spectacularly confirmed by Tonegawa.[27]

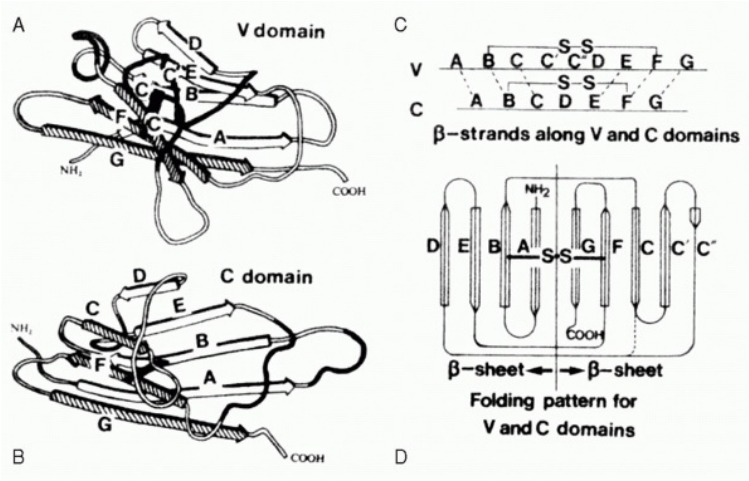

FIG. 5.4. The Immunoglobulin Domain. A: A typical variable domain structure. Note the projection of the C-C′ strands and loop away from the core. **B:** A typical compact constant domain structure. **C:** The cysteines used to pin the two b-sheets together are found in the B and F strands. **D:** The folding pattern for variable and constant domains.[2]

THE IMMUNOGLOBULIN DOMAIN

The Ig domain is the core unit that defines members of the Ig superfamily (reviewed in Williams and Barclay[2] and Harpaz and Chothia[28]). This section describes the Ig domain in detail.

The Immunoglobulin Superfamily

Each Ig domain consists of two sandwiched β-pleated sheets “pinned” together by a disulfide bridge between two conserved cysteine residues (Fig. 5.4). The structure of the β-pleated sheets in an Ig domain varies depending on the number and conformation of strands in each sheet. Two such structures, V and C, are typically found in Igs. C-type domains, which are the most compact, have seven antiparallel strands distributed as three strands in the first sheet and four strands in the second. Each of these strands has been given an alphabetical designation ranging from amino terminal A to carboxy-terminal G. Side chains positioned to lie sandwiched between the two strands tend to be nonpolar in nature. This hydrophobic core helps maintain the stability of the structure to the point that V domains engineered to replace the conserved cysteines with serine residues retain their ability to bind antigen. The residues that populate the external surface of the Ig domain and the residues that form the loops that link strands can vary greatly in sequence. These solvent exposed residues offer multiple targets for docking with other molecules.

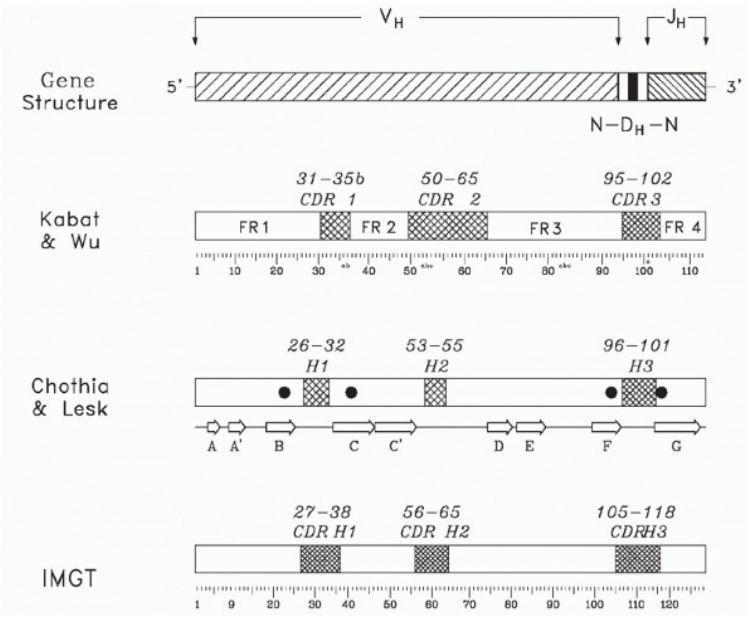

FIG. 5.5. Sequence Conservation and Hypervariability within a Heavy Chain Variable (V) Domain. The primary sequence the V domain can be divided into four regions of sequence conservation, termed framework regions (FRs), and three regions of hypervariability, termed complementarity determining regions (CDRs). A schematic of the genomic origin of the variable domain is shown at the top of the figure. The classic separation of the sequence into FR and CDR by Kabat et al.[31] is shown below the gene structure. The letter designation for individual β strands is given beneath the Chothia and Lesk nomenclature,[32] which focused more on structure. The positions of each of the four invariant residues of the V_H chain (FR1 Cys22, FR2 Trp36, FR3 Cys92, and FR4 Trp103) are shown as *darkened circles* on the Chothia and Lesk model. The ImMunoGeneTics information system® designation has attempted to rationalize sequence variability with structure and is the current nomenclature of choice.[33]

The V Domain

V-type domains add two additional antiparallel strands to the first sheet, creating a five strand-four strand distribution. Domain stability results from the tight packing of alternately inward-pointing residue side chains enriched for the presence of hydrophobic moieties to create a hydrophobic domain core. The H and L variable domains are held together primarily through noncovalent interaction between the inner faces of the β sheets.[29,30]

Early comparisons of the primary sequences of the V domains of different antibodies identified four intervals of relative sequence stability, termed framework regions (FRs), which were separated by three hypervariable intervals, termed complementarity determining regions (CDRs) (Figs. 5.5 and 5.6).[31] The exact location of these intervals has been adjusted over the years, first by a focus on the primary sequence,[31] then by a focus on the three-dimensional structure,[32] and, more recently, by a consensus integration of the two approaches by the international ImMunoGeneTics information system® (IMGT)[33] (see Fig. 5.5). (For students of the Ig repertoire, IMGT maintains an extremely useful website, http://www.imgt.org, which contains a large database of sequences as well as a multiplicity of software tools.)

The C and C′ strands that define a V domain form FR2. These strands project away from the core of the molecule (see Figs. 5.4 and 5.6) where they take on a conserved structure that is parallel and opposite to the FR2 of the companion V and adjacent to the FR4 of the complementary chain. Approximately 50% of the interdomain contacts in the hydrophobic

core of the V domain are formed by contacts between the FR2 of one chain and the FR4 of the complementary chain.[30] Another 30% to 45% is contributed by contacts between the CDR3 and the FR2 or CDR3 of the complementary domain. The overall interdomain contact includes between 12 and 21 residues from the L chain V domain and 16 to 22 residues from H chain V domain, most of which are contributed by the FR2, CDR3, and FR4 regions.

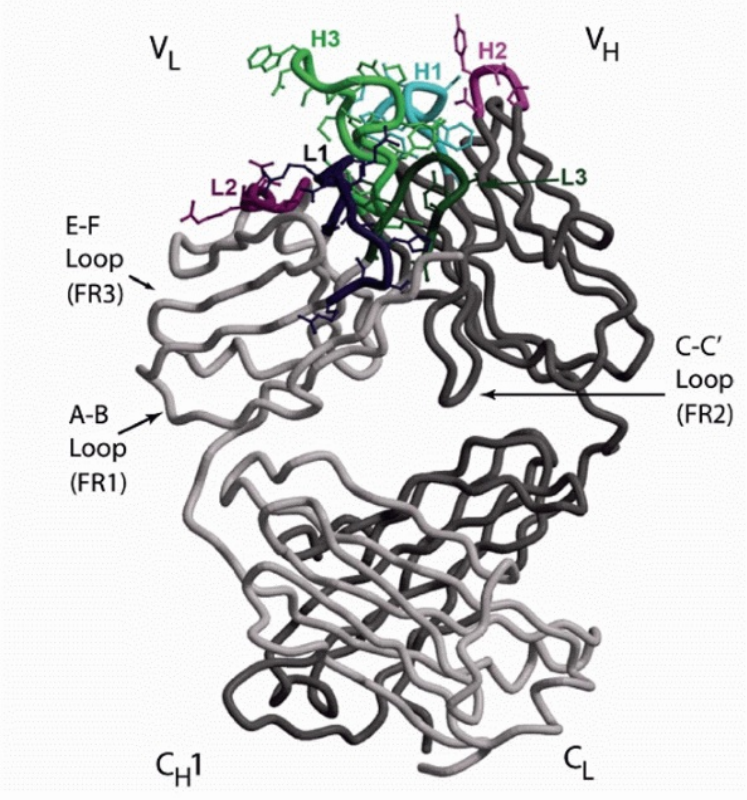

FIG. 5.6. The Structure of an Fab. The antigen-binding site is formed by the heavy (H) and light (L) chain B-C, C′-C″, and F-G loops. Each loop encodes a separate complementarity determining region (CDR). The location of CDRs H1, H2, H3, L1, L2, and L3 are shown. The opposing H and L chain C-C′ strands and loop help stabilize the interaction between VH and VL. This C-C′ structure is encoded by the second V framework region, FR2. The inclusion of this structure permits the variable (V) domains to interact in a head-to-head fashion. The E-F strands and loop are encoded by the FR3 region and lie directly below the antigen-binding site. The A-B strands and loop encode FR1 and lie between the CH1 and CL domains and the rest of the Vs. The beta sheet strands of the CH1 and CL domains rest crosswise to each other. The illustration is modified.[203]

There are approximately 40 crucial sequence sites that influence variable domain inter- and intradomain interactions.[32,34] Four of these sites are relatively invariant: the two cysteines that form the disulphide bridge between the beta sheets and two tryptophan (phenylalanine in Jκ) residues, one near the beginning of the C strand and the second near the beginning of the G strand, that pack against the bridge to add stability. Beyond these and other common core residues, Ig domains can vary widely in their primary amino acid sequences. However, a common secondary and tertiary structure characteristic of the core Ig V domain tends to be preserved.

FAB STRUCTURE AND FUNCTION

Introduction

It is the Fab domain that allows Ig to discriminate between antigens. The Fab is individually manufactured to precise specifications by individual developing B cells. It shows an amazing array of binding capabilities while maintaining a highly homologous scaffold. This section describes the characteristics of the Fab domain, its component V domains, and the *paratope*, which is the part of the Fab that actually binds antigen.

Fab, Fv, and Fb

The antigen-binding fragment (Fab) is a heterodimer that contains an L chain in its entirety and the V and C_H1 portions of the H chain (see Figs. 5.1 and 5.6). The Fab in turn can be divided into a variable fragment (Fv) composed of the V_H and V_L domains, and a constant fragment (Fb) composed of the C_L and C_H1 domains (see Table 5.1). Single Fv fragments can be produced in the laboratory through genetic engineering techniques.[35] They recapitulate the monovalent antigen binding characteristics of the original, parent antibody. Other than minor allotypic differences, the sequences of the constant domains do not vary for a given H chain or L chain isotype. The eponymous V domain, however, is quite variable.

Generation of Immunoglobulin Variable Domains by Recombination

Ig V domain genes are assembled in an ordered fashion by a series of recombination events.[27,36,37,38,39] The elegant mechanisms used for the assembly of these genes and the Fvs they create are fully discussed in Chapter 6. However, in order to understand the relationship between antibody structure and function, a brief review is in order.

In BALB/c mice, Ig V assembly begins with the joining of one of 13 diversity (D_H) gene segments to one of four joining (J_H) gene segments. This is followed by the joining of one of 110 functional variable (V_H) gene segments.[40,41,42] Each D_H gene segment has the potential to rearrange in any one of six reading frames (RFs), three by deletion and three by inversion. Thus, these 127 gene segments can come together in 3.4×10^4 combinations. The numbers of gene segments can vary quite widely between different mouse strains, but the process of assembly and the multiplicative effect of combinatorial diversity is the same.

In the final assembled V domain, the V_H gene segment encodes FRs H1 to H3 and CDRs H1 and H2 in their entirety (see Figs. 5.1 and 5.5), and the J_H encodes FR-H4. CDR-H3 is created de novo in developing B cells by the joining process. CDR-H3 contains the D_H gene segment in its entirety, as well as portions of the V_H and J_H gene segments.

After a functional H chain has been created, L chain assembly begins. Mice contain two L chain loci, κ and λ. In C57BL/6 mice, the κ locus includes five Jκ gene segments and 140 Vκ gene segments, of which 4 and 73 have been shown to be functional, respectively.[43,44,45] This provides 292 combinations. There is only one Cκ.[46] The BALB/c λ locus contains three Vλ, three functional Jλ, and two or three functional Cλ chains.[47,48] The λ constant domains are functionally indistinguishable from each other. Due to gene organization, the λ repertoire provides at most seven combinations. Each V_L encodes FRs L1 to L3, CDR L1 and L2, and two thirds of CDR-L3 (see Fig. 5.1). Each J_L encodes one-third of CDR-L3 and FR4 in its entirety. Any one

H chain can combine with any one L chain, thus 211 V, D, and J gene segments can provide approximately 1×10^7 different H:L combinations.

At the V→D and D→J junctions, the potential for CDRH3 diversity is amplified by imprecision in the site of joining, allowing exonucleolytic loss as well as palindromic (P junction) gain of terminal V_H, D_H, or J_H germline sequence. B cells that develop after birth express the enzyme terminal deoxynucleotidyl transferase (TdT) during the H chain rearrangement process.[27,36] TdT catalyzes the relatively random incorporation of non-germline-encoded nucleotides between V_H and D_H, and between D_H and J_H. Each three nucleotides of N

addition increase the potential diversity of CDR-H3 20-fold. Thus sequences with nine nucleotides of N addition each between the V→D and D→J junctions would enhance the potential for diversity by $(20)^6$, or by 6×10^7; six-fold greater than the potential diversity provided by VDJ gene segment combinations. These genomic gymnastics permit the length of CDR-H3 to vary from 5 to 20 amino acids among developing B cells in BALB/c bone marrow.[49] Together, imprecision in the site of VDJ joining and N addition provides the opportunity to create nearly random CDR-H3 sequence, potentially freeing the CDR-H3 repertoire from germline sequence constraints. Although a limited amount of N addition is observed between V_L and J_L in human,[50,51] N addition in murine L chains is distinctly uncommon. Moreover, the length of CDR-L3 appears to be under relatively strict control, greatly limiting the potential for somatic L chain junctional diversity. [48,50] Thus, CDR-H3 represents the greatest focus for the initial somatic diversification of the antibody repertoire.

Segmental Conservation and Diversity within the V Domain

Although the large numbers of V gene segments might give the impression of a smooth incremental range of available diversity, multigene families are thought to evolve in concert through mechanisms of gene conversion, and V gene segments are no exception. Sequence relationships allow grouping them into families and clans of sequences that share nucleotide homology,[52] as well as structural features. Close inspection of the V_H gene repertoire has shown that these family relationships reflect segmental gene conversion coupled with selection for function.[4,53,54]

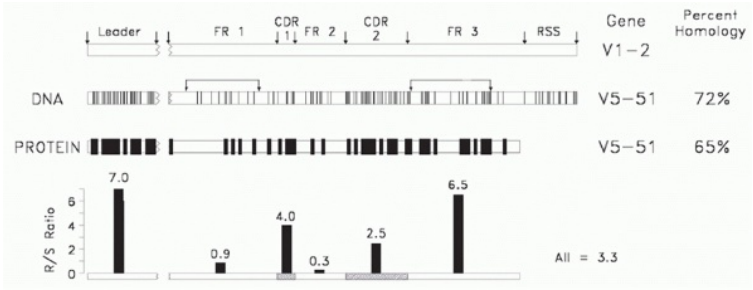

FIG. 5.7. A comparison of two human Clan I V_H sequences that belong to different V_H families (modified[4]). Shown is a comparison of the deoxyribonucleic acid and amino acid sequences of the V5-51 and V1-2 gene segments. Each line depicts a divergence in a nucleotide or amino acid at that position. Shown at the bottom is a replacement/silent site substitution analysis by interval. Random mutation tends to exhibit an R/S ratio of 2.9. The smaller the ratio, the greater the preservation of sequence. The intervals identified by the arrows predict the family and clan of origin.

Due to the need to maintain a common secondary and tertiary core Ig V domain structure capable of associating randomly with a complementary V chain to form a stable Fv, the core sequence of FR2, which is encoded by the V_H gene segment, and the core sequence of FR4, which is encoded by the J_H gene segment, are highly conserved among all Ig V domains. Conversely, the need to generate a diverse repertoire of antigen-binding sites has led to extensive diversity in the CDR-1 and CDR-2 intervals. One might presume that the FR1 and FR3 intervals, which form the external surface of the antibody, would not be under any particular constraints, but sequence comparisons suggest otherwise.

Given the need to diversify the CDRs and the need to preserve FR2, it is not surprising that family identity, which might reflect ancestral relationships, can be assigned by the extent of FR1 and FR3 similarity.[55] Of these, FR1 appears to be under the greatest constraints, with V_H gene segments belonging to different families both within and across species barriers

exhibiting extensive similarities in FR1 sequence (Fig. 5.7). Sequence similarities in FR1 and, to a lesser extent, FR3 allow grouping of human and murine V_H families into three clans of related sequences, presumably reflecting an early divergence in sequence from a primordial V_H gene sequence (Fig. 5.8).

Constraints on the Sequence and Structure of Variable-Encoded Complemenatarity Determining Regions

The antigen-binding site of an Ig is formed by the juxtaposition of the six hypervariable H and L chain V domain

intervals: CDRs-H1, -H2, and -H3; and CDRs-L1, -L2, and -L3.[31] The CDR sequences of V gene segments tend to be enriched for codons where mutations maximize replacement substitutions.[56] This includes the RGYW motif that facilitates somatic hypermutation.[57,58] While evolution appears to favor CDR1 and CDR2 sequences that facilitate codon diversity, it also appears to preserve specific loop structures.

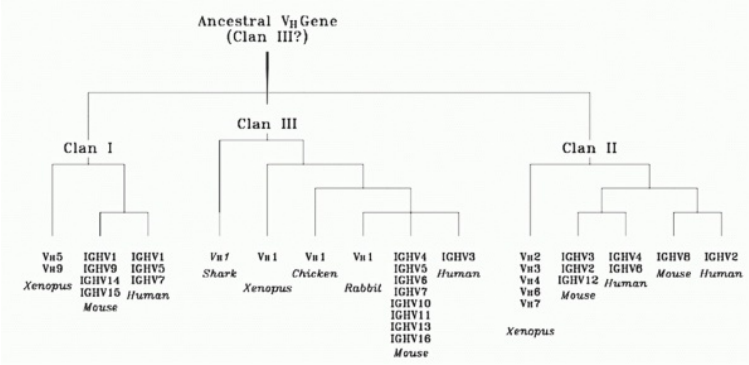

FIG. 5.8. Evolutionary Relationships among Vertebrate V_H Families. The sizes or relative placements of the evolutionary connecting lines are not to scale. V_H sequences from all mammalian species analyzed to date can be placed into one of these three clans (modified[4]).

Although there is great variation in the sequence and size of these CDRs, it has been shown that five of them, CDR-H3 being the notable exception, possess one of a small set of main-chain conformations termed canonical structures.[32,34,59,60,61,62] Each canonical structure is determined by the loop size and by the presence of certain residues at key positions in both the loop and framework regions. It can be calculated that the total number of possible combinations of canonical structures, or structure classes, is 300.[63] However, only 10 of these combinations, or classes, are sufficient to describe the majority of human and mouse Fab sequences. Among specific classes, the lengths of CDR-H3 and -L1 appear to correlate with the type of recognized antigen. Antibodies with short loops in -H2 and -L1 appear to be preferentially specific for large antigens (proteins), whereas antibodies with long loops in -H2 and -L1 appear to be preferentially specific for small molecules (haptens).[63]

Given that the sequence and structure of the framework regions, which define families, influences the canonical structure of V_H-encoded CDRs, it is not surprising that the structure repertoire of canonical structures is strongly associated with family and clan identity.[64] This implies restrictions to the random diversification of the hypervariable loop structures (canonical structures) and their combinations within the same V_H gene segment (canonical structure classes). It further suggests evolutionarily and structurally imposed restrictions operating to counteract the random diversification of these CDRs.

Diversity and Constraints on the Sequence and Structure of CDR-H3

The combination of VDJ assortment, variation in the site of gene segment rearrangement, and N nucleotide addition makes CDR-H3 the most variable of the six hypervariable regions. In some cases, the sequence of CDR-H3 appears designed to provide optimal flexibility.[65] Correspondingly, it has been more difficult to assign canonical structures to the CDR-H3 loops similar to those observed for the V-encoded CDRs. However, insight into a gradient of possible structures has been gained.

CDR-H3 can be separated into a base, which is adjacent to the frameworks, and a loop. The base tends to be stabilized by two common residues, an arginine at Kabat position 94 (IMGT 106) and an aspartic acid at Kabat 101 (IMGT 116).[32,33] These form a salt bridge which, together with the adjacent residues, tends to create one of three backbone conformations, termed kinked, extrakinked, and extended.[66] In some sequences with kinked or extrakinked bases, it is possible to predict whether an intact hydrogen-bond ladder may be formed within the loop of the CDR-H3 region, or whether the hydrogen-bond ladder is likely to be broken.[67,68] However, for many CDR-H3 sequences, especially those that are longer, current tools provide less than optimal predictions for the structures of individual CDR-H3s.

Despite the potential for totally random sequence provided by the introduction of N nucleotides, close inspection has shown that the distribution of amino acids in the

CDR-H3 loop is enriched for tyrosine and glycine,[31,69] and relatively depleted of highly polar (charged) or nonpolar (hydrophobic) amino acids, although the precise pattern depends on the species of origin.[70] This pattern of amino acid utilization is established early in B-cell development, prior to the expression of Ig on the surface of the cell (Fig. 5.9)[49,71,72,73] and reflects evolutionary conservation of J_H and D_H gene segment sequences. In particular, although the absolute sequence of the D_H is not the same, the pattern of amino acid usage by RF is highly conserved. Of the six potential RFs, RF1 by deletion is enriched for tyrosine and glycine. RF2 and RF3 by deletion are enriched for hydrophobic amino acids, as they are by inversion. RF1 by inversion tends to encode highly polar, often positively charged, amino acids.[69] Various species use different mechanisms to bias for use of RF1 by deletion, to limit use of hydrophobic RFs, and to restrict or prevent use of RFs enriched for charged amino acids. Forced rearrangement into RFs with charged amino acids yields an altered repertoire enriched for charge and depleted of tyrosine and glycine.[71]

The distribution of CDR-H3 lengths can also be regulated both as a function of differentiation and as a function of ontogeny.[49,74] In association with long V-encoded CDRs, short CDR-H3s create an antigen-binding cavity at the center of the antigen-binding site, and CDRs of intermediate length can create an antigen-binding groove. Each species appears to prefer a specific range of CDR-H3 lengths.[75] Long CDR-H3s, which can create “knobs” at the center of the antigen-binding site, are unevenly distributed between species and reflect both divergence in germline sequence and somatic selection.

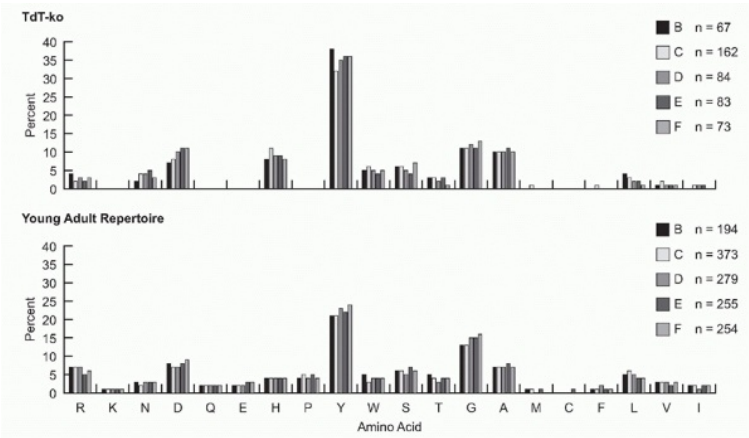

FIG. 5.9. Both in the absence or presence of N-addition, the preference for tyrosine and glycine in complementarity determining region-H3 begins early and intensifies with B-cell development. V_H 7183DJCμ transcripts were cloned and isolated from fractions B (pro-B cells) through F (mature B cells) from the bone marrow of 8- to 10-week-old terminal deoxynucleotidyl transferase-sufficient and terminal deoxynucleotidyl transferase-knockout BALB/c mice.[73] The amino acids are arranged by relative hydrophobicity, as assessed by a normalized Kyte-Doolittle scale.[204,205] Use is reported as the percent of the sequenced population. The number of unique sequences per fraction is shown.

The Antigen-Binding Site is the Product of a Nested Gradient of Regulated Diversity

The tension between the need to conserve essential structure and the need to emphasize diversity in an environment subject to unpredictable antigen challenge appears to create a gradient of regulated diversity in the Fv. The most highly conserved components of the Fv are FR2 and FR4, which form the hydrophobic core of the V_H:V_L dimer (see Figs. 5.4 and 5.5). FR1, which in the H chain presents with three conserved structures, helps form the ball and socket joint between the V_H and C_H1. FR3, which in the H chain defines the family and provides 7 different structures in human versus 16 different structures in mouse, frames the antigen-binding site (Fig. 5.10). The V-encoded CDRs, -H1, -H2, -L1, -L2, and most of -L3, are programmed for diversity. However, conserved residues within these CDRs, which interact with V family-associated FR3 residues, constrain diversity within a

preferred range of canonical structures. CDR-H3, the focus of junctional diversity, lies at the center of the antigen-binding site. The conformation of its base tends to fit within three basic structures. The loop varies greatly in sequence, yet still maintains a bias for the use of tyrosine and glycine. Thus, diversity increases with proximity to the tip of the antigenbinding site but appears to be held within regulated limits.

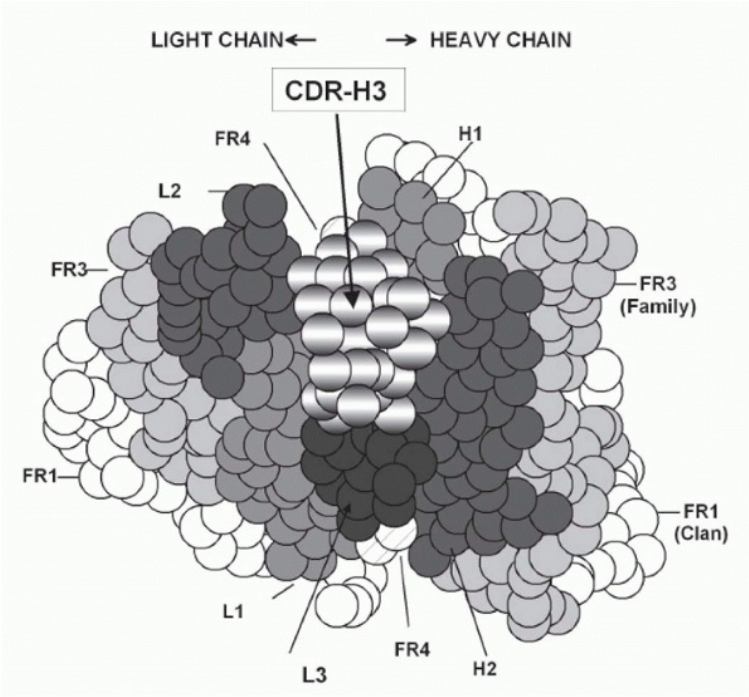

FIG. 5.10. Location and Generation of Complementarity Determining Region-H3.

A: A cartoon of the classic antigen-binding site (modified [4]). Due to its central location, most antigens bound to the antibody will interact with complementarity determining region-H3.

The extent and pattern of diversity in CDR-H3 can have a critical effect in the biologic function of Ig as a soluble effector molecule. Absence of N addition, with its enhancement of tyrosine and glycine usage (see Fig. 5.9) facilitates B-cell development, whereas enrichment for charged amino acids impairs it.[71,76,77] However, both the absence of N addition and enrichment for charged amino acids impair immune responses and protection in vivo.[71,76,78]

Somatic Hypermutation and Affinity Maturation

Following exposure to antigen and T-cell help, the V domain genes of germinal center lymphocytes can undergo mutation at a rate of up to 10^{-3} changes per base pair per cell cycle,[79] a process termed *somatic hypermutation*. Somatic hypermutation allows *affinity maturation* of the antibody repertoire in response to repeated immunization or exposure to antigen. Although affinity maturation often preserves the canonical structure of the CDR loops, the distribution of diversity appears to differ between the primary and antigenselected repertoire.[80] In the primary repertoire, diversity is focused at the center of the binding site in CDR-H3. With hypermutation, somatic diversity appears to spread to the V-encoded CDRs in the next ring of the binding site (see Fig. 5.10), enabling a more custom-tailored fit.

Binding of "Superantigens" to Nonclassic Variable Domain Antigen-Binding Sites

Not all antigens bind to the paratope created by the classic antigen-binding site. Antigens that can bind to public idiotopes on V domain frameworks[81] and recognize large portions of the available repertoire are termed superantigens. There are indications that B cell superantigens influence the pathogenesis of some common infections, such as those caused by *Staphylococcus aureus*.

ANTIGEN-ANTIBODY INTERACTIONS

Technological advances in biomolecular structure determination, analysis of molecular

dynamics, protein expression and mutagenesis, and biophysical investigation of receptor-ligand complex formation have facilitated significant advances in the understanding of antigen-antibody interactions. Particularly valuable has been the integration of high-resolution structural data with thermodynamic and kinetic analyses on a number of antigen-monoclonal antibody complexes. In this section, some of the key insights arising from these studies will be reviewed.

Molecular Flexibility

Like many other protein domains, V domains exhibit varying degrees and modes of molecular flexibility. Evidence suggests that some V modules (ie, V_L-V_H pairs) can adopt two or more conformations with meaningful frequencies in the unbound state. Because these different conformational states can exhibit distinguishable binding proclivities, molecular flexibility provides monoclonal antibodies with a mechanism for polyspecificity.[82] Molecular flexibility can also play a role in binding a single ligand, which, in such instances, may be better understood as a process of binding rather than as a simple event.[83,84] The extent of conformational adjustment by antibody or antigen required for complex formation can influence both the thermodynamics and kinetics of that process.

Role of Water

A significant role for water molecules has become clear from the study of high-resolution structures and thermodynamic analyses of antigen-antibody complexes. Water molecules exhibit a broad range of association times with protein surfaces. Thus some of the more tightly protein-associated of these solvent molecules effectively behave as parts of the protein.[85] Water molecules found in the antigen-antibody interface, whether constitutively bound or newly recruited, can make important contributions to both the intrinsic (ie, monovalent) affinity of the complex and to the differential affinities for different ligands (ie, specificity).[86] Amino acid residues of antigen and antibody can interact, indirectly, through hydrogen bonds to one or more water molecules.[86]

Thermodynamics and Antigen-Antibody Interactions

Specific residues in the antibody V domains or the antigen can contribute to complex formation in different ways, and some residues can contribute in multiple ways.[87] In addition

to making van der Waals contact, residues can be important due to contributions to the free energy of complex formation or to the differential free energy of complex formation for two or more different ligands. Some residues may contribute primarily to modulation of the association rate, the “relaxation” of the forming complex, or the dissociation rate.[83,88] Other contact residues contribute minimally to the energetics of complex formation, and yet other noncontact residues can be significant thermodynamic contributors.[89,90]

Structural and thermodynamic/kinetic comparisons of antibodies with germline V domain sequences and with somatically mutated V domain sequences have provided new insights into the structural and energetic bases for affinity maturation.[89,90] Mutations in both contact and noncontact residues can have major consequences, positive or negative, for the affinity with which an antibody binds an antigen. They can also favor tighter binding by enhancing V domain rigidity, thereby reducing the entropic penalty associated with complex formation.[90] Antibodies derived from secondary or later responses that have incorporated somatic mutations have been shown to exhibit less than absolute specificity. Even these “mature” antibodies can bind multiple ligands when screened on libraries of peptides or proteins,[91,92] a lesson likely to be relevant to most biomolecules.

It has been routine to distinguish between interactions in which antibodies bind to protein antigens versus those in which antibodies bind to carbohydrate antigens. Recent studies of antibodies that bind to human immunodeficiency virus-1 gp120 with high affinity and that exhibit potent and broad neutralizing activity suggest that antibodies can bind to epitopes

composed of both peptide and glycan elements.[93,94]

IMMUNOGLOBULIN "ELBOW JOINTS" AND "HINGES"

The structure of the constant domains can affect antibodyantigen interactions by influencing the range of molecular flexibility permitted between the two Fabs. In this section, the role of the Ig hinge will be discussed.

Elbow Joints

Individual Ig V and C domains tend to create rather rigid dimers. However, the antibody molecule as a whole, which consists of four or more such dimeric modules linked like beads on a string, can be viewed as a paradigm of molecular flexibility.[95] Flexibility begins between the Fv and the Fb of the Fab at what is termed the elbow bend or elbow angle.[96] This reflects both a ball and socket interaction between the FR1 of the H chain and the C_H1 domain,[97] and the identity of the L chain.[96] Five residues (three in V_H and two in C_H1) that are highly conserved in both antibodies and T-cell receptors make the key contacts that constitute this "joint." Elbow angles, assessed from crystal structures of homogeneous Fab fragments, range from 130 degrees to 180 degrees. λ light chains appear to permit an Fv to adopt a wider range of elbow angles than their κ chain counterparts.

Hinges

Some Ig isotypes contain a structural element that does not strictly correspond to the canonical structural motifs of Ig superfamily V and C domains, which is termed the hinge. Where it occurs, it is located between the C-terminus of the C^H1 and the N-terminus of the C^H2 domains. In isotypes such as IgG and IgA, the hinge is encoded by one (or more) separate exon(s). In isotypes with four C^H domains (ie, IgM and IgE), the C^H2 domain serves in place of a classical hinge.

The hinge, or the C^H2 domain in Igs that lack a hinge, permits an Fab arm to engage in an angular motion relative both to the other Fab arm and to its Fc stem (Figs. 5.3 and 5.11). This permits the two Fab arms to cover a range from maximal extension to an almost parallel alignment. The range of motion of the Fab arms reflects the nature of the hinge region, which in some C genes is rigid and in others, such as human IgA1, functions more as a tether for each individual Fab than as a support. This flexibility has major implications for antibody function, because it enables a bivalent antibody molecule to bind epitopes in a variety of relative spatial arrangements.

Among human IgG subclasses, the most unusual hinge region is that of IgG3. Unlike other human IgG hinge regions, the IgG3 hinge is encoded by a quadruplicated hinge exon, making it the longest hinge (62 amino acids) by far (Table 5.2). The primary structure of this hinge has been divided into upper, core (or middle), and lower hinge regions with somewhat different functional associations. The upper hinge in particular has been associated with the magnitude of segmental flexibility as assessed by fluorescence emission anisotropy kinetics[98]

and with the magnitude of Fab-Fab flexibility by immunoelectron microscopy.[99] The core or middle hinge appears to serve, at least in part, a spacer function. The lower hinge functions primarily to facilitate C_H2-C_H2 interactions.

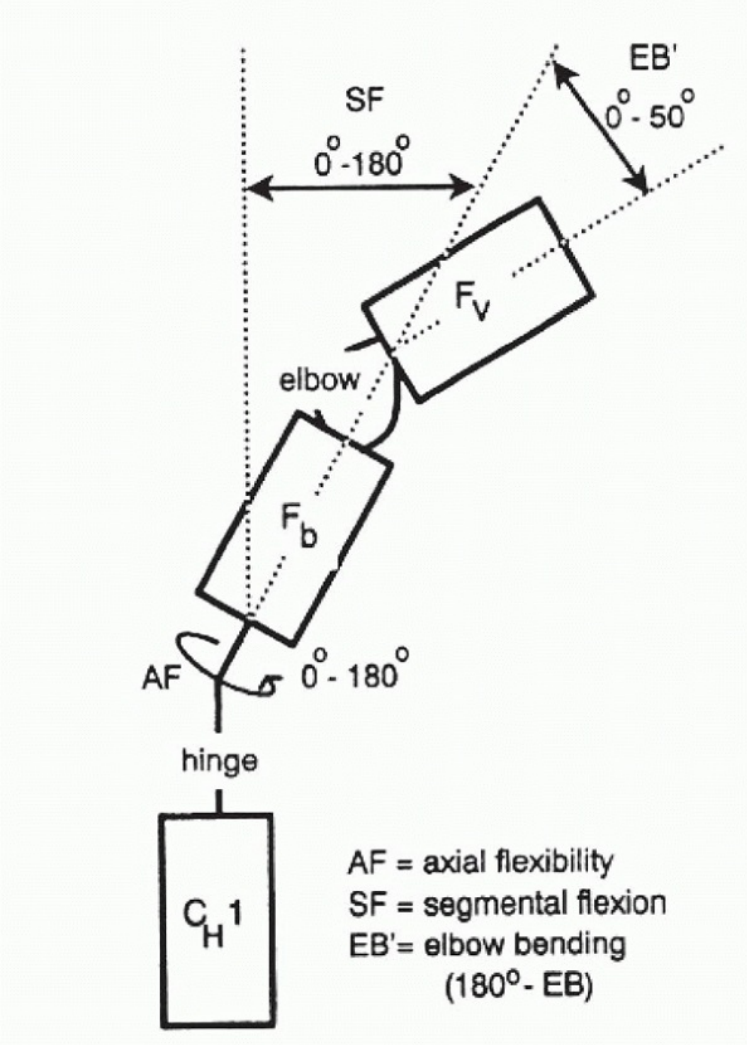

FIG. 5.11. Illustration of the Motions and Flexibility of the Immunoglobulin. Axial and segmental flexibility are determined by the hinge. The switch peptide (elbow) also contributes flexibility to the Fab. The measure of the elbow angle is defined with respect to the Fv and Fb axes of two-fold symmetry. From Carayannopoulos and Capra[206] with permission.

TABLE 5.2 Properties of Hinges in Immunoglobulin G, A, and D

Ig Type	Upper Hinge Length	Middle Hinge Length	Lower Hinge Length	Genetic Hinge Configuration (Amino Acids/Exon)	Susceptibility to Proteolysis	Special Features
IgG1	4	10	6	15		
IgG2	3	8	6	12		
IgG3	12	49	6	17-15-15-15		
IgG4	7	4	6	12		

IgA1	1	23	2	19	High	Heavy O-linked glycosylation
IgD				34-24	High	Extensive charged amino acids; heavy O-linked glycosylation at N terminus

Segmental flexibility of the Ig molecule, conferred mainly by the hinge, permits or facilitates simultaneous binding through two or more Fab arms. Such monogamous bivalency or multivalency, which enhances overall binding,[100,101] is a crucial factor permitting biosynthetically feasible antibody concentrations to offer adequate immunity against replicating pathogens. While it is more speculative why the Fab-Fc geometry needs to vary, it may have to do with optimizing effector function activation when antigen is bound, such as maintaining antigen binding when Fc receptors are simultaneously engaged.

The attribution of flexibility control to the hinge is supported by protein engineering studies in which V domain- identical IgGs of different subclasses were analyzed.[98,102] This basic conclusion is also supported by studies in which hinge regions have been selectively mutated or swapped among human or mouse IgG subclasses.[103,104,105] One of these studies indicated that structural variation among subclasses in the C_H1 domain also influenced segmental flexibility as assessed by nanosecond fluorescence polarization measurements.[103] Early suggestions that IgG subclass-related differences in activating the classical pathway of complement were explained by differences in segmental flexibility[98] were not confirmed by the studies in which mutant hinge regions were created.[103,104,105]

There are several types of molecular motion attributable to the hinge region that contribute to overall segmental flexibility (see Fig. 5.11). These include flexing between Fab arms (motion toward or away from one another in the same plane), Fab arms moving in and out of the same plane, Fab arms rotating along their long axes, and Fab arms moving in or out of the same plane as the Fc region.[106,107] The inter-Fab angles observed by electron microscopy range from O degrees to 180 degrees.[108,109] Similarly, Fab arm long-axis rotations can extend up to 180 degrees.[110]

Another key role of the hinge is the maintenance of the C_H2-C_H2 interaction (ie, effectively constraining molecular mobility within the Fc region itself). The lower hinge stabilizes C_H2-C_H2 contacts by providing the key cysteine residues involved in inter-H chain disulfide bonds. Experiments in which IgG molecules were modified to eliminate the hinge region demonstrate that covalent linkage between the hinge regions just “upstream” of the two C_H2 domains is critical for the preservation of IgG effector function.[111]

HEAVY CHAIN STRUCTURE AND FUNCTION

What might be termed the **fundamental strategy of humoral immunity** is a two-step process that begins with the identification by antibodies (of appropriate binding specificity) of the molecules or molecular complexes that should be eliminated. Following such identification (ie, noncovalent complex formation), antibodies can then trigger other molecular systems (eg, complement) or cells (eg, phagocytes) to destroy or remove the antigenic material—guilt by association at the molecular level. Thus, antigen specificity, determined primarily by the V

domains in the Fab arms, is physically and functionally linked to effector function, the activation of which is primarily attributable to the C domains of the Fc region. The effector functions associated with the humoral immune response primarily involve either complement or Fc receptor-bearing cells, such as neutrophils, macrophages, and mast cells. As might be expected, therefore, the Fc contains sites for noncovalently interacting with complement components, such as C1q, and with Fc receptors. This section focuses on the structure and function of the Fc regions of the various Ig classes and subclasses.

Structure and Function of the Fc

The necessity for interacting effectively with relatively conserved molecules such as C1q and Fc receptors provides a selective basis for maintaining the primary structures of the Fc region, at least where changes would undermine such intermolecular contacts. The degeneracy possible in noncovalent molecular recognition events permits selected primary sequence variations without catastrophic alterations in function. However, there are allotypic differences in the H chain constant domains among both human and mouse Igs. In some cases, these allotypic differences are associated with variation in function, at least in vitro. For example, two recombinant V domain-identical IgG3 antibodies, of

different allotypes, exhibited differential abilities to bind C1q or initiate antibody-dependent cell-mediated cytotoxicity.[112] Nevertheless, the H chains of the human IgG subclasses are particularly well conserved, with > 90% identity of amino acid sequences. In other mammalian species that have two or more IgG subclasses, they tend to exhibit less amino acid sequence identity than the human IgG subclasses.

By convention, the H chain constant domains are numbered from N-terminal to C-terminal, with C_H1 residing in the Fab arm and the remaining two (IgG, IgA, IgD) or three (IgM, IgE) C domains (C_H2, C_H3, and, if relevant, C_H4) residing in the Fc. In IgM and IgE, the C_H2 domain largely plays the role of the hinge region. The C domains of different isotypes and from different species share several key structural features. In distinction from V domains, which consist of four- and five-stranded β-pleated sheets linked through an intrachain disulfide bond, C domains consist of three- and four-stranded β-pleated sheets linked through an intrachain disulfide bond (see Fig. 5.4). Across isotypes, amino acid sequence identity for C_H domains is approximately 30%, while for subclasses (within an isotype) the amino acid sequence identity for CH domains is in the range of 60% to 90%. Important physical and biological properties of the human Ig isotypes are summarized in Table 5.3.

As noted previously, the traditional and accepted functional anatomy of Igs attributes antigen binding (both specificity and affinity) to the V modules in the Fab arms and effector function activation to the Fc region. While this scheme is both well supported and appealing, there is considerable (perhaps not widely appreciated) evidence that in some cases structural variations in H chain domains (ie, C_H domains and hinge) can influence both the affinity of the antibody for antigen and the discrimination among antigens.[113,114,115,116,117,118] Although these instances of C domain influence on ligand binding (through the V domains) primarily involve multivalent antigens, there are also reports that suggest C domain influence in instances of monovalent recognition.[119,120,121] Mechanisms for these effects in the context of binding multivalent antigens include isotype-related differences in segmental flexibility as well as the tendency for self-association. Results from a study comparing resistance to pneumococcal infection for IgG3-deficient and IgG3-producing mice are consistent with the notion that the cooperative binding permitted by murine IgG3 antibodies contributes to the effectiveness of humoral immunity.[122]

Fc Glycosylation

All Igs contain N-linked oligosaccharides, and it is becoming increasingly clear that this glycosylation plays significant roles in Ig structure and function. Though the type and extent of glycosylation varies among isotypes, an N-linked oligosaccharide on Asn 297 in the C_H2 domain is conserved on all mammalian IgGs and homologous portions of IgM, IgD, and IgE.

As the average serum IgG contains 2.8 oligosaccharides, there is often glycosylation present in the V domain as well.[123] The consensus sequence for the V domain N-linked oligosaccharides is not present in the germline, but it can be created during somatic hypermutation.[124] Glycosylation in the Fc region has been shown to be important for antibody half-life and effector functions.[125,126,127] Glycosylation of the Fc domain influences complement activation as Ig hypoglycosylation influences affinity for C1q as well as Ig binding to the FcR, possibly due to its effects on Ig structure.[128] Differential sialylation of the core Fc polysaccharide has recently been shown to have dramatic effects on the proinflammatory versus anti-inflammatory activity of IgG.[129,130] Furthermore, IgD N-linked glycans are necessary for IgD to bind to the IgD receptor on T cells.[131]

V domain glycosylation potentially affects the affinity for antigen, antibody half-life, antibody secretion, and organ targeting. Interestingly, glycosylation has been shown to be capable of both positively and negatively affecting antigen binding.[132,133,134,135] The biologic significance of Ig glycosylation can be seen from studies demonstrating that IgG from patients with rheumatoid arthritis is galactosylated to a lesser extent (termed IgG G0) than IgG from normal controls. In some cases, hypogalactosylation correlates with disease activity.[136] Hypogalactosylation of IgG has also been found to occur in other chronic inflammatory diseases such as Crohn's disease and systemic lupus erythematosus.[137]

Immunoglobulin M

IgM is an isotype of firsts. It is ontogenetically primary, being expressed first on developing B lineage cells. IgM is also the isotype that initially dominates the primary humoral immune response. It is probably, along with IgD, a phylogenetically primitive isotype in jawed vertebrates (an almost first) and may be the most phylogenetically stable isotype.[138]

IgM serves important immunological functions both on the surfaces of B-lymphocytes and in the fluid phase in the blood and in the mucosal secretions. On the cell surface, IgM consists of two identical μ H chains and two identical L chains (μ_2L_2). It is initially expressed on B lineage cells in noncovalent association with surrogate L chains, and subsequently, following successful L chain gene rearrangement, with κ or λ light chains. On the mature B-cell surface, IgM is noncovalently associated with two other polypeptide chains, Ig-α (CD79a) and Ig-β (CD79b).[139,140,141] These integral membrane proteins serve to transduce signals when surface IgM binds to and is cross-linked by cognate antigen.

In the secreted form, IgM can consist of either pentamers $(\mu_2L_2)_5$ or, less often, hexamers $(\mu_2L_2)_6$.[142] The μ_2L_2 monomers of the pentameric form are linked one to another by disulfide bonds in the C_H4 domains. Two of these monomers are, on one side, disulfide bonded not to another μ chain but to a 15,000 Da-polypeptide, called J chain. J chain is also found in polymeric IgA. There may be multiple patterns of such disulfide bonding, such that different cysteines participate in different monomeric units.[143]

Application of electron microscopy to polymeric IgM molecules has suggested that IgM can adopt two different quaternary arrangements: star and staple.[144,145] All of the antigen-binding sites are arrayed in radial fashion, in the same plane as one another and the Fc regions, in the star arrangement. In the staple form, the Fab arms bend out of the plane of the Fc regions. It has been conjectured that the staple form is utilized in binding simultaneously to two or more epitopes on multivalent antigens, such as bacterial or viral surfaces.

TABLE 5.3 Properties of Immunoglobulin Isotypes

Class or subclass	IgM	IgD	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	IgE

properties									
Molecular weight of secreted form (kDa)[a]	950(p)	175	150	150	160	150	160(m), 300(d)	160(m), 350(d)	190
Sedimentation coefficient	19S	7S		6.6S			7S	11S	8S
Functional valency 5 or	10	2	2	2	2	2	2 or 4	2 or 4	2
Interheavy disulphide bonds per monomer	1	1	2	4	11	2	2	2	1
Membrane Ig cytoplasmic region	3	3	28	28	28	28	14	14	28
Secreted Ig tailpiece	20	9	2	2	2	2	20	20	2
Other chain	J chain (16 kDa)	9	—	—	—	—	J chain (16 kDa) secretory component (70 kDa)		—
N-glycosylation sites	5	3	1	1	2	1	2	4	7
O-glycosylation sites	0	7	0	0	0	0	8	0	0
Carbohydrate average (%)	10-12	9-14	2-3	2-3	2-3	2-3	7-11	7-11	12-13
Adult level range (age 16-60) in serum (mg/ml)[b]	0.25-3.1	0.03-0.4	5-12	2-6	0.5-1	0.2-1	1.4-4.2	0.2-0.5	0.0001-0.0002
Approximate % total Ig in adult serum	10	0.2	45-53	11-15	3-6	1-4	11-14	1-4	0.004
Synthetic rate (mg/kg weight/day)	3.3	0.2	33	33	33	33	19-29	3.3-5.3	0.002

Biological half-life (days)	5-10	2-8	21-24	21-24	7-8	21-24	5-7	4-6	1-5
Transplacental transfer	0	0	++	+	++	++	0	0	0
Complement activation classical pathway (Clq)	++++	0	+++	+	++++	0	0	0	0
Complement activation alternative pathway	0	0	0	0	0	0	+	0	0
Reactivity with protein A via Fc	0	0	++	++	+/-	++	0	0	0
Allotypes	—	—	G1m	G2m	G3m	—	—	A2m	Em
Biological properties	Primary antibody response, some binding to pIgR, some binding to phagocytes	Mature B-cell marker	Placental transfer, secondary antibody for most responses to pathogen, binds macrophages and other phagocytic cells by FcγR				Secretory Ig, binds pIgR		Allergy and parasite reactivity, binds FcεR

Ig, immunoglobulin; d, dimer; m, monomer; p, pentamer.

[a] Light chain molecular weight is 25 kDa.

[b] Total = 9.5-21.7 mg/ml.

Compiled from Carayannopoulos and Capra,206 Lefranc and Lefranc,209 Kuby,210 and Janeway et al.211

A major pathway through which soluble IgM mediates immunity or immunopathology is the activation of the classical pathway of complement. On a per molecule basis, relative to other isotypes, IgM is highly active in activating the classical pathway and can thereby effectively opsonize bacterial pathogens. In select cases (eg, *Neisseria meningitidis*), binding of IgM to bacterial surfaces, followed by complement activation, can cause direct lysis of the bacteria through the insertion of the membrane attack complex into the bacterial membrane.[146] IgM, like polymeric IgA, can reduce the effective number of colony- or plaque-forming units for, respectively, bacteria and viruses, through agglutination. Significant physical and biological properties of IgM and the other Ig isotypes are shown in Table 5.3.

Immunoglobulin D

IgD is primarily of interest in its membrane form, as the soluble form of IgD is found in relatively modest concentrations in the blood and other body fluids. The cell surface form of IgD is found along with IgM on all mature, naïve B cells, where it appears capable of

transducing activating and tolerizing signals.[147] As is true for IgM, the membrane form of IgD associates noncovalently with Ig-α (CD79a) and Ig-β (CD79b). Simultaneous cell surface expression of two H chain isotypes expressing the same V_H domains and the same L chains occurs via differential ribonucleic acid splicing.[148]

IgD exhibits greater sensitivity to proteolytic cleavage than IgM, which is consistent with a relatively short serum half-life of only 2.8 days. The relatively long hinge region is a primary target for proteolysis. Relatively modest efforts have historically been devoted to investigating of the functional roles of IgD antibodies, especially in the secreted form, in comparison to the other isotypes. However, new information suggests that IgD antibodies are produced in the upper respiratory mucosa by an unanticipated mechanism of class switch recombination and that these antibodies participate in host defense against pathogens relevant to this anatomical environment. In addition, secreted IgD antibodies can bind to basophils, through a receptor that is yet to be identified, and when these antibodies are crosslinked by cognate antigen, the basophils release potent mediators that influence immune reactivity, inflammation, and pathogen viability.[149]

Immunoglobulin G

IgG is the predominant isotype (approximately 70% to 75% of the total Ig) in the blood and extravascular compartments. The four human IgG subclasses (IgG1, IgG2, IgG3, and IgG4) are named in order of their relative serum concentrations, with IgG1 the most prevalent and IgG4 the least. There are differences in effector functions (eg, complement activation and Fc receptor binding) and other biological properties (such as serum half-life) among these subclasses. However, there are also crucial functional commonalities, such as placental passage (see Table 5.3).

IgG antibodies are the hallmark of immunological memory in the humoral immune response. In addition to the isotype switch from IgM to IgG in a secondary antibody response, somatic hypermutation can lead to affinity maturation, a process by which the average affinity of antibody for the antigen eliciting the immune response can increase.

IgG antibodies contribute to immunity directly and through the activation of complement or FcR-bearing cells. Important examples of immunity mediated directly through antibody binding include neutralization of toxins (eg, diphtheria toxin) and viruses (eg, poliovirus). Medically important examples of IgG-induced complement activation include immunity to encapsulated bacterial pathogens leading either to opsonization and destruction within phagocytes (eg., *Streptococcus pneumoniae*) or to direct complementmediated lysis (eg, *Neisseria meningitidis*). Activation of FcR-bearing cells by IgG antibodies has also been implicated in immunity to pathogens (eg, *Cryptococcus neoformans*).[150] The consensus view is that human IgG1 and IgG3 isotypes are effective activators of the classical complement pathway. While some older sources state that IgG2 and IgG4 are weak or nonactivators of the classical complement pathway, more recent evidence suggests that when epitope density is high, IgG2 is effective in activating complement.[151,152] One possible source for the isotype-related variation in complementactivating ability is variation in affinity for C1q (IgG3>IgG1> IgG2>IgG4), the portion of the first component in the classical pathway that physically contacts the C_H2 domains of antibodies. However, isotype-associated differences in complement activation have also been found to occur at steps of the cascade subsequent to the binding of C1q to antibody.[112,153] For example, in one study of chimeric monoclonal antibodies engineered to express identical V domains and representing all four human IgG subclasses, the IgG3 antibody fixed C1q better than the IgG1 antibody, but the IgG1 molecule was more effective in mediating complement-dependent cell lysis than the IgG3 molecule.[153] Thus, it is probably not possible to rank the relative abilities of the IgG subclasses to activate complement in a single absolute hierarchy.

The affinities of IgG subclasses for Fc receptors vary from about 5×10^5 M^{-1} to about 10^8 M^{-1}. Recent studies in the mouse suggest that the relative contributions of IgG subclasses to various immunopathological processes depend on their relative affinities for the activating

versus inhibiting isoforms of FcR.[154]

A remarkable attribute of IgG (for three of the four subclasses) is its serum half-life of about 23 days. This property, attributable to the Fc region and its interaction with the neonatal Fc receptor (FcγRn), has been exploited for therapeutics through the genetic fusion of solubilized receptors, (eg, cytotoxic T-lymphocyte antigen 4) to IgG Fc regions.[155]

A recently derived insight into the function of IgG4 antibodies relates to their apparently unique ability to spontaneously dissociate into half-molecules and to form antibodies composed of different half-molecules with distinct antigen

specificities (ie, bispecific antibodies that cannot cross-link antigen and thus have anti-inflammatory activity).[156] This phenomenon may be of particular relevance to specific immunotherapy in the setting of allergy.[157]

Immunoglobulin A

While IgG is the clearly predominant isotype in the blood, IgA is the dominant Ig isotype in the mucosal secretions as well as in breast milk and colostrum.[158]

In the blood, 10% to 15% of the Ig is IgA (vs. 65% to 75% IgG). Moreover, IgA has a shorter half-life than IgG in serum. The predominant form of IgA in human serum or plasma is monomeric (ie, α_2L_2), but there are small quantities of dimers [$(\alpha_2L_2)_2$] and fewer still trimers and tetramers. Secretory IgA consists of dimers and lesser amounts of trimers and tetramers associated with one joining (J) chain (distinct from the J region in the heavy chain V domain) and one SC chain (see following discussion). The latter is the extracellular portion of the polymeric Ig receptor (pIgR), which is expressed by mucosal epithelial cells and transfers polymeric IgA or IgM from basolateral to apical surfaces, thereby providing most of the Ig content of the mucosal secretions. The J chain is disulfide bonded to the tail pieces, short C-terminal extensions of the C_H3 domains, of the two IgA monomers of a dimer, while SC forms a disulfide bond to a cysteine in one C_H2 domain of one monomer.

The two α H chain C region genes correspond to two IgA subclasses, IgA1 and IgA2. IgA1 is the predominant (> 80%) IgA subclass in the serum. While IgA2 is the major form in some human mucosal secretions, such as those in the large intestine and the female genital tract, there is variation in the relative proportion of IgA1 and IgA2 in different secretions. The shorter hinge region of IgA2 confers increased resistance to bacterial proteases that might be encountered in the mucosal environment. The extended hinge region of IgA1 is believed to permit molecules of this isotype to accommodate variable epitope spacings on multivalent antigens. While the long hinge region of IgA1 molecules might be expected to confer relatively high susceptibility to proteolysis, relative protection against the activity of bacterial proteases is provided by heavy O-linked glycosylation in the hinge (Fig. 5.12). Nevertheless, the IgA1 hinge is uniquely susceptible to IgA proteases produced by certain pathogenic bacteria.[159]

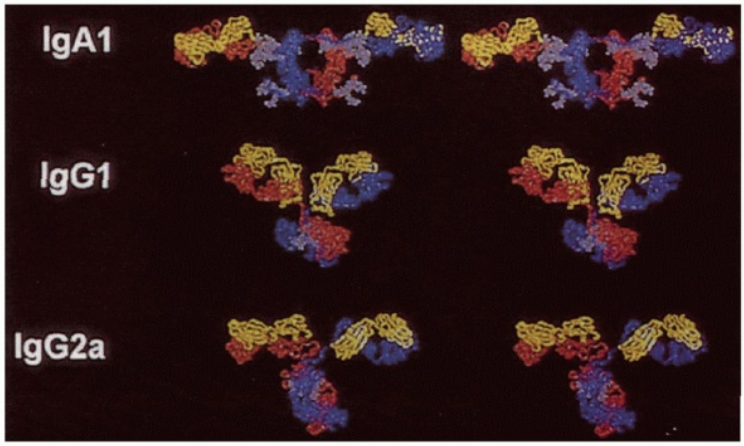

FIG. 5.12. A comparison of an x-ray and neutron-solution-scattering theoretical model (human immunoglobulin (Ig)A1) and x-ray crystal (murine IgG1 and IgG2a) structures. Light chains (*yellow*), heavy chains (*red* and *dark blue*), and glycosylation (*light blue*) are illustrated. The extended length of IgA1 over that of IgG can be seen along with extensive glycosylation that characterizes this isotype. From Boehm et al.[207] with permission.

Secretory IgA (S-IgA) has been shown to participate in immunity against a range of viral, bacterial, and parasitic pathogens at mucosal surfaces. The relative absence of functional complement and phagocytes in mucosal secretions is consistent with a different mix of mechanisms for mediating immune effects associated with S-IgA versus, for example, serum IgG. Mechanisms associated with IgA are less dependent on inflammation-producing molecules or cells, such as inhibition of microbial adherence through V module-mediated specific binding to microbial adhesins, agglutination of microbes, blocking of microbial receptors for cell surface carbohydrates with IgA-associated glycans, and mucus trapping (in which binding of S-IgA to bacteria makes them more adherent to host-generated mucus). There is also evidence that polymeric IgA can neutralize viruses

in some circumstances by interfering with steps postattachment, such as internalization. In some cases, posttranslational modifications of viral surface molecules, related to proteolytic events associated with epithelial cell transit, lead to IgA antibodies expressing protection-related antigen specificities that have no parallel in the IgG pool.[160,161] Some of the contributions of IgA to immunity are mediated through binding to FcαRI (CD89) on human neutrophils, monocytes/macrophages, and eosinophils. For example, cells bearing FcαRI on their plasma membranes can phagocytose IgA-antigen complexes. FcαRI binds to the IgA Fc region between the Cα2 and the Cα3 domains.[162] Amino acid residues in the IgA Fc region critically involved in the interaction with CD89 are indicated in Figure 5.13.

The ability of IgA to activate complement is controversial. At present, the preponderance of evidence suggests that IgA does not activate the classical complement pathway and only weakly, and under some pathophysiological circumstances, activates the alternative complement pathway. However, there is evidence suggesting that, in vitro at least, polymeric, but not monomeric, IgA can activate the complement pathway dependent on mannose-binding lectin.[163]

Additional properties of the polymeric forms of IgM and IgA are considered in the following section.

Immunoglobulin E

IgE is best known for its association with hypersensitivity reactions and allergy, but this isotype is also of interest in the context of immunity to parasites. In the blood, IgE is present

at the lowest concentration of any of the Ig isotypes (with roughly five orders of magnitude less IgE than IgG) and has the shortest half-life. The unimpressive quantitative representation of IgE in the blood is related to the high affinity of IgE antibodies for FcεRI, often referred to as the high-affinity Fc receptor for IgE. FcεRI is expressed on mast cells, basophils, Langerhans cells, and eosinophils. Due to the high affinity of FcεRI for its IgE ligand, mast cells and basophils are covered with relatively long-lived FcεRI-IgE complexes.

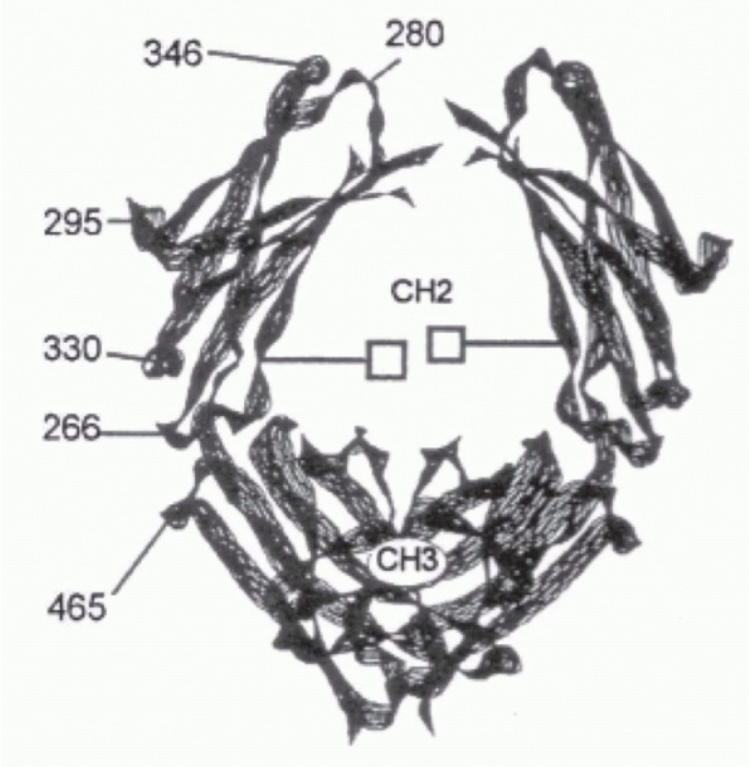

FIG. 5.13. Illustration of the Residues Essential for the Binding of immunoglobulin (Ig)A to FcαR (CD89). Residues represent mutations made in IgA C_H regions as mapped on an Fcy fragment. Of the residues mutated, L465 and L266 were found to be important for binding to CD89. From Carayannopoulos et al.[162] with permission.

The interaction between IgE and FcεRI has an affinity of ~10^{10} L/M. It primarily involves contacts between FcεRI and amino acids in the C_H3 domains, with some contributions from amino acids in the C_H2 domains. Although each IgE potentially has two sites for interacting with the FcεRI, the stoichiometry has been shown to be 1:1. Furthermore, it has been suggested that IgE binds to FcεRI in a kinked conformation.[164]

Upon ligation with bivalent or multivalent antigens specifically recognized by the bound IgE molecules, the FcεRI molecules transduce signals that activate the mast cells or basophils to secrete potent mediators of inflammation, such as histamine. These mediators are responsible for the symptoms associated with asthma, allergic rhinitis, and anaphylaxis.

There is a second receptor for IgE. FcεRII (CD23), a type II membrane protein, is expressed on monocytes/macrophages, B-lymphocytes, natural killer cells, follicular dendritic cells, Langerhans cells, eosinophils, activated epithelial cells, and platelets. It binds monomeric IgE with an affinity of ∽10^7 L/M, roughly three orders of magnitude lower than the affinity of FcεRI for IgE. Functional consequences of FcεRII-IgE interaction on macrophages include secretion of mediators of immediate hypersensitivity as well as cytokines and chemokines. There is also evidence suggesting that the FcεRII-IgE interaction can contribute to antigen capture and presentation to both B and T cells.

Fc Receptor Immunoglobulin Interactions

There are four receptors that bind the Fc regions of IgG molecules in humans and five such receptors in mice. Three of the human receptors are expressed primarily on hematopoietic cells directly involved in immune responses: FcγRI, FcγRII, and FcγRIII. Among the FcγR involved in antibody effector functions, there is important variation in affinity for IgG molecules. FcγRI binds IgG with relatively high affinity, permitting the binding of monomeric IgG. In contrast, FcγRII and FcγRIII bind to IgG with relatively low affinity. Consequently, these latter two receptors do not bind significant quantities of monomeric IgG but preferentially interact with IgG that has been effectively aggregated through interaction with bivalent or multivalent antigens (ie, immune complexes). Key amino acid residues involved in the binding FcγRs and FcεR1 with their corresponding Ig can be seen in Figure 5.14. Allotypic variations in FcγRIIA can also influence affinity for IgG ligands and subsequent effector function.[165]

There are different isoforms of FcγRII and FcγRIII. Of particular functional relevance, FcγRIIA is activating,

whereas FcγRIIB is inhibiting. In mouse models, deficiency of FcγRIIB can be associated with an autoimmune syndrome similar to human lupus.[166] Studies in mice have also suggested that the effectiveness of antibodies of the various murine IgG subclasses in mediating FcγR-dependent effector functions are correlated with the ratio of affinities of antibodies of those subclasses for activating versus inhibiting FcγR.[154]

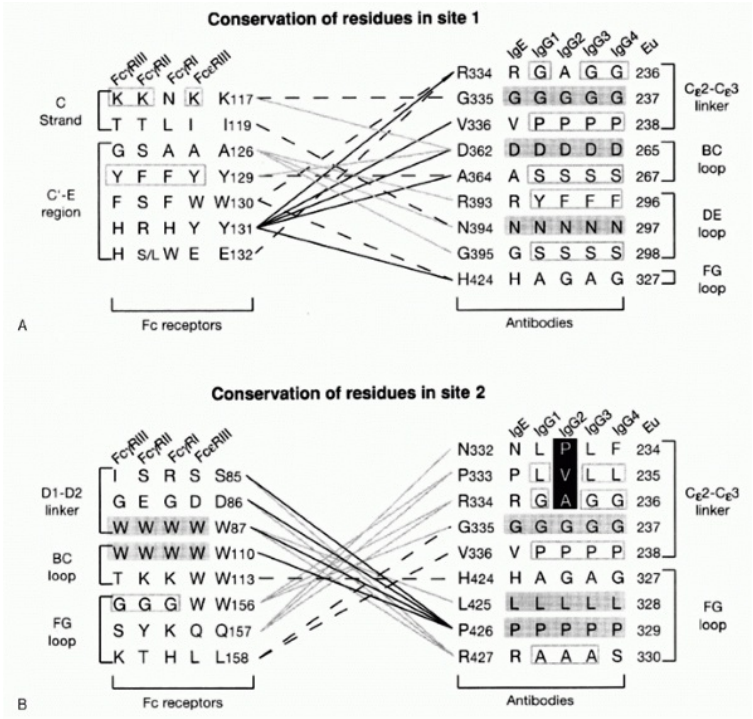

FIG. 5.14. Certain residues are conserved between FcγRs and FcεRI as well as between immunoglobulin (Ig)G and IgE that facilitate binding. Two sites participate: site 1 in **(A)** and site 2 in **(B)**. *Heavy lines* indicate the highest number of contacts and *dashed lines* indicate the least. Of considerable note are residues W87 and W110 in site 2 of the receptors and P426 in the Ig that form a core "praline sandwich" in the interaction between Ig and receptor. From Garman et al.[208] with permission.

FcγRIIIA is expressed on natural killer cells, whereas FcγRIIIB is expressed on neutrophils. The former receptor is attached to the membrane by a standard transmembrane polypeptide,

whereas the latter is attached via a glycophospholipid tail.

The fourth human receptor, the neonatal FcγR, or FcγRn (sometimes referred to as the Brambell receptor), transports IgG across the placenta. It also plays a crucial role in protecting IgG from proteolytic degradation, thereby prolonging serum half-life. While FcγRI, FcγRII, and FcγRIII are members of the immunoglobulin superfamily, FcγRn is structurally similar to major histocompatibility complex class I molecules, including noncovalent association with β2-microglobulin. The interaction between FcγRn and IgG involves amino acid residues in the C_H2-C_H3 interface[167] and is pH-sensitive. This latter property is consistent with the ability of FcγRn to bind IgG in acidic vesicular compartments and then release it into the neutral-pH environment of the blood. A crystallographic structure of the rat FcγRn in a 1:1 complex with a heterodimeric Fc (containing only one FcγRn-binding site) reveals that there are conformational changes in the Fc on binding to FcγRn.[168] The investigators also identified three titratable

salt bridges that confer pH-dependent binding of the IgG Fc to the FcγRn.

There are other FcR that interact with the non-IgG isotypes: IgM, IgD, IgA, and IgE. The FcR for IgA and IgE are covered in the respective sections devoted to the corresponding isotypes. In mice, an additional Fcα/μR binds both IgA and IgM, whereas in human these receptors are distinct.[169,170] Functional attributes of these receptors and the FcδR are continuing to be studied.

There are functional as well as structural parallels among FcR for IgG and non-IgG isotypes. Some of the features of Fc-FcR interaction that are conserved across isotypes are illustrated in Figure 5.14.

Transmembrane and Cytoplasmic Domains

Igs are expressed in both membrane and secreted forms. In contrast to the secreted form, the membrane Ig contains a transmembrane and a cytoplasmic domain. The transmembrane domain is a typical single-pass polypeptide segment consisting of 26 hydrophobic residues, extending from the C-terminus end of the C-terminal C^H domain and which forms an alphahelix followed by a variable number of basic amino acids. The cytoplasmic portion of the Ig H chain ranges in length from 3 amino acids for IgG to 30 amino acids for IgE.

Both membrane expression of Ig and the integrity of the cytoplasmic domain are important in antibody function. As the cytoplasmic domain is rather short, membrane-bound IgM is not thought to "signal" directly, but through the associated Igα and Igβ molecules.[171] However, disruption of either membrane expression of IgG1 in mice or the cytoplasmic tail results in the failure to generate an effective IgG1 response and IgG1 memory.[172] Mice lacking IgE membrane expression exhibit significant impairment in IgE responses and have extremely low levels of secreted IgE.[173] For those Igs with longer membrane:cytoplasmic tails, membrane Ig affects signaling beyond its association with Igα and Igβ. Specific residues in the transmembrane domain have been identified that are crucial for signal transduction while having no effect on the association with Igα and Igβ.[174]

HIGHER ORDER STRUCTURE

Many of the biological functions of IgA and IgM are dependent on their ability to form multimeric structures. This section will discuss the role of multimeric Ig in immune function.

Dimers, Pentamers, and Hexamers

The majority of multimeric IgA exists as dimers and, less commonly, trimers and tetramers, while IgM forms pentamers and occasionally hexamers. The polymeric structures of these antibodies enhances their functional affinity (avidity) for antigen, is essential for their active transport (both IgA and IgM) across epithelial cells to mucosal secretions, and in the case of IgM, enhances the activation of the classical pathway of complement. Once multimerized, IgA or IgM in a complex with J chain can bind to pIgR and cross mucosal epithelial cells.[175,176]

Though IgM can undergo transcytosis to the mucosal secretions, its principal action is in the serum.

The ability of IgA and IgM to multimerize is due to a tailpiece, an additional C-terminal segment of 18 amino acids in the secreted forms of the μ and α heavy chains. Tailpieces of both IgM and IgA contain a penultimate cysteine (residue 575 in IgM and 495 in IgA) that forms two different disulfide bonds important for multimer formation. In an Ig monomeric unit containing two identical H chains, one cysteine residue forms intermonomeric subunit bonds, whereas the remaining cysteine residue on the other heavy chain bonds to a cysteine on the J chain.[177,178,179,180]

Another cysteine residue, Cys414, also forms intermonomeric subunit disulfide bonds in IgM, and this bond is important in hexamer formation.[181] Besides disulfide bonds, the highly conserved glycan linked to Asn563 in IgM (and the homologous region in IgA) is also important for multimerization.[182]

Domain-swapping experiments demonstrate that the tailpiece regulates multimerization in the context of the specific H c hain. While addition of the α tailpiece to IgM has little effect on IgM polymerization, the introduction of the μ tailpiece to IgA leads to higher-order IgA polymers.[183] Based on this finding, it has been proposed that IgM polymerization is more efficient than IgA polymerization.

The J Chain

The J chain, an evolutionarily conserved 137 amino acid polypeptide produced by B-lymphocytes, functions to regulate multimer formation and to promote linkage of multimeric Ig to pIgR on epithelial cells. The J chain consists of a single domain in a beta barrel conformation and does not show sequence similarity to Ig domains.[184] It contains eight cysteine residues that participate in disulfide bonds with two tailpiece cysteines, as described previously, as well as function to stabilize its own structure through intramolecular bonds.[183,185] The J chain influences the polymerization of the multimers, as in the absence of J chain, IgA forms fewer dimers and IgM forms fewer pentamers.[186]

The J chain exists in all polymeric forms of IgA and is important in IgA polymerization and secretion across the mucosa. J chain is not required for IgM polymers but is required for external secretion. While IgM pentamers contain J chain, hexamers almost always lack it. The makeup of IgM is biologically significant because IgM hexamers have about 20-fold greater complement-activating activity than IgM pentamers. The presence of increased levels of hexameric IgM has been postulated to play a role in the pathogenesis of Waldenström macroglobulinemia and cold agglutinin disease.[187]

Immunoglobulin Transport

Transport of dimeric IgA and pentameric IgM to the mucosal secretions occurs after binding to pIgR that is present on the basolateral surface of the lining epithelial cells. The J chain is essential for the secretion of IgA and IgM and,

as described previously, influences the polymeric structure of the Ig.[175] pIgR is a transmembrane receptor synthesized by mucosal epithelial cells that contains seven domains including five extracellular V-like domains, a transmembrane domain, and a cytoplasmic domain.[188] Once bound to pIgR, polymeric Ig is endocytosed and transported to the apical surface of the cell. pIgR is then proteolytically cleaved between the fifth and sixth domains to release a complex (termed secretory Ig) containing the H, L, and J chains, and the SC, which represents the cleaved extracellular portion of pIgR.[189,190] As pIgR undergoes constitutive transcytosis in the absence of polymeric Ig, free SC is also released into the mucosal secretions. SC has several biological functions, including protecting the Ig from degradation by proteases and binding bacterial antigens such as the *Clostridium difficile* toxin A.[191,192] SC also functions to localize sIgA to the mucus layer to help protect against invasion by

pathogens.[193] Results from pIgR-null mice demonstrate that alternate pathways exist to transport polymeric Ig to the mucosal secretions as some secretory Ig still crosses the epithelial cells in the absence of pIgR.[194] Results from pIgR-null mice also demonstrate the importance of high levels of secretory antibodies as these mice are more susceptible to mucosal infections with pathogens such as *Salmonella typhimurium and Streptococcus pneumoniae*.[195,196]

The epithelial transcytosis of polymeric Ig has several biological implications. First by delivering the Ig to the mucosal surface, it enables antibodies to bind to pathogenic agents and prevent them from penetrating the mucosa, a process termed immune exclusion. Second, transcytosing antibody can neutralize viruses intracellularly.[197,198] Finally, polymeric Ig can bind to antigens in the mucosal lamina propria and excrete them to the mucosal lumen (where they can be removed from the body) by the same pIgR-mediated transcytosis process.[199,200] Some pathogens can exploit the pIgR-mediated transcytosis process in reverse to penetrate the mucosa. For example, the pneumococcal adhesin, CbpA, can bind pIgR at the epithelial apical surface, leading to bacterial penetration of the mucosa.[201]

CONCLUSION

Igs are extremely versatile molecules that can carry out many biological activities at the same time. The need to be able to recognize unique antigen structures prior to any previous exposure coupled with the need to maintain host cell receptor or complement recognition properties presents a truly unique challenge for the system. As has been described, the system incorporates diversity within specific constraints. The precise biological niches may differ, but the overall design for these molecules is the same.

The flexibility and biologic properties of Igs have made them a major focus of molecular engineering. Igs are being used as therapeutic agents, as well as for biotechnology applications. These opportunities have led to a resurgence of interest in the structure-function aspects of antibodies as we approach “designer antibodies.” Both the variable and constant portions of these molecules are current substrates for engineering purposes, offering the potential for altering both receptor and effector function. The study of antibodies began with the need to understand how sera could neutralize toxins. It is likely that antibodies will continue to be a major focus for those who seek to take fundamental principles of protein chemistry to the bedside.

REFERENCES

1. Schroeder HW Jr, Wald D, Greenspan NS. Immunoglobulins: structure and function. In: Paul W, ed. *Fundamental Immunology*. Philadelphia, PA: Lippincott Wiliams & Wilkins; 2008:125-151.

2. Williams AF, Barclay AN. The immunoglobulin superfamily—domains for cell surface recognition. *Ann Rev Immunol*. 1988;6:381-405.

3. Sakano H, Rogers JH, Huppi K, et al. Domains and the hinge region of an immunoglobulin heavy chain are encoded in separate DNA segments. *Nature*. 1979;277(5698):627-633.

4. Kirkham PM, Schroeder HW Jr. Antibody structure and the evolution of immunoglobulin V gene segments. *Sem Immunol*. 1994;6(6):347-360.

5. Rogers J, Early P, Carter C, et al. Two mRNAs with different 3′ ends encode membrane-bound and secreted forms of immunoglobulin mu chain. *Cell*. 1980;20:303-312.

6. Cohn M. The immune system: a weapon of mass destruction invented by evolution to

even the odds during the war of the DNAs. *Immunol Rev*. 2002;185:24-38.

7. Cohn M. What are the commonalities governing the behavior of humoral immune recognitive repertoires? *Dev Comp Immunol*. 2006;30:19-42.

8. Ravetch JV. Fc receptors. *Curr Opin Immunol* . 1997;9(1):121-125.

9. Crowley JJ, Goldfien RD, Schrohenloher RE, et al. Incidence of three cross-reactive idiotypes on human rheumatoid factor paraproteins. *J Immunol*. 1988;140:3411-3418.

10. Nimmerjahn F, Ravetch JV. Divergent immunoglobulin g subclass activity through selective Fc receptor binding. *Science*. 2005;310(5753) 1510-1512.

11. Sanchez-Mazas A, Fernandez-Vina M, Middleton D, et al. Immunogenetics as a tool in anthropological studies. *Immunology*. 2011;133(2): 143-164.

12. Wright A, Morrison SL. Effect of glycosylation on antibody function: implications for genetic engineering. *Trends Biotechnol*. 1997;15(1):26-32.

13. Lux A, Nimmerjahn F. Impact of differential glycosylation on IgG activity. *Adv Exp Med Biol*. 2011;780:113-124.

14. Jefferis R, Lund J, Goodall M. Recognition sites on human IgG for Fc gamma receptors: the role of glycosylation. *Immunol Lett*. 1995;44(2-3):111-117.

15. Yoo EM, Morrison SL. IgA: an immune glycoprotein. *Clin Immunol*. 2005;116(1):3-10.

16. Suzuki H, Kiryluk K, Novak J, et al. The pathophysiology of IgA nephropathy. *JASN*. 2011;22(10):1795-1803.

17. Silverstein AM. *A History of Immunology* . San Diego, CA: Academic Press; 1989.

18. Behring E, Kitasato S. Ueber das Zustandekommen der Diphtherie-Immunitat und der Tetanus-Immunitat bei thieren. *Deutsche medizinsche Wochenschrift*. 1890;16:1145-1148.

19. Ehrlich P. Die Wertbemessung des Diphtherieheilserums. *Klinisches Jahrbuch*. 2006;6:299-326.

20. Lindenmann J. Origin of the terms “antibody” and “antigen.” *Scand J Immunol* . 1984;19:281-285.

21. Tiselius A, Kabat EA. An electrophoretic study of immune sera and purified antibody preparations. *J Exp Med*. 1939;69:119-131.

22. Porter RR. Separation and isolation of fractions of rabbit gammaglobulin containing the antibody and antigenic combining sites. *Nature*. 1958;182(4636):670-671.

23. Nisonoff A, Wissler FC, Lipman LN. Properties of the major component of a peptic digest of rabbit antibody. *Science*. 1960;132:1770-1771.

24. Edelman GM, Poulik MD. Studies on the structural units of the gamma globulins. *J Exp Med*. 1961;113:861-884.

25. Putnam FW. Immunoglobulin structure: variability and homology. *Science*. 1969;163:633-644.

26. Dreyer WJ, Bennett JC. The molecular basis of antibody formation: a paradox. *Proc Natl Acad Sci U S A*. 1965;54:864-869.

27. Hozumi N, Tonegawa S. Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions. *Proc Natl Acad Sci U S A* . 1976;73(10):3628-3632.

28. Harpaz Y, Chothia C. Many of the immunoglobulin superfamily domains in cell adhesion molecules and surface receptors belong to a new structural set which is close to that containing variable domains. *J Mol Biol* . 1994;238(4):528-539.

29. Chothia C, Novotny J, Bruccoleri R, Karplus M. Domain association in immunoglobulin molecules. The packing of variable domains. *J Mol Biol* . 1985;186:651-663.

30. Padlan EA. Anatomy of the antibody molecule. *Mol Immunol* . 1994;31(3):169-217.

31. Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C. *Sequences of Proteins of Immunological Interest*. Bethesda, MD: U.S. Department of Health and Human Services; 1991.

32. Chothia C, Lesk AM. Canonical structures for the hypervariable regions of immunoglobulins. *J Mol Biol* . 1987;196:901-917.

33. Ehrenmann F, Kaas Q, Lefranc MP. IMGT/3Dstructure-DB and IMGT/DomainGapAlign: a database and a tool for immunoglobulins or antibodies, T cell receptors, MHC, IgSF and MhcSF. *Nucleic Acids Res*. 2010;38:D301-D307.

34. Chothia C, Lesk AM, Tramontano A, et al. Conformations of immunoglobulin hypervariable regions. *Nature*. 1989;342:877-883.

35. Huston JS, McCartney J, Tai MS, et al. Medical applications of singlechain antibodies. *Int Rev Immunol* . 1993;10(2-3):195-217.

36. Alt FW, Baltimore D. Joining of immunoglobulin heavy chain gene segments: Implications from a chromosome with evidence of three D-J heavy fusions. *Proc Natl Acad Sci U S A*. 1982;79:4118-4122.

37. Rajewsky K. Clonal selection and learning in the antibody system. *Nature*. 1996;381(6585):751-758.

38. Hood L, Galas D. The digital code of DNA. *Nature*. 2003;421:444-448.

39. Nossal GJV. The double helix and immunology. *Nature*. 2003;421:440-444.

40. Ichihara Y, Hayashida H, Miyazawa S, Kurosawa Y. Only DFL16, DSP2, and DQ52 gene families exist in mouse immunoglobulin heavy chain diversity gene loci, of which DFL16 and DSP2 originate from the same primordial DH gene. *Eur J Immunol* . 1989;19:1849-1854.

41. Feeney AJ, Riblet R. DST4: a new, and probably the last, functional DH gene in the BALB/c mouse. *Immunogenetics*. 1993;37(3):217-221.

42. Johnston CM, Wood AL, Bolland DJ, Corcoran AE. Complete sequence assembly and characterization of the C57BL/6 mouse Ig heavy chain V region. *J Immunol*. 2006;176(7):4221-4234.

43. Max EE, Seidman JG, Leder P. Sequences of five potential recombination sites encoded close to an immunoglobulin kappa constant region gene. *Proc Natl Acad Sci U S A*. 1979;76(7):3450-3454.

44. Sakano H, Huppi K, Heinrich G, Tonegawa S. Sequences at the somatic recombination sites of immunoglobulin light-chain genes. *Nature*. 1979;280(5720):288-294.

45. Thiebe R, Schable KF, Bensch A, et al. The variable genes and gene families of the mouse immunoglobulin kappa locus. *Eur J Immunol* . 1999;29(7):2072-2081.

46. Seidman JG, Max EE, Leder P. A kappa-immunoglobulin gene is formed by site-specific recombination without further somatic mutation. *Nature*. 1979;280(5721):370-375.

47. Selsing E, Miller J, Wilson R, Storb U. Evolution of mouse immunoglobulin lambda genes. *Proc Natl Acad Sci U S A* . 1982;79(15): 4681-4685.

48. Sanchez P, Marche PN, Rueff-Juy D, Cazenave PA. Mouse V lambda X gene sequence generates no junctional diversity and is conserved in mammalian species. *J Immunol*. 1990;144:2816-2820.

49. Ivanov II, Schelonka RL, Zhuang Y, Gartland GL, Zemlin M, Schroeder HW Jr. Development of the expressed immunoglobulin CDR-H3 repertoire is marked by focusing of constraints in length, amino acid utilization, and charge that are first established in early B cell progenitors. *J Immunol*. 2005;174:7773-7780.

50. Lee SK, Bridges SL Jr, Koopman WJ, Schroeder HW Jr. The immunoglobulin kappa light chain repertoire expressed in the synovium of a patient with rheumatoid arthritis. *Arthritis Rheum*. 1992;35:905-913.

51. Victor KD, Capra JD. An apparently common mechanism of generating antibody diversity: length variation of the VL-JL junction. *Mol Immunol*. 1994;31(1):39-46.

52. Brodeur PH, Riblet RJ. The immunoglobulin heavy chain variable region (IgH-V) locus in the mouse. I. One hundred Igh-V genes comprise seven families of homologous genes. *Eur J Immunol* . 1984;14: 922-930.

53. Tutter A, Brodeur PH, Shlomchik MJ, Riblet RJ. Structure, map position, and evolution of two newly diverged mouse Ig VH gene families. *J Immunol*. 1991;147:3215-3223.

54. Perlmutter RM, Berson B, Griffin JA, Hood LE. Diversity in the germline antibody repertoire: molecular evolution of the T15 VH gene family. *J Exp Med*. 1985;162:1998-2016.

55. Kirkham PM, Mortari F, Newton JA, Schroeder HW Jr. Immunoglobulin VH clan and family identity predicts variable domain structure and may influence antigen binding. *EMBO J*. 1992;11:603-609.

56. Chang B, Casali P. The CDR1 sequences of a major proportion of human germline Ig V H genes are inherently susceptible to amino acid replacement. *Immunol Today*.

1994;15:367-373.

57. Rogozin IB, Kolchanov NA. Somatic hypermutagenesis in immunoglobulin genes. II. Influence of neighbouring base sequences on mutagenesis. *Biochim Biophys Acta*. 1992;1171(1):11-18.

58. Dorner T, Brezinschek HP, Brezinschek RI, Foster SJ, Domiati-Saad R, Lipsky PE. Analysis of the frequency and pattern of somatic mutations within nonproductively rearranged human variable heavy chain genes. *J Immunol* . 1997;158(6):2779-2789.

59. Tramontano A, Chothia C, Lesk AM. Framework residue 71 is a major determinant of the position and conformation of the second hypervariable region in the VH domains of immunoglobulins. *J Mol Biol* . 1990;215:175-182.

60. Al Lazikani B, Lesk AM, Chothia C. Canonical structures for the hypervariable regions of T cell alphabeta receptors. *J Mol Biol* . 2000;295(4):979-995.

61. Kuroda D, Shirai H, Kobori M, Nakamura H. Systematic classification of CDR-L3 in antibodies: implications of the light chain subtypes and the VL-VH interface. *Proteins*. 2009;75(1):139-146.

62. North B, Lehmann A, Dunbrack RL Jr. A new clustering of antibody CDR loop conformations. *J Mol Biol* . 2011;406(2):228-256.

63. Vargas-Madrazo E, Lara-Ochoa F, Almagro JC. Canonical structure repertoire of the antigen-binding site of immunoglobulins suggests strong geometrical restrictions associated to the mechanism of immune recognition. *J Mol Biol* . 1995;254(3):497-504. Eratta: *J Mol Biol* . 1996;258(5):893.

64. Almagro JC, Hernandez I, del Carmen RM, Vargas-Madrazo E. The differences between the structural repertoires of VH germ-line gene segments of mice and humans: implication for the molecular mechanism of the immune response. *Mol Immunol* . 1997;34(16-17):1199-1214.

65. Babor M, Kortemme T. Multi-constraint computational design suggests that native sequences of germline antibody H3 loops are nearly optimal for conformational flexibility. *Proteins*. 2009;75(4):846-858.

66. Shirai H, Kidera A, Nakamura H. Structural classification of CDR-H3 in antibodies. *FEBS Lett*. 1996;399(1-2):1-8.

67. Shirai H, Kidera A, Nakamura H. H3-rules: identification of CDR-H3 structures in antibodies. *FEBS Lett*. 1999;455(1-2):188-197.

68. Kuroda D, Shirai H, Kobori M, Nakamura H. Structural classification of CDR-H3 revisited: a lesson in antibody modeling. *Proteins*. 2008;73(3):608-620.

69. Ivanov II, Link JM, Ippolito GC, Schroeder HW Jr. Constraints on hydropathicity and sequence composition of HCDR3 are conserved across evolution. In: Zanetti M, Capra JD, eds. *The Antibodies*. London, United Kingdom: Taylor and Francis Group; 2002:43-67.

70. Zemlin M, Klinger M, Link J, et al. Expressed murine and human CDRH3 intervals of equal length exhibit distinct repertoires that differ in their amino acid composition and predicted range of structures. *J Mol Biol* . 2003;334(4):733-749.

71. Ippolito GC, Schelonka RL, Zemlin M, et al. Forced usage of positively charged amino acids in immunoglobulin CDR-H3 impairs B cell development and antibody production. *J Exp Med*. 2006;203(6):1567-1578.

72. Schelonka RL, Ivanov II, Jung D, et al. A single DH gene segment is sufficient for B cell development and immune function. *J Immunol*. 2005;175:6624-6632.

73. Schelonka RL, Ivanov, II, Vale AM, et al. The CDR-H3 repertoire from TdT-deficient adult bone marrow is a close, but not exact, homologue of the CDR-H3 repertoire from perinatal liver. *J Immunol*. 2010;185(10):6075-6084.

74. Schroeder HW Jr, Zhang L, Philips JB III. Slow, programmed maturation of the immunoglobulin HCDR3 repertoire during the third trimester of fetal life. *Blood*. 2001;98(9):2745-2751.

75. Link JM, Larson JE, Schroeder HW Jr. Despite extensive similarity in germline DH and JH sequence, the adult Rhesus macaque CDR-H3 repertoire differs from human. *Mol Immunol*. 2005;42:943-955.

76. Schelonka RL, Ivanov, II, Vale AM, Dimmitt RA, Khaled M, Schroeder HW Jr. Absence of N addition facilitates B cell development, but impairs immune responses. *Immunogenetics*. 2011;69(9):599-609.

77. Carey JB, Moffatt-Blue CS, Watson LC, Gavin AL, Feeney AJ. Repertoire-based selection into the marginal zone compartment during B cell development. *J Exp Med*. 2008;205(9):2043-2052.

78. Nguyen HH, Zemlin M, Vu HL, et al. Heterosubtypic immunity to influenza A virus infection requires a properly diversified antibody repertoire. *J Virol*. 2007;81:9331-9338.

79. Shlomchik MJ, Marshak-Rothstein A, Wolfowicz CB, Rothstein TL, Weigert MG. The role of clonal selection and somatic mutation in autoimmunity. *Nature*. 1987;328:805-811.

80. Tomlinson IM, Walter G, Jones PT, Dear PH, Sonnhammer EL, Winter G. The imprint of somatic hypermutation on the repertoire of human germline V genes. *J Mol Biol*. 1996;256(5):813-817.

81. Goodyear CS, Silverman GJ. B cell superantigens: a microbe's answer to innate-like B cells and natural antibodies. *Springer Semin Immunopathol*. 2005;26(4):463-484.

82. James LC, Roversi P, Tawfik DS. Antibody multispecificity mediated by conformational diversity. *Science*. 2003;299(5611):1362-1367.

83. Li Y, Lipschultz CA, Mohan S, Smith-Gill SJ. Mutations of an epitope hot-spot residue alter rate limiting steps of antigen-antibody proteinprotein associations. *Biochemistry*. 2001;40(7):2011-2022.

84. Lipschultz CA, Yee A, Mohan S, Li Y, Smith-Gill SJ. Temperature differentially affects encounter and docking thermodynamics of antibody—antigen association. *J Mol Recognit*. 2002;15(1):44-52.

85. Bhat TN, Bentley GA, Boulot G, et al. Bound water molecules and conformational stabilization help mediate an antigen-antibody association. *Proc Natl Acad Sci U S A*. 1994;91(3):1089-1093.

86. Levitt M, Park BH. Water: now you see it, now you don't. *Structure*. 1993;1(4):223-226.

87. Greenspan NS, Di Cera E. Defining epitopes: it's not as easy as it seems. *Nature Biotechnol*. 1999;17(10):936-937.

88. Li Y, Urrutia M, Smith-Gill SJ, Mariuzza RA. Dissection of binding interactions in the complex between the anti-lysozyme antibody HyHEL-63 and its antigen. *Biochemistry*. 2003;42(1):11-22.

89. Hawkins RE, Russell SJ, Baier M, Winter G. The contribution of contact and non-contact residues of antibody in the affinity of binding to antigen. The interaction of mutant D1.3 antibodies with lysozyme. *J Mol Biol* . 1993;234(4):958-964.

90. Patten PA, Gray NS, Yang PL, et al. The immunological evolution of catalysis. *Science*. 1996;271(5252):1086-1091.

91. Kramer A, Keitel T, Winkler K, Stocklein W, Hohne W, Schneider-Mergener J. Molecular basis for the binding promiscuity of an anti-p24 (HIV-1) monoclonal antibody. *Cell*. 1997;91(6):799-809.

92. Michaud GA, Salcius M, Zhou F, et al. Analyzing antibody specificity with whole proteome microarrays. *Nature Biotechnol*. 2003;21(12):1509-1512.

93. Pejchal R, Doores KJ, Walker LM, et al. A potent and broad neutralizing antibody recognizes and penetrates the HIV glycan shield. *Science*. 2011;334(6059):1097-1103.

94. McLellan JS, Pancera M, Carrico C, et al. Structure of HIV-1 gp120 V1/V2 domain with broadly neutralizing antibody PG9. *Nature*. 2011;480(7377):336-343.

95. Huber R, Bennett WS. Antibody-antigen flexibility. *Nature*. 1987;326(6111):334-335.

96. Stanfield RL, Zemla A, Wilson IA, Rupp B. Antibody elbow angles are influenced by their light chain class. *J Mol Biol* . 2006;357(5):1566-1574.

97. Lesk AM, Chothia C. Elbow motion in immunoglobulins involves a molecular ball-and-socket joint. *Nature*. 1988;335:188-190.

98. Dangl JL, Wensel TG, Morrison SL, Stryer L, Herzenberg LA, Oi VT. Segmental flexibility and complement fixation of genetically engineered chimeric human, rabbit and mouse antibodies. *EMBO J*. 1988;7: 1989-1994.

99. Roux KH, Greenberg AS, Greene L, et al. Structural analysis of the nurse shark (new) antigen receptor (NAR): molecular convergence of NAR and unusual mammalian immunoglobulins. *Proc Natl Acad Sci U S A* . 1998;95(20):11804-11809.

100. Gopalakrishnan PV, Karush F. Antibody affinity. VI. Synthesis of bivalent lactosyl haptens and their interaction with anti-lactosyl antibodies. *Immunochemistry*. 1974;11(6):279-283.

101. Hornick CL, Karush F. Antibody affinity. 3. The role of multivalance. *Immunochemistry*. 1972;9(3):325-340.

102. Phillips ML, Oi VT, Schumaker VN. Electron microscopic study of ring-shaped,

bivalent hapten, bivalent antidansyl monoclonal antibody complexes with identical variable domains but IgG1, IgG2a and IgG2b constant domains. *Mol Immunol*. 1990;27(2):181-190.

103. Schneider WP, Wensel TG, Stryer L, Oi VT. Genetically engineered immunoglobulins reveal structural features controlling segmental flexibility. *Proc Natl Acad Sci U S A* . 1988;85(8):2509-2513.

104. Tan LK, Shopes RJ, Oi VT, Morrison SL. Influence of the hinge region on complement activation, C1q binding, and segmental flexibility in chimeric human immunoglobulins. *Proc Natl Acad Sci U S A* . 1990;87(1):162-166.

105. Shopes B. A genetically engineered human IgG with limited flexibility fully initiates cytolysis via complement. *Mol Immunol*. 1993;30(6):603-609.

106. Nezlin R. Internal movements in immunoglobulin molecules. *Adv Immunol*. 1990;48:1-40.

107. Schumaker VN, Phillips ML, Hanson DC. Dynamic aspects of antibody structure. *Mol Immunol*. 1991;28(12):1347-1360.

108. Roux KH, Monafo WJ, Davie JM, Greenspan NS. Construction of an extended three-dimensional idiotope map by electron microscopic analysis of idiotope-anti-idiotope complexes. *Proc Natl Acad Sci U S A* . 1987;84(14):4984-4988.

109. Valentine RC, Green NM. Electron microscopy of an antibody-hapten complex. *J Mol Biol*. 1967;27(3):615-617.

110. Wade RH, Taveau JC, Lamy JN. Concerning the axial rotational flexibility of the Fab regions of immunoglobulin G. *J Mol Biol* . 1989;206(2):349-356.

111. Brekke OH, Michaelsen TE, Sandin R, Sandlie I. Activation of complement by an IgG molecule without a genetic hinge. *Nature*. 1993;363(6430):628-630.

112. Bruggemann M, Williams GT, Bindon CI, et al. Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. *J Exp Med*. 1987;166(5):1351-1361.

113. Greenspan NS, Monafo WJ, Davie JM. Interaction of IgG3 antistreptococcal group A carbohydrate (GAC) antibody with streptococcal group A vaccine: enhancing and inhibiting effects of anti-GAC, anti-isotypic, and anti-idiotypic antibodies. *J Immunol*. 1987;138(1):285-292.

114. Cooper LJ, Robertson D, Granzow R, Greenspan NS. Variable domain-identical antibodies exhibit IgG subclass-related differences in affinity and kinetic constants as determined by surface plasmon resonance. *Mol Immunol*. 1994;31(8):577-584.

115. Cooper LJ, Shikhman AR, Glass DD, Kangisser D, Cunningham MW, Greenspan NS. Role of heavy chain constant domains in antibody-antigen interaction. Apparent specificity differences among streptococcal IgG antibodies expressing identical variable domains. *J Immunol*. 1993;150(6):2231-2242.

116. Morelock MM, Rothlein R, Bright SM, et al. Isotype choice for chimeric antibodies affects binding properties. *J Biol Chem*. 1994;269(17):13048-13055.

117. Fulpius T, Spertini F, Reininger L, Izui S. Immunoglobulin heavy chain constant region determines the pathogenicity and the antigenbinding activity of rheumatoid factor. *Proc Natl Acad Sci U S A* . 1993;90(6):2345-2349.

118. Casadevall A, Scharff MD. The mouse antibody response to infection with Cryptococcus neoformans: VH and VL usage in polysaccharide binding antibodies. *J Exp Med*. 1991;174(1):151-160.

119. Pritsch O, Hudry-Clergeon G, Buckle M, et al. Can immunoglobulin C(H)1 constant region domain modulate antigen binding affinity of antibodies? *J Clin Invest*. 1996;98(10):2235-2243.

120. Pritsch O, Magnac C, Dumas G, Bouvet JP, Alzari P, Dighiero G. Can isotype switch modulate antigen-binding affinity and influence clonal selection? *Eur J Immunol* . 2000;30(12):3387-3395.

121. Torres M, May R, Scharff MD, Casadevall A. Variable-region-identical antibodies differing in isotype demonstrate differences in fine specificity and idiotype. *J Immunol* . 2005;174(4):2132-2142.

122. McLay J, Leonard E, Petersen S, Shapiro D, Greenspan NS, Schreiber JR. Gamma 3 gene-disrupted mice selectively deficient in the dominant IgG subclass made to bacterial polysaccharides. II. Increased susceptibility to fatal pneumococcal sepsis due to absence of antipolysaccharide IgG3 is corrected by induction of anti-polysaccharide IgG1. *J Immunol* . 2002;168(7):3437-3443.

123. Kinoshita N, Ohno M, Nishiura T, et al. Glycosylation at the Fab portion of myeloma immunoglobulin G and increased fucosylated biantennary sugar chains: structural analysis by high-performance liquid chromatography and antibody-lectin enzyme immunoassay using Lens culinaris agglutinin. *Cancer Res* . 1991;51(21):5888-5892.

124. Dunn-Walters D, Boursier L, Spencer J. Effect of somatic hypermutation on potential N-glycosylation sites in human immunoglobulin heavy chain variable regions. *Mol Immunol* . 2000;37(3-4):107-113.

125. Tao MH, Morrison SL. Studies of aglycosylated chimeric mousehuman IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. *J Immunol* . 1989;143(8):2595-25601.

126. Wawrzynczak EJ, Cumber AJ, Parnell GD, Jones PT, Winter G. Blood clearance in the rat of a recombinant mouse monoclonal antibody lacking the N-linked oligosaccharide side chains of the CH2 domains. *Mol Immunol* . 1992;29(2):213-220.

127. Tsuchiya N, Endo T, Matsuta K, et al. Effects of galactose depletion from oligosaccharide chains on immunological activities of human IgG. *J Rheumatol* . 1989;16(3):285-290.

128. Krapp S, Mimura Y, Jefferis R, Huber R, Sondermann P. Structural analysis of human IgG-Fc glycoforms reveals a correlation between glycosylation and structural integrity. *J Mol Biol* . 2003;325(5):979-989.

129. Kaneko Y, Nimmerjahn F, Ravetch JV. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science*. 2006;313(5787):670-673.

130. Anthony RM, Kobayashi T, Wermeling F, Ravetch JV. Intravenous gammaglobulin

suppresses inflammation through a novel T(H)2 pathway. *Nature*. 2011;475(7354):110-113.

131. Amin AR, Tamma SM, Oppenheim JD, et al. Specificity of the murine IgD receptor on T cells is for N-linked glycans on IgD molecules. *Proc Natl Acad Sci U S A* . 1991;88(20):9238-9242.

132. Leibiger H, Wustner D, Stigler RD, Marx U. Variable domain-linked oligosaccharides of a human monoclonal IgG: structure and influence on antigen binding. *Biochem J*. 1999;338(2):529-538.

133. Wright A, Tao MH, Kabat EA, Morrison SL. Antibody variable region glycosylation: position effects on antigen binding and carbohydrate structure. *EMBO J*. 1991;10:2717-2723.

134. Coloma MJ, Trinh RK, Martinez AR, Morrison SL. Position effects of variable region carbohydrate on the affinity and in vivo behavior of an anti-(1- >6) dextran antibody. *J Immunol*. 1999;162(4):2162-2170.

135. Gala FA, Morrison SL. V region carbohydrate and antibody expression. *J Immunol* . 2004;172(9):5489-5494.

136. Parekh RB, Roitt IM, Isenberg DA, Dwek RA, Ansell BM, Rademacher TW. Galactosylation of IgG associated oligosaccharides: reduction in patients with adult and juvenile onset rheumatoid arthritis and relation to disease activity. *Lancet*. 1988;1(8592):966-969.

137. Tomana M, Schrohenloher RE, Koopman WJ, Alarcon GS, Paul WA. Abnormal glycosylation of serum IgG from patients with chronic inflammatory diseases. *Arthritis Rheumat*. 1988;31:333-338.

138. Ohta Y, Flajnik M. IgD, like IgM, is a primordial immunoglobulin class perpetuated in most jawed vertebrates. *Proc Natl Acad Sci U S A* . 2006;103(28):10723-10728.

139. Hombach J, Tsubata T, Leclercq L, Stappert H, Reth MG. Molecular components of the B-cell antigen receptor complex of the IgM class. *Nature*. 1990;343:760-762.

140. Venkitaraman AR, Williams GT, Dariavach P, Neuberger MS. The B-cell antigen receptor of the five immunoglobulin classes. *Nature*.1991;352(6338):777-781.

141. Campbell MA, Sefton BM. Protein tyrosine phosphorylation is induced in murine B lymphocytes in response to stimulation with antiimmunoglobulin. *EMBO J*. 1990;9(7):2519-2526.

142. Davis AC, Roux KH, Shulman MJ. On the structure of polymeric IgM. *Eur J Immunol*. 1988;18(7):1001-1008.

143. Davis AC, Roux KH, Pursey J, Shulman MJ. Intermolecular disulfide bonding in IgM: effects of replacing cysteine residues in the mu heavy chain. *EMBO J*. 1989;8(9):2125-2131.

144. Feinstein A, Munn EA. Conformation of the free and antigen-bound IgM antibody molecules. *Nature*. 1969;224(226):1307-1309.

145. Svehag SE, Bloth B, Seligmann M. Ultrastructure of papain and pepsin digestion

fragments of human IgM globulins. *J Exp Med*. 1969;130(4):691-705.

146. Griffiss JM, Jarvis GA, O'Brien JP, Eads MM, Schneider H. Lysis of Neisseria gonorrhoeae initiated by binding of normal human IgM to a hexosamine-containing lipooligosaccharide epitope(s) is augmented by strain-specific, properdin-binding-dependent alternative complement pathway activation. *J Immunol* . 1991;147(1):298-305.

147. Lutz C, Ledermann B, Kosco-Vilbois MH, et al. IgD can largely substitute for loss of IgM function in B cells. *Nature*. 1998;393(6687):797-801.

148. Cheng HL, Blattner FR, Fitzmaurice L, Mushinski JF, Tucker PW. Structure of genes for membrane and secreted murine IgD heavy chains. *Nature*. 1982;296(5856):410-415.

149. Chen K, Xu W, Wilson M, et al. Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cellstimulating programs in basophils. *Nat Immunol*. 2009;10(8):889-898.

150. Yuan R, Clynes R, Oh J, Ravetch JV, Scharff MD. Antibody-mediated modulation of Cryptococcus neoformans infection is dependent on distinct Fc receptor functions and IgG subclasses. *J Exp Med*. 1998;187(4):641-648.

151. Garred P, Michaelsen TE, Aase A. The IgG subclass pattern of complement activation depends on epitope density and antibody and complement concentration. *Scand J Immunol* . 1989;30(3):379-382.

152. Michaelsen TE, Garred P, Aase A. Human IgG subclass pattern of inducing complement-mediated cytolysis depends on antigen concentration and to a lesser extent on epitope patchiness, antibody affinity and complement concentration. *Eur J Immunol* . 1991;21(1):11-16.

153. Bindon CI, Hale G, Bruggemann M, Waldmann H. Human monoclonal IgG isotypes differ in complement activating function at the level of C4 as well as C1q. *J Exp Med*. 1988;168(1):127-142.

154. Nimmerjahn F, Ravetch JV. Fcgamma receptors: old friends and new family members. *Immunity*. 2006;24(1):19-28.

155. Gribben JG, Guinan EC, Boussiotis VA, et al. Complete blockade of B7 family-mediated costimulation is necessary to induce human alloantigen-specificanergy: a method to ameliorate graft-versus-host disease and extend the donor pool. *Blood*. 1996;87(11):4887-4893.

156. van der Neut Kolfschoten M, Schuurman J, Losen M, et al. Antiinflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. *Science*. 2007;317(5844):1554-1557.

157. Aalberse R. The role of IgG antibodies in allergy and immunotherapy. *Allergy*. 2011;66(Suppl 95):28-30.

158. Mestecky J, Moro I, Kerr MA, Woof JM. Mucosal immunoglobulins. In: Mestecky J, Lamm ME, Strober W, eds. *Mucosal Immunology*. Amsterdam, The Netherlands: Elsevier Academic Press; 2005:153-181.

159. Plaut AG, Wistar R Jr, Capra JD. Differential susceptibility of human IgA immunoglobulins to streptococcal IgA protease. *J Clin Invest*. 1974;54(6):1295-1300.

160. Sharpe AH, Fields BN. Pathogenesis of viral infections. Basic concepts derived from the reovirus model. *New Eng J Med* . 1985;312(8):486-497.

161. Zhaori G, Sun M, Faden HS, Ogra PL. Nasopharyngeal secretory antibody response to poliovirus type 3 virion proteins exhibit different specificities after immunization with live or inactivated poliovirus vaccines. *J Infect Dis*. 1989;159(6):1018-1024.

162. Carayannopoulos L, Hexham JM, Capra JD. Localization of the binding site for the monocyte immunoglobulin (Ig) A-Fc receptor (CD89) to the domain boundary between Calpha2 and Calpha3 in human IgA1. *J Exp Med*. 1996;183(4):1579-1586.

163. Roos A, Bouwman LH, van Gijlswijk-Janssen DJ, Faber-Krol MC, Stahl GL, Daha MR. Human IgA activates the complement system via the mannan-binding lectin pathway. *J Immunol*. 2001;167(5):2861-2868.

164. Zheng Y, Shopes B, Holowka D, Baird B. Conformations of IgE bound to its receptor Fc epsilon RI and in solution. *Biochemistry*. 1991;30(38):9125-9132.

165. Sanders LA, van de Winkel JG, Rijkers GT, et al. Fc gamma receptor IIa (CD32) heterogeneity in patients with recurrent bacterial respiratory tract infections. *J Infect Dis*. 1994;170(4):854-861.

166. Bolland S, Ravetch JV. Spontaneous autoimmune disease in Fc(gamma) RIIB-deficient mice results from strain-specific epistasis. *Immunity*. 2000;13(2):277-285.

167. West AP Jr, Bjorkman PJ. Crystal structure and immunoglobulin G binding properties of the human major histocompatibility complexrelated Fc receptor(,). *Biochemistry*. 2000;39(32):9698-9708.

168. Martin WL, West AP Jr, Gan L, Bjorkman PJ. Crystal structure at 2.8 A of an FcRn/heterodimeric Fc complex: mechanism of pH-dependent binding. *Mol Cell*. 2001;7(4):867-877.

169. Kubagawa H, Oka S, Kubagawa Y, et al. Identity of the elusive IgM Fc receptor (FcmuR) in humans. *J Exp Med*. 2009;206(12):2779-2793.

170. Maliszewski CR, March CJ, Schoenborn MA, Gimpel S, Shen L. Expression cloning of a human Fc receptor for IgA. *J Exp Med*. 1990;172(6):1665-1672.

171. Blum JH, Stevens TL, DeFranco AL. Role of the mu immunoglobulin heavy chain transmembrane and cytoplasmic domains in B cell antigen receptor expression and signal transduction. *J Biol Chem*. 1993;268(36):27236-27245.

172. Kaisho T, Schwenk F, Rajewsky K. The roles of gamma1 heavy chain membrane expression and cytoplasmic tail in IgG1 responses. *Science*. 1997;276(5311):412-415.

173. Achatz G, Nitschke L, Lamers MC. Effect of transmembrane and cytoplasmic domains of IgE on the IgE response. *Science*. 1997;276(5311):409-411.

174. Pleiman CM, Chien NC, Cambier JC. Point mutations define a mIgM transmembrane region motif that determines intersubunit signal transduction in the antigen receptor. *J Immunol*. 1994;152(6):2837-2844.

175. Brandtzaeg P, Prydz H. Direct evidence for an integrated function of J chain and

secretory component in epithelial transport of immunoglobulins. *Nature*. 1984;311(5981):71-73.

176. Rojas R, Apodaca G. Immunoglobulin transport across polarized epithelial cells. *Nat Rev Mol Cell Biol*. 2002;3(12):944-955.

177. Mestecky J, Schrohenloher RE. Site of attachment of J chain to human immunoglobulin M. *Nature*. 1974;249(458):650-652.

178. Mestecky J, Schrohenloher RE, Kulhavy R, Wright GP, Tomana M. Site of J chain attachment to human polymeric IgA. *Proc Natl Acad Sci U S A* . 1974;71(2):544-548.

179. Bastian A, Kratzin H, Fallgren-Gebauer E, Eckart K, Hilschmann N. Intra- and inter-chain disulfide bridges of J chain in human S-IgA. *Adv Exp Med Biol* . 1995;371A:581-583.

180. Frutiger S, Hughes GJ, Paquet N, Luthy R, Jaton JC. Disulfide bond assignment in human J chain and its covalent pairing with immunoglobulin M. *Biochemistry*. 1992;31(50):12643-12647.

181. Sorensen V, Sundvold V, Michaelsen TE, Sandlie I. Polymerization of IgA and IgM: roles of Cys309/Cys414 and the secretory tailpiece. *J Immunol* . 1999;162(6):3448-3455.

182. de Lalla C, Fagioli C, Cessi FS, Smilovich D, Sitia R. Biogenesis and function of IgM: the role of the conserved mu-chain tailpiece glycans. *Mol Immunol* . 1998;35(13):837-845.

183. Sorensen V, Rasmussen IB, Sundvold V, Michaelsen TE, Sandlie I. Structural requirements for incorporation of J chain into human IgM and IgA. *Int Immunol* . 2000;12(1):19-27.

184. Zikan J, Novotny J, Trapane TL, et al. Secondary structure of the immunoglobulin J chain. *Proc Natl Acad Sci U S A* . 1985;82(17):5905-5909.

185. Mole JE, Bhown AS, Bennett JC. Primary structure of human J chain: alignment of peptides from chemical and enzymatic hydrolyses. *Biochemistry*. 1977;16(16):3507-3513.

186. Randall TD, Brewer JW, Corley RB. Direct evidence that J chain regulates the polymeric structure of IgM in antibody-secreting B cells. *J Biol Chem*. 1992;267(25):18002-18007.

187. Hughey CT, Brewer JW, Colosia AD, Rosse WF, Corley RB. Production of IgM hexamers by normal and autoimmune B cells: implications for the physiologic role of hexameric IgM. *J Immunol* . 1998;161(8):4091-4097.

188. Mostov KE, Friedlander M, Blobel G. The receptor for transepithelial transport of IgA and IgM contains multiple immunoglobulin-like domains. *Nature*. 1984;308:37-43.

189. Mostov KE, Blobel G. A transmembrane precursor of secretory component. The receptor for transcellular transport of polymeric immunoglobulins. *J Biol Chem*. 1982;257(19):11816-11821.

190. Kaetzel CS. The polymeric immunoglobulin receptor: bridging innate and adaptive immune responses at mucosal surfaces. *Immunol Rev*. 2005;206:83-99.

191. Perrier C, Sprenger N, Corthesy B. Glycans on secretory component participate in innate protection against mucosal pathogens. *J Biol Chem*. 2006;281(20):14280-14287.

192. Dallas SD, Rolfe RD. Binding of Clostridium difficile toxin A to human milk secretory component. *J Med Microbiol* . 1998;47(10):879-888.

193. Phalipon A, Cardona A, Kraehenbuhl JP, Edelman L, Sansonetti PJ, Corthesy B. Secretory component: a new role in secretory IgAmediated immune exclusion in vivo. *Immunity*. 2002;17(1):107-115.

194. Shimada S, Kawaguchi-Miyashita M, Kushiro A, et al. Generation of polymeric immunoglobulin receptor-deficient mouse with marked reduction of secretory IgA. *J Immunol*. 1999;163(10):5367-5373.

195. Wijburg OL, Uren TK, Simpfendorfer K, Johansen FE, Brandtzaeg P, Strugnell RA. Innate secretory antibodies protect against natural Salmonella typhimurium infection. *J Exp Med*. 2006;203(1):21-26.

196. Sun K, Johansen FE, Eckmann L, Metzger DW. An important role for polymeric Ig receptor-mediated transport of IgA in protection against Streptococcus pneumoniae nasopharyngeal carriage. *J Immunol* . 2004;173(7):4576-4581.

197. Huang YT, Wright A, Gao X, Kulick L, Yan H, Lamm ME. Intraepithelial cell neutralization of HIV-1 replication by IgA. *J Immunol* . 2005;174(8):4828-4835.

198. Mazanec MB, Kaetzel CS, Lamm ME, Fletcher D, Nedrud JG. Intracellular neutralization of virus by immunoglobulin A antibodies. *Proc Natl Acad Sci U S A* . 1992;89(15):6901-6905.

199. Kaetzel CS, Robinson JK, Chintalacharuvu KR, Vaerman JP, Lamm ME. The polymeric immunoglobulin receptor (secretory component) mediates transport of immune complexes across epithelial cells: a local defense function for IgA. *Proc Natl Acad Sci U S A*. 1991;88(19):8796-8800.

200. Banks WA, Freed EO, Wolf KM, Robinson SM, Franko M, Kumar VB. Transport of human immunodeficiency virus type 1 pseudoviruses across the blood-brain barrier: role of envelope proteins and adsorptive endocytosis. *J Virol* . 2001;75(10):4681-4691.

201. Zhang JR, Mostov KE, Lamm ME, et al. The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells. *Cell*. 2000;102(6):827-837.

202. Harris LJ, Skaletsky E, McPherson A. Crystallographic structure of an intact IgG1 monoclonal antibody. *J Mol Biol*. 1998;275(5):861-872.

203. Manivel V, Sahoo NC, Salunke DM, Rao KV. Maturation of an antibody response is governed by modulations in flexibility of the antigencombining site. *Immunity*. 2000;13(5):611-620.

204. Kyte J, Doolittle RF. A simple method for displaying the hydropathic character of a protein. *J Mol Biol* . 1982;157:105-132.

205. Eisenberg D. Three-dimensional structure of membrane and surface proteins. *Ann Rev Biochem*. 1984;53:595-623.

206. Carayannopoulos L, Capra JD. Immunoglobulins: structure and function. In: Paul WE, ed. *Fundamental Immunology*. New York, NY: Raven Press; 1993:283-314.

207. Boehm MK, Woof JM, Kerr MA, Perkins SJ. The Fab and Fc fragments of IgA1 exhibit a different arrangement from that in IgG: a study by X-ray and neutron solution scattering and homology modelling. *J Mol Biol* . 1999;286(5):1421-1447.

208. Garman SC, Wurzburg BA, Tarchevskaya SS, Kinet JP, Jardetzky TS. Structure of the Fc fragment of human IgE bound to its high-affinity receptor Fc epsilonRI alpha. *Nature*. 2000;406(6793):259-266.

209. Lefranc MP, Lefranc G. *The Immunoglobulin FactsBook*. San Diego, CA: Academic Press; 2001.

210. Kuby J. *Immunology*. New York, NY: W.H. Freeman and Company;1997.

211. Janeway CA Jr, Travers P, Walport M, Shlomchik M. *Immunobiology*. New York, NY: Garland Publishing; 1999.

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Chapter 6

Immunoglobulins: Molecular Genetics

Edward E. Max

Sebastian Fugmann

INTRODUCTION

To respond to a foreign molecule (antigen) on an invading pathogen, the “humoral” immune system generates antibodies, or immunoglobulins (Igs), that can bind specifically to the offending antigen. Each antibody molecule is composed of two identical light (L) chains and two identical heavy (H) chains, all linked by disulfide bonds to form a symmetric Y-shaped tetramer. The ability of the immune system to generate an antigenspecific antibody depends on the fact that, before exposure to antigen, millions of naïve resting B cells circulate in the individual, each cell displaying on its membrane several thousand identical copies of a single unique species of antibody; these serve as B-cell receptors (BCRs) for that lymphocyte. Only a tiny fraction of the B cells express a BCR capable of binding to any particular antigen. When these B cells bind their antigen, they become activated to proliferate and mature into antibodysecreting plasma cells, which manufacture large amounts of antibody specific for the activating antigen. To be able to generate antibodies against a universe of diverse pathogens, this “clonal selection” mechanism for specific antibody secretion requires an enormous diversity of Ig species expressed on naïve B cells prior to antigen exposure. Indeed, in the 1960s the number of different antibody sequences in the repertoire of typical mouse was estimated in the millions. To encode this many sequences seemed to require an unreasonably high percentage of the mammalian genome (now estimated to contain only about 30,000 genes). Understanding the genetic source of Ig diversity—Ig gene assembly —was the first major challenge and achievement of the molecular biologic investigations of antibody genes, and this will be discussed first in this chapter.

A week or so after antigen administration, the B-cell response changes in two ways that generally improve the protective functions of antibodies. B cells initially express antibodies of the IgM isotype, but cells that migrate into germinal centers receive T-cell-derived stimuli that can induce them to switch to production of IgG, IgA, or IgE without changing their antigen specificity; this switch results from a deoxyribonucleic acid (DNA) recombination event known as class switch recombination (CSR). In addition, over the course of an immune response, the affinity of antibody for antigen gradually improves as a result of somatic hypermutation (SHM) of antibody genes, coupled to selection for B cells expressing high-affinity antibodies. CSR and SHM are discussed later in this chapter.

In this chapter, well-established facts about Ig genes are summarized concisely, while areas currently under investigation are considered in more detail, with particular attention to topics expected to interest immunologists.

OVERVIEW OF IMMUNOGLOBULIN GENE ASSEMBLY

In the 1960s, investigators determined the amino acid sequences of Igs secreted by several mouse myelomas (clonal tumors of B-lymphocytes that secrete a single pure species of Ig). The N-terminal domains of the L and H chains— each roughly 100 amino acids—were highly diverse between different myeloma proteins and were designated variable (V) regions. In contrast, sequences of the remaining domains of the proteins were essentially identical for every myeloma Ig of a given class (and so they were designated constant [C] region domains). The advent of recombinant DNA technology allowed comparisons of V region genes expressed in different myelomas with the corresponding sequences in nonlymphoid

DNA (commonly referred to as “germline” DNA). It was found that each myeloma V gene is composed of several segments that are separated in germline DNA; these germline segments must undergo one or more DNA recombination events to assemble a complete V region.[1] For example, each complete Vκ gene from a myeloma or B-lymphocyte encodes roughly 108 amino acids and is assembled by linking one of about 40 germline Vκ segments (encoding amino acids 1 through 95) to one (of five) “joining” or Jκ segments encoding residues 96 to 108. Similarly, a complete Vλ gene is assembled from one germline Vλ segment and one Jλ segment. H chains are assembled from three segments; a diversity (D) segment is interposed between V_H and J_H. In developing B cells, the germline gene segments are assembled into functional V exons by a process named V(D)J recombination (Fig. 6.1).

V(D)J recombination is a “cut and paste” process in which the DNA between two recombining V, D, or J gene segments is excised from the chromosome, and the two remaining DNA segments are joined together to reseal the DNA break. The two principal proteins executing the “cut” phase of this process are encoded by the recombination activating genes (RAG)1 and 2. These proteins recognize unique sequences, known as recombination signal sequences (RSSs), that flank and mark each eligible V, D, and J gene segment (RSSs are described further in the following). After the RAG proteins cut the DNA, the subsequent “joining” of the gene segments relies largely on ubiquitous DNA repair factors.

How Recombination Contributes to Diversity

V(D)J recombination contributes in several distinct ways to the diversity of antigen-binding specificities. First, there is

combinatorial diversity, as each Ig locus contains multiple V, D (in case of the IgH locus), and J segments that can be combined in many ways. The total number of theoretically possible combinations of V_H, D_H, J_H, V_L, and J_L, is the multiplication product of the numbers of possible H chains— about 40 (V_H) × 27 (D_H) × 6 (J_H) or 6480 combinations in humans —times the number of possible L chain combinations (about 290), or almost 2 million. This repertoire is vastly larger than could be achieved by devoting the same total lengths of DNA sequence to preassembled variable region exons. Second, there is junctional diversity generated by flexibility in the position of joining between gene segments. This was initially recognized by comparisons of nucleotide sequences of various myeloma Vκ genes to their germline Vκ and Jκ precursors. As shown in Figure 6.2A, these comparisons revealed that the crossover point between sequence derived from a germline Vκ region and a Jκ region could vary in different myelomas, increasing the diversity of amino acids around codons 95 and 96. H chain VDJ exons exhibit this flexibility at both V-D and D-J junctions, yielding striking variation in the lengths D regionderived segments, from zero to about 14 amino acids. And additional junctional diversity is produced by the addition of nucleotides not present in any germline elements: “N” and “P” nucleotides, discussed below. Importantly, the three-dimensional structures of Igs established by X-ray crystallography reveal that the VL-JL junction and the V_H-D_H-J_H junction each encode one of the three “complementarity determining region” loops of L or H chain that can contact antigen; thus, this junctional diversity is directly functionally relevant for diversifying antigen binding.

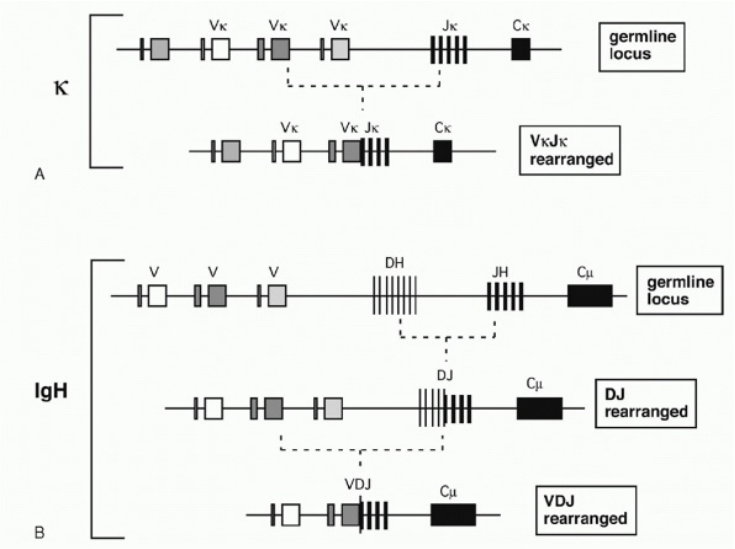

FIG. 6.1. Variable Assembly Recombination. A: In the κ locus, a single recombination event joins a germline Vκ region with one of the Jκ segments. **B:** In the immunoglobulin H locus, an initial recombination joins a diversity segment to a J_H segment. A second recombination completes the variable assembly by joining a V_H to DJ_H.

The imprecision of V(D)J recombination increases Ig diversity, but at a cost. Because the precise boundaries between V, D, and J result from independent stochastic events, only about one-third of all recombination events maintain the correct reading frame through the J segments. Gene rearrangements leading to functional Ig genes are often referred to as "productive," while out-of-frame rearrangements are labeled "nonproductive."

Function of Recombination Signal Sequences

Analysis of DNA sequences flanking the germline V, D, and J gene segments revealed highly similar sequence motifs that have subsequently been shown to define targets for V(D)J recombination: the RSSs, which serve as the recognition sequences for the V(D)J recombinase proteins RAG1 and RAG2, as mentioned previously. Notably, RSSs lie adjacent to L- and H-chain Ig gene segments and to T-cell-receptor (TCR) gene elements throughout phylogeny. RSSs consist of a conserved seven base pairs (bps) long "heptamer" (consensus: CACAGTG) and a nine bp long "nonamer" sequences

(consensus: ACAAAAACC) that are separated by less wellconserved spacers of either approximately 12 or 23 bp in length (Fig. 6.3). Based on the spacer lengths, the two classes of RSSs are referred to as 12-RSSs and 23-RSSs, respectively. (Note that some laboratories use the term recombination signal instead of RSS in their publications.)

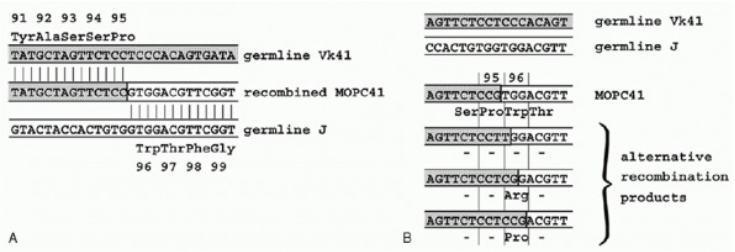

FIG. 6.2. Vκ-Jκ Recombination at Single Base Resolution. A: The sequence of the recombined MOPC41 κ gene around the VJ junction is shown (*center*) with the sequences of the two germline precursors (Vκ41 and Jκ1) shown above and below. The

germline origins of the recombined gene are indicated by the vertical lines and the shading of the V-derived sequence. **B:** The consequences of joining the same germline sequences (from part *A*) at four different positions are shown. Of the four alternative recombination products illustrated, the top one is that actually found in MOPC41. The second example has a single nucleotide difference but no change in encoded amino acid sequence. The third and fourth alternatives yield Arg or Pro at position 96; both of these amino acids have been found at this position in sequenced mouse κ chains.

Recombination occurs almost exclusively between coding sequences associated with RSSs of different spacer lengths, a requirement referred to as the "12/23-rule" (i.e., the recombination between two 12-RSSs [or two 23-RSSs] is "forbidden" and does not occur *in vivo*). Within each gene locus, all gene segments of one class (e.g., all Vs in the Igκlocus) carry RSSs with the same spacer length. Thus the 12/23 rule drives appropriate recombination events leading to functional VJ and VDJ products, and prevents futile recombination events, such as between two V or two J gene segments. While the heptamer and nonamer are the major determinants of RSS function necessary for V(D)J recombination, increasing evidence suggests that spacer sequences can modulate recombination efficiencies of compatible gene segments (e.g., they affect the non-random usage of human Vκ elements[2]).

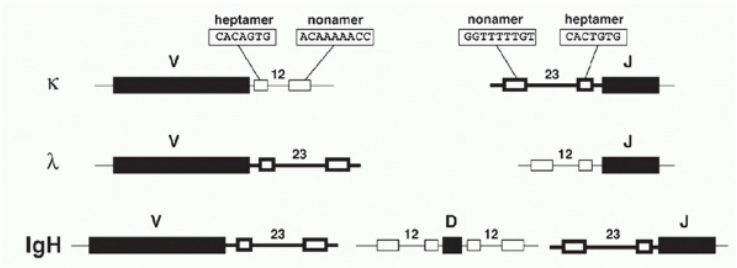

FIG. 6.3. Conserved Elements Flank Germline Variable (V), Diversity (D), and Joining (J) Region Genes. Conserved heptamer and nonamer recombination signal sequences (RSSs) lie adjacent to V, D, and J coding sequences and are important for targeting V(D)J recombination. The heptamer and nonamer elements are separated by spacer regions of about 12 basepairs (bp) (*thin lines*) or 23 bp (*thick lines*). Depending on the locus, V regions may be flanked by 12 bp or 23 bp RSS; and similarly for J regions. But one of each type of element must be present for recombination to occur, a requirement that prevents futile recombination events (e.g., J to J).

THE THREE IMMUNOGLOBULIN GENE LOCI

To understand the contribution of the germline V, D, J element repertoire to Ig diversity, several laboratories undertook cloning and sequence analysis of individual V region genes from the IgH, Igκ, and Igλ loci of human and mouse. More recently, the complete sequences of all human and mouse Ig loci have been determined as part of the genome sequencing projects for these two species (available online at www.ncbi.nlm.nih.gov, though annotation that describes

function and refers to earlier literature is incomplete). It is important to point out that Ig gene loci are not identical between individuals (humans) or between individual strains of inbred mice. Several Internet resources are devoted to providing convenient updated access to Ig germline gene sequences. The international ImMunoGeneTics database (http://imgt.org) includes a database for Ig and TCR genes from a variety of species, and includes maps, sequences, lists of chromosomal translocations, and multiple helpful links. IgBLAST (www.ncbi.nlm.nih.gov/igblast/) is a service of the National Center for Biotechnology Information and allows a submitted sequence to be searched against known annotated

germline V, D, and J sequences.

TABLE 6.1 Overview of the Number of Variable, Diversity, and Joining Segments in Each of the Three Immunoglobulin Loci in Humans and Mice

Locus	Species	V		D		J	
		Functional	*Pseudogenes*	*Functional*	*Pseudogenes*	*Functional*	*Pseudogenes*
IgH	Mouse	110	85	10		4	
	Human	40	83	24	3	6	3
Igκ	Mouse	95	45			4	1
	Human	46	87			5	
Igλ	Mouse	3				3	1
	Human	36	56			4-5	2-3

D, diversity; Ig, immunoglobulin; J, joining; V, variability.

Pseudogenes are recognized based on sequence defects that would preclude function (premature stop codons, defective recombination signal sequences, defective splice sites). The numbers in this table are approximate, owing to variation between mouse strains and between individual humans.

The Murine Immunoglobulin H Germline Variable, Diversity, and Joining Gene Segments

V_H Segments

The murine V_H region extends over about 2.5 megabases on chromosome 12 and includes roughly 100 functional segments (depending on mouse strain) plus additional V_H pseudogene segments (Table 6.1). All V_H elements are in the same transcriptional orientation as the D, J_H, and C_H regions.[3] The V_H segments are classified into 16 distinct families based on sequence similarity; V_H elements within a family show more than 80% nucleotide sequence identity. Elements of individual V_H families are largely clustered together, though some interdigitation occurs (Fig. 6.4). The V_H families can be grouped into three “clans” based on sequence conservation primarily of their framework regions (framework region 1, codons 6 to 24, and framework region 3, codons 67 to 85, respectively), which form the more conserved structural backbone of the Ig variable region. Importantly, these clans are conserved between man, mouse, and frog, suggesting that their emergence in the repertoire preceded the amphibian-reptile divergence.[4]

FIG. 6.4. Maps of the Murine and Human V_H Loci. The 15 known murine V_H gene families are shown in their approximate map positions. Each *rectangle* represents a cluster of V_H genes of the indicated family; the clan identification of the V_H families is indicated by the color of the rectangle: *black* for clan I, *gray* for clan II, and *white* for clan III. Although some interdigitation is shown by overlapping families (e.g., the Q52 and 7183 families), the murine V_H families are largely clustered. In contrast, all human V_H genes (*vertical lines*) of a prototypic haplotype are shown in the right panel; extensive interdigitation of families is apparent.

Diversity and J_H

About 50 kb downstream of the most 3′ V_H element resides the murine D cluster spanning about 80 kb, depending on

the mouse strain (see Table 6.1). Each D segment is flanked by 12 RSSs on both sides, so that the 12/23 rule ensures that all assembled V genes carry a D element between their V and J segments (which are both flanked by 23 RSSs that prevent direct V to J rearrangements).

The murine D elements are classified into four families: DSP2, DFL16, DST4, and DQ52. Although D regions could theoretically contribute to Ig diversity by being read in all three frames, the mouse has evolved mechanisms that strongly favor one of them.[5] Four functional germline J_H sequences reside about 0.7 kb downstream of the most 3′ D region, DQ52.

The Human Immunoglobulin H Germline Variable, Diversity, and Joining Gene Segments

V_H Segments

The human V_H locus spans 1.1 Mb at the telomeric end of chromosome 14 (14q32.33) (see Table 6.1). The human germline V_H segments—numbering roughly 40 to 45—fall into seven families that, in contrast to the family clusters characteristic of the murine locus, are extensively interdigitated (see Fig. 6.4). Some human V_H sequences are polymorphic owing to V_H insertions or deletions in different allelic chromosomes. Twenty-four additional germline V_H sequences have been mapped to chromosome 15 and 16 and represent nonfunctional "orphans" that were apparently duplicated from the IgH locus on chromosome 14.[6]

Diversity and J_H Regions

Twenty-six human D elements are located in an ˜40 kb region about 20 kb downstream of VH6, the most 3′ of the V_H genes.[7] This D cluster is comprised of four tandem duplications of a 9.5 kb segment containing a representative of each of six D families. The twenty-seventh D element—DHQ52— is the only one showing sequence similarity to a mouse segment (DQ52) and shares a homologous location just 5′ to J_H1. In contrast to mice, humans use all reading frames of D elements.[7] One reading frame encodes primarily hydrophilic residues, one encodes hydrophobic residues, and one includes frequent stop codons. Some D

elements contain stop codons that can be removed by nuclease trimming during VDJ assembly. As in mice, the human J_H cluster is immediately downstream of DHQ52.

Heavy Chain Constant Regions

Murine and human genomic clones containing C region H-chain (C_H) genes include separate exons encoding the ˜100 to 110 amino acid Ig domains. These domains were independently identified by internal homologies of amino acid sequences and by three-dimensional structural analysis (X-ray crystallography). The exons are separated from each other by introns of roughly 0.1 to 0.3 kb. Thus, for example, the mouse γ2b protein has three major domains (CH1, CH2, and CH3) with a small hinge domain between CH1 and CH2. The gene structure may be summarized as follows:

CH1 - intron - **hinge** - intron - **CH2** - intron - **CH3**

(292) (314) (64) (106) (328) (119) (322)

where the numbers in parentheses represent the number of nucleotides in each segment. As an interesting contrast, the hinge region of the α gene is encoded contiguously with the CH2 domain with no intervening intron, while the unusually long human γ3 hinge is encoded by three or four hinge exons.

Genomic Organization of the C_H Region

Each B-lymphocyte initially produces IgM by expressing an assembled variable region linked to Cμ, but may use CSR (discussed later in this chapter) to replace Cμ with one of the several C_H regions lying downstream, thereby allowing expression of IgG, IgA, or IgE (Fig. 6.5A). Eight murine C_H genes span about 200 kb of DNA on chromosome 12; these genes were linked by contiguous clones in 1982.[8] Several γ pseudogenes lie within the clustered γ functional genes[9] (Fig. 6.5B). The coding sequences of all C_H genes are oriented in the same direction.

The human C_H genes were similarly cloned, and then eventually completely linked by the Human Genome Project. The human IgH locus contains a large duplication, with two copies of a γ-γ-ε-α unit separated by a γ pseudogene (see Fig. 6.5B). One of the duplicated ε sequences is also a pseudogene, and a third closely homologous ε-related sequence—a "processed" pseudogene—is present on chromosome 9.

The IgH locus has also been examined in several other species besides mouse and human, and several notable differences have been observed. Rabbits, for example, have 13 Cα sequences and only a single Cγ gene[10]; this unusual expansion of genes contributing to mucosal immunity may be related to the peculiar habit of coprophagy in these animals. In contrast to the multiplicity of rabbit Cα genes, pigs have only one Cα gene and eight Cγ genes. Camels are unusual in having H chains that function in the absence of L chains.[11]

Membrane versus Secreted Immunoglobulin

Igs are found either as secreted molecules in the serum or as membrane-bound receptors. The membrane-bound μ chains contain a C-terminal hydrophobic transmembrane domain consisting of 26 uncharged hydrophobic amino acids encoded by additional membrane exons, and these residues anchor the protein in the cell membrane lipid bilayer. The membrane (μ_m) and secreted (μ_s) forms are derived from the same gene by alternative splicing (Fig. 6.6). The same general gene structure has been found for other C_H genes, suggesting that differential splicing accounts for the two forms of all Ig isotypes.

Early B cells make roughly similar quantities of both μm and μ_s, whereas maturation to the plasma cell stage is associated with strong predominance of μ_s production, facilitating high-level secretion of circulating Ig. The balance between the two ribonucleic acid (RNA) splice forms of μ has been interpreted as a competition between splicing of the CH4 and M1 exons versus the cleavage/polyadenylation at the upstream μ_s poly(A) site. These processes are mutually exclusive because CH4-M1 splice removes the μ_s poly(A) site, while cleavage at the μ_s poly(A) site removes the membrane exons.

Cis-regulatory elements (and corresponding transacting RNA binding proteins) control the balance between these

processes. They include a GU-rich element downstream of the μs poly(A) site,[12] the polyadenylation factor cleavage stimulator factor 64,[13] and the U1A protein.[14] These factors likely function downstream of B-lymphocyte-induced maturation protein-1 (BLIMP-1) whose expression in Ig-secreting plasma cells was also found to be critical for μ_S poly(A) site utilization.[15] *Cis*-acting sequences affecting the ratio of alternative splice forms have been described for other isotypes besides Cμ, particularly Cα.[16]

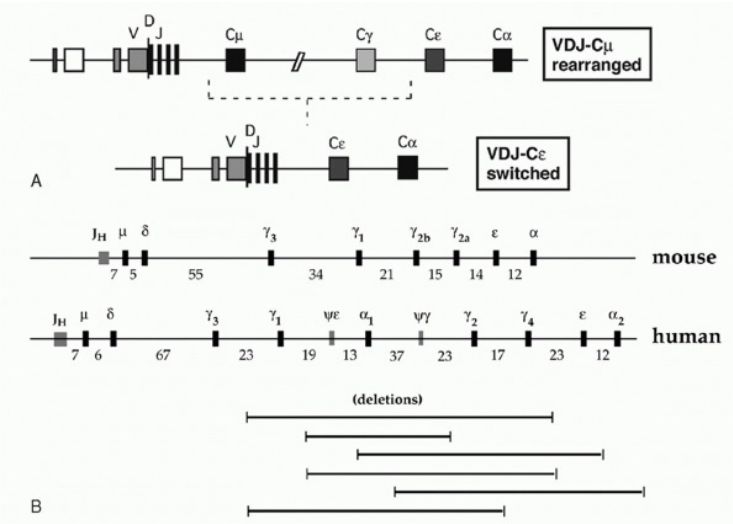

FIG. 6.5. Deletional Isotype Switch Recombination. A: The expression of "downstream" heavy chain genes is accomplished by a recombination event that replaces the Cμ gene with the appropriate heavy chain constant gene (Cε is shown as an example), deleting the deoxyribonucleic acid between the recombination breakpoints. **B:** The murine and human heavy chain constant region genes are diagrammed with the approximate intergene distance indicated below (in kb); various literature values for these distances differ somewhat, possibly due to allelic polymorphisms. The human locus shows a large duplication of γγεα sequences.

Membrane Ig serves as the antigen-recognition component of the BCR that is critical for initiating the signal for lymphocyte activation following contact with antigen. Transduction is mediated by an associated protein dimer composed of the BCR components Igα and Igβ (CD79a and CD79b) whose cytoplasmic domains contain immunoreceptor tyrosine-based activation motifs similar to those found in the CD3 chains mediating TCR signaling. Additional signaling is mediated by conserved tyrosines in the cytoplasmic tails of the IgG and IgE H chains, which serve as a phosphorylation-dependent docking sites for the signaling adapter Grb2.[17] Binding of Grb2 enhances BCR signaling and subsequent B-cell proliferation.

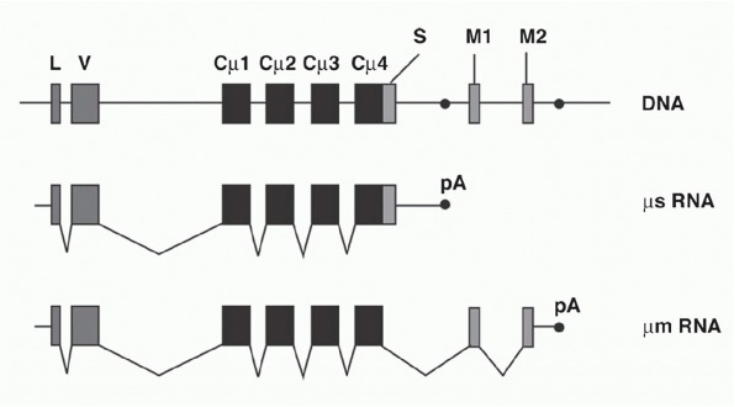

FIG. 6.6. Two Ribonucleic Acids (RNAs) Generated from the μ Gene by Alternative Processing. The *top line* illustrates the exons of the μ gene (*black rectangles*) in an expressed, rearranged μ gene. A primary transcript including all the exons present in the deoxyribonucleic acid can be processed as shown to yield either μ_S RNA (containing a C-terminal "secreted" sequence) or μ_m RNA (containing the two membrane exons).

Kappa Light Chain Genes

Murine Germline Vκ Locus

The murine Vκ locus spans about 3.2 mb on chromosome 6[18] and contains 20 Vκ families, some of which are shared by human and mouse (see Table 6.1). Vκ sequences within a single family are largely clustered together. Some Vκ elements lie in the opposite orientation to that of the Jκ and Cκ elements, and these Vκ segments undergo VJ recombination by an inversion rather than deletion (Fig. 6.7). A few Vκ sequences have been localized to chromosome 16 and 19 and are considered orphan genes.

Human Germline Vκ Locus

The human Vκ locus (see Table 6.1) lies on the short arm of chromosome 2 (2p11-2) spanning ~2 mb of DNA.[19] The locus includes a large inverted duplication, so that most Vκ sequences exist in pairs with one copy lying in the cluster proximal to Jκ (and in the same orientation) and a second copy (inverted) in the distal cluster. The average sequence similarity between duplicates is 98.9%, suggesting the duplication occurred less than 5 million years ago. This is consistent with the absence of such duplication in chimpanzees, which diverged from the human lineage approximately 6 million years ago. Interestingly, about 5% of human alleles also lack the distal duplication.

Outside the Igκ locus, at least 25 orphan Vκ segments have been identified in clusters on chromosome 1, 2, and 22. The orphan cluster located in the long arm of chromosome 2 was probably separated from the major locus—on the short arm of this chromosome—by a pericentric inversion (which must have occurred rather recently in evolution as it is absent from chimpanzee and gorilla).

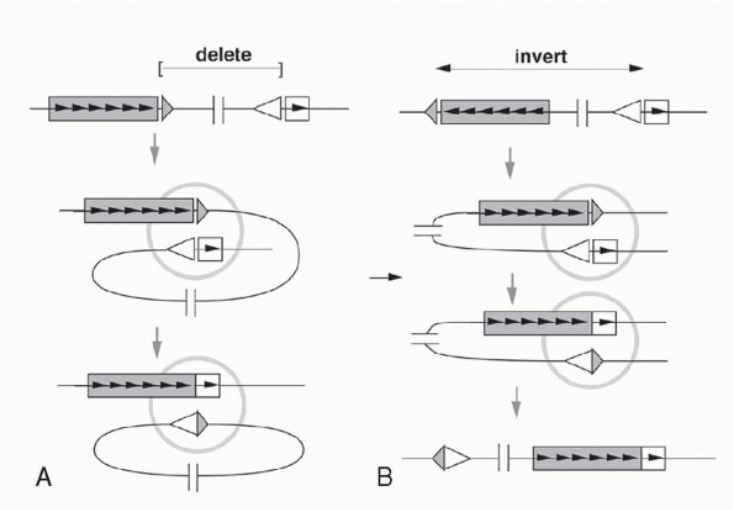

FIG. 6.7. The Same "Micro" Mechanism of Recombination can Join Vκ and Jκ by Deletion or Inversion, Depending on the Relative Orientation of the Two Precursors in Germline DNA. A: When V coding sequence (*shaded rectangle*) and J coding sequence (*white rectangle*) are oriented in the same 5′ → 3′ direction in germline DNA (as indicated by the *internal arrowheads*), the recombination yields a VJ coding joint plus a DNA circle containing the signal joint (*apposed triangles*). **B:** If V is oriented in the opposite direction in germline DNA, an identical recombination reaction at the "micro" level (*inside shaded circle*) leaves the signal joint linked to the recombined VJ coding joint.

Jκ and Cκ Elements

In comparison to the H chain genes, the organization of the C region segments in the κ locus is relatively simple (see Table 6.1). A single Cκ gene with a single exon and with no reported alternative splice products is found in both mouse and human. While all five Jκ elements are functional in humans, the third J element in mice has not been observed in functional κ L chains.

Apart from the typical Vκ-Jκ rearrangements, an additional recombination event occurs uniquely in the κ locus. The event is mediated by V(D)J recombination utilizing a 23-RSS element—designated Recombining Sequence in the mouse[20] and Kappa Deleting Element in the human[21]—that is positioned in an intergenic region downstream of Cκ; the recombination results in the deletion of the Cκ exon. Hence, Cκ fragments are undetectable on Southern blots of DNA from λ-expressing human lymphoid cells,[22] as in most B cells the Cκ genes are apparently deleted from both chromosomes before Igλ gene rearrangement begins.

Lambda Light Chain Genes

Murine λ Locus

In laboratory mouse strains, only about 5% of the B-lymphocytes utilize Igλ L chain, and the diversity of these L chains is meager due to the very small number of V region genes (Fig. 6.8). Complete sequence analysis[23] of the murine locus revealed two V-J-C clusters (Vλ2-Vλx-Jλ2Cλ2-Jλ4Cλ4 and Vλ1-Jλ3Cλ3-Jλ1Cλ1) separated by about 110 kb. Each Jλ is linked to its own Cλ region gene, but Jλ4 is nonfunctional. Recombination occurs largely within each cluster, although Vλ2Cλ1 products are occasionally observed. The ancestry of the Vλx element is uncertain, as it is rather dissimilar to the other Vλ segments; indeed, it resembles Vκ as much as Vλ. In contrast to the Igκ locus, the Vλ segments are flanked by 23-RSS and the Jλ gene segments by 12-RSS (see Fig 6.3).

Human λ Locus

The human Vλ region was characterized by intensive cloning, sequencing, and mapping of Vλ elements and ultimately by the complete sequence analysis of 1 Mb covering the entire locus[24] (see Table 6.1). Within the Vλ cluster lies the human VpreB gene (discussed below), as well as several genes and pseudogenes unrelated to the Igλ system.

λ L chains are much more abundant in man than in mouse (about 40% of human L chains are λ versus about 5% in mouse). Four forms of human λ chains have been classified serologically, with differences residing in a small number of amino acids in the C region. The serologic classification of Kern+ corresponds to a glycine at position 152 versus a serine in Kern-. The Oz+ designation corresponds to a lysine at position 190 versus an arginine in the Oz- variant. Similarly, Mcg+ λ chains (versus Mcg-) contain asparagine 112 (versus alanine), threonine 114 (versus serine) and lysine 163 (versus threonine).

Four functional human Jλ-Cλ segments and three pseudogenes are clustered within an approximately 33 kb region of DNA (see Fig. 6.8) and the four major expressed human λ isotypes correspond to the functional JCλ1, JCλ2, JCλ3, and

JCλ7, with the latter encoding an isotype provisionally designated Mcp.[25] JCλ6 may be functional in some individuals, and the common allele—which has a 4 bp insertion leading to a deletion of the C-terminal third of the Cλ region— can nevertheless undergo Vλ-Jλ recombination, encoding a truncated protein that can associate with H chains. A variety of polymorphic variants of the human λ locus have been detected, apparently the result of gene duplication, as shown in Figure 6.8.[26] Lastly, three Cλ-related sequences have been discovered near the major Jλ-Cλ cluster. One of these, designated λ14.1, represents the human homolog of the murine "surrogate" L chain λ5 (see following discussion).

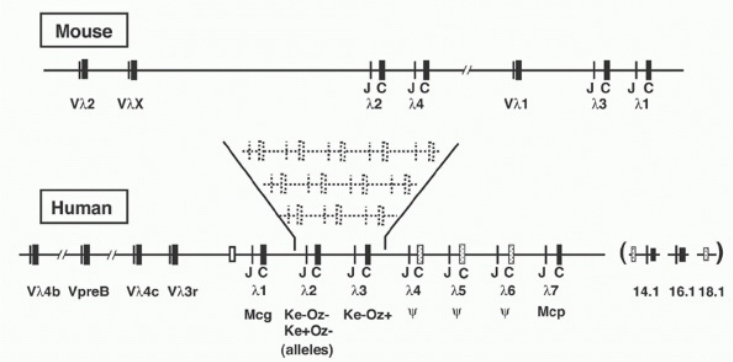

FIG. 6.8. Germline λ Genes. The maps in this figure are schematic (i.e., not to scale). **A:** The murine λ gene system includes four JC complexes and three V genes, as shown. **B:** The human λ locus includes multiple V genes, of which only three are shown. The human VpreB "surrogate" light chain gene is located within the Vλ cluster. The Cλ locus includes a segment of seven JC complexes plus three additional unlinked sequences. The hatched JC complexes diagrammed above the seven linked λ sequences represent polymorphic variants with additional duplications of the JC unit. The 14.1 sequence—the human λ5 "surrogate" light chain homolog—lies downstream of the JC cluster. Exon 1 of the 14.1 gene is homologous to an exon upstream of Jλ1 (as indicated by the *unlabeled white rectangle*).

λ-Related "Surrogate" Light Chains

Ig H chains cannot reach the cell surface without pairing with Ig L chains. However, Ig μ H chains can be detected on the surface of pre-B cells whose Igκ and Igλ loci are still in their germline configuration and thus do not produce L chains. In these cells, a "surrogate L chain" (SLC) composed of two smaller proteins, VpreB and λ5, facilitates the surface expression of the μ H chain protein. The first component (λ5) was identified as the product of a gene expressed exclusively in pre-B cells that showed high sequence similarity to the J and C

regions of the λ locus,[27] As four murine Cλ genes were already known, it was designated λ5. The second component of the SLC was identified as a gene residing about 4.7 kb upstream of λ5 in the mouse genome. Based on its similarities to both Vλ and Vκ (and its expression in pre-B cells), it was called VpreB1. A second, nearly identical sequence in the mouse genome is named VpreB2 and appears to be functional,[28] and a less similar VpreB3 has also been described. Neither λ5 nor VpreB genes show evidence of gene rearrangement in B or pre-B cells, and homologs have been found in every mammalian species examined.

The two SLC proteins form a L chain-like heterodimer that is able to fulfill some functions of a true L chain, including association with μ H chains to permit surface μ expression prior to the availability of κ or λ L chains. Thus, when a μ H chain gene was transfected into an Ig-negative myeloma line, no surface μ expression was observed unless λ5 and VpreB genes were also transfected.[29] Surface μ chains are covalently linked to the λ5 protein, while the VpreB1 protein is noncovalently associated. The expression of μ-SLC on the surface of pre-B cells triggers the onset of Vκ-Jκ rearrangement, as discussed below.

In humans, three λ5-like sequences are located downstream of the human Cλ cluster on chromosome 22 (see Fig. 6.8), but only one—designated 14.1—appears to be functional. The human VpreB homolog lies within the Vλ cluster[30] in contrast to murine VpreB, which lies close upstream of λ5.

V(D)J RECOMBINATION

The mechanism by which germline variable region segments (V_L and J_L, or V_H, D, and J_H) are assembled in the DNA to form a complete active V region has been pursued ever since Ig gene recombination was first discovered. In this section we will address 1) the molecular mechanism of the reaction, 2) the topology of the recombination events, 3) the components of the recombinase machinery, and 4) the regulation of that machinery during B-cell development.

Molecular Mechanism of V(D)J Recombination

Recombination Model Overview

A model for the detailed mechanism of the V(D)J recombination event must account for the observed features of the recombination *products* and of their germline *precursors*. In the germline *precursors*, the RSSs with their heptamer, nonamer, and appropriate 12 or 23 bp spacers are necessary and sufficient to create efficient recombination targets; model substrates in which RSSs flank DNA sequences completely unrelated to Ig genes are competent to undergo recombination. The model shown in Figure 6.9 will serve as a framework for discussion of the recombination mechanism. The recombination is thought to begin with binding of the RAG1-RAG2 complex to the RSSs that flank the two gene segments to be recombined. Simultaneous DNA cleavage occurs precisely between the RSSs and the gene segments. The two ends of the RSSs (frequently named “signal ends”) are joined directly, forming “signal joints.” In contrast, the ends of the gene segments (also referred to as “coding ends”) are processed prior to joining and are ultimately ligated together, giving rise to “coding joints” and completing the recombination event.

Recombination Products: Coding Joints and Signal Joints

In the recombination *products*, signal joints are typically direct ligation products of the signal ends: the RSSs are joined directly at the heptamers (“back-to-back”), and nucleotide additions or deletions at these junctions are quite rare. The properties of the coding joints, however, are more complex, as the joining reaction at these DNA ends is “imprecise.” The following features are frequently present:

1. Deletions: variable number of bases are deleted from the ends of the coding regions (in comparison to the “complete” sequence in the germline precursor)
2. Nongermline (“N”) nucleotides: random nucleotides (with a bias toward G and C) are added by a templateindependent DNA polymerase (discussed below). The sequence of

these N nucleotides has no relationship to the germline V, D, or J sequences.

3. Palindromic ("P") nucleotides: the ends of the coding gene segments are sealed by a DNA hairpin structure (see Fig. 6.9, and discussed in the following). "Opening" of these hairpins frequently occurs by nicking at some distance away from the hairpin tip leading to single-stranded overhangs. Filling in of such overhangs by DNA polymerases generates DNA palindromes that mirror the nucleotides at the end of the V, D, or J segment.[31] P nucleotides are generally only one or two bps, but they can be longer, especially in mice with the severe combined immunodeficiency defect (SCID) disorder in which the opening of the hairpins occurs in an aberrant manner.

Recombination Intermediates: Blunt Signal Ends and Hairpin Coding Ends

To study broken DNA ends as intermediates in V(D)J recombination, several laboratories employed ligation-mediatedpolymerase chain reaction (LM-PCR) to detect signal ends. This technique involves ligating blunt double-stranded oligonucleotide linkers to blunt genomic DNA breaks, and then amplifying the ligation junctions between a primer in the ligated oligonucleotide and a primer based on known sequence from the ligated genomic DNA; amplification products can

then be cloned and sequenced. LM-PCR analyses of both TCR and Ig genes undergoing V(D)J recombination showed the signal ends to be blunt double strand breaks (dsbs), usually exactly at the heptamer border.[32] Similar LM-PCR experiments failed to detect the coding ends unless they were pretreated with mung bean nuclease, a single-strandspecific endonuclease that recognizes the distortion of DNA at a hairpin structure. Sequences of these LM-PCR products from coding ends suggested that the hairpins are precisely at the end of the coding elements, usually without loss or gain of a single nucleotide.[33] By Southern blot analyses, coding ends were found to have two properties suggestive of a hairpin-like structure: 1) resistance to exonuclease treatment, and 2) doubling of the apparent length of restriction fragments under denaturing electrophoresis conditions.[34]

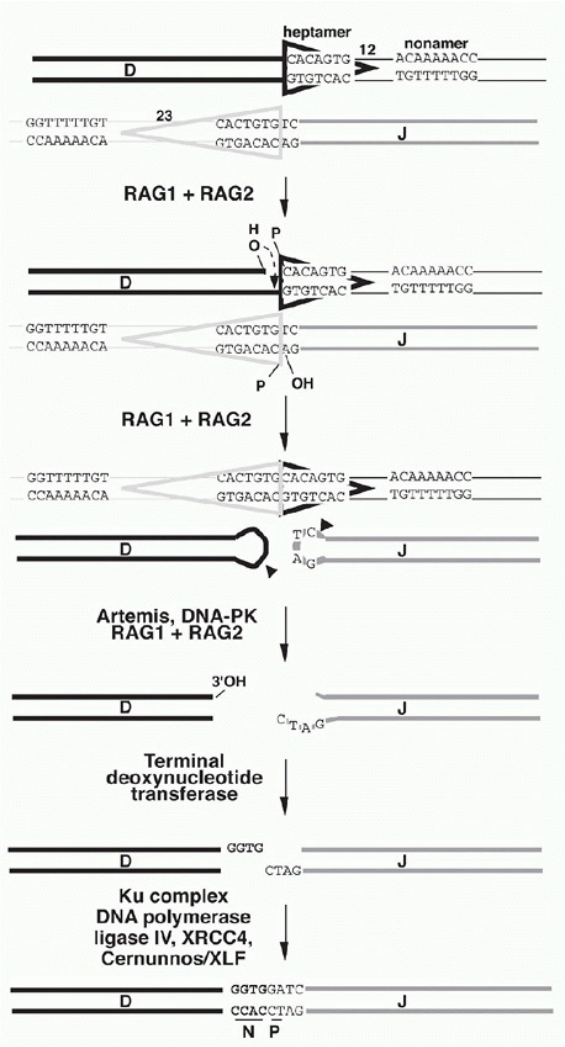

FIG. 6.9. Model for V Assembly Recombinations. All V assembly recombination reactions (in immunoglobulin and T-cell receptor genes) may proceed by a common mechanism, illustrated here by D-J recombination. The recombination signal sequences (RSSs) are included in *triangles*, which is the conventionally used RSS graphic. Hairpin loops are created on coding ends dependent on the action of the two Recombination Activating Genes: RAG1 and RAG2. After the opening of the hairpin loops, the pictured diversity coding sequence shows the effects of "nibbling" by exonuclease, but the joining coding sequence is spared and shows P nucleotide generation; N region addition is pictured in this example as occurring only on the diversity region end. In reality, exonuclease digestion and N nucleotide addition can occur on either (or both) ends. The steps in the proposed mechanism are discussed in the text.

Hairpin ends represent V(D)J recombination intermediates that, in wild-type cells, are opened at the hairpin tip (or a few nucleotides away from it) by the Artemis nuclease (discussed below). P nucleotides result from opening the loop at an asymmetric position (see Fig. 6.9); this model would explain why P nucleotides are never observed at coding ends that have been "nibbled" after opening of the hairpin. P nucleotide segments in the rare coding joints observed in SCID mice are unusually long and likely result from resolution of hairpins by nicking enzymes that, unlike Artemis, do not focus on the area near the tip of hairpin loops but instead nick in variable positions in the double-stranded hairpin "stem."[34]

Topology of V(D)J Recombination

Deletion versus Inversion

If a V segment and a J segment are both oriented in the same direction, they can recombine by excising the DNA between the coding sequences and ligating the two coding ends. Ligation of the two signal ends produces a DNA circle that generally lacks replication origins and therefore fails to replicate as cells divide after V(D)J recombination. Such excision circles are therefore generally absent in mature B-lymphocytes that have already undergone several rounds of proliferation after completing the Ig gene assembly. By isolating circular DNA from cells actively undergoing Vκ-Jκ rearrangement, it is possible to isolate and characterize the circular molecules bearing signal joints.[35]

As mentioned previously, some germline Vκ genes are oriented in the opposite direction from the Jκ-Cκ region. In these cases, VJ recombination occurs by an inversion of the DNA between the recombining V and J segments, leaving both the VκJκ coding joint and the signal joint (formed by ligating the RSSs) retained in the chromosome (see Fig. 6.7). This demonstrates that the enzymatic machinery "sees" only the DNA in the immediate vicinity of the recombination site and is insensitive to the topology of the DNA strands far from this site.

Nonstandard Joints

In addition to the canonical coding and signal joints, several "nonstandard" recombination joints have been documented, that, though not contributing to physiologic Ig gene assembly, represent tell-tale signs of a recombination event.[36] In the first phase of V(D)J recombination, the DNA is cut at both gene segment-RSS boundaries that participate in the reaction, thereby generating four DNA ends. In principle, there are three possible topologies in which these DNA ends can be rejoined:

1. "Signal and coding joints": the standard reaction product in which the two coding ends get joined generating the assembled VJ gene and the 12-RSS/23-RSS signal joint.
2. "Open and shut joints": the RSSs get ligated back to the gene segments from which they were released. These joints are topologically identical to the starting DNAs, but can be distinguished from them if nucleotides have been added or deleted at the junctions.
3. "Hybrid joints": joints in which the RSSs have traded places so that the 23-RSS that was flanking the Vκ segment is now linked to the Jκ segment, and vice versa.

"Hybrid" and "open and shut" joints have been observed in transfected plasmids bearing artificial recombination substrates[36] as well as in endogenous Ig loci *in vivo*.[37]

Secondary V(D)J Recombination

As discussed previously, imprecise joining of gene segments causes about two-thirds of all recombination products to be out-of-frame. Thus, a B-lymphocyte could end up with nonproductively rearranged Igκ genes on both alleles. However, germline Vκ segments lying upstream of an initial Vκ-Jκ recombination junction can recombine with Jκ segments lying downstream of the junction, producing a "secondary" recombination event, as shown in Figure 6.10A.

Such secondary recombination also occurs in cells that have assembled a productive Vκ-Jκ joint if the encoded antigen binding domain recognizes an autoantigen. This type of secondary recombination, known as "receptor editing," is considered in more detail later in this chapter.

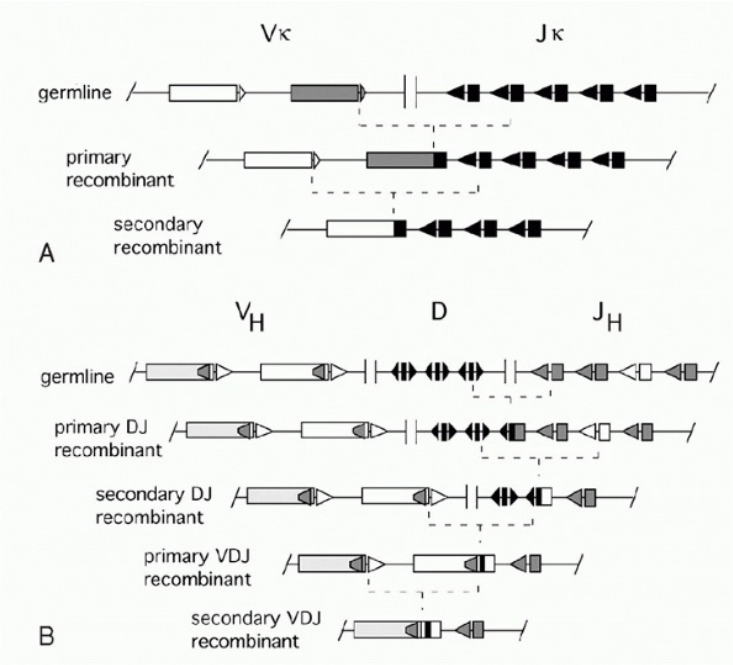

FIG. 6.10. Secondary Recombinations. A: In the κ light chain system, a primary recombination can be followed by recombination between an upstream V and a downstream J. **B:** Analogous secondary recombinations can occur in the heavy chain system between upstream D and downstream J segments. After V(D)J recombination eliminates all 12-RSS signal elements from the chromosome, secondary recombination can still occur between VH (23-RSS) and an internal heptamer within the VH coding sequence of the VDJ unit.

In the IgH locus, secondary D-J rearrangements sometimes occur, but only until V_H-DJ_H recombination removes all unused upstream D_H segments (Fig. 6.10B). VDJ rearrangement eliminates all the 12-RSSs from the IgH locus that could pair with the 23-RSSs flanking the upstream V_H elements. Sometimes, these V_H segments do, however, recombine with an established VDJ unit, displacing most of the originally assembled V_H element,[38] a process sometimes called V_H replacement. Such events are mediated by cryptic RSSs (mainly a heptamer sequence) that is present near the 3′ end of about 70% of all V_H genes (see Fig. 6.10B). Such internal cryptic RSSs are not generally found in L chain genes. As discussed previously for the L chain, secondary recombination represents a rescue mechanism for cells with nonproductive rearrangements on both H chain chromosomes, and for cells whose encoded antibody recognizes an autoantigen.

The V(D)J Machinery

Since the discovery of V(D)J recombination as the process that assembles the germline antigen receptor gene segments into functional genes, one major question was the identity of the enzymatic machinery catalyzing this complex set of reactions. Genetic and biochemical work by a large number of laboratories led to identification of a total of 13 different proteins that have been shown to be directly involved in V(D)J recombination: RAG1, RAG2, HMG1, Ku70, Ku80, DNA-PKcs, Artemis, pol μ, pol λ, TdT, XRCC4, Cernunnos/XRCC4-like factor (XLF), and DNA ligase IV. The only lymphoid-specific factors are RAG1, RAG2, and TdT; all others are ubiquitously expressed in all cell types, and this feature allows investigators to study aspects of V(D)J recombination by ectopically expressing the RAG proteins in nonlymphoid cells. A recent biochemical tour de force study showed that coding joint formation could be recapitulated *in vitro* using artificial recombination substrates and highly purified preparations of all 13 proteins.[39] The respective coding joints showed all of the features typically observed *in vivo* (nucleotide deletion, N nucleotide, and P nucleotide

addition), suggesting that most, if not all, of the factors involved in the coding end processing steps of V(D)J recombination have been identified. In contrast, signal joint formation was not observed. This step seems to require the removal of the RAG proteins after the cleavage reaction and is likely to require additional factors as yet unidentified.

Recombination Activating Gene Proteins: Mediators of Early Steps in V(D)J Recombination

A major advance in the investigation of V(D)J recombination was the identification of two genes whose products are critical for this process in the B and T cell lineages. In the pioneering experiments, Schatz and Baltimore[40] stably transfected fibroblasts with a construct containing a selectable marker whose expression was dependent on V(D)J recombination; as expected, no measurable recombination occurred in this nonlymphoid cell. However, when either human or murine genomic DNA was transfected into these fibroblasts, a small fraction of recipient cells stably expressed recombinase activity, activating the selectable marker. This suggested that a single transfected genomic DNA fragment was able confer recombinase activity in a fibroblast. (Presumably the fibroblast contained endogenous copies of the same genes, but their expression was repressed by mechanisms that could not repress the transfected genes.) This active fragment was cloned and turned out to contain two closely linked genes, designated RAG1 and RAG2, respectively. Both RAG1 and RAG2 are essential for recombination; therefore, these genes would not have been discovered by this transfection technique if they had not been closely linked in the genome. The genes are notable for having no introns splitting up their open reading frame in most species, and for their opposite transcriptional orientation in all species examined.

A crucial role for the RAG genes in V(D)J recombination was supported by the conservation of these genes in all jawed vertebrate species analyzed thus far, from shark through man. RAG1 and RAG2 are expressed together in developing B and T cells, specifically at the stages at which V(D)J recombinase activity is required for the assembly of Ig and TCR genes. Moreover, mouse strains in which either gene has been eliminated by homologous recombination (gene “knockouts”) have no mature B or T cells, as the result of their inability to initiate V(D)J recombination.[41,42] Similarly, a subset of human patients with SCID syndrome characterized by the complete absence of T- or B-lymphocytes have been found to have null mutations in RAG genes.[43] Patients with hypomorphic alleles often have a complex set of features (oligoclonal T cells, hepatosplenomegaly, eosinophilia, decreased serum Ig but elevated IgE) known as the Omenn syndrome, which can also be caused by defects in other genes involved in V(D)J recombination. Interestingly, the same RAG mutation in different patients can cause either Omenn syndrome or SCID, depending on unknown factors.[44]

RAG1 shows intrinsic binding affinity for the RSS nonamer sequence via its nonamer binding domain even in the absence of RAG2. Exhaustive mutational analysis has revealed that RAG1 contains the catalytic center of the RAG complex, composed of three amino acids critical for all enzymatic activity: D600, D708, and E962.[45,46] RAG2, on the other hand, serves as a regulatory cofactor; it has no intrinsic binding affinity for RSSs, but once bound to RAG1 improves the strength and specificity of RAG1 RSS contacts.[47,48] It is also enhances RAG activity on chromosomal substrates and it restricts V(D)J recombination to the G0/G1 stage of the cell cycle (both features are discussed below).

Attempts to determine the molecular role of the RAG proteins in cell-free recombination assays were initially hampered by poor solubility of the proteins, but functional analyses of truncated RAG genes (using RAG expression vectors cotransfected into fibroblasts along with recombination substrate plasmids) revealed that surprisingly large segments of both proteins could be deleted without eliminating recombinase activity, and some of the remaining core regions were

soluble and could be handled relatively easily in experiments. This work allowed the demonstration that in a cell-free *in vitro* system, core regions of the two RAG proteins together are capable of carrying out cleavage of substrate DNAs as well as hairpin formation

on the coding end.[49]

The RAG-mediated cleavage occurs in two steps: first a nick is introduced on the top strand between a gene segment and the adjacent heptamer (see Fig. 6.9), then the 3′-hydroxyl group participates as the nucleophile in a direct transesterification reaction to attack the phosphodiester bond adjacent to the heptamer on the bottom strand (see Fig. 6.9), yielding a DNA hairpin structure on the coding end and a new 3′-hydroxyl group on the 3′ end of the bottom heptamer strand.[50] After DNA cleavage, the RAG proteins remain in a complex with the DNA ends and facilitate aspects of the joining phase. Mutant forms of RAG1 or RAG2 have been reported that are competent for cleavage but show impairment in coding or signal joint formation.[51]

While nicking can occur asynchronously at the 12-RSS and 23-RSS, hairpin formation is "coupled" and occurs synchronously at both RSSs. *In vitro*, coupled cleavage requires only the RAG proteins, HMG1/2 (discussed below) and Mg^{2+} as the divalent metal ion in the reaction buffer. *In vivo*, DNA dsb formation at an individual RSS is dangerous as it could give rise to translocations, and it is thought that Mg^{2+} promotes an optimal molecular "architecture" for controlled V(D)J recombination. *In vivo* experiments indeed suggest that RAG proteins may bind to and introduce a nick at a single 12-RSS, but do not complete DNA cleavage until a matching 23-RSS is captured into the RAG-RSS complex.[52]

In addition to the "classical" activities of RAG proteins on DNA segments containing RSSs, these proteins can also catalyze DNA strand cleavage on "nonstandard" substrates.

1. Transposition. *In vitro*, purified recombinant core RAG proteins can catalyze the excision and insertion of a DNA fragment with signal ends into foreign DNA, acting as a transposase.[53,54] This property provides additional support for the early speculation that the V(D)J recombination system may have originated by insertion of transposon-like DNA fragment encoding RAG genes (and bearing RSSs at its ends) into a primordial antigen receptor gene, thereby generating a pair of separated V and J gene segments. This model of the origin of V(D)J recombination is consistent with the many mechanistic similarities at the molecular level between Ig gene rearrangements and transposition,[55] and the recent identification of the Transib transposase family that shows striking sequence similarity to RAG1 and is widespread in insect, echinoderm, helminth, coelenterate, and fungal genomes.[56] The recent finding of an apparent homolog of the entire RAG1 and RAG2 gene locus in a sea urchin genome suggests that the two RAG genes may have entered the genome of a common ancestor of all deuterostomes far earlier than the Ig-/TCR-based adaptive immune system developed.[57] It remains unclear whether the primordial RAG transposon encoded solely RAG1 (which would then have integrated next to the primordial RAG2 gene) or both RAG1 and RAG2. The transposase activity of RAGs, however, seems to be almost completely suppressed *in vivo*, and the C-terminus of RAG2 may have evolved to control this potentially deleterious activity.[51,58,59,60]
2. V_H replacement. As mentioned previously, recombination events can occur between a V_H 23-RSS and cryptic RSS within rearranged V_H coding sequences. An *in vitro* model suggests that in V_H replacement, the RAG proteins nick both DNA strands without forming a hairpin coding end.[61] Whether this is indeed a completely different activity is unclear.
3. Translocations at non-RSS sequences. The RAG complex also generates two nicks to cleave within the major breakpoint region of the *Bcl2* gene. This 150-bp segment is the target of a common RAG-catalyzed translocation between the IgH locus and the *Bcl2* gene occurring in most follicular lymphomas. In this segment, there are no RSSs, and the RAG proteins recognize an unusual sequence-dependent DNA conformation different from the normal B-form double helix.[62]

Although the "core" RAG proteins have been useful for elucidating the molecular mechanism of the cleavage step of V(D)J recombination in biochemical studies, it is clear that the "noncore" portions of each protein confer important functions, as expected from their sequence conservation across species. Broadly speaking, the "noncore" regions ensure

regulated and efficient recombination on the physiological substrates (i.e., imperfect RSSs deviating from the perfect consensus heptamer and nonamer) in the context of chromatin. The functions of the “noncore” regions have largely been inferred by comparing V(D)J recombination products from cells expressing core RAG proteins versus full-length versions, and more recently by *in vitro* studies using full-length RAG proteins that are now available for such analyses.

The C-terminal region of RAG2 has multiple functions and is important for achieving normal numbers of B- and T-lymphocytes *in vivo*,[63] for the formation of precise signal joints during IgH recombination,[64] and for protecting against RAG-mediated DNA transposition.[51,65] These functions are thought to be conferred at least in part, by a plant homeo domain (PHD) zinc finger fold that is formed by amino acids 414 to 487 in murine RAG2. This PHD domain binds specifically to the tails of histone H3 that are trimethylated at lysine 4 (H3K4Me3),[66,67,68] a histone modification that is associated with “open” chromatin and that is uniquely present on “accessible” RSSs in Ig loci (discussed below). *In vitro* studies suggest that the binding of the RAG2 PHD domain to histone tails causes a conformational change that increases the catalytic activity of the RAG complex.[69]

Furthermore, the RAG2 C terminus regulates RAG2 protein levels—and hence V(D)J recombinase activity—across the cell cycle to prevent dsbs during DNA synthesis or mitosis, when such breaks could lead to chromosomal deletions.[32] RAG1 protein and messenger RNA (mRNA) transcript levels of both RAG genes vary little across the cell cycle, but phosphorylation of RAG2 at Thr490 by the cyclin-dependent

kinase cdk2 mediates its destruction via ubiquitination and proteasomal degradation during S phase.[70] Mice expressing RAG2 with a T490A mutation (which cannot be phosphorylated) showed RAG2 protein and dsbs throughout the cell cycle, demonstrating the importance of the RAG2 degradation signal in cell-cycle control of V(D)J recombination.[71,72]

The N-terminal noncore region of RAG1 is required *in vivo* for optimal RAG1 activity and for the formation of precise signal joints in D-J recombination.[64] This region of RAG1 contains a RING finger domain that seems to be required for ubiquitination of several proteins, including histone H3.[73]

Apart from the obvious importance of the RAG proteins in understanding the initial steps of V(D)J recombination, knowledge of these proteins and their genes has allowed two major technical advances that have opened the way to many additional experiments. First, various nonlymphoid cell lines with known defects in various DNA repair genes have been transfected with the RAG genes to identify genes involved V(D)J recombination (these factors are described below). Second, availability of the RAG1 and RAG2 knockout mice has been instrumental in a large number of immunology studies. These mice completely lack functional B cells or T cells, and are not “leaky” like SCID mice, which develop some functional B and T cells, especially as the animals age. Thus the RAG-deficient mice can be used to study the importance of the “innate” immune system (i.e., responses that occur in the absence of antigen-specific lymphocytes) in particular immune responses. They can also be used as recipients for various lymphocyte populations to explore the roles of different cell types. They can also be used as recipients for various lymphocyte populations to explore the roles of different cell types. They can be transfected with transgenes encoding specific Ig genes to study the roles of specific antibodies in B cell development and in immune responses. Finally, they can be used in “RAG complementation” experiments designed to assess the phenotype —in lymphocytes—of various other gene knockouts.[74] In RAG complementation, embryonic stem cells in which the gene of interest has been knocked out by homologous recombination are injected into homozygous RAG2 knockout (RAG2-/-) blastocysts. This procedure yields chimeric mice in which all B and T cells derive from the embryonic stem cells deleted for the gene of interest, as these are the only source of intact RAG genes to support lymphocyte development. Such animals can be made more easily than a knockout mouse line, and can be used to study the effect of gene deletion in lymphocytes independent of effects the deletion may have in other cells. In particular, for cases where the gene knockout causes

embryonic lethality due to effects on nonlymphoid cells, RAG complementation allows the selective knockout in lymphocytes to be studied in the background normal gene expression in nonlymphoid cells.

High Mobility Group Proteins

The search for RAG cofactors that stimulate cleavage activity in biochemical assays led to the identification of HMG1.[75] HMG1 (and the closely related HMG2) are abundant and ubiquitous proteins that bind DNA in a non-sequence-specific manner and to cause a local bend in DNA. The two RAG proteins can form a stable signal complex with a 12-RSS, but efficient complex formation with a 23-RSS requires the addition of either HMG1 or HMG2.[76] HMG1/2 apparently stabilizes the bending of the 23-RSS that is induced by the RAG proteins themselves.[77]

Nonhomologous End Joining Components

The RAG proteins are the essential lymphocyte-specific factors in the DNA cleavage phase of V(D)J recombination, but DNA repair factors that are part of a DNA repair pathway known as nonhomologous end joining (NHEJ) are essential for the joining phase. NHEJ is the major pathway for repair of dsbs (such as those induced by ionizing radiation or reactive oxygen species) during the G0-G1 phases of the cell cycle. (In the S and G2 phases, the additional chromatid genome copy enables breaks to be repaired by homologous recombination.) The six classical core components of NHEJ are Ku70, Ku80, DNA-PKcs, XRCC4, DNA Ligase IV, Artemis, and Cernnunos/XLF, but additional proteins play a role in some models of NHEJ.

The DNA-PK Complex. The first gene for an NHEJ component to be recognized as participating in V(D)J recombination was the SCID gene. This gene was originally identified as being mutated in the *scid* mouse strain that is immunodeficient due to a marked impairment in V(D)J recombination of both Ig and TCR genes. Lymphocytes from *scid* mice are able to perform the RAG-mediated cleavage reaction, and can also form signal joints, but are markedly defective in coding joint formation. Subsequently, it was found that the *scid* mutation also impairs NHEJ, causing radiosensitivity.

The gene mutated in the *scid* mouse strain encodes DNAPKcs, a large protein (460 kD) with a kinase domain near its C terminus that is related to phosophoinositide-3-kinase (PI3K). This kinase is DNA-dependent and represents the catalytic subunit (hence “cs”) of a heterotrimer known as the DNA-PK complex. The other components are Ku70 and Ku80 (also referred to as Ku86), which were originally identified as the autoantigens recognized by a patient antiserum (Ku was the coded name of the patient, and the numbers refer to the approximate size of the proteins, 70 kD and 80 to 86 kD, respectively). Together, these two very abundant proteins form a heterodimer that binds to the ends of double-stranded DNA independent of the nucleotide sequence of the DNA. The DNA-Ku complex can then recruit DNAPKcs and activate autophosphorylation of this protein.[78] *In vitro* activation of DNA-PKcs was found to be efficient when DNA ends either were at high concentration or, if at low concentration, were on DNA fragments long enough to circularize readily. In contrast, when the DNA-PKcs was located on the ends of DNA fragments too short to circularize (and too dilute for efficient intermolecular interactions with other DNA ends), the DNA-PKcs activation was much reduced. These observations suggest that kinase activation can occur only after two DNA ends are brought together by DNA-PKcs in “synapsis.”[79,80] Further phosphorylation of

DNA-PKcs inactivates the protein and may prepare it for removal once DNA ends have been sealed.

Ku genes are highly conserved through evolution, and homologs are even found encoded in the genome of some bacteria, consistent with a function in general NHEJ not restricted to V(D)J recombination. While mice with a targeted deletion of DNA-PKcs resemble the original *scid* mutation (i.e., defective coding but functional signal joint formation[81,82]), Ku70 and Ku80 mutant cell lines are defective in both signal and coding joint formation, and Ku70- and Ku80-deficient mice exhibit a complete block in B- and T-cell development due to their inability to undergo V(D)J recombination.[83,84,85]

DNA Ligase IV and XRCC4. An important role of activated Ku-DNA-PKcs complex is to recruit the additional components of NHEJ. One such component is DNA ligase IV, which is recruited to the Ku complex and activated by the protein XRCC4.[86,87] The evidence suggests that DNA ligase IV is the essential ligase that joins DNA ends in V(D)J recombination and NHEJ. Human patients with ligase IV deficiency (characterized by hypomorphic alleles) have a severe phenotype including chromosomal instability, developmental and growth retardation, radiosensitivity, and immunodeficiency with a T-B-NK+ phenotype.[88] The rare D_H-J_H junctions detected show extensive nucleotide deletion consistent with delayed ligation and prolonged exonuclease digestion.[89] In mice, disruption of either the XRCC4 or the DNA ligase IV gene causes embryonic lethality associated with neuronal apoptosis. Crossing these mice with p53 mutants does not improve V(D)J recombination, but rescues the mice from embryonic lethality, suggesting that neuronal cells may be unusually susceptible to p53-triggered apoptosis induced by normal low-level DNA damage during brain development; a similar mechanism may explain the severe human phenotype.[90] DNA ligase IV is the only NHEJ component absolutely required to join compatible sticky DNA ends *in vitro*, though XRCC4 can stimulate this activity significantly.[87]

Cernunnos/XRCC4-like Factor. The next NHEJ component was independently discovered by two laboratories. One group used yeast two-hybrid screening to search for proteins interacting with XRCC4.[91] The other group searched for the gene causing a syndrome of T+ B lymphocytopenia, increased radiosensitivity, and microcephaly in a Turkish family; these investigators used functional cDNA rescue of a patient's cell line from a radiomimetic drug to identify the gene.[92] The protein identified by both groups is a 299 amino acid nuclear protein, which was named Cernunnos or XLF. The protein has a predicted secondary structure similar to that of XRCC4, to which it binds in cells[93] as expected from its isolation via two-hybrid screen. When Cernunnos/XLF-deficient fibroblasts were transfected with RAG genes and a recombination substrate, imprecise signal joining was observed, similar to the defect in patients with hypomorphic DNA ligase IV mutations. These experiments all suggest a role for Cernunnos/XLF linked to the function of XRCC4 and ligase IV.

Artemis. The coding ends generated by RAG cleavage cannot be directly ligated because of their hairpin structure, and therefore V(D)J recombination requires a single-strand endonuclease activity to cleave the hairpins. This activity is conferred by the protein named Artemis, which was discovered through positional cloning of the genetic defect in a group of human SCID patients with defects in V(D)J recombination and increased radiation sensitivity.[94] Patients with homozygous null mutations of Artemis survive (no embryonic lethality) and show sensitivity to γ irradiation as well as defects in coding joints, while signal joint formation is normal. Hypomorphic Artemis mutations can cause features of the Omenn syndrome similar to those observed with hypomorphic RAG gene mutations.[95] Purified recombinant Artemis protein has an intrinsic exonuclease activity *in vitro*; however, when complexed with DNA-PKcs in the presence of DNA ends, it gains a single-strand endonuclease activity and, in an ATP-dependent step, becomes phosphorylated at multiple sites in the C-terminal region of the protein.[96,97] The Artemis endonuclease can cleave synthetic and RAG-generated hairpin ends as well as other singlestranded DNA near a transition to double-strand DNA.[98]

DNA Polymerase X Family Members. If a hairpin opening leaves blunt ends or complementary sticky ends (like the ends generated by many restriction enzymes), *in vitro* joining experiments suggest that these ends can be joined by ligase IV without any additional processing.[99] However, as Artemis probably opens most hairpins noncomplementary DNA overhangs, further processing of DNA ends generally occurs before ligation completes the recombination. This processing may include further nuclease digestion (by Artemis or exonucleases) and apparently also involves variable DNA extension by three DNA polymerases—polymerase λ, polymerase μ, and terminal deoxynucleotidyl transferase (TdT)—all of which are members of the polymerase X family. Interestingly, all three proteins contain a Brca1-C-terminus domain, which is thought to confer binding to Ku.[100]

Terminal Deoxynucleotidyl Transferase and N Regions. TdT, the primary source of untemplated "N region" additions in VDJ junctions, is an enzyme uniquely expressed in the thymus and bone marrow; in the B lineage, it is expressed almost exclusively in pro-B cells. It catalyzes the nontemplated addition of nucleotides to the 3′ end of DNA strands. Though no template determines the nucleotides added, the enzyme adds dG residues preferentially, consistent with N region sequences observed in VDJ joints. Both TdT expression and N nucleotide addition are characteristically absent from fetal lymphocytes.[101] N region addition is common in H chain genes (recombined in pro-B cells) but rare in murine L chain genes (recombined in pre-B cells), though perhaps somewhat less rare in human.[102] This is consistent with the observation that in mice the expression of a μ H chain may downregulate TdT expression,[103] contributing to the reduced level during the stage of L chain recombination.

Lymphocytes with engineered defects in their TdT genes produced rearranged Ig V regions with almost no N additions. Conversely, when TdT expression was engineered in cells undergoing κ or λ L chain rearrangement, the level of

N nucleotide addition to these coding joints was dramatically increased. Furthermore, mice engineered to undergo premature Vκ-Jκ joining in pro-B cells show an increased frequency of N region nucleotides in their recombined Vκ genes.[104] These results suggest that the low frequency of N region sequences in normal κ or λ recombinations is caused by the reduced levels of TdT at this stage of B-cell development (see following discussion).

The absence of N region addition in TdT mutant mice, as well as in normal fetal lymphocytes, is associated with an increase in the frequency of recombination junctions with microhomologies. These are short stretches of nucleotides that are present close to the end of both germline gene segments involved in the recombination event. These junctions suggest a joining intermediate in which the complementary single-stranded regions from the two coding ends hybridize to each other, much as "sticky ends" generated by restriction endonucleases can facilitate ligation of DNA fragments. This alternative joining pathway may restrict the diversity of neonatal antibodies; the resulting antibodies are possibly enriched in specificities for commonly encountered pathogens, or have broadened specificity, as has been reported for TCRs lacking N regions.[105] Decreased N region nucleotides and a high incidence of homology-mediated recombination have also been found in the rare coding joints formed in Ku80-/- mice, consistent with a role for Ku in recruiting TdT or supporting its action.[106]

Polymerase μ and Polymerase λ. Polymerase μ and polymerase λ are ubiquitously expressed polymerases. Both readily fill in single-strand gaps in DNA and apparently participate in V(D)J recombination by filling in single-strand 3′ overhangs generated by asymmetric hairpin opening. Without this filling in, such overhangs might be resected by nucleases. Indeed, when *in vitro* NHEJ reconstitution experiments are performed using purified proteins and DNA fragments with overhanging ends, the omission of polymerase μ or polymerase λ increases the deletional trimming at junctions.[100] Similar excessive deletions at VDJ junctions are observed in mice lacking polymerase μ or polymerase λ. Remarkably, however, polymerase μ knockout mice show abnormalities only in their L chains,[107] whereas the deletions in polymerase λ knockouts are restricted to their H chains.[108] This selectivity may be explained by corresponding changes in the relative mRNA levels for these two polymerases at different stages of B-cell development.

Other Participants in V(D)J Recombination

DNA Damage Response Factors. In eukaryotic cells, DNA breaks initiate signals that halt cell division, induce DNA repair, and in some cases trigger apoptosis. Several proteins apart from NHEJ components can be detected at DNA breaks induced by V(D)J recombination or irradiation, including γ-H2AX, a phosphorylated form of the histone H2AX; ATM, the product of the gene mutated in the disease ataxia telangiectasia; Nbs1 (or nibrin), the product of the gene mutated in Nijmegen breakage syndrome; and 53BP1, p53 binding protein 1. The importance of these proteins in V(D)J recombination is not clear because defects in all three

are compatible with near normal V(D)J recombination. Possibly, they participate in backup mechanisms to prevent aberrant V(D)J recombination and thus translocations.

Pax5/B-Cell-Specific Activator Protein. Pax5 (also known as B-cell-specific activator protein; BSAP) is a transcription factor required for normal B-cell development. Pax5-deficient mice are able to complete DJH recombination, but V_H to DJ_H recombination is impaired except for certain V_H genes located proximal to the D regions. Interestingly, 94% of human and mouse V_H coding genes were found to have potential Pax5 binding sites. Surprisingly, Pax5 was found to coimmunoprecipitate with RAG proteins, to potentiate *in vitro* cleavage of a V_H gene RSS, and to enhance V_H to DJ_H recombination in RAG-transfected fibroblasts; the latter enhancement required intact Pax5 binding sites in the V_H sequence.[109]

REGULATION OF V(D)J RECOMBINATION IN B-CELL DEVELOPMENT

The expression of only one antigen binding specificity by each B-lymphocyte is a crucial requirement of the clonal selection model of the humoral immune response. Thus, the recombination events that occur between Ig gene segments are carefully regulated so that most B cells express only one L chain isotype, either Igκ or Igλ (isotype exclusion), and use only one of the two alleles of H and L chain genes (allelic exclusion). These constraints ensure that each B cell expresses a single H_2L_2 combination. Current evidence suggests that V(D)J recombination is controlled largely at two levels: regulation of the RAG protein activity and regulation of accessibility of the germline V, D, and J elements to the recombinase machinery. Both of these are controlled by the stage of B-cell development; conversely, the expression of Ig provides a signal critical for regulating maturation of B cells. A brief scheme of B-cell development is presented in the following as background.

B- and T-lymphocytes differentiate from pluripotent hematopoietic stem cells in the fetal liver and bone marrow (Fig. 6.11). The primordial lymphoid progenitor has the potential to differentiate into B- or T-lymphocytes or natural killer cells. Among the earliest markers that indicate B-lineage specificity are the non-Ig components of the pre-BCR: Igα, Igβ, and λ5. CD19, which functions as a coreceptor in signal transduction, first appears in large proliferating “pro-B” cells, which also express several other distinguishing surface markers including c-kit, B220, TdT, and CD43. RAG gene expression in pro-B cells initiates D to J rearrangements on both alleles. Subsequently, recombination with germline V_H elements occurs; if the recombination is “productive” (i.e., yielding an “in-frame” VDJ junction), a μ H chain protein can be produced. This protein appears on the B-cell surface along with SLC in a pre-BCR (also named μ-SLC) complex that also includes Igα and Igβ. As the resulting large pre-B cells proliferate, RAG gene expression declines. After several rounds of division, the cells become smaller, stop dividing, turn up RAG gene expression once more, undergo L chain recombination, and express surface

IgM. These “immature B cells” again turn down RAG expression. In these IgM+IgD- immature B cells, contact with autoantigens may upregulate RAG expression again to facilitate receptor editing (discussed in more detail below). When immature B cells eventually also express surface IgD, they become “mature B cells” and migrate into the periphery, ready to be triggered by antigen exposure.

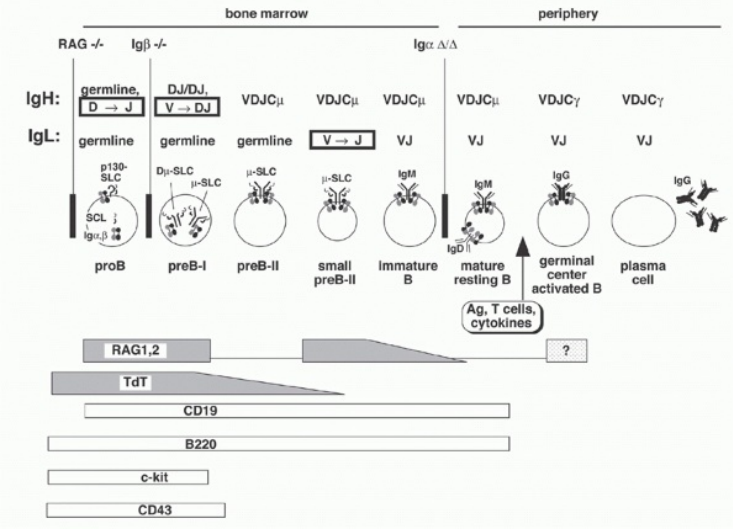

FIG. 6.11. Immunoglobulin (Ig) Gene Recombination in B-Cell Development. A simplified scheme of B-cell development is presented as a background for discussion of Ig gene recombination. The stages occurring in the bone marrow versus in the periphery (e.g., lymph nodes, spleen) are shown, along with the status of IgH and IgL genes at each stage. A graphic depicting the Ig-related proteins displayed on the surface at each stage is presented; at the bottom, the stage-dependent expression of recombination activating genes and terminal deoxynucleotidyl transferase—both important in V(D)J recombination—is schematically depicted.

Allelic Exclusion and Regulated V(D)J Recombination

The previous description of B-cell development serves as a background to understand an explanation of allelic exclusion that was first proposed by Alt and colleagues[110] and has been supported by subsequent experiments. According to this model the functional rearrangement of an L (or H) chain gene in a particular B cell would inhibit further L (or H) chain gene rearrangement in the same cell. If the inhibition occurred promptly after the first functional rearrangement, then two functional Igs could never be produced in the same cell. An initial nonproductive rearrangement would have no inhibitory effect, so recombination could continue until a functional product resulted or until the cell used up all its germline precursors.

In pro-B cells, the first Ig gene rearrangements join D to J_H segments (commonly on both chromosomes), and this is followed by V_H to DJ_H recombination. If the first V_H to DJ_H recombination in a pro-B cell produces a functional VDJ gene, a functional μ H chain will be expressed on the cell surface paired with the SLCs. The expression of this pre-BCR complex has been shown to have two consequences. First, it blocks further H chain recombination by decreasing RAG gene expression[111] and by reducing target accessibility, as reflected in decreased V_H gene transcription.[112] The latter is important for rendering the IgH locus inaccessible during subsequent rearrangement of the Igκ and Igλ loci. If the initial V_H to DJ_H rearrangement is nonfunctional (e.g., out of frame), subsequent V_H to DJ_H recombination occurs on the other allele. If the VDJ recombination product on the

second chromosome is also nonproductive, then the cell has reached a dead end and is eliminated by apoptosis.[113]

The second consequence of pre-BCR expression is the initiation of Ig L chain recombination. This effect was originally deduced from the rarity of κ-expressing cells without H chain gene rearrangement, suggesting that H chain expression is required for κ recombination. As additional evidence, a functional μ gene introduced into early B-lineage cells can cause RAG gene expression and turn on transcription of unrearranged Vκ genes. These are designated

"sterile" transcripts because they cannot encode a κ protein, but they are required for Vκ-Jκ recombination. When this recombination ensues, the possibilities for functional and nonproductive Vκ-Jκ rearrangements resemble those discussed previously for the H chain. Expression of a functional κ chain that can associate with μ to form a surface-expressed IgM molecule results in the downregulation of RAG gene expression and suppression of further κ rearrangements. By this mechanism, functional rearranged VκJ-Cκ transgenes can suppress rearrangement of endogenous κ genes.[114]

Most B cells show isotypic exclusion (i.e., they express either κ or λ but not both). Furthermore, κ rearrangement seems to occur before λ. Thus in normal and malignant human B-lymphoid cells, κ-expressing cells generally have their λ genes in germline configuration, while in λ-expressing cells, κ genes are either rearranged (rarely) or deleted (most commonly) by recombination signal recombination events discussed previously in this chapter.[22] The mechanisms that dictate the order of L chain recombination remain unknown. Plausible models include either the selective suppression of λ recombination until all options on the Igκ locus are exhausted or differences in the timing of the developmental programs controlling κ and λ accessibility.

Regulation of RAG Expression

A complete explanation of RAG gene expression would explain its lymphoid specificity, the two waves of RAG expression (during IgH and IgL rearrangements) and the autoantigen-induced upregulation associated with receptor editing. Although our current knowledge is still incomplete, several *cis*-regulatory elements that regulate RAG expression have been characterized. Surprisingly, the elements and mechanism for regulating expression during B- and T-cell development are distinct. RAG1 and RAG2 are transcribed toward each other in opposite directions, driven by promoters near the respective transcription start sites. Three B-cell-specific enhancers—designated Erag, D3, and Ep—have been reported, lying about 23 kb, 8 kb, and 1.6 kb, respectively, upstream of RAG2.[115,116,117] The B-cell-specific function of these regulatory regions is likely explained by the intersecting specificities of transcription factors that interact with them, including Pax5, E2A, FoxP1, FoxO1, NFATc1, and Ikaros. NFκB, which binds at several locations in the RAG enhancers, and FoxO1 (binding to Erag) were found to be important mediators of the upregulation of RAG expression in cells undergoing receptor editing.[118,119] Regulation of RAG2 protein across the cell cycle has been discussed previously in this chapter.

Parameters Affecting Recombinational Accessibility and Transcription

V(D)J recombination is triggered by RAG expression in the development of both B and T cells, yet Ig gene recombination is largely confined to B cells (exception: early T cells typically show D-J_H recombination); TCR gene recombination is exclusive to T cells. A widely accepted explanation for this locus specificity is provided by the "accessibility" model.[120] This model proposes that only those gene segments programmed for recombination at a given stage of B- and T-cell development are "accessible" to the RAG recombinase. One clue suggesting this model was that susceptibility to recombination and transcription of germline gene elements seem to be tightly correlated.[120] For example, many germline V_H genes are transcribed at the pre-B cell stage, just at the time when these genes are targets for recombination; these transcripts— designated "sterile" like the Vκ transcripts mentioned previously —are not seen in more mature B cells in which H chain recombination has been terminated. In support of the accessibility model, recombinant RAG proteins incubated with nuclei purified from pro-B cells (which generate sterile transcripts in the IgH locus) were found to cleave DNA at Ig J_H RSSs, but not at TCRδ RSSs; conversely, in pro-T nuclei the TCRδ RSS was cleaved, but not an Ig gene RSS.[121]

One molecular correlate of accessibility is the epigenetic state of DNA in the nuclear chromatin. The minimal repeat unit of chromatin is the nucleosome, which consists of eight core histones (two copies each of H2A, H2B, H3, and H4) with 146 bp DNA wrapped around it. *In vitro*, RAG proteins are unable to bind to and cut DNA wrapped around nucleosomes,[122,123] and hence nucleosomes have to be shifted or removed (a process

called chromatin remodeling) to allow access. An alternative but not mutually exclusive approach to gain access is posttranslational modification of the histone tails, which regulates the tightness of DNA-nucleosome contacts. The following section provides an overview of how accessibility of the Ig gene loci for RAG activity is regulated by several distinct but interconnected epigenetic mechanisms. We discuss a few important examples for each mechanism and refer to comprehensive review articles for an in-depth discussion.

Subnuclear Localization

In general, inactive genes tend to be located in the periphery of nuclei, while active genes are recruited to a more central nuclear location.[124] It is unclear whether the location per se dictates the chromatin state of a locus or whether the movement is a consequence of a locus being "opened." Fluorescence in situ hybridization (FISH) with large (~100 kb) probes specific for Ig loci is routinely used to reveal the position of Ig gene loci and control genes in the nucleus. The IgH and Igκ loci are located at the nuclear periphery in hematopoietic progenitors and pro-T cells, but move to central areas of the nucleus in pro-B cells.[125] As only the IgH locus gets rearranged at this stage, the correlation of position with accessibility is not perfect.

Transcription

Transcription typically occurs in "open" chromatin, and an emerging theme suggests that while some locus "opening" has

to precede transcription, transcription per se also positively reinforces this chromatin state. As mentioned previously, transcription of individual elements within Ig gene loci correlates well with their availability for V(D)J recombination at that stage. In early pro-B cells when D to J_H recombination takes places a "μo" transcript starts upstream of DQ52 and proceeds all the way through the J_H elements[126,127]; and only after DJ_H rearrangement do V_H sterile transcripts appear.[120] It is currently unclear whether transcription per se is directly linked to recombination or whether the correlation is largely mediated by similar requirements for gaining access to chromatinized DNA.

Histone Modifications

Posttranslational modifications of histone tails are important epigenetic marks of the chromatin state (also referred to as the "histone code"). Distinct marks correlate well with actively transcribed and inactive (or repressed) gene loci. As these patterns hold true for Ig loci as well, active marks (e.g., histone acetylation) and inactive marks (e.g., the methylation of lysine 9 on histone H3, H3K9Me2) correlate well with both transcription and recombination accessibility.[128] Importantly, one particular mark for open chromatin, H3K4Me3, is directly linked to the RAG recombinase. As discussed previously, histone tails with this modification are recognized by the PHD domain of RAG2; strikingly, the distribution of RAG2 in Ig loci matches exactly the pattern of H3K4Me3 (which mark accessible gene segments).[129] Beyond facilitating the recruitment of the recombinase, this histone modification also increases cleavage activity *in vitro*.[69]

Methylation

Most cytosine residues within CpG dinucleotides are methylated in mammalian DNA, but genes that are actively expressed in a particular cell are generally relatively hypomethylated in that cell type, implying that DNA methylation inhibits transcription. DNA methylation also seems to inhibit V(D)J recombination. The developmental maturation from pro-B to pre-B cells is associated with progression from a κ locus that is largely methylated, nontranscribed, and nonrearranging to one that is hypomethylated, transcribed, and rearranging.[130,131] Furthermore, methylation of artificial recombination substrates blocked V(D)J recombination when transfected into a recombination competent B-cell line[132]; V(D)J recombination of a transgenic construct occurred only when it was unmethylated.[133] Methylation and histone acetylation are interrelated; for example, the methyl-CpG-binding protein MeCP2 recruits

histone deacetylases, which reduce acetylation of histones.

Gene Localization in the Nucleus

While accessibility allows the RAG proteins to selectively bind to appropriate sets of RSSs at each developmental stage, V(D)J recombination also requires that a pair of compatible gene segments (and their RSSs) are in close physical proximity. This becomes a particularly daunting requirement for gene segments > 1 mb apart in linear DNA sequence. Such distant segments are apparently brought close together in the nucleus, a process of "locus compaction" that loops out large regions of intervening DNA. Evidence for this model derives from FISH experiments showing greater compaction of the IgH locus in pro-B cells poised to undergo V(D)J recombination than in their earlier hematopoietic progenitors.[125] B cells deficient in Pax5 are impaired in recombination of the most distal V_H regions and show less movement of these regions towards the J_H-C locus than normal cells.[134] Data from a recently developed high-resolution FISH method provide a detailed model of the three-dimensional structure of the IgH locus, revealing rosette-like structures with central hubs from which several loops extend.[135]

cis Mediators of Accessibility and Looping

All previously described properties are dependent on *cis*-regulatory elements within the Ig loci, including classic promoters and enhancers. Individual promoters are present upstream of all V elements in all Ig loci, while the downstream D and J elements share a smaller number of promoters. Enhancers are present in each Ig locus, and they are thought to confer the transcriptional activation of each locus at the appropriate stage of B-cell development. Murine κ and IgH loci have intronic enhancers in the intron between J and C (iEκ and Eμ, respectively), and all three loci have enhancers downstream of C coding regions. (For example, downstream of the murine IgH locus is a complex of four enhancers, collectively known as the 3′ regulatory region.) Promoters and enhancers were originally defined based on their role in regulating transcription, but these and other recently reported elements appear to play additional roles in Ig gene recombination. Transcriptional activation and the correlated locus "opening" is mediated by the recruitment of transcription factors that in turn recruit histone modifying and chromatin-remodeling enzymes. In addition, promoters and enhancers regulate transcription through the formation of DNA loops, some of which are critical determinants of the three-dimensional structure of Ig loci, thereby affecting V(D)J recombination as well as CSR (as discussed below).

An enhancer may activate transcription of several genes within a given gene locus, but its effects may be deleterious if it can activate other nearby genes requiring different patterns of expression. To prevent enhancer function beyond appropriate domains, boundary elements known as insulators establish borders between gene loci that are differentially regulated. The protein CCCTC binding factor (CTCF) is commonly found at insulators and also functions by creating DNA loops. One such insulator apparently lies downstream of a complex of enhancers at the 3′ end of the IgH locus, the 3′-regulatory region, where it may protect genes further downstream from being regulated by the Ig enhancer elements.[136] A recently discovered regulatory element with insulator properties is the intergenic control region-1 (IGCR1), which participates in CTCF-dependent looping between the Eμ and 3′ regulatory region enhancers.[137,138,139] Based on results of deletion of this region, the IGCR1 element suppresses recombination of V_H to D segments not already joined to J_H, prevents V_H to DJ_H recombination in T cells, and mediates the BCR-induced signal that terminates sterile V_H transcription and recombinational accessibility of V_H segments after a productive VDJ recombination leads to expression of a μ protein.

Late RAG Expression: Receptor Editing and Receptor Revision

Although the RAG genes are generally downregulated by a signal mediated by the appearance of membrane IgM at the end of the pre-B-cell stage, RAG gene expression and V(D)J recombination can recur later during "receptor editing" of autoreactive B cells in the

bone marrow. One estimate suggests that about 25% of Igs are products of receptor editing.[140] After production of an initial Igκ. protein, receptor editing by L chain rearrangement can occur three ways: an initial VκJκ junction could be deleted by recombination between an upstream V and downstream J on the same chromosome ("secondary recombination," as discussed previously), VκJκ recombination could occur on the other (allelic) copy of the κ locus, or Vλ-Jλ recombination could be activated.

Most replacement of productively rearranged L or H chain genes likely serves to extinguish an antibody that was autoreactive, thus complementing two other mechanisms to silence autoantibodies: anergization and cell deletion by apoptosis. Early studies with transgenic autoantibodies suggested that anergy or deletion were the main fates of self-reactive B cells, but these conclusions may have depended on the nonphysiologic inability of the cells to silence the transgenic autoantibody by receptor editing. More recent studies involving autoantibody "knockin" genes—i.e., productive V[D]J recombined genes swapped into the physiologic positions within the IgH or Igκ gene loci using homologous recombination —have shown that receptor editing is the major mechanism for B cell tolerance.[141,142] This conclusion was also supported by a study of mice expressing a transgenic antibody against the murine Cκ constant region, a model of a self-superantigen; these mice provided evidence of receptor editing leading to virtually 100% λ L chain expression.[143]

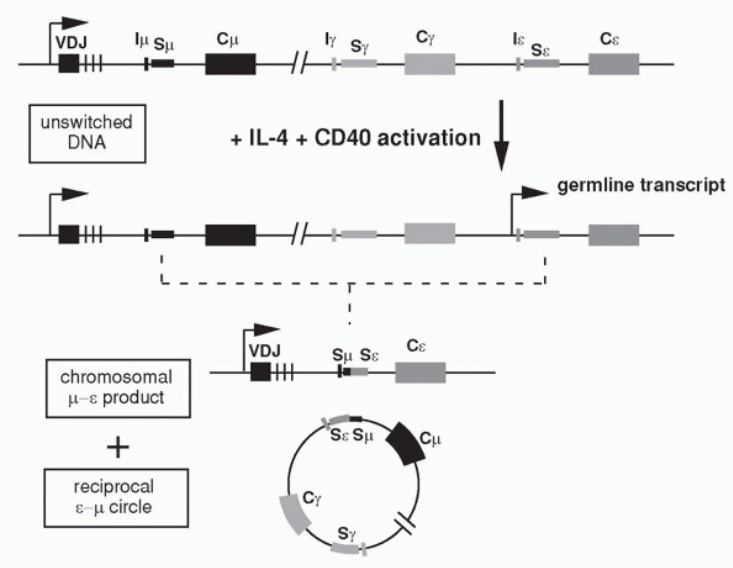

FIG. 6.12. Switch Regions and Composite Switch Junctions. The recombination breakpoints in isotype switch recombination fall within repetitive "switch" (S) regions. Stimuli that activate switch recombination (IL-4 and CD40 activation in the example shown) generally promote transcription across the target S region, initiating just upstream at the "I" exon. Recombination between Sμ and Sε produces two composite switch junctions: an Sμ-Sε junction retained in chromosomal DNA, and a reciprocal Sε-Sμ junction found in fractions of circular DNA. Polymerase chain reaction amplification across either composite junction can be used to study switch recombination.

IMMUNOGLOBULIN GENE ALTERATIONS IN GERMINAL CENTERS

Several days after exposure to an antigen, B cells accumulate in local lymph nodes, gut-associated lymphoid tissue, and spleen, and begin additional maturation steps in germinal centers (GCs). During the GC response, antigen-driven B cells undergo cycles of proliferation and their Ig genes undergo two unique alterations. 1) Lymphocytes switch from making IgM to making a new H chain isotype by the process of CSR. This process introduces dsbs upstream of Cμ in a specific repetitive noncoding DNA segment—the "switch region" —and in a similar switch region upstream of the new target Cx region; the DNA between the breaks is then deleted, and the ends of the remaining chromosomal DNA are rejoined so that

the assembled VDJ region now lies upstream of the new Cx gene (Fig. 6.12). 2) In the other GC-associated gene alteration, the affinity of the antibody for its antigen increases by a process that introduces random mutations in VH and VL—somatic hypermutation (SHM)—and then selects for B cells producing higher-affinity antibodies. For many years, CSR and SHM were considered to be unrelated processes, but several lines of evidence provided hints that they might share some mechanistic features. First, while dsbs are expected intermediates for the DNA recombination underlying class switching, DNA breaks in V regions were also detected accompanying SHM. Second (and conversely), in addition to the mutations occurring in V regions associated with SHM, mutations were also observed surrounding the recombination junctions of CSR. Third, RNA transcription was found to be required for both processes.

Activation-Induced Deaminase

A fourth and dramatic link between CSR and SHM was the discovery that both processes require the protein known as activation-induced deaminase (AID). The gene encoding AID (known as *Aicda*, for *a*ctivation *i*nduced *c*ytosine *dea*minase) was discovered[144] by a subtractive strategy designed to screen for transcripts that were expressed in a murine B-cell line when induced to undergo CSR, but that were not expressed in uninduced cells. AID is expressed almost exclusively in GC B cells and in B cells activated *in vitro*, though exceptions to this generalization will be discussed later. Mice engineered with a targeted defect in the *Aicda* gene are completely deficient in CSR and SHM. The same defects are seen in patients with a homozygous defect in the human *Aicda* gene, a condition known as the hyper-IgM syndrome-2.[145] These patients have elevated serum levels of IgM because their B cells cannot undergo efficient CSR. AID is not only necessary for CSR and SHM, but apparently sufficient (in a mammalian cell at least), as overexpression of AID in fibroblasts can confer transcription-dependent CSR of an artificial switch construct[146] and transcriptiondependent SHM of a transfected model mutation target DNA.[147] These experiments suggest that AID is the only B-cell-specific protein required for SHM and CSR. AID is also required for somatic Ig gene conversion in those species (e.g., rabbit and chicken) that use that process to somatically diversify Ig genes.

As translated from the cDNA, AID is a ˜24 kD 198 amino acid protein that forms homodimers. AID shows 34% amino acid identity with the RNA editing enzyme APOBEC1, which catalyzes the deamination of a cytosine residue to uracil in a specific position in the mRNA encoding apolipoprotein B. The human *Apobec1* and *Aicda* genes are genetically linked, both lying at chromosome 12p13. Three other APOBEC1-related genes on other chromosomes have also been identified, but are not thought to participate in SHM or CSR.

Like APOBEC1, recombinant AID protein has a cytidine deaminase activity *in vitro*; it was initially proposed that, by analogy with APOBEC1, AID functions by deaminating cytidines in specific RNAs to produce novel edited transcripts encoding one or more proteins required for CSR and SHM. However, no evidence of AID-dependent edited RNAs has been reported, nor does AID deaminate RNA cytidines *in vitro.* Instead, current evidence indicates that AID acts on DNA, deaminating cytidines to uracil—in V regions for SHM, and in switch regions for CSR. The resulting uracils would be then recognized as both Watson-Crick mismatches and abnormal DNA bases by the cell's genetic surveillance machinery, triggering error-prone repair of V regions to produce SHM, or DNA cleavage to mediate CSR.

This DNA-deamination model for AID is consistent with various properties reported for this protein *in vitro* or in cells.

1. In transfected *Escherichia coli* cells, AID mutates cytidine to uracil in DNA, a result that would be unexpected by the RNA-deamination model, as bacteria presumably lack the specific mammalian RNA targets predicted by that model.[148,149]
2. Defective SHM and CSR are observed with inhibition or genetic inactivation of uracil-N-glycosylase (UNG), an enzyme that removes uracil residues from DNA,[150,151] consistent with the idea that CSR and SHM involve a step in which AID-produced uracil in DNA is

removed by UNG. Moreover, B cells from *Ung*-/- mice accumulate uracils in V and in switch regions in an AID-dependent manner.[152]

3. AID isolated from B-lymphocytes can deaminate cytidine to uracil in single-strand DNA *in vitro*[153,154,155] or in doublestrand DNA that is being transcribed *in vitro*, presumably because transcription causes localized regions of singlestrandedness.[155,156,157]
4. 4. AID is found to be associated with IgH genes *in vivo* in B cells undergoing CSR,[158,159,160] as assessed by Chromatin ImmunoPrecipitation (ChIP) using an anti-AID antibody.
5. A DNA sequence motif WRC—where W is A or T (*w*eak Watson-Crick pairs) and R (pu*r*ine) is A or G—that has been recognized as a hotspot target of SHM is also a preferred target for AID deamination *in vitro*,[154,157] and the hotspot preference can be altered by engineering amino acid changes in the segment of the AID protein thought to recognize the hotspot target in DNA.[161,162,163]

In light of these and other observations, the DNA deamination model for AID action is now almost universally accepted. The AID protein is highly conserved from fish to human, with all species having approximately 200 amino acids and sharing sequence similarities throughout the protein. In all species, AID contains a motif common to the active site of all cytidine deaminases: H[A/V]E - $X_{(24-36)}$ - PCXXC. This motif is also found in other members of the AID-APOBEC gene family, including APOBEC2, APOBEC3 (with several distinct paralogs in human), and APOBEC4.[164] AID is encoded in the five exons of the *Aicda* gene.

Structure-function relationships of AID have been probed by examining cross-species sequence comparisons and the effects of natural and engineered mutations in the protein. Remarkably, mutations in the N-terminus of AID impaired SHM but not CSR, whereas mutations or deletion in the C-terminus selectively impaired CSR,[165,166,167] suggesting the possibility that the N- and C-terminal regions of the protein contact specific cofactors required (respectively) for SHM and CSR function. Other functional features of the protein include a dimerization domain, several phosphorylatable residues, segments affecting nuclear localization, and target sequences for association with other proteins.

Although AID was initially recognized for its participation in SHM and CSR in GC cells, more recent reports have reported several other roles for this protein, both beneficial and deleterious.

1. *Epigenetic methylation.* Selective DNA methylation of cytidines is an epigenetic mechanism that participates in the regulation of transcription, and the methylation pattern of DNA is normally replicated when DNA is replicated prior to cell division. In primordial germ cells of

early embryogenesis, a global erasure of methylation occurs as part of the reprogramming to pluripotency, and AID apparently participates in this process.[168,169]

2. *Germline mutation.* AID can deaminate methylcytosine to thymine, which unlike uracil is a natural DNA base. This reaction in germ cells may contribute to the most frequent germline point mutation observed in mammals: the transition from CpG to TpG.[170]
3. *Genomic protection.* AID and other APOBEC family members protect cells against retroviral infections and against the spread of endogenous retroviruses.[171,172,173]
4. *Tolerance, apoptosis.* AID appears to be important for the establishment of B-cell tolerance in humans and mice, in that the low levels of AID expression observed in immature and transitional B cells appear to be necessary for suppressing the appearance of autoantibodies.[174,175,176] This effect may be related to the apparent requirement of AID for normal levels of apoptosis, which contribute to the elimination of B cells expressing autoantibodies.
5. *Oncogenic mutations and translocations.* Soon after AID was discovered, it was observed

that overexpression of the protein caused tumors in transgenic mice,[177] and even normal expression of AID in B cells contributes to tumorigenic translocations and oncogenic mutations.[178] A notable example of AID-stimulated translocation is the recurrent *c-myc/Ig* translocation seen in Burkitt lymphoma and murine myelomas. Other cancers where AID plays a role through mutation include intestinal and lung cancer.

Based on studies of AID+/- heterozygous mice expressing roughly 50% of normal AID, the level of AID activity seems to be the limiting factor for both SHM and CSR, but is also limiting for oncogenic translocations and mutations.[179,180] Therefore, AID activity must be regulated robustly to balance between its multiple beneficial and deleterious actions. The regulation of AID activity is extremely complex, involving multiple levels of control, and is still incompletely understood.

Transcriptional and Posttranscriptional Regulation of AID Expression

Early studies showed that AID expression in B cells is upregulated by IL-4 and CD40 engagement. In more recent investigations, four DNA regions that regulate *Aicda* gene expression have been identified. They include an upstream enhancer responsive to T-cell signals, a promoter, a regulatory region just downstream of exon 1, and an additional enhancer downstream of the *Aicda* gene.[181,182,183,184,185,186]

The microRNA miR-155 has been shown to suppress AID expression.[187,188] MicroRNAs are short (21 to 23 nt) RNAs that hybridize to complementary sites in numerous target RNAs, triggering their degradation or inhibiting their translation. A target for miR-155 lies in the 3′ untranslated region of the AID transcript. AID expression was increased in miR-155-deficient mice and in mice whose *Aicda* gene was mutated to disrupt the 3′UTR target sequence. In both cases, consequences of the increase in AID expression included increased off-target mutations and increased *c-myc-IgH* translocations. Perhaps to protect against such effects, miR-155 is itself upregulated by the same signals that induce *Aicda* transcription in B cells.

Posttranslational Regulation

The level of AID activity in the nucleus is affected by its distribution between cytoplasm and nucleus, by the protein half-life, and by phosphorylation at various positions in the protein.

AID was found to shuttle between nucleus and cytoplasm.[189] Most of the protein is cytoplasmic and only a small fraction is in the nucleus, where it can act on DNA to cause CSR and SHM as well as “off-target” deleterious effects. Movement between cytoplasm and nucleus is controlled by at least three independent mechanisms: active nuclear import mediated by an N-terminal nuclear localization signal,[190] active nuclear export mediated by a C-terminal nuclear export signal,[189,191] and a cytoplasmic retention mechanism apparent when active import and export mechanisms are both blocked. Two proteins that may facilitate AID import independently of the nuclear localization signal are GANP[192] and CTNNBL1.[193] Nuclear-cytoplasmic distribution affects AID stability, because in the nucleus AID is targeted for ubiquitination and proteasomal degradation, shortening its halflife.[194] Conversely, in the cytoplasm, the chaperone protein Hsp90 protects AID from proteasomal degradation.[195]

In addition to ubiquitination, AID is also subject to posttranslational phosphorylation at several distinct residues in the protein, including Ser3, Thr27, Ser38, Thr140, and Tyr184. Of these, the functional relevance of the Ser38 site is best understood. Phosphorylation of AID Ser38 is essential to recruit replication protein A (RPA), a trimeric singlestrand DNA binding protein[159,196] that may be required to stabilize a single-strand DNA target for AID. Preparations of AID purified from transfected *E. coli* or from nonlymphoid eukaryotic cells lack Ser38 phosphorylation; they can deaminate single-strand DNA *in vitro*, but are inactive in an assay for deamination of double-strand DNA transcribed *in vitro* by T7 polymerase. Activity in the latter assay requires AID that is phosphorylated at Ser38 and associated with RPA. In the AID protein, Ser38 lies within a conserved phosphorylation site [RRXX(T/S)] for the cyclic-AMP dependent protein kinase A (PKA), and multiple experiments have confirmed that PKA phosphorylates AID at Ser38 *in vitro* and physiologically *in vivo*.[196,197] The physiologic

importance of Ser38 phosphorylation is strongly supported by observations of the effect of mutating this serine to alanine. The AID^{S38A} protein has essentially normal activity in deaminating single-stranded DNA *in vitro*, but severely reduced activity in deaminating transcribed double-strand DNA *in vitro*.

Less is known about the other sites of AID phosphorylation. At Thr140, alanine replacement causes more modest inhibition of AID activity than Ser38Ala, preferentially affecting SHM *in vivo*, but without significant effect on *in vitro* catalytic activity.[198] In contrast to Ser38 and Thr140, phosphorylation of Ser3 apparently suppresses *in vivo* AID activity; alanine replacement at this position increases SHM,

CSR, and oncogenic translocation, also without affecting *in vitro* catalytic activity.[199]

Clearly, multiple mechanisms regulate the level of activity of AID in B-cell nuclei, but these mechanisms cannot explain how—within nuclei—AID acts on Ig V genes and switch regions at a much higher frequency than on the rest of the genome. Targeting mechanisms differ somewhat between SHM and CSR, and will be discussed in the context of those processes.

Somatic Hypermutation

The hypothesis that antibody genes inherited in the germline might be subject to somatic mutation in lymphocytes during the life of an individual was suggested as an explanation for the diversity of antibodies several years before recombinant DNA technology became available to clarify the role of V(D)J recombination. Persuasive evidence for somatic mutation was reported in the 1970s: analyses of Vλ1 amino acid sequences of murine myeloma antibodies showed many instances of a particular prototype sequence, plus several variants containing unique single amino acid substitutions that could be explained by single nucleotide changes. The prototype was interpreted as reflecting the germline sequence, with the variants arising by somatic mutation.[200] Subsequent investigations at the DNA level revealed myeloma V region sequences that deviated from their germline counterparts, verifying the principle of somatic mutation.

Somatic mutations are much rarer in IgM than in antibodies with "switched" isotypes (IgG, IgA, and IgE) made by B cells that have been exposed to AID in GCs, but they do occur; antibodies with "switched" isotypes without mutations are also found. These observations suggest that though both SHM and CSR normally occur in GC B cells, the two processes are unlinked.

Role of Hypermutation in Immune Responses

To understand the role of SHM in the antibody response, several groups have studied the extent of Ig gene mutation at different times after the immunization of mice. Studies of the responses to p-azophenylarsonate (Ars), phosphorylcholine, influenza hemagglutinin, oxazalone, and several other antigens have all indicated that the initial response after primary immunization is established by antibodies showing no somatic mutation. About 1 week after immunization, mutated sequences begin to be observed, increasing during the next week or so. Booster immunizations yield sequences showing additional mutations.

Many hybridomas made late in the immune response produce mutated antibodies with a higher antigen affinity than the unmutated (sometimes loosely called "germline") antibodies made early after immunization. The shift to higher affinity is a phenomenon long recognized at the level of (polyclonal) antisera and has been termed "affinity maturation." This phenomenon can now be explained as the result of an "evolutionary" mechanism selecting antibodies of progressively higher affinity from the pool of randomly mutated V sequences.

According to this model, at the time of initial antigen exposure an animal has a set of naïve B-lymphocytes expressing IgM with germline (unmutated) versions of Ig variable regions resulting from gene rearrangements that occurred prior to immunization. Because of the diversity of available V_H, D,J_H, V_L, and J_L sequences as well as the impressive recombinational potential described previously, some B cells will express Ig molecules capable of binding the antigen with modest affinity. Antigen binding stimulates these B cells to proliferate and to move to lymphoid follicles, where they form GCs. In the GC environment,

AID is expressed and SHM machinery is activated, generating random mutations in the Ig genes of stimulated GC B cells. Many of these mutations reduce the affinity of the encoded antibody for antigen,[201] and some may induce autoantibody specificities (i.e., the ability to bind to self-molecules[202]). As clearance of the antigen lowers the antigen concentrations, only the cells displaying high affinity for antigen will be stimulated effectively; cells displaying lower-affinity antibodies or antibodies with affinity for self-antigens are subjected to programmed cell death ("apoptosis").[203,204] The preferential proliferation of the high-affinity cells and their maturation to secreting plasma cells causes an increase in the average affinity of the antibodies in the serum. Some high-affinity cells become memory cells, persisting long after the initial antigen exposure, ready to respond to a subsequent antigen exposure with rapid production of high-affinity antibody. In this model, the driving force for affinity maturation— analogous to natural selection in the evolution of species—is selection for high-antibody affinity in the face of falling antigen concentration. The importance of this selective force is suggested by the observation that affinity maturation can be inhibited by repeated injection of antigen (which removes the selective pressure for high affinity)[205] or by overexpression of the antiapoptotic protein Bcl-XL (which allows survival of B cells expressing low affinity antibody).[206]

Cellular Context of Somatic Mutation

Each GC appears to be populated by a small number of antigen-specific founder B cells[207] and an unusual Thy-1 negative T-cell population, also antigen-specific,[208] The GC environment promotes contact between the B cell and follicular dendritic cells— which store, process, and present antigen—and T-lymphocytes, which activate somatic mutation in part via CD40-CD40Ligand interaction.[209] In a widely accepted model of GC function, SHM occurs in a subpopulation of B cells known as centroblasts. These cells proliferate in the "dark zone" of the GC and bear characteristic surface markers including IgD, CD38, and the receptor for peanut agglutinin. Proliferating GC centroblasts give rise to nondividing centrocytes in the "light zone" of the GC; there centrocytes are programmed for apoptosis unless they are rescued by follicular dendritic cell-presented antigen and T-cell activation via CD40 engagement. Selection for high-affinity antibodies occurs because cells expressing high-affinity antibodies are most efficiently rescued from apoptosis. Surviving centrocytes may return to the dark zone to undergo several successive cycles of mutation and proliferation followed by selection. This model is supported by direct observation (by two-photon microscopy) of B cells moving between light zone and dark zone.[210] Migration of

B cells to follicles or GC zones is thought to be controlled by a complex system of chemotactic receptor /ligand pairs including CXCR5/CXCL13, CXCR4/SDF1, CCR7/CCL9 (or CCL21), S1P1/sphingosine-1-phosphate, and EBI2/its lipid ligand.[211] Although certain features of this classical model have been challenged, the broad outlines have received strong support from studies in which cells in the dark zone or light zone of individual GCs can be marked by a photo-activatable green fluorescent protein (GFP) and then followed for several hours using two-photon imaging as the cells migrate through a mounted lymph node.[212]

The notion that GC B cells compete to avoid apoptosis on the basis of antigen affinity of their BCR has been has been supported by a study that directly compared caspase activation (an apoptosis marker) in B cells expressing transgenic antibodies with higher versus lower affinity for the same antigen; the lower-affinity cells were found to undergo a significantly higher rate of apoptosis.[213] The mechanism by which high-affinity antigen binding selects for survival is not fully understood. One possibility is that a higher-affinity BCR directly delivers a stronger activation signal to the B cell, inhibiting apoptosis. However, a second possibility is that a higher-affinity BCR could capture antigen more efficiently, enabling stronger antigen presentation to GC T cells, which could then deliver stronger survival signals to the B cell via secreted or surface protein interactions. Consistent with the second model, experiments using two-photon microscopy have documented that that B cells from mice immunized with a

fluorescent antigen can capture that antigen from follicular dendritic cells in the GC.[214] To test whether B-cell antigen capture might confer survival independently of the BCR, investigators engineered B cells in ovalbumin-primed mice to deliver ovalbumin via a surface lectin instead of via the BCR; they observed that the internalized ovalbumin antigen was capable of conferring a B-cell survival advantage in the GC in the absence of BCR engagement.[212] However, effective BCR signaling can enhance antigen processing and presentation,[215] so internalization/presentation and BCR signaling may work together to promote GC B-cell survival and mediate selection for high affinity.

Most experiments on SHM have focused on GC cells, as does the discussion in this chapter. However, T-cell-independent SHM may also occur in a population of less mature cells, which may populate the splenic marginal zone and which may increase the repertoire of circulating lymphocytes prior to antigen exposure, especially in young individuals,[216,217] or perhaps function in tolerance induction, as discussed previously. Also, mice lacking histologically detectable GCs as a result of lymphotoxin-α deficiency are capable of SHM and affinity maturation.[218] T-cell-independent antigens can induce a low level of SHM in B cells.[219]

Molecular Mechanism of Hypermutation

AID deaminates cytidine to uracil, an analog of thymine. Thus, if replicated before repair, an original C:G base pair would, in the daughter cell receiving the uracil-bearing DNA strand, mutate to a T:A. But it was initially not clear how cytidine deamination could affect A:T base pairs, which are targeted in 50% to 60% of mutations observed in SHM. By analyzing abnormalities in SHM that are observed in various mutant B cells, Di Noia and Neuberger[150] formulated a model for SHM that has explained this question and has gained wide acceptance (Fig. 6.13). The model proposes that after AID-catalyzed deamination creates a uracil residue in the target DNA, the possible outcomes depend on how the resulting mismatch is resolved. 1) The U:G mismatch may be replicated as described above, resulting in what are known as phase 1A mutations. 2) The uracil base may, before replication, be excised by UNG, creating an abasic site. Normally, the creation of such abasic sites is the first step of the base excision repair pathway, in which subsequent steps remove the sugar-phosphate backbone, leaving a single nucleotide gap that is then restored to a C:G bp by DNA polymerase β and DNA ligase. If the DNA replicates before the abasic site is repaired, the strand with the abasic site may directly engage translesional polymerases (which are error-prone, as discussed below) to insert an unpaired nucleotide (i.e., any nucleotide) opposite the abasic lesion, leading to phase 1B mutations (see Fig. 6.13). 3) The original U:G mismatch— or possibly the abasic site created by UNG action—may be recognized by the mismatch repair (MMR) system of the cell. MMR triggers excision of the DNA strand for several nucleotides surrounding the mismatch, and this strand is then resynthesized by polymerase β, in the case of faithful repair, or by error-prone polymerases, in the case of SHM. Error-prone repair inserts mispaired bases, which may become fixed on one strand by replication, creating mutations (designated phase 2) from both A:T and G:C bps at some distance from the position of the original U:G mismatch. If a mispaired base is recognized by MMR before replication, a new cycle of MMR may be initiated, extending mutation even farther from the initial deamination event.

Role of Uracil-N-Glycosylase in Somatic Hypermutation. The UNG gene encodes two proteins that differ in their N termini as a result of alternative promoters that generate different initial coding exons. UNG1 is expressed in mitochondria, whereas UNG2 is nuclear. Hydrolytic deamination of cytosines to uracil occurs at a significant rate in all eukaryotic and prokaryotic cells, and misincorporation of dUTP during replication further contributes to the load of uracil in DNA. UNG2 plays a major role in mitigating this load by initiating faithful base excision repair.

In SHM, the faithful repair of uracil is somehow subverted to introduce mutations. *Ung*-/- mice and human patients with a rare form of HyperIgM immunodeficiency due to *UNG* mutations have similar immunologic phenotypes. First, as discussed in the following, *Ung*-/- individuals are profoundly defective in CSR, as expected if this process requires AID-catalyzed deamination of cytidine followed by *UNG*-catalyzed removal of uracil. Although the frequency

of mutation is roughly normal in *UNG*-deficient individuals, mutations at C are almost exclusively transitions of C to T (phase IA). As suggested by Figure 6.13, *Ung*-/- individuals would not create the abasic sites that lead to C → G and C → A mutations by replication, though some mutations of these kinds could be produced by MMR. Indeed, the frequencies of mutations

from A, T, and G in *Ung*-/- individuals are normal, apparently resulting from MMR activated by the U:G mismatch in many cells.

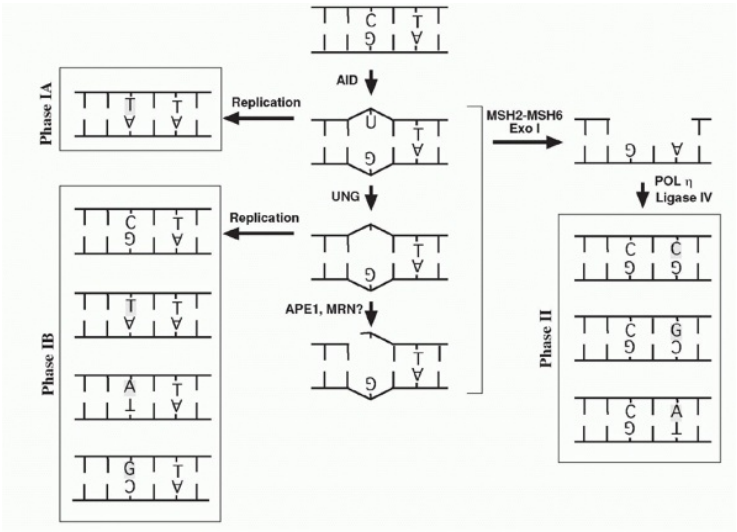

FIG. 6.13. Mechanistic Model of Reactions Triggered by Activation-Induced Deaminase (AID) in Somatic Hypermutation and Class Switch Recombination (CSR). The graphic at the top depicts a small region in a VH gene showing a targeted C:G basepair and a nearby T:A pair. After AID-catalyzed deamination of a C residue, the DNA may be subjected to various modifications as shown, leading to mutations indicated by nucleotides in gray rectangles. The model is described in the text. The Phase II mutations occur when mismatched bases incorporated by an errorprone polymerase are replicated before they can be corrected by MMR. The same mechanism explains two switchregion -associated events occurring during CSR: DNA strand breaks (see center panel at bottom) and mutations.

Mismatch Repair in Somatic Hypermutation. MMR is a highly conserved mechanism that detects abnormalities in DNA, including mispaired nucleotides and abnormal bases, and repairs them. The eukaryotic MMR system has two main components. MutS binds tightly and specifically to DNA defects and recruits MutL. The Mut complex then activates a latent nuclease activity to remove a segment of the DNA strand including the mismatched base; this gapped strand is then resynthesized by a DNA polymerase. Mammals have several MutS homologs, including three reported in somatic cells—MSH2, MSH3, and MSH6—which exist in the cell as heterodimers. MSH2-MSH6 (also known as MutSα) is the most abundant form and is specialized for recognizing single base gaps or mismatches, while MSH2-MSH3 (MutSβ) recognizes larger gaps and insertion/deletion loops. The mammalian MutL proteins are heterodimers consisting of MLH1 paired with PMS1, PMS2, or MLH3. Apart from their effects on SHM and CSR, mutations in MMR genes, especially MSH2 and MLH1, underly hereditary nonpolyposis colorectal cancer. Recent efforts in several laboratories have led to *in vitro* reconstitution of mammalian MMR with purified components, enabling powerful analysis of this complex mechanism.[220] In addition to the MutS and MutL proteins, the system requires the following components: Exo1 to excise the gap; replication protein A, which binds to the single strand DNA in the gapped region (and is known to bind to phosphorylated AID, as discussed previously); proliferating cell nuclear antigen (PCNA), which promotes processivity by encircling DNA in a sliding ring clamp and by recruiting other

components; DNA polymerase δ; DNA ligase I; and several other proteins.

Knockouts of MSH2, MSH6, and Exo1 have been studied by several laboratories and show consistent decrease in B-cell SHM from A:T bps (with a corresponding increase in the percentage of mutations from G:C bps).[221,222] These results, in the context of the model of Figure 6.13, suggest that MMR triggered by the MSH2-MSH6 heterodimer primarily introduces mutation at A:T bps. The predilection for

mutating A:T bps matches the activity of polymerase η, as discussed in the next section, implying that this polymerase may be the one most frequently engaged by the MMR machinery in repairing AID-generated lesions. Indeed, MSH2-MSH6 is capable of binding to a U:G mismatch, MSH2 can bind to polymerase η in cell extracts, and MSH2-MSH6 can stimulate the activity of polymerase η *in vitro*.[223]

Error-Prone Polymerases. Although avoidance of error is a high priority for most DNA replication, error-prone DNA synthesis served an important function long before SHM evolved to mutate Ig genes; indeed, error-prone polymerases have been most thoroughly studied in *E. coli* and yeast. These polymerases are useful in all cells for replication of DNA containing focal lesions that would block replication by stringent high-fidelity polymerases. However, the tolerance of these error-prone polymerases to abnormalities in DNA structure is accompanied by tolerance of non-Watson-Crick basepairing in the replication of normal DNA and by absence of DNA proofreading activity. More than a dozen of these polymerases have evolved in eukaryotes, each specializing in different aspects of "translesion DNA synthesis" (TLS) and showing differing spectra of infidelity in replication of normal DNA. Clear evidence for participation of several TLS polymerases in SHM has come from comparing the known *in vitro* activities of a particular polymerase with the SHM abnormalities seen in mice or humans with mutations in the corresponding polymerase gene.

Polymerase η characteristically inserts mismatched bases opposite T nucleotides, and individuals lacking this polymerase show the predicted abnormality: decreased mutations at A:T base pairs. This was first demonstrated in patients with xeroderma pigmentosum variant disease, whose defects in polymerase η subject them to sunlight-induced skin cancers in addition to their abnormal SHM; a similar defect in mutations from A:T bps was subsequently shown for polymerase η knockout mice.[224,225] Polymerase η binds to MSH2-MSH6, as mentioned previously, and is upregulated in cells undergoing SHM.

In addition to polymerase η, the TLS polymerase REV1 has been shown to participate in SHM. REV1 is known to preferentially insert cytosine residues opposite uracil or abasic sites, and *Rev1* knockout B cells were found to be impaired in C to G mutations, especially in the nontranscribed (coding) strand.[226] Polymerase ζ,[227,228] polymerase κ,[229] and polymerase θ[230,231,232] knockouts have also been reported to show altered SHM patterns, but these effects are smaller or not seen in all circumstances.

Error-prone repair apparently operates in competition with faithful repair carried out by DNA polymerase β. The human B-cell line BL2 is known to undergo SHM, but in subclones of this line with higher SHM proficiency, polymerase β levels were very low; overexpression of polymerase β in a proficient subclone suppressed SHM activity.[233] Compared to wild-type B cells, polymerase β-deficient B cells (developing in a wild-type recipient) showed increased switch region mutation after induction of CSR *in vitro*.[234]

A fundamental question about the action of error-prone polymerases is how they are specifically engaged for SHM of Ig genes, given that most spontaneous cytosine deamination in nonlymphoid cells is accurately repaired. One apparent clue is that some TLS polymerases are upregulated in B cells undergoing SHM. In addition, when the sliding clamp protein PCNA is monoubiquitinated at lysine residue K164, it is known to bind and activate TLS polymerases including polymerase η. When this ubiquitination is prevented, either by knockout of the specific E3 ubiquitin ligase of PCNA or by a K164R mutation at the ubiquitination site in PCNA, SHM is abnormal. $PCNA^{K164P}$ mice were found to have a dramatically decreased ability to mutate A:T sites during SHM, similar to the phenotype of the polymerase η knockout, as though polymerase η requires monoubiquinated PCNA in order to

participate in SHM.[235] However, these clues do not fully explain why Ig genes in GC B cells are less faithfully repaired than other genes in the same cells. This question is part of the larger issue of mutational targeting, as discussed below.

Targeting and Distribution of Mutations

The mutation rate of Ig genes in GC B cells undergoing SHM may reach as high as 10^{-3} mutations/bp/generation, or about 10^6 times higher than the normal genomic mutation rate;[236] this elevated rate could be lethal to B cells if mutations were not carefully targeted specifically to Ig genes. Several other genes highly expressed in GC B cells were also found to be mutated at lower levels (e.g., *Bcl6* and *Cd95*[237]), though several other genes expressed in GC B cells at comparable levels are not mutated. Apparently, some of the features that target SHM to Ig genes may be shared by other genes.

Recently, a genome-wide analysis has made it clear that AID binds much more widely to the genome than was previously appreciated. When chromatin from mouse B cells incubated with lipopolysaccharide (LPS) and IL-4 was analyzed by ChIP with an anti-AID antibody, 5910 genes were found by deep sequencing of the immunoprecipitated DNA (ChIP-seq),[160] including many previously described AID targets. Although this is a large number, it is still a small minority of the genome. As judged by the number of sequence tags recovered, AID binding to the IgH locus was substantially higher than binding to any other region. A sample of genes identified as AID-binding by ChIP-seq showed significant mutation frequency, whereas control genes did not. The AID-binding genes were associated with high levels of mRNA transcription (by RNA-seq), by a chromatin mark commonly associated with transcription—histone H3 trimethylated on Lys4 (H3K4me3)—and by density of Pol II binding. However, only the IgH locus, with its uniquely high levels of AID occupancy and mutations, bound to RPA; this binding was AID-dependent and was diminished in the S38A and T140A mutants of AID described previously.

The difference between AID-targeting and SHM frequency has been reinforced by analysis of SHM in panels of non-Ig genes isolated from murine Peyer patch B cells.[238] All the non-Ig genes were mutated at levels much lower than the 2.2×10^{-2} mutations per bp detected near J_H4, but substantial numbers of mutations were found at *Bcl6*, *Pim1*, *CD79B*, and several other previously reported targets of SHM. The mutations were almost completely AID-dependent, as in cells from *Aicda*-/- mice, all genes showed very low mutation

frequencies that were barely above the sequencing error rate. However, different genes varied widely in the way their mutation rates were affected by the double knockout *Ung-/- Msh2-/-,* in which only phase 1A mutations should be possible. At one extreme, *c-myc*, which is known to mutate at a very low rate in GC B cells, showed a mutation rate in the *Ung-/- Msh2-/-* cells almost 17-fold higher than in the wild-type, as though almost all AID-induced deamination in the wild-type B cells had been faithfully repaired by UNG- and Msh2-dependent mechanisms. In contrast, *Bcl6* mutations were only 1.3-fold higher in the double knockout, suggesting that faithful repair of this gene was dramatically less active.

Thus SHM susceptibility depends not only on differential AID targeting across the genome but also on differential ratios of error-prone versus faithful repair. The distinction between these two variables (deamination and repair) has only recently been recognized, so it was not taken into account in many earlier studies described in the following.

RNA Transcription. A relationship between SHM and RNA transcription is suggested by the observation that unrearranged V_H and Vκ genes are generally neither transcribed nor mutated, but become susceptible to both processes when V(D)J recombination moves them close to their (respective) intronic enhancers, Eμ and iEκ. In contrast, the λ locus lacks an enhancer between J and C; unrearranged Vλ regions are transcribed in B cells[239] and can be mutated.[240] The Ig coding sequences are apparently not specifically required to target SHM, as transgenic V coding sequence can be replaced by a human β-globin gene or prokaryotic *neo* or *gpt* gene without affecting the hypermutation rate downstream of the promoter.

Mutations around a given V region are distributed in a domain that begins roughly 100 to 200 bps downstream of the promoter, extends for ~1.5 to 2 kb downstream, and then tapers off long before the RNA transcriptional termination. This distribution led to the hypothesis that after transcriptional initiation, a "mutator factor" attaches to the transcriptional machinery, attacks DNA as the transcription complex moves downstream, and eventually falls off, so that further transcription proceeds without mutations.[241] Consistent with this model, a VκJκ-Cκ transgene bearing a second Vκ promoter engineered upstream of the Cκ region was found to incur mutations over a second domain extending into the Cκ region, in addition to the usual V region mutations.[242] Conversely, the insertion of an irrelevant 2 kb DNA fragment between a Vκ promoter and the leader (signal peptide) exon prevented mutation within the Vκ transgene, which now apparently lay downstream of the mutational domain.[243] (Mutations also occur in domains surrounding repetitive switch regions upstream of C_H genes. The frequency of these mutations is similar to that in V regions, but the domains are larger, correlating to some extent with the length of the switch region, as discussed in the following.) The key mutator factor is apparently AID, which can deaminate double-strand DNA *in vitro* only when the DNA is transcribed. One hypothesis suggests that DNA near the advancing RNA polymerase complex becomes negatively supercoiled or partially single-stranded, which might facilitate access of the DNA to AID.[244] Another explanation, involving R loops, is described below. Evidence for patches of single-stranded DNA in transcribed V regions undergoing SHM has been obtained using a bisulfite technique.[245]

In vivo dependence of SHM frequency on transcriptional activity has been confirmed by several findings. In one study, SHM was studied in knockin mice with a prerecombined V_HDJ_H region driven by either of two V region promoters; SHM rates in these knockin strains were highly correlated with transcription driven by the different promoters.[246] In another study, a tetracycline-inducible GFP reporter gene engineered with a stop codon was stably transfected into a B-cell line. The rate of reversion of the stop codon by SHM (allowing GFP expression) was found to be directly related to the transcription rate, as regulated by a tetracycline analog.[247] Other studies examining SHM in models where transcription was altered by mutating enhancers have supported the relationship between SHM and enhancer-induced transcription. Some non-Ig enhancers could support SHM in a stably transfected cell line,[248] but others could not, leading to the suggestion that specific elements in enhancers might confer susceptibility to SHM. One candidate element is the sequence CAGGTG, which is a target for E-box transcription factors and which is found in several Ig enhancers. When a murine κ enhancer containing CAGGTG was linked to a GFP gene inactivated by a premature stop codon, stable transfectants of chicken B-cell line DT40 were found to produce mutants that expressed GFP, but a single mutation in the CAGGTG motif prevented SHM without changing transcription.[249] Inactivation of the E-box transcription factor E2A in DT40 cells was found to reduce SHM of endogenous Ig genes without significant effects on Ig gene (or AID) transcription.[250]

However, some conflicting conclusions have been drawn about enhancer-dependence of SHM depending on whether experiments investigated transgenes versus constructs created in the endogenous context using homologous recombination (i.e., knockouts or knockins). For example, the downstream IgH enhancers HS3b and HS4 were found important for SHM in transgenes[251] but dispensable in the context of endogenous genes.[252]

In one case, transcriptional enhancer activity has been clearly separated from an associated SHM stimulating activity. Just downstream of the chicken Ig λ 3′ enhancer lies a DNA segment whose deletion in the DT40 B-cell line severely impaired SHM without dramatically affecting transcription; this segment could confer SHM when inserted into a non-Ig locus.[253,254]

To summarize, it appears that transcription is necessary but not sufficient for targeting hypermutation, but the additional elements required for SHM have not been defined as of this writing (though E2A function may be among them).

Chromatin Marks. A parameter related to transcription of Ig genes (i.e., their context in chromatin) has also been studied in relation to SHM targeting. Culture of a B-cell line under conditions that upregulate SHM (in the presence of T cells and anti-IgM) led to increased acetylation of histone H3 and H4 at V region but not C region DNA, as assessed by ChIP analysis[255] paralleling the distribution of mutations. The increased histone acetylation was not a consequence of AID action as it occurred when AID expression (and SHM) was inhibited by antisense treatment; moreover, when cells were treated with the deacetylase inhibitor trichostatin A, the C region was both acetylated and subjected to SHM. Other histone modifications that may be correlated with SHM are phosphorylation or monoubiquitination of histone H2B.[256,257]

Hotspot Focusing. Mutations occurring within V region genes expressed *in vivo* in B cells may be highly selected for antigen-binding function of the expressed antibody. To analyze the spectrum of mutations produced by SHM unbiased by selection, investigators have studied nonproductively rearranged VDJ alleles or "passenger" transgenes engineered with stop codons to prevent expression as a protein. In these genes, mutational "hot spots" as well as "cold spots" have been recognized, apparently due to local sequence features that may promote or suppress somatic mutation. The consensus sequence WRC (i.e., [A/T][G/A][C]) is the most consistent hotspot for mutation, presumably reflecting the predilection of AID for *in vitro* deamination of this sequence. It is possible that evolution has concentrated mutational hot spot frequencies in CDR regions to enhance the potential for diversity generation in the parts of the protein critical for antigen contact.[258]

The previous discussion has identified several factors influencing the targeting of SHM, a process in which a unique triggering event—AID-dependent deamination of DNA—is followed by a cascade of other events that depend on mechanisms common to most cells. All of these steps, including the AID-dependent trigger, can be affected by biologic parameters common to all cells, such as transcription, enhancers, epigenetic state, DNA repair mechanisms, etc. Many of these same mechanisms affect CSR as well as SHM. Recently, three additional biologic parameters have been found to influence AID-triggered events in CSR: RNA polymerase stalling, the RNA splicing in spliceosomes, and RNA degradation by exosomes. It is possible that these three processes impinge on both SHM and CSR, but because they were discovered in the context of CSR, they are discussed in the following section.

Heavy Chain Switch

Switch Regions and Switch Junctions

As briefly mentioned previously in this chapter, isotype switching involves removal of Cμ from downstream of the rearranged H chain VDJ gene and its replacement by a new downstream C_H region. This occurs by a deletional recombination—CSR—in which the recombinational breakpoints generally occur within G-rich repetitive DNA sequences known as switch (or S) regions lying 5′ of each C_H region (except Cδ). While most switch breakpoints fall in the S regions, some are in nearby nonrepetitive DNA. The S region of the mouse μ gene, Sμ, is located about 1 to 2 kb 5′ to the Cμ coding sequence and is composed of numerous tandem repeats of sequences of the form (GAGCT) n(GGGGT), where n is usually 2 to 5 but can range as high as 17. All of the S regions of downstream isotypes include pentamers similar to GAGCT and GGGGT embedded in larger repeat units rather than precisely tandemly repeated as in Sμ. In support of the critical role of S regions for CSR, knockout of Sγ1 by homologous recombination essentially abolished expression of IgG1 from that allele,[259] and mice with a deletion of Sμ were also dramatically impaired in CSR.[260,261]

A switch recombination between, for example, μ and ε genes produces a composite Sμ-Sε sequence (see Fig. 6.12). From a comparison between the sequence of an Sμ-Sε composite switch region and the sequences of the germline Sμ and Sε, one can localize the exact recombination sites between Sμ and Sε that occurred in each allele. Such comparisons have indicated that there is no specific site, either in Sμ or in any other S region, where the recombination always occurs, although clusters of recombination sites have been reported at

two specific regions within the tandem repeats of murine Sγ3 region.[262] Thus, unlike the enzymatic machinery of V-J recombination, the switch machinery can break and join sequences in a broad target region, and as the recombination targets are in intronic DNA, there are no reading frame complications. Often both IgH alleles in a single cell undergo switching to the same downstream isotype. Some alleles undergo sequential switching events; for example, a common pathway to IgE expression is an initial μ → γ1 switch, followed by a CSR between the composite Sμ-Sγ1 switch region and the Sε region.[263] Sγ1- Sε switching may even occur independently of Sμ.[264] Although most CSR occurs as a deletion within a single IgH allele, switching between two allelic chromosomes was detected at a frequency of roughly 7% to 10% in mouse and rabbit.[265,266]

DNA fragments excised by switch recombination have been cloned from fractions of circular DNA isolated from cells actively undergoing isotype switch recombination. Thus, at least some of the excised DNA segments ligate their ends to form "switch circles"; these contain composite switch junctions that are in theory reciprocal to the composite switch junction retained on chromosomal DNA (see Fig. 6.12). Because switch circles are not linked to centromeres and do not apparently contain origins of replication, they are not efficiently replicated. Therefore, they are not found in cells that have divided many times after switching (e.g., in myelomas or hybridomas).

Many composite switch junction sequences show mutations near the recombination breakpoint when compared to the corresponding germline switch sequences. Indeed, many features of CSR are shared by SHM (as indicated previously in this chapter), including the requirements for transcription, AID, UNG2, and MMR components for normal CSR and SHM. However, CSR is more complex in that it involves simultaneous targeting to two DNA regions (i.e., switch

regions of Cμ and the target Cx), it requires dsbs, and it consequently requires mechanisms to repair the breaks. AID can theoretically trigger single-strand breaks on both DNA strands by the mechanisms discussed for SHM, including cleavage by APE-1. If these breaks are close enough together, they can effectively form a dsb with staggered ends, and if dsbs occur in two switch regions, the DNA repair mechanisms common to all cells may rejoin the broken ends to complete a CSR event. Indeed, B cells in which Sμ and Sγ1 were deleted and replaced by recognition sites for the yeast homing endonuclease I-SceI were capable of mediating some μ → γ1 CSR in the absence of AID if I-SceI was expressed,[267] highlighting the participation of ubiquitous AID-independent repair mechanisms for CSR. These mechanisms depend on the NHEJ components described previously in this chapter plus backup participation by alternative end-joining mechanisms discussed below.

Regulation of Isotype Switching: Proliferation and AID Expression

Isotype switching occurs physiologically in animals about 1 week after immunization with T-dependent antigens, at about the same time that somatic mutation of Ig genes begins. Both processes normally occur in GCs of lymphoid organs, a location that facilitates interactions between B cells, T cells, and follicular dendritic cells presenting antigen. As demonstrated by *in vitro* switching experiments, T cells promote switching by secretion of cytokines (especially IL-4 and transforming growth factor β) as well as by cell-to-cell contact.

A major component of the cell contact signal is mediated by an interaction between the B-cell surface marker CD40 and its ligand—designated CD40L, CD154, or gp39—which is expressed on activated T cells (primarily CD4+). CD40 is a member of the TNF-receptor family, while CD40L belongs to the TNF-ligand family. The dependence of switching on the CD40-CD40L interaction is highlighted by the genetic disease known as the X-linked hyper-IgM syndrome-1, which was found to be caused by a defect in the human gene encoding the CD40L/gp39.[268] Like AID-deficient patients with hyper-IgM syndrome-2 described previously, patients with X-linked hyper-IgM syndrome-1 have elevated concentrations of IgM in their serum and almost no Igs of other isotypes. In addition, their antibodies fail to show affinity maturation or evidence of B-cell memory responses. Similar defects are seen in humans with mutations in their CD40 gene, an autosomal recessive disease designated hyper-IgM 3.[269]

CSR impairment may also be caused by abnormal function of CD40 signaling pathway components including IKKγ (also known as NEMO), NFκB proteins, and C-Jun N-terminal kinase. Mouse strains with engineered defects in CD40 are defective in SHM and T-dependent CSR, but respond with normal isotype switching to T-independent antigens. The T-independent switching pathway may be especially important for gut-associated switching to IgA.[270] One role of the CD40 engagement is to induce B-cell proliferation. Indeed, other proliferative stimuli (e.g., LPS or IgM or IgD crosslinking) can support cytokine-induced isotype switching *in vitro* in the absence of T cells and CD40 activation. The relationship of CSR to cell division is supported by evidence that switching is linked to the cell cycle[271] and to the number of cell divisions after stimulation,[272] a phenomenon which may reflect cell division-related regulation of AID expression.[273] However, apart from activating proliferation, CD40 has additional effects that may facilitate switching, including upregulation of IL-4 responsiveness and IL-4 receptor number,[274] upregulation of sterile "switch transcripts" (discussed below), and upregulation of AID expression. Activated B cells also express CD40L, which can not only trigger CD40 signaling but also transduce a "reverse" signal affecting B-cell function.[275] Independent of CD40, B-cell activation can independently be stimulated by TLR ligands and cytokines such as APRIL, another member of the TNF family.[276]

Regulation of Class Switch Recombination to Specific Isotypes: Promoters, Enhancers, and Chromatin

Different isotypes are known to predominate in different immune responses depending on the antigen, route of antigen administration, and several other parameters. These different parameters act in part by influencing the cytokine milieu of the B cells. IL-4, for example, promotes the expression of IgE and IgG1, whereas TNF-β promotes switching to IgA. These lymphokines have been proposed to act by making the targetisotype "accessible" to switch recombinase machinery that may be largely non-isotype-specific. The accessibility is associated with expression of a "sterile" or "germline" RNA transcript that initiates upstream of a target S region (see Fig. 6.12) and extends through the target C region. The germline transcript is spliced so that a noncoding upstream exon known as an I ("intron") region is joined to the first coding exon of the C region. (This contrasts with the "productive" transcript containing VDJ spliced to the C region.) The same experimental conditions—particularly the same cytokines—that favor the accumulation of germline transcripts from a particular isotype generally also stimulate switch recombination to the same isotype. In many cases, the signals transduced by the cytokine receptor have been elucidated. For example, IL-4 stimulates germline transcription by activating the transcription factor STAT6, which binds to one of several IL-4 response motifs in the promoter region upstream of Iε and Iγ1. CD40 engagement also acts in part through NFκB-mediated binding to I region promoters.[277]

Gene targeting experiments have shown that mouse strains lacking the I region (and its promoter) of a particular isotype do not switch to that isotype, reinforcing the idea that sterile transcription is necessary for CSR.[278] The low extent of sequence conservation of the I exons and the lack of consistent open reading frames suggest that these transcripts do not encode a functional protein. Indeed, the exact sequence of the I region may be irrelevant as an I region can be replaced by an unrelated sequence and still support CSR.[279] However, the transcribed exon upstream of the S region apparently needs a splice donor site allowing splicing to the downstream C region, as a targeted construct lacking such a splice donor site was reportedly unable to support CSR even though transcription through the S region occurred.[280]

Apart from I region promoters, germline transcription and isotype switching are also regulated by IgH enhancers. A combined deletion of the murine 3′-regulatory region enhancers HS3b and HS4 was found to cause a significant impairment in switching to most isotypes, although switching to IgG1 was unaffected (and IgA only moderately decreased).[281] The diminished switching was associated with diminished germline transcription of the same isotypes,

suggesting that an important function of the enhancers in CSR is to increase germline transcription. The relative independence of γ1 from regulation by enhancers may be related to the putative locus control region associated with that gene.[282]

Enhancers are believed to function via physical interactions between enhancer-bound proteins and promoterbound proteins, creating a DNA loop that brings enhancers into close proximity to promoters. Such looping may have special significance in promoting DNA recombination between segments of DNA lying great linear distances apart in the chromosome, as has been discussed in the context of V(D)J recombination. Chromosome conformation capture experiments have shown that Eμ and 3′-regulatory region enhancers are in close proximity in mature resting B cells, and that when B cells are stimulated to switch to γ1 by LPS + IL4 (or to γ3 by LPS alone), the corresponding I region promoter moves close to the two enhancers.[283,284] This looping would bring Sμ, which lies just downstream of Eμ, close to the Iγ1 (or Iγ3) promoter, presumably facilitating recombination between Sμ and the Sγ region.

Another parameter correlated with regional transcriptional regulation is the chromatin context of the genes, including specific modifications of histone proteins, as discussed previously in this chapter. For example, acetylation of histone H3 (H3Ac) is associated with DNA regions of increased "accessibility" to transcription (as well as to experimental digestion by restriction enzymes), while transcriptional promoters tend to be marked with trimethylation of H3 at lysine4 (H3K4me3). In B cells stimulated to switch to γ1 by LPS + IL4 (or to γ3 by LPS alone), the corresponding I region and switch regions show increased H3Ac and H3K4me3 marks.[285,286] The importance of the H3K4me3 mark for transcription and CSR is highlighted by evidence that preventing this mark—by B cell-specific knockout of PTIP, a component of the machinery that catalyzes this modification—leads to impaired germline γ transcription and defective CSR to γ isotypes.[287] PTIP knockout B cells show decreased DNA looping between the 3′ enhancer region and Iγ region promoters, suggesting that PTIP contributes to this looping.[288] Knockdown of other factors that are necessary to maintain H3K4me3 also reduce CSR efficiency, including the components of the complex known as FACT (*fa*cilitates *c*hromatin *t*ranscription).[289]

Surprisingly, a histone modification generally associated with gene silencing—trimethylation of H3 lysine 9 (H3K9me3)—has also been reported to mark switch regions targeted for CSR in both mouse and human B cells.[290,291] Recently, a screen for nuclear proteins associating with AID *in vitro* identified KAP1 (KRAB domain-associated protein 1),[292] a transcriptional repressor that associates with heterochromatin protein 1, as binding to H3K9me-modified chromatin. By coimmunoprecipitation, KAP1 was confirmed to bind to AID *in vivo*, and B-cell-specific conditional knockout of KAP1 was found to diminish AID binding to Sμ and to impair CSR efficiency by about 50%.

Switch Region Targeting of AID in Class Switch Recombination: R Loops, Paused Polymerase II, and AGCT

A model in which AID loads onto an RNA polymerase complex and acts on DNA as the transcription complex travels downstream was discussed previously in the context of AID function in SHM, and a similar model apparently applies in the context of CSR. The domain of AID susceptibility in switch regions can be deduced from the distribution of C:G → T:A mutations in B cells that are defective for both UNG and MMR, because (as shown in Fig 6.13) in these cells the only AID-dependent mutations would be U:G mismatches resolved by replication to T:A. A study of the distribution of such mutations in clones from *Ung*-/-, *Msh2*-/- B cells found that a domain of mutations began about 150 bp 3′ of the Iμ transcription start site and extended 4 to 5 kb downstream, with diminishing mutation frequency near Cμ.[293] The location of AID binding in B-cell DNA can also be directly determined by ChIP-seq analysis, as discussed previously. AID was found to bind to many loci that are transcribed in B cells; indeed, the patterns of AID and RNA polymerase II binding detected by ChIP are very similar.[160] RPA, however, was efficiently bound only at IgH switch regions, and this binding was inhibited by the Ser38Ala mutation that blocks the critical Ser38 phosphorylation

discussed previously.

One likely consequence of germline transcription in facilitating CSR involves the formation of a stable RNA:DNA complex known as an R-loop. In this structure, RNA transcribed from the template strand of a DNA molecule binds tightly to that strand with Watson-Crick basepairing, displacing the other DNA strand, which forms a single-stranded loop. In support of the R-loop model, cell-free transcription across G-rich switch regions was found to generate a stable association of the transcript RNA with the template DNA[294]; significantly, no substantial association occurred when the switch region was transcribed in reverse orientation, leading to a C-rich transcript, or when the transcribed template was a DNA fragment other than a switch region. The displaced DNA strand in S region R loops was susceptible to deamination by AID.[155] Evidence that such R-loops form *in vivo* over S regions has been obtained from bisulfite analysis of singlestrand DNA regions in B cells.[295,296]

The tightly bound RNA-DNA complex on the template strand of an R-loop may explain both the high level of polymerase II accumulation and the AID-induced mutations in the vicinity of switch region DNA.[297] Progression of the polymerase II complex transcribing through the switch region might be impeded by the RNA bound to the template strand, leading to an accumulation of "stalled" polymerase II molecules; the "stalling" of AID molecules associated with the transcription complex might prolong exposure of the DNA to deamination by AID. Some support for this scheme was reported from an *in vitro* model in which AIDtriggered mutations in a transcribed DNA substrate were

increased when transcription was slowed by reducing the nucleotide triphosphate concentration.[298] The importance of stalled polymerase II for CSR was reinforced by the results of a screen for factors whose knockdown by specific shRNAs would inhibit CSR[299]: one protein recovered in this screen was the murine homolog of suppressor of Ty 5 (Spt5), a transcription elongation factor known to be associated with stalled polymerase II. Spt5 knockdown decreased both CSR and switch region hypermutation, as well as AID occupancy of the Sμ region, without affecting cellular levels of AID protein or germline transcripts. By coimmunoprecipitation experiments, AID and Spt5 were found to associate. And genes with high Spt5 occupancy by ChIP analysis were found to be most susceptible to mutations induced by AID overexpression. Polymerase II stalling has been extensively studied in yeast and drosophila, and is found in many genes that require more rapid changes in expression than can be achieved by modulating transcriptional initiation.[300] Apparently, the complex of proteins mediating polymerase II stalling has been adapted in B cells for the special function of regulating CSR of IgH genes.

The R-loop model explains how the "upper" nontranscribed DNA strand would become single-stranded and accessible to AID, but it raises the question of how AID might gain access to the "lower" template DNA strand, held in a tight RNA-DNA hybrid. This strand is known to be accessible to AID because it undergoes C → T mutations in *Ung*-/- Msh2-/- double knockout B cells.[293] A likely answer to this question has come from an analysis of proteins bound to AID when mixed with a B cell extract plus *in vitro*-transcribed switch region DNA.[301] The complex of AID and transcribed DNA was found to bind to components of the multisubunit RNA exosome. The exosome is an evolutionarily conserved structure containing nine core proteins that can associate with RNA nucleases, leading to degradation of RNA from template DNA; the exosome could thus potentially expose the template DNA strand to AID. Indeed, shRNA knockdown of one exosome component in the murine B cell line CH12F3 inhibited CSR. Moreover, exosome components were found to associate with AID *in vivo* by immunoprecipitation experiments and were detected (by ChIP) bound to switch region DNA in cells activated for CSR. Finally, in a deamination assay of a model switch region DNA transcribed *in vitro* by T7 polymerase, the addition of AID + RPA + PKA led to deamination of only the nontemplate strand, but the further addition of exosome components led to deamination on the template strand as well. These experiments highlight the exosome as a likely candidate for explaining AID action on the template strand of R-loops, though an unhybridized "lower strand" may also be produced as a result of transcription-dependent supercoiling or antisense transcription.

However, the propensity for R-loop formation is not the only property of switch regions that facilitates CSR. When the Sγ1 region was inverted, it retained about 25% of the wildtype CSR activity.[302] As this inverted, and now C-rich, DNA segment could not form an R-loop, this result suggests that while the R-loop contributes to CSR, other features of the S region that are preserved in the inverted sequence also play a role. Replacement of a natural murine Sμ sequence with the AT-rich frog Sμ sequence, which cannot form an R-loop, supported somewhat reduced but still substantial frequencies of CSR, and it functioned equally well in either orientation. Significantly, in either orientation, the recombination junctions were clustered in a portion of the S region that is rich in repeats of the sequence AGCT, which is a special case of the WRC consensus sequence for AID targeting, being present on both strands as a self-complementary palindrome. Indeed, the AGCT-rich region was a good substrate for *in vitro* deamination by AID when transcribed in association with RPA. The AGCT motif is enriched in all mammalian S regions and may be particularly effective as a target for CSR because its presence in clusters on both strands promotes closely spaced nicks on opposite strands, or even a dsb with a single base overhang if the cytosines in both strands of the same AGCT motif are targeted.[303] The density of AGCT in S regions correlates with the location of switch junctions better than the density of WRC or the boundaries of G-richness or R-loops.[296] These results all suggest that clusters of AGCT may represent a target for CSR that evolved in amphibians, with R-loop formation evolving later in mammals to further enhance AID accessibility to S region DNA.

DNA Breaks as Intermediates in Class Switch Recombination

The recombination event that underlies isotype switching includes DNA breaks and rejoining events that must involve both strands of DNA. Although the RAG-induced DNA breaks that initiate V(D)J recombination occur at the corresponding position on the two strands (yielding a blunt end and a hairpin), the nature of the ends in the initial CSR cleavage is not so clear. An early compilation of switch junctions[304] found only infrequent instances of microhomology at the junction (i.e., short sequence segments that are identical in the unrearranged S sequences near the recombination breakpoint). As these microhomology examples would be consistent with invasion of one DNA strand from Sμ targeting a short homologous region in a downstream S region (or vice versa), the rarity of such junctions has been interpreted as an indication that CSR only rarely occurs by strand invasion and instead usually proceeds by ligation of blunt DNA ends. However, dsbs with staggered ends would generally be the result of the widely accepted mechanism shown in Figure 6.14 (bottom left): a DNA break on one strand might result from AID-catalyzed cytosine deamination, removal of the resulting uracil by UNG, and singlestrand cleavage 5′ to the abasic site by an endonuclease, probably apurinic-apyridinic endonuclease 1 (APE1). The initial staggered ends could be converted to blunt ends through exonuclease trimming of a 5′ or 3′ single strand overhang, through filling in of a shorter 3′ end by a DNA polymerase, or through a combination of both processes. Filling in by error-prone polymerases could explain the mutations commonly observed around the switch junction, as mentioned previously.

Evidence supporting initially staggered DNA breaks in CSR was reported from occasional switch junctions observed in a model CSR substrate designed with two oppositely

oriented S regions such that CSR would occur by inversion, preserving both recombination junctions on the same chromosome. Several duplications at the ends of inverted DNA after CSR suggested that complementary overhangs at dsbs had been filled in before joining.[305] Additional evidence for staggered breaks has come from several investigators using ligation-mediated-polymerase chain reaction (LM-PCR) to detect double-strand DNA ends at switch regions in cells undergoing CSR.[306,307,308] LM-PCR protocols involving ligation of a blunt linker directly to blunt ends from genomic DNA were successful in amplifying blunt ends from DNA of B cells activated for CSR, but when the DNA was pretreated with T4 polymerase, which would convert staggered end cuts to blunt, the yield of amplified LM-PCR products was significantly increased, suggesting that most of the ends in the isolated genomic DNA (before T4 polymerase treatment) were staggered.

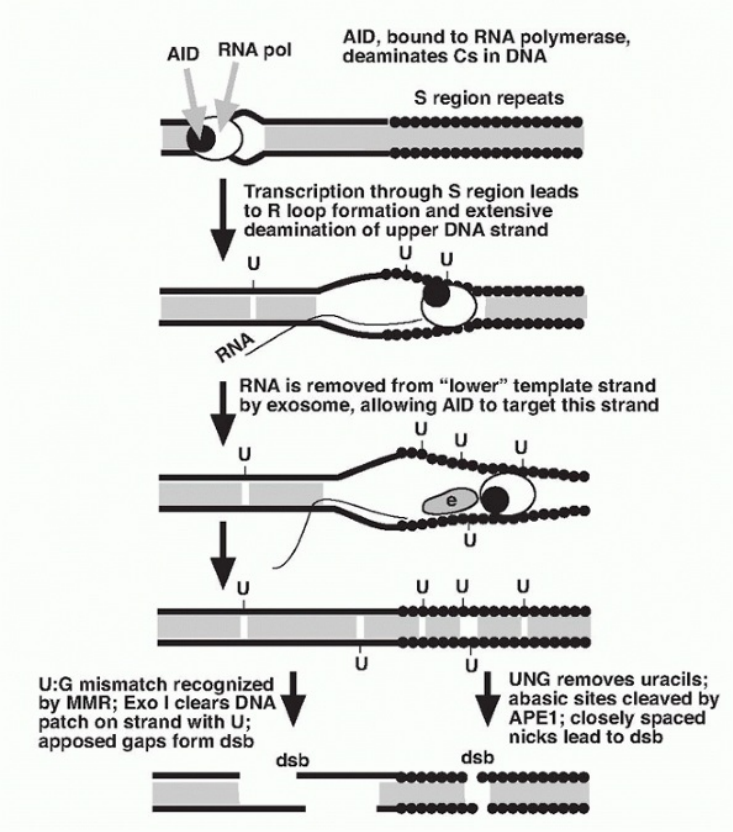

FIG 6.14. Mechanistic Model of Class Switch Recombination. This figure incorporates features of the most widely accepted models, but some aspects are uncertain at present. Two strands of deoxyribonucleic acid (DNA) near the 5′ boundary of a switch region are shown in *black*, with the S region repeats indicated by *dots*. DNA with normal Watson-Crick basepairing is indicated by the *gray shading* between the DNA strands. Ribonucleic acid (RNA) polymerase moves to the right, transcribing the sequence into an RNA strand; multiple other proteins accompany the RNA polymerase, but only activation-induced deaminase (AID) (*black circle*) is shown. AID can deaminate C residues to U in single-stranded R-looped DNA displaced by the RNA-DNA complex. The exosome complex (*e* in the drawing) degrades RNA in the RNA-DNA hybrid, leaving an unpaired lower strand where AID can deaminate C residues. DNA cleavage triggered by processing of uracils can lead to double-strand breaks in pathways dependent or independent of mismatch repair components as shown.

When the CSR-associated dsbs in Sμ were examined by LMPCR in B cells from normal and AID-/- individuals, the dsbs were significantly fewer in AID-/- B cells, though not completely absent. Furthermore, microscopic foci of the modified histone γH2AX (which rapidly accumulates at dsbs) were found at IgH genes (localized by FISH) in B cells undergoing CSR; these foci were strikingly diminished in AID-/- B cells, consistent with AID-dependence of dsbs in CSR.[309] These foci, as well as switch region dsbs, occur predominantly in the G1 phase of the cell cycle.[310] Like the off-target deamination by AID discussed previously in the context of SHM, off-target DNA breaks are another potential consequence of AID. B cells from mice overexpressing AID showed a high incidence of chromosomal translocations and DNA breaks compared with *Aicda*-/- mice; in the context of homozygous p53 deficiency (which allows cell growth despite DNA damage), many of the mice developed B-cell lymphomas.[311] In a study designed to systematically examine the locations of dsbs in mouse B cells stimulated to undergo CSR, ChIP was used to identify DNA associated with Nbs1, a protein that rapidly binds to dsbs *in vivo*, as discussed below.[312] This analysis detected hundreds of reproducible AIDdependent DNA break locations, some of which were syntenic

with DNA rearrangements found in human B-cell lymphomas. Many of the AID-dependent dsbs occurred in nontranscribed regions, unlike the CSR-related dsbs in IgH switch regions. Evidence indicates that AID-dependent off-target dsbs are normally repaired largely by homologous recombination: B cells defective for the homologous recombination component XRCC2 were found to harbor many more γ-H2AX foci than were found in *Xrcc2*+/+ cells or in *Aicda*-/- Xrcc2-/- cells.[313] B cells apparently protect against these breaks by upregulating *Xrcc2* transcription when activated for CSR.

Ubiquitously Expressed Components of Class Switch Recombination Machinery

UNG and Mismatch Repair. The model of Figure 6.13 suggests that after AID-catalyzed deamination of a cytidine residue, the resulting uracil is removed by *UNG*, creating an abasic site that is converted by APE1 to a single-strand DNA break (nick), which can become double-stranded if there is a nearby nick on the opposite strand. This model predicts that both CSR and the creation of dsbs would be severely impaired in the absence of *UNG*. Indeed, B cells from *Ung*-/- mice showed almost complete inability to switch *in vitro* to IgG1 or IgG3 secretion, and significant impairment in IgA secretion.[151] dsbs in *Ung*-/- B cells, as detected by LM-PCR, were also significantly reduced, but not abolished.[308] Human patients with homozygous *UNG* deficiency due to mutations in both *UNG* alleles showed a hyper-IgM phenotype, with a more profound defect than in *Ung*-/- mice: the patients showed essentially no IgG, IgE, or IgA secretion by stimulated B cells, and no dsbs (by LM-PCR) in Sμ.[314]

A role for APE1 or APE2 in CSR is supported by the observation that in mice with engineered deficiencies these genes, B cells induced for CSR show decreased switching and decreased induction of dsbs.[315]

The complementary pathways of UNG and MMR action in SHM were discussed previously (see Fig. 6.13); current evidence suggests a similar participation of MMR in CSR. Although

CSR was dramatically impaired in *Ung*-/- B cells (e.g., *in vitro* switching to IgG1 was reduced to about 6% of wild-type), the double knockout *Ung*-/- Msh2-/- caused significant further impairment (to 1.5% of wild-type IgG1 switching).[316] Evidence suggests that the C-terminal 10 amino acids of AID that are required for CSR but not SHM may function by stabilizing the interaction of UNG2 and the MSH2-MSH6 dimer to DNA.[317]

If a single-strand break created on one strand by UNG and APE1 is too far away from the the closest single-strand break on the opposite strand to create a dsb, then Exo1 engaged by MMR can chew from a nick on one strand toward a nick on the opposite strand, creating a dsb.[310,318] Consistent with this idea, mice engineered with a knockout of the MMR component Exo1 show a significantly decreased efficiency of CSR, to roughly 15% to 30% of normal.[319]

Thus, many of the same AID-triggered mechanisms that induce mutations in V_H regions operate in switch regions to induce dsbs. Once created, these dsbs are resolved by mechanisms distinct from the mechanisms resolving mismatches in SHM; however, as discussed in the following, some of the components that resolve dsbs generated in V(D)J recombination play a similar role in CSR.

End Joining Proteins in Class Switch Recombination. If AID triggers DNA cleavage at switch regions, ubiquitous DNA repair and ligation enzymes could participate in the subsequent DNA repair steps of CSR, as in V(D)J recombination. This possibility has been tested by engineered gene knockouts. However, as NHEJ knockouts would impair V(D)J recombination, thus blocking B-cell development prior to the stage of CSR, investigators have studied NHEJ in CSR using mouse strains with knockins of productive recombined H and L chain genes, which can undergo CSR. IgH/IgL knockin mice with intact Ku genes were able to switch to downstream isotypes, but the corresponding Ku-70- or Ku-80-deficient mice were reported to be dramatically impaired in CSR, although decreased cell proliferation could have contributed to this effect as more recent studies using different conditions report 30% to 50% residual CSR activity.[320]

The other “core” factors of NHEJ—XLF/Cernunnos, Ligase4, and XRCC4—catalyze the DNA ligation in NHEJ and have also been investigated for participation in CSR.[320,321,322,323,324] B cells deficient in any one of these genes show variably decreased CSR efficiency, with increased junctional microhomologies compared with normal B cells. This observation suggests that one or more fairly robust backup pathways—commonly called alternative NHEJ—can repair dsbs using microhomology-based ligation when “classic” NHEJ is inoperative. This conclusion is consistent with studies of alternative NHEJ repair of dsbs unrelated to CSR.[78] The proteins CtIP,[325] PARP1,[326,327] and XRCC1[328] apparently contribute to alternative NHEJ during CSR, as experimentally reduced expression of each protein decreases microhomology at CSR junctions.

Apart from these core NHEJ components, Artemis and DNA-PKcs are required for joining ends that require processing, like the hairpin coding ends produced in V(D)J recombination discussed previously in this chapter. Artemisdeficient murine B cells have no obvious impairment in CSR efficiency[329] but show several-fold increases (compared to wild-type) in the number of chromosome aberrations in the IgH locus detected by FISH assays[330]; these aberrations are AID-dependent and only observed after activation of B cells for CSR. In humans, Artemis deficiency results in a SCID syndrome with severe defects in B- and T-cell development, but rare Sμ-Sα recombination junctions amplified from patient B cells were found to show a high index of microhomologies,[331] suggesting that Artemis is required for normal classic-NHEJ resolution of dsbs in human CSR.

Studies on the role of DNA-PKcs in CSR have yielded somewhat conflicting conclusions on whether this protein is required for maximally efficient CSR, with the results of individual studies perhaps depending on the knocked-in Ig genes used, the specific mutations of DNA-PKcs, or the genetic background of mice studied.[332,333] However, *DNAPKcs-/-* B cells showed evidence of AID-dependent chromosome aberrations in the IgH locus, similar to those seen in *Artemis-/-* cells but more numerous.[330] Thus it appears that both Artemis and DNA-PKcs are required for efficient repair of at least a subset of the dsbs associated with CSR.

DNA Damage Response Proteins in Class Switch Recombination. NHEJ is one component of a larger mechanism for detecting and repairing dsbs, collectively known as the DNA damage response (DDR). Because DNA dsbs are potentially damaging to the cell, and occur in all cells through accidents of DNA metabolism, toxic chemicals, and radiation, DDR appeared early in evolution, and many components are conserved from yeast to mammals. Defects in DDR can cause developmental abnormalities, cancer predisposition, and sensitivity to radiation, as well as immunodeficiency resulting from impaired V(D)J recombination or CSR. DDR factors participate in a baroque cascade of interactions to cluster at dsbs and initiate repair. The complexities of the DDR are beyond the scope of this chapter, but are described in several recent reviews.[78,334]

Several DDR components have been documented to participate in CSR and are listed below; mutations in some of these cause specific genetic syndromes in humans, often associated with immunodeficiency.

1. Nbs1, product of the gene that is defective in the human disease Nijmegen breakage syndrome.[309,335,336] Nbs1 functions as part of a complex that also includes Mre11 and Rad50 (MRN complex). Mre11 has a nuclease activity, and may contribute to DNA cleavage in CSR; mice and humans with defective Mre11 also show impaired CSR.[337,338]
2. ATM, product of the gene *m*utated in the human disease *a*taxia *t*elengectasia and a member of the phosphatidylinositol-3′-kinase family.[339,340]
3. γ-H2AX—the phosphorylated form of histone H2AX— which rapidly accumulates in foci at dsbs, and helps to assemble other proteins at dsbs to prevent the breaks from progressing to chromosome translocations.[341]
4. The E3 ubiquitin ligase RNF8 (Ring Finger 8), which is known to monoubiquitinate

histones at dsbs.[342]

5. The E3 ubiquitin ligase RNF168, found to be mutated in the RIDDLE (*r*adiosensitivity, *i*mmuno*d*eficiency, *d*ysmorphic features, *l*earning difficulties) syndrome.[343]

6. 53BP1 (p53 binding protein 1) originally discovered as a protein binding to the tumor suppressor p53, but subsequently found to function in checkpoint control and to localize rapidly to DNA breaks *in vivo*. *53BP1*-/- murine B cells show dramatic impairment in CSR (to 55 to 25% of wild-type), but increases in the frequency of AID-triggered deletions within Sμ,[344] suggesting that formation of dsbs and repair of closely-spaced breaks does not require 53BP1, but joining of more distant dsbs depends on this protein.

Although the we currently lack a detailed model explaining the functions of all the DDR factors in dsb repair and specifically in CSR, the emerging evidence suggests that these proteins have mutual interactions and distinct but related roles, so that the elimination of any one protein reduces CSR efficiency but permits residual CSR to occur by pathways that remain intact.

Other Proteins that may Target AID for Class Switch Recombination. As discussed in the context of SHM, accurate targeting of AID is important since off-target activities of this protein can be deleterious, and off-target dsbs triggered by AID could be particularly dangerous. Several searches for proteins that might contribute to targeting of AID to switch regions have uncovered candidates for this function. In one study,[345] biotinylated AID was used as bait to fish for *in vitro* binding proteins, which were identified by mass spectrometry. One protein identified by this screen was PTBP2 (polypyrimidine tract-binding protein 2) which is considered to be a regulator of RNA splicing. Knockdown of PTBP2 by shRNA in CH12 B cells caused a substantial decrease in binding of AID to Sμ (determined by ChIP) and significant impairment of CSR. The possible role of a splicing regulator in CSR could be related to the puzzling observation that germline transcripts must be spliced in order for efficient CSR,[280] as mentioned previously.

A screen for proteins that could bind selectively *in vitro* to AGCT sequences—which are found clustered in many switch regions—identified another protein that might target AID in CSR: the 14-3-3 proteins, a family of seven members widely expressed and known to bind to many signaling proteins.[346] ChIP assays on B cells stimulated for CSR showed that 14-3-3 proteins bind to switch regions in an isotypespecific way (depending on stimulus conditions) in both normal and *Aicda*-/- cells, and reduction of 14-3-3 activity by either a peptide inhibitor or by genetic engineering decreased both CSR efficiency and AID binding to switch region DNA. Finally, a bimolecular fluorescence complementation assay revealed that in B-cell nuclei AID and 14-3-3 molecules form a complex that is dependent on the AID C-terminal amino acids required for CSR. These experiments suggest that 14-3-3 proteins are additional candidates for AID targeting molecules.

PKA, the cAMP-regulated protein kinase responsible for phosphorylation of AID Ser38 discussed previously, has also been suggested as a candidate that targets AID-dependent DNA cleavage to switch regions.[347] PKA was found by ChIP to bind to switch regions of stimulated B cells from normal and *Aicda*-/- mice, whereas the binding of RPA depended on Ser38-phosphorylated AID. Cellular concentrations of cAMP were found to rise rapidly after stimulation to CSR, and a genetic inactivation of PKA activity that prevented its activation by cAMP reduced both RPA binding and CSR. These data suggest a model in which CSR stimuli recruit both PKA and nonphosphorylated AID to switch regions, and AID gains the ability to recruit RPA and trigger dsbs efficiently only when cAMP-activated PKA phosphorylates Ser38.

Additional components of CSR may be discovered by analysis of patients with a hyper-IgM phenotype unexplained by defects in known components.[314]

CONCLUSION

Recombinant DNA technology has revolutionized the study of the antibody response. Initial

investigations used powerful cloning and sequencing methods to define the structure of the Ig genes as they exist in the germline and in actively secreting B-lymphocytes. Subsequent experiments have begun to shed light on the mechanisms of the processes unique to these genes: V(D)J recombination, CSR, and SHM.

The knowledge of Ig genes gained so far has answered some of the most puzzling mysteries about antibody diversity, as discussed previously, and has also led to many practical ramifications involving these genes that are beyond the scope of this chapter. As one example, cloned Ig genes have allowed the production of recombinant monoclonal antibodies and the bioengineering of Ig-fusion proteins that exploit the exquisite specificity of antibody V region binding (e.g., antibody-toxin fusions) or the ability of Ig C region domains to extend serum half-life or engage Fc receptors. Other engineered derivatives utilizing Ig genes include single-chain antibodies, bispecific antibodies, and “intrabodies” designed not to be secreted from a cell but rather to bind to intracellular targets.[348,349,350] Ig V gene fragments cloned into bacteriophage so as to express single-chain V regions on the phage surface (phage display libraries) can be used to obtain specific monoclonal antibodies without immunization or use of mammalian cells, and *in vitro* mutation and selection protocols can mimic affinity maturation to yield high-affinity antibodies.[351] Even Ig gene regulatory regions have been exploited to achieve B-cell-specific expression of oncogenes[352] and of intracellular toxins that could be used to target B lymphomas.[353] Apart from these biotechnology applications, Ig gene probes have led to the identification of numerous proto-oncogenes that become activated by translocation into Ig gene loci.[354] For instance, Bcl2 was initially discovered as the target of Ig H chain translocation in follicular lymphoma, and provided an entry into an entire family of apoptosis-related genes. A final example of medical benefit from Ig gene technology has been the use of patient-specific Ig gene rearrangements of leukemias or lymphomas to monitor disease status by PCR.[355,356]

Further practical applications of Ig genes can be anticipated in the future, as well as a deeper scientific understanding of their molecular biology and their contribution to the immune system.

REFERENCES

1. Tonegawa S. Somatic generation of antibody diversity. *Nature*. 1983; 302:575-581.

2. Nadel B, Tang A, Escuro G, Lugo G, Feeney AJ. Sequence of the spacer in the recombination signal sequence affects V(D)J rearrangement frequency and correlates with nonrandom Vkappa usage in vivo. *J Exp Med*. 1998;187:1495-1503.

3. Johnston CM, Wood AL, Bolland DJ, Corcoran AE. Complete sequence assembly and characterization of the C57BL/6 mouse Ig heavy chain V region. *J Immunol*. 2006;176:4221-4234.

4. Nei M, Gu X, Sitnikova T. Evolution by the birth-and-death process in multigene families of the vertebrate immune system. *Proc Natl Acad Sci U S A* . 1997;94:7799-7806.

5. Gu H, Kitamura D, Rajewsky K. B cell development regulated by gene rearrangement: arrest of maturation by membrane-bound D mu protein and selection of DH element reading frames. *Cell*. 1991;65:47-54.

6. Tomlinson IM, Cook GP, Carter NP, et al. Human immunoglobulin VH and D segments on chromosomes 15q11.2 and 16p11.2. *Hum Mol Gen*. 1994;3:853-860.

7. Corbett S, Tomlinson I, Sonnhammer E, Buck D, Winter G. Sequence of the human immunoglobulin diversity (D) segment locus: a systematic analysis provides no evidence for the use of DIR segments, inverted D segments, “minor” D segments or D-D recombination. *J Molec Biol*. 1997;271:587-597.

8. Shimizu A, Takahashi N, Yaoita Y, Honjo T. Organization of the constant-region gene family of the mouse immunoglobulin heavy chain. *Cell*. 1982;28:499-506.

9. Akahori Y, Kurosawa Y. Nucleotide sequences of all the gamma gene loci of murine immunoglobulin heavy chains. *Genomics*. 1997;41:100-104.

10. Burnett RC, Hanly WC, Zhai SK, Knight KL. The IgA heavy-chain gene family in rabbit: cloning and sequence analysis of 13 C alpha genes. *EMBO J*. 1989; 8:4041-4047.

11. Hamers-Casterman C, Atarhouch T, Muyldermans S, et al. Naturally occurring antibodies devoid of light chains. *Nature*. 1993;363:446-448.

12. Phillips C, Kyriakopoulou CB, Virtanen A. Identification of a stem-loop structure important for polyadenylation at the murine IgM secretory poly(A) site. *Nucleic Acids Res*. 1999;27:429-438.

13. Takagaki Y, Seipelt RL, Peterson ML, Manley JL. The polyadenylation factor CstF-64 regulates alternative processing of IgM heavy chain pre-mRNA during B cell differentiation. *Cell*. 1996;87:941-952.

14. Phillips C, Pachikara N, Gunderson SI. U1A inhibits cleavage at the immunoglobulin M heavy-chain secretory poly(A) site by binding between the two downstream GU-rich regions. *Mol Cell Biol*. 2004;24:6162-6171.

15. Shapiro-Shelef M, Lin KI, McHeyzer-Williams LJ, Liao J, McHeyzer-Williams MG, Calame K. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity*. 2003;19:607-620.

16. Coyle JH, Lebman DA. Correct immunoglobulin alpha mRNA processing depends on specific sequence in the C alpha 3-alpha M intron. *J Immunol*. 2000;164:3659-3665.

17. Engels N, Konig LM, Heemann C, et al. Recruitment of the cytoplasmic adaptor Grb2 to surface IgG and IgE provides antigen receptorintrinsic costimulation to class-switched B cells. *Nat Immunol*. 2009;10:1018-1025.

18. Brekke KM, Garrard WT. Assembly and analysis of the mouse immunoglobulin kappa gene sequence. *Immunogenetics*. 2004;56:490-505.

19. Kawasaki K, Minoshima S, Nakato E, et al. Evolutionary dynamics of the human immunoglobulin kappa locus and the germline repertoire of the Vkappa genes. *Eur J Immunol*. 2001;31:1017-1028.

20. Durdik J, Moore MW, Selsing E. Novel kappa light-chain gene rearrangements in mouse lambda light chain-producing B lymphocytes. *Nature*. 1984;307:749-752.

21. Siminovitch KA, Bakhshi A, Goldman P, Korsmeyer SJ. A uniform deleting element mediates the loss of kappa genes in human B cells. *Nature*. 1985;316:260-262.

22. Hieter PA, Korsmeyer SJ, Waldmann TA, Leder P. Human immunoglobulin kappa light-chain genes are deleted or rearranged in lambdaproducing B cells. *Nature*. 1981;290:368-372.

23. Gerdes T, Wabl M. Physical map of the mouse lambda light chain and related loci. *Immunogenetics*. 2002;54:62-65.

24. Kawasaki K, Minoshima S, Nakato E, et al. One-megabase sequence analysis of the human immunoglobulin lambda gene locus. *Genome Res*. 1997;7:250-261.

25. Niewold TA, Murphy CL, Weiss DT, Solomon A. Characterization of a light chain product of the human JC lambda 7 gene complex. *J Immunol* . 1996;157:4474-4477.

26. van der Burg M, Barendregt BH, van Gastel-Mol EJ, Tumkaya T, Langerak AW, van Dongen JJ. Unraveling of the polymorphic C lambda 2-C lambda 3 amplification and the Ke+ Oz- polymorphism in the human Ig lambda locus. *J Immunol* . 2002;169:271-276.

27. Kudo A, Sakaguchi N, Melchers F. Organization of the murine Igrelated lambda 5 gene transcribed selectively in pre-B lymphocytes. *EMBO J*. 1987;6:103-107.

28. Dul JL, Argon Y, Winkler T, Ten BE, Melchers F, Martensson IL. The murine VpreB1 and VpreB2 genes both encode a protein of the surrogate light chain and are co-expressed during B cell development. *Eur J Immunol* . 1996;26:906-913.

29. Tsubata T, Reth M. The products of pre-B cell-specific genes (lambda 5 and VpreB) and the immunoglobulin mu chain form a complex that is transported onto the cell. *J Exp Med*. 1990;172:973-976.

30. Frippiat JP, Williams SC, Tomlinson IM, et al. Organization of the human immunoglobulin lambda light-chain locus on chromosome 22q11.2. *Hum Mol Genet*. 1995;4:983-991.

31. McCormack WT, Tjoelker LW, Carlson LM, et al. Chicken IgL gene rearrangement involves deletion of a circular episome and addition of single nonrandom nucleotides to both coding segments. *Cell*. 1989;56:785-791.

32. Schlissel M, Constantinescu A, Morrow T, Baxter M, Peng A. Doublestrand signal sequence breaks in V(D)J recombination are blunt, 5′-phosphorylated, RAG-dependent, and cell cycle regulated. *Genes Dev*. 1993;7:2520-2532.

33. Zhu C, Roth DB. Characterization of coding ends in thymocytes of scid mice: implications for the mechanism of V(D)J recombination. *Immunity*. 1995;2:101-112.

34. Roth DB, Menetski JP, Nakajima PB, Bosma MJ, Gellert M. V(D)J recombination: broken DNA molecules with covalently sealed (hairpin) coding ends in scid mouse thymocytes. *Cell*. 1992;70:983-991.

35. Shimizu T, Iwasato T, Yamagishi H. Deletions of immunoglobulin C kappa region characterized by the circular excision products in mouse splenocytes. *J Exp Med*. 1991;173:1065-1072.

36. Lewis SM, Hesse JE, Mizuuchi K, Gellert M. Novel strand exchanges in V(D)J recombination. *Cell*. 1988;55:1099-1107.

37. Lew S, Franco D, Chang Y. Activation of V(D)J recombination induces the formation of interlocus joints and hybrid joints in scid pre-B-cell lines. *Mol Cell Biol* . 2000;20:7170-7177.

38. Reth M, Gehrmann P, Petrac E, Wiese P. A novel VH to VHDJH joining mechanism in heavy-chain-negative (null) pre-B cells results in heavy-chain production. *Nature*. 1986;322:840-842.

39. Lu H, Shimazaki N, Raval P, et al. A biochemically defined system for coding joint

formation in V(D)J recombination. *Mol Cell*. 2008;31:485-497.

40. Schatz DG, Baltimore D. Stable expression of immunoglobulin gene V(D)J recombinase activity by gene transfer into 3T3 fibroblasts. *Cell*. 1988;53:107-115.

41. Shinkai Y, Rathbun G, Lam KP, et al. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell*. 1992;68:855-867.

42. Mombaerts P, Iacomini J, Johnson RS, et al. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*. 1992;68:869-877.

43. Schwarz K, Gauss GH, Ludwig L, et al. RAG mutations in human B cell-negative SCID. *Science*. 1996;274:97-99.

44. Corneo B, Moshous D, Gungor T, et al. Identical mutations in RAG1 or RAG2 genes leading to defective V(D)J recombinase activity can cause either T-B-severe combined immune deficiency or Omenn syndrome. *Blood*. 2001;97:2772-2776.

45. Landree MA, Wibbenmeyer JA, Roth DB. Mutational analysis of RAG1 and RAG2 identifies three catalytic amino acids in RAG1 critical for both cleavage steps of V(D)J recombination. *Genes Dev*. 1999;13:3059-3069.

46. Kim DR, Dai Y, Mundy CL, Yang W, Oettinger MA. Mutations of acidic residues in RAG1 define the active site of the V(D)J recombinase. *Genes Dev*. 1999;13:3070-3080.

47. Akamatsu Y, Oettinger MA. Distinct roles of RAG1 and RAG2 in binding the V(D)J recombination signal sequences. *Mol Cell Biol*. 1998;18:4670-4678.

48. Swanson PC, Desiderio S. V(D)J recombination signal recognition: distinct, overlapping DNA- protein contacts in complexes containing RAG1 with and without RAG2. *Immunity*. 1998;9:115-125.

49. McBlane JF, van GD, Ramsden DA, et al. Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. *Cell*. 1995;83:387-395.

50. van Gent DC, Mizuuchi K, Gellert M. Similarities between initiation of V(D)J recombination and retroviral integration. *Science*. 1996;271:1592-1594.

51. Tsai CL, Drejer AH, Schatz DG. Evidence of a critical architectural function for the RAG proteins in end processing, protection, and joining in V(D)J recombination. *Genes Dev*. 2002;16:1934-1949.

52. Curry JD, Geier JK, Schlissel MS. Single-strand recombination signal sequence nicks in vivo: evidence for a capture model of synapsis. *Nat Immunol*. 2005;6:1272-1279.

53. Agrawal A, Eastman QM, Schatz DG. Transposition mediated by RAG1 and RAG2 and its implications for the evolution of the immune system. *Nature*. 1998;394:744-751.

54. Hiom K, Melek M, Gellert M. DNA transposition by the RAG1 and RAG2 proteins: a possible source of oncogenic translocations. *Cell*. 1998;94:463-470.

55. Zhou L, Mitra R, Atkinson PW, Hickman AB, Dyda F, Craig NL. Transposition of hAT elements links transposable elements and V(D)J recombination. *Nature*. 2004;432:995-1001.

56. Kapitonov VV, Jurka J. RAG1 core and V(D)J recombination signal sequences were

derived from Transib transposons. *PLoS Biol*. 2005;3:e181.

57. Fugmann SD, Messier C, Novack LA, Cameron RA, Rast JP. An ancient evolutionary origin of the Rag1/2 gene locus. *Proc Natl Acad Sci U S A* . 2006;103:3728-3733.

58. Messier TL, O'Neill JP, Hou SM, Nicklas JA, Finette BA. In vivo transposition mediated by V(D)J recombinase in human T lymphocytes. *EMBO J*. 2003;22:1381-1388.

59. Chatterji M, Tsai CL, Schatz DG. Mobilization of RAG-generated signal ends by transposition and insertion in vivo. *Mol Cell Biol* . 2006;26:1558-1568.

60. Reddy YV, Perkins EJ, Ramsden DA. Genomic instability due to V(D)J recombination-associated transposition. *Genes Dev*. 2006;20:1575-1582.

61. Rahman NS, Godderz LJ, Stray SJ, Capra JD, Rodgers KK. DNA cleavage of a cryptic recombination signal sequence by RAG1 and RAG2. Implications for partial V(H) gene replacement. *J Biol Chem*. 2006;281:12370-12380.

62. Raghavan SC, Swanson PC, Ma Y, Lieber MR. Double-strand break formation by the RAG complex at the bcl-2 major breakpoint region and at other non-B DNA structures in vitro. *Mol Cell Biol* . 2005;25:5904-5919.

63. Akamatsu Y, Monroe R, Dudley DD, et al. Deletion of the RAG2 C terminus leads to impaired lymphoid development in mice. *Proc Natl Acad Sci U S A* . 2003;100:1209-1214.

64. Talukder SR, Dudley DD, Alt FW, Takahama Y, Akamatsu Y. Increased frequency of aberrant V(D)J recombination products in core RAGexpressing mice. *Nucleic Acids Res*. 2004;32:4539-4549.

65. Elkin SK, Matthews AG, Oettinger MA. The C-terminal portion of RAG2 protects against transposition in vitro. *EMBO J*. 2003;22:1931-1938.

66. Matthews AG, Kuo AJ, Ramon-Maiques S, et al. RAG2 PHD finger couples histone H3 lysine 4 trimethylation with V(D)J recombination. *Nature*. 2007;450:1106-1110.

67. Ramon-Maiques S, Kuo AJ, Carney D, et al. The plant homeodomain finger of RAG2 recognizes histone H3 methylated at both lysine-4 and arginine-2. *Proc Natl Acad Sci U S A*. 2007;104:18993-18998.

68. Liu Y, Subrahmanyam R, Chakraborty T, Sen R, Desiderio S. A plant homeodomain in RAG-2 that binds Hypermethylated lysine 4 of histone H3 is necessary for efficient antigen-receptor-gene rearrangement. *Immunity*. 2007;27:561-571.

69. Shimazaki N, Tsai AG, Lieber MR. H3K4me3 stimulates the V(D)J RAG complex for both nicking and hairpinning in trans in addition to tethering in cis: implications for translocations. *Mol Cell*. 2009;34:535-544.

70. Jiang H, Chang FC, Ross AE, et al. Ubiquitylation of RAG-2 by Skp2-SCF links destruction of the V(D)J recombinase to the cell cycle. *Mol Cell*. 2005;18:699-709.

71. Li Z, Dordai DI, Lee J, Desiderio S. A conserved degradation signal regulates RAG-2 accumulation during cell division and links V(D)J recombination to the cell cycle. *Immunity*. 1996;5:575-589.

72. Zhang L, Reynolds TL, Shan X, Desiderio S. Coupling of V(D)J recombination to the cell cycle suppresses genomic instability and lymphoid tumorigenesis. *Immunity*.

2011;34:163-174.

73. Grazini U, Zanardi F, Citterio E, Casola S, Goding CR, McBlane F. The RING domain of RAG1 ubiquitylates histone H3: a novel activity in chromatin-mediated regulation of V(D)J joining. *Mol Cell*. 2010;37:282-293.

74. Chen J, Lansford R, Stewart V, Young F, Alt FW. RAG-2-deficient blastocyst complementation: an assay of gene function in lymphocyte development. *Proc Natl Acad Sci U S A*. 1993;90:4528-4532.

75. van Gent DC, Hiom K, Paull TT, Gellert M. Stimulation of V(D)J cleavage by high mobility group proteins. *EMBO J*. 1997;16:2665-2670.

76. Hiom K, Gellert M. Assembly of a 12/23 paired signal complex: a critical control point in V(D)J recombination. *Mol Cell*. 1998;1:1011-1019.

77. Aidinis V, Bonaldi T, Beltrame M, Santagata S, Bianchi ME, Spanopoulou E. The RAG1 homeodomain recruits HMG1 and HMG2 to facilitate recombination signal sequence binding and to enhance the intrinsic DNA-bending activity of RAG1-RAG2. *Mol Cell Biol*. 1999;19:6532-6542.

78. Lieber MR. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu Rev Biochem*. 2010;79:181-211.

79. DeFazio LG, Stansel RM, Griffith JD, Chu G. Synapsis of DNA ends by DNA-dependent protein kinase. *EMBO J*. 2002;21:3192-200.

80. Weterings E, Verkaik NS, Bruggenwirth HT, Hoeijmakers JH, van Gent DC. The role of DNA dependent protein kinase in synapsis of DNA ends. *Nucleic Acids Res*. 2003;31:7238-7246.

81. Gao Y, Chaudhuri J, Zhu C, Davidson L, Weaver DT, Alt FW. A targeted DNA-PKcs-null mutation reveals DNA-PK-independent functions for KU in V(D)J recombination. *Immunity*. 1998;9:367-376.

82. Taccioli GE, Amatucci AG, Beamish HJ, et al. Targeted disruption of the catalytic subunit of the DNA-PK gene in mice confers severe combined immunodeficiency and radiosensitivity. *Immunity*. 1998;9:355-366.

83. Zhu C, Bogue MA, Lim DS, Hasty P, Roth DB. Ku86-deficient mice exhibit severe combined immunodeficiency and defective processing of V(D)J recombination intermediates. *Cell*. 1996;86:379-389.

84. Gu Y, Jin S, Gao Y, Weaver DT, Alt FW. Ku70-deficient embryonic stem cells have increased ionizing radiosensitivity, defective DNA endbinding activity, and inability to support V(D)J recombination. *Proc Nat Acad Sci U S A* . 1997;94:8076-8081.

85. Nussenzweig A, Chen C, da Costa Soares V, et al. Requirement for Ku80 in growth and immunoglobulin V(D)J recombination. *Nature*. 1996;382:551-555.

86. Critchlow SE, Bowater RP, Jackson SP. Mammalian DNA doublestrand break repair protein XRCC4 interacts with DNA ligase IV. *Curr Biol* . 1997;7:588-598.

87. Grawunder U, Wilm M, Wu XK, et al. Activity of DNA ligase IV stimulate by complex formation with XRCC4 in mammalian cells. *Nature*. 1997;388:492.

88. O'Driscoll M, Cerosaletti KM, Girard PM, et al. DNA ligase IV mutations identified in patients exhibiting developmental delay and immunodeficiency. *Mol Cell*. 2001;8:1175-1185.

89. van der Burg M, van Veelen LR, Verkaik NS, et al. A new type of radiosensitive T-B-NK+ severe combined immunodeficiency caused by a LIG4 mutation. *J Clin Invest*. 2006;116:137-145.

90. Gao Y, Ferguson DO, Xie W, et al. Interplay of p53 and DNA-repair protein XRCC4 in tumorigenesis, genomic stability and development. *Nature*. 2000;404:897-900.

91. Ahnesorg P, Smith P, Jackson SP. XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining. *Cell*. 2006;124:301-313.

92. Buck D, Malivert L, de Chasseval R, et al. Cernunnos, a novel nonhomologous end-joining factor, is mutated in human immunodeficiency with microcephaly. *Cell*. 2006;124:287-299.

93. Callebaut I, Malivert L, Fischer A, Mornon JP, Revy P, de Villartay JP. Cernunnos interacts with the XRCC4 x DNA-ligase IV complex and is homologous to the yeast nonhomologous end-joining factor Nej1. *J Biol Chem*. 2006;281:13857-13860.

94. Moshous D, Callebaut I, de Chasseval R, et al. Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency. *Cell*. 2001;105:177-186.

95. Ege M, Ma Y, Manfras B, et al. Omenn syndrome due to ARTEMIS mutations. *Blood*. 2005;105:4179-4186.

96. Drouet J, Frit P, Delteil C, de Villartay JP, Salles B, Calsou P. Interplay between Ku, Artemis, and the DNA-dependent protein kinase catalytic subunit at DNA ends. *J Biol Chem*. 2006;281:27784-27793.

97. Ma Y, Pannicke U, Lu H, Niewolik D, Schwarz K, Lieber MR. The DNA-dependent protein kinase catalytic subunit phosphorylation sites in human Artemis. *J Biol Chem*. 2005;280:33839-33846.

98. Ma Y, Schwarz K, Lieber MR. The Artemis:DNA-PKcs endonuclease cleaves DNA loops, flaps, and gaps. *DNA Repair (Amst)* . 2005;4:845-851.

99. Budman J, Chu G. Processing of DNA for nonhomologous end-joining by cell-free extract. *EMBO J*. 2005;24:849-860.

100. Ma Y, Lu H, Tippin B, et al. A biochemically defined system for mammalian nonhomologous DNA end joining. *Mol Cell*. 2004;16:701-713.

101. Feeney AJ. Lack of N regions in fetal and neonatal mouse immunoglobulin V-D-J junctional sequences. *J Exp Med*. 1990;172:1377-1390.

102. Victor KD, Capra JD. An apparently common mechanism of generating antibody diversity: length variation of the VL-JL junction. *Mol Immunol*. 1994;31:39-46.

103. Wasserman R, Li YS, Hardy RR. Down-regulation of terminal deoxynucleotidyl transferase by Ig heavy chain in B lineage cells. *J Immunol*. 1997;158:1133-1138.

104. Hiramatsu R, Akagi K, Matsuoka M, et al. The 3' enhancer region determines the

B/T specificity and pro-B/pre-B specificity of immunoglobulin V kappa-J kappa joining. *Cell*. 1995;83:1113-1123.

105. Gavin MA, Bevan MJ. Increased peptide promiscuity provides a rationale for the lack of N regions in the neonatal T cell repertoire. *Immunity*. 1995;3:793-800.

106. Bogue MA, Wang C, Zhu C, Roth DB. V(D)J recombination in Ku86-deficient mice: distinct effects on coding, signal, and hybrid joint formation. *Immunity*. 1997;7:37-47.

107. Bertocci B, De Smet A, Berek C, Weill JC, Reynaud CA. Immunoglobulin kappa light chain gene rearrangement is impaired in mice deficient for DNA polymerase mu. *Immunity*. 2003;19:203-211.

108. Bertocci B, De Smet A, Weill JC, Reynaud CA. Nonoverlapping functions of DNA polymerases mu, lambda, and terminal deoxynucleotidyltransferase during immunoglobulin V(D)J recombination in vivo. *Immunity*. 2006;25:31-41.

109. Zhang Z, Espinoza CR, Yu Z, et al. Transcription factor Pax5 (BSAP) transactivates the RAG-mediated V(H)-to-DJ(H) rearrangement of immunoglobulin genes. *Nat Immunol*. 2006;7:616-624.

110. Alt FW, Enea V, Bothwell AL, Baltimore D. Activity of multiple light chain genes in murine myeloma cells producing a single, functional light chain. *Cell*. 1980;21:1-12.

111. Grawunder U, Leu TM, Schatz DG, et al. Down-regulation of RAG1 and RAG2 gene expression in preB cells after functional immunoglobulin heavy chain rearrangement. *Immunity*. 1995;3:601-608.

112. Schlissel MS, Morrow T. Ig heavy chain protein controls B cell development by regulating germ-line transcription and retargeting V(D)J recombination. *J Immunol*. 1994;153:1645-1657.

113. Fang W, Mueller DL, Pennell CA, et al. Frequent aberrant immunoglobulin gene rearrangements in pro-B cells revealed by a bcl-xL transgene. *Immunity*. 1996;4:291-299.

114. Ma A, Fisher P, Dildrop R, et al. Surface IgM mediated regulation of RAG gene expression in E mu-N-myc B cell lines. *EMBO J*. 1992;11:2727-2734.

115. Hsu LY, Lauring J, Liang HE, et al. A conserved transcriptional enhancer regulates RAG gene expression in developing B cells. *Immunity*. 2003;19:105-117.

116. Wei XC, Kishi H, Jin ZX, et al. Characterization of chromatin structure and enhancer elements for murine recombination activating gene-2. *J Immunol*. 2002;169:873-881.

117. Kuo TC, Schlissel MS. Mechanisms controlling expression of the RAG locus during lymphocyte development. *Curr Opin Immunol*. 2009;21:173-178.

118. Verkoczy L, Ait-Azzouzene D, Skog P, et al. A role for nuclear factor kappa B/rel transcription factors in the regulation of the recombinase activator genes. *Immunity*. 2005;22:519-531.

119. Amin RH, Schlissel MS. Foxo1 directly regulates the transcription of recombination-activating genes during B cell development. *Nat Immunol*. 2008;9:613-622.

120. Yancopoulos GD, Alt FW. Developmentally controlled and tissue-specific expression of unrearranged VH gene segments. *Cell*. 1985;40:271-281.

121. Stanhope-Baker P, Hudson KM, Shaffer AL, Constantinescu A, Schlissel MS. Cell type-specific chromatin structure determines the targeting of V(D)J recombinase activity in vitro. *Cell*. 1996;85:887-897.

122. Golding A, Chandler S, Ballestar E, Wolffe AP, Schlissel MS. Nucleosome structure completely inhibits in vitro cleavage by the V(D) J recombinase. *EMBO J*. 1999;18:3712-3723.

123. McBlane F, Boyes J. Stimulation of V(D)J recombination by histone acetylation. *Curr Biol*. 2000;10:483-486.

124. Osborne CS, Chakalova L, Brown KE, et al. Active genes dynamically colocalize to shared sites of ongoing transcription. *Nat Genet*. 2004;36:1065-1071.

125. Kosak ST, Skok JA, Medina KL, et al. Subnuclear compartmentalization of immunoglobulin loci during lymphocyte development. *Science*. 2002;296:158-162.

126. Alessandrini A, Desiderio SV. Coordination of immunoglobulin DJH transcription and D-to-JH rearrangement by promoter-enhancer approximation. *Mol Cell Biol* . 1991;11:2096-2107.

127. Schlissel MS, Corcoran LM, Baltimore D. Virus-transformed pre-B cells show ordered activation but not inactivation of immunoglobulin gene rearrangement and transcription. *J Exp Med*. 1991;173:711-720.

128. Chakraborty T, Chowdhury D, Keyes A, et al. Repeat organization and epigenetic regulation of the DH-Cmu domain of the immunoglobulin heavy-chain gene locus. *Mol Cell*. 2007;27:842-850.

129. Ji Y, Resch W, Corbett E, Yamane A, Casellas R, Schatz DG. The in vivo pattern of binding of RAG1 and RAG2 to antigen receptor loci. *Cell*. 2010;141:419-431.

130. Storb U, Arp B. Methylation patterns of immunoglobulin genes in lymphoid cells: correlation of expression and differentiation with undermethylation. *Proc Natl Acad Sci U S A*. 1983;80:6642-6646.

131. Goodhardt M, Cavelier P, Doyen N, Kallenbach S, Babinet C, Rougeon F. Methylation status of immunoglobulin kappa gene segments correlates with their recombination potential. *Eur J Immunol* . 1993;23:1789-1795.

132. Hsieh CL, Lieber MR. CpG methylated minichromosomes become inaccessible for V(D)J recombination after undergoing replication. *EMBO J*. 1992;11:315-325.

133. Engler P, Haasch D, Pinkert CA, et al. A strain-specific modifier on mouse chromosome 4 controls the methylation of independent transgene loci. *Cell*. 1991;65:939-947.

134. Fuxa M, Skok J, Souabni A, Salvagiotto G, Roldan E, Busslinger M. Pax5 induces V-to-DJ rearrangements and locus contraction of the immunoglobulin heavy-chain gene. *Genes Dev*. 2004;18:411-422.

135. Jhunjhunwala S, van Zelm MC, Peak MM, et al. The 3D structure of the immunoglobulin heavy-chain locus: implications for long-range genomic interactions. *Cell*. 2008;133:265-279.

136. Garrett FE, Emelyanov AV, Sepulveda MA, et al. Chromatin architecture near a potential 3′ end of the igh locus involves modular regulation of histone modifications during B-Cell development and in vivo occupancy at CTCF sites. *Mol Cell Biol* . 2005;25:1511-1525.

137. Degner SC, Verma-Gaur J, Wong TP, et al. CCCTC-binding factor (CTCF) and cohesin influence the genomic architecture of the Igh locus and antisense transcription in pro-B cells. *Proc Natl Acad Sci U S A* . 2011;108:9566-9571.

138. Featherstone K, Wood AL, Bowen AJ, Corcoran AE. The mouse immunoglobulin heavy chain V-D intergenic sequence contains insulators that may regulate ordered V(D)J recombination. *J Biol Chem*. 2010;285:9327-9338.

139. Guo C, Yoon HS, Franklin A, et al. CTCF-binding elements mediate control of V(D)J recombination. *Nature*. 2011;477:424-430.

140. Casellas R, Shih TA, Kleinewietfeld M, et al. Contribution of receptor editing to the antibody repertoire. *Science*. 2001;291:1541-1544.

141. Hippen KL, Schram BR, Tze LE, Pape KA, Jenkins MK, Behrens TW. In vivo assessment of the relative contributions of deletion, anergy, and editing to B cell self-tolerance. *J Immunol* . 2005;175:909-916.

142. Halverson R, Torres RM, Pelanda R. Receptor editing is the main mechanism of B cell tolerance toward membrane antigens. *Nat Immunol* . 2004;5:645-650.

143. Ait-Azzouzene D, Verkoczy L, Peters J, et al. An immunoglobulin C kappa-reactive single chain antibody fusion protein induces tolerance through receptor editing in a normal polyclonal immune system. *J Exp Med*. 2005;201:817-828.

144. Muramatsu M, Sankaranand VS, Anant S, et al. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J Biol Chem*. 1999;274:18470-18476.

145. Revy P, Muto T, Levy Y, et al. Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* . 2000;102:565-575.

146. Okazaki IM, Kinoshita K, Muramatsu M, Yoshikawa K, Honjo T. The AID enzyme induces class switch recombination in fibroblasts. *Nature*. 2002;416:340-345.

147. Yoshikawa K, Okazaki I, Eto T, et al. AID enzyme-Induced hypermutation in an actively transcribed gene in fibroblasts. *Science*. 2002;296:2033-2036.

148. Petersen-Mahrt SK, Harris RS, Neuberger MS. AID mutates E. coli suggesting a DNA deamination mechanism for antibody diversification. *Nature*. 2002;418:99-103.

149. Sohail A, Klapacz J, Samaranayake M, Ullah A, Bhagwat AS. Human activation-induced cytidine deaminase causes transcription-dependent, strand-biased C to U deaminations. *Nucleic Acids Res*. 2003;31:2990-2994.

150. Di Noia J, Neuberger MS. Altering the pathway of immunoglobulin hypermutation by inhibiting uracil-DNA glycosylase. *Nature*. 2002;419:43-48.

151. Rada C, Williams GT, Nilsen H, Barnes DE, Lindahl T, Neuberger MS. Immunoglobulin isotype switching is inhibited and somatic hypermutation perturbed in

UNG-deficient mice. *Curr Biol* . 2002;12:1748-1755.

152. Maul RW, Saribasak H, Martomo SA, et al. Uracil residues dependent on the deaminase AID in immunoglobulin gene variable and switch regions. *Nat Immunol* . 2011;12:70-76.

153. Bransteitter R, Pham P, Scharff MD, Goodman MF. Activationinduced cytidine deaminase deaminates deoxycytidine on singlestranded DNA but requires the action of RNase. *Proc Natl Acad Sci U S A* . 2003;100:4102-4107.

154. Yu K, Huang FT, Lieber MR. DNA substrate length and surrounding sequence affect the activation-induced deaminase activity at cytidine. *J Biol Chem*. 2004;279:6496-6500.

155. Chaudhuri J, Tian M, Khuong C, Chua K, Pinaud E, Alt FW. Transcription-targeted DNA deamination by the AID antibody diversification enzyme. *Nature*. 2003;422:726-730.

156. Ramiro AR, Stavropoulos P, Jankovic M, Nussenzweig MC. Transcription enhances AID-mediated cytidine deamination by exposing single-stranded DNA on the nontemplate strand. *Nat Immunol* . 2003;4:452-456.

157. Pham P, Bransteitter R, Petruska J, Goodman MF. Processive AID-catalysed cytosine deamination on single-stranded DNA simulates somatic hypermutation. *Nature*. 2003;424:103-107.

158. Nambu Y, Sugai M, Gonda H, et al. Transcription-coupled events associating with immunoglobulin switch region chromatin. *Science*. 2003;302:2137-2140.

159. Chaudhuri J, Khuong C, Alt FW. Replication protein A interacts with AID to promote deamination of somatic hypermutation targets. *Nature*. 2004;430:992-998.

160. Yamane A, Resch W, Kuo N, et al. Deep-sequencing identification of the genomic targets of the cytidine deaminase AID and its cofactor RPA in B lymphocytes. *Nat Immunol* . 2011;12:62-69.

161. Wang M, Rada C, Neuberger MS. Altering the spectrum of immunoglobulin V gene somatic hypermutation by modifying the active site of AID. *J Exp Med* . 2010;207:141-153.

162. Kohli RM, Maul RW, Guminski AF, et al. Local sequence targeting in the AID/APOBEC family differentially impacts retroviral restriction and antibody diversification. *J Biol Chem*. 2010;285:40956-40964.

163. Carpenter MA, Rajagurubandara E, Wijesinghe P, Bhagwat AS. Determinants of sequence-specificity within human AID and APOBEC3G. *DNA Repair* . 2010;9:579-587.

164. Conticello SG. The AID/APOBEC family of nucleic acid mutators. *Genome Biol* . 2008;9:229.

165. Ta VT, Nagaoka H, Catalan N, et al. AID mutant analyses indicate requirement for class-switch-specific cofactors. *Nat Immunol* . 2003;4:843-848.

166. Barreto V, Reina-San-Martin B, Ramiro AR, McBride KM, Nussenzweig MC. C-terminal deletion of AID uncouples class switch recombination from somatic hypermutation and gene conversion. *Mol Cell* . 2003;12:501-508.

167. Shinkura R, Ito S, Begum NA, et al. Separate domains of AID are required for somatic hypermutation and class-switch recombination. *Nat Immunol* . 2004;5:707-712.

168. Popp C, Dean W, Feng S, et al. Genome-wide erasure of DNA methylation in mouse primordial germ cells is affected by AID deficiency. *Nature*. 2010;463:1101-1105.

169. Bhutani N, Brady JJ, Damian M, Sacco A, Corbel SY, Blau HM. Reprogramming towards pluripotency requires AID-dependent DNA demethylation. *Nature*. 2010;463:1042-1047.

170. Morgan HD, Dean W, Coker HA, Reik W, Petersen-Mahrt SK. Activation-induced cytidine deaminase deaminates 5-methylcytosine in DNA and is expressed in pluripotent tissues: implications for epigenetic reprogramming. *J Biol Chem*. 2004;279:52353-52360.

171. Esnault C, Millet J, Schwartz O, Heidmann T. Dual inhibitory effects of APOBEC family proteins on retrotransposition of mammalian endogenous retroviruses. *Nucleic Acids Res*. 2006;34:1522-1531.

172. Gourzi P, Leonova T, Papavasiliou FN. A role for activation-induced cytidine deaminase in the host response against a transforming retrovirus. *Immunity*. 2006;24:779-786.

173. MacDuff DA, Demorest ZL, Harris RS. AID can restrict L1 retrotransposition suggesting a dual role in innate and adaptive immunity. *Nucleic Acids Res*. 2009;37:1854-1867.

174. Meyers G, Ng YS, Bannock JM, et al. Activation-induced cytidine deaminase (AID) is required for B-cell tolerance in humans. *Proc Natl Acad Sci U S A* . 2011;108:11554-11559.

175. Kuraoka M, Holl TM, Liao D, et al. Activation-induced cytidine deaminase mediates central tolerance in B cells. *Proc Natl Acad Sci U S A* . 2011;108:11560-11565.

176. Zaheen A, Boulianne B, Parsa JY, Ramachandran S, Gommerman JL, Martin A. AID constrains germinal center size by rendering B cells susceptible to apoptosis. *Blood*. 2009;114:547-554.

177. Okazaki IM, Hiai H, Kakazu N, et al. Constitutive expression of AID leads to tumorigenesis. *J Exp Med*. 2003;197:1173-1181.

178. Ramiro AR, Jankovic M, Eisenreich T, et al. AID is required for c-myc/IgH chromosome translocations in vivo. *Cell*. 2004;118:431-438.

179. Sernandez IV, de Yebenes VG, Dorsett Y, Ramiro AR. Haploinsufficiency of activation-induced deaminase for antibody diversification and chromosome translocations both in vitro and in vivo. *PLoS One*. 2008;3:e3927.

180. Takizawa M, Tolarova H, Li Z, et al. AID expression levels determine the extent of cMyc oncogenic translocations and the incidence of B cell tumor development. *J Exp Med*. 2008;205:1949-1957.

181. Yadav A, Olaru A, Saltis M, Setren A, Cerny J, Livak F. Identification of a ubiquitously active promoter of the murine activation-induced cytidine deaminase (AICDA) gene. *Mol Immunol*. 2006;43:529-541.

182. Tran TH, Nakata M, Suzuki K, et al. B cell-specific and stimulationresponsive enhancers derepress Aicda by overcoming the effects of silencers. *Nat Immunol*. 2010;11:148-154.

183. Park SR, Zan H, Pal Z, et al. HoxC4 binds to the promoter of the cytidine deaminase AID gene to induce AID expression, class-switch DNA recombination and somatic hypermutation. *Nat Immunol*. 2009;10:540-550.

184. Crouch EE, Li Z, Takizawa M, et al. Regulation of AID expression in the immune response. *J Exp Med*. 2007;204:1145-1156.

185. Pauklin S, Sernandez IV, Bachmann G, Ramiro AR, Petersen-Mahrt SK. Estrogen directly activates AID transcription and function. *J Exp Med*. 2009;206:99-111.

186. Mai T, Zan H, Zhang J, Hawkins JS, Xu Z, Casali P. Estrogen receptors bind to and activate the HOXC4/HoxC4 promoter to potentiate HoxC4-mediated activation-induced cytosine deaminase induction, immunoglobulin class switch DNA recombination, and somatic hypermutation. *J Biol Chem*. 2010;285:37797-37810.

187. Teng G, Hakimpour P, Landgraf P, et al. MicroRNA-155 is a negative regulator of activation-induced cytidine deaminase. *Immunity*. 2008;28:621-629.

188. Dorsett Y, McBride KM, Jankovic M, et al. MicroRNA-155 suppresses activation-induced cytidine deaminase-mediated Myc-Igh translocation. *Immunity*. 2008;28:630-638.

189. Ito S, Nagaoka H, Shinkura R, et al. Activation-induced cytidine deaminase shuttles between nucleus and cytoplasm like apolipoprotein B mRNA editing catalytic polypeptide 1. *Proc Natl Acad Sci U S A* . 2004;101:1975-1980.

190. Patenaude AM, Orthwein A, Hu Y, et al. Active nuclear import and cytoplasmic retention of activation-induced deaminase. *Nat Struct Mol Biol* . 2009;16:517-527.

191. McBride KM, Barreto V, Ramiro AR, Stavropoulos P, Nussenzweig MC. Somatic hypermutation is limited by CRM1-dependent nuclear export of activation-induced deaminase. *J Exp Med*. 2004;199:1235-1244.

192. Maeda K, Singh SK, Eda K, et al. GANP-mediated recruitment of activation-induced cytidine deaminase to cell nuclei and to immunoglobulin variable region DNA. *J Biol Chem*. 2010;285:23945-23953.

193. Han L, Masani S, Yu K. Cutting edge: CTNNBL1 is dispensable for Ig class switch recombination. *J Immunol* . 2010;185:1379-1381.

194. Aoufouchi S, Faili A, Zober C, et al. Proteasomal degradation restricts the nuclear lifespan of AID. *J Exp Med*. 2008;205:1357-1368.

195. Orthwein A, Patenaude AM, Affar el B, Lamarre A, Young JC, Di Noia JM. Regulation of activation-induced deaminase stability and antibody gene diversification by Hsp90. *J Exp Med*. 2010;207:2751-2765.

196. Basu U, Chaudhuri J, Alpert C, et al. The AID antibody diversification enzyme is regulated by protein kinase A phosphorylation. *Nature*. 2005;438:508-511.

197. Pasqualucci L, Kitaura Y, Gu H, Dalla-Favera R. PKA-mediated phosphorylation regulates the function of activation-induced deaminase (AID) in B cells. *Proc Natl Acad Sci U S A*. 2006;103:395-400.

198. McBride KM, Gazumyan A, Woo EM, Schwickert TA, Chait BT, Nussenzweig MC. Regulation of class switch recombination and somatic mutation by AID phosphorylation. *J*

Exp Med. 2008;205:2585-2594.

199. Gazumyan A, Timachova K, Yuen G, et al. Amino-terminal phosphorylation of activation-induced cytidine deaminase suppresses c-myc/IgH translocation. *Mol Cell Biol*. 2011;31:442-449.

200. Weigert M, Riblet R. Genetic control of antibody variable regions. *Cold Spring Harb Symp Quant Biol*. 1977;41(pt 2):837-846.

201. Wiens GD, Brown M, Rittenberg MB. Repertoire shift in the humoral response to phosphocholine-keyhole limpet hemocyanin: VH somatic mutation in germinal center B cells impairs T15 Ig function. *J Immunol*. 2003;170:5095-5102.

202. Ray SK, Putterman C, Diamond B. Pathogenic autoantibodies are routinely generated during the response to foreign antigen: a paradigm for autoimmune disease. *Proc Natl Acad Sci U S A* . 1996;93:2019-2024.

203. Choe J, Kim HS, Zhang X, Armitage RJ, Choi YS. Cellular and molecular factors that regulate the differentiation and apoptosis of germinal center B cells. Anti-Ig down-regulates Fas expression of CD40 ligandstimulated germinal center B cells and inhibits Fas-mediated apoptosis. *J Immunol*. 1996;157:1006-1016.

204. Hennino A, Berard M, Krammer PH, Defrance T. FLICE-inhibitory protein is a key regulator of germinal center B cell apoptosis. *J Exp Med*. 2001;193:447-458.

205. Eisen HN, Siskind GW. Variations in affinities of antibodies during the immune response. *Biochemistry*. 1964;3:996-1008.

206. Takahashi Y, Cerasoli DM, Dal Porto JM, et al. Relaxed negative selection in germinal centers and impaired affinity maturation in bcl-xL transgenic mice. *J Exp Med*. 1999;190:399-410.

207. Jacob J, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. II. A common clonal origin for periarteriolar lymphoid sheath-associated foci and germinal centers. *J Exp Med*. 1992;176:679-87.

208. Zheng B, Han S, Kelsoe G. T helper cells in murine germinal centers are antigen-specific emigrants that downregulate Thy-1. *J Exp Med*. 1996;184:1083-1091.

209. Razanajaona D, van Kooten C, Lebecque S, et al. Somatic mutations in human Ig variable genes correlate with a partially functional CD40-ligand in the X-linked hyper-IgM syndrome. *J Immunol*. 1996;157:1492-1498.

210. Allen CD, Okada T, Tang HL, Cyster JG. Imaging of germinal center selection events during affinity maturation. *Science*. 2007;315:528-31.

211. Allen CD, Ansel KM, Low C, et al. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol*. 2004;5:943-952.

212. Victora GD, Schwickert TA, Fooksman DR, et al. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell*. 2010;143:592-605.

213. Anderson SM, Khalil A, Uduman M, et al. Taking advantage: highaffinity B cells in the germinal center have lower death rates, but similar rates of division, compared to low-affinity cells. *J Immunol*. 2009;183:7314-7325.

214. Suzuki K, Grigorova I, Phan TG, Kelly LM, Cyster JG. Visualizing B cell capture of cognate antigen from follicular dendritic cells. *J Exp Med*. 2009;206:1485-1493.

215. Sharma S, Orlowski G, Song W. Btk regulates B cell receptor-mediated antigen processing and presentation by controlling actin cytoskeleton dynamics in B cells. *J Immunol*. 2009;182:329-339.

216. Mao C, Jiang L, Melo-Jorge M, et al. T cell-independent somatic hypermutation in murine B cells with an immature phenotype. *Immunity*. 2004;20:133-144.

217. Weller S, Braun MC, Tan BK, et al. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood*. 2004;104:3647-3654.

218. Matsumoto M, Lo SF, Carruthers CJ, et al. Affinity maturation without germinal centres in lymphotoxin-alpha-deficient mice. *Nature*. 1996;382:462-466.

219. Toellner KM, Jenkinson WE, Taylor DR, et al. Low-level hypermutation in T cell-independent germinal centers compared with high mutation rates associated with T cell-dependent germinal centers. *J Exp Med*. 2002;195:383-389.

220. Jiricny J. The multifaceted mismatch-repair system. *Nat Rev Mol Cell Biol* . 2006;7:335-346.

221. Wiesendanger M, Kneitz B, Edelmann W, Scharff MD. Somatic hypermutation in MutS homologue (MSH)3-, MSH6-, and MSH3/MSH6-deficient mice reveals a role for the MSH2-MSH6 heterodimer in modulating the base substitution pattern. *J Exp Med*. 2000;191:579-584.

222. Martomo SA, Yang WW, Gearhart PJ. A role for Msh6 but not Msh3 in somatic hypermutation and class switch recombination. *J Exp Med*. 2004;200:61-68.

223. Wilson TM, Vaisman A, Martomo SA, et al. MSH2-MSH6 stimulates DNA polymerase eta, suggesting a role for A:T mutations in antibody genes. *J Exp Med*. 2005;201:637-645.

224. Martomo SA, Yang WW, Wersto RP, et al. Different mutation signatures in DNA polymerase eta- and MSH6-deficient mice suggest separate roles in antibody diversification. *Proc Natl Acad Sci U S A* . 2005;102:8656-8661.

225. Delbos F, De Smet A, Faili A, Aoufouchi S, Weill JC, Reynaud CA. Contribution of DNA polymerase eta to immunoglobulin gene hypermutation in the mouse. *J Exp Med*. 2005;201:1191-1196.

226. Jansen JG, Langerak P, Tsaalbi-Shtylik A, van den Berk P, Jacobs H, de Wind N. Strand-biased defect in C/G transversions in hypermutating immunoglobulin genes in Rev1-deficient mice. *J Exp Med*. 2006;203:319-323.

227. Zan H, Komori A, Li Z, et al. The translesion DNA polymerase zeta plays a major role in Ig and bcl-6 somatic hypermutation. *Immunity*. 2001;14:643-653.

228. Diaz M, Verkoczy LK, Flajnik MF, Klinman NR. Decreased frequency of somatic hypermutation and impaired affinity maturation but intact germinal center formation in mice expressing antisense RNA to DNA polymerase zeta. *J Immunol*. 2001;167:327-335.

229. Faili A, Stary A, Delbos F, et al. A backup role of DNA polymerase kappa in Ig gene hypermutation only takes place in the complete absence of DNA polymerase eta. *J Immunol*. 2009;182:6353-6359.

230. Masuda K, Ouchida R, Hikida M, et al. Absence of DNA polymerase theta results in decreased somatic hypermutation frequency and altered mutation patterns in Ig genes. *DNA Repair (Amst)* . 2006;5:1384-1391.

231. Zan H, Shima N, Xu Z, et al. The translesion DNA polymerase theta plays a dominant role in immunoglobulin gene somatic hypermutation. *EMBO J*. 2005;24:3757-3769.

232. Masuda K, Ouchida R, Hikida M, et al. DNA polymerases eta and theta function in the same genetic pathway to generate mutations at A/T during somatic hypermutation of Ig genes. *J Biol Chem*. 2007;282:17387-17394.

233. Poltoratsky V, Prasad R, Horton JK, Wilson SH. Down-regulation of DNA polymerase beta accompanies somatic hypermutation in human BL2 cell lines. *DNA Repair*. 2007;6:244-253.

234. Wu X, Stavnezer J. DNA polymerase beta is able to repair breaks in switch regions and plays an inhibitory role during immunoglobulin class switch recombination. *J Exp Med*. 2007;204:1677-1689.

235. Bachl J, Ertongur I, Jungnickel B. Involvement of Rad18 in somatic hypermutation. *Proc Natl Acad Sci U S A* . 2006;103:12081-1206.

236. Kleinstein SH, Louzoun Y, Shlomchik MJ. Estimating hypermutation rates from clonal tree data. *J Immunol*. 2003;171:4639-4649.

237. Shen HM, Michael N, Kim N, Storb U. The TATA binding protein, c-Myc and survivin genes are not somatically hypermutated, while Ig and BCL6 genes are hypermutated in human memory B cells. *Int Immunol*. 2000;12:1085-1093.

238. Liu M, Duke JL, Richter DJ, et al. Two levels of protection for the B cell genome during somatic hypermutation. *Nature*. 2008;451:841-845.

239. Picard D, Schaffner W. Unrearranged immunoglobulin lambda variable region is transcribed in kappa-producing myelomas. *EMBO J*. 1984;3:3031-3035.

240. Weiss S, Wu GE. Somatic point mutations in unrearranged immunoglobulin gene segments encoding the variable region of lambda light chains. *EMBO J*. 1987;6:927-932.

241. Peters A, Storb U. Somatic hypermutation of immunoglobulin genes is linked to transcription initiation. *Immunity*. 1996;4:57-65.

242. Longerich S, Tanaka A, Bozek G, Nicolae D, Storb U. The very 5' end and the constant region of Ig genes are spared from somatic mutation because AID does not access these regions. *J Exp Med*. 2005;202:1443-1454.

243. Winter DB, Sattar N, Mai JJ, Gearhart PJ. Insertion of 2 kb of bacteriophage DNA between an immunoglobulin promoter and leader exon stops somatic hypermutation in a kappa transgene. *Mol Immunol*. 1997;34:359-366.

244. Shen HM, Storb U. Activation-induced cytidine deaminase (AID) can target both DNA strands when the DNA is supercoiled. *Proc Natl Acad Sci U S A* . 2004;101:12997-13002.

245. Ronai D, Iglesias-Ussel MD, Fan M, Li Z, Martin A, Scharff MD. Detection of chromatin-associated single-stranded DNA in regions targeted for somatic hypermutation. *J Exp Med*. 2007;204:181-190.

246. Fukita Y, Jacobs H, Rajewsky K. Somatic hypermutation in the heavy chain locus correlates with transcription. *Immunity*. 1998;9:105-114.

247. Bachl J, Carlson C, Gray-Schopfer V, Dessing M, Olsson C. Increased transcription levels induce higher mutation rates in a hypermutating cell line. *J Immunol*. 2001;166:5051-5057.

248. Tumas-Brundage K, Vora KA, Giusti AM, Manser T. Characterization of the cis-acting elements required for somatic hypermutation of murine antibody V genes using conventional transgenic and transgene homologous recombination approaches. *Semin Immunol*. 1996;8:141-150.

249. Tanaka A, Shen HM, Ratnam S, Kodgire P, Storb U. Attracting AID to targets of somatic hypermutation. *J Exp Med*. 2010;207:405-415.

250. Schoetz U, Cervelli M, Wang YD, Fiedler P, Buerstedde JM. E2A expression stimulates Ig hypermutation. *J Immunol*. 2006;177:395-400.

251. Terauchi A, Hayashi K, Kitamura D, Kozono Y, Motoyama N, Azuma T. A pivotal role for DNase I-sensitive regions 3b and/or 4 in the induction of somatic hypermutation of IgH genes. *J Immunol*. 2001;167:811-820.

252. Morvan CL, Pinaud E, Decourt C, Cuvillier A, Cogne M. The immunoglobulin heavy-chain locus hs3b and hs4 3′ enhancers are dispensable for VDJ assembly and somatic hypermutation. *Blood*. 2003;102:1421-1427.

253. Kothapalli N, Norton DD, Fugmann SD. Cutting edge: a cis-acting DNA element targets AID-mediated sequence diversification to the chicken Ig light chain gene locus. *J Immunol*. 2008;180:2019-2023.

254. Blagodatski A, Batrak V, Schmidl S, et al. A cis-acting diversification activator both necessary and sufficient for AID-mediated hypermutation. *PLoS Genet*. 2009;5:e1000332.

255. Woo CJ, Martin A, Scharff MD. Induction of somatic hypermutation is associated with modifications in immunoglobulin variable region chromatin. *Immunity*. 2003;19:479-489.

256. Odegard VH, Kim ST, Anderson SM, Shlomchik MJ, Schatz DG. Histone modifications associated with somatic hypermutation. *Immunity*. 2005;23:101-110.

257. Borchert GM, Holton NW, Edwards KA, Vogel LA, Larson ED. Histone H2A and H2B are monoubiquitinated at AID-targeted loci. *PLoS One*. 2010;5:e11641.

258. Shapiro GS, Aviszus K, Murphy J, Wysocki LJ. Evolution of Ig DNA sequence to target specific base positions within codons for somatic hypermutation. *J Immunol*. 2002;168:2302-2306.

259. Zarrin AA, Tian M, Wang J, Borjeson T, Alt FW. Influence of switch region length on immunoglobulin class switch recombination. *Proc Natl Acad Sci U S A* . 2005;102:2466-2470.

260. Luby TM, Schrader CE, Stavnezer J, Selsing E. The mu switch region tandem repeats are important, but not required, for antibody class switch recombination. *J Exp Med*. 2001;193:159-168.

261. Khamlichi AA, Glaudet F, Oruc Z, Denis V, Le Bert M, Cogne M. Immunoglobulin class-switch recombination in mice devoid of any S mu tandem repeat. *Blood*. 2004;103:3828-3836.

262. Du J, Zhu Y, Shanmugam A, Kenter AL. Analysis of immunoglobulin Sgamma3 recombination breakpoints by PCR: implications for the mechanism of isotype switching. *Nucleic Acids Res*. 1997;25:3066-3073.

263. Yoshida K, Matsuoka M, Usuda S, Mori A, Ishizaka K, Sakano H. Immunoglobulin switch circular DNA in the mouse infected with Nippostrongylus brasiliensis: evidence for successive class switching from mu to epsilon via gamma 1. *Proc Natl Acad Sci U S A* . 1990;87:7829-7833.

264. Zhang T, Franklin A, Boboila C, et al. Downstream class switching leads to IgE antibody production by B lymphocytes lacking IgM switch regions. *Proc Natl Acad Sci U S A*. 2010;107:3040-3045.

265. Reynaud S, Delpy L, Fleury L, Dougier HL, Sirac C, Cogne M. Interallelic class switch recombination contributes significantly to class switching in mouse B cells. *J Immunol*. 2005;174:6176-6183.

266. Kingzette M, Spieker-Polet H, Yam PC, Zhai SK, Knight KL. Transchromosomal recombination within the Ig heavy chain switch region in B lymphocytes. *Proc Natl Acad Sci U S A*. 1998;95:11840-11845.

267. Zarrin AA, Del Vecchio C, Tseng E, et al. Antibody class switching mediated by yeast endonuclease-generated DNA breaks. *Science*. 2007;315:377-381.

268. Allen RC, Armitage RJ, Conley ME, et al. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science*. 1993;259:990-993.

269. Ferrari S, Giliani S, Insalaco A, et al. Mutations of CD40 gene cause an autosomal recessive form of immunodeficiency with hyper IgM. *Proc Natl Acad Sci U S A* . 2001;98:12614-12619.

270. Bergqvist P, Stensson A, Lycke NY, Bemark M. T cell-independent IgA class switch recombination is restricted to the GALT and occurs prior to manifest germinal center formation. *J Immunol* . 2010;184:3545-3553.

271. Hodgkin PD, Lee JH, Lyons AB. B cell differentiation and isotype switching is related to division cycle number. *J Exp Med*. 1996;184:277-281.

272. McCall MN, Hodgkin PD. Switch recombination and germ-line transcription are division-regulated events in B lymphocytes. *Biochim Biophys Acta*. 1999;1447:43-50.

273. Rush JS, Liu M, Odegard VH, Unniraman S, Schatz DG. Expression of activation-induced cytidine deaminase is regulated by cell division, providing a mechanistic basis for division-linked class switch recombination. *Proc Natl Acad Sci U S A* . 2005;102:13242-13247.

274. Siepmann K, Wohlleben G, Gray D. CD40-mediated regulation of interleukin-4 signaling pathways in B lymphocytes. *Eur J Immunol* . 1996;26:1544-1552.

275. Grammer AC, McFarland RD, Heaney J, Darnell BF, Lipsky PE. Expression, regulation, and function of B cell-expressed CD154 in germinal centers. *J Immunol*. 1999;163:4150-4159.

276. Ozcan E, Rauter I, Garibyan L, Dillon SR, Geha RS. Toll-like receptor 9, transmembrane activator and calcium-modulating cyclophilin ligand interactor, and CD40 synergize in causing B-cell activation. *J Allergy Clin Immunol* . 2011;128:601-609.

277. Mao CS, Stavnezer J. Differential regulation of mouse germline Ig gamma 1 and epsilon promoters by IL-4 and CD40. *J Immunol*. 2001;167:1522-1534.

278. Stavnezer J. Antibody class switching. *Adv Immunol*. 1996;61:79-146.

279. Qiu G, Harriman GR, Stavnezer J. Ialpha exon-replacement mice synthesize a spliced HPRT-C(alpha) transcript which may explain their ability to switch to IgA. Inhibition of switching to IgG in these mice. *Int Immunol*. 1999;11:37-46.

280. Hein K, Lorenz MG, Siebenkotten G, Petry K, Christine R, Radbruch A. Processing of switch transcripts is required for targeting of antibody class switch recombination. *J Exp Med*. 1998;188:2369-2374.

281. Pinaud E, Khamlichi AA, Le Morvan C, et al. Localization of the 3' IgH locus elements that effect long-distance regulation of class switch recombination. *Immunity*. 2001;15:187-199.

282. Adams K, Ackerly H, Cunningham K, Dunnick W. A DNase I hypersensitive site near the murine gamma1 switch region contributes to insertion site independence of transgenes and modulates the amount of transcripts induced by CD40 ligation. *Int Immunol*. 2000;12:1705-1713.

283. Wuerffel R, Wang L, Grigera F, et al. S-S synapsis during class switch recombination is promoted by distantly located transcriptional elements and activation-induced deaminase. *Immunity*. 2007;27:711-722.

284. Chatterjee S, Ju Z, Hassan R, Volpi SA, Emelyanov AV, Birshtein BK. Dynamic changes in binding of immunoglobulin heavy chain 3' regulatory region to protein factors during class switching. *J Biol Chem*. 2011;286:29303-29312.

285. Li Z, Luo Z, Scharff MD. Differential regulation of histone acetylation and generation of mutations in switch regions is associated with Ig class switching. *Proc Natl Acad Sci U S A*. 2004;101:15428-15433.

286. Wang L, Wuerffel R, Feldman S, Khamlichi AA, Kenter AL. S region sequence, RNA polymerase II, and histone modifications create chromatin accessibility during class switch recombination. *J Exp Med*. 2009;206:1817-1830.

287. Daniel JA, Santos MA, Wang Z, et al. PTIP promotes chromatin changes critical for immunoglobulin class switch recombination. *Science*. 2010;329:917-923.

288. Schwab KR, Patel SR, Dressler GR. Role of PTIP in class switch recombination and long-range chromatin interactions at the immunoglobulin heavy chain locus. *Mol Cell Biol*. 2011;31:1503-1511.

289. Stanlie A, Aida M, Muramatsu M, Honjo T, Begum NA. Histone3 lysine4 trimethylation regulated by the facilitates chromatin transcription complex is critical for

DNA cleavage in class switch recombination. *Proc Natl Acad Sci U S A* . 2010;107:22190-22195.

290. Kuang FL, Luo Z, Scharff MD. H3 trimethyl K9 and H3 acetyl K9 chromatin modifications are associated with class switch recombination. *Proc Natl Acad Sci U S A* . 2009;106:5288-5293.

291. Chowdhury M, Forouhi O, Dayal S, et al. Analysis of intergenic transcription and histone modification across the human immunoglobulin heavy-chain locus. *Proc Natl Acad Sci U S A*. 2008;105:15872-15877.

292. Jeevan-Raj BP, Robert I, Heyer V, et al. Epigenetic tethering of AID to the donor switch region during immunoglobulin class switch recombination. *J Exp Med*. 2011;208:1649-1660.

293. Xue K, Rada C, Neuberger MS. The in vivo pattern of AID targeting to immunoglobulin switch regions deduced from mutation spectra in msh2-/- ung-/- mice. *J Exp Med*. 2006;203:2085-2094.

294. Daniels GA, Lieber MR. RNA:DNA complex formation upon transcription of immunoglobulin switch regions: implications for the mechanism and regulation of class switch recombination. *Nucleic Acids Res*. 1995;23:5006-5011.

295. Yu K, Chedin F, Hsieh CL, Wilson TE, Lieber MR. R-loops at immunoglobulin class switch regions in the chromosomes of stimulated B cells. *Nat Immunol*. 2003;4:442-451.

296. Huang FT, Yu K, Hsieh CL, Lieber MR. Downstream boundary of chromosomal R-loops at murine switch regions: implications for the mechanism of class switch recombination. *Proc Natl Acad Sci U S A* . 2006;103:5030-5035.

297. Rajagopal D, Maul RW, Ghosh A, et al. Immunoglobulin switch mu sequence causes RNA polymerase II accumulation and reduces dA hypermutation. *J Exp Med*. 2009;206:1237-1244.

298. Canugovi C, Samaranayake M, Bhagwat AS. Transcriptional pausing and stalling causes multiple clustered mutations by human activationinduced deaminase. *FASEB J*. 2009;23:34-44.

299. Pavri R, Gazumyan A, Jankovic M, et al. Activation-induced cytidine deaminase targets DNA at sites of RNA polymerase II stalling by interaction with Spt5. *Cell*. 2010;143:122-133.

300. Margaritis T, Holstege FC. Poised RNA polymerase II gives pause for thought. *Cell*. 2008;133:581-584.

301. Basu U, Meng FL, Keim C, et al. The RNA exosome targets the AID cytidine deaminase to both strands of transcribed duplex DNA substrates. *Cell*. 2011;144:353-363.

302. Shinkura R, Tian M, Smith M, Chua K, Fujiwara Y, Alt FW. The influence of transcriptional orientation on endogenous switch region function. *Nat Immunol*. 2003;4:435-441.

303. Han L, Masani S, Yu K. Overlapping activation-induced cytidine deaminase hotspot motifs in Ig class-switch recombination. *Proc Natl Acad Sci U S A* . 2011;108:11584-11589.

304. Dunnick W, Hertz GZ, Scappino L, Gritzmacher C. DNA sequences at immunoglobulin switch region recombination sites. *Nucleic Acids Res*. 1993;21:365-372. Errata *Nucleic Acids Res*. 1993;21(9):2285.

305. Chen X, Kinoshita K, Honjo T. Variable deletion and duplication at recombination junction ends: implication for staggered double-strand cleavage in class-switch recombination. *Proc Natl Acad Sci U S A* . 2001;98:13860-13865.

306. Rush JS, Fugmann SD, Schatz DG. Staggered AID-dependent DNA double strand breaks are the predominant DNA lesions targeted to S mu in Ig class switch recombination. *Int Immunol* . 2004;16:549-557.

307. Catalan N, Selz F, Imai K, Revy P, Fischer A, Durandy A. The block in immunoglobulin class switch recombination caused by activationinduced cytidine deaminase deficiency occurs prior to the generation of DNA double strand breaks in switch mu region. *J Immunol* . 2003;171:2504-2509.

308. Schrader CE, Linehan EK, Mochegova SN, Woodland RT, Stavnezer J. Inducible DNA breaks in Ig S regions are dependent on AID and UNG. *J Exp Med*. 2005;202:561-568.

309. Petersen S, Casellas R, Reina-San-Martin B, et al. AID is required to initiate Nbs1/gamma-H2AX focus formation and mutations at sites of class switching. *Nature*. 2001;414:660-665.

310. Schrader CE, Guikema JE, Linehan EK, Selsing E, Stavnezer J. Activation-induced cytidine deaminase-dependent DNA breaks in class switch recombination occur during G1 phase of the cell cycle and depend upon mismatch repair. *J Immunol* . 2007;179:6064-6071.

311. Robbiani DF, Bunting S, Feldhahn N, et al. AID produces DNA double-strand breaks in non-Ig genes and mature B cell lymphomas with reciprocal chromosome translocations. *Mol Cell* . 2009;36:631-641.

312. Staszewski O, Baker RE, Ucher AJ, Martier R, Stavnezer J, Guikema JE. Activation-induced cytidine deaminase induces reproducible DNA breaks at many non-Ig Loci in activated B cells. *Mol Cell* . 2011;41:232-242.

313. Hasham MG, Donghia NM, Coffey E, et al. Widespread genomic breaks generated by activation-induced cytidine deaminase are prevented by homologous recombination. *Nat Immunol* . 2010;11:820-826.

314. Imai K, Slupphaug G, Lee WI, et al. Human uracil-DNA glycosylase deficiency associated with profoundly impaired immunoglobulin classswitch recombination. *Nat Immunol* . 2003;4:1023-1028.

315. Schrader CE, Guikema JE, Wu X, Stavnezer J. The roles of APE1, APE2, DNA polymerase beta and mismatch repair in creating S region DNA breaks during antibody class switch. *Philos Trans R Soc Lon B Biol Sci* . 2009;364:645-652.

316. Rada C, Di Noia JM, Neuberger MS. Mismatch recognition and uracil excision provide complementary paths to both Ig switching and the A/T-focused phase of somatic mutation. *Mol Cell* . 2004;16:163-171.

317. Ranjit S, Khair L, Linehan EK, et al. AID binds cooperatively with UNG and Msh2-

Msh6 to Ig switch regions dependent upon the AID C terminus. *J Immunol*. 2011;187:2464-2475.

318. Stavnezer J, Schrader CE. Mismatch repair converts AID-instigated nicks to double-strand breaks for antibody class-switch recombination. *Trends Genet*. 2006;22:23-28.

319. Bardwell PD, Woo CJ, Wei K, et al. Altered somatic hypermutation and reduced class-switch recombination in exonuclease 1-mutant mice. *Nat Immunol*. 2004;5:224-229.

320. Boboila C, Yan C, Wesemann DR, et al. Alternative end-joining catalyzes class switch recombination in the absence of both Ku70 and DNA ligase 4. *J Exp Med*. 2010;207:417-427.

321. Li G, Alt FW, Cheng HL, et al. Lymphocyte-specific compensation for XLF/cernunnos end-joining functions in V(D)J recombination. *Mol Cell*. 2008;31:631-640.

322. Yan CT, Boboila C, Souza EK, et al. IgH class switching and translocations use a robust non-classical end-joining pathway. *Nature*. 2007;449:478-482.

323. Soulas-Sprauel P, Le Guyader G, Rivera-Munoz P, et al. Role for DNA repair factor XRCC4 in immunoglobulin class switch recombination. *J Exp Med*. 2007;204:1717-1727.

324. Boboila C, Jankovic M, Yan CT, et al. Alternative end-joining catalyzes robust IgH locus deletions and translocations in the combined absence of ligase 4 and Ku70. *Proc Natl Acad Sci U S A*. 2010;107:3034-3039.

325. Lee-Theilen M, Matthews AJ, Kelly D, Zheng S, Chaudhuri J. CtIP promotes microhomology-mediated alternative end joining during classswitch recombination. *Nat Struct Mol Biol*. 2011;18:75-79.

326. Wang M, Wu W, Rosidi B, Zhang L, Wang H, Iliakis G. PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. *Nucleic Acids Res*. 2006;34:6170-6182.

327. Robert I, Dantzer F, Reina-San-Martin B. Parp1 facilitates alternative NHEJ, whereas Parp2 suppresses IgH/c-myc translocations during immunoglobulin class switch recombination. *J Exp Med*. 2009;206:1047-1056.

328. Saribasak H, Maul RW, Cao Z, et al. XRCC1 suppresses somatic hypermutation and promotes alternative nonhomologous end joining in Igh genes. *J Exp Med*. 2011;208:2209-2216.

329. Rooney S, Alt FW, Sekiguchi J, Manis JP. Artemis-independent functions of DNA-dependent protein kinase in Ig heavy chain class switch recombination and development. *Proc Natl Acad Sci U S A* . 2005;102:2471-2475.

330. Franco S, Murphy MM, Li G, Borjeson T, Boboila C, Alt FW. DNAPKcs and Artemis function in the end-joining phase of immunoglobulin heavy chain class switch recombination. *J Exp Med*. 2008;205: 557-564.

331. Du L, van der Burg M, Popov SW, et al. Involvement of Artemis in nonhomologous end-joining during immunoglobulin class switch recombination. *J Exp Med*. 2008;205:3031-3040.

332. Manis JP, Dudley D, Kaylor L, Alt FW. IgH class switch recombination to IgG1 in DNA-PKcs-deficient B cells. *Immunity*. 2002;16:607-617.

333. Kiefer K, Oshinsky J, Kim J, Nakajima PB, Bosma GC, Bosma MJ. The catalytic subunit of DNA-protein kinase (DNA-PKcs) is not required for Ig class-switch recombination. *Proc Natl Acad Sci U S A* . 2007;104:2843-2848.

334. Polo SE, Jackson SP. Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. *Genes Dev*. 2011;25:409-433.

335. Reina-San-Martin B, Nussenzweig MC, Nussenzweig A, Difilippantonio S. Genomic instability, endoreduplication, and diminished Igclassswitch recombination in B cells lacking Nbs1. *Proc Natl Acad Sci U S A* . 2005;102:1590-1595.

336. Kracker S, Bergmann Y, Demuth I, et al. Nibrin functions in Ig classswitch recombination. *Proc Natl Acad Sci U S A* . 2005;102:1584-1589.

337. Dinkelmann M, Spehalski E, Stoneham T, et al. Multiple functions of MRN in end-joining pathways during isotype class switching. *Nat Struct Mol Biol* . 2009;16:808-813.

338. Lahdesmaki A, Taylor AM, Chrzanowska KH, Pan-Hammarstrom Q. Delineation of the role of the Mre11 complex in class switch recombination. *J Biol Chem*. 2004;279:16479-16487.

339. Lumsden JM, McCarty T, Petiniot LK, et al. Immunoglobulin class switch recombination is impaired in Atm-deficient mice. *J Exp Med*. 2004;200:1111-1121.

340. Reina-San-Martin B, Chen HT, Nussenzweig A, Nussenzweig MC. ATM is required for efficient recombination between immunoglobulin switch regions. *J Exp Med*. 2004;200:1103-1110.

341. Reina-San-Martin B, Difilippantonio S, Hanitsch L, Masilamani RF, Nussenzweig A, Nussenzweig MC. H2AX is required for recombination between immunoglobulin switch regions but not for intraswitch region recombination or somatic hypermutation. *J Exp Med*. 2003;197:1767-1778.

342. Ramachandran S, Chahwan R, Nepal RM, et al. The RNF8/RNF168 ubiquitin ligase cascade facilitates class switch recombination. *Proc Natl Acad Sci U S A* . 2010;107:809-814.

343. Stewart GS, Panier S, Townsend K, et al. The RIDDLE syndrome protein mediates a ubiquitin-dependent signaling cascade at sites of DNA damage. *Cell*. 2009;136:420-434.

344. Reina-San-Martin B, Chen J, Nussenzweig A, Nussenzweig MC. Enhanced intra-switch region recombination during immunoglobulin class switch recombination in 53BP1-/- B cells. *Eur J Immunol* . 2007;37:235-239.

345. Nowak U, Matthews AJ, Zheng S, Chaudhuri J. The splicing regulator PTBP2 interacts with the cytidine deaminase AID and promotes binding of AID to switch-region DNA. *Nat Immunol* . 2011;12:160-166.

346. Xu Z, Fulop Z, Wu G, Pone et al. 14-3-3 adaptor proteins recruit AID to 5′-AGCT-3′-rich switch regions for class switch recombination. *Nat Struct Mol Biol* . 2010;17:1124-1135.

347. Vuong BQ, Lee M, Kabir S, et al. Specific recruitment of protein kinase A to the immunoglobulin locus regulates class-switch recombination. *Nat Immunol* . 2009;10:420-426.

348. Holliger P, Hudson PJ. Engineered antibody fragments and the rise of single domains. *Nat Biotechnol*. 2005;23:1126-1136.

349. Chames P, Van Regenmortel M, Weiss E, Baty D. Therapeutic antibodies: successes, limitations and hopes for the future. *Br J Pharmacol* . 2009;157:220-233.

350. Nieri P, Donadio E, Rossi S, Adinolfi B, Podesta A. Antibodies for therapeutic uses and the evolution of biotechniques. *Curr Med Chem*. 2009;16:753-779.

351. Pande J, Szewczyk MM, Grover AK. Phage display: concept, innovations, applications and future. *Biotechnol Adv*. 2010;28:849-858.

352. Schmidt EV, Pattengale PK, Weir L, Leder P. Transgenic mice bearing the human c-myc gene activated by a4n immunoglobulin enhancer: a pre-B-cell lymphoma model. *Proc Natl Acad Sci U S A*. 1988;85:6047-6051.

353. Sarris A, Ford R. Recent advances in the molecular pathogenesis of lymphomas. *Curr Opin Oncol* . 1999;11:351-363.

354. Bende RJ, Smit LA, van Noesel CJ. Molecular pathways in follicular lymphoma. *Leukemia*. 2007;21:18-29.

355. Garcia-Castillo H, Barros-Nunez P. Detection of clonal immunoglobulin and T-cell receptor gene recombination in hematological malignancies: monitoring minimal residual disease. *Cardiovasc Hematol Disord Drug Targets*. 2009;9:124-135.

356. Chesi M, Kuehl WM, Bergsagel PL. Recurrent immunoglobulin gene translocations identify distinct molecular subtypes of myeloma. *Ann Oncol*. 2000;11(suppl 1):131-135.

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Chapter 7

Antigen-Antibody Interactions and Monoclonal Antibodies

Jay A. Berzofsky

Ira J. Berkower

INTRODUCTION

The basic principles of antigen-antibody interaction are those of any bimolecular reaction. Moreover, the binding of antigen by antibody can, in general, be described by the same theories and studied by the same experimental approaches as the binding of a hormone by its receptor, of a substrate by enzyme, or of oxygen by hemoglobin. There are several major differences, however, between antigen-antibody interactions and these other situations. First, unlike most enzymes and many hormone-binding systems, antibodies do not irreversibly alter the antigen they bind. Thus, the reactions are, at least in principle, always reversible. Second, antibodies can be raised, by design of the investigator, with specificity for almost any substance known. In each case, one can find antibodies with affinities as high as and specificities as great as those of enzymes for their substrates and receptors for their hormones. The interaction of antibody with antigen can thus be taken as a prototype for interactions of macromolecules with ligands in general. In addition, these same features of reversibility and availability of a wide variety of specificities have made antibodies invaluable reagents for identifying, quantitating, and purifying a growing number of substances of biologic and medical importance.

One other feature of antibodies that in the past proved to be a difficulty in studying and using them, compared to, say, enzymes, is their enormous heterogeneity. Even “purified” antibodies from an immune antiserum, all specific for the same substance and sharing the same overall immunoglobulin (Ig) structure, will be a heterogeneous mixture of molecules of different subclass, different affinity, and different fine specificity and ability to discriminate among crossreacting antigens. The advent of hybridoma monoclonal antibodies[1,2,3] has made available a source of homogeneous antibodies to almost anything to which antisera can be raised. Nevertheless, heterogeneous antisera are still in widespread use and even have advantages for certain purposes, such as precipitation reactions. Therefore, it is critical to keep in mind throughout this chapter, and indeed much of the volume, that the principles derived for the interaction of one antibody with one antigen must be modified and extended to cover the case of heterogeneous components in the reaction.

In this chapter, we examine the theoretical principles necessary for analyzing, in a quantitative manner, the interaction of antibody with antigen and the experimental techniques that have been developed to study these interactions as well as to make use of antibodies as quantitative reagents. Furthermore, we discuss the derivation, use, and properties of

monoclonal antibodies.

THERMODYNAMICS AND KINETICS

The Thermodynamics of Affinity

The basic thermodynamic principles of antigen-antibody interactions, as we indicated previously, are the same as those for any reversible bimolecular binding reaction. We review these as they apply to this particular immunologic reaction.

Chemical Equilibrium in Solution

For this purpose, let S = antibody binding sites, L = ligand (antigen) sites, and SL = the complex of the two. Then for the reaction

$$S + L \rightleftharpoons SL \qquad (1)$$

the mass action law states

$$K_A = \frac{[SL]}{[S][L]} \qquad (2)$$

where K_A = association constant (or affinity) and square brackets = molar concentration of the reactants enclosed. The import of this equation is that for any given set of conditions such as temperature, pH, and salt concentration, the ratio of the concentration of the complex to the product of the concentrations of the reactants at equilibrium is always constant. Thus, changing the concentration of either antibody or ligand will invariably change the concentration of the complex, provided neither reactant is limiting, that is, neither has already been saturated, and provided sufficient time is allowed to reach a new state of equilibrium. Moreover, because the concentrations of antibody and ligand appear in this equation in a completely symmetrical fashion, doubling either the antibody concentration or the antigen concentration results in a doubling of the concentration of the antigen-antibody complex, provided the other reactant is in sufficient excess. This proviso, an echo of the one just discussed, is inherent in the fact that [S] and [L] refer to the concentrations of free S and free L, respectively, in solution, not the total concentration, which would include that of the complex. Thus, if L is not in great excess, doubling [S] results in a decrease in [L] as some of it is consumed in the complex, so the net result is less than a doubling of [SL]. Similarly, halving the volume results in a doubling of the total concentration of both antibody and

ligand. If the fraction of both reactants tied up in the complex is negligibly small (as might be the case for low-affinity binding), the concentration of the complex quadruples. However, in most practical cases, the concentration of complex is a significant fraction of the total concentration of antigen or antibody or both, so the net result is an increase in the concentration of complex but by a factor of <4. The other important, perhaps obvious, but often forgotten, principle to be gleaned from this example is that because it is concentration, not amount, of each reactant that enters into the mass action law (Equation 2), putting the same amount of antigen and antibody in a smaller volume will increase the amount of

complex formed, and diluting them in a larger volume will greatly decrease the amount of complex formed. Moreover, these changes go approximately as the square of the volume, so volumes are critical in the design of an experiment.

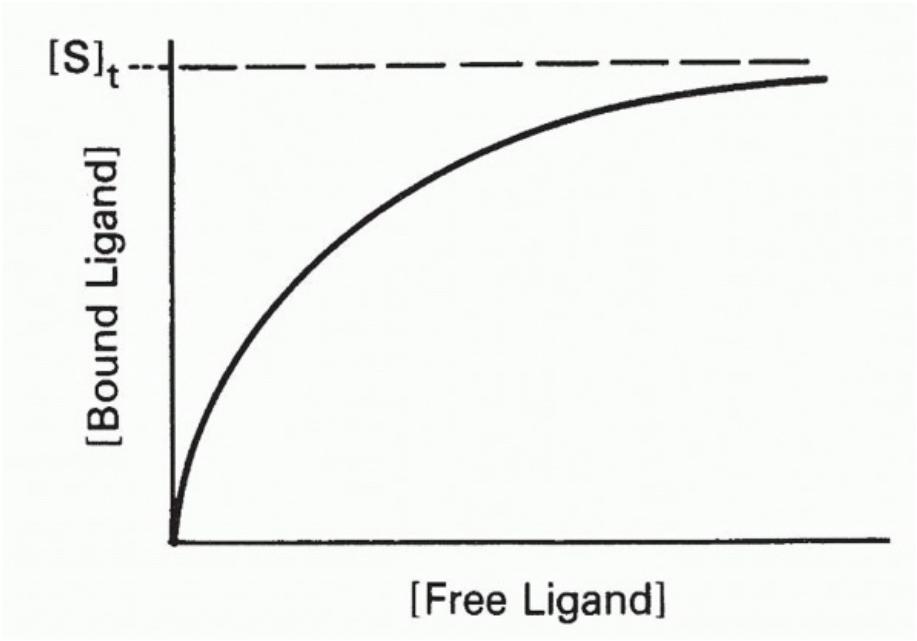

FIG. 7.1. Schematic Plot of Bound Ligand Concentration as a Function of Free Ligand Concentration at a Constant Total Concentration of Antibody-Combining Sites, $[S]_t$. The curve asymptotically approaches a plateau at which [bound ligand] =[S_t.]

The effect of increasing free ligand concentration [L], at constant total antibody concentration, on the concentration of complex, [SL], is illustrated in Figure 7.1. The mass action law (Equation 2) can be rewritten as

$$[SL] = K_A[S][L] = K_A([S]_t - [SL])[L] \qquad (3)$$

or

$$[SL] = \frac{K_A[S]_t[L]}{(1 + K_A[L])} \qquad (3')$$

where $[S]_t$ = total antibody site concentration; that is, [S] + [SL]. Initially, when the complex [SL] is a negligible fraction of the total antibody $[S]_t$, the concentration of complex increases nearly linearly with increasing ligand. However, as a larger fraction of antibody is consumed, the slope tapers off and the concentration of complex, [SL], asymptotically approaches a plateau value of $[S]_t$ as all the antibody becomes saturated. Thus, the concentration of antibody-binding sites can be determined from such a saturation binding curve (see Fig. 7.1), taking the concentration of (radioactively or otherwise labeled) ligand bound at saturation as a measure of the concentration of antibody sites.* This measurement is sometimes referred

to as antigen-binding capacity.

The total concentration of ligand at which the antibody begins to saturate is a function not only of the antibody concentration but also of the association constant, KA, also called the affinity. This constant has units of molar (M^{-1}) or L/mol, if all the concentrations in Equation 2 are molar. Thus the product $K_A[L]$ is unitless. It is the value of this product relative to 1 that determines how saturated the antibody is, as can be seen from Equation 3′. For example, an antibody with an affinity of 10^7 M^{-1} will not be saturated if the ligand concentration is 10^{-8} M (product $K_A[L] = 0.1$) even if the total amount of ligand is in great excess over the total amount of antibody. From Equation 3′ the fraction of antibody occupied would be only 0.1/1.1, or about 9%, in this example. These aspects of affinity and the methods for measuring affinity are analyzed in greater detail in the next section.

Free Energy

Regarding thermodynamics, the affinity, KA, is also the central quantity because it is directly related to the free energy, ΔF, of the reaction by the equations

$$\Delta F° = -RT \ln K_A \qquad (4)$$

$$K_A = e^{-\Delta F°/RT} \qquad (4')$$

where R = so-called gas constant (1.98717 cal/°K•mol), T = absolute temperature (in degrees Kelvin), ln = natural logarithm, and e = base of the natural logarithms. The minus sign is introduced because of the convention that a negative change in free energy corresponds to positive binding. The ΔF° is the standard free-energy change defined as the ΔF for 1 mol antigen + 1 mol antibody sites combining to form 1 mol of complex at unit concentration.

It is also instructive to note an apparent discrepancy in Equations 4 and 4′. As defined in Equation 2, K_A has dimensions of M^{-1} (ie, L/mol), whereas in Equation 4′, it is dimensionless. The reason is that for Equation 4′ to hold strictly, K_A must be expressed in terms of mole fractions rather than concentrations. The mole fraction of a solute is the ratio of moles of that solute to the total number of moles of all components in the solution. Because water (55 M) is by far the predominant component of most aqueous solutions, for practical purposes, one can convert K_A into a unitless ratio of mole fractions by dividing all concentrations in Equation 2 by 55 M. This transformation makes Equation 4′ strictly correct, but it introduces an additional term, -RT ln 55 (corresponding to the entropy of dilution), into Equation 4. This constant term cancels out when one is subtracting ΔF values but not when one discusses ratios of ΔF values.

An important rule of thumb can be extracted from these equations. Because ln 10 = 2.303, a 10-fold increase in affinity of binding corresponds to a free-energy change ΔF of only 1.42 kcal/mol at 37°C (310.15°K). (The corresponding values for 25 and 4°C are 1.36 and 1.27 kcal/mol, respectively.) This is less than one-third the energy of a single hydrogen bond (about 4.5 kcal/mol). Looked at another way, a very high affinity of 10^{10} M^{-1} corresponds to a ΔF of only 14.2 kcal/mol, approximately the bonding energy of three hydrogen bonds. (Of course, because hydrogen bonds with water are broken during the formation of hydrogen bonds between

antigen and antibody, the net energy per hydrogen bond is closer to 1 kcal/mol.) It is apparent from this example that of the many interactions (hydrophobic and ionic as well as hydrogen bonding) that occur between the contact residues in an antibody-combining site and the contacting residues of an antigen (such as a protein), almost as many are repulsive as attractive. It is this small difference of a few kilocalories between much larger numbers corresponding to the total of attractive and the total of repulsive interactions that leads to net "high-affinity" binding. If ΔF were any larger, binding reactions would be of such high affinity as to be essentially irreversible. Viewed in this way, it is not surprising that a small modification of the antigen can result in an enormous change in affinity. A single hydrogen bond can change the affinity manyfold, and similar arguments apply to hydrophobic interactions and other forms of bonding. This concept is important when we discuss specificity and antigen structure later.

Effects of Temperature, pH, Salt Concentration, and Conformational Flexibility

It was mentioned previously that K_A is constant for any given set of conditions such as temperature, pH, and salt concentration. However, it varies with each of these conditions. We have already seen that the conversion of free energy to affinity depends on temperature. However, the free energy itself is also a function of temperature

$$\Delta F^\circ = \Delta H^\circ - T\,\Delta S^\circ \qquad (5)$$

where ΔH = change in enthalpy (the heat of the reaction),† ΔS = entropy (a term related to the change in disorder produced by the reaction),[2] and T = absolute temperature (in degrees Kelvin).

It can be shown that the association constant KA will thus vary with temperature as follows:

$$\frac{d \ln K_A}{dT} = \frac{\Delta H^\circ}{RT^2} \qquad (6)$$

or equivalently,

$$\frac{d \ln K_A}{d(1/T)} = \frac{-\Delta H^\circ}{R} \qquad (6')$$

The derivation of these equations is beyond the scope of this book.[4] However, the practical implications are as follows. First, one can determine the standard ΔH° of the reaction from the slope of a plot of ln K_A versus 1/T. Second, for an interaction that is primarily exothermic (ie, driven by a large negative ΔH, such as the formation of hydrogen bonds and polar bonds), the affinity decreases with increasing temperature. Thus many antigen-antibody interactions have a higher affinity at 4°C than at 25°C or 37°C, so maximum binding for a given set of concentrations can be achieved in the cold. In contrast, apolar or hydrophobic interactions are driven largely by the entropy term T ΔS, and ΔH° is near zero. In this case, there is little effect of temperature on the affinity.

As for the effects of pH and salt concentration (or ionic strength) on the affinity, these vary depending on the nature of the interacting groups. Most antigen-antibody reactions are studied near neutral pH and at physiologic salt concentrations (0.15 M NaCl). If the interaction is dominated by ionic interactions, high salt concentration lowers the affinity.

Conformational flexibility of an antigen can also affect the affinity by affecting the entropy term in Equation 5. An outstanding example comes from the thermodynamics of binding of a series of broadly neutralizing monoclonal antibodies to the human immunodeficiency virus (HIV) envelope protein gp120. The energetics of binding between HIV gp120 and its cellular receptor cluster of differentiation (CD)4[5] or to a panel of monoclonal antibodies[6] has been studied in detail. The results provide insight into the ways protein flexibility can help the virus to evade antibody immunity. They may also explain part of the difficulty in eliciting antibodies of this type when immunizing with the native protein.

GP120 is a glycoprotein that exhibits at least two conformational states: one exists on the virus, and it changes to the other when it binds the CD4 receptor on the cell surface. Monoclonal antibodies F105 and b12 bind residues within the CD4 binding site on gp120 while monoclonal 2G12 binds the opposite surface. The CD4 binding site defines a neutralizing surface that is conserved among a broad range of HIV isolates.

As shown in Table 7.1, CD4 and each antibody demonstrated a strongly negative ΔF, corresponding to high affinity binding. However, as shown by microcalorimetry, they arrived at the affinity in different ways. The CD4 and F105 have a strong negative ΔH of binding, but they also have a large negative entropy due to the conformational change required, as shown by strongly positive values for -TΔS. This entropy effect greatly reduces the overall ΔF of binding, so they depend on a very large negative ΔH to bind. In contrast, monoclonal b12 has moderate levels of both ΔH and -TΔS, resulting in a nearly identical ΔF. Monoclonal 2G12 binds gp120 with a less favorable ΔH but no entropy cost.

The two discrete conformations of gp120 may be called the open and closed forms. The excursion between these two conformations affects the entropy of binding. CD4 and F105 only bind the open form, which reduces randomness and creates an entropy barrier. Monoclonal b12 is less dependent on one conformation, so it binds with less entropy cost, and 2G12 is indifferent to the two forms, so it has no entropy effect at all.

The strong entropy effect observed for these monoclonals may illustrate one way for the virus to evade antibody binding, through a mechanism called conformational masking.

The randomness of gp120, as found on the virus, means that it is rarely in the open conformation, making it difficult for most antibodies such as F105 to bind. Only the rare antibody that is b12-like can bind multiple forms and eventually pull gp120 into its most favorable conformation.

TABLE 7.1 Energetics of Antibody Binding to gp120 Core Structure

Ligand	ΔF	ΔH	-TΔS
CD4	-10.4	-48.4	38.2

F105	-11.4	-30.0	18.6
b12	-12.3	-18.0	5.7
2G12	-7.8	-6.2	-1.6

CD, cluster of differentiation. From Kwong, et al.6

The same effect may explain the difficulty in eliciting antibodies to this site using native gp120. If it is rarely in the open form, it will be unable to trigger B cells to make antibodies that require this form, and it will deliver only a weak antigenic stimulus to those that bind partially to different conformations, such as b12. These considerations suggest that a more favorable vaccine antigen could be made if gp120 could be anchored in the open conformation, so it could stimulate B cells to make antibodies that require this form. The hallmark of this structure would be its ability to bind F105 and b12 with a good ΔH and reduced values of -TΔS.

Kinetics of Antigen-Antibody Reactions

A fundamental connection between the thermodynamics and kinetics of antigen-antibody binding is expressed by the relationship

$$K_A = \frac{k_1}{k_{-1}} \qquad (7)$$

where k_1 and k_{-1} are the rate constants for the forward (association) and backward (dissociation) reactions.

The forward reaction is determined largely by diffusion rates (theoretical upper limit 10^9 L/mol/sec) and by the probability that a collision will result in binding, that is, largely the probability that both the antigen and the antibody will be oriented in the right way to produce a good fit as well as the activation energy for binding. The diffusive rate constant can be shown[7] to be approximated by the Smoluchowski equation

$$k_{dl} = 4\pi a D(6 \times 10^{20}) \qquad (7a)$$

where *a* = sum of the radii in centimeters of the two reactants, D = sum of the diffusion constants in cm^2/sec for the individual reactants, and the constant 6×10^{20} is necessary to convert the units to M^{-1}. • sec^{-1}. For example, if $a = 10^{-6}$ cm and $D = 10^{-7}$ cm^2/sec, then $k_{d1} \approx 7.5 \times 10^8$ M^{-1}. • sec^{-1}. Association rates will generally be slower for large protein antigens than for small haptens. This observation may be due to the smaller value of D, to the orientational effects in the collision, and to other nondiffusional aspects of protein-protein interactions. Therefore, association rates for protein antigens are more frequently on the

order of 10^5 to 10^6 M^{-1}. • sec^{-1} (see following discussion). However, this observation can also be partly understood from diffusion-limited rates alone. If the radii of hypothetically spherical reactants are r_1 and r_2, then in Equation 7a, $a = r_1 + r_2$, whereas D is proportional to $1/r_1 + 1/r_2$. The diffusive rate constant is therefore proportional to

$$(r_1 + r_2)\left(\frac{1}{r_1} + \frac{1}{r_2}\right) = \frac{(r_1 + r_2)^2}{r_1 r_2} \qquad (7b)$$

From this result, it can be seen that if $r_1 = r_2 = r$, then r cancels out and the whole term in Equation 7b is simply equal to 4. Thus, for the interaction between two molecules of equal size, the diffusive rate constant is the same regardless of whether those molecules are large or small.[8] However, if one molecule is large and the other small, the rate is greater than if both molecules are large. This difference occurs because reducing the radius r_1 while keeping r_2 constant (and larger than r_1) has a greater effect on increasing the diffusion constant term D, proportional to $(1/r_1 + 1/r_2)$, in which the smaller radius produces the larger term than it has on the term *a*, which is still dominated by the larger radius r_2. For example, if $r_2 = r$ as shown, but $r_1 = 0.1r$, then the numerator in Equation 7b is only reduced from $4r^2$ to $1.21r^2$, whereas the denominator is reduced from $1r^2$ to $0.1r^2$. Thus, the ratio is increased from 4 to 12.1. Viewed another way, the greater diffusive mobility of the small hapten outweighs its diminished target area relative to a large protein antigen because the larger target area of the antibody is available to both.

The dissociation rate (or "off rate") k_{-1} is determined by the strength of the bonds (as it affects the activation energy barriers for dissociation) and the thermal energy kT (where k is Boltzmann constant), which provides the energy to surmount this barrier. The activation energy for dissociation is the difference in energy between the starting state and the transition state of highest energy to which the system must be raised before dissociation can occur.

As pointed out by Eisen,[9] if one compares a series of related antigens, of similar size and other physical properties, for binding to an antibody, the association rates are all very similar. The differences in affinity largely correspond to the differences in dissociation rates.

A good example is that of antibodies to the protein antigen staphylococcal nuclease.[10] Antibodies to native nuclease were fractionated on affinity columns of peptide fragments to isolate a fraction specific for residues 99 through 126. The antibodies had an affinity of 8.3×10^8 M^{-1} for the native antigen and an association rate constant, k_{on}, of 4.1×10^5 M^{-1}. • sec^{-1}. This k_{on} was several orders of magnitude lower than had been observed for small haptens,[11] as discussed previously. A value of k_{off} of 4.9×10^{-4} sec^{-1} was calculated using these results in Equation 7. This is a first-order rate constant from which one can calculate a halftime for dissociation (based on $t_{1/2} = \ln 2/k_{off}$) of 23 minutes. These rates are probably typical for high-affinity ($K_A \approx 10^9$ M^{-1}) antibodies to small protein antigens such as nuclease (molecular weight [MW] ≈ 17,000). The dissociation rate is important to know in designing experiments to measure binding because if the act of measurement perturbs the equilibrium,

the time one has to make the measurement (eg, to separate bound and free) is determined by this halftime for dissociation. For instance, a 2-minute procedure that involves dilution of the antigen-antibody mixture can be completed before significant dissociation has occurred if the dissociation halftime is 23 minute. However, if the on rate is the same, but the affinity 10-fold lower, still a respectable $8 \times 10^7\ M^{-1}$, then the complex could be 50% dissociated in the time required to complete the procedure. This caution is relevant when we discuss methods of measuring binding and affinity in the following.

Because knowledge of the dissociation rate can be so important in the design of experiments, a word should be said about techniques to measure it. Perhaps the most widely applicable one is the use of radiolabeled antigen. After equilibrium is reached and the equilibrium concentration of bound radioactivity determined, a large excess of unlabeled antigen is added. Because any radioactive antigen molecule that dissociates is quickly replaced by an unlabeled one, the probability of a radioactive molecule associating again is very small. Therefore, one can measure the decrease in radioactivity bound to antibody with time to determine the dissociation rate.‡

AFFINITY

It is apparent from the previous discussion that a lot of information about an antigen-antibody reaction is packed into a single value, its affinity. In this section, we examine affinity more closely, including methods for measuring affinity and the heterogeneity thereof, the effects of multivalency of antibody and/or of antigen, and the special effects seen when the antigen-antibody interaction occurs on a solid surface (two-phase systems).

Interaction in Solution with Monovalent Ligand

The simplest case is that of the interaction of antibody with monovalent ligand. We may include in this category both antihapten antibodies reacting with truly monovalent haptens and antimacromolecule antibodies, which have been fractionated to obtain a population that reacts only with a single, nonrepeating site on the antigen.** In the latter case, the antigen behaves as if monovalent in its interaction with the particular antibody population under study. The proviso that the site recognized (antigenic determinant) be nonrepeating, that is, occur only once per antigen molecule, of course, is critical.

If the combining sites on the antibody are independent (ie, display no positive or negative cooperativity for antigen binding), then for many purposes one can treat these combining sites, reacting with monovalent ligands, as if they were separate molecules. Thus, many, but not all, of the properties we discuss can be analyzed in terms of the concentration of antibody-combining sites, independent of the number of such sites per antibody molecule (2 for IgG and IgA, 10 for IgM).

To determine the affinity of an antibody, one generally determines the equilibrium concentrations of bound and free ligand, at increasing total ligand concentrations, but at constant antibody concentration. Alternatively, one can vary the antibody concentration, but then, the analysis is slightly more complicated. Perhaps the theoretically most elegant experimental method to determine these quantities is equilibrium dialysis,[12,13] depicted and explained in Figure 7.2, in which ligand (antigen) is allowed to equilibrate between two

chambers, only one of which contains antibody, separated by a semipermeable membrane impermeable to antibody. The important feature of this method, as opposed to most others, is that the concentrations of ligand in each chamber can be determined without perturbing the equilibrium. The disadvantage of this method is that it is applicable only to antigens small enough to permeate freely a membrane that will exclude antibody. Another technical disadvantage is that bound antigen, determined as the difference between bound plus free antigen in one chamber and free antigen in the other, is not measured independently of free antigen.

Another category of method uses radiolabeled ligand in equilibrium with antibody and then physically separates free antigen bound to antibody and quantitates each separately. The methods used to separate bound and free antigen are discussed in the section on radioimmunoassay (RIA). These methods generally allow independent measurement of bound and free antigen but may perturb the equilibrium.

Scatchard Analysis

Once data are obtained, there are a number of methods of computing the affinity, of which we shall discuss two.

Perhaps the most widely used is that described by Scatchard[14] (Fig. 7.3[15]). The mass action equilibrium law is plotted in the form of Equation 3

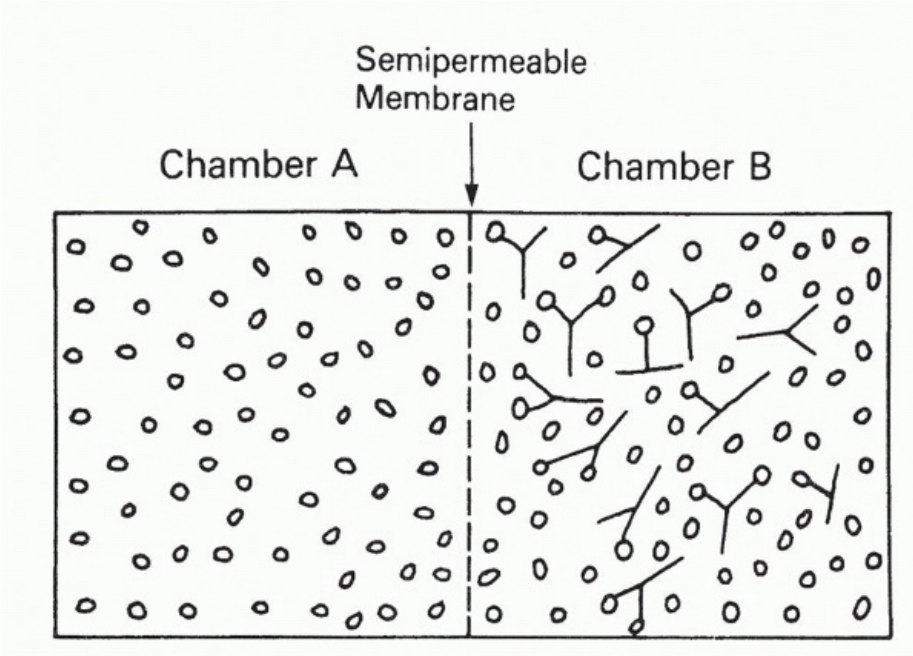

FIG. 7.2. Equilibrium Dialysis. Two chambers are separated by a semipermeable membrane that is freely permeable to ligand but not at all to antibody. Antibody is placed in one chamber (B) and ligand in one or both chambers. Regardless of how the ligand is distributed initially, after sufficient time to reach equilibrium, it will be distributed as follows. The concentration of free ligand will be identical in both chambers, but chamber B will have additional ligand bound to antibody. The concentration of bound ligand is thus the difference between the ligand concentration in the two chambers, whereas the

free concentration is the concentration in chamber A. Because these concentrations must obey the mass action law, Equation 2, they can be used to determine the affinity KA, from Equation 3 or 3′, by any of several graphical procedures, such as Scatchard analysis (described in the text).

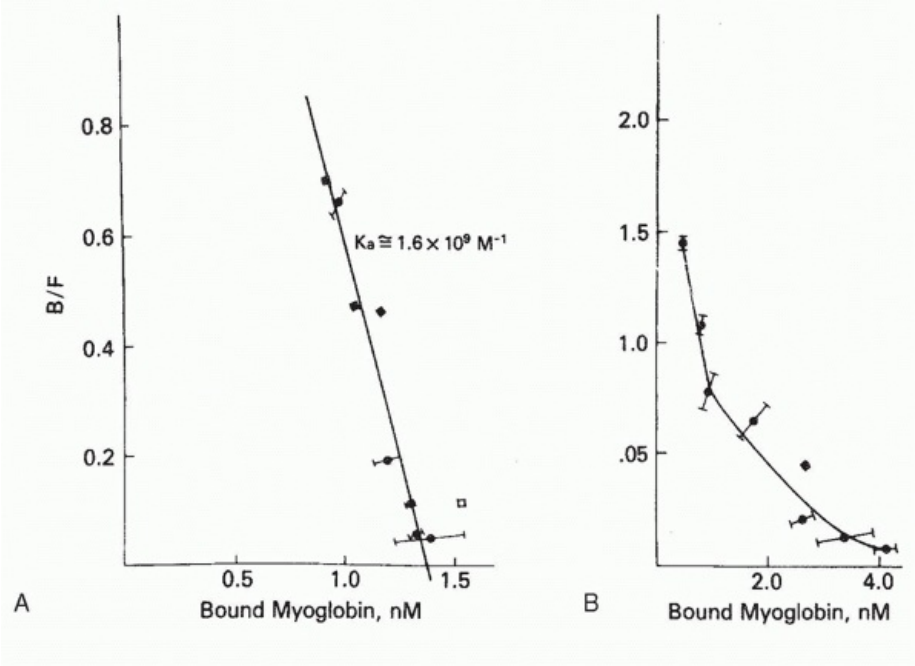

FIG. 7.3. Scatchard analysis of the binding of [3H]-sperm whale myoglobin by a monoclonal antibody to myoglobin **(A)** and by the serum antibodies from the same mouse whose spleen cells were fused to prepare the hybridoma **(B)**. The monoclonal antibody (clone HAL 43-201E11, clone 5) produces a linear Scatchard plot, whose slope, -1.6 × 10^9 M^{-1}, equals -K_A, and whose intercept on the abscissa gives the concentration of antibodybinding sites. In contrast, the serum antibodies produce a curved (concave up) Scatchard plot, indicative of heterogeneity of affinity. From Berzofsky et al.,[15] with permission.

$$[SL] = K_A([S]_t - [SL])[L] \qquad (3)$$

and B is substituted for [SL] and F for [L], referring to bound and free ligand, respectively. Then the Scatchard equation is

$$\frac{B}{F} = K_A([S]_t - B) \qquad (8)$$

Note that a very critical implicit assumption was made in this seemingly very simple conversion. The [SL] within the parentheses in Equation 3 was intended to be the concentration of bound antibody sites so that ($[S]_t$ - [SL]) = free [S]. However, in Equation 8,

we have substituted B, the concentration of bound ligand. If the ligand behaves as monovalent, then this substitution is legitimate, as every bound ligand molecule corresponds to an occupied antibody site. However, if the ligand is multivalent and can bind more than one antibody site, then Equation 8 is valid only in ligand excess where the frequency of ligands with more than one antibody bound is very low. In this section, we are discussing only monovalent ligands, but this proviso must be kept in mind when the Scatchard analysis is applied in other circumstances.

From Equation 8, we see that a plot of B/F versus B should yield a straight line (for a single affinity), with a slope of $-K_A$ and an intercept on the abscissa corresponding to antibody-binding site concentration (see Fig. 7.3). This is the so-called Scatchard plot. An alternative version that is normalized for antibody concentration is especially useful if the data were obtained at different values of total antibody concentration, $[A]_t$, instead of constant $[A]_t$. However, for this version, one requires an independent measure of total antibody concentration, other than the intercept of the plot. Then one divides Equation 8 by the total concentration of antibody molecules (making no assumptions about the number of sites per molecule) to obtain

$$\frac{R}{c} = K_A(n - r) \qquad (9)$$

where r = the number of occupied sites per antibody molecule, n = the total number of sites per antibody molecule, and c = free ligand concentration, that is, c = F. Thus,

$$r = \frac{B}{[\text{total antibody}]} = \frac{B}{[A]_t}$$

$$n = \frac{[\text{total sites}]}{[\text{total antibody}]} = \frac{[S]_t}{[A]_t}$$

where $[A]_t$ = total molar antibody concentration. In this form of the Scatchard plot, r/c versus r, the slope is still $-K_A$ and the intercept on the r axis is n. Thus one can determine the number of sites per molecule. Of course, if one determines $[S]_t$ from the intercept of Equation 8, one can also calculate the number of sites per molecule by dividing $[S]_t$ by any independent measure of antibody concentration. Thus, the only advantage of normalizing all the data points first to plot the r/c form arises when the data were obtained at varying antibody concentrations. If the antibody concentration is unknown but held constant, then the B/F form is more convenient and actually provides one measure of antibody (site) concentration. Because today we know the value of n for each class of antibody (2 for IgG and serum IgA, 10 for IgM), the concentration of sites and that of antibody are easily converted in many cases.

Heterogeneity of Affinity

The next level of complexity arises when one is dealing with a mixture of antibodies of

varying affinity for the ligand. This is the rule, rather than the exception, when one deals with antibodies from immune serum, even if they are fractionated to be monospecific, that is, all specific for the same site

on the antigen. Contrast, for example, the linear Scatchard plot for a homogeneous monoclonal antibody to myoglobin (see Fig. 7.3A), with the curved Scatchard plot for the serum antibodies from the same mouse used to prepare the hybridoma monoclonal antibody (see Fig. 7.3B). This concave up Scatchard plot is typical for heterogeneous antibodies. In a system such as hormone receptor-hormone interaction, in which negative cooperativity can occur between receptor sites (ie, occupation of one site lowers the affinity of its neighbor), a concave up Scatchard plot can be produced by negative cooperativity in the absence of any intrinsic heterogeneity in affinity. However, in the case of antibodies, where no such allosteric effect has been demonstrated, a concave up Scatchard plot indicates heterogeneity of affinity.

Ideally, one would like to imagine that the tangents all along the curve correspond (in slope) to the affinities of the many subpopulations of antibodies. Mathematically, this is not strictly correct, but it is true that the steeper part of the curve corresponds to the higher affinity antibodies and the shallower part of the curve to the lower affinity antibodies. Graphical methods have been developed to analyze more quantitatively the components of such curves,[16,17] and a very general and versatile computer program (LIGAND) has been developed by Munson and Rodbard[18] that can fit such curves using any number of subpopulations of different affinity. For purposes of this chapter, we discuss only the case of two affinities and then examine the types of average affinities that have been proposed when one is dealing with much greater heterogeneity. We also examine mathematical estimates of the degree of heterogeneity (analogous to a variance).

When an antibody population consists of only two subpopulations of different affinities, K_1 and K_2, then we can add the component Equations 3′ to obtain

$$r = r_1 + r_2 = \frac{n_1 K_1 c}{(1 + K_1 c)} + \frac{n_2 K_2 c}{(1 + K_2 c)} \qquad (10)$$

so that

$$\frac{r}{c} = \frac{n_1 K_1}{(1 + K_1 c)} + \frac{n_2 K_2}{(1 + K_2 c)} \qquad (10')$$

where the subscripts correspond to the two populations. Then the graph of r/c versus r can be shown to be a hyperbola whose asymptotes are, in fact, the linear Scatchard plots of the two components (Fig. 7.4). This situation has been analyzed graphically by Bright.19 Taking the limits as $c \rightarrow 0$ and as $c \rightarrow \infty$, it can easily be shown that the intercept on the abscissa is just $n_1 + n_2$ (or, in the form B/F versus B, the intercept is the total concentration of binding sites $[S]_t$), and the intercept on the ordinate is $n_1K_1 + n_2K_2$. Thus, one can still obtain the total value of n or $[S]_t$ from the intercept on the abscissa. The problem is in obtaining the two affinities, K_1 and K_2, and the concentrations of the individual antibody subpopulations (corresonding to n_1 and n_2). If K_1 is greater than K_2, one can approximate the affinities from

the slopes of the tangents at the two intercepts (see Fig. 7.4), but these will not, in general, be exactly parallel to the two asymptotes, which give the true affinities, so some error is always introduced, depending on the relative values of n_1 and n_2 and K_1 and K_2. A graphical method for solving for these exactly has been worked out by Bright[19] and computer methods by Munson and Rodbard.[18]

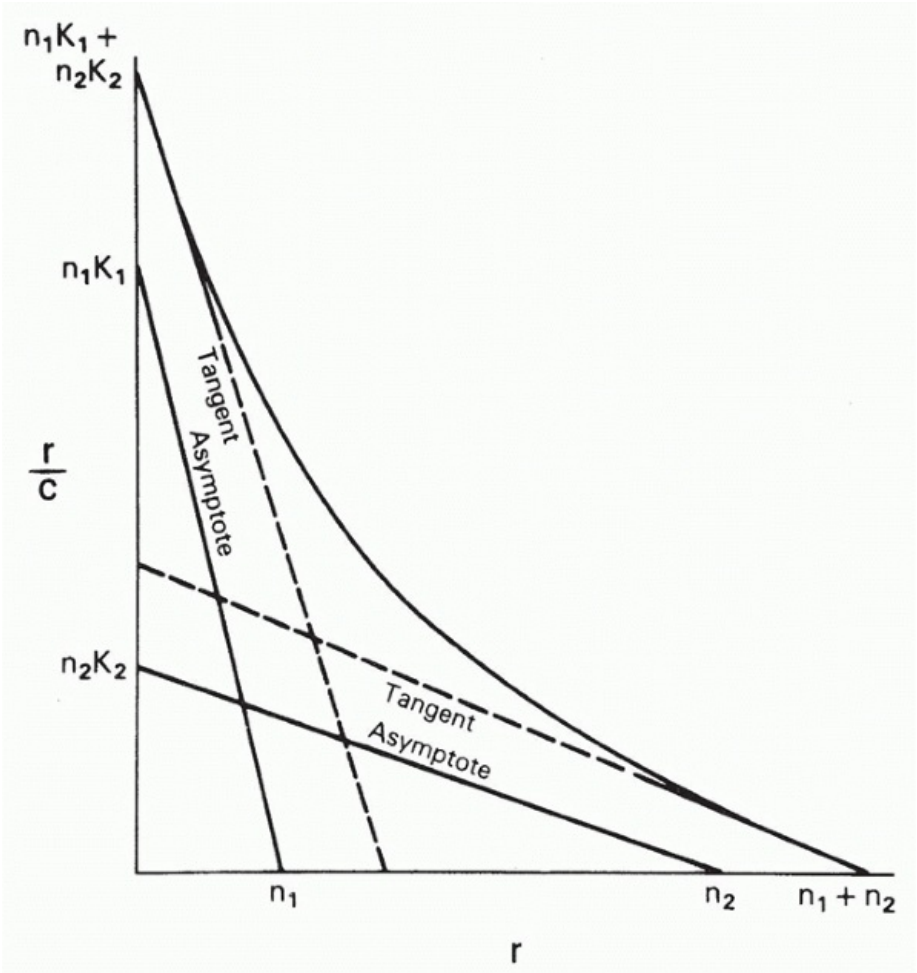

FIG. 7.4. Analysis of a Curved Scatchard Plot Produced by a Mixture of Two Antibodies with Different Affinities. The antibodies have affinities K_1 and K_2 and have n_1 and n_2 binding sites per molecule, respectively. The r is the concentration of bound antigen divided by the total antibody concentration (ie, bound sites per molecule), and c is the free antigen concentration. The curve is a hyperbola that can be decomposed into its two asymptotes, which correspond to the linear Scatchard plots of the two components in the antibody mixture. The tangents to the curve at its intercepts only approximate these asymptotes so that the slopes of the tangents estimate but do not accurately correspond to the affinities of the two antibodies. However, the intercept on the r axis corresponds to $n_1 + n_2$. Note that in this case n_1 and n_2 must be defined in terms of the total antibody concentration, not that of each component.

Average Affinities

In practice, of course, one rarely knows that one is dealing with exactly two subpopulations, and most antisera are significantly more heterogeneous than that. Therefore, the previously mentioned case is more illustrative of principles than of practical value. When faced with a curved Scatchard plot, one usually asks what the average affinity is, and perhaps some measure of the variance of the affinities, without being able to define exactly how many different affinity populations exist.

Suppose one has m populations each with site concentration $[S_i]$ and affinity K_i, so that at free ligand concentration [L], the fraction of each antibody that has ligand bound will be given by an equation of the form of Equation 3′:

$$B_i = \frac{K_i[S_i]_t[L]}{(1 + K_i[L])} \qquad (11)$$

Then the bound concentrations sum to give

$$B = \sum_{i=1}^{m} B_i = \sum_{i=1}^{m} \frac{K_i[S_i]_t[L]}{(1 + K_i[L])} \qquad (11')$$

Substituting F for [L] and dividing through by this quantity, one obtains

$$\frac{B}{F} = \sum_{i=1}^{m} \frac{K_i[S_i]_t}{(1 + K_iF)} \qquad (12)$$

or equivalently,

$$\frac{r}{c} = \sum_{i=1}^{m} \frac{K_in_i}{(1 + K_ic)} \qquad (12')$$

These can be seen to be generalizations of Equations 10 and 10′. Taking the limits as $F \rightarrow 0$ and $F \rightarrow \infty$, one again sees that the

$$\text{intercept on ordinate} = \sum_{i=1}^{m} K_i[S_i]_t \qquad (13)$$

and the

$$\text{intercept on abscissa} = \sum_{i=1}^{m} [S_i]_t = [S]_t \qquad (14)$$

Therefore, one can still obtain the total antibody site concentration from the intercept on the abscissa (Fig. 7.5).[20]

Two types of average affinity can be obtained graphically from the Scatchard plot.[20] Perhaps the more widely used K_0 is actually more accurately a median affinity rather than a mean affinity. It is defined as the slope of the tangent at the point on the curve where half the sites

are bound, that is, where B = $[S_t]/2$ (see Fig. 7.5). A second type of average affinity, which we call Kav, is a weighted mean of the affinities, each affinity weighted by its proportional representation in the antibody population. Thus, we take the ratio

$$K_{av} = \sum_{i=1}^{m} \frac{K_i[S_i]_t}{[S]_t} \qquad (15)$$

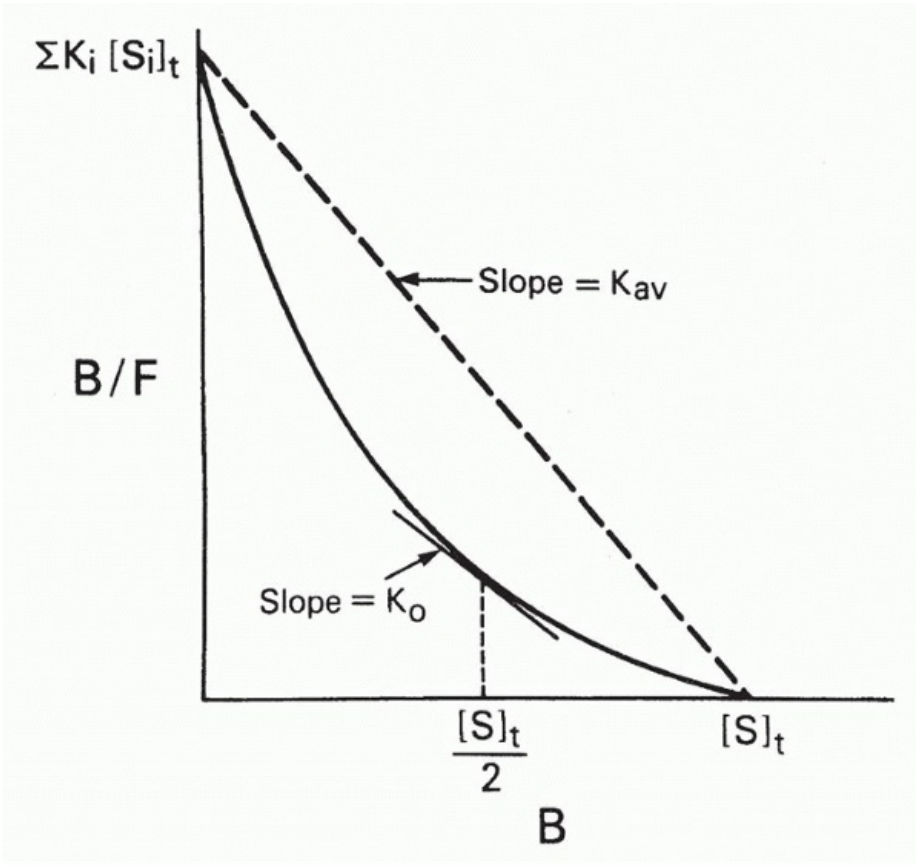

FIG. 7.5. Types of Average Affinities for a Heterogeneous Population of Antibodies, as Defined on a Scatchard Plot. K_0 is the slope of the tangent to the curve at a point where B = $[S]_t/2$, that is, where half the antibody sites are bound. Thus, K_0 corresponds to a median affinity. The K_{av} is the slope of the chord between the intercepts and corresponds to a weighted average of the affinities weighted by the concentrations of the antibodies with each affinity. Adapted from Berzofsky.[20]

From Equations 13 and 14, it is apparent that K_{av} is simply the ratio of the two intercepts on the B/F and B axes, that is, the slope of the chord (see Fig. 7.5). This type of weighted mean affinity, K_{av}, is therefore actually easier to obtain graphically in some cases than K_0, and we shall see that it is useful in other types of plots as well.

Indices of Heterogeneity: The Sips Plot

For a heterogeneous antiserum, one would also like to have some idea of the extent of heterogeneity of affinity. For instance, if the affinities were distributed according to a normal (Gaussian) distribution, one would like to know the variance.[21,22] More complex analyses have been developed that do not require as many assumptions about the shape of the distribution,[23,24,25] but the first and most widely used index of heterogeneity arbitrarily assumes that the affinities fit a distribution, first described by Sips,[26] which is similar in shape to a normal distribution. This was applied to the case of antibody heterogeneity by Nisonoff and Pressman,[27] and is summarized by Karush and Karush.[28] One fits the data to the assumed binding function

$$r = \frac{n(K_0 c)^a}{(1 + (K_0 c)^a)} \qquad (16)$$

which is analogous to Equations 3′ and 11 (the Langmuir adsorption isotherm) except for the exponent *a*, which is the index of heterogeneity. This index, *a*, is allowed to range from 0 to 1. For *a* = 1, Equation 16 is equivalent to Equation 3, and there is no heterogeneity. As *a* decreases toward 0, the heterogeneity increases. To obtain a value for a graphically, one plots the algebraic rearrangement of Equation 16:

$$\log\left(\frac{r}{n-r}\right) = a \log c + a \log K_0 \qquad (17)$$

so that the slope of log [r/(n - r)] versus log c is the heterogeneity index *a*.

C. DeLisi (personal communication) has derived the variance (second moment) of the Sips distribution in terms of the free energy RT ln K_0, about the mean of free energy. The result (normalized to RT) gives the dispersion or width of the distribution as a function of *a*:

$$\frac{\sigma^2_{Sips}}{R^2T^2} = \frac{\pi^2(1-a^2)}{3a^2} \qquad (18)$$

This is useful for determining a quantity, δ_{Sips}, which can be thought of as analogous to a standard deviation, if one keeps in mind that this is not a true Gaussian distribution. In addition, as noted previously, the use of the Sips distribution requires the assumption that the affinities (really the free energies) are continuously distributed symmetrically about a mean, approximating a Gaussian distribution. This assumption frequently is not valid.

The Plot of B/F versus F or T

Another graphical method that is useful for estimating affinities is the plot of bound/free versus free or total ligand concentration, denoted F and T, respectively[20] (Fig. 7.6). To simplify the discussion, let us define the bound/free ratio, B/F, as R, and define R_0 as the intercept, or limit, as free

ligand F → 0. First, for the case of a homogeneous antibody, from Equation 3′,

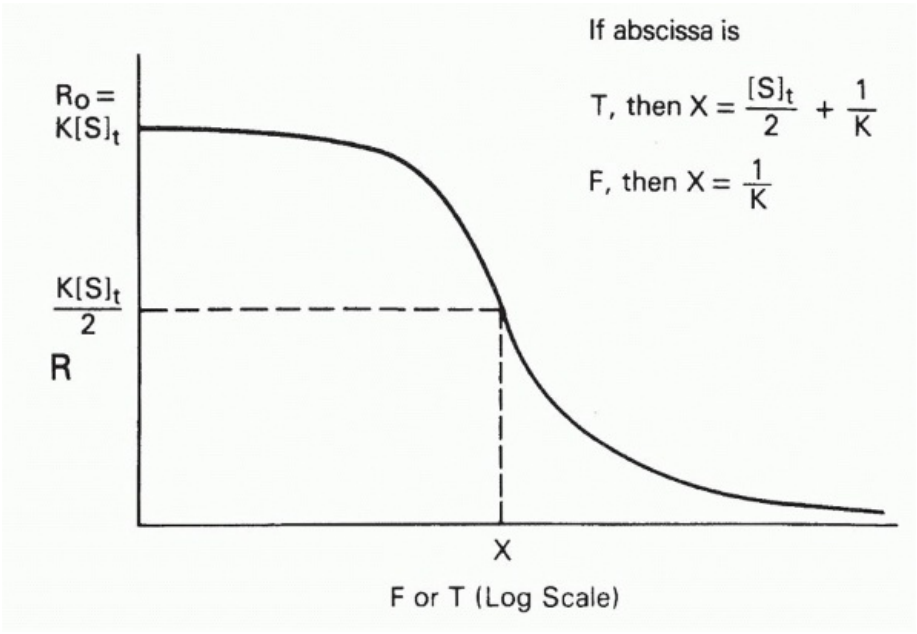

FIG. 7.6. Schematic Plot of R, the Bound/Free Ratio, as a Function of Free (F) or Total (T) Antigen Concentration. The curves have a similar sigmoidal shape, but the midpoint (where R = $R_0/2$) of the plot of R versus T has a term dependent on antibody site concentration ([S]t), whereas the midpoint of the plot of R versus F is exactly 1/K, independent of antibody concentration. Adapted from Berzofsky.[20]

$$R = \frac{B}{F} = \frac{K[S]_t}{(1 + KF)} \qquad (19)$$

and

$$R_0 = \lim_{F \to 0} \frac{B}{F} = K[S]_t \qquad (20)$$

Let us define the midpoint of the plot (see Fig. 7.6) as the point at which R decreases to half its initial value, R_0, that is, at which R = $K[S]_t/2$. For the case of homogeneous antibody (ie, a single affinity), simple algebraic manipulation,[20] substituting $K[S]_t/2$ (ie, $R_0/2$) for B/F in Equation 8, will show that at this midpoint††

$$F = \frac{1}{K} \qquad (21)$$

and

$$B = \frac{[S]_t}{2} \qquad (22)$$

so that the total concentration, T, is

$$T = B + F = \frac{[S]_t}{2} + \frac{1}{K} \qquad (23)$$

Thus, if one plots B/F versus F, the midpoint directly yields 1/K. However, it is frequently more convenient experimentally to plot B/F versus T. In this case, the midpoint is no longer simply the reciprocal of the affinity. As seen from Equation 23, the assumption that the midpoint is 1/K will result in an error equal to half the antibody-binding site concentration. Thus, in plots of B/F versus T, the midpoint will be a good estimate of the affinity only if $[S]_t/2 << 1/K$, that is, if the antibody concentration is low compared to the dissociation constant. In fact, if the affinity is so high that $1/K << [S]_t/2$, then one will merely be measuring the antibody concentration, not the affinity at all[20] (see Fig. 7.6).

In the case of a heterogeneous antiserum, we have already seen that

$$R_0 = \sum_i K_i[S_i]_t \qquad (13)$$

Therefore, at the midpoint, when $B/F = R_0/2$, it is easy to see that

$$K_{av} = \left(\frac{B}{F}\right)\left(\frac{2}{[S]_t}\right) = \frac{R_0}{[S]_t} \qquad (24)$$

Thus, one can still obtain the average affinity, as defined previously.[20]

Regardless of average affinities, the effect of affinity heterogeneity is to broaden the curve or to make the slope shallower. This can be seen by visualizing the curve of B/F versus F as a step function. Each antibody subpopulation of a given affinity, K_i, will be titrated to 50% of its microscopic B/F at a free ligand concentration $F = 1/K_i$. The high-affinity antibodies will be titrated at low F, but the low-affinity antibodies will require much higher F to be titrated. The resulting step function is analogous to the successive transitions corresponding to different pK values in a pH titration.

Intrinsic Affinity

The affinity, K_A, that we have been discussing so far is what has been termed the intrinsic affinity, that is, the affinity of each antibody-combining site treated in isolation. We have been able to do this, regardless of the valence of the antibodies, by using the concentration of combining sites, [S], in our equations rather than the concentration of antibody molecules, [A], which may have more than one site. Even without any cooperativity between combining sites, there is a statistical effect that makes the actual affinity different from the intrinsic

affinity if the antibody is multivalent and one uses whole antibody concentration rather than site concentration. The way this difference arises can best be seen by examining the case of a bivalent antibody, such as IgG. We assume that the two sites are equivalent and neither is affected by events at the other. The ligand, as in this whole section, is monovalent. Then there are two binding steps

$$A + L \overset{K_1}{\rightleftharpoons} AL, \qquad AL + L \overset{K_2}{\rightleftharpoons} AL_2 \tag{25}$$

and the corresponding actual affinities are

$$K_1 = \frac{[AL]}{[A][L]}, \qquad K_2 = \frac{[AL_2]}{[AL][L]} \tag{26}$$

If the intrinsic affinity of both equivalent sites is K, then K1 will actually be twice K because the concentration of available sites [S] will be twice the antibody concentration when the first ligand is about to bind in step 1. However,

once one site is bound, the reverse (dissociation) reaction of step 1 can occur from only one site, namely, that which is occupied. Conversely, for the second step, the forward reaction has only one remaining available site; however, in the reverse reaction, $AL_2 \rightarrow AL + L$, either site can dissociate to go back to the AL state. The second site bound need not be the first to dissociate, and because the sites are identical, one cannot tell the difference. Thus, for step 2, the apparent concentration of sites for the reverse reaction is twice that available for the forward reaction, so the affinity K_2 for the second step will be only half the intrinsic affinity, K.

It is easy to see how this statistical effect can be extrapolated to an antibody with n sites[29]:

$$K_1 = nK \quad \text{and} \quad K_n = \left(\frac{1}{n}\right) K \tag{27}$$

For the steps in between, two derivations are available,9,29 which yield

$$K_i = \frac{(n - i + 1)}{i} K \tag{28}$$

The actual affinity, rather than the intrinsic affinity, becomes important with monovalent ligands when one is interested in the effective affinity (based on a molar antibody concentration) under conditions where [L] is so low that only one site can bind antigen. Then for IgG or IgM (with 2 or 10 sites per molecule, respectively), the apparent affinity will be theoretically 2 or 10 times the intrinsic affinity. For most purposes, it is easier to use site concentrations and intrinsic affinities. The analyses given previously, such as B/F versus F or the Scatchard plot, whether B/F versus B or r/c versus r, will all yield intrinsic affinities. It is the intrinsic affinity that tells us something about the nature of the antibody-ligand interaction.

Once one enters the realm of multivalent ligands, the actual affinity or effective affinity involving multipoint binding between multivalent antibody molecule and multivalent ligand molecule can be much greater than the intrinsic affinity for binding at each site. This case is

the subject of the next section.

Interaction with Multivalent Ligands

So far, we have discussed only situations in which the ligand is monovalent or effectively monovalent with respect to the particular antibody under study. However, in many situations, the ligand molecule has multiple repeating identical determinants, each of which can bind independently to the several identical combining sites on a divalent or multivalent antibody.‡‡ Although the intrinsic affinity for the interaction of any single antibody-combining site with any single antigenic determinant may be the same as that discussed in the preceding section, the apparent or effective affinity may be much higher due to the ability of a single antibody molecule to bind more than one identical determinant of a multivalent antigen molecule. Karush[30] has termed this phenomenon "monogamous bivalency." Such monogamous binding can occur between two molecules in solution, or between a molecule in solution and one on a solid surface, such as a cell membrane or microtiter plate. We first discuss the situation in solution and then discuss the additional considerations that apply when one of the reactants is bound to a solid surface.

Monogamous Bivalency

Suppose a divalent antibody molecule reacts with antigen that has two identical determinants. This situation has been treated in detail by Crothers and Metzger,[31] and by Karush.[30] Let us call the two antibody sites S and S′, and the antigenic determinants D and D′, with the understanding that, in actuality, we cannot distinguish S from S′ or D from D′. The interaction can be broken up into two steps, a bimolecular reaction

$$\begin{array}{cccc} S & D & \overset{K_1}{\rightleftharpoons} & S-D \\ | & + & | & | \\ S' & D' & & S' \quad D' \end{array} \qquad (29)$$

followed by an intramolecular reaction

$$\begin{array}{cccc} S-D & & \overset{K_2}{\rightleftharpoons} & S-D \\ | & | & | & | \\ S' & D' & & S'-D' \end{array} \qquad (30)$$

The association constant for the first step, K_1, is related to the intrinsic affinity, K, simply by a statistical factor of 4 due to the degeneracy (equivalence) between S and S′ and between D and D′. This is a typical second-order reaction between antigen and antibody. However, the second step (Equation 30) is a first-order reaction because it is effectively an interconversion between two states of a single molecular complex, the reactants S′ and D′ being linked chemically (albeit noncovalently) through the S-D bond formed in the first step. Thus, the first-order equilibrium constant, K_2, is not a function of the concentrations of S-S and D-D in solution, as K_1 would be. Rather, the forward reaction depends on the geometry of the complex and the flexibility of the arms; in other words, the probability that S′ and D′ will encounter each other and be in the right orientation to react if they do come in contact

depends on the distances and freedom of motion along the chain S′-S-D-D′ rather than on the density of molecules in solution (ie, concentration).

The reverse reaction for step 2, on the other hand, will have a rate constant similar to that for the simple monovalent S-D → S + D reaction, as the dissociation reaction depends on the strength of the S′-D′ (or S-D) bond and is not influenced by the other S-D interaction unless there is strain introduced by the angles required for simultaneous bonds between S and D and S′ and D′. Note that K_2 will inherently have a statistical factor of 1/2 compared to the intrinsic K'_2 for the analogous reaction if the S′-S-D-D′ link were all covalent because in the forward reaction of Equation 30 only one pair can react, whereas in the reverse reaction either S′-D′ or S-D could dissociate to produce the equivalent result.

We would like to know the apparent or observed affinity for the overall reaction

$$\begin{array}{ccc} S & D & S-D \\ | & | & | \\ + & \overset{K_{obs}}{\rightleftharpoons} & \\ | & | & | \\ S & D & S-D \end{array} \qquad (31)$$

Because the free energies, ΔF_1 and ΔF_2, for the two steps are additive, the observed affinity will be the product of K_1 and K_2

$$K_{obs} = K_1 K_2 \qquad (32)$$

where we have defined K_1 and K_2 to include the statistical degeneracy factors.*** The equilibrium constants K_1 and K_2 are each the ratios of forward and reverse rate constants, as in Equation 7. Of these four rate constants, all are directly related to the corresponding terms for the intrinsic affinity between S and D except for the intramolecular forward reaction of step 2, as noted previously. Thus, the difficulty in predicting K_{obs} is largely a problem of analyzing the geometric (steric) aspects of K_2, assuming one already knows the intrinsic affinity, Crothers and Metzger[31] have analyzed this problem for particular situations. Qualitatively, we can say that whether K_2 will be larger or smaller than K will depend on factors such as the enforced proximity of S′ and D′ in step 2 and the distance between D and D′ compared to the possible distances accessible between S and S′, which in turn depends on the length of the antibody arms and the flexibility of the hinge between them. Thus, because K_1 can be approximated by K, except for statistical factors, the apparent affinity for this “monogamous bivalent” binding interaction, K_{obs}, may range from significantly less than to significantly greater than K^2. If K_2 is of the same order of magnitude as K, then K_{obs} will be of the order of K^2, which can be huge (eg, if $K \approx 10^9\ M^{-1}$, Kobs could be $\approx 10^{18}\ M^{-1}$). The halftime for dissociation would be thousands of years. It is easy to see how such monogamous bivalent interactions can appear to be irreversible, even though in practice the observed affinity is rarely more than a few orders of magnitude larger than the K for a single site, possibly due to structural constraints.[32]

If apparent affinities this high can be reached by monogamous bivalency, even greater ones

should be possible for the multipoint binding of an IgM molecule to a multivalent ligand. Although IgM is decavalent for small monovalent ligands, steric restrictions often make it behave as if pentavalent for binding to large multivalent ligands. However, even five-point binding can lead to enormously tight interactions. Therefore, even though the intrinsic affinity of IgM molecules tends to be lower than that of IgG molecules for the same antigen,[30] the apparent affinity of IgM can be quite high.

Two-Phase Systems

The same enhanced affinity seen for multipoint binding applies to two-phase systems. Examples include the reaction of multivalent antibodies with antigen attached to a cell surface or an artificial surface (such as Sepharose or the plastic walls of a microtiter plate), the reaction of a multivalent ligand with antibodies on the surface of a B cell, a Sepharose bead, or a plastic plate, and the reaction of either component with an antigen-antibody precipitate. For the reasons outlined previously, "monogamous" binding can make the apparent affinity of a multivalent antibody or antigen for multiple sites on a solid surface be quite large to the point of effective irreversibility.

However, another effect also increases the effective affinity in a two-phase system. This effect applies even for monovalent antibodies (Fab fragments) or monovalent ligands. The effect arises from the enormously high effective local concentration of binding sites at the surface, compared to the concentration if the same number of sites were distributed in bulk solution.[33] Looked at another way, the effect is due to the violation, at the liquid-solid interface, of the basic assumption in the association constants, K_A, discussed previously, that the reactants are all distributed randomly in the solution. (To some extent, the latter is involved in the enhanced affinity of multivalency as well.) This situation has been analyzed by DeLisi34 and DeLisi and Wiegel,[35] who break the reaction down into two steps: the diffusive process necessary to bring the antigen and antibody into the right proximity and orientation to react, and the reactive process itself. The complex between antigen and antibody, when positioned but not yet reacted, is called the encounter complex. The reaction can then be written

$$S + D \underset{k_-}{\overset{k_+}{\rightleftharpoons}} S \cdots D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} SD \qquad (33)$$

where S = antibody site, D = antigenic determinant, k+ and k_ = forward and reverse diffusive rate constants, and k_1 and k_{-1} = forward and reverse reactive rate constants once the encounter complex is formed. If the encounter complex is in a steady state, the overall rate constants will be given by

$$k_f = \frac{k_1 k_+}{(k_1 + k_-)} \qquad (34)$$

$$k_r = \frac{k_{-1} k_-}{(k_1 + k_-)} \qquad (35)$$

where subscripts f and r = forward and reverse.[34] The association constant, according to Equation 7, is the ratio of these two, or

$$K_A = \frac{k_1 k_+}{k_{-1} k_-} \qquad (36)$$

The relative magnitudes of k_1 and k_- determine the probable fate of the encounter complex. Is it more likely to react to form SD or to break up as the reactants diffuse apart?

Now suppose that k is slow compared to k_1. Then the SD bound complex and the encounter complex, S … D, may interconvert many times before the encounter complex breaks up and one of the reactants diffuses off into bulk solution. If the surface has multiple antigenic sites, D, then even a monovalent antibody (Fab) may be much

more likely, when SD dissociates to S … D, to rereact with the same or nearby sites than to diffuse away into bulk solution, again depending on the relative magnitudes of these rate constants. This greater probability to rereact with the surface rather than diffuse away is the essence of the effect we are describing. A more extensive mathematical treatment of reactions with cells is given in DeLisi34 and DeLisi and Wiegel.[35]

A somewhat different, and very useful, analysis of the same or a very similar effect was given by Silhavy et al.[36] These authors studied the case of a ligand diffusing out of a dialysis bag containing a protein for which the ligand had a significant affinity. Once the ligand concentration became low enough that there was an excess of free protein sites, then the rate of exit of ligand from the dialysis bag was no longer simply its diffusion rate nor was it simply the rate of dissociation of protein-ligand complex. These authors showed that under these conditions the exit of ligand followed quasi-first-order kinetics, but with a half-life longer than the half-life in the absence of protein by a factor of $(1 + [P]K_A)$

$$t_+ = t_-(1 + [P]K_A) \qquad (37)$$

where [P] = protein site concentration, K_A = affinity, and t_+ and t_- = half-lives in the presence and absence of protein in the bag.

In this case, the protein was in solution, so the authors could use the actual protein concentration and the actual intrinsic affinity, K_A. In the case of protein on a two-dimensional surface, it is harder to know what to use as the effective concentration. However, the high local concentration of protein compartmentalized in the dialysis bag can be seen to be analogous to the high local concentration attached to the solid surface. The underlying mechanism of the two effects is essentially the same and so are the implications. For instance, in the case of dialysis, a modest 10 μM concentration of antibody sites with an affinity of 10^8 M^{-1} can reduce the rate of exit of a ligand 1000-fold. A dialysis that would otherwise take 3 hours would take 4 months. It is easy to see how this “retention effect” can make even modest affinities appear infinite (ie, the reactions appear irreversible). This retention effect applies not only to immunologic systems but also to other interactions at a cell surface or between cell compartments where the local concentration of a protein may be

high. In particular, these principles of two-phase systems should also govern the interaction between antigen specific receptors on the surface of T cells and antigen-major histocompatibility complex (MHC) molecule complexes on the surface of antigen-presenting cells, B cells, or target cells.

One final point is useful to note. Because these retention effects depend on a localized abundance of unoccupied sites, addition of a large excess of unlabeled ligand to saturate these sites will diminish or abolish the retention effect and greatly accelerate the dissociation or exit of labeled ligand. This effect of unlabeled ligand can be used as a test for the retention effect, although one must be aware that in certain cases the same result can be an indication of negative cooperativity among receptor sites.

RADIOIMMUNOASSAY AND RELATED METHODS

Since it was first suggested in 1960 by Yalow and Berson,[37] RIA has rapidly become one of the most widespread, widely applicable, and most sensitive techniques for assessing the concentration of a whole host of biologic molecules. Most of the basic principles necessary to understand and apply RIA have been covered previously in this chapter. In this section, we examine the concepts and methodologic approaches used in RIA. For a detailed methods book, we refer the reader to Chard,[38] Rodbard,[39] and Yalow.[40]

The central concept of RIA is that the binding of an infinitesimal concentration of highly radioactive tracer antigen to low concentrations of a high-affinity-specific antibody is very sensitive to competition by unlabeled antigen and is also very specific for that antigen. Thus, concentrations of antigen in unknown samples can be determined by their ability to compete with tracer for binding to antibody. The method can be used to measure very low concentrations of a molecule, even in the presence of the many impurities in biologic fluids. Accomplishment of this requires an appropriate high-affinity antibody and radiolabeled antigen, a method to distinguish bound from free-labeled antigen, optimization of concentrations of antibody and tracer-labeled antigen to maximize sensitivity, and generation of a standard curve, using known concentrations of competing unlabeled antigen, from which to read off the concentrations in unknown samples as well as the best method for representing the data. We review all these steps and pitfalls in this procedure except the preparation of antibodies and labeled antigens.

Separation of Bound and Free Antigen

Whatever parameter one uses to assess the amount of competition by the unlabeled antigen in the unknown sample to be tested, it will always be a function of bound versus free, radiolabeled antigen. Therefore, one of the most critical technical requirements is the ability to distinguish clearly between antibody-bound radioactive tracer and free radioactive tracer. This distinction usually requires physical separation of bound and free ligand. If the bound fraction is contaminated by free ligand, or vice versa, enormous errors can result, depending on the part of the binding curve on which the data fall.

Solution Methods

Solution RIA methods have the advantage that binding can be related to the intrinsic affinity of the antibody. However, bound and free antigen must be separated by a method that does not perturb the equilibrium. Three basic types of approaches have been used: precipitate the

antibody with bound antigen, leaving free antigen in solution; precipitate the free antigen, leaving antibody and bound antigen in solution; or separate free from antibody-bound antigen molecules in solution on the basis of size by gel filtration. This last method is too cumbersome to use for large numbers of samples and is too slow, in general, to be sure the equilibrium is not perturbed in the process. Therefore, gel filtration columns are not widely used for RIA. Methods that precipitate antibody are perhaps the most widely used. If the antigen is sufficiently smaller (<30,000 MW) than the antibody that it will remain in solution at

concentrations of either ammonium sulfate[41] or polyethylene glycol, 6000 MW (10% W:W),[42] which will precipitate essentially all the antibody, then these two reagents are frequently the most useful. Precipitation with polyethylene glycol and centrifugation can be accomplished before any significant dissociation has occurred due to dilutional effects.[43] However, if the antigen is much larger than about 30,000 to 40,000 MW, these methods will produce unacceptably high background control values in the absence of specific antibody. If the antibody is primarily of a subclass of IgG that binds to staphylococcal protein A or G, one can take advantage of the high affinity of protein A or G for IgG by using either protein A (or G)-Sepharose or formalinkilled staphylococcal organisms (Cowan I strain) to precipitate the antibody.[44] Finally, one can precipitate the antibody using a specific second antibody, an anti-Ig raised in another species. Maximal precipitation occurs not at antibody excess but at the "point of equivalence" in the middle of the titration curve where antigen (in this case, the first antibody) and the (second) antibody are approximately equal in concentration. Thus, one must add carrier Ig to keep the Ig concentration constant and determine the point of equivalence by titrating with the second antibody. Even worse, the precipitin reaction is much slower than the antigen-antibody reaction itself, allowing reequilibration of the antigen-antibody interaction after dilution by the second antibody. Some of these problems can be reduced by enhancing precipitation with low concentrations of polyethylene glycol.

The other type of separation method is adsorption of free antigen to an agent, such as activated charcoal or talc, which leaves antigen bound to antibody in solution. Binding of antigen by these agents depends on size and hydrophobicity. Although these methods are inexpensive and rapid, they require careful adjustment and monitoring of pH, ionic strength, and temperature to obtain reproducible results and to avoid adsorption of the antigen-antibody complex. Furthermore, because these agents have a high affinity for antigen, they can compete with a low-affinity antibody and alter the equilibrium. Also, as charcoal quenches beta scintillation counting, it can be used only with gamma-emitting isotopes such as ^{125}I.

Solid-Phase Methods

Solid-phase RIA methods have the advantages of high throughput and increased apparent affinity due to the effects at the solid-liquid interface noted previously. However, they have the concomitant disadvantage that one is not measuring the true intrinsic affinity because of these same effects. The method itself is fairly simple. One binds the antibody in advance to a solid surface such as a Sepharose bead or the walls of a microtiter plate well. To avoid competition from other serum proteins for the solid phase, one must use purified antibody in this coating step. Once the wells (or Sepharose beads) are coated, one can incubate them with labeled tracer antigen with or without unlabeled competitor, wash and count directly the radioactivity bound to the plastic wells or to the Sepharose. The microtiter plate method is

particularly useful for processing large numbers of samples. However, because the concentration, or even the amount, of antibody coating the surface is unknown and because the affinity is not the intrinsic affinity, one cannot use these methods for studying the chemistry of the antigen-antibody reaction itself. A detailed analysis of the optimum parameters in this method is given by Zollinger et al.[43]

A variation that does allow determination of affinity, based on the enzyme-linked immunosorbant assay (ELISA) described in the following, but equally applicable to RIA, was described by Friguet et al.[45] This uses antigen-coated microtiter wells and free antibody but measures competition by free antigen to prevent the antibody in solution from binding to the antibody on the plate (see Fig. 7.9B). Thus, the antibody bound to the plastic is antibody that was free in the solution equilibrium. The affinity measured is that between the antibody and antigen in solution, not that on the plastic, so it is not directly influenced by the multivalency of the surface. However, as pointed out by Stevens,46 the determination of affinity is strictly accurate only for monovalent Fab fragments because a bivalent antibody with only one arm bound to the plastic and one bound by antigen in solution will still be counted as free. Therefore, there will be an underestimate of the ligand occupancy of the antibody combining sites and thus an underestimate of affinity. Stevens also points out a method to correct for this error based on binomial analysis. Subsequently, Seligman[47] showed that the nature and density of the antigen on the solid surface can also influence the estimate of affinity.

Optimization of Antibody and Tracer Concentrations for Sensitivity

The primary limitations on the sensitivity of the assay are the antibody affinity and concentration, the tracer concentration, and the precision (reproducibility) of the data. In general, the higher the affinity of the antibody, the more sensitive the assay can be made. Once one prepares the highest affinity antibody available, this parameter limits the extent to which the other parameters can be manipulated. For instance, because the unlabeled antigen in the unknown sample is going to compete against labeled tracer antigen, the lower the tracer concentration, the lower the concentration of the unknown sample, which can be measured up to a point. That point is determined by the affinity, K_A, as can be seen from the theoretical considerations discussed previously.[38] The steepest part of the titration curve will occur in the range of concentrations around $1/K_A$. Concentrations of ligand much below $1/K_A$ will leave most of the antibody sites unoccupied so that competition will be less effective. Thus, there is no value in reducing the tracer concentration more than a few-fold lower than $1/K_A$. Therefore, although it is generally useful to increase the specific radioactivity of the tracer and reduce its concentration, it is important to be aware of this limit of $1/K_A$. Increasing the specific activity more than necessary can result in denaturation of antigen.

Similarly, lowering the antibody concentration will also increase sensitivity, up to a point. This limit also depends on $1/K_A$ and on the background “nonspecific binding.” Decreasing the antibody concentration to the point that binding of tracer is too close to background will result in

loss of sensitivity due to loss of precision. In general, the fraction of tracer bound in the absence of competitor should be kept greater than 0.2, and in general closer to 0.5.[48]

A convenient procedure to follow to optimize tracer and antibody concentrations is first to choose the lowest tracer concentration that results in convenient counting times and counting precision for bound values of only one-half to onetenth the total tracer. Then, keeping this tracer concentration constant, one dilutes out the antibody until the bound/free antigen ratio is close to 1.0 (bound/total = 0.5) in the absence of competitor. This antibody concentration in conjunction with this tracer concentration will generally give near-optimal sensitivities, within the limits noted previously. It is important to be aware that changing the tracer concentration will require readjusting the antibody concentration to optimize sensitivity.

Analysis of Data: Graphic and Numerical Representation

We have already examined the Scatchard plot (bound/free versus bound) and the plot of bound/free versus free or total antigen concentration as methods of determining affinity. The latter lends itself particularly to the type of competition curves that constitute an RIA. In fact, the independent variable must always be antigen concentration, as that is the known quantity one varies to generate the standard curve. Let us use B, F, and T to represent the concentrations of bound, free, and total antigen, respectively. We have seen that the plot of B/F versus F is more useful for determining the affinity, K_A, than the plot of B/F versus T. However, in RIA, the quantity one wants to determine is T, and correspondingly, the known independent variable in generating the standard curve is T. Another difference between the situation in RIA and that discussed previously is that, in RIA, one has both labeled and unlabeled antigen. The dependent variable, such as B/F, is the ratio of bound tracer over free tracer, as only radioactive antigen is counted. The B/F for the unlabeled antigen will be the same at equilibrium, assuming that labeled and unlabeled antigen bind the antibody equivalently, that is, with the same K_A. This assumption is not always valid and requires experimental testing.

The sigmoidal shape of B/F versus F or T, when F or T (the “dose”) is plotted on a log scale, has been seen in Figure 7.6. The shape for B/T versus F or T would be similar. Note that because B + F = T,

$$\frac{B}{F} = \frac{B}{(T - B)} = \frac{B/T}{(1 - B/T)} \qquad (38)$$

and

$$\frac{B}{T} = \frac{B/F}{(1 + B/F)} \qquad (39)$$

These transformations can be useful. If one plots B/F or B/T versus F or T on a linear scale, then the shape is approximately hyperbolic, as in Figure 7.7. The plot of B/F versus T (log scale) was one of the first methods used to plot RIA data and is still among the most useful. The most sensitive part of the curve is the part with the steepest slope.

It has been shown by probability analysis that if the antigen has multiple determinants, each capable of binding antibody molecules simultaneously and independently of one another, then the more such determinants capable of being recognized by the antibodies in use, the steeper will be the slope.[49] This effect of multideterminant binding on steepness arises

because, in RIA, an antigen molecule is scored as bound whether it has one antibody molecule attached or several. It is scored as free only if no antibody molecules are attached. Thus, the probability that an antigen molecule is scored as free is the product of the probabilities that each of its determinants is free. The effect can lead to quite steep slopes and has been confirmed experimentally.[49]

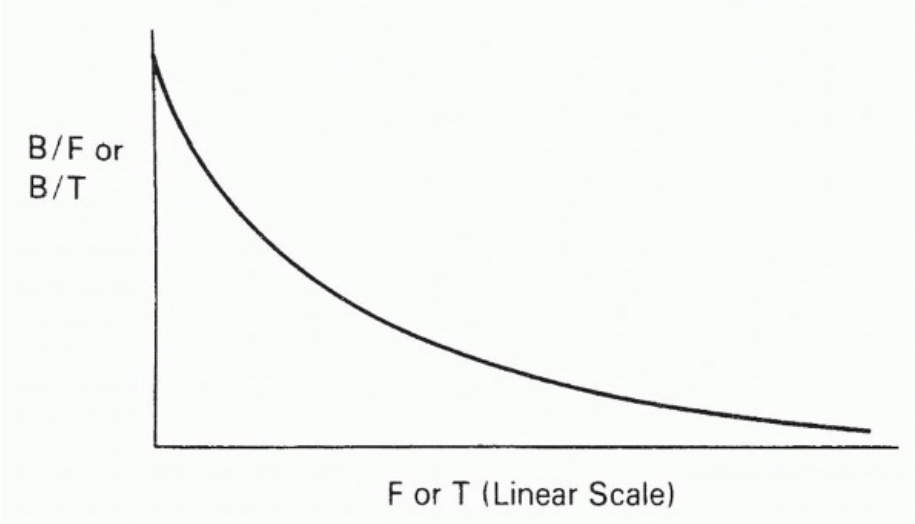

FIG. 7.7. Schematic Plot of B/F or B/T (the Bound Over Free or Total Antigen Concentration) as a Function of Free (F) or Total (T) Antigen Concentration, when Plotted on a Linear Scale. Contrast with similar plot on a log scale in Figure 7.6.

A transform that allows linearization of the data in most cases is the logit transform.[50,51] To use this, one first expresses the data as B/B_0, where B_0 is the concentration of bound tracer in the absence of competitor. One then takes the logit transform of this ratio, defined as

$$\text{logit}\,(Y) = \ln\left[\frac{Y}{(1-Y)}\right] \qquad (40)$$

where ln = the natural log (log to the base e). The plot of logit (B/B^0) versus ln T is usually a straight line (Fig. 7.8).

The slope is usually -1 for the simplest case of a monoclonal antibody binding a monovalent antigen. The linearity of this plot obviously makes it very useful for graphical interpolation, which one would like to do to read antigen concentration off a standard curve. One additional advantage is that linearity facilitates tests of parallelism. If the unknown understudy is identical to the antigen used to generate the standard curve, then a dilution curve of the unknown should be parallel to the standard curve in this logit-log coordinate system. If not, the assay is not valid.

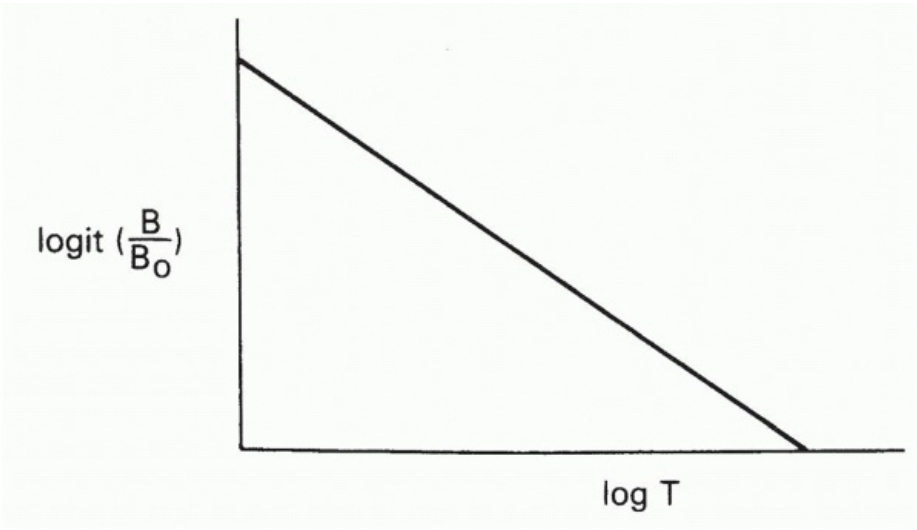

FIG. 7.8. Schematic Logit. Log plot used to linearize radioimmunoassay data. B and T are bound tracer and total antigen concentration, respectively, and B_0 is the value of B when no unlabeled antigen is added to tracer. The logit function is defined by Equation 40, logit (Y) = ln [Y/(1 - Y)].

These and other methods of analyzing the data are discussed further by Feldman and Rodbard[52] and Rodbard,[39] including statistical treatment of data. Although a number of computer programs have become available for rapid analysis of RIA data without using manual plots of standard curves, they are all based on these and similar methods, and their accurate interpretation depends on an understanding of these concepts.

Corrections for B, F, and T

Before we leave this section on analysis of RIA data, we must point out a few controls and corrections to the data without which the results may be fallacious.

First, in any method that precipitates antibody and bound antigen (or uses a solid-phase antibody), there may always be a fraction of antigen that precipitates or binds nonspecifically in the absence of specific antibody. Thus, one must always run controls with normal serum or Ig to determine this background. The nonspecific binding usually increases linearly with antigen dose, that is, it does not saturate. This control value should be subtracted from B but does not affect F when measured independently, only F determined as T minus B. The total antigen that is meaningful is the sum of that which is specifically bound and that which is free. Nonspecifically bound antigen should be deleted from any term in which it appears.

A second correction is that for immunologically inactive radiolabel, that is, either free radioisotope, or isotope coupled to an impurity or to denatured antigen. The fraction of radioactive material that is immunologically reactive with the antibodies in the assay can be determined by using a constant, low concentration of labeled antigen and adding increasing concentrations of antibody. If there is no contamination with inactive material, all the radioactivity should be able to be bound by sufficient antibody. If the fraction of tracer bound reaches a plateau at <100% bound, then only this fraction is active in the assay. The importance of this correction can be seen from the example in which the tracer is only 80%

active. Then, when the true B/F is 3 (B/T = 0.75), applying only to the active 80% of the tracer, the remaining 20%, which can never be bound, will mistakenly be included in the free tracer, doubling the amount that is measured as free. Thus, the measured B/F will be only 1.5 (ie, 0.6/0.4) instead of the true value of 3 (ie, 0.6/0.2). This factor of 2 will make a serious difference in the calculation of affinity, for instance, from a Scatchard plot. Also, it will result in a plateau in the Scatchard plot at high values of B/F, as with 20% of the tracer obligatorily free, B/F can never exceed 4 (ie, 0.8/0.2). To correct for this potentially serious problem, the inactive fraction must always be determined and subtracted from both F and T.

Nonequilibrium Radioimmunoassay

So far, we have assumed that tracer and unlabeled competitor are added simultaneously, and sufficient incubation time is allowed to achieve equilibrium. To measure the affinity, of course, equilibrium must be assured. However, suppose one's sole purpose is to measure the concentration of competitor by RIA. Then one can actually increase the sensitivity of the assay by adding the competitor first, allowing it to react with the antibody, and then intentionally adding the tracer for too short a time to reach a new equilibrium. One is essentially giving the competitor a competitive advantage. It can be shown that the slope of the dose-response curve, B/T versus total antigen added, is increased in the low-dose range —a mathematical measure of increased sensitivity. A detailed mathematical analysis of this procedure may be found in Rodbard et al.[53] Note, however, that use of such nonequilibrium conditions requires very careful control of time and temperature.

Enzyme-Linked Immunosorbent Assay

An alternative solid-phase readout system for the detection of antigen-antibody reactions is the ELISA.[54] In principle, the only difference from RIAs is that antibodies or antigen are covalently coupled to an enzyme instead of a radioisotope so that bound enzyme activity is measured instead of bound cpm. In practice, the safety and convenience of nonradioactive materials and the commercial availability of plate readers that can measure the absorbance of 96 wells in a few seconds account for ELISA's widespread use. Because both ELISA and RIA are governed by the same thermodynamic constraints, and the enzyme can be detected in the same concentration range as commonly used radioisotopes, the sensitivity and specificity are comparable. We consider three basic strategies for using ELISA assays to detect specific antibody or antigen.

As shown in Figure 7.9A, the indirect antibody method is the simplest way to detect and measure specific antibody in an unknown antiserum. Antigen is noncovalently attached to each well of a plastic microtiter dish. For this purpose, it is fortunate that most proteins bind nonspecifically to plastic. Excess free antigen is washed off, and the wells are incubated with an albumin solution to block the remaining nonspecific protein binding sites. The test antiserum is then added, and any specific antibody binds to the solid-phase antigen. Washing removes unbound antibodies. Enzyme-labeled anti-Ig is added. This binds to specific antibody already bound to antigen on the solid phase, bringing along covalently attached enzyme. Unbound antiglobulin-enzyme conjugate is washed off; substrate is then added. The action of bound enzyme on substrate produces a colored product, which is detected as increased absorbance in a spectrophotometer.

Although this method is quick and very sensitive, it is often difficult to quantitate. Within a

defined range, the increase in optical density is proportional to the amount of specific antibody added in the first step. However, the amount of antibody bound is not measured directly. Instead, the antibody concentration of the sample is estimated by comparing it with a standard curve for a known amount of antibody. It is also difficult to determine affinity by this method because the solid-phase antigen tends to

increase the apparent affinity. The sensitivity of this assay for detecting minute amounts of antibody is quite good, especially when affinity-purified antiglobulins are used as the enzyme-linked reagent. A single preparation of enzymelinked antiglobulin can be used to detect antibodies to many different antigens. Alternatively, class-specific antiglobulins can be used to detect how much of a specific antibody response is due to each Ig class. Obviously, reproducibility of the assay depends on uniform antigen coating of each well, and the specificity depends on using purified antigen to coat the wells.

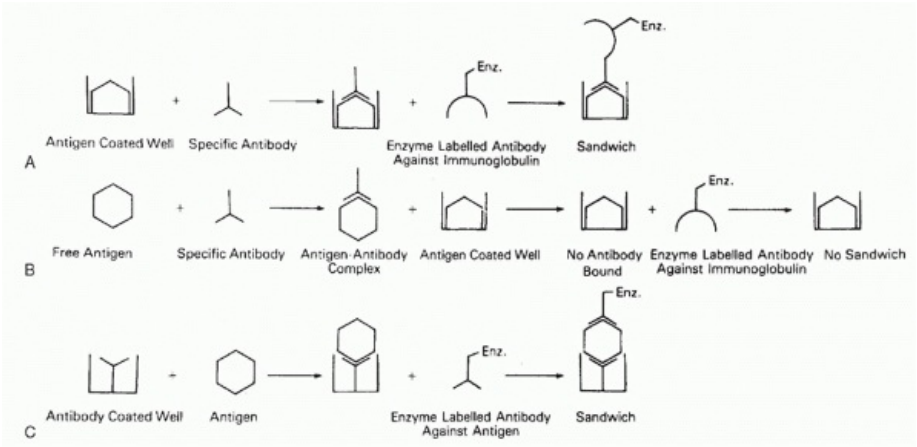

FIG. 7.9. Three Strategies for the Detection of Specific Antibody-Antigen Reactions Using the Enzyme-Linked Immunosorbant Assay Technique. A: Direct binding. **B:** Hapten inhibition. **C:** Antigen sandwich.

Figure 7.9B shows the competition technique for detecting antigen. Soluble antigen is mixed with limiting amounts of specific antibody in the first step. Then the mixture is added to antigen-coated wells and treated as described in Figure 7.9A. Any antigen-antibody complexes formed in the first step will reduce the amount of antibody bound to the plate, and hence will reduce the absorbance measured in the final step. This method permits the estimate of affinity for free antigen, which is related to the half-inhibitory concentration of antigen. Mathematical analysis of affinity by this approach was described by Friguet et al.[45] with modification by Stevens,[46] as discussed under RIA solid-phase methods. In addition, some estimate of cross-reactivity between the antigen in solution and that on the plate can be obtained.

Figure 7.9C shows the sandwich technique for detecting antigen. Microtiter plates are coated with specific antibody. Antigen is then captured by the solid-phase antibody. A second antibody specific for the antigen, and coupled to enzyme, is added. This binds to the solid-phase antigen-antibody complex, carrying enzyme along with it. Excess second antibody is

washed off, and substrate is added. The absorbance produced is a function of the antigen concentration of the test solution, which can be determined from a standard curve. Specificity of the assay depends on the specificity of the antibodies used to coat the plate and detect antigen. Sensitivity depends on the affinity as well as amount of the first antibody coating the well, which can be increased by using affinity-purified antibodies or monoclonal antibodies in this step. The sandwich method depends on divalency of the antigen or else the two antibodies must be specific for different antigenic determinants on the same antigen molecule. When comparing two monoclonal antibodies to the same antigen, this technique can be used to ascertain whether they can bind simultaneously to the same molecule or whether they compete for the same site or sites close enough to cause steric hindrance.[55]

When antibodies are serially diluted across a plate, the last colored well indicates the titer. Specificity of binding can be demonstrated by coating wells with albumin and measuring antibody binding in parallel with the antigen-coated wells. Because it can be used to test many samples in a short time, ELISA is often used to screen culture supernatants in the production of hybridoma antibodies. The sensitivity of the method allows detection of clones producing specific antibodies at an early stage in cell growth.

An important caution when using native protein antigens to coat solid-phase surfaces (see Fig. 7.9A) is that binding to a surface can alter the conformation of the protein. For instance, using conformation specific monoclonal antibodies to myoglobin, Darst et al.[56] found that binding of myoglobin to a surface altered the apparent affinity of some antibodies more than others. This problem may be avoided by using the solution phase methods of Figure 7.9B or 7.9C.

ELIspot Assay

The normal ELISA assay can be modified to measure antibody production at the single cell level. In this method, tissue culture plates are coated with antigen, and various cell populations are cultured on the plate for 4 hours. During that time, B cells settle to the bottom and secrete antibodies, which bind antigen nearby and produce a footprint of the antibody-secreting cell. The cells are then washed off, and a second antibody, such as enzyme-labeled goat antihuman IgG, is added. Finally, unbound antibody is washed off, and enzyme substrate is added in soft agar. Over the next 10 minutes, each footprint of enzyme activity converts the substrate to a dark spot of insoluble dye, corresponding to the localized zone where the B cell originally secreted its antibody.

Using this method, it is possible to detect as few as 10 to 20 antibody-producing B cells in the presence of 10^6 spleen cells, and typical results for immunized mice range from 200 to 500 spot-forming cells per 10^6 spleen cells.[57,58] Clearly, to work at all, this assay must be capable of detecting the amount of antibody secreted by a single immune B cell and specific enough to exclude nonspecific antibodies produced by most nonimmune B cells. Sensitivity depends on the affinity and amount of antibodies secreted and may be optimized by titering the amount of antigen on the plate.

This type of assay is useful in analyzing the cellular requirements for antibody production in vitro, as the number of responding B cells is measured directly. It can also be used to detect antibodies made in the presence of excess antigen. For example, during acute infections[59]

and in autoimmunity,[60] when antigen may be in excess over antibody, this assay makes it possible to measure antibody-producing B cells, even though free antibody may not be detectable in circulation. It can also be used to measure local production of self-reactive antibodies in a specific tissue, such as synovium. By using two detecting antibodies, each specific for a different Ig class and coupled to a different enzyme, and two substrates producing different colored dyes, cells secreting IgA and IgG simultaneously can be detected.[61] Recently, ELIspot was used to show that bacterial deoxyribonucleic acid (DNA)-containing CpG sequences is a polyclonal B-cell mitogen.[62]

ELIspot can also detect secreted cytokines, as opposed to antibodies, by coating the plate with a capture antibody and detecting antigen with an enzyme-coupled second antibody (as in a sandwich ELISA; see Fig. 7.9C). For example, using plates coated with monoclonal antibody to interleukin (IL)-4, T cells secreting IL-4 could be detected,[63] providing one measure of T helper 2 cells.

SPECIFICITY AND CROSS-REACTIVITY

The specificity of an antibody or antiserum is defined by its ability to discriminate between the antigen against which it was made (called the homologous antigen or immunogen) and any other antigen one might test. In practice, one cannot test the whole universe of antigens but only selected antigens. In this sense, specificity can only be defined experimentally within that set of antigens one chooses to compare. Karush[30] has defined a related term, selectivity, as the ability of an antibody to discriminate, in an all-or-none fashion, between two related ligands. Thus, selectivity depends not only on the relative affinity of the antibody for the two ligands but also on the experimental lower limit for detection of reactivity. For instance, an anticarbohydrate antibody with an affinity of 10^5 M^{-1} for the immunogen may appear to be highly selective, as reaction with a related carbohydrate with a 100-fold lower affinity, 10^3 M^{-1}, may be undetectable. On the other hand, an antibody with an affinity of 10^9 M^{-1} for the homologous ligand may appear to be less selective because any reaction with a related ligand with a 100-fold lower affinity would still be quite easily detectable.

Conversely, cross-reactivity is defined as the ability to react with related ligands other than the immunogen. More usually, this is examined from the point of view of the ligand. Thus, one might say that antigen Y cross-reacts with antigen X because it binds to anti-X antibodies. Note that in this sense, it is the two antigens that are cross-reactive, not the antibody. However, the cross-reactivity of two antigens, X and Y, can be defined only with respect to a particular antibody or antiserum. For instance, a different group of anti-X antibodies may not react at all with Y so that with respect to these antibodies, Y would not be cross-reactive with X. One can also use the term in a different sense, saying that some anti-X antibodies cross-react with antigen Y.

In most cases, cross-reactive ligands have lower affinity than the immunogen for a particular antibody. However, exceptions can occur in which a cross-reactive antigen binds with a higher affinity than the homologous antigen itself. This phenomenon is called heterocliticity, and the antigen that has a higher affinity for the antibody than does the immunogen is said to be heteroclitic. Antibodies that manifest this behavior are also described as heteroclitic antibodies. A good example is the case of antibodies raised in C57BL/10 mice against the

hapten nitrophenyl acetyl. These antibodies have been shown by Mäkelä and Karjalainen[64] to bind with higher affinity to the cross-reactive hapten, nitroiodophenyl acetyl, than to the immunogen itself. Another example is the case of retro-inverso or retro-D peptides.[65,66,67,68,69] By reversing the chirality from L to D amino acids, and simultaneously reversing the sequence of amino acids, one can produce a peptide that is resistant to proteolysis and has its side chains approximately in the same position as the original L amino acid peptide, with the exception of some amino acids with secondary chiral centers such as Thr and Ile. However, the backbone NH_2 and COOH moieties are reversed. Antibodies that interact with only the side chains might not distinguish these peptides, whereas antibodies that interact with the main chain as well as side chains might distinguish them and have potentially higher or lower affinity. In a study of monoclonal antibodies to a hexapeptide from histone H3, some bound the retro-D form with higher affinity than the native sequence and some did not.[67,68] The former are examples of heterocliticity. In addition to greater binding affinity, the retro-D peptides may have even greater activity in vivo because of their resistance to proteolysis.[65,66,67,68,69] This stability makes them more useful as drugs as well.[65,66,70]

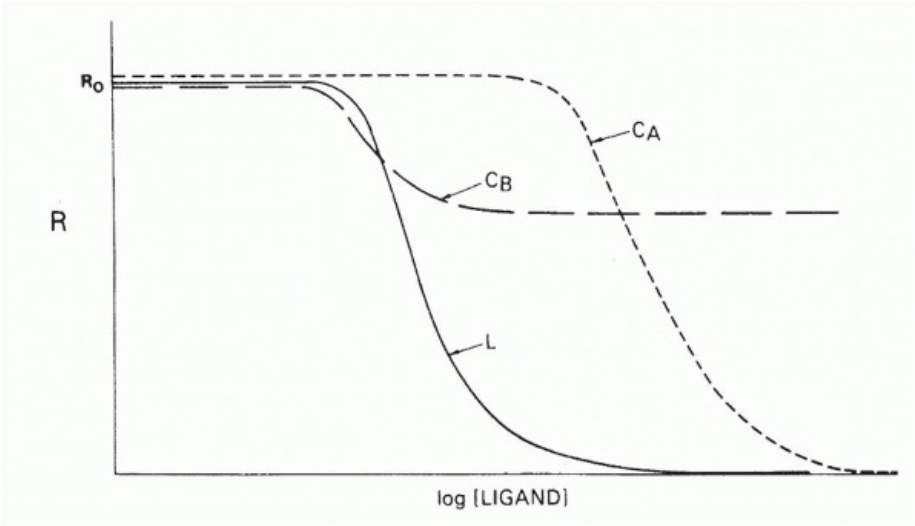

FIG. 7.10. Schematic Radioimmunoassay Binding Curves for Homologous Ligand L and Cross-Reacting Ligands. Cross-reacting ligand C_A manifests type 1 or true cross-reactivity demonstrated by complete inhibition of tracer ligand binding and a lower affinity. Ligand C_B displays type 2 cross-reactivity or determinant sharing, as recognized from the plateau at less than 100% inhibition, but not necessarily a lower affinity. The ordinate R is the ratio of bound/free radiolabeled tracer ligand, and R_0 is the limit of R as the concentration of all ligands, including tracer, approaches zero. From Berzofsky and Schechter,[71] with permission.

Cross-reactivity has often been detected by methods such as the Ouchterlony test, or hemagglutination (see the following for descriptions of both of these) or similar methods,

which have in common the fact that they do not distinguish well between differences in affinity and differences in concentration. This practical aspect, coupled with the heterogeneity of immune antisera, has led to ambiguities in the usage of the terms “cross-reactivity” and “specificity.” With the advent of RIA and ELISA techniques, this ambiguity in the terminology, as well as in the interpretation of data, has become apparent.

For these reasons, Berzofsky and Schechter[71] have defined two forms of cross-reactivity and, correspondingly, two forms of specificity. These two forms of cross-reactivity are illustrated by the two prototype competition RIA curves in Figure 7.10. In reality, most antisera display both phenomena simultaneously.

Type 1 cross-reactivity, or true cross-reactivity, is defined as the ability of two ligands to react with the same site on the same antibody molecule, possibly with different affinities. For example, the related haptens dinitrophenyl and trinitrophenyl may react with different affinity for antibodies raised to dinitrophenyl hapten. In protein antigens, such differences could occur with small changes in primary sequence (eg, the conservative substitution of threonine for serine), or with changes in conformation, such as the cleavage of the protein into fragments (Fig. 7.11).[71,72,73,74,75] If a peptide fragment contained all the contact residues in an antigenic determinant (ie, those that contact the antibody-combining site), it might cross-react with the native determinant for antibodies against the native form but with lower affinity because the peptide would not retain the native conformation. This type of affinity difference is illustrated by competitor C_A in Figure 7.10, in which complete displacement of tracer can be achieved at high enough concentrations of C_A, but higher concentrations of C_A than of the homologous ligand, L, are required to produce any given degree of inhibition.

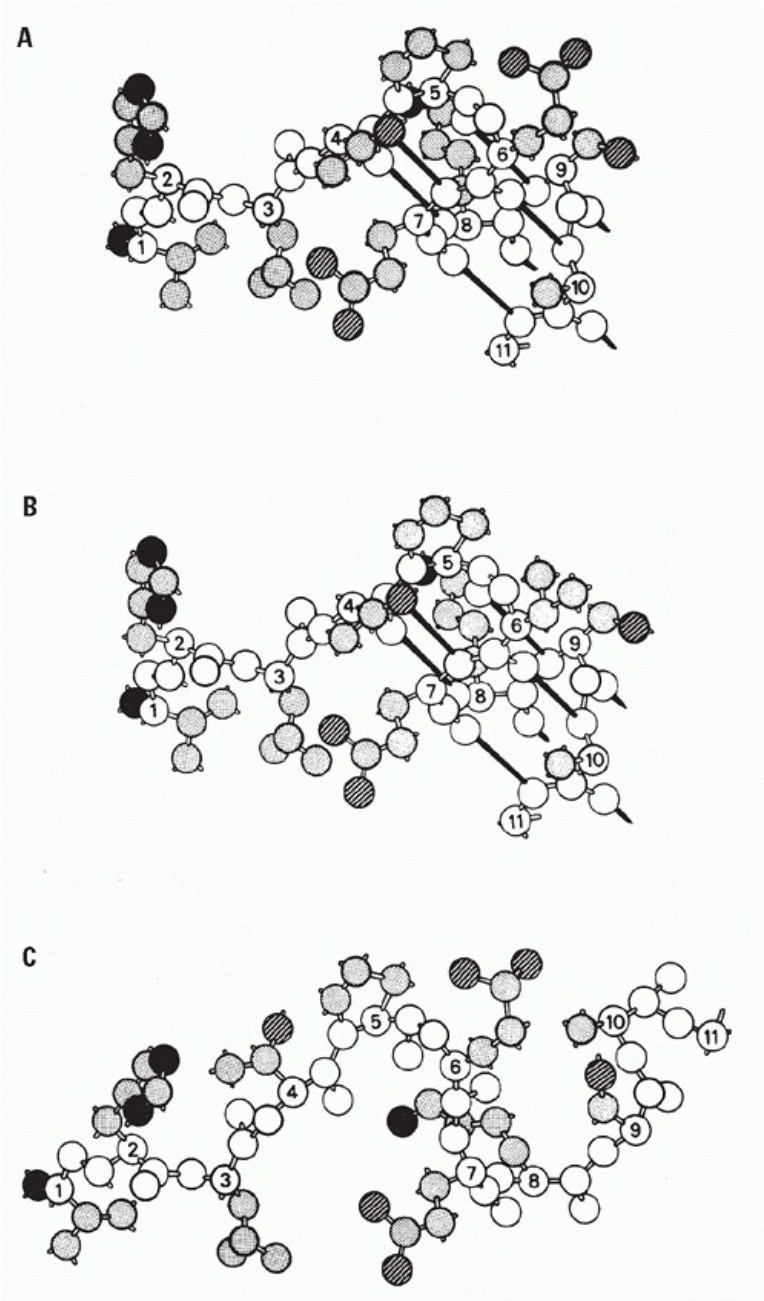

FIG. 7.11. An Artist's Drawing of the Amino Terminal Region of the β Chain of Hemoglobin. A: The first 11 residues of the β^A chain. **B:** The comparable regions of the β^S chain. The substitution of valine for the normal glutamic acid at position 6 makes a distinct antigenic determinant to which a subpopulation of antibodies may be isolated.[72,73] **C:** A schematic diagram of the sequence in (**A**) unfolded as occurs when the protein is denatured. This region may be cleaved from the protein, or the peptide synthesized,[74] resulting in changed antigenic reactivity. An antiserum prepared to hemoglobin (or the β chain thereof) might exhibit cross-reactivity with the structures shown in (**B**) and (**C**), but the molecular mechanisms would be different. Polypeptide backbone atoms are in *white* in the side chains, oxygen atoms are *hatched*, nitrogen atoms are *black*, and carbon atoms are *lightly stippled*. Adapted from Berxofsky and Schechter[71] and Dean and Schecter.[75]

A separate issue from affinity differences is the issue of whether the cross-reactive ligand reacts with all or only a subpopulation of the antibodies in a heterogeneous serum.

This second type of cross-reactivity, which we call type 2 cross-reactivity or shared reactivity, therefore can occur only when the antibody population is heterogeneous, as in most conventional antisera. In this case, the affinity of the cross-reactive ligand may be greater than, less than, or equal to that of the homologous ligand for those antibodies with which it interacts. Therefore, the competition curve is not necessarily displaced to the right, but the inhibition will reach a plateau at less than complete inhibition, as illustrated by competitor C_B in Figure 7.10. As an example, let us consider the case of a protein with determinants X and Y, and an antiserum against this protein containing both anti-X and anti-Y antibodies. Then a mutant protein in which determinant Y was so altered as to be unrecognizable by anti-Y, but determinant X was intact, would manifest type 2 crossreactivity.

It would compete with the wild-type protein only for anti-X antibodies (possibly even with equal affinity), but not for anti-Y antibodies.

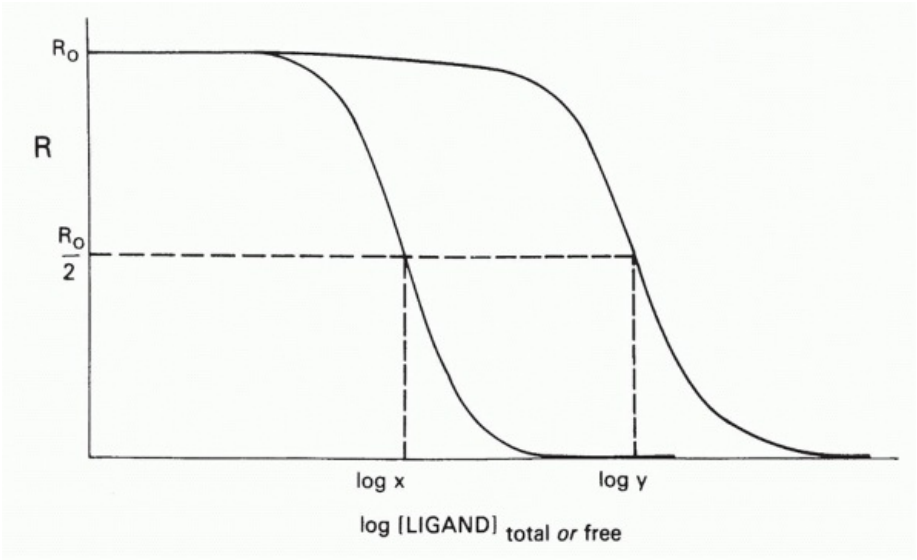

FIG. 7.12. Schematic Radioimmunoassay Binding Curves Showing the Effect of Affinity on the Midpoint and the Slope at the Midpoint and the Value of Using Free (Ligand) Rather than Total (Ligand). Ordinate R is the ratio of bound/free radiolabeled tracer ligand, and R_0 the limit of R as all ligand concentrations approach zero. If x and y are the concentrations of ligands X and Y that reduce R to exactly $R_0/2$, then if the abscissa is total ligand concentration, $x = 1/K_X + [S]_t/2$ and $y = 1/K_Y + [S]_t/2$, where $[S]_t$ is the concentration of antibody binding sites and K_X and K_Y the affinities of the antibody for the respective ligands. However, if the abscissa is free ligand concentration, $x = 1/K_X$ and $y = 1/K_Y$ so that the ratio x/y (or the difference log x - log y on a log plot) corresponds to the ratio of affinities K_Y/K_X. Note that the slopes at the midpoints are the same on a log scale, but that for Y would be only K_Y/K_X that for X on a linear scale. From Berxofsky and Schechter,[71] with permission.

Of course, both types of cross-reactivity could occur simultaneously. A classic example would be the peptide fragment previously discussed in the case of type I cross-reactivity. Suppose the fragment contained the residues of determinant X, albeit not in the native conformation, but did not contain the residues of a second determinant, Y, which was also expressed on the native protein. If the antiserum to the native protein consisted of anti-X and anti-Y, the peptide would compete only for anti-X antibodies (type 2 cross-reactivity) but would have a lower affinity than the native protein even for these antibodies. Thus, the competition curve would be shifted to the right and would plateau before reaching complete inhibition.†††

In the case of a homogeneous (eg, monoclonal) antibody in which only type 1 or true cross-reactivity can occur, one can quantitate the differences in affinity for different crossreactive ligands by a method analogous to the B/F versus F method described previously. Suppose that ligands X and Y cross-react with homologous ligand L for a monoclonal antibody. If one plots the bound/free (B/F = R) ratio for radiolabeled tracer ligand L as a function of the log of the concentration of competitors X and Y, one obtains two parallel competition curves (Fig. 7.12),[71] under the appropriate conditions (see subsequent discussion). The first condition is that the concentration of free tracer be less than $1/K_L$, the affinity for tracer. In this case, it can be shown[71] that

$$K_X \approx \frac{1}{[X]_{free}} \qquad (41)$$

at the midpoint where $R = R_0/2$, where K_X = affinity for X. This is analogous to Equation 21 for the case in which unlabeled homologous ligand is the competitor. Also, in

analogy with Equation 23, it can be shown that if the total concentration of competitor, $[X]_t$, is used instead of the free concentration, [X]free, an error term will arise, giving

$$[X]_t \text{ (at } R = R_0/2) = \frac{1}{K_X} + \frac{[S]_t}{2} \qquad (42)$$

Thus, with competitor on a linear scale, the difference in midpoint for competitors X and Y will correspond to the difference $1/K_X - 1/K_Y$ regardless of whether free or total competitor is plotted, but the ratio of midpoint concentrations will equal K_X/K_Y only if the free concentrations are used.

This last point is important if one plots the log of competitor concentration, as is usually done, as the horizontal displacement between the two curves on a log scale corresponds to the ratio [X] / [Y], not the difference.[71]

If a second condition also holds, namely, that the concentration of bound tracer is small compared to the antibody site concentration $[S]_t$, then the slopes (on a linear scale) of the curves at their respective midpoints (where $R = R_0/2$) will be proportional to the affinity for that competitor, K_X or K_Y (71). (Both conditions can be met by keeping tracer L small relative to both K_L and $[S]_t$.) When $[X]_{free}$ and $[Y]_{free}$ are plotted on a log scale, the slopes will

appear to be equal (ie, the curves will appear parallel) because a parallel line shifted m-fold to the right on a log scale will actually be 1/m as steep, at any point, in terms of the antilog as abscissa.

When the antibodies are heterogeneous in affinity, the curves will be broadened and in general will not be parallel. When heterogeneity of specificity is present, and type 2 cross-reactivity occurs, it should be pointed out that the fractional inhibition achieved at the plateau in a B/F versus free competitor plot will not be proportional to the fraction of antibodies reacting with that competitor but will be proportional to a weighted fraction, where the antibody concentrations are weighted by their affinity for the tracer.[71]

These two types of cross-reactivity lead naturally to two definitions of specificity.[71] The overall specificity of a heterogeneous antiserum is a composite of both of these facets of specificity. Type 1 specificity is based on the relative affinities of the antibody for the homologous ligand and any cross-reactive ligands. If the affinity is much higher for the homologous ligand than for any cross-reactive ligand tested, then the antibody is said to be highly specific for the homologous ligand (ie, it discriminates very well between this ligand and the others). If the affinity for cross-reactive ligands is below the threshold for detection in an experimental situation, then type 1 specificity gives rise to selectivity as was discussed previously.[30] The specificity can even be quantitated in terms of the ratio of affinities for the homologous ligand and a cross-reactive ligand.[76] It is this type 1 specificity that most immunochemists would call true specificity, just as we have called type 1 cross-reactivity true cross-reactivity.

The common use of the term “cross-reactivity” to include type 2 or partial reactivity leads to a second definition of specificity, which applies only to heterogeneous populations of antibodies such as antisera. We call this type 2 specificity. If all the antibodies in the mixture react with the immunogen, but only a small proportion react with any single cross-reactive antigen, then the antiserum would be said to be relatively specific for the immunogen. Note that it does not matter whether the affinity of a subpopulation that reacts with a cross-reactive antigen is high or low (type 1 cross-reactivity). As long as that subpopulation is a small fraction of the antibodies, the mixture is specific. Thus, type 2 specificity depends on the relative concentrations of antibodies in the heterogeneous antiserum, not just on their affinities. Also note that one can use these relative concentrations of antibody subpopulations to compare the specificity of a single antiserum for two cross-reactive ligands. However, it would not be meaningful to compare the specificity of two different antisera for the same ligand by comparing the fraction of antibodies in each serum which reacted with that ligand. Although type 2 specificity may appear to some a less classic concept of specificity than type 1, it is type 2 specificity that one primarily measures in such assays as the Ouchterlony double immunodiffusion test, and it carries equal weight with type 1 specificity in such assays as hemagglutination, discussed in the following section. Type 2 specificity also leads naturally to the concept of “multispecificity,” described in the following section.

Multispecificity

The theory of multispecificity, introduced and analyzed by Talmadge[77] and Inman,[78,79] and discussed on a structural level by Richards et al.,[80] suggests a mechanism by which the diversity and specificity of antisera can be expanded and also understood. The idea is that

each antibody may actually bind, with high affinity, a variety of diverse antigens. When one immunizes with immunogen A, one selects for many distinct antibodies, which have in common only that they all react with A. In fact, each antibody may react with other compounds, but if fewer than 1% of the antibodies bind B, and fewer than 1% bind C, and so on, then by type 2 specificity, the whole antiserum will appear to be highly specific for A. Note that the subpopulation that binds B may react with an affinity for B as high as or higher than that for A so that the population would not be type 1 specific for A. This same population would presumably be selected if one immunized with B. The net result would be that the diversity of highly (type 2) specific antisera an organism could generate would be even greater than the diversity of B-cell clones (or antibody structures). This principle can explain how polyclonal antisera can sometimes appear paradoxically more specific than a monoclonal antibody.

OTHER METHODS

We mention only a few of the other methods for measuring antigen-antibody interactions. Other useful techniques include quenching of the tryptophan fluorescence of the antibody by certain antigens on binding[81] (a sensitive method useful for such experiments as fast kinetic studies), antibody-dependent cellular cytotoxicity, immunofluorescence including flow cytometry, immunohistochemistry, and inhibition by antibody of plaque formation by antigenconjugated bacteriophage[82] (a method as sensitive as RIA as inhibition of even a few phage virions can be detected).

Quantitative Precipitin

Among the earliest known properties of antibodies were their ability to neutralize pathogenic bacteria and their ability to form precipitates with bacterial culture supernatants. Both activities of each antiserum were highly specific for the bacterial strain against which the antiserum was made. The precipitates contained antibody protein and bacterial products. The supernatants contained decreased amounts of antibody protein and, under the right conditions, had lost the ability to neutralize bacteria. However, quantitation of the antibody precipitated was difficult because the precipitate contained antigen protein as well as antibody protein.

Heidelberger and Kendall[83,84] solved this problem when they found that purified pneumococcal cell wall polysaccharide could precipitate with antipneumococcal antibodies. In this case, the amount of protein nitrogen measured in the precipitate was entirely due to antibody protein, and the amount of reducing sugar was mostly due to the antigen. Plotting the amount of antibody protein precipitated from a constant volume of antiserum by increasing amounts of carbohydrate antigen gives the curve shown in Figure 7.13.

As shown in Figure 7.13A, the amount of antibody precipitated rises initially, reaches a plateau, and then declines. The point of maximum precipitation was found to coincide with the point of complete depletion of neutralizing antibodies and is called the equivalence point. The amount of antibody protein in the precipitate at equivalence is considered to equal the total amount of specific antibody in that volume of antiserum. The rising part of the curve is called the antibody excess zone (antigen limiting), and the part of the curve beyond the equivalence point is called the antigen excess zone.

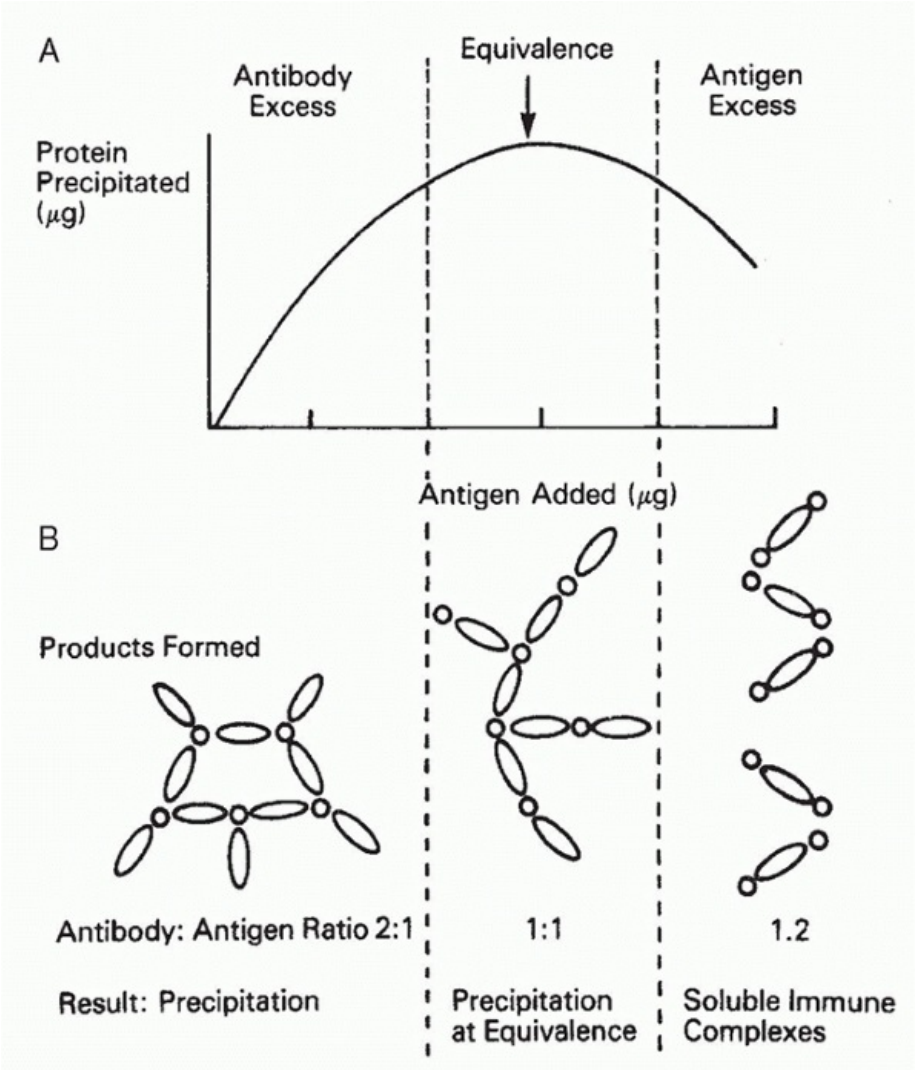

FIG. 7.13. Quantitative Immunoprecipitation. To a fixed amount of specific antibody are added increasing amounts of nonprotein antigen. The figure shows the amount of antibody protein **(A)** and the ratio of antibody to antigen **(B)** found in the precipitate. At antigen excess, soluble immune complexes are found in the supernatant, and the precipitate is decreased.

Supernatants and precipitates were carefully analyzed for each zone of antibody or antigen excess, as shown in Figure 7.13B. When antigen was limiting, the precipitate contained high ratios of antibody to antigen. The supernatant in this zone contained free antibody with no detectable antigen. As more antigen was added, the amount of antibody in the precipitate rose, but the ratio of antibody to antigen fell. At equivalence, no free antibody or antigen could be detected in the supernatant. As more antigen was added, the precipitate contained less antibody, but the ratio of antibody to antigen remained constant. The supernatant now contained antigen-antibody complexes because the complexes at antigen excess were small enough to remain in solution. No unbound antibody was detected.

The lattice theory[83,84] is a model of the precipitation reaction that explains these observations. It assumes that antibodies are multivalent and antigens are bivalent or

polyvalent. Thus, long chains can form consisting of alternating antibody linked to antigen linked to antibody, and so on. The larger the size of the aggregate, the less soluble the product. In the antibody excess zone, branch points can form whenever three antibodies bind to a single antigen, resulting in a three-dimensional lattice structure, which precipitates. In the equivalence zone, when equimolar amounts of antibody and antigen are mixed, the likelihood of more than two antibodies binding the same antigen molecule decreases. With fewer branch points, the product is more likely to consist of long chains of alternating antibody and antigen molecules in the molar ratio of 1:1. At even higher antigen ratios, each antigen molecule will have 0 or 1 antibody bound, so shorter chain lengths are found, until the product is small enough to remain in solution. Such soluble antigen-antibody “immune complexes” are detectable in the antigen excess zone, where no free antibody is found.

Besides explaining the observed precipitation phenomena on a statistical basis, the lattice theory made the important prediction that antibodies are bivalent or multivalent. The subsequent structural characterization of antibodies revealed their molecular weight and valency. Antibodies are indeed bivalent, except for IgM, which is functionally pentavalent and forms precipitates even more efficiently.

Antigens can be polyvalent either by having multiple copies of the same determinant or by having many different determinants, each of which reacts with different antibodies in a polyclonal antiserum. The predominant antigenic determinants of polysaccharides are often the nonreducing end of the chain. Branched chain polysaccharides have more than one end and are polyvalent. Nonbranched chains such as dextran (polymer of glucose) are monovalent for end specific antidextran antibodies and do not precipitate them.[85] However, a second group of antidextran antibodies is specific for internal glucose moieties. Because each dextran polymer consists of many internal units, it is polyvalent for

internal α(1 → 6)-linked glucose-specific antibodies. Thus, unbranched dextran polymer can be used to distinguish between end-specific and internal-specific antibodies, as it will precipitate with the latter antibodies but not the former.[85,86] Monomeric protein antigens, such as myoglobin or lysozyme, behave as if they were polyvalent for heterogeneous antisera but monovalent for monoclonal antibodies. This results from the fact that each antigen molecule has multiple antigenic determinants but only one copy of each determinant. Thus, a polyspecific antiserum can bind more than one antibody to different determinants on the same molecule and form a lattice. However, using antibodies directed against a single determinant (such as a monoclonal antibody), no precipitate will form. In this case, antigen-antibody reactions must be measured by some other binding assay, such as RIA or ELISA.

Immunodiffusion and the Ouchterlony Method

One of the most useful applications of immunoprecipitation is in combination with a diffusion system.[87] Diffusion could be observed by gently adding a drop of protein solution to a dish of water without disturbing the liquid. The rate of migration of protein into the liquid is proportional to the concentration gradient times the diffusion coefficient of the protein according to Fick's law,

$$\frac{dQ}{dt} = -DA\frac{dc}{dx} \qquad (43)$$

where Q = amount of substance that diffuses across an area A per unit time t; D = diffusion coefficient, which depends on the size of the molecule; and dc/dx = concentration gradient. Because antibody molecules are so large, their diffusion coefficients are quite low, and diffusion often takes 1 day or more to cover the 5 to 20 mm required in most systems. In order to stabilize the liquid phase for such long periods of time, a gel matrix is added to provide support without hindering protein migration. In practice, 0.3% to 1.5% agar or agarose will permit migration of proteins up to the size of antibodies while preventing mechanical and thermal currents. By carefully adjusting the concentration of antibody and antigen, these systems can provide a simple analysis of the number of antigenic components and the concentration of a given component. By adjusting the geometry of the reactants entering the gel, immunodiffusion can provide useful information concerning antigenic identity or difference, or partial cross-reaction, as well as the purity of antigens and the specificity of antibodies.

In single diffusion methods,[88,89,90,91] antibody is incorporated in the gel, and antigen is allowed to diffuse from one end of a tube gel in one dimension or from a hole in a gel in a petri dish in two dimensions. Over time, the antigen concentration reaches equivalence with the antibody in the gel, and a precipitin band forms. As more antigen diffuses, antigen excess is achieved at this position, so the precipitate dissolves and the boundary of equivalence moves farther. By integrating Fick's law, we find that the distance moved is proportional to the square root of time. If two species of antigen a and b are diffusing, and the antiserum contains antibodies to both, two independent bands will form. These will move at independent rates, depending on antigen concentration in the sample, diffusion coefficient (size), and antibody concentration in the agar. Similarly, in the two-dimensional method, at a given radius of diffusion, antigen concentration will be equivalent to the antibody in the gel, and a precipitin ring will form. The higher the initial antigen concentration, the farther the antigen will diffuse before precipitating and the wider the area of the ring will be. The area of the ring is directly proportional to the initial antigen concentration. This method provides a convenient quantitative assay that can be used to measure Ig classes by placing test serum in the well and antiserum to each class of human Ig in the agar. Sensitivity can be increased by lowering the concentration of antiserum in the gel, giving wider rings, as the antigen must reach a lower concentration to be at equivalence. However, the antiserum cannot be diluted too far, or no precipitate will form.

The double diffusion methods use the same principles, except that instead of having one reactant incorporated in the gel at a constant concentration, both antigen and antibody are loaded some distance apart in a gel of pure agarose alone and are allowed to diffuse toward each other. At some point in the gel, antigen diffusion and antibody diffusion will provide sufficient concentrations of both reactants for immunoprecipitation to occur. The line of precipitation becomes a barrier for the further diffusion of the reactants, so the precipitin band is stable. If the antigen preparation is heterogeneous, and the antiserum is a heterogeneous mixture of antibodies, different bands will form for each pair of antigen and antibody reacting at positions dependent on concentration and molecular weight of each. The number of lines indicates the number of antigen-antibody systems reacting in the gel. The ability of

immunodiffusion to separate different antigen-antibody systems gives a convenient estimate of antigen purity or antibody specificity.

In the most widely used Ouchterlony method of double diffusion in two dimensions,[87] three or more wells are cut in an agarose gel in a dish in the pattern shown in Figure 7.14. Antigen a or b is placed in the upper wells, whereas antiserum containing anti-a or anti-b is placed in the lower well. Each antigen-antibody reaction system will form its own precipitin line between the wells. As shown in Figure 7.14A, this should extend an equal distance to the left and right of the wells. When different antigens are present in different wells (see Fig. 7.14C), the precipitating systems do not interact immunochemically, so the precipitin lines cross. However, when the same antigen is present in both wells (see Fig. 7.14B), each line of precipitation becomes a barrier preventing the other antigen and antibody from diffusing past the precipitin line. This shortens the precipitin line on that side of the well. In addition, antigen diffusion from the neighboring wells shifts the zone of antigen excess, causing the equivalence line to deviate downward and meet between the two wells. Complete fusion of precipitin lines with no spurs is called a line of identity, indicating that the antigen in each well reacts with all the antibody capable of reacting with antigen in the other well.

The analytical power of this method is shown in Figure 7.14D. When a mixed antigen sample is placed in one well and pure antigen b is placed in the other well, antiserum to a plus b gives the pattern shown. Two precipitin lines form with the left well and one precipitin line with the right well. The line of complete fusion allows us to identify the second band as antigen b; the first band is antigen a. From their relative distance of migration, we can conclude that antigen a is in excess over antigen b, assuming their diffusion coefficients are comparable and both antibodies are present in equal amounts. Finally, because the precipitin line of antigen a-anti-a is not shortened at all, there is no contamination of the right sample with antigen a, and the two antigens do not cross-react.

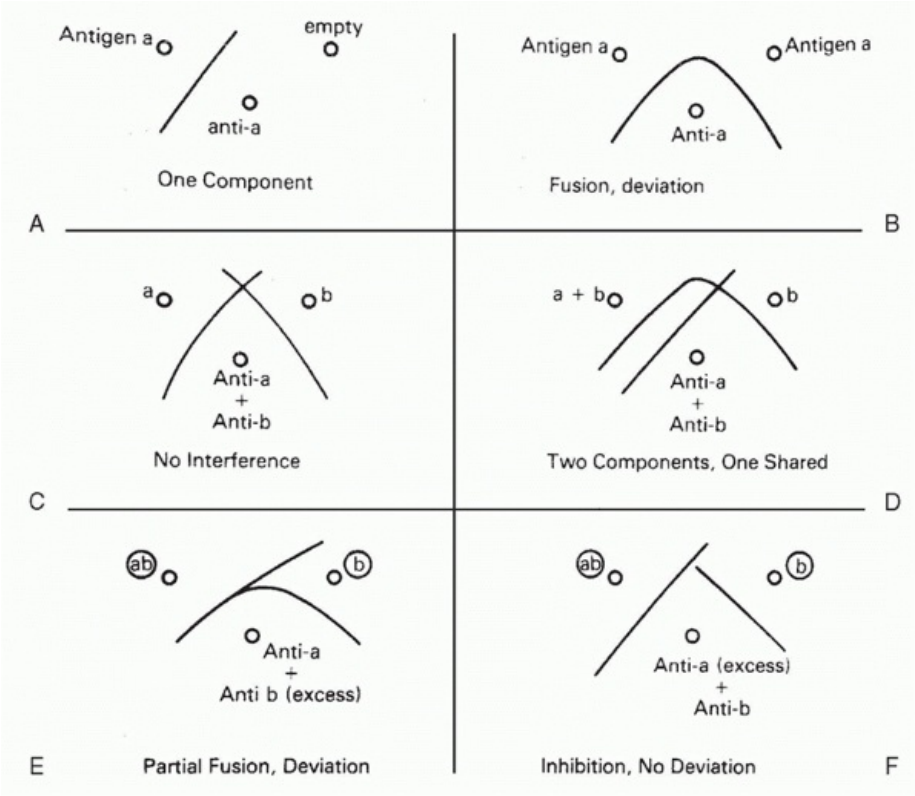

FIG. 7.14. Immunodiffusion of Two Components in Two Dimensions. Cross-reactions produce inhibition *(shortened bands)* or deviation *(curved bands)*. Lines of identity are shown in **(B)** and **(D)**.[87]

The type of cross-reactivity detected by this Ouchterlony double immunodiffusion in agar is what we have defined previously as type 2 cross-reactivity. The method is not suitable for measuring affinity differences required for quantitating type 1 cross-reactivity. Sensitivity can be increased by use of radioactive antigen and detection of the precipitate by autoradiography.

Immunoelectrophoresis

Some antigen-antibody systems are too complex for double immunodiffusion analysis, either because there are too many bands or they are too close together. Immunoelectrophoresis combines electrophoresis in one dimension (Fig. 7.15) with immunodiffusion in the perpendicular direction. In the first step, electrophoresis separates the test antigens according to charge and size, in effect, separating the origin of diffusion of different antigens. This is equivalent to having each antigen start in a different well, as shown in the right-hand panel. A horizontal trough is then cut into the agar and filled with antiserum to all the components. Immunodiffusion occurs between the separated antigens and the linear source of antibody. The results for a mixture of three antigens approximate those shown for three antigens in separate wells.87 Fusion, deviation, and inhibition between precipitin lines can be analyzed as described previously. The resolution of each band is somewhat decreased due to widening of the origin of diffusion during electrophoresis. However, the immunodiffusion of unseparated human serum proteins, for example, is greatly facilitated by prior electrophoresis. Starting from a single well, only the heavier bands would be visible.

However, prior electrophoresis makes it possible for each electrophoretic species to make its own precipitin line. Monospecific antiserum can be placed in a parallel horizontal trough so that each band of precipitation can be identified. Immunoelectrophoresis is commonly used to diagnose myeloma proteins in human serum. The unknown serum is electrophoresed, followed by immunodiffusion against antibodies to human Ig heavy or light chains. A widening arc of IgG suggests the presence of an abnormal Ig species. At this same electrophoretic mobility, a precipitin line with anti-κ, but not anti-λ, reactivity strongly suggests the diagnosis of myeloma or monoclonal gammopathy, as these proteins arise from a single clone that synthesizes only one light chain. All normal electrophoretic species of human Igs contain both light chain isotypes, although κ exceeds λ by the ratio of 2:1 in humans. As shown in Figure 7.15C, the abnormal arc with γ mobility reacts

with anti-IgG and anti-κ but not anti-λ antiserum. Thus, it is identified as an IgG-κ monoclonal protein.

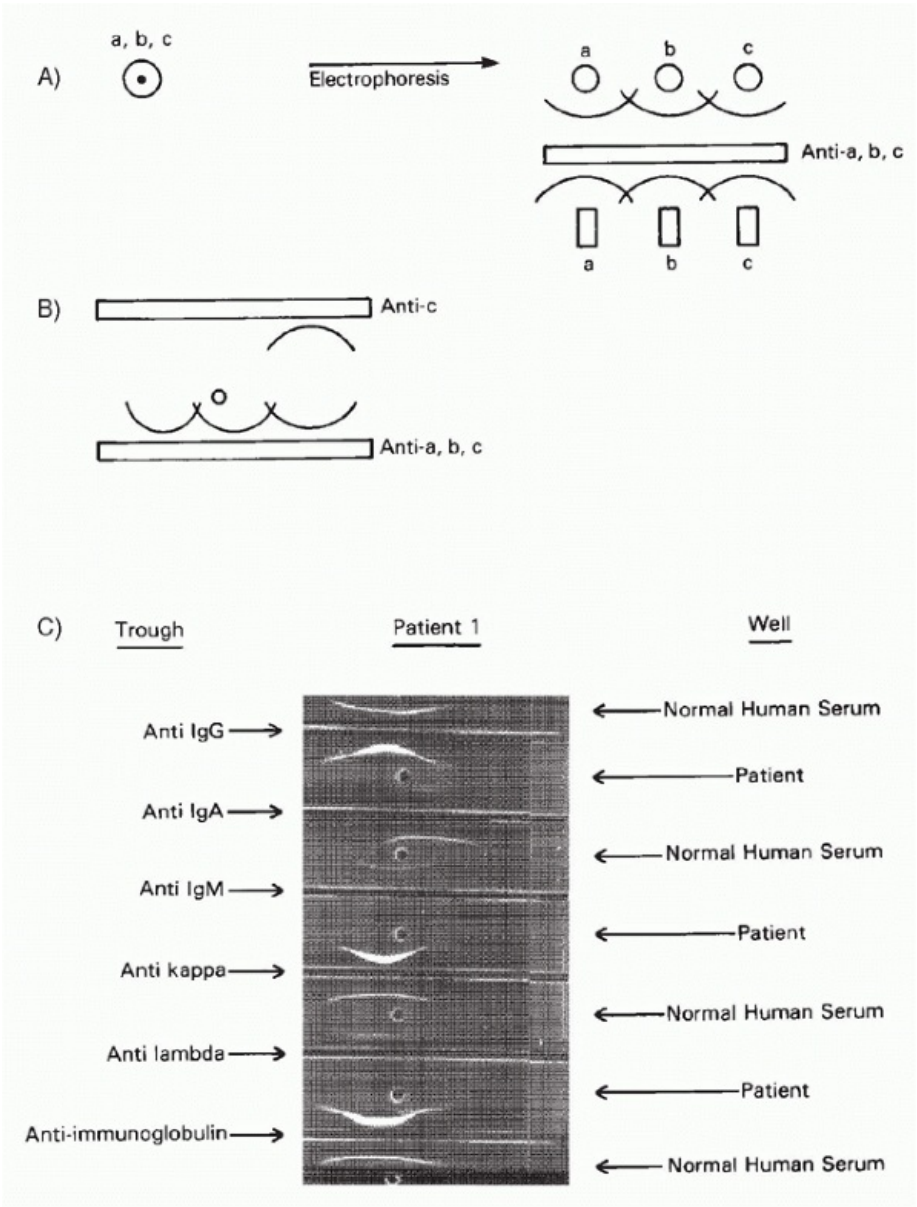

FIG. 7.15. Immunoelectrophoresis. A sample containing multiple components is

electrophoresed in an agarose gel, separating the antigens in the horizontal dimension. Then a horizontal trough is cut into the gel and antiserum is added. Immunodiffusion between the separated antigens and the trough is equivalent to having separate wells, each with a different antigen.[87] This technique is used to identify a myeloma protein in human serum. Sera from the patient or normal individual were placed in the circular wells and electrophoresed. Antisera were then placed in the rectangular troughs and immunodiffusion proceeded perpendicular to the direction of electrophoresis. The abnormally strong reaction with anti-immunoglobulin (Ig) G and anti-κ, but no reaction with anti-λ antibodies, indicate a monoclonal protein (IgG,κ), as polyclonal Ig should react with both antilight-chain antisera. Failure to form a band with anti-IgM and a reduced band with anti-IgA show typical reduction of normal Igs in this disease. (Photographs courtesy of Theresa Wilson, NIH Clinical Chemistry Section.)

Hemagglutination and Hemagglutination Inhibition

Hemagglutination

A highly sensitive technique yielding semiquantitative values for the interaction of antibody with antigen involves the agglutination by antibodies of red blood cells coated with the antigen.[92] For exogenous antigens that are adsorbed to the red blood cell surface, the reaction is called passive hemagglutination. Untreated red blood cells are negatively charged, and electrostatic forces keep them apart. Following treatment with tannic acid (0.02 mg/mL for 10 minutes at 37°C), however, they clump readily.

Untreated red blood cells are easily coated with polysaccharide antigens, which they adsorb readily. After tanning, the uptake of some protein antigens is good, giving a sensitive reagent, whereas for others it tends to be quite variable; coating red blood cells has been the limiting factor in the usefulness of this method for certain antigens. Apparently, slightly aggregated or partially denatured protein antigens are adsorbed preferentially.[92]

The test for specific antibodies is done by serially diluting the antiserum in the U-shaped wells of a microtiter plate and adding antigen-coated red cells. When cross-linked by specific antibodies, agglutinated cells settle into an even carpet spread over the round bottom of the well. Unagglutinated red cells slide down the sides and form a much smaller button at the very bottom of the well. The titer of a sample is the highest dilution at which definite agglutination occurs. With hyperimmune antisera, inhibition of agglutination is often observed at high doses of antibody, termed a *prozone effect*. Two interpretations have been offered: One is that, at great antibody excess, each cell is coated with antibody, so cross-linking by the same antibody molecule becomes improbable. The second interpretation is the existence of some species of inefficient or “blocking” antibodies that occupy

antigen sites without causing aggregation of cells.[9] To assure antigen specificity, the antiserum should be absorbed against uncoated red cells prior to the assay, and an uncoated red cell control should be included with each assay. IgM is up to 750 times more efficient than IgG at causing agglutination, which may affect interpretation of data based on titration. The titer may vary by a factor of 2 simply due to subjective estimates of the endpoint.

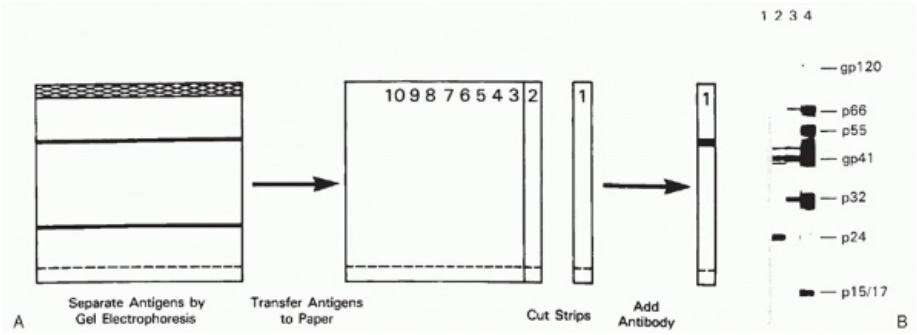

FIG. 7.16. A: Western blot technique. The antigen preparation is run through a polyacrylamide gel, which separates its components into different bands. These bands are then transferred to paper by electrophoresis in the horizontal dimension. The paper is cut into strips. Each strip is incubated with test antibodies, followed by further incubation with enzyme-labeled second antibodies. If the test antibodies bind to the component antigens, they will produce discrete dark bands at the corresponding positions on the strip. **B:** Clinical specimens from patients with acquired immunodeficiency syndrome tested on strips bearing human immunodeficiency virus-1 viral antigens, showing antibodies to viral gag (p15/17, p24, and p55 precursor), pol (p66 and possibly p32), and env (gp41 and gp120) proteins. Lane 1 is the negative control, and lanes 2 to 4 are sera from three different patients.

Once the hemagglutination titer of an antiserum is determined, its interaction with antigen-coated red blood cells can be used as a sensitive assay for antigen. To constant amounts of antibody (diluted to a concentration twofold higher than the limiting concentration producing agglutination) are added varying amounts of free antigen. Agglutination will be inhibited when half or more of the antibody sites are occupied by free antigen. In a similar fashion, the assay can be used for the detection and quantitation of anti-idiotype antibodies that react with the variable region of antibodies and sterically block antigen binding.

Immunoblot (Western Blot)

A most useful technique in the analysis of proteins is polyacrylamide gel electrophoresis, in which charged proteins migrate through a gel in response to an electric field. When ionic detergents such as sodium dodecyl sulfate are used, the distance traveled is inversely proportional to the logarithm of molecular weight. The protein components of complex structures, such as viruses, appear as distinct bands, each at its characteristic molecular weight. Because antibodies may be unable to diffuse into the gel, it is necessary to transfer the protein bands onto a nitrocellulose membrane support first, where they are exposed for antibody binding.[93]

The immunoblot is often used to detect viral proteins with specific antibodies that bind these proteins on the nitrocellulose blot. Then a second antibody, which is either enzyme conjugated or radiolabeled, is used to detect the antigen -antibody band. Crude viral antigen preparations can be used, as only those bands that correspond to viral antigens will be detected.

Typical results are shown in Figure 7.16. HIV-1 was cultured in susceptible H9 cells. The viral proteins were separated by polyacrylamide gel electrophoresis and detected by immunoblot, using the serum of infected patients. Each antigen band recognized by the antiserum has been identified as a viral component or precursor protein. The gp160 precursor is processed to mature gp120 and gp41 envelope proteins, whereas a p66 precursor is processed to the p51 mature form of reverse transcriptase, and a p55 precursor becomes the p24 and p17 gag and matrix proteins of the virus.[94] The practical uses of the HIV western blot include diagnosing infection, screening blood units to prevent HIV transmission, and testing new vaccines.

Surface Plasmon Resonance

Surface plasmon resonance (SPR) uses the electromagnetic properties of light to measure the binding affinity of a variety of biologic molecules, including antigen-antibody pairs. In this method, polarized light passes through a glass plate coated on the back surface with a thin metal film, usually gold. Biologic materials binding to the metal film behind the plate can alter its refractive index in ways that affect the angle and intensity of reflected light.

At angles close to perpendicular, light will pass through the glass, although it will bend at the interface due to

differences in the refractive index of glass and what is behind it. Above a certain angle, called the critical angle, bending will be so great that total internal reflection will occur. Small changes in refractive index behind the glass can be detected as significant changes in the critical angle, where light reflection occurs, and in the intensity of reflected light at this angle. By reading the reflected light intensity in a diode array detector, the critical angle and intensity can be determined simultaneously. Due to the wave nature of light, the effect of refractive index in the gold film extends about one wavelength beyond the glass or about 300 to 700 nm.[95] Within this layer, if an antigen is covalently attached to the gold, then antibody binding can be detected as a change in refractive index, resulting in a different critical angle and intensity of the reflected light.

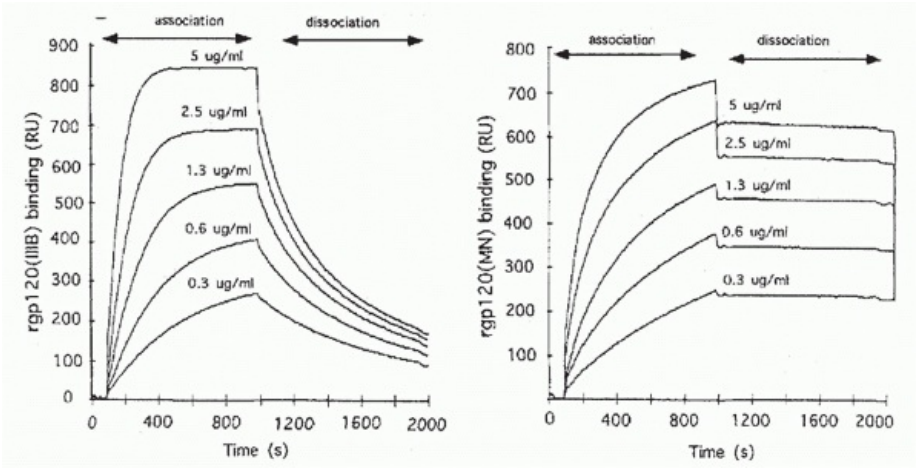

FIG. 7.17. Monoclonal Antibody to gp120 was Introduced into the Flow Cell at Time 0, and Antibody Binding was Followed Over Time as a Change in the

Critical Angle, Measured in Response Units. After 1000 seconds, free antibody was washed out, and the release of bound antibody was measured as a decrease in refractive index. Lower affinity binding to gp120 from the IIIB strain *(left)* was shown as a faster "off rate," as compared to the very slow rate of antibody release from the MN strain *(right)*. These results, obtained under nonequilibrium conditions, provide direct measurement of the forward and reverse rate constants for antibody binding, and the ratio of these two gives the affinity constant. Modified from VanCott et al.,[97] with permission.

SPR systems have three essential features[96]: an optical system that allows determination of the critical angle and light intensity at the same time, a coupling chemistry that links antigen or antibody to the gold surface, and a flow system that rapidly delivers the binding molecule in the mobile phase, so SPR can measure the rate of binding, rather than the rate of diffusion. Because binding causes a physical change in the gold film, there is no need for radioactive labels or enzyme conjugates. Molecular binding interactions can be followed in real time.

A typical SPR experiment is shown in Figure 7.17. HIV gp120 of type IIIB (*left panel*) or MN (*right panel*) were fixed to the gold layer, and various concentrations of monoclonal antibody to gp120 were added to the flow cell.[97] Over the first 1000 seconds, antibody binding was measured as a change in reflected light (in response units), allowing a calculation of the rate constant for the forward reaction of antibody binding. Once the signal reached a plateau, antibody was washed out of the flow cell, and the decrease in SPR signal over time indicated the rate at which antibody came off the antigen. The "on rate" for antibody binding to IIIB gp120 (*left*) was about twice as fast as for MN (*right*) at each antibody concentration. However, the "off rate" was about 50-fold less for MN than for IIIB. Combining these kinetic results indicates much greater binding affinity for gp120 of MN type, which may explain the observation that MN type virus was 10-fold more sensitive to neutralization by this antibody than was the IIIB strain.

MONOCLONAL ANTIBODIES

Homogeneous Igs have long played important roles in immunologic research. Starting in the 1950s, Slater et al.[98] studied sera from human patients with multiple myeloma and recognized the relationship between abnormal myeloma proteins and normal serum globulins. Potter[99] characterized numerous mouse myeloma tumors and identified the antigenic specificities of some of them. Human and mouse myeloma proteins were studied as representative Igs

and recognized for the advantages they had with proteins as diverse as antibodies for studies of Ig structure, function, and genetics. It was not yet possible, however, to induce monoclonal Igs of desired specificity.

This goal was achieved by the introduction of hybridoma technology by Köhler and Milstein[1,100] and by Margulies et al.[101] in the 1970s. Since that time, monoclonal antibodies have come to play an enormous role in biologic research and applications. They offer as

advantages the relative ease of the production and purification of large quantities of antibody, the uniformity of antibody batches, and the ready availability of Ig messenger ribonucleic acid and DNA from the hybrid cell lines.

Derivation of Hybridomas

Hybridomas producing monoclonal antibodies are generated by the somatic cell fusion of two cell types: antibodyproducing cells from an immunized animal, which by themselves die in tissue culture in a relatively short time, and myeloma cells, which contribute their immortality in tissue culture to the hybrid cell. The myeloma cells are variants carrying drug selection markers so that only those myeloma cells that have fused with spleen cells providing the missing enzyme will survive under selective conditions. Initial work used myeloma cells that secreted their own Ig products, but later such fusion partners were replaced by myeloma variants that fail to express Ig[102,103] so that the fused cell secretes exclusively antibody of the desired specificity. Successful hybridoma production is influenced by the characteristics of the cell populations (immune lymphocytes and myeloma fusion partner), the fusion conditions, and the subsequent selection and screening of the hybrids. A diagrammatic version of the overall process of hybridoma derivation is presented in Figure 7.18.

This section will not attempt to provide a detailed, stepby-step protocol for laboratory use. For that purpose, the reader is referred to monographs and reviews on the subject, including a detailed laboratory protocol with many hints and mention of problems to avoid.[104]

Hybridomas Derived from Species Other than Mice

Laboratory mice are the most common species immunized for hybridoma production, but for a variety of reasons, other animal species often have advantages. If an antigen of interest is nonpolymorphic in the mouse, the mouse component might be immunogenic in other species while mice would be tolerant to it. In the case of hybridomas for clinical use, mouse antibodies have the drawback of inducing antimouse Ig immune responses with possible deleterious effects.

Several approaches have been taken to the derivation of hybridomas in species other than mouse. First, interspecies hybridization can be performed using mouse myeloma fusion partners. The resulting hybrids are often unstable and throw off chromosomes, but clones can sometimes be selected that produce antibody in a stable fashion. Examples of this would be rat-mouse fusion to produce antibody to the mouse Fc receptor[105] and hamster-mouse fusion to produce antibody to the mouse CD3 equivalent.[106] Rabbit-mouse hybridomas have also been described.[107]

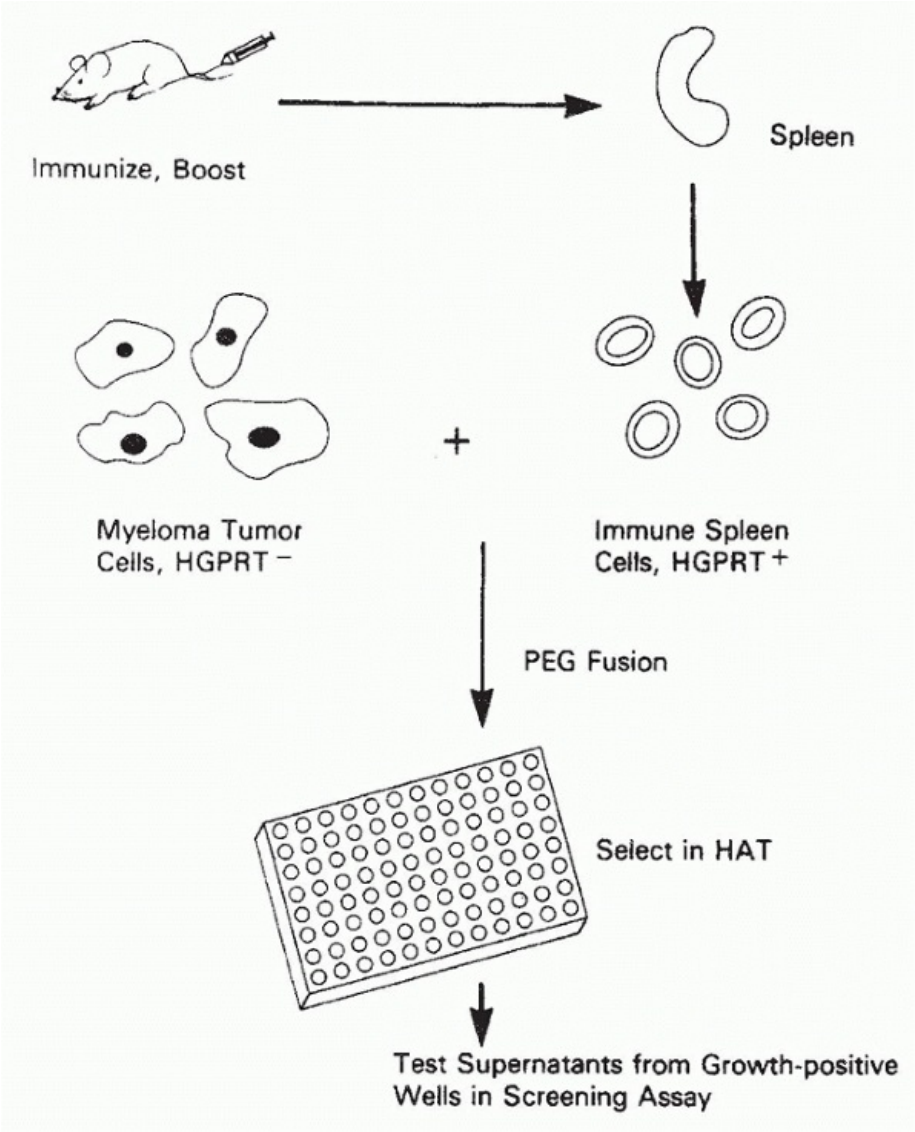

FIG. 7.18. Production of Hybridomas. Steps in the derivation of hybridomas can be outlined as shown. Spleen cells from immunized donors are fused with myeloma cells bearing a selection marker. The fused cells are then cultured in selective medium until visible colonies grow, and their supernatants are then screened for antibody production. HAT, hypoxanthine, aminopterin, thymidine; HGPRT, Hypoxanthine-guanine phosphoribosyltransferase; PEG, polyethylene glycol.

A second approach is the use of fusion partner cells from the desired species. Myeloma variants carrying drug selection markers are available in a number of species. A rat myeloma line adapted for this purpose, IR983F, was described by Bazin.[108] Production of human hybridomas is of special importance because their use in therapies would avoid the problem of human immune responses to Ig derived from other animal species, as discussed in detail in the later section on applications.

Use of Gene Libraries to Derive Monoclonal Antibodies

Monoclonal antibodies produced by hybridoma technology are derived from B cells of immunized animals. A recent alternative technology uses gene libraries and expression

systems instead. This approach has the advantages of avoiding laborintensive immunizations of animals and the screening of antibody-containing supernatants. Another advantage of the approach is circumventing tolerance. One can derive monoclonal antibodies to antigens expressed in the animal species

that donated the gene library, including highly conserved antigens for which there may be no available responder that does not express the antigen.

The first version of such an approach involved preparation of VH and Vκ libraries and expression of the libraries in bacteria. Further development of the system led to use of VH and VL libraries (see Chapters 5 & 6) made separately, and then preparation of a combinatorial library by cleaving, mixing, and religating the libraries at a restriction site.[109,110] A linker can be used so that VH and VL can both be expressed on one covalent polypeptide; the flexibility of the linker allows association of the VH and VL in a normal three-dimensional configuration and thus formation of an antigen-binding site.[110]

Another innovation involves expression of V_H and Vκ genes on the surface of bacteriophage as fusion proteins with a phage protein to permit rapid screening of large numbers of sequences.[110,111,112] Adsorption of antibody-bearing phage on antigen-coated surfaces allows positive selection of phage containing DNA encoding the desired variable region fragment (Fv) from combinatorial variable region gene libraries.[111,112]

Human antibody gene sequences can be recovered by polymerase chain reaction (PCR) from peripheral blood cells,[113] bone marrow,[114] or human cells reimmunized in severe combine immunodeficiency-hu mice.[115] The phage display technique can then be used to select antigen-binding clones and derive human reagents of desired specificity, such as antibody to hepatitis surface antigen[113] or HIV envelope.[114]

One limitation in the phage library technique initially was low affinity of the monoclonal antibodies derived because they were generated by a random process and not subject to further somatic mutation. Several approaches have now been used to improve affinities. Hypermutation and selection has now been achieved in vitro by a strategy using a bacterial mutator strain.[116] The process involves multiple rounds of mutation followed by growth in nonmutator bacteria and then selection for high-affinity binding led to an overall 100-fold increase in affinity.[116] Improved affinity has also been achieved by use of site-directed mutagenesis to alter residues in hypervariable regions affecting dissociation rates.[117]

Recombinant Monoclonal Antibodies Derived from Single B Cells After Cell Sorting

A new and very productive approach to making monoclonal antibodies is based on amplification of both the V_H and V_L chains of single B cells by reverse transcription-PCR.[118,119] In the first step, B cells are sorted by fluorescence-activated cell sorting, based on antigen specificity or other characteristics, and then distributed in 96 well plates at a dilution of one B cell per well. The cells are lysed, followed by reverse transcription of the messenger ribonucleic acid of a single cell and amplification of the V_H and V_L chains by nested PCR of each chain separately. The two chains are cloned into expression vectors in

frame with the appropriate constant regions. Both chains are coexpressed to reconstitute the antibody. The resulting monoclonal antibodies are then analyzed for sequence, antigen specificity, affinity, and biologic activity. The efficiency of antibody production has been as high as 30% to 60% per well in different experiments.

Advantages include the ability to focus on a particular B-cell compartment, such as memory B cells, during the sorting step, the large numbers of monoclonals produced in a single experiment (typically more than 100), and the absence of antigen boosting prior to cloning. Due to the high efficiency of cloning, these monoclonals are thought to represent the expressed B-cell repertoire at a given time, location, and stage of differentiation. For example, human monoclonals had the same ratio of kappa to lambda light chains (60% to 40%) as found in circulating B cells, and they showed allelic exclusion in 95% of wells. Key features of the method include the choice of fluorescent antigen for B-cell sorting, called the "bait," and the choice of primers for nested PCR. The bait should capture as many antigen specific B cells as possible; different bait will capture different monoclonals from similar populations of B cells (compare Pietzsch et al.[120] and Scheid et al.[121]). A complex bait with multiple epitopes, such as HIV gp140, will capture antibodies to different parts of the antigen.[119,122,123] In order to amplify highly variable antibodies, the forward (and reverse) PCR primers should be chosen upstream (or downstream) of the V_H region so they will capture sequence variation near both ends of the coding sequence.

In one study, 1.5 × 105 memory B cells from a chronically HIV-infected subject were sorted based on binding to a stabilized form of gp120.[121] Individual antigen-specific B cells were distributed in microtiter wells, and they produced 576 monoclonal antibodies that bound gp120 by ELISA. Sequencing the V_H and V_L chains showed that each monoclonal was often clonally related to several others, which differed only by somatic mutation. Overall, these monoclonals represented 200 distinct B-cell clones. The monoclonals could be divided into groups, based on binding four distinct epitopes on gp120. Within the same clonally related group, some monoclonals had potent HIV-neutralizing activity while other closely related monoclonals were inactive. The B-cell donor was a long-term nonprogressor. The analysis of a significant sampling of the antibody repertoire may provide insight into how these patients control their circulating virus, and these findings may lead to improved vaccine antigens.

Applications of Monoclonal Antibodies

Because monoclonal antibodies can be made easily and reproducibly in large quantities, they allow many experiments that were not possible or practical previously. Affinity chromatography based on monoclonal antibodies can be used as a step in purification of molecular species that are difficult to purify chemically. Homogeneous antibody can be crystallized and can also be crystallized together with antigen to permit the study of the structure of antibody and of antigen -antibody complexes by x-ray diffraction. Homogeneous antibodies are also valuable in the study of antibody diversity. Such analyses have revealed much about the roles of somatic mutation, changes in affinity, and changes in clonal dominance in antibody responses.

Catalytic Antibodies

One area of recent interest is the use of antibody molecules to catalyze chemical reactions.[124] In this role, antibodies serve as an alternative to enzymes, an alternative that can be customized and manipulated more easily in some cases.

The concept of antibodies as catalysts had been proposed a long time ago by Woolley.[124] Use of homogeneous antibodies permitted identification of some with significant catalytic effects; MOPC167 accelerates the hydrolysis of nitrophenyl phosphorylcholine by 770-fold.[125] Polyclonal antibodies have also been reported to possess detectable enzymatic activity.[126] With the advent of hybridoma technology, purposeful selection of antibodies with potent enzymatic function became possible. Antibodies have been characterized that catalyze numerous chemical reactions, with rates nearing 10^8-fold above the spontaneous rate.[124] One common strategy for elicitation of such antibodies is immunization with transition state analogs,[127] although there are other strategies.[128] Antibodies function as catalysts in a stereospecific manner,[129] a valuable property.

Molecular mechanisms of antibody-mediated catalysis vary, as do enzymatic reactions.[128,130] To accelerate a reaction, an antibody has to lower the activation energy barrier to the reaction, which means lowering the energy of the transition state by stabilizing it. For this reason, an antibody that recognizes the transition state is favorable, and immunizations with analogs of the transition state have advantages.

Antibodies can serve as what has been termed an *entropy trap*[124]; binding to the antibody "freezes out" the rotational and translational degrees of freedom of the substrate and thus makes a chemical reaction far more favorable energetically. Interactions with chemical groups on the antibody can neutralize charges or bury hydrophobic groups, thereby stabilizing a constrained transition state.

Discovery of such catalytic antibodies opens practical opportunities: Antibodies can be customized for an application by appropriate selection and can be produced relatively cheaply and purified easily. Catalytic antibodies can be developed to perform chemical reactions for which no enzyme is available. They can shield intermediates from solvent, for example, allowing reactions that do not occur in aqueous solution.[131] They can form peptide bonds,[132] suggesting a new approach to polypeptide synthesis. Thus, catalytic antibodies will likely have many practical applications.

Bispecific and Bifunctional Antibodies

Antibodies produced naturally by a single B cell have only one binding site specificity, and their effector functions are determined by the structure of the Fc domain. The availability of monoclonal antibodies made possible the generation in quantity of artificial antibodies as cross-linking reagents by linking binding sites of two specificities to form bispecific antibodies. A variety of techniques have been used to prepare such hybrid or bispecific antibodies, and they have been put to a variety of uses. In addition, antibody-binding sites can be linked to other functional domains such as toxins, enzymes, or cytokines to create "bifunctional antibodies."[133]

One of the most powerful uses of hybrid antibodies is in redirecting cytolytic cells to targets

of a defined specificity. In one early demonstration of this use,[134] a monoclonal antibody specific for the Fcγ receptor and one specific for the hapten dinitrophenyl were chemically cross-linked. In the presence of this hybrid antibody, FcγR-bearing cells were able to lyse haptenated target cells specifically. The FcγR played a critical role; antibody to MHC class I antigens on the cell could not be substituted. Antibody to the T-cell receptor complex has also been used extensively to redirect T-cell lysis to desired targets. For example, anti-CD3 was cross-linked to antitumor antibodies and mixed with effector cells. These “targeted T cells” were able to inhibit the growth of human tumor cells in vivo in nude mice.[135] Bispecific antibodies have also been used recently to alter the tropism of a viral gene therapy vector to target specific cells.[136]

Cumbersome cross-linking chemistry can now be replaced by genetic engineering for creation of designer antibodies.[133] Bifunctional and bispecific antibodies can be engineered as single chain variable fragment constructs or by specialized strategies using two chains. A wide variety of configurations are possible and can be used to make multivalent reagents as well as reagents with one site of each specificity. Tags can be built in by fusion of additional sequence such as streptavidin, or, as mentioned previously, antibody domains can be combined with other functional domains such as toxins, enzymes, or cytokines.

Clinical Applications

The possible clinical uses of monoclonal antibodies are many. In vitro, they are widely used in RIA and ELISA measurements of substances in biologic fluids, from hormones to toxins. They are also extremely valuable in flow cytometric assays of cell populations using antibodies specific for differentiation antigens expressed on cell surfaces. Monoclonal antibodies plus complement or toxin-conjugated monoclonal antibodies have also been used to remove T cells from bone marrow prior to transplantation.[137]

In vivo, although it took more than two decades for the original promise of hybridoma technology to be translated into widespread clinical applications, a number of monoclonal antibodies are now in use or in trials for a variety of purposes.[138,139,140] Monoclonal antibody OKT3 directed to a marker on human T lymphocytes is used as a treatment for rejection reactions in patients undergoing kidney transplant.[141] Other monoclonal antibodies, for example, ^{111}In-labeled CYT-103 referred to as Oncoscint (Cytogen Corp, Princeton, NJ),[142] are used as diagnostic tumor imaging reagents. Monoclonal antibodies have now been approved for various therapeutic uses.[140,143] Cancer therapies use either unconjugated monoclonal antibody[138,144,145,146,147,148] or toxin-coupled[149,150] or radiolabeled monoclonal antibody.[144,148,151] Molecules targeted in cancer therapies include CD25 (IL-2 receptor alpha chain) in adult T-cell leukemia,[138,140,148,152,153,154] CD20 in non-Hodgkin lymphoma using either unlabeled[147,155] or more recently radionuclide-labeled anti-CD20,[156,157] the HER-2/neu oncoprotein in breast and

ovarian cancer,[145,146,158,159] CD22 in hairy cell leukemia,[150] vascular endothelial growth factor to limit angiogenesis in diverse tumors (especially colorectal, renal, and non-smallcell

lung cancer),[160,161,162,163,164,165] antiepidermal growth factor receptor in colorectal carcinoma and others,[166] anti-CD52 in chronic lymphocytic leukemia,[167] and anti-CD33 in acute myelogenous leukemia.[168] Other therapies studied include antilipopolysaccharide for treatment of sepsis, anti-IL-6 receptor for treatment of multiple myeloma and for rheumatoid arthritis, anti-IgE for treatment of allergy,[139] anti-tumor necrosis factor for treatment of arthritis,[169,170] anti-respiratory syncytial virus for prevention of respiratory syncytial virus morbidity and mortality in infants,[171,172] and anti-IL-2 receptor (CD25) for prevention of graft rejection[148,173] as well as for treatment of autoimmune diseases such as uveitis[174] and multiple sclerosis.[175] Other monoclonal antibodies have been developed to modulate immune responses. On the one hand, antibodies to block checkpoint inhibitors in the immune response, especially against cancer, have been developed, such as the first licensed one, ipilimumab, against cytotoxic T-lymphocyte antigen-4 (CD152), an inhibitory receptor expressed on activated T cells,[176,177,178,179,180,181] and anti-PD-1, another inhibitory receptor on activated T cells that is currently in trials.[182,183,184,185,186] An antibody to transforming growth factor-beta, a soluble inhibitor of immune responses, is also in clinical trials in both cancer and fibrosis.[187,188,189,190] On the other hand, monoclonal antibodies are being developed to amplify immune responses and synergize with vaccines, such as agonist anti-CD40 antibodies.[191,192,193]

In the specialized case of B-cell lymphoma, monoclonal anti-idiotypes against the idiotype expressed by the patient's tumor have been tested as a “magic bullet” therapy.[194] Active immunization of the patient with idiotype[195,196,197,198,199] has the advantage that escape mutants[200] are less likely to emerge because multiple idiotopes are recognized. Another approach under study is immunization using not idiotype as protein but plasmid DNA-encoding patient idiotype.[201] This approach would have additional advantages, such as ease of preparing customized reagents for each patient.

Production of Human or Humanized Monoclonal Antibodies

Many of the side effects of monoclonal antibodies in clinical use are due to the foreign Ig constant regions. Recognition of foreign Ig epitopes can lead to sensitization and so preclude subsequent use in the same individual of different monoclonal antibodies. Thus, monoclonal antibodies with some or all structure derived from human Ig have advantages. Several approaches have been taken employing fusion of human cells with animal myelomas or with human tumor cells of various kinds[202,203] and use of Epstein-Barr virus to immortalize antibody-producing cells.[204] Production of populations of sensitized human cells to be fused presents another special problem, as the donors cannot be immunized at will. In one example, in vitro stimulation of lymphocytes with antigen followed by fusion with mouse myeloma cells has been used to generate a series of antibodies to varicella zoster.[205]

Another approach to production of monoclonal antibodies with human characteristics involves application of genetic engineering. The part of the antibody structure recognized as foreign by humans can be minimized by combining human constant regions with mouse variable regions[206,207] or even just mouse hypervariable segments[208] by molecular genetic

techniques. Antigen-binding specificity is retained in some cases, and the “humanized” chimeric molecules have many of the advantages of human hybridomas.

Production of fully human monoclonal antibodies in transgenic mice has now been achieved by multiple laboratories. The strategy has involved insertion into the mouse germ line of constructs containing clusters of human Ig V, D, J, and C genes (see Chapter 6) to generate one transgenic line and targeted disruption of the mouse heavy chain and κ chain loci to generate another transgenic line. From these two lines, mice are then bred that express only human antibodies.

To show feasibility of this approach, cosmids carrying parts of the human heavy chain locus were used to make transgenic mice.[209] The next step was to produce mice carrying human genes for both heavy and light chains to generate a functional human repertoire. Several groups using different technologies constructed heavy chain miniloci containing functional V segments representing several major V region families, D and J segments, constant and switch regions, and enhancers. The κ chain constructs were made that contained multiple functional Vκ segments, the J segments, Cκ, and enhancers.[210,211] Mice were bred that were homozygous both for the transgene loci and for disruption of the mouse heavy chain and κ light chain loci; note that the mouse λ locus was left intact. The human Ig genes could rearrange in the mouse genome, and expression of human Ig resulted. If these mice were immunized with a fragment of tetanus toxin, resulting antibodies included some that were fully human.[211] In one of the studies,[210] serum contained human μ, γ1, and κ as well as mouse λ and γ. Immunization of such mice with various antigens led to class switching, somatic mutation, and production of human antibodies of affinities of almost 10^8.

Ig expression in these mice demonstrates cross-species compatibility of the components involved in antibody gene rearrangement and diversification. The mice also provide a responder able to provide fully human antibodies to clinically important antigens, and they have the advantage that they are not tolerant to human antigens, such as the human IgE and human CD4 used by Lonberg et al.[210]

Nucleotide Aptamers: An Alternative to Monoclonal Antibodies

Antibodies are not the only biologic macromolecules that have evolved to permit an enormous range of specific structures. Oligonucleotides selected for ability to bind a ligand with high affinity and specificity are termed “aptamers” and can be used in many of the ways antibodies have been used. Selection, properties, and uses of aptamers have been reviewed.[212] Aptamers have the advantage that their

production does not require animals or cell culture. These well-defined reagents may be used increasingly in diagnostic testing and are also being tested in clinical trials for use as imaging agents or therapeutics.

Specificity and Cross-Reactivity

Specificity of Monoclonal Antibodies

Because all the molecules in a sample of monoclonal antibody have the same variable region structure, barring variants arising after cloning, they all have the same specificity. This

uniformity has the advantage that batches of monoclonal antibody do not vary in specificity as polyclonal sera often do. The most obvious fact about cross-reactions of monoclonal antibodies is that they are characteristic of all molecules and cannot be removed by absorption without removing all activity. An exception would be an apparent cross-reaction due to a subset of denatured antibody molecules, which could be removed on the basis of that binding. The homogeneity of monoclonal antibodies allows refinement of specificity analysis that was not possible with polyclonal sera. A few examples follow.

First, one can use monoclonal antibodies to distinguish closely related ligands in cases where most antibodies in a polyclonal serum would cross-react, and so absorption of a serum would not leave sufficient activity to define additional specificities. This ability is useful in designing clinical assays for related hormones, for example. Such fine discrimination also allows the definition of new specificities on complex antigens. When large numbers of monoclonal antibodies specific for class I and class II MHC antigens were analyzed, some defined specificities that could not be defined with existing polyclonal antisera.[213,214,215]

On the other hand, monoclonal antibodies are also a powerful tool for demonstrating similarities rather than distinctions between two antigens. In some cases, only a minor portion of an antibody response detects a cross-reaction, and so it is not detected by polyclonal reagents. For example, determinants shared by the I-A and I-E class II MHC antigens in the mouse were demonstrated using monoclonal antibodies215 while they had been suspected but were difficult to demonstrate using polyclonal sera.

Another type of fine specificity analysis possible only with monoclonal antibodies is the discrimination of spatial sites (epitope clusters) by competitive binding. In some cases, such epitope clusters correspond to specificities that are readily distinguished by other means. However, in other cases, the epitope clusters may not be distinguishable by any serologic or genetic means. An example is the splitting of the classical specificity Ia.7 into three epitope clusters by competitive binding with monoclonal antibodies.[215] The epitopes cannot be distinguished genetically, as all three are expressed on cells of all Ia.7-positive mouse strains. Thus, polyclonal sera cannot be absorbed to reveal the different specificities. Only with the use of monoclonal antibodies were the epitopes resolved from each other.

The importance of this type of analysis is shown by another example, the definition of epitope clusters on CD4, a surface molecule on a subset of human T cells that also functions as the receptor for HIV. Monoclonal antibodies to CD4 can be divided into several groups based on competitive inhibition.[216] The cluster containing the site recognized by OKT4A is closely related to virus infection, as antibodies to this site block syncytium formation. The cluster recognized by OKT4, however, is not related to infection because antibodies to it do not block syncytium formation,[216] and cells expressing variant forms of the CD4 molecule lacking the OKT4 epitope can still be infected by HIV.[217] This information about the sites on the molecule is important in understanding the molecular interactions of virus with its receptor and may be useful in designing vaccine candidates.

While most antibodies are not MHC-restricted in their recognition of antigens, distinguishing them from T-cell receptors, antibodies can be selected that recognize peptide -MHC complexes[218,219,220] (MHC-restricted antipeptide antibodies or peptide-dependent anti-MHC class I antibodies). Several monoclonal antibodies have been selected that require both

a certain MHC class I antigen and a particular peptide for reactivity. Such monoclonal antibodies are useful reagents capable of detecting cells presenting the appropriate peptide-MHC complexes on their surfaces.[218] Such monoclonal antibodies may also be useful in dissection of T-cell responses. In one study, the monoclonal antibodies could inhibit IL-2 secretion by a T-cell hybridoma of corresponding specificity and could also block induction of cytotoxic T lymphocyte recognizing that epitope when given in vivo during priming.[220] Such monoclonal antibodies have been used to address structural questions about antigen recognition by T and B cells.[219] Such antibodies also appeared to skew the repertoire of T cells for this particular HIV peptide MHC complex to specific T-cell receptor Vβ types and T-cell avidities.[221] However, only very rare monoclonal antibodies have this type of specificity and they were purposely selected in the fusions, so they do not provide a general comparison of T-cell receptor and Ab characteristics.

Cross-Reactions of Monoclonal Antibodies

Monoclonal antibodies display many type 1 cross-reactions, emphasizing that antibody cross-reactions represent real similarities among the antigens, not just an effect of heterogeneity of serum antibodies. Even antigens that differ for most of their structure can share one determinant, and a monoclonal antibody recognizing this site would then give a 100% cross-reaction. An example is the reactivity of autoantibodies in lupus with both DNA and cardiolipin.[222]

It should be emphasized that sharing a “determinant” does not mean that the antigens contain identical chemical structures, but rather that they bear a chemical resemblance that may not be well understood, for example, a distribution of surface charges. Antibodies to the whole range of antigens can react with Igs in idiotype anti-idiotype reactions, showing a cross-reactivity of the same antibodies with proteins (the anti-idiotypes) and with the carbohydrates, nucleic acids, lipids, or haptens against which they were raised.

Polyclonal versus Monoclonal Antibodies

When monoclonal antibodies first became available, some people expected that they would be exquisitely specific and would be superior to polyclonal sera for essentially all purposes. Further thought about the issues discussed previously, however, suggests that this is not always the case and depends on the intended use of the antibodies. Not only do monoclonal antibodies cross-react, but when they do, the cross-reaction is not minor and cannot be removed by absorption. A large panel of monoclonal antibodies may be needed before one is identified with the precise range of reactivity desired for a study.

In polyclonal sera, on the other hand, each different antibody has a distinct range of reactivity, and the only common feature would be detectable reactivity with the antigen used for immunization or testing. Thus, the serum as a whole may show only a low-titered cross-reaction with any particular other antigen, and that cross-reaction can be removed by absorption, leaving substantial activity against the immunizing antigen. For the purposes of an experiment, a polyclonal serum may be “more specific” than any one of its clonal parts and may be more useful. This concept is the basis of the theory of “multispecificity” (see previous discussion).

Polyclonal sera also have advantages in certain technical situations such as immunoprecipitation in which multivalency is important. Many antigens are univalent with respect to monoclonal antibody binding but display multiple distinct sites that can be recognized by different components of polyclonal sera. Thus, a greater degree of cross-linking can be achieved.

The ultimate serologic reagent in many cases may well be a mixture of monoclonal antibodies that have been chosen according to their cross-reactions. The mixture would be better defined and more reproducible than a polyclonal antiserum and would have the same advantage of overlapping specificities.

CONCLUSION

In conclusion, antibodies, whether monoclonal or polyclonal, provide a unique type of reagent that can be made with high specificity for almost any desired organic or biochemical structure, often with extremely high affinity. These can be naturally divalent, for example in the case of IgG or multivalent, for example in the case of IgM or can be made as monovalent molecules such as Fab or recombinant Fv fragments. They serve not only as a major arm of host defense playing a major role in the protective efficacy of most existing antiviral and all antibacterial vaccines but also as very versatile tools for research and clinical use. RIAs and ELISAs have revolutionized the detection of minute quantities of biologic molecules, such as hormones and cytokines, and thus have become indispensable for clinical diagnosis and monitoring of patients as well as for basic and applied research. Current solid-phase versions of these take advantage not only of the intrinsic affinity and specificity of the antibodies but also of the implicit multivalency and local high concentration on a solid surface. Cross-reactivity of antibodies often provides the first clue to relationships between molecules that might not otherwise have been compared. Conversely, methods that use antigens to detect the presence of antibodies in serum have become widespread in testing for exposure to a variety of pathogens, such as HIV. Antibodies also provide specific reagents invaluable in the rapid purification of many other molecules by affinity chromatography. They have also become indispensable reagents for other branches of biology, for example, in histocompatibility typing and phenotyping of cells using a myriad of cell-surface markers that were themselves discovered with monoclonal antibodies and for separating these cells by fluorescence-activated cell sorting, panning, or chromatographic techniques. Monoclonal antibodies have also finally emerged as clinically important therapeutics in cancer, arthritis, organ graft rejection, and infectious diseases. Thus, antibodies are among the most versatile and widely used types of reagents today, and their use is constantly growing. Understanding the fundamental concepts in antigen-antibody interactions thus has become essential not only to an understanding of immunology but also to the effective use of these valuable molecules in many other fields.

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REFERENCES

1. Köhler G, Milstein C. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur J Immunol* . 1976;6:511-519.

2. Melchers F, Potter M, Warner NC. *Lymphocyte Hybridomas*. Berlin, Germany: Springer-Verlag; 1978.

3. Kennett RH, McKearn TJ, Bechtol KB. *Monoclonal Antibodies. Hybridomas: A New Dimension in Biological Analyses*. New York, NY: Plenum Press; 1980.

4. Moore WJ. *Physical Chemistry*. 3rd ed. Englewood Cliffs, NJ: Prentice-Hall; 1962.

5. Myszka DG, Sweet RW, Hensley P, et al. Energetics of the HIV gp120-CD4 binding reaction. *Proc Natl Acad Sci U S A* . 2000;97:9026-9031.

6. Kwong PD, Doyle ML, Casper DJ, et al. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. *Nature*. 2002;420:678-682.

7. DeLisi C. The biophysics of ligand-receptor interactions. *Q Rev Biophys*. 1980;13:201-230.

8. Fersht A. *Enzyme Structure and Mechanisms*. New York, NY: Freeman; 1977.

9. Eisen HN. *Immunology*. 2nd ed. Baltimore, MD: Harper & Row; 1980.

10. Sachs DH, Schecter AN, Eastlake A, Anfinsen CB. Inactivation of staphylococcal nuclease by the binding of antibodies to a distinct antigenic determinant. *Biochem*. 1972;11:4268-4273.

11. Hammes GG. Relaxation spectrometry of biological systems. *Adv Protein Chem*. 1968;23:1-57.

12. Eisen HN, Karush F. The interaction of purified antibody with homologous hapten. Antibody valence and binding constant. *J Am Chem Soc*. 1949;71:363-364.

13. Pinckard RN. Equilibrium dialysis and preparation of hapten conjugates. 1978;1:1-17.

14. Scatchard G. The attractions of proteins for small molecules and ions. *Ann N Y Acad Sci*. 1949;51:660-672.

15. Berzofsky JA, Hicks G, Fedorko J, Minna J. Properties of monoclonal antibodies specific for determinants of a protein antigen, myoglobin. *J Biol Chem*. 1980;255:11188-11191.

16. Rodbard D, Munson PJ, Thakur AK. Quantitative characterization of hormone

receptors. *Cancer*. 1980;46:2907-2918.

17. Thakur AK, Jaffe ML, Rodbard D. Graphical analysis of ligandbinding systems: evaluation by Monte Carlo studies. *Anal Biochem*. 1980;107:279-295.

18. Munson PJ, Rodbard D. Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem*. 1980; 107:220-239.

19. Bright DS. *On Interpreting Spectrophotometric Measurements of Two Quinoline-DNA Complexes (Doctoral Dissertation)*. Fort Collins, CO: Colorado State University; 1974.

20. Berzofsky JA. The assessment of antibody affinity from radioimmunoassay. *Clin Chem*. 1978;24:419-421.

21. Pauling L, Pressman D, Grossberg AL. Serological properties of simple substances. VII. A quantitative theory of the inhibition by haptens of the precipitation of heterogeneous antisera with antigens, and comparison with experimental results for polyhaptenic simple substances and for azoproteins. *J Am Chem Soc*. 1944;66:784-792.

22. Karush F. The interaction of purified antibody with optically isomeric haptens. *J Am Chem Soc*. 1956;78:5519-5526.

23. Thakur AK, DeLisi C. Theory of ligand binding to heterogeneous receptor populations: characterization of the free-energy distribution function. *Biopolymers*. 1978;17:1075-1089.

24. DeLisi C. Characterization of receptor affinity heterogeneity by Scatchard plots. *Biopolymers*. 1978;17:1385-1386.

25. Thakur AK, Munson PJ, Hunston DL, Rodbard D. Characterization of ligand-binding systems by continuous affinity distributions of arbitrary shape. *Anal Biochem*. 1980;103:240-254.

26. Sips R. On the structure of a catalyst surface. *J Chem Phys*. 1948;16:490-495.

27. Nisonoff A, Pressman D. Heterogeneity and average combining site constants of antibodies from individual rabbits. *J Immunol*. 1958;80:417-428.

28. Karush F, Karush SS. Equilibrium dialysis. 3. Calculations. In: Williams CA, Chase MW, eds. *Methods in Immunology and Immunochemistry*. New York, NY: Academic Press; 1971:389-393.

29. Klotz IM. Protein interactions. In: Neurath H, Bailey K, eds. *The Proteins*. New York, NY: Academic Press; 1953:727-806.

30. Karush F. The affinity of antibody: range, variability, and the role of multivalence. 1978:85-116.

31. Crothers DM, Metzger H. The influence of polyvalency on the binding properties of antibodies. *Immunochemistry*. 1972;9:341-357.

32. Hornick CL, Karush F. Antibody affinity - III. The role of multivalence. *Immunochemistry*. 1972;9:325-340.

33. DeLisi C, Metzger H. Some physical chemical aspects of receptor-ligand interactions. *Immunol Commun*. 1976;5:417-436.

34. DeLisi C. The effect of cell size and receptor density on ligand-receptor reaction rate constants. *Mol Immunol*. 1981;18:507-511.

35. DeLisi C, Wiegel FW. Effect of nonspecific forces and finite receptor number on rate constants of ligand-cell bound-receptor interactions. *Proc Natl Acad Sci U S A* . 1981;78:5569-5572.

36. Silhavy TJ, Szmelcman S, Boos W, Schwartz M. On the significance of the retention of ligand by protein. *Proc Natl Acad Sci U S A* . 1975;72:2120-2124.

37. Yalow RS, Berson SA. Immunoassay of endogenous plasma insulin in man. *J Clin Invest*. 1960;39:1157-1175.

38. Chard T. *An Introduction to Radioimmunoassay and Related Techniques*. Amsterdam, The Netherlands: North Holland; 1978.

39. Rodbard D. Mathematics and statistics of ligand assays: an illustrated guide. In: Langan J, Clapp JJ, eds. *Ligand Assay: Analysis of International Developments on Isotopic and Nonisotopic Immunoassay*. New York: Masson; 1981:45-101.

40. Yalow R. Radioimmunoassay. *Rev Biophys Bioeng*. 1980;9:327-345.

41. Farr RS. A quantitative immunochemical measure of the primary interaction between I*BSA and antibody. *J Infect Dis*. 1958;103:239-262.

42. Desbuquois B, Aurbach GD. Use of polyethylene glycol to separate free and antibody-bound peptide hormones in radioimmunoassays. *J Clin Endocrinol Metab*. 1971;33:732-738.

43. Zollinger WD, Dalrymple JM, Artenstein MS. Analysis of parameters affecting the solid phase radioimmunoassay quantitation of antibody to meningococcal antigens. *J Immunol*. 1976;117:1788-1798.

44. Kessler SW. Rapid isolation of antigens from cells with a staphylococcal protein-A-antibody adsorbent: parameters of the interaction of antibodyantigen complexes with protein A. *J Immunol*. 1975;115:1617-1624.

45. Friguet B, Chaffotte AF, Djavadi-Ohaniance L, Goldberg ME. Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. *J Immunol Methods*. 1985;77:305-319.

46. Stevens FJ. Modification of an elisa-based procedure for affinity determination: correction necessary for use with bivalent antibody. *Mol Immunol*. 1987;24:1055-1060.

47. Seligman SJ. Influence of solid-phase antigen in competition enzymelinked immunosorbent assays (ELISAs) on calculated antigen-antibody dissociation constants. *J Immunol Methods*. 1994;168:101-110.

48. Ekins RP. Basic principles and theory. *Br Med Bull* . 1974;30:3-11.

49. Berzofsky JA, Curd JG, Schechter AN. Probability analysis of the interaction of antibodies with multideterminant antigens in radioimmunoassay: application to the amino terminus of the beta chain of hemoglobin S. *Biochemistry*. 1976;15:2113-2121.

50. von Krogh M. Colloidal chemistry and immunology. *J Infect Dis*. 1916;19:452-477.

51. Rodbard D, Lewald JE. Computer analysis of radioligand assay and radioimmunoassay data. *Acta Endocrinol*. 1970;64:79-103.

52. Feldman H, Rodbard D. Mathematical theory of radioimmunoassay. In: Odell WD, Daughaday WH, eds. *Principles of Competitive Protein-Binding Assays* . Philadelphia, PA: Lippincott; 1971:158-203.

53. Rodbard D, Ruder JH, Vaitukaitis J, Jacobs HS. Mathematical analysis of kinetics of radioligand assays: improved sensitivity obtained by delayed addition of labeled ligand. *J Clin Endocrinol Metab*. 1971;33:343-355.

54. Voller A, Bidwell D, Bartlett A. Enzyme-linked immunosorbent assay. In: Rose NR, Friedman H, eds. *Manual of Clinical Immunology* . Washington: American Society of Microbiology; 1980:359-371.

55. Kohno Y, Berkower I, Minna J, Berzofsky JA. Idiotypes of antimyoglobin antibodies: shared idiotypes among monoclonal antibodies to distinct determinants of sperm whale myoglobin. *J Immunol*. 1982; 128:1742-1748.

56. Darst SA, Robertson CR, Berzofsky JA. Adsorption of the protein antigen myoglobin affects the binding of conformation-specific monoclonal antibodies. *Biophysical J*.

1988;53:533-539.

57. Sedgwick J. A solid phase immunoenzymatic technique for the enumeration of specific antibody-secreting cells. *J Imm Methods*. 1983;57:301-309.

58. Czerkinsky CC, Nilsson L, Nygren H, Ouchterlony O, Tarkowski A. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. *J Immunol Methods*. 1983;65:109-121.

59. Bocher WO, Herzog-Hauff S, Herr W, et al. Regulation of the neutralizing anti-hepatitis B surface (HBs) antibody response in vitro in HBs vaccine recipients and patients with acute or chronic hepatitis virus (HBV) infection. *Clin Exp Immunol*. 1996;105:52-58.

60. Ronnelid J, Huang YH, Norrlander T, et al. Short-term kinetics of the humoral anti-C1q response in SLE using the ELISPOT method: fast decline in production in resonse to steroids. *Scand J Immunol* . 1994;40:243-250.

61. Czerkinsky C, Moldoveanu Z, Mestecky J, Nilsson L, Ouchterlony O. A novel two colour ELISPOT assay I. Simultaneous detection of distinct types of antibody-secreting cells. *J Immunol Methods*. 1988;115:31-37.

62. Krieg AM, Yi A, Matson S, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature*. 1995;374:546-549.

63. Ronnelid J, Klareskog L. A comparison between ELISPOT methods for the detection of cytokine producing cells: greater sensitivity and specificity using ELISA plates as compared to nitrocellulose membranes. *J Immunol Methods*. 1997;200:17-26.

64. Mäkelä O, Karjalainen K. Inherited immunoglobulin idiotypes of the mouse. *Immunol Rev*. 1977;34:119-138.

65. Jameson BA, McDonnell JM, Marini JC, Korngold R. A rationally designed CD4 analogue inhibits experimental allergic encephalomyelitis. *Nature*. 1994;368:744-746.

66. Brady L, Dodson G. Reflections on a peptide. *Nature*. 1994;368:692-693.

67. Guichard G, Benkirane N, Zeder-Lutz G, Van Regenmortel MHV, Briand J, Muller S. Antigenic mimicry of natural L-peptides with retroinverso- peptidomimetics. *Proc Natl Acad Sci U S A*. 1994;91:9765-9769.

68. Benkirance N, Guichard G, Van Regenmortel MHV, Briand J, Muller S. Cross-reactivity of antibodies to retro-inverso peptidomimetics with the parent protein histone H3 and chromatin core particle. *J Biol Chem*. 1995;270:11921-11926.

69. Briand J, Guichard G, Dumortier H, Muller S. Retro-inverso peptidomimetics as new immunological probes. *J Biol Chem*. 1995;270:20686-20691.

70. Häyry P, Myllärniemi M, Aavik E, et al. Stabile D-peptide analog of insulin-like growth factor-1 inhibits smooth muscle cell proliferation after carotid ballooning injury in the rat. *FASEB J*. 1995;9:1336-1344.

71. Berzofsky JA, Schechter AN. The concepts of cross-reactivity and specificity in immunology. *Mol Immunol*. 1981;18:751-763.

72. Young NS, Curd JG, Eastlake A, Furie B, Schechter AN. Isolation of antibodies specific to sickle hemoglobin by affinity chromatography using a synthetic peptide. *Proc Natl Acad Sci U S A*. 1975;72:4759-4763.

73. Young NS, Eastlake A, Schecter AN. The amino terminal region of the sickle hemoglobin beta chain. II. Characterization of monospecific antibodies. *J Biol Chem*. 1976;251:6431-6435.

74. Curd JG, Young N, Schecter AN. Antibodies to an amino terminal fragment of beta globin. II. Specificity and isolation of antibodies for the sickle mutation. *J Biol Chem*. 1976;251:1290-1295.

75. Dean J, Schecter AN. Sickle-cell anemia: molecular and cellular bases of therapeutic approaches. *N Engl J Med*. 1978;299:752-763.

76. Johnston MFM, Eisen HN. Cross-reactions between 2,4-dinitrophenyl and nemadione (vitamin K3) and the general problem of antibody specificity. *J Immunol*. 1976;117:1189-1196.

77. Talmadge D. Immunological specificity. *Science*. 1959;129:1643-1648.

78. Inman JK. Multispecificity of the antibody combining region and antibody diversity. In: Sercarz EE, Williamson AR, Fox CF, eds. *The Immune System: Genes, Receptors, Signals*. New York, NY: Academic Press; 1974:37-52.

79. Inman JK. The antibody combining region: speculations on the hypothesis of general multispecificity. In: Bell GI, Perelson AS, Pimbley GH Jr, eds. *Theoretical Immunology*. New York, NY: Marcel Dekker; 1978:243-278.

80. Richards FF, Konigsberg WH, Rosenstein RW, Varga JM. On the specificity of antibodies. *Science*. 1975;187:130-137.

81. Parker CW. Spectrofluorometric methods. In: Weir DM, ed. *Handbook of Experimental Immunology*. Oxford, United Kingdom: Blackwell; 1978:18.1-18.25.

82. Haimovich J, Hurwitz E, Novik N, Sela M. Preparation of protein-bacteriophage conjugates and their use in detection of antiprotein antibodies. *Biochim Biophys Acta*. 1970;207:115-124.

83. Heidelberger M, Kendall FE. The precipitin reaction between type III pneumococcus polysaccharide and homologous antibody. *J Exp Med*. 1935;61:563-591.

84. Heidelberger M, Kendall FE. A quantitative theory of the precipitin reaction. II. A study of an azoprotein-antibody system. *J Exp Med*. 1935;62:467-483.

85. Kabat EA. *Structural Concepts in Immunology and Immunochemistry* . 2nd ed. New York, NY: Hold, Rinehart, and Winston; 1976.

86. Cisar J, Kabat EA, Dorner MM, Liao J. Binding properties of immunoglobulin containing sites specific for terminal or nonterminal antigenic determinants in dextran. *J Exp Med*. 1975;142:435-459.

87. Ouchterlony O, Nilsson LA. Immunodiffusion and Immunoelectrophoresis. In: Weir DM, ed. *Handbook of Experimental Immunology* . Oxford, United Kingdom: Blackwell; 1978:19.1-19.44.

88. Feinberg JG. Identification, discrimination and quantification in Ouchterlony gel plates. *Int Arch Allergy* . 1957;11:129-152.

89. Tomasi TB Jr, Zigelbaum S. The selective occurrence of gamma1A globulins in certain body fluids. *J Clin Invest*. 1963;42:1552-1560.

90. Fahey JL, McKelvey EM. Quantitative determination of serum immunoglobulins in antibody-agar plates. *J Immunol* . 1965;94:84-90.

91. Mancini G, Carbonara AO, Heremans JF. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochem*. 1965;2:235-254.

92. Herbert WJ. Passive haemagglutination with special reference to the tanned cell technique. In: Weir DM, ed. *Handbook of Experimental Immunology* . Oxford, United Kingdom: Blackwell; 1978:20.1-20.20.

93. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A*. 1979;76:4350-4354.

94. Schupbach J, Popovic M, Gilden RV, Gonda MA, Sarngadharan MG, Gallo RC. Serological analysis of a subgroup of human T-lymphotropic retroviruses (HTLV-III) associated with AIDS. *Science*. 1984;224:503-505.

95. Feynman RP, Leighton RB, Sands M. *The Feynman Lectures on Physics*. Vol II. Reading, MA: Addison-Wesley Publishing Co; 1964.

96. Mullett WM, Lai EP, Yeung JM. Surface plasmon resonance-based immunoassays. *Methods*. 2000;22:77-91.

97. VanCott TC, Bethke FR, Polonis VR, et al. Dissociation rate of antibody-gp120 binding interactions is predictive of V3-mediated neutralization of HIV-1. *J Immunol*. 1994;153:449-459.

98. Slater RJ, Ward SM, Kunkel HG. Immunological relationships among the myeloma proteins. *J Exp Med*. 1955;101:85-108.

99. Potter M. Immunoglobulin-producing tumors and myeloma proteins of mice. *Physiol Rev*. 1972;52:631-719.

100. Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*. 1975;256:495-497.

101. Margulies DH, Kuehl WM, Scharff MD. Somatic cell hybridization of mouse myeloma cells. *Cell*. 1976;8:405-415.

102. Shulman M, Wilde CD, Kohler G. A better cell line for making hybridomas secreting specific antibodies. *Nature*. 1978;276:269-270.

103. Kearney JF, Radbruch A, Liesegang B, Rajewsky K. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. *J Immunol*. 1979;123:1548-1550.

104. Yokoyama WM. Production of monoclonal antibodies. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, eds. *Current Protocols in Immunology*. Vol I. New York, NY: John Wiley & Sons, Inc; 2001:2.5.1.

105. Unkeless JC. Characterization of monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *J Exp Med*. 1979;150:580-596.

106. Leo O, Foo M, Sachs DH, Samelson LE, Bluestone JA. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc Natl Acad Sci U S A*. 1987;84:1374-1378.

107. Yarmush ML, Gates FT, Weisfogel DR, Kindt TJ. Identification and characterization of rabbit-mouse hybridomas secreting rabbit immunoglobulin chains. *Proc Natl Acad Sci U S A*. 1980;77:2899-2903.

108. Bazin H. Production of rat monoclonal antibodies with the Lou rat nonsecreting IR983F myeloma cell line. *Prot Biol Fluids*. 1981;29:615-618.

109. Huse WD, Sastry L, Iverson SA, et al. Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science*. 1989;246:1275-1281.

110. Clackson T, Hoogenboom HR, Griffiths AD, Winter G. Making antibody fragments using phage display libraries. *Nature*. 1991;352:624-628.

111. McCafferty J, Griffiths AD, Winter G, Chiswell DJ. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature*. 1990;348:552-554.

112. Kang AS, Barbas CF, Janda KD, Benkovic SJ, Lerner RA. Linkage of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surfaces. *Proc Natl Acad Sci U S A* . 1991;88:4363-4366.

113. Zebedee SL, Barbas CF, III, Hom Y, et al. Human combinatorial antibody libraries to hepatitis B surface antigen. *Proc Natl Acad Sci U S A* . 1992;89:3175-3179.

114. Burton DR, Barbas CF III, Persson MAA, Koenig S, Chanock RM, Lerner RA. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. *Proc Natl Acad Sci U S A*. 1991;88:10134-10137.

115. Duchosal MA, Eming SA, Fischer P, et al. Immunization of hu-PBLSCID mice and the rescue of human monoclonal Fab fragments through combinatorial libraries. *Nature*. 1992;355:258-262.

116. Low NM, Holliger P, Winter G. Mimicking somatic hypermutation: Affinity maturation of antibodies displayed on bacteriophage using a bacterial mutator strain. *J Mol Biol* . 1996;260:359-368.

117. Thompson J, Pope T, Tung J, et al. Affinity maturation of a high-affinity human monoclonal antibody against the third hypervariable loop of human immunodeficiency virus: use of phage display to improve affinity and broaden strain reactivity. *J Mol Biol* . 1996;256:77-88.

118. Tiller T, Meffre E, Yurasov S, Tsuiji M, Nussenzweig MC, Wardemann H. Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J Immunol Methods*. 2008;329:112-24.

119. Scheid JF, Mouquet H, Feldhahn N, et al. Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals. *Nature*. 2009;458:636-640.

120. Pietzsch J, Scheid JF, Mouquet H, et al. Human anti-HIV-neutralizing antibodies

frequently target a conserved epitope essential for viral fitness. *J Exp Med*. 2010;207:1995-2002.

121. Scheid JF, Mouquet H, Ueberheide B, et al. Sequence and structural convergence of broad and potent HIV antibodies that mimic CD4 binding. *Science*. 2011;333:1633-1637.

122. Pietzsch J, Scheid JF, Mouquet H, Seaman MS, Broder CC, Nussenzweig MC. Anti-gp41 antibodies cloned from HIV-infected patients with broadly neutralizing serologic activity. *J Virol*. 2010;84:5032-5042.

123. Mouquet H, Klein F, Scheid JF, et al. Memory B cell antibodies to HIV-1 gp140 cloned from individuals infected with clade A and B viruses. *PLoS One*. 2011;6:e24078.

124. Lerner RA, Benkovic SJ, Schultz PG. At the crossroads of chemistry and immunology: catalytic antibodies. *Science*. 1991;252:659-667.

125. Pollack SJ, Jacobs JW, Schultz PG. Selective chemical catalysis by an antibody. *Science*. 1986;234:1570-1573.

126. Shuster AM, Gololobov GV, Kvashuk OA, Bogomolova AE, Smirnov IV, Gabibov AG. DNA hydrolyzing autoantibodies. *Science*. 1992;256:665-667.

127. Tramontano A, Janda KD, Lerner RA. Catalytic antibodies. *Science*. 1986;234:1566-1570.

128. Shokat KM, Leumann CJ, Sugasawara R, Schultz PG. A new strategy for the generation of catalytic antibodies. *Nature*. 1989;338:269-271.

129. Pollack SJ, Hsiun P, Schultz PG. Stereospecific hydrolysis of alkyl esters by antibodies. *J Am Chem Soc*. 1989;111:5961-5962.

130. Wirsching P, Ashley JA, Benkovic SJ, Janda KD, Lerner RA. An unexpectedly efficient catalytic antibody operating by ping-pong and induced fit mechanisms. *Science*. 1991;252:680-685.

131. Shabat D, Itzhaky H, Reymond J, Keinan E. Antibody catalysis of a reaction otherwise strongly disfavoured in water. *Nature*. 1995;374:143-146.

132. Hirschmann R, Smith AB, Taylor CM, et al. Peptide synthesis catalyzed by an antibody containing a binding site for variable amino acids. *Science*. 1994;265:234-237.

133. Kriangkum J, Xu BW, Nagata LP, Fulton RE, Suresh MR. Bispecific and bifunctional single chain recombinant antibodies. *Biomol Eng*. 2001;18:31-40.

134. Karpovsky B, Titus JA, Stephany DA, Segal DM. Production of targetspecific effector cells using hetero-cross-linked aggregates containing anti-target cell and anti-Fcg receptor antibodies. *J Exp Med*. 1984;160:1686-1701.

135. Titus JA, Garrido MA, Hecht TT, Winkler DF, Wunderlich JR, Segal DM. Human T cells targeted with anti-T3 cross-linked to antitumor antibody prevent tumor growth in nude mice. *J Immunol*. 1987;138:4018-4022.

136. Wickham TJ, Segal DM, Roelvink PW, et al. Targeted adenovirus gene transfer to endothelial and smooth muscle cells by using bispecific antibodies. *J Virol*. 1996;70:6831-6838.

137. Vallera DA, Ash RC, Zanjani ED, et al. Anti-T-cell reagents for human bone marrow transplantation: Ricin linked to three monoclonal antibodies. *Science*. 1983;222:512-515.

138. Waldmann TA. Monoclonal antibodies in diagnosis and therapy. *Science*. 1991;252:1657-1662.

139. Berkower I. The promise and pitfalls of monoclonal antibody therapeutics. *Curr Opinion Biotech*. 1996;7:622-628.

140. Waldmann TA. Immunotherapy: past, present and future. *Nat Med*. 2003;9:269-277.

141. Ortho Multicenter Transplant Study Group. A randomized clinical trial of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. *N Eng J Med*. 1988;313:337-342.

142. Collier BD, Abdel-Nabi H, Doerr RJ, et al. Immunoscintigraphy performed with In-111-labeled CYT-103 in the management of colorectal cancer: comparison with CT. *Radiology*. 1992;185:179-186.

143. Ezzell C. Magic bullets fly again. *Scientific American*. 2001;285:34-41.

144. Sears HF, Herlyn D, Steplewski Z, Koprowski H. Effects of monoclonal antibody immunotherapy on patients with gastrointestinal adenocarcinoma. *J Biol Resp Mod*. 1984;3:138-150.

145. Baselga J, Tripathy D, Mendelsohn J, et al. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J Clin Oncol*. 1996;14:737-744.

146. Baselga J, Norton L, Albanell J, Kim YM, Mendelsohn J. Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. *Cancer Res*. 1998;58:2825-2831.

147. Maloney DG, Grillo-Lopez AJ, Bodkin DJ, et al. IDEC-C2B8: results of a phase I multiple-dose trial in patients with relapsed non-Hodgkin's lymphoma. *J Clin Oncol*. 1997;15:3266-3274.

148. Morris JC, Waldmann TA. Advances in interleukin 2 receptor targeted treatment. *Ann Rheum Dis*. 2000;59(suppl 1):i109-i114.

149. Frankel AE, Houston LL, Issell BF. Prospects for immunotoxin therapy in cancer. *Ann Rev Med*. 1986;37:125-142.

150. Kreitman RJ, Wilson WH, Bergeron K, et al. Efficacy of the anti-CD22 recombinant immunotoxin BL22 in chemotherapy- resistant hairy-cell leukemia. *N Engl J Med*. 2001;345:241-247.

151. Carrasquillo JA, Krohn JA, Beaumier P, et al. Diagnosis and therapy for solid tumors with radiolabeled antibodies and immune fragments. *Cancer Treat Rep*. 1984;68:317-328.

152. Waldmann TA, Goldman CK, Bongiovanni KF, et al. Therapy of patients with human T-cell lymphotrophic virus I-induced adult T-cell leukemia with anti-Tac, a monoclonal antibody to the receptor for interleukin-2. *Blood*. 1988;72:1805-1816.

153. Waldmann TA. Multichain interleukin-2 receptor: a target for immunotherapy in lymphoma. *J Natl Cancer Inst*. 1989;81:914-923.

154. Zhang M, Zhang Z, Garmestani K, et al. Activating Fc receptors are required for antitumor efficacy of the antibodies directed toward CD25 in a murine model of adult t-cell leukemia. *Cancer Res*. 2004;64:5825-5829.

155. van Oers MH, Klasa R, Marcus RE, et al. Rituximab maintenance improves clinical outcome of relapsed/resistant follicular non-Hodgkin's lymphoma, both in patients with and without rituximab during induction: results of a prospective randomized phase III intergroup trial. *Blood*. 2006;108(10):3295-3301.

156. Witzig TE, Gordon LI, Cabanillas F, et al. Randomized controlled trial of yttrium-90-labeled ibritumomab tiuxetan radioimmunotherapy versus rituximab immunotherapy for patients with relapsed or refractory low-grade, follicular, or transformed B-cell non-Hodgkin's lymphoma. *J Clin Oncol*. 2002;20:2453-2463.

157. Gibson AD. Updated results of a Phase III trial comparing ibritumomab tiuxetan with rituximab in previously treated patients with non-Hodgkin's lymphoma. *Clin Lymphoma*. 2002;3:87-89.

158. Robert N, Leyland-Jones B, Asmar L, et al. Randomized phase III study of

trastuzumab, paclitaxel, and carboplatin compared with trastuzumab and paclitaxel in women with HER-2-overexpressing metastatic breast cancer. *J Clin Oncol*. 2006;24:2786-2792.

159. Coudert BP, Arnould L, Moreau L, et al. Pre-operative systemic (neo-adjuvant) therapy with trastuzumab and docetaxel for HER2-overexpressing stage II or III breast cancer: results of a multicenter phase II trial. *Ann Oncol*. 2006;17:409-414.

160. Yang JC, Haworth L, Sherry RM, et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N Engl J Med*. 2003;349:427-434.

161. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med*. 2004;350:2335-2342.

162. Rini BI, Halabi S, Taylor J, Small EJ, Schilsky RL. Cancer and Leukemia Group B 90206: a randomized phase III trial of interferon-alpha or interferon-alpha plus anti-vascular endothelial growth factor antibody (bevacizumab) in metastatic renal cell carcinoma. *Clin Cancer Res*. 2004;10:2584-2586.

163. Johnson DH, Fehrenbacher L, Novotny WF, et al. Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. *J Clin Oncol*. 2004;22:2184-2191.

164. Starling N, Cunningham D. Monoclonal antibodies against vascular endothelial growth factor and epidermal growth factor receptor in advanced colorectal cancers: present and future directions. *Curr Opin Oncol* . 2004;16:385-390.

165. Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discov* . 2004;3:391-400.

166. Gibson TB, Ranganathan A, Grothey A. Randomized phase III trial results of panitumumab, a fully human anti-epidermal growth factor receptor monoclonal antibody, in metastatic colorectal cancer. *Clin Colorectal Cancer*. 2006;6:29-31.

167. Wendtner CM, Ritgen M, Schweighofer CD, et al. Consolidation with alemtuzumab in patients with chronic lymphocytic leukemia (CLL) in first remission—experience on safety and efficacy within a randomized multicenter phase III trial of the German CLL Study Group (GCLLSG). *Leukemia*. 2004;18:1093-1101.

168. Tsimberidou AM, Giles FJ, Estey E, O'Brien S, Keating MJ, Kantarjian HM. The role of gemtuzumab ozogamicin in acute leukaemia therapy. *Br J Haematol* . 2006;132:398-

409.

169. Lipsky PE, van der Heijde DM, St Clair EW, et al. Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. *N Engl J Med* . 2000;343:1594-1602.

170. Pisetsky DS. Tumor necrosis factor blockers in rheumatoid arthritis. *N Engl J Med* . 2000;342:810-811.

171. Johnson S, Oliver C, Prince GA, et al. Development of a humanized monoclonal antibody (MEDI-493) with potent in vitro and in vivo activity against respiratory syncytial virus. *J Infect Dis*. 1997;176:1215-1224.

172. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. The IMpact-RSV Study Group. *Pediatrics*. 1998;102:531-537.

173. Bumgardner GL, Hardie I, Johnson RW, et al. Results of 3-year phase III clinical trials with daclizumab prophylaxis for prevention of acute rejection after renal transplantation. *Transplantation*. 2001;72:839-845.

174. Nussenblatt RB, Fortin E, Schiffman R, et al. Treatment of noninfectious intermediate and posterior uveitis with the humanized anti-Tac mAb: a phase I/II clinical trial. *Proc Natl Acad Sci U S A* . 1999;96:7462-7466.

175. Bielekova B, Richert N, Howard T, et al. Humanized anti-CD25 (daclizumab) inhibits disease activity in multiple sclerosis patients failing to respond to interferon beta. *Proc Natl Acad Sci U S A*. 2004;101:8705-8708.

176. Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med*. 1995;182:459-465.

177. Leach DR, Krummel MF, Allison JP. Enhancement of anitumor immunity by CTLA-4 blockade. *Science*. 1996;271:1734-1736.

178. Phan GQ, Yang JC, Sherry RM, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci U S A* . 2003;100:8372-8377.

179. Wolchok JD, Neyns B, Linette G, et al. Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study. *Lancet Oncol* . 2010;11:155-164.

180. Weber J, Thompson JA, Hamid O, et al. A randomized, double-blind, placebo-controlled, phase II study comparing the tolerability and efficacy of ipilimumab

administered with or without prophylactic budesonide in patients with unresectable stage III or IV melanoma. *Clin Canc Res*. 2009;15:5591-5598.

181. Yuan JD, Gnjatic S, Li H, et al. CTLA-4 blockade enhances polyfunctional NY-ESO-1 specific T cell responses in metastatic melanoma patients with clinical benefit. *Proc Natl Acad Sci U S A*. 2008;105:20410-20415.

182. Barber DL, Wherry EJ, Masopust D, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*. 2006;439:682-687.

183. Freeman GJ, Wherry EJ, Ahmed R, Sharpe AH. Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade. *J Exp Med*. 2006;203:2223-2227.

184. Velu V, Titanji K, Zhu B, et al. Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature*. 2008;458(7235):206-210.

185. Hasan A, Ghebeh H, Lehe C, Ahmad R, Dermime S. Therapeutic targeting of B7-H1 in breast cancer. *Expert Opin Ther Targets* . 2011;15:1211-1225.

186. Rosenblatt J, Glotzbecker B, Mills H, et al. PD-1 blockade by CT-011, anti-PD-1 antibody, enhances ex vivo T-cell responses to autologous dendritic cell/myeloma fusion vaccine. *J Immunother* . 2011;34:409-418.

187. Morris JC, Shapiro GI, Tan AR, et al. Phase I/II Study of GC1008: A human anti-transforming growth factor-beta (TGFb) monoclonal antibody (MAb) in patients with advanced malignant melanoma (MM) or renal cell carcinoma (RCC). *J Clin Oncol.* 2008;26.

188. Terabe M, Matsui S, Park J-M, et al. Transforming growth factorb production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *J Exp Med*. 2003;198:1741-1752.

189. Terabe M, Ambrosino E, Takaku S, et al. Synergistic enhancement of CD8+ T cell-mediated tumor vaccine efficacy by an anti-transforming growth factor-beta monoclonal antibody. *Clin Cancer Res*. 2009;15:6560-6569.

190. Takaku S, Terabe M, Ambrosino E, et al. Blockade of TGF-beta enhances tumor vaccine efficacy mediated by CD8(+) T cells. *Int J Cancer* . 2010;126:1666-1674.

191. Weiss JM, Back TC, Scarzello AJ, et al. Successful immunotherapy with IL-2/anti-CD40 induces the chemokine-mediated mitigation of an immunosuppressive tumor microenvironment. *Proc Natl Acad Sci U S A* . 2009;106:19455-19460.

192. Weiss JM, Ridnour LA, Back T, et al. Macrophage-dependent nitric oxide expression

regulates tumor cell detachment and metastasis after IL-2/anti-CD40 immunotherapy. *J Exp Med*. 2010;207:2455-2467.

193. Fransen MF, Sluijter M, Morreau H, Arens R, Melief CJM. Local activation of CD8 T cells and systemic tumor eradication without toxicity via slow release and local delivery of agonistic CD40 antibody. *Clin Canc Res*. 2011;17:2270-2280.

194. Miller RA, Maloney DG, Warnke R, Levy R. Treatment of B-cell lymphoma with monoclonal anti-idiotype antibody. *N Eng J Med* . 1982;306:517-522.

195. Kwak LW, Young HA, Pennington RW, Weeks SD. Vaccination with syngeneic, lymphoma-derived immunoglobulin idiotype combined with granulocyte/macrophage colony-stimulating factor primes mice for a protective T-cell response. *Proc Natl Acad Sci U S A*. 1996;93:10972-10977.

196. Hsu FJ, Caspar CB, Czerwinski D, et al. Tumor -specific idiotype vaccines in the treatment of patients with B-cell lymphoma—long-term results of a clinical trial. *Blood*. 1997;89:3129-3135.

197. Timmerman JM, Czerwinski DK, Davis TA, et al. Idiotype-pulsed dendritic cell vaccination for B-cell lymphoma: clinical and immune responses in 35 patients. *Blood*. 2002;99:1517-1526.

198. Davis TA, Hsu FJ, Caspar CB, et al. Idiotype vaccination following ABMT can stimulate specific anti-idiotype immune responses in patients with B-cell lymphoma. *Biol Blood Marrow Transplant*. 2001;7:517-522.

199. Neelapu SS, Kwak LW, Kobrin CB, et al. Vaccine-induced tumor-specific immunity despite severe B-cell depletion in mantle cell lymphoma. *Nat Med*. 2005;11:986-991.

200. Meeker T, Lowder J, Cleary ML, et al. Emergence of idiotype variants during treatment of B-cell lymphoma with anti-idiotype antibodies. *N Eng J Med* . 1985;312:1658-1665.

201. Hakim I, Levy S, Levy R. A nine-amino acid peptide from IL-1á augments antitumor immune responses induced by protein and DNA vaccines. *J Immunol* . 1996;157:5503-5511.

202. Cole RJ, Morrisey DM, Houghton AN, Beattie JEJ, Oettgen HF, Old LJ. Generation of human monoclonal antibodies reactive with cellular antigens. *Proc Natl Acad Sci U S A*. 1983;80:2026-2030.

203. Olsson L, Kaplan HS. Human-human monoclonal antibody-producing hybridomas: technical aspects. *Meth Enzymol*. 1983;92:3-16.

204. Seigneurin JM, Desgranges C, Seigneurin D, et al. Herpes simplex virus glycoprotein D: human monoclonal antibody produced by bone marrow cell line. *Science*. 1983;221:173-175.

205. Sugano T, Matsumoto Y, Miyamoto C, Masuho Y. Hybridomas producing human monoclonal antibodies against varicella-zoster virus. *Eur J Immunol* . 1987;17:359-364.

206. Morrison SL. Transfectomas provide novel chimeric antibodies. *Science*. 1985;229:1202-1207.

207. Morrison SL, Johnson MJ, Herzenberg LA, Oi VT. Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. *Proc Natl Acad Sci U S A.* 1984;81:6851-6855.

208. Jones PT, Dear PH, Foote J, Neuberger MS, Winter G. Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature*. 1986;321:522-525.

209. Brüggemann M, Spicer C, Buluwela L, et al. Human antibody production in transgenic mice: expression from 100 kb of the human IgH locus. *Eur J Immunol* . 1991;21:1323-1326.

210. Lonberg N, Taylor LD, Harding FA, et al. Antigen-specific human antibodies from mice comprising four distinct genetic modifications. *Nature*. 1994;368:856-859.

211. Green LL, Hardy MC, Maynard-Currie CE, et al. Antigen-specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs. *Nature Genet*. 1994;7:13-21.

212. Jayasena SD. Aptamers: an emerging class of molecules that rival antibodies in diagnostics. *Clin Chem*. 1999;45:1628-1650.

213. Klein J, Huang HS, Lemke H, Hämmerling GJ, Hämmerling U. Serological analysis of H-2 and Ia molecules with monoclonal antibodies. *Immunogenetics*. 1979;8:419-432.

214. Ozato K, Mayer N, Sachs DH. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J Immunol* . 1980;124:533-540.

215. Pierres M, Devaux C, Dosseto M, Marchetto S. Clonal analysis of B-and T-cell responses to Ia antigens. I. Topology of epitope regions on I-Ak and I-Ek molecules analyzed with 35 monoclonal alloantibodies. *Immunogenetics*. 1981;14:481-495.

216. Sattentau QJ, Dalgleish AG, Weiss RA, Beverley PCL. Epitopes of the CD4 antigen and HIV infection. *Science*. 1986;234:1120-1123.

217. Hoxie JA, Flaherty LE, Haggarty BS, Rackowski JL. Infection of T4 lymphocytes by HTLV-III does not require expression of the OKT4 epitope. *J Immunol*. 1986;136:361-363.

218. Porgador A, Yewdell JW, Deng YP, Bennink JR, Germain RN. Localization, quantitation, and in situ detection of specific peptide MHC class I complexes using a monoclonal antibody. *Immunity*. 1997;6:715-726.

219. Messaoudi I, LeMaoult J, Nikolic-Zugic J. The mode of ligand recognition by two peptide : MHC class I-specific monoclonal antibodies. *J Immunol*. 1999;163:3286-3294.

220. Polakova K, Plaksin D, Chung DH, Belyakov IM, Berzofsky JA, Margulies DH. Antibodies directed against the MHC-I molecule H-2Dd complexed with an antigenic peptide: similarities to a T cell receptor with the same specificity. *J Immunol*. 2000;165:5703-5712.

221. Chung DH, Belyakov IM, Derby MA, et al. Competitive inhibition in vivo and skewing of the T cell repertoire of antigen-specific CTL priming by an anti-peptide-MHC mAb. *J Immunol*. 2001;167:699-707.

222. Koike T, Tomioka H, Kumagai A. Antibodies cross-reactive with DNA and cardiolipin in patients with systemic lupus erythematosus. *Clin Exp Immunol*. 1982;50:298-302.

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Chapter 8

B-Lymphocyte Development and Biology

Richard R. Hardy

INTRODUCTION

B lymphocytes constitute one of the major arms of the immune system, being responsible for humoral immunity. B cells in humans and mice are produced throughout life, primarily in the fetal liver before birth and in the bone marrow after birth. Their development from hematopoietic stem cells (HSCs) has been extensively characterized in mice, and the generation of numerous gene-targeted and transgenic lines in many cases has provided crucial information on the role of transcription factors, cellular receptors, and interactions that are critical in their generation. Recently, the role of microribonucleic acids (miRNAs) in regulating hematopoietic development has also emerged. The complexity of this process is now apparent, and B-cell progenitor differentiation into multiple peripheral subsets with distinctive functions is also widely appreciated. This chapter will focus on B-cell development and function in the mouse, touching more briefly on aspects of human B cells that are similar or distinctive, with a focus on immunodeficiency and B-cell neoplasias. It will conclude with a brief description of novel aspects of B-lymphocyte development in other species, highlighting differences from development in mouse and human.

B-CELL DEVELOPMENT IN MICE

In mice, B cells are produced from HSCs through a complex process of differentiation that has been uncovered over the past 30 years or so. One of the goals of classical hematology has been the delineation of differentiation pathways for different lineages of blood cells. There has been considerable progress in utilizing the ordered expression of a diverse set of cell surface and internal proteins, some with known functions, others whose roles are only suspected, to construct a description of the intermediate stages that cells transit as they develop into B lymphocytes. A simplified example of such a description is presented in Figure 8.1. Thus, HSCs with the capacity to generate all the cell types in blood generate progeny with a more restricted capacity, recognizable in this example by expression of the receptor for interleukin (IL)-7. These in turn produce yet more restricted progenitor cells identified by expression of cluster of differentiation (CD)45R/B220 (and, importantly, by absence of CD19). This kind of pathway can be constructed by isolation and short-term culture of intermediate stages, allowing progression to occur, which helps to define the order. This framework for development serves as a starting point for analysis of the effects of transcription factors, microenvironmental interactions, cytokines, and natural or engineered mutations. It can also be extended by analysis of gene or protein expression at distinct intermediate stages. Critical processes, such as D-J rearrangement and immunoglobulin (Ig)

heavy chain expression, can also be mapped onto this framework. Progress in this work facilitates experiments that address additional issues, such as identification of key regulatory interactions, developmental checkpoints, and the mechanism of B-lineage commitment.

The following sections will cover the sites of B-lineage development at different stages of ontogeny, then focus on what is known about their development in the bone marrow of adult mice, highlighting the function of the pre-B-cell receptor and the crucial role of Ig heavy and light chains in guiding development. Later sections will consider their differentiation into various specialized peripheral populations and emphasize insights into B-cell selection gained from various transgenic models of tolerance.

Early Development

Sites of B Lymphopoiesis during Ontogeny

In the mouse, hematopoiesis occurs predominantly in the fetal liver prior to birth, in the spleen just prior to and shortly after birth, and in the bone marrow thereafter. Prior to liver hematopoiesis, the blood islands of the yolk sac (YS) contain the first identifiable hematopoietic cells, nucleated erythrocytes with embryonic forms of hemoglobin.[1] However, these early YS precursors appear incapable of generating other blood cell lineages, and generation of all blood cell types, including lymphocytes,[2,3] initiates at around 9 to 10 dpc in an embryonic region referred to as the splanchnopleura/AGM (or simply Sp/AGM). Cells from this site are capable of longterm repopulation of lethally irradiated adult recipients with all blood lineages.[4,5] These cells colonize the fetal liver at about 11 dpc, initiating hematopoiesis there. Thus, there are two sites of very early hematopoietic precursors, with one in the YS largely limited to erythropoiesis and the other in the Sp/AGM capable of complete (referred to as “definitive”) hematopoiesis. However, it may be that precursors in the YS have a broader lineage potential in the fetal microenvironment, as when they are injected directly into the newborn liver.[6] HSCs capable of developing into all the blood cell types are produced in the Sp/AGM and migrate to the fetal liver at about d10. Thereafter, B-lineage cells develop largely in a wave, with earlier stages present at earlier times and later stage predominating at later times, close to (and shortly after) birth.[7,8] This progression with gestation day is easily visualized by staining with antibodies that delineate B-cell

development, as shown in Figure 8.2. Early precursors can also be found in the fetal omentum.[9] In contrast with the bone marrow, cells at most differentiation stages in the fetal liver appear to be rapidly proliferating so that larger and larger numbers of B-lineage cells are detected at progressive days of gestation. Another distinction of fetal liver from bone marrow development in the adult is the absence of terminal deoxynucleotidyl transferase (TdT),[10,11] an enzyme that mediates nontemplated addition of nucleotides at the D-J and V-D junctions of Ig heavy chain. [12,13,14] Therefore, heavy chains produced during fetal development have little or no N-region addition, and CDR3 diversity is constrained even further by favoring of short stretches of homology at the V-D and D-J junctions.[15,16] Rearrangement of certain V or D elements may also differ between fetal and adult development, as for example, the reported high utilization of the DFL16.1 segment in fetal liver.[17] Differential expression of genes other than TdT also distinguish B-cell development during fetal life from that in the

adult including precursor lymphocyte regulated myosin light chain like PLRLC transcripts[11,18] and major histocompatibility complex (MHC) class II.[19,20] Interestingly, although absence of the cytokine IL-7 completely eliminates bone marrow B-lineage development,[21] it nevertheless spares some fetal development,[22] suggesting a difference in growth requirements. The B-cell progeny of this early fetal wave may largely consist of B cells quite distinct from adult-derived cells, populating the B-1 subset.[23]

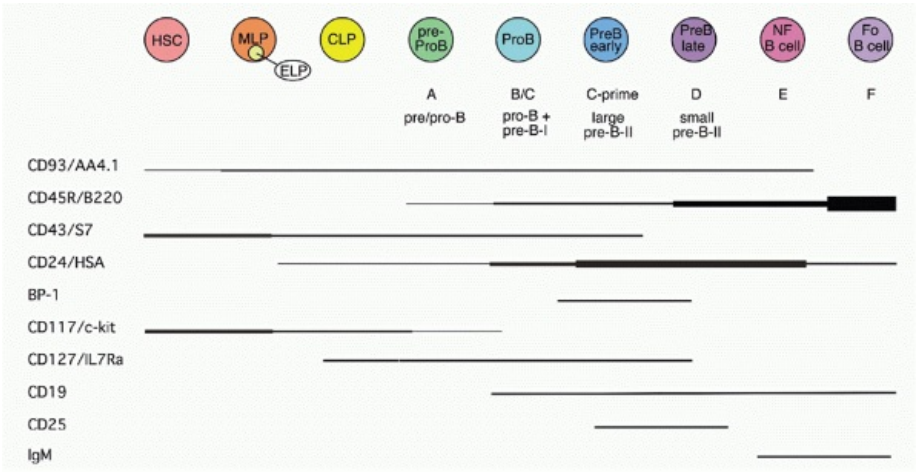

FIG. 8.1. Differentiation Diagram for Development of B Cells from Hematopoietic Stem Cells. Expression of the each surface protein is indicated by a line. Changes in level of expression is indicated by line thickness. CLP, common lymphoid progenitor; ELP, early lymphoid progenitor; Fo B, follicular B cell; HSC, hematopoietic stem cell; MLP, multilineage progenitor; NF B, newly formed B cell.

At birth, B-cell development can also be detected in spleen, but development at this site gradually decreases to very low levels by 2 to 4 weeks of age. Over this same period, B-cell development shifts to the bone marrow and thereafter it continues for the life of the animal. B lymphopoiesis decreases in aged mice. This may be due to diminished responsiveness of precursors to IL-7.[24,25]

Stem Cells, Commitment, and Early B-Cell Progenitors in Bone Marrow

B cells are continually generated from HSCs in the bone marrow of adult mice. Considerable effort has focused on evaluating the functional capacity of fractions of bone marrow cells to repopulate different lineages of blood cells; this work has progressed to the stage of defining a phenotype for such cells, with expression of c-KIT constituting an important marker in the so-called lineage negative subset.[26,27] This is the small fraction of bone marrow cells (< 5%) that lacks expression of a panel of “differentiation markers,” cell surface molecules that are expressed on later stages of various hematopoietic cell lineages. Careful analysis of this HSC fraction using additional markers has shown that it represent perhaps 1/30,000 of nucleated bone marrow cells with as few as 10 mediating multilineage repopulation in cell transfer assays.[28,29,30] An important capacity of “true” or “long-term repopulating” HSCs is their ability to give rise to cells in a recipient mouse that can also repopulate all the blood cell

lineages upon retransfer into a second host, indicating a capacity for extensive self-renewal without differentiation into more restricted progenitors.

A major focus in research on hematopoiesis has been defining and characterizing lineage-restricted progenitors, such as the common myeloid and common lymphoid

progenitors (CLPs).[31,32,33] The CLP cell fraction was identified by lack of a panel of "lineage markers," expression of the IL-7Rα chain, and distinctive, intermediate levels of c-KIT, compared to higher levels on HSCs. Initial characterization of these cells in various functional assays suggested that these cells could generate B, T, natural killer (NK), and a subset of dendritic cells but no other blood cell lineages. The reason for this restriction has been intensively studied, and downregulation of the receptor for granulocyte-myeloid colony stimulating factor has been suggested to be a key event in this process.[34] Cells with the phenotype of CLP constitute about 1/3000 of bone marrow cells. Prior to the CLP stage, multipotent progenitors exhibit low-level expression of genes characteristic of diverse cell lineages, leading to the idea that such promiscuous expression indicates chromatin accessibility that facilitates flexibility in cell fate decisions.[35]

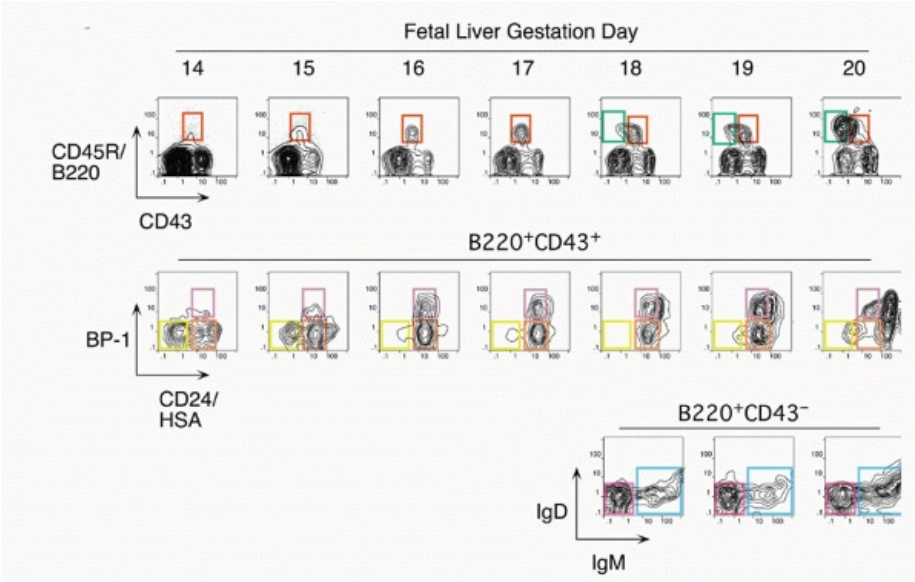

FIG. 8.2. Phenotypic Progression of Developing B-Lineage Cells in Mouse Fetal Liver Analyzed at Different Days of Gestation. Note that B220+cluster of differentiation (CD)43+ cells precede B220+CD43- cells and that within the B220+CD43+ fraction, heat stable antigen (HSA)- cells precede HSA+ cells, and BP-1- cells precede BP-1+ cells. Within the B220+CD43- fraction, the immunoglobulin M+ percentage increases until birth (at about day 20).

CLP cells can give rise in short-term cultures to cells of the B lineage, naturally raising the issue of when cells become restricted to the B lineage. Most cells growing in stromal cultures give rise only to B cells upon transfer into mice, and the phenotype of these cells has been well characterized. [36] Most have at least some heavy chain rearrangement and bear the B-

lineage marker CD19.[37,38] There is less certainty concerning the cells isolated directly from primary lymphoid tissues, as such cells are quite rare similar to CLPs and HSCs. Most of the CD45R/B220+ cells in bone marrow are also CD19+; such cells are committed to the production of B cells.[39] However, a subset of B220+ cells lacks detectable CD19 expression; cells within this fraction can generate CD19+ cells in short-term stromal culture with IL-7. Such cells are included in the CD43+CD24^{low} fraction (Fr. A, 1% of bone marrow) of B220+ cells in bone marrow, but this fraction also contains other cell types including NK-lineage precursors.[38,40] Thus, it is necessary to exclude cells lacking AA4.1 (about half[38]) and expressing Ly6c.[41] Many of these Ly6c+ cells also express CD4; recent work suggests that these are plasmacytoid dendritic cells.[42,43] A phenotypic approach for enriching and fractionating very early B-lineage subsets is shown in Figure 8.3A.

Careful analysis of the LIN− (including CD19) IL7Rα+cKIT+ CD45/B220− (CLP) and CD45/B220+ (Fr. A), as delineated in Figure 8.3, modified our understanding of the earliest stages of B-cell development in bone marrow.[44] First, while CLP stage cells fail to efficiently generate myeloid cells upon transfer into irradiated hosts, they nevertheless retain significant capacity to produce such cells in short-term cultures, likely due to continued (albeit reduced) expression of receptors for myeloid growth factors. In contrast, this myeloid capacity is greatly reduced as cells begin to express CD45R/B220 (ie, become “Fr. A”), concomitant with reduced expression of receptors for myeloid growth factor receptors. Yet these Fr. A cells, while poorly reconstituting T cells in cell transfer assays, nevertheless retain the capacity to generate T-lineage cells in culture, mediated by engagement of Notch by its ligand DL1.[45] Thus, the potential for alternate hematopoietic lineages appears to be lost somewhat later in progression down the B-lineage pathway in mouse bone marrow than previously thought. On the other hand, it appears that initiation of Ig rearrangement is initiated earlier than some studies have indicated. Determination of the extent of germline deoxyribonucleic acid (DNA) segments lost upon D-J rearrangement, and the formation of such D-J segments in individual cells isolated by electronic cell sorting showed that 30% to 50% of cells in CLPs and more than 80% of cells in Fr. A contained a D-J rearrangement on at least one chromosome.[44] This is consistent with high-level expression of genes important in Ig rearrangement, including TdT, Rag-1, and Rag-2, in CLP[46] and Fr. A stage cells.

An emerging view of CLPs (and possibly even the earlier multilineage progenitor [MLP]) stage cells considers them to be early B-lineage precursors rather than branch points in the production of other hematopoietic cell lineages. Thus, analysis of CD4/CD8/CD3 “triple-negative” cells in thymus failed to identify cells with a surface phenotype comparable to CLPs, and mutant mice lacking CLPs in bone marrow nevertheless have relatively intact thymic development, leading these authors to suggest a distinct “early T progenitor” different from CLPs.[47] Furthermore, cells with T/myeloid potential, but lacking B-lineage capacity, have been described.[48] It seems reasonable to hypothesize that MLP, CLP, and Fr. A stage cells occupy a distinctive microenvironmental niche in bone marrow where they receive signals that guide them along the early stages of B-lineage development culminating in CD19+ pro-B cells that are irreversibly committed to becoming B cells due to expression of PAX5[39] (see following section). Interestingly, cells considered to be progressing down a

lymphoid/B-lineage path can be redirected to become dendritic cells by signals through toll-like receptors (TLRs), suggesting that infection can profoundly alter early stages in hematopoietic development.[49]

Additional issues remain regarding the lineage restriction of cells at these early stages in B-cell development. For example, there is evidence that cells restricted to generating B and myeloid/macrophage (but not T) lineage may exist in the fetal liver[50] and even in bone marrow.[51] There is also apparently a different dependence of fetal liver B lymphopoiesis on expression of the transcription factor BSAP compared to bone marrow, as determined by analysis of PAX5 null mice.[52] Further comparison of B-cell development in fetal liver with that in bone marrow is needed to clarify this point. Finally, the precise delineation and characterization of B-cell precursors earlier than CLPs, prior to IL-7 expression, remains imprecise. It seems likely that at least some of the MLP stage cells mentioned previously are initiating a B-lineage program based on their expression of E2A, Rag-1, Rag-2, and TdT.[44] However, potential heterogeneity in this fraction needs to be assessed. Determination of Rag-1 transcriptional activity at the single cell level by a green fluorescent protein reporter, used for identification of the early lymphoid progenitor fraction,[53] may provide a key approach for such studies.

Transcription Factors Regulating B-Lineage Development

The GATA-2 and Runx1/AML1 transcription factors are required for the development of HSCs that are the precursors of all the blood cell lineages, including B cells[54,55,56,57,58] (Fig. 8.4). Experiments with the core-binding factor-associated leukemia fusion protein CBFbeta-SMMHC, whose expression

inhibits RUNX function, have revealed that its expression negatively impacts pre-pro-B-cell through pre-B-cell populations in bone marrow.[59] Although the frequency of CLP stage cells was unaffected, the expression of B-lineage associated genes (such as CD79a and λ5) was decreased, demonstrating the key importance of early RUNX activity.

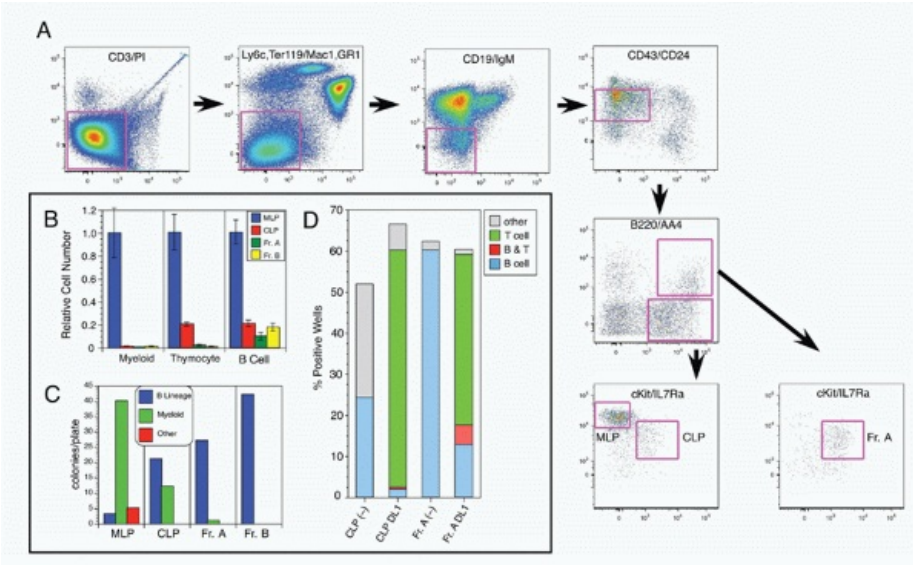

FIG. 8.3. A: An approach for purifying the earliest stage of B-lineage cells in mouse bone marrow. Bone marrow cells expressing cell surface proteins characteristic of differentiated stages of T, myeloid, erythroid, and B lineages are depleted sequentially by electronic gating in the first three panels. Cells with low-level expression of cluster of differentiation (CD)24/heat stable antigen (HSA) and intermediate levels of CD43(S7) are selected in the fourth and the distribution of CD45R/B220 versus CD93/AA4 is shown in the fifth. AA4+B220− cells contain multilineage progenitors (MLPs) and common lymphoid progenitors (CLPs), resolved by analysis for c-Kit versus interleukin (IL)-7Rα in panel six. AA4+B220+ cells, shown in the final panel, are enriched for c-Kit+IL-7Rα+ cells, termed Fr. A. CLP stage cells resemble Fr. A, but lack detectable expression of CD45R/B220. In contrast with CLP and Fr. A, MLP stage cells have higher levels of c-Kit and lack IL-7Rα expression. **B:** Functional analysis of early B-lineage cells by in vivo competition assay, showing absence of myeloid or T-lineage generation, but production of B-lineage cells from Fr.A. In contrast, CLP stage cells generate B and T cells, whereas MLPs repopulate B, T, and myeloid lineage cells. MLP, CLP, and Fr. A as identified in **A**. Fr. B stage cells are DJ/DJ rearranged pro-B cells, identified as CD19+CD43+CD24(HSA)+. **C:** Functional analysis of early B-lineage cells by in vitro S17 stromal cell assay, showing predominant B-lineage colony formation from Fr. A, but some myeloid generation from CLP stage cells. **D:** Functional analysis of early B-lineage cells by in vitro DL1-OP9 stromal cell culture, revealing significant T-lineage potential in Fr. A stage cells.

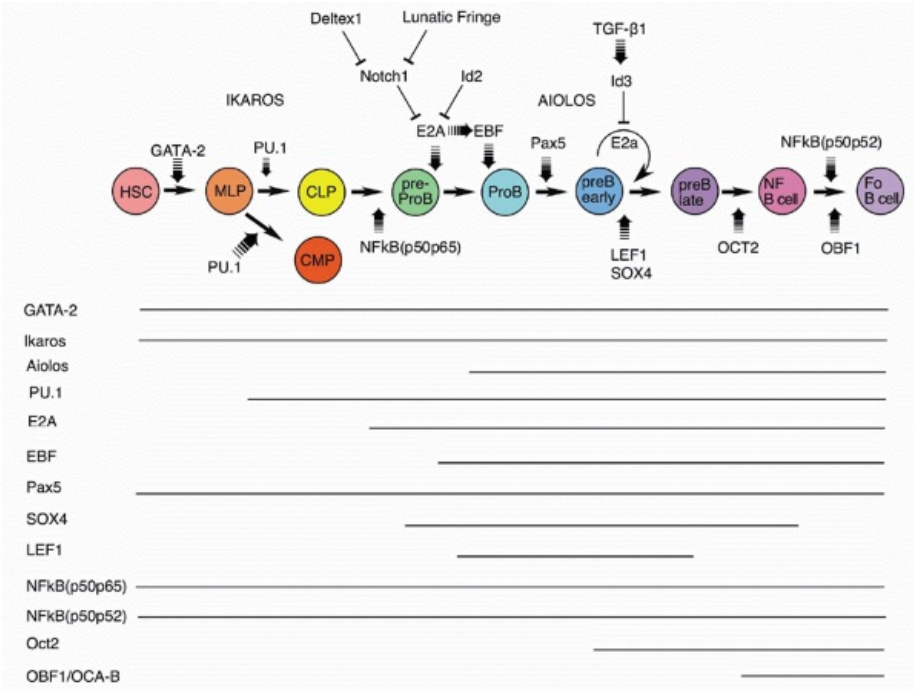

FIG. 8.4. Transcription Factors Important at Different Stages in B-Cell Development in Mouse Bone Marrow. Some regulatory networks are also shown. Positive/activating activity is indicated by *arrows*, whereas negative or blocking activity is indicated by *bars*. The rapidly cycling stage, early pre-B cell, is also indicated.

Predominant stages of expression are indicated below the diagram.

Somewhat later acting, but still very early in development, is the Ikaros transcription factor.[60,61,62] Ikaros and the related transcription factor Aiolos[63] play important roles in lymphocyte development. Ikaros is expressed early in hematopoietic precursors. Ikaros null mice lack B-lineage cells[62]; a different Ikaros mutant that acts as a dominant negative completely blocks lymphoid development.[60] Ikaros activates numerous early B-lineage genes, including TdT, Rag-1, λ5, and VpreB. Expression of EBF1 in Ikaros−/− hematopoietic progenitor cells restored generation of CD19+ cells, but these cells were not committed to the B-cell fate and failed to rearrange IgH genes.[64] Thus, Ikaros acts in a transcription factor pathway, inducing EBF1 expression, and acting in concert with PAX5 to maintain B-lineage commitment, but also altering chromatin compaction around the IgH locus, which together with Rag expression results in heavy chain rearrangement. Aiolos is detected somewhat later in development at about the stage of B-lineage commitment, and its expression increases further at later stages. It is induced by pre-B-cell receptor (BCR) signaling (see section on the role of Ig heavy chain and the pre-BCR), and it acts to down-regulate transcription of λ5 (initially induced by action of Ikaros), a part of the pre-BCR complex. In this way, Ikaros family members play key roles both initiating and terminating pre-BCR signaling, a critical checkpoint in B-cell development.

The PU.1, an Ets family transcription factor, is critical for progression to the earliest stage of lymphoid development, as demonstrated by the inability of PU.1 null precursors to generate lymphocytes.[65,66] An important target of PU.1 for B-lineage development is the gene for Igβ, known as MB-1. The level of PU.1 appears to be critical for development

along the B lineage, as, while low-level expression induced in PU.1 null mice allowed B-lineage development, high-level expression blocked this and fostered myeloid lineage development,[67] likely due to differential induction of the IL-7Rα and macrophage colony-stimulating factor receptor chains.[68] In fact, retroviral mediated expression of the IL-7Rα chain complements defective B lymphopoiesis in PU.1 null bone marrow HPCs.[69] Surprisingly, recent work from several groups indicates that some B-cell development can occur in the absence of PU.1 expression.[70] Furthermore, analysis of conditional PU.1 knockout mice showed that expression of this transcription factor was not required after the pre-B-cell stage.[71]

E2A codes for two proteins, E12 and E47, members of the basic helix-loop-helix family of transcription factors; its induction is crucial from the earliest stages of B-lineage development, as all stages after CD19 expression are absent from E2A null mice.[72,73] These mice lack detectable D-J rearrangements, and, interestingly, such rearrangements can be induced in nonlymphoid cells by introduction of the Rag genes and ectopic expression of E2A,[74] implicating this transcription factor in the process of chromatin remodeling of the Ig heavy chain locus that permits accessibility by the recombinase machinery.[75] The regulation of E2A is crucial for B-lineage development, as negative regulators such as Notch1 and ID2 have been show to block this lineage and induce alternate cell fates, the T and NK

lineages.[76,77,78,79] Consistent with this picture, ectopic expression of genes that negatively regulate Notch1, lunatic fringe, and Deltex-1 induce the B-cell fate.[80,81,82]

Expression of the early B-cell factor, EBF1, a member of the O/E protein transcription factor family, is requisite for progression of early B-lineage progenitors to the D-J rearranged pro-B stage (Fr. B), as shown in EBF1 null mice.[83] The expression of EBF1 is induced by action of the epigenetic histone H2A deubiquitinse MYSM1, as revealed by targeting this gene.[84] EBF1 and E2A act at a similar stage in early in B-lineage development; these two transcription factors can act together to upregulate a family of early B-lineage-specific genes, including Ig-α/β, VpreB/λ5, and Rag-1/2.[85,86] There is evidence that E2A upregulates expression of EBF1, found by transfection of E2A in a macrophage cell line,[87] suggesting an ordering of these two in development. Furthermore, recent studies showed that there are two distinct promoters for EBF1 that are regulated differently.[88] A distal promoter is activated by IL-7 signaling, E2A and EBF1, whereas a proximal promoter is regulated by PAX5, Ets1, and PU.1. Such complex regulation indicates that B-cell development occurs through the action of several feedback loops in a regulatory network that is becoming understood.[89,90]

BSAP, the product of the PAX5 gene, is expressed throughout B-cell development until the plasma cell stage.[91] PAX5/BSAP transcriptional targets include CD19 and BLNK; expression of this transcription factor acts to upregulate V to D-J heavy chain rearrangement.[52] Analysis of chromatin structure around the Ig heavy chain locus revealed that PAX5 induces V to D-J locus contraction, thereby promoting rearrangement.[92] PAX5 null mutant mice show an arrest in bone marrow development at the pro-B stage, likely due to the lack of complete heavy chain rearrangements and also due to the absence of the critical B-cell adaptor protein BLNK that serves to link the pre-BCR to the intracellular signaling pathway via the tyrosine kinase Syk.[93] BSAP/PAX5 also acts to repress alternate cell fates, as pro-B phenotype cells isolated from PAX5 null bone marrow can generate diverse hematopoietic cell lineages, in contrast with such cells from wild-type mice that are B-lineage restricted.[39,94] This occurs by repression of the myeloid growth factor receptor gene c-fms[95] and by repression of the Notch1 signaling pathway[96] critical for T-cell fate specification.[45,97] Conditional targeting of PAX5 in more mature B-cell stages shows that its continued expression is necessary for maintenance and function of mature B cells.[98] Finally, as mentioned previously, in contrast with bone marrow, the absence of BSAP/PAX5 arrests B-cell development prior to the B220+ stage in fetal liver, suggesting a crucial difference in the early dependence on this transcription factor.[52]

The Forkhead family transcription factor FoxO1 plays important roles at several stages of B-cell development.[99] Early in development, it acts to induce expression of the receptor for IL-7 at the CLP stage. It also functions to regulate Rag-1 and Rag-2 expression during heavy and light chain rearranging stages of development.[100] Finally, in mature B cells, it is needed for normal expression of L-selectin, a homing receptor important for normal recirculation of peripheral B cells through the lymphatics. GA binding protein, a ubiquitously expressed Ets family transcription factor, is another player regulating expression of the IL-7R.[101]

Importantly, through interaction with PAX5, it acts in concert to induce expression of critical PAX5 target genes such as CD79a. There is recent evidence that FoxO1 regulates Ikaros activity by altering splicing of its messenger ribonucleic acid (mRNA), rather than altering Ikaros transcription.[102] FoxO1 activation of Ikaros was sufficient for induction of rearrangement of proximal VH genes, but expression of PAX5 was required for rearrangement of distal VH genes. Thus, FoxO1, Ikaros, and PAX5 appear to function in a network to coordinate the ordered rearrangement of Ig genes during B-cell development.

Lymphoid enhancer binding factor (LEF-1) shows a pattern of expression restricted to the pro-B and pre-B stages of B-cell development.[103] Targeted inactivation of the LEF-1 gene allows B-cell development but with reduced numbers.[104] This is because LEF-1 regulates transcription of the Wnt/β-catenin signaling pathway whose activation increases proliferation and decreases apoptosis of early B-lineage cells. In fact, exposure of normal pro-B cells to Wnt protein induces their proliferation.[104] Interestingly, there is a counterproliferative signal that can act at the pre-B proliferative stage, mediated by transforming growth factor-β1.[105] It appears that this occurs due to induction of the ID3 inhibitor that negatively regulates the activity of E2A.[106] Another transcription factor whose expression is similar to LEF-1 is SOX-4; its inactivation also results in the inability of normal early B-lineage cell expansion and a block at the pro-B stage.[107]

Several forms of NF-κb subunits are expressed throughout B-cell development; this transcription factor can regulate kappa light chain expression and also growth factor signaling.[108] Mice lacking the p65 subunit die before birth, so development must be analyzed by transfer of fetal liver precursors into wild-type recipients. Such experiments showed diminished B-lineage cell numbers, but the major defect was in mature B-cell mitogenic responses.[109] Mice lacking the p50 subunit showed relatively normal B-cell development but again poor response to mitogen by mature B cells.[109] However, mice lacking both the p50 and p65 subunits failed to generate any B220+ B-lineage cells. Curiously, when mixed with wild-type fetal liver cells, normal numbers of mature B cells could be generated from the double-defective precursors, suggesting that the defect could be overcome by secreted or membrane-bound signals provided by the wild-type precursors. Another double mutant, p50p52, showed a late stage defect in B-cell development, with a failure to generate mature B cells in spleen.[110]

Inactivation of the Oct-2 transcription factor results in neonatal lethality, but transfers of fetal precursors can reconstitute lymphoid cells in wild-type recipients, allowing assessment of effects on the B lineage. Such studies have shown that fewer mature follicular B cells are generated in these mice and B-1 (CD5+) B cells are completely eliminated.[111,112,113] Similarly, the Oct binding factor, OBF-1, also known as OCA-B and BOB-1, appears to function in the maturation of newly formed B cells in the bone marrow to become follicular B cells in the periphery, as inactivation of this gene resulted in a significant deficit in mature B cells.[114,115,116] Both of these transcription factors have been shown to regulate the follicular B-cell chemokine receptor CXCR5, which may explain at least part of the defect.[117] Curiously, unlike Oct-2 null mice, there was reportedly no deficit in B-1 B cells in OBF-1 null

mice. Interestingly, when the OBF-1 mutant mouse is crossed with btk-deficient mice, there is a complete lack of peripheral B-cell generation,[118] suggesting that this transcription factor may function in the BCR-mediated selection of mature B cells.

Impact of Microribonucleic Acids on B-Cell Development

The miRNAs are small noncoding ribonucleic acids that facilitate the degradation of mRNAs and thereby act at a posttranscriptional level to regulate gene expression. The generation of mature functional miRNAs requires action of Dicer, a protein that cleaves pre-miRNAs, so the targeting of Dicer allows assessment of the global effect of miRNA on B-cell development. Ablation of Dicer in early B-lineage progenitors results in a block at the pro- to pre-B cell stage, likely due to upregulation of the proapoptotic molecule Bim.[119] Counteraction of Bim function by a Bcl-2 transgene reveal further dysregulation of normal development, including nontemplated nucleotide addition (N-regions) at the Ig light chain V-J junction, due to aberrant expression of the terminal deoxynucleotidyl transferase gene that is normally extinguished at the pre-B-cell stage.

Another approach for assessing the importance of specific miRNAs is direct overexpression or knockdown of expression; such a study with miR-150 reveal its role in regulating c-Myb, a transcription factor that regulates pro-B to pre-B progression and also the survival of mature B cells.[120] Transgenic overexpression of miR-17-92, a miRNA often found to be amplified in lymphoma, resulted in a lympho-proliferative syndrome and autoimmune disease, resulting in premature death.[121] One target of miR-17-92 is Bim; its decrease in the transgenic animals may have resulted in excessive cell survival and a loss of normal tolerance to self-antigens. The potential relevance to normal growth regulation is quite interesting, considering the amplification of this miRNA in some lymphomas. Another miRNA with a cancer association, miR-21, has been studied as a transgene in mice, where it results in tumors with a pre-B malignant lymphoid-like phenotype.[122] The miRNAs that are amplified in cancers and likely contribute to the neoplastic process are now termed “oncomirs.”

miRNAs can also influence lineage choice or act at key checkpoints during hematopoietic development. Retroviral provision of miR-34a in bone marrow hematopoietic progenitors blocked B-cell development at the pro-B to pre-B checkpoint, resulting in reduced numbers of mature B cells in mice repopulated with such precursors. A possible explanation for the developmental block is action of miR-34a on Foxp1, a transcription factor that appears to be important at this stage, as cotransfection of FoxO1 lacking its normal 3' UTR target of miR-34a restored B-cell development. A novel regulator of lineage choice appears to be Let-7, a family of miRNAs regulated by the highly conserved ribonucleic acid-binding protein Lin28.[123] While Lin28 has been studied for its role in pluripotency, developmental timing, and oncogenesis, a recent study indicates that it may regulate a developmental switch in both B and T cells, such that its expression in adult hematopoietic progenitors results in reprograming development toward a more “innate-like” pattern, normally only seen during fetal/neonatal timing.[124]

Bone Marrow Developmental Stages

Functional Definition

Distinct stages of developing B-lineage cells can be delineated based on their capacity for growth under different culture conditions. That is, the earliest precursors require cell contact with the stromal microenvironment in addition to specific cytokines, notably IL-7.[105,125] This stromal cell/precursor adhesive interaction is mediated, at least in part, by binding of VLA-4 to intercellular adhesion molecule-1.[126,127] Later stage cells do not require cell contact but maintain a need for cytokines.[128,129] Both cell types can undergo considerable cell proliferation in culture. Interestingly, the difference between cell contact requirement and independence is linked to the expression of heavy chain protein.[128,130] A population of cytoplasmic heavy chain-expressing B-lineage cells later than either of these, the so-called late or small pre-B cells, does not proliferate in culture. These cells likely require different culture conditions for survival, as they usually do not persist for extended periods, but rather die with a half-life of less than 24 hours unless protected from apoptosis by a Bcl-2 transgene.[131]

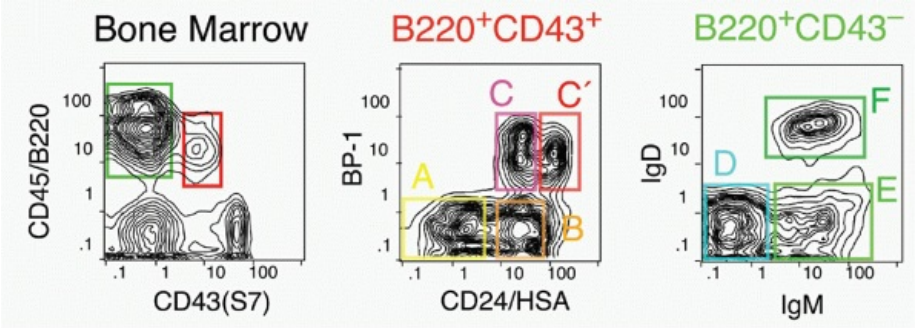

FIG. 8.5. Flow Cytometry Approach for Analyzing Different Stages of Cluster of Differentiation (CD)19+ B-Cell Development in Mouse Bone Marrow. Note that the antibody used for CD24/heat stable antigen (HSA) staining, 30F1, is important, as other monoclonal antibodies that recognize HSA do not resolve high from low-level expression as well. The cells expressing low levels of CD24/HSA, labeled “A”, are CD19- and is enriched for very early B-lineage precursors but also is contaminated by other cell types that can be detected by staining for AA4, NK1.1, DX5, and Ly6c; early B-lineage precursors are AA4+NK1.1-DX5-Ly6c-.

Phenotypic Definition

Further clarification of the heterogeneity in bone marrow can be achieved by analysis using fluorescent staining reagents and either microscopic or flow cytometric analysis. For example, the earliest determination that there were both heavy chain surface-positive B cell and cytoplasmic-positive pre-B cells was through microscopic examination using anti-Ig staining.[132,133] Later studies in mouse showed that there were specific surface proteins or “markers” that could be useful in identifying these populations, notably a restricted isoform (CD45Ra) of the common leukocyte antigen, CD45.[134] This largely B-lineage-restricted 200

kDa molecular mass isoform is often referred to as “B220.” Some highly B-lineage-restricted monoclonal antibodies, such as RA3-6B2, recognize a specific glycosylation of the CD45Ra isoform.[135] However, as described previously, even highly specific antibodies such as 6B2 may also recognize other cell types, such as particular differentiation stages or subsets of NK or dendritic cells.

The application of multiparameter/multicolor flow cytometry and additional monoclonal antibodies specific for other cell surface proteins differentially expressed during B-lineage development has facilitated delineation of multiple additional intermediate stages in this pathway.[129] For example, the B220+ population in bone marrow can be further fractionated into an earlier subset expressing CD43 (about 3% to 5% of marrow cells) and a later fraction with much lower CD43 expression (20% to 30% of marrow). The precursor/progeny relationship of cells in these two fractions can be readily demonstrated by short-term culture, with CD43+ cells giving rise to CD43− cells. These two populations can be further subfractionated based on additional developmentally regulated surface proteins, such as CD24/heat stable antigen (HSA), BP-1 (a zinc-dependent cell surface metallopeptidase also known as aminopeptidase A[136]), and the surface Ig molecules IgM and IgD.[129] This is shown in Figure 8.5. Again, these cell populations can be isolated and short-term culture used to determine their order in the pathway. Alternative approaches based on other developmental markers can be correlated with this framework of cell stages, notably the system developed by Melchers' group[137] using expression of CD45R/B220, CD19, c-KIT, and the IL-2Rα chain. A diagram summarizing this type of phenotypic subdivision and relating different nomenclatures is shown in Figure 8.6.

Culture Systems and Critical Microenvironmental Interactions

The combination of phenotypic characterization coupled with analysis of growth and differentiation in culture has provided a powerful approach for the further understanding of B-cell development, as employed by many different investigators. Bone marrow cultures developed by Whitlock and Witte,[138,139,140] and fetal liver cultures developed by Melchers' group[141,142] have allowed determination of the critical cytokines and some of the cell adhesion molecules important in the in vivo development of these cells. Many of these are summarized in Table 8.1. A typical B-lineage colony proliferating on S17 stromal cells in the presence of IL-7 is shown in Figure 8.7.

Survival and growth of the earliest stages of developing B-lineage cells require cell contact with nonlymphoid adherent cells that can be isolated from bone marrow, cells referred to generically as “stromal cells.” A number of lines have been derived from primary cultures of bone marrow adherent cells and characterized in terms of their capacity to support B lymphopoiesis in vitro.[143] This work has led to the discovery of adhesion molecules that play important roles in mediating the organization of clusters of developing B-lineage cells on stromal layers, including CD44 interacting with hyaluronate and VLA-4 interacting with vascular cell adhesion molecule 1.[126,127,144,145] Both of these interactions could be disrupted by addition of blocking antibodies

to CD44 and VLA-4 on B-cell precursors, resulting in a disruption of normal pre-B proliferation

in vitro.[146] Such adhesion interactions may serve to transmit signals directly to the stromal cells or B precursors, or both. There is some evidence that stromal cells are induced to elaborate specific growth mediators after interaction with B-cell precursors or soluble regulators.[147]

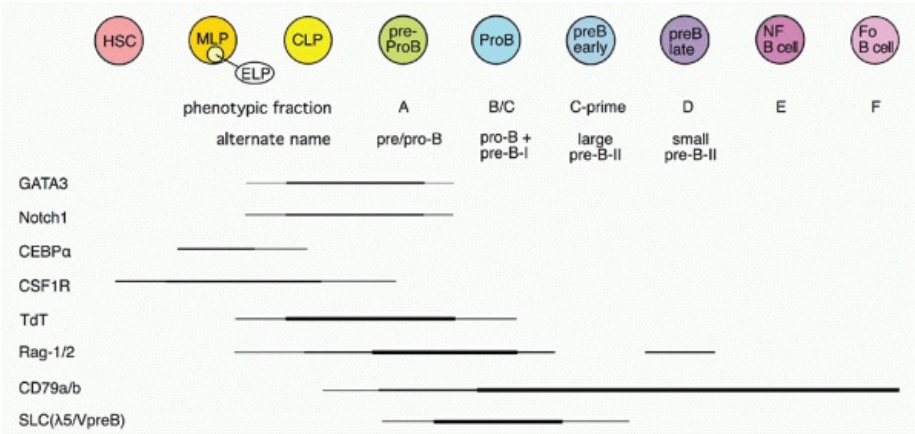

FIG. 8.6. Diagram of Distinct Phenotypic Stages and Characterization of Terminal Deoxynucleotidyl Transferase, Biphasic Rag Expression, Immunoglobulin-α/β, and Surrogate Light Chain Expression. Genes characteristic of myeloid and T-cell lineage are also shown. The cell type descriptions are cross-referenced to the alphabetic phenotypic fraction nomenclature and also to the Basel nomenclature. The early lymphoid progenitor population is identified by activation of the Rag-1 locus in a green fluorescent protein reporter mouse.

Another function of the stromal cells is to produce growth factors critical to B-lineage survival, proliferation, and differentiation; the most important of these for mouse B-cell development is IL-7.[105,125,148,149] IL-7 promotes the survival and proliferation of pro-B and pre-B stage cells, both in vivo and in vitro.[150,151] Neutralizing antibody to IL-7 can block B-cell development in vitro,[129] and IL-7 expressed as a transgene can deregulate normal B-cell development, leading to B-cell lymphadenopathy.[152] The IL-7 receptor consists of a unique IL-7Rα chain[153] paired with the common gamma chain (λc) that is also found in the receptors for

IL-2, IL-4, IL-9, IL-15, and IL-21.[154] IL-7Rα null mice have a severe deficit of both B and T cells in the periphery and lack most B-lineage cells in bone marrow.[155] Mice with targeted inactivation of the γc or IL-7 do have some B-lineage development, suggesting an alternate cytokine; this appears to be thymic stromal lymphopoietin (TSLP). This protein was first identified as a pre-B-cell growth factor produced by a thymic stromal line[156] and shows some of the same effects in culture as IL-7, although possibly inducing less proliferation and more differentiation.[157] Its receptor has been cloned; it consists of two chains, the TSLP receptor and IL-7Rα.[158] The TSLP receptor shares both sequence homology and genomic exon

organization with the common gamma chain.[159] Signaling through the IL-7 receptor requires JAK3 and activates the transcription factor STAT5, whereas signaling through TSLP is JAK3 independent but also activates STAT5.[157,160] Unexpectedly, the growth response to TSLP requires synergy with the pre-BCR in bone marrow but not in fetal liver,[161] leading some to propose that this might be a marker for distinctive B1 B-cell development in bone marrow.[162] When TSLP is overexpressed from an inducible transgene, B1 B cells expand, apparently at the expense of marginal zone (MZ) B cells.[163]

TABLE 8.1 Regulators of Growth of Early B-Lineage Cells

Mediator	Effect	Reference
L-7	Stimulates CLP and B-precursor proliferation	105,148,149,590
TSLP	Alternate IL-7-like cytokine	156,157
IGF-1	Stimulates accumulation of Cμ+cells in culture	185
FLT-3/FLK2-L	Critical for earliest stages of B-lineage development	168,172,591,592
c-KIT-L	Synergizes in IL-7-induced proliferation	26,166
IL-3	Substitute for IL-7 in proliferation of pre-B clones	176
CXCL12/CXCR4	Crucial chemokine interaction for early B-lineage precursors	194,196,198,199
Hemokinin	Novel regulator of B lymphopoiesis	187
VLA-4/VCAM-1	Adhesive interaction; antibodies to either block B lymphopoiesis	126,127,593
CD44/hyaluronate	Adhesive interaction; mediates association of B-lineage/stromal cells	144,146
TGF-β	Inhibits proliferation stimulated by IL-7	105
Sex steroids	Decrease B-lineage precursors in bone	594,595

	marrow	
Growth hormones	Required for normal B lymphopoiesis	188,189,596
TLRs	Innate immune system regulation	190

CD, cluster of differentiation; CLP, common lymphoid progenitor; IL, interleukin; TGF, transforming growth factor; TLR, toll-like receptor; TSLP, thymic stromal lymphopoietin; VCAM, vascular cell adhesion molecule.

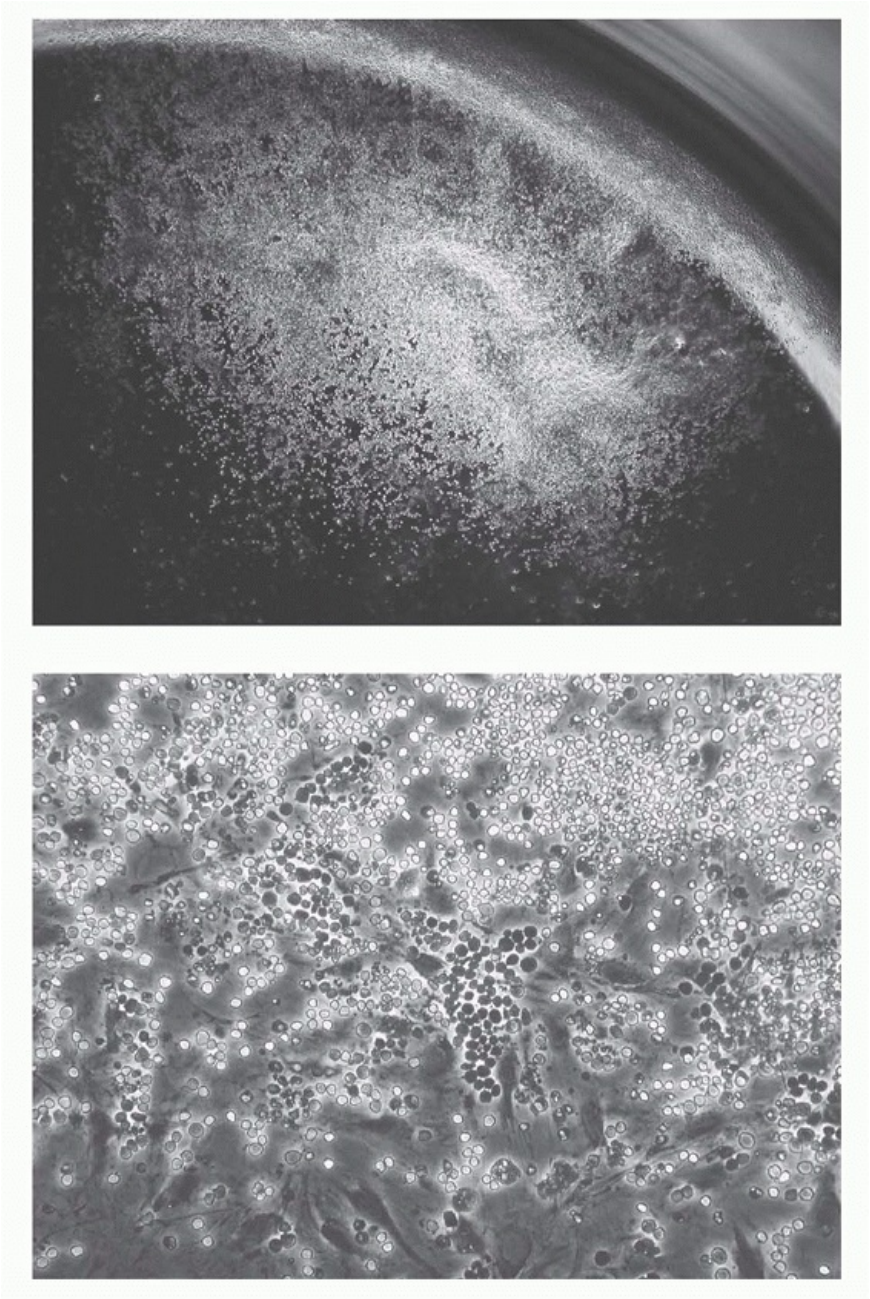

FIG. 8.7. Photomicrographs of B-Lineage Colony Proliferating on S17 Stromal Layer (in the Well of a 96-Well Microplate) in the Presence of Interleukin-7. Day

10 colony derived from a single Fr. A phenotype (see Fig. 8.5) cell. Low power and high power views. All of these cells now express CD19 and many have progressed to BP-1+.

The earliest precursors in the B-lineage pathway, probably including cells that are not B-lineage committed but that can efficiently give rise to B cells in a short time in vitro, have receptors for SCF/c-KIT-ligand[26,27,164,165,166,167] and FLK2/FLT3-ligand.[168,169,170,171,172] Thus, the most permissive cultures for expanding precursors of B-lineage cells will include these cytokines, in addition to IL-7 and a stromal adherent cell layer, such as S17.[173,174,175] IL-3 has also occasionally been suggested as playing a support role for pre-B cells in vitro,[176] although its role in vivo may be at a much earlier stage. While culture conditions have been reported that can support B-lineage development in the absence of stromal cells,[177,178] the clear-cut alteration of contact-dependence prior and post heavy chain expression[128,129,130,179,180] argues that the most physiologic model for early B-lineage growth will include stromal cells. Besides providing important cell-cell contacts that may signal survival, proliferation, and differentiation, it is also likely that stromal cells bind at least some cytokines to their surface, providing higher local concentrations to the clusters of B-lineage precursors that adhere.[181,182] B-lineage development may be modified by exposure to hormones; considerable interest has focused on sex steroids released during pregnancy that serve to depress B lymphopoiesis, particularly the pre-B-cell pool.[183] This may be important to avoid autoimmune responses by the mother but could have negative consequences due to possible transient immunodeficiency. Interestingly, fetal B lymphopoiesis is not similarly depressed due to the absence of hormone receptors on fetal B-lineage cells.[184] Insulin-like growth factor has been reported to potentiate progression in vitro to the Cμ+stage,[185,186] and, more recently, there is a report of a bioactive peptide, a type of tachykinin, that synergizes with IL-7 to enhance the growth of IL-7-dependent cultures.[187] Besides insulin-like growth factor, other pituitary hormones, thyroxine, and growth hormone have effects on B lymphopoiesis.[188] For example, thyroxine treatment can restore normal B-cell development in dwarf Pit-1 mutant mice with deficient pituitary function.[189] Recent work has highlighted the effect that activation of TLRs during infection may have on altering development.[190] Thus, it is likely that more detail remains to be filled in to complete our picture of the growth requirements and modulating influences of B-lineage cells in mouse bone marrow. Another function of cell-cell interaction is cell fate determination during the lineage commitment stage, very early in development of B-lineage cells. The Notch signaling pathway is implicated in cell fate determination in invertebrates and more recently has been shown to function in lymphoid lineage specification.[45,76,191] Notch family transmembrane receptors regulate transcription by being cleaved upon ligand binding to release an intracellular cytoplasmic domain that translocates to the nucleus where it interacts with the transcriptional repressor CSL.[192] Recent studies have shown that Notch1 can play a pivotal role in commitment of common lymphoid progenitors to the T-cell

lineage.[76] That is, expression of Notch1 by retroviral transduction has been shown to redirect B-lineage differentiation in bone marrow along the T lineage. Furthermore, a reciprocal result was found in conditional Notch1 null mice, blocking T-cell development in the thymus to be replaced by B-cell development.[97] Finally, altering the Notch1 modifier lunatic fringe by overexpressing this molecule under regulation of an lck promoter resulted in B-cell development in the thymus.[80] Differentiation of lymphoid precursors to NK or dendritic cell lineages was unaffected in Notch1 null CLP cells, so Notch apparently affects only the B/T-lineage decision.

Role of Chemokines in Migration of B-Cell Precursors

One of the most distinctive features of B-cell development in bone marrow is the migration of developing precursors from early stages nearest the bone endosteum layer to latter stages progressively closer to the central arteriole, where they will eventually exit.[193] This migration is likely due to differential expression of specific adhesion molecules and also to expression of chemokine receptors. Analysis of B-cell migration has identified a critical chemokine that is important in this process, SDF-1, now known as CXCL12,[194,195] and its receptor CXCR4.[196] CXCL12 is expressed by fetal liver and bone marrow stromal cells, whereas CXCR4 is found on hematopoietic precursors and B-cell progenitors.[197] Deletion of either the receptor or ligand results in severely impaired B lymphopoiesis.[198,199,200] Interestingly, the critical defect appears to be failure to retain precursors in the primary lymphoid organ, as progenitors and precursors can be found in the blood of mutant mice.[201]

Gene Expression and Immunoglobulin Rearrangement

In addition to delineation of developmental stages based on changes in protein surface expression, B-lineage cells can also be characterized for expression of internal proteins related to critical processes in their progression along this pathway, specifically those related to rearrangement and expression of the B-cell antigen receptor (Fig. 8.8). Thus, expression of μ heavy chain constant region, prior to Ig rearrangement, from a cryptic promoter generates a “sterile transcript” that reflects an open chromatin structure important for the onset of rearrangement,[202,203,204] and so analysis of sterile μ expression can be used to investigate very early stages of B-cell development. Classical northern analysis can be done with transformed lines, but much work analyzing RNA levels in B-lineage cell fractions, whether directly isolated or cultured, has depended on polymerase chain reaction amplification of complementary DNA.[38] For example, using this approach, sterile μ can be detected in a very early fraction of B220+CD43+CD19- (Fr. A) cells. Expression of the recombinase activating genes Rag-1 and Rag-2, which together make the double-strand breaks in DNA required for Ig rearrangement,[205,206,207] also occurs in Fr. A stage cells, which also have high levels of TdT, the enzyme responsible for adding nontemplated nucleotides at the D-J and V-D junctions of the heavy chain.[12,13] Rag-1 binds in a highly specific fashion to discrete sites within the IgH locus, recombination signal sequences, defining “recombination centers.”[208] The extent of heavy chain or light chain rearrangement can be quantitated either in bulk isolated populations[129] or in individual cells.[137,209,210] At the heavy chain locus, D-J rearrangement occurs prior to V-DJ rearrangement, and cells with

extensive D-J but little V-DJ rearrangement can be detected at the B220+CD43+CD19- (Fr. A) stage, where Rag-1/2 and TdT are strongly expressed.[44] V-DJ rearrangements are readily detected in the abundant B220+CD43− (Fr. D) stage small pre-B cells, although productive rearrangement has already completed by the large pre-B (Fr. C-prime) stage (see following discussion). Single-cell sequence analysis of rearrangements in Fr. C stage cells shows a large proportion with nonproductive rearrangements on both chromosomes, suggesting that this may represent a dead-end fraction.[209] Some light chain rearrangement is detectable in early stage B220+CD43− (Fr. B) cells, and this is consistent with the observation that low-level kappa light chain rearrangement is detectable in bone marrow of mice where μ heavy chain has been crippled by deletion of the membrane exon.[211] However, much higher levels of kappa rearrangement can be detected in B220+CD43− (Fr. D) stage cells consistent with the finding of sterile kappa mRNA increase just prior to this stage likely induced by pre-BCR signaling (see following discussion).

Role of Immunoglobulin Heavy Chain and the Pre-B-Cell Receptor

Many years ago, the analysis of severe combined immunodeficiency (SCID) mouse[212] bone marrow revealed the presence of a population of B220+ cells, all with a very early CD43+ phenotype, suggesting a block in B-cell development at this stage.[213] SCID mice have a defect in the catalytic subunit of the DNA-dependent protein kinase DNA-PKcs,[214,215] and, as a result, B-lineage cells in these mice are very ineffective at completing productive Ig heavy chain rearrangements. This block could be overcome by introduction of an Ig heavy chain transgene, indicating a critical role for μ protein in progressing past an early developmental checkpoint.[216] Furthermore, a gene targeting experiment that eliminated the membrane exon of μ heavy chain (μ-mt) also generated a block at this stage.[217,218]

The μ heavy chain is associated with a set of B-cell-specific peptides at the early pre-B-cell stage,[219] and this complex is referred to as the pre-BCR. It seems clear that pre-BCR mediates a type of signaling function analogous to the BCR in mature B cells. Prior to light chain expression, two peptides known as λ5 and VpreB, originally isolated as B-lineage-specific complementary DNAs,[220,221] associate with heavy chain. The λ5 shows homology to a lambda constant region and VpreB is so-called because it has homology to a variable region domain. Together, these peptides constitute a pseudo- or surrogate light chain (SLC). The critical role of λ5 was demonstrated unambiguously in genetargeted mice, where B-cell development was blocked at the B220+CD43+ stage.[222] The production of some mature cells that accumulate in this mutant is likely due to early kappa rearrangement, with light chain substituting for SLC, as demonstrated in light chain transgenic experiments.[223]

The μ heavy chain has a very short cytoplasmic region consisting of only three amino acids; signal transduction

through the BCR is mediated by accessory peptides, similar to the CD3 components of the T-cell receptor, known as Ig-α and Ig-β.[224,225,226,227] Inactivation of Ig-β[228] results in a block at the B220+CD43+ stage in mouse bone marrow, similar to that seen in μ-mt and λ5

null mice. Finally, the Syc tyrosine kinase plays a critical role in transducing BCR cross-linking signals in mature B cells and inactivation of this gene results in a “leaky” block at this same stage.[229,230] Thus, any mutation that affects this pre-BCR complex (see following section; see Fig. 8.10A) precludes efficient progression past the earliest stages of B-cell development.

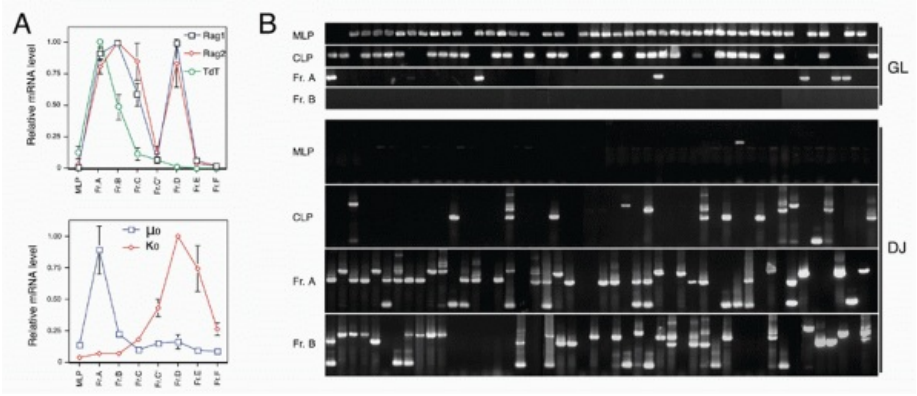

FIG. 8.8. A: Profiles of immunoglobulin (Ig) rearrangement-related gene expression. Cells isolated using fractionation scheme in Figure 8.3 for multilineage progenitors and Fr. A; in Figure 8.5 for Fr. B through Fr. F. Relative messenger ribonucleic acid levels assessed by performing semiquantitative reverse transcription-polymerase chain reaction using limited numbers of cycles, blotting, then probing, and quantitating the probe signal. Note the biphasic expression of Rag genes and the early expression of sterile μ (labeled *μ0*) during the first wave, where heavy chain rearranges, and the upregulation of sterile kappa transcripts *(κ0)* during the second Rag wave, when most light chain rearrangement takes place. **B:** Single-cell polymerase chain reaction analysis of Ig heavy chain rearrangement in four early stages of developing B-lineage cells. Deoxyribonucleic acid prepared from individual cells isolated following the scheme shown in Figure 8.3 was divided into two aliquots and analyzed for retention of a deoxyribonucleic acid segment lost upon any D-J rearrangement (labeled *GL*, germline) and also for D-J rearrangement. Note that D-J rearrangement initiates at the common lymphoid progenitor stage, where 30% to 50% of cells show a rearrangement.

Careful examination of B-cell development in normal mice shows that heavy chain is first expressed at a late fraction of the B220+CD43+ stage, termed Fr. C-prime (Fig. 8.9A). This fraction is also interesting because it shows a much higher proportion of cells in cycle (revealed by a high frequency of cells with greater than 2N DNA content; Fig. 8.9B), compared with any other B220+ stage in bone marrow.[129] Mice unable to assemble a pre-BCR, due to inability to rearrange heavy chain (Rag-1 null), show a block in development at the CD43+ stage that can be complemented by introduction of a functionally rearranged μ heavy chain as a transgene[180] (Fig. 8.9C). Analysis of several types of pre-BCR defective mutant mice shows a complete absence of Fr. C-prime stage cells, suggesting that pre-BCR signaling results in the upregulation of CD24/HSA and also entry into rapid cell proliferation. Thus, a model of pre-BCR function is that it signals the clonal expansion phase of pre-B-cell development, amplifying cells with in-frame VDJ rearrangements capable of making heavy

chain protein. The precise nature of pre-BCR signaling remains to be completely understood. An early model suggested that cross-linking of heavy chain was mediated through interaction of SLC with a bone marrow expressed ligand. However, subsequent experiments showed that normal light chain could substitute for SLC, and that even a V_H truncated μ heavy chain could mediate progression past this stage. Furthermore, intensive searches for the putative ligand over a 10-year period have been fruitless, leading to the model

that pre-BCR signaling is more akin to "tonic" signaling in mature B cells.[231,232,233] That is, simple assembly of the complex (or possibly some degree of multimerization fostered by the self-aggregating nature of SLC[234]) probably is sufficient for the cell to pass this developmental checkpoint (Fig. 8.10B). One clear-cut finding is that pre-BCR signaling in a transformed pro-B-cell model system can occur in the absence of any additional cell type, suggesting that if a ligand exists, it must be expressed on B-lineage cells rather than stromal cells.[235] Possibly, pre-BCR homodimerization is mediated through glycosylation at a conserved asparagine residue in the first μ constant region domain.[236] Studies of the T-cell analog of the pre-BCR, pre-Tα, provide strong evidence that it signals through spontaneous dimerization, without requirement for an external ligand.[237,238,239]

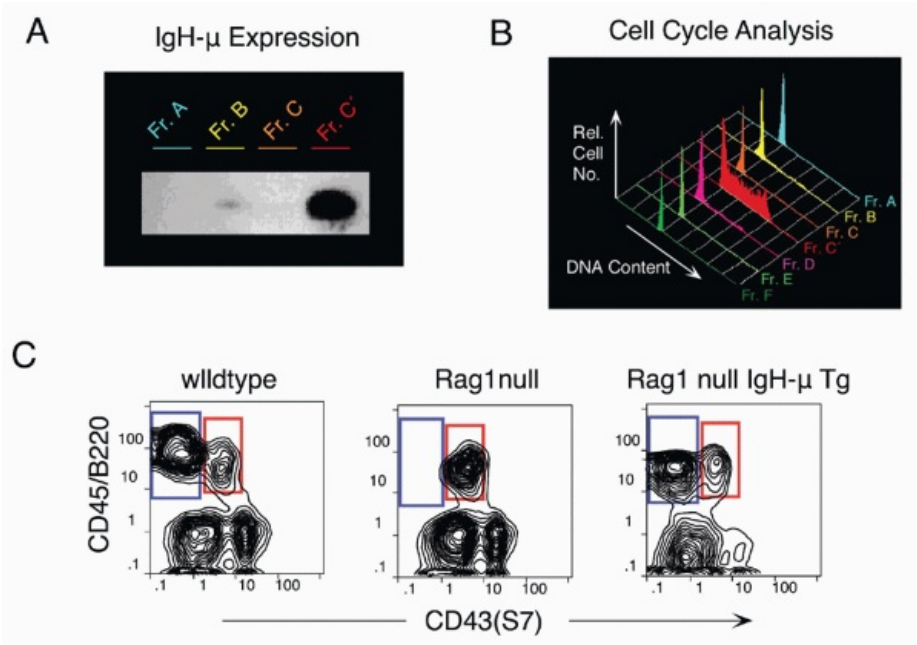

FIG 8.9. A: Western blot of immunoglobulin μ heavy chain expression showing high-level expression in Fr. C-prime. **B:** Cell cycle analysis of individual fractions shows most cells in Fr. C-prime are cycling. Propidium iodide staining of permeabilized sorted cells allows determination of deoxyribonucleic acid content per cell using flow cytometry. **C:** Block in B-cell development in Rag-1-deficient mice can be overcome by introduction of an immunoglobulin μ heavy chain transgene.

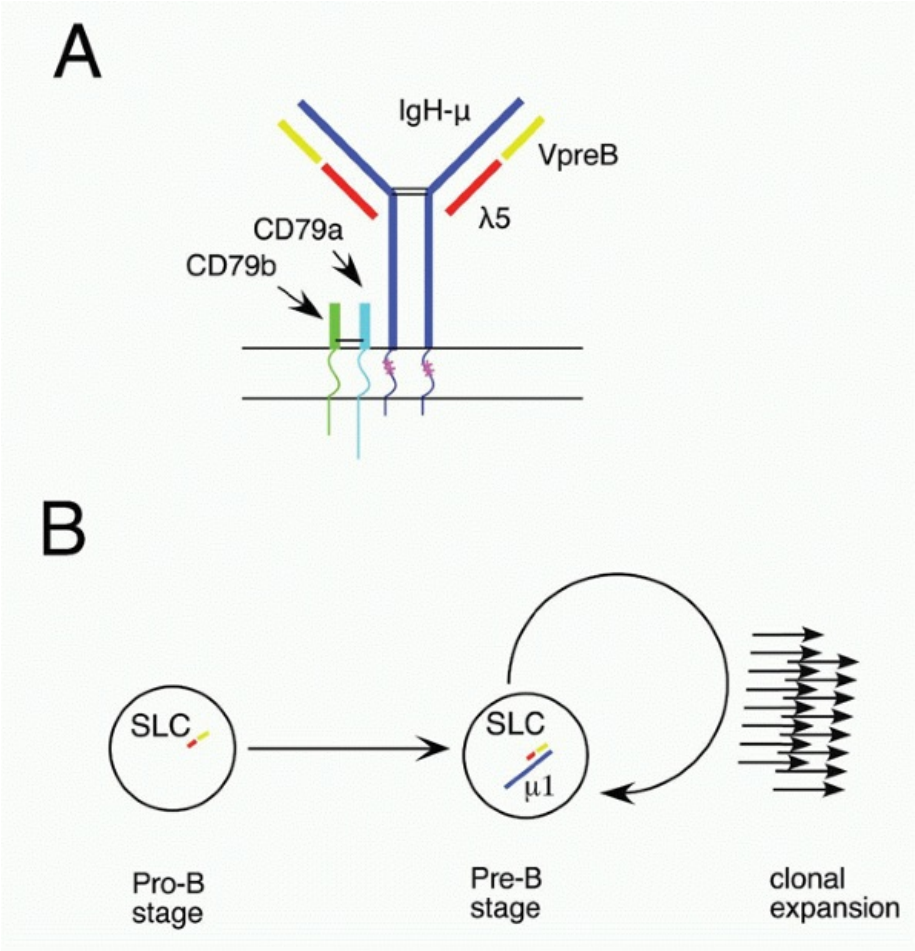

FIG 8.10. A: Diagram of the pre-B-cell receptor (BCR) μ heavy chain with surrogate light chain (λ5 and VpreB) in place of conventional light chain. As in the BCR, immunoglobulin (Ig)-α and Ig-β serve to couple signals between the receptor and cytoplasmic components, such as BLNK and Syk. Starred m transmembrane residues are important in mediating interaction with Ig-α/Ig-β, as mutation of these diminishes BCR function. **B:** Clonal expansion mediated by pre-BCR assembly. Association of newly generated μ heavy chain with pre-existing surrogate light chain leads to a burst of proliferation at the pre-B stage.

Mutations in other molecules in the pre-BCR signaling pathway have been shown to affect B-cell development and pre-B-cell clonal expansion. Although the Btk mutation is less severe in mouse than in human, there nevertheless is an alteration in pre-B-cell expansion in Btk-deficient mice.[240] Also, X-linked immunodeficiency (xid) B cells (deficient in Btk) have been reported to proliferate more in stromal cell cultures, possibly due to decreased differentiation to later nonproliferative stages.[241,242] The role of Btk is thought to modulate BCR signaling strength,[243] and this is probably also the case for pre-BCR signaling, allowing only strongly signaling pre-BCRs to progress in the mutant mice. BLNK/SLP65 serves to link the pre-BCR to the Syk kinase critical in BCR signaling.[244,245] Mutant mice lacking BLNK show a partial block in B-cell development at the pro-B to pre-B transition.[246] Curiously, whereas pre-BCR signaling is thought to mediate allelic exclusion (expression of a single heavy chain allele),

this remains intact in BLNK-deficient mice.[247] Animals deficient in both BLNK and Btk develop an extensive pre-B expansion that progresses to lymphoma, leading to study of such mice as a model for human pre-B acute lymphoblastic leukemia (ALL).[248,249]

Syk-deficient mice show a more severe block at the pro-B to pre-B transition and a lack of allelic exclusion.[229,230,247] There is also evidence that pre-BCR signals through Erk to induce proliferation.[250]

Outcomes of pre-BCR signaling, in addition to pre-B proliferation, are downregulation of the Rag genes,[251] down-regulation of TdT,[235] and transcriptional activation of the kappa locus, detected as upregulation of sterile kappa transcripts.[252] A control element for regulating Rag expression has been identified.[253,254] Extinction of recombinase activity is probably important for chromosomal stability during the clonal burst period of B-cell development[255,256,257] and is also at least a part of the mechanism that assures allelic exclusion, the expression of a single heavy chain by any given B cell.[258] There is evidence that pre-BCR selection requires low levels of IL-7[259] and probably occurs naturally as the developing precursors migrate through different stromal cell microenvironments in bone marrow.

The function of the pre-BCR may be more complex than simply to sense whether an in-frame VDJ rearrangement has occurred. This possibility is suggested by the observation that heavy chains with different VDJ segments vary in their capacity to assembly with SLC components.[260,261,262,263] V regions are classified into families based on sequence homology, and many members of two of these families, the 7183 and Q52, appear to frequently generate heavy chains that assemble poorly with SLC.[262] A consequence of this will likely be poor pre-BCR signaling and little clonal expansion; such cells will become underrepresented at later stages of B-cell development relative to cells containing heavy chains that signal effectively. One explanation for this SLC assembly-mediated clonal expansion is that it serves a quality control function to test heavy chain V regions for their potential to fold with real Ig light chain, a critical requirement if the cell is to express a complete BCR. An alternative (not necessarily mutually exclusive) explanation is that making pre-B-cell proliferation dependent on pre-BCR expression provides a simple mechanism for regulating the extent of clonal expansion, as an immediate consequence of pre-BCR signaling is to terminate SLC expression. Thereafter, SLC protein levels decay and are diluted by cell division so that after several rounds of proliferation, pre-BCR levels will decrease to below the threshold required to provide the signal to maintain the cell in cycle. Figure 8.11 illustrates a model for bone marrow B-cell development, showing the pre-BCR checkpoint.

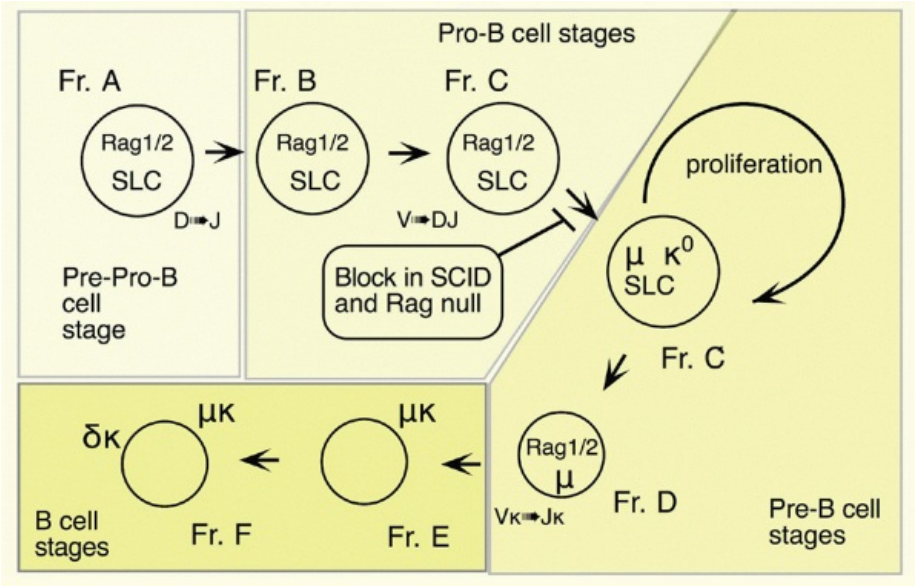

FIG. 8.11. Model of Mouse Bone Marrow B-Cell Development Showing Relationship of Immunoglobulin Rearrangement with Progression and Proliferation.

One of the most striking examples of pre-BCR selection is seen with the D-proximal V_H gene, V_H81X, where early precursors show biased overutilization due to preferential rearrangement of this V_H gene.[11,264,265] In fact, regulatory sequences interspersed throughout the distal VH region, termed PAX5-activated intergenic repeat elements, appear to enhance utilization of distal J558 family VH genes.[266] Furthermore, a recombination regulatory region, the intergenic control region 1, which lies between the VH and D clusters, has been shown to inhibit proximal and promote distal VH gene utilization.[267]

Curiously, although it is abundant in early B-cell precursors, V_H81X is rarely seen in the mature B-cell compartment. The demonstration of the decrease in representation of cells with V_H81X rearrangements at the pre-B clonal expansion phase in bone marrow,[268,269] together with the finding that heavy chains utilizing VH81X frequently fail to assemble functional pre-BCRs,[260,261,270] explained this paradox. Models of pre-BCR three-dimensional structures based on x-ray crystallography may help to explain variation in the ability of different heavy chains to assemble with SLC.[271]

However, it is still puzzling that the most frequently rearranged V_H gene is so strongly selected *against* at the clonal expansion stage. A possible explanation may lie in comparisons of V_H utilization during fetal development. That is, in contrast with bone marrow precursor cultures, the ratio of productive/nonproductive V_H81X does not decrease during cultures of fetal precursors.[272,273] Furthermore, the proliferative burst that pre-BCR assembly provides to bone marrow pre-B cells may instead result in exit from cell cycle in fetal precursors,[263] leading to selection of very different BCR repertoires during fetal and adult B lymphopoiesis. The possible significance of this is discussed subsequently in the section on B1 B cells.

Leaving aside such developmental difference, the role of the pre-BCR in B-cell selection remains subject to debate, with evidence suggesting it functions to eliminate[274] or enrich[275] self-reactivity.

Light Chain Rearrangement and Generation of Immature B Cells

Besides termination of TdT and SLC gene expression, pre-BCR signaling also results in the downregulation of Rag-1 and Rag-2 expression, mediated by activity of Gfi1b on Erag, the B cell-specific Rag regulatory element,[254] and the repression of FoxO1.[276] It appears that induction of Bcl-6 by pre-BCR signaling functions in this progression by repressing Myc and Ccnd2, thereby promoting exit from cell cycle.[277,278] As mentioned previously, there is evidence that the pre-BCR signals through ERK and this Ras-MEK-ERK signaling acts to silence transcription of Ccd3, encoding the cell cycle protein cyclin D3, thus promoting cell quiescence.[279] Opposing cell cycle exit, IL-7R signaling activates STAT5, maintaining Ccd3 expression. Eventually, the pre-B cell escapes from

IL-7 signaling, exits the cell cycle, and the Rag genes are reexpressed at high levels. Sterile kappa transcripts become detectable during the cycling stage, likely reflecting chromatin remodeling to make the kappa light chain locus accessible,[252] so induction of the recombinase machinery can initiate kappa light chain V to J rearrangement.

An interesting feature of the Vκ locus is that the approximately 100 genes are in both transcriptional orientations, and so these genes can rearrange either by deletion (generating an extrachromosomal excision circle) or by inversion.[280] The absence of intervening D segments also means that it is possible for upstream V kappa genes to rearrange to downstream J kappa segments, “leapfrogging” the initial rearrangement, assuming it was to any Jκ other than Jκ5. The successive association of different kappa chains with the same heavy chain in a B cell is referred to as BCR “editing” and was originally observed in the context of autoreactivity, which maintains Rag expression even at the B-cell stage[281,282] (see following section on B-cell tolerance). Because assembly and expression of a complete BCR (that is not self-reactive) terminates Rag expression, an additional reason for light chain editing in the bone marrow may be to replace an initial light chain that fails to assemble effectively with the particular heavy chain present in that pre-B cell. This is probably the explanation for multiple light chain rearrangements detected in single early B-lineage cells.[283] Complete failure of kappa rearrangement, possibly after receptor editing to avoid self-reactivity, leads the pre-B cell to a second phase of light chain rearrangement, dependent on IKK-mediated NF-κB signaling, where the λ light chain locus rearranges.[284]

Newly formed B cells can be distinguished from mature B cells on the basis of their inability to proliferate in response to BCR cross-linking (ie, they are functionally immature). This is also the stage where negative selection is reported in transgenic models of autoreactivity.[285,286] Cells at this stage have a short half-life of only a few days, compared to mature follicular B cells with a half-life measured in months. They can be distinguished by surface phenotype from other B cells based on expression of certain combinations of markers, such as IgM+IgD−, absence of CD23, and high-level expression of CD24/HSA.[287] Recently, there have been reports of single markers that are useful in distinguishing newly formed cells from

any mature subset, such as the molecules recognized by monoclonal antibodies 493[288] and AA4.1.[289] The AA4.1 target molecule has been cloned and identified as the mouse ortholog of a component of the human C1q receptor.[290]

Cells similar to newly formed B cells can be generated to varying extent during stromal cell culture of B-cell precursors, although the more primitive cycling pre-B or pro-B cells are usually more abundant and tend to increase in frequency with prolonged culture. It is possible to induce differentiation of B cells in these cultures by withdrawing IL-7, which induces a wave of small pre-B and then newly formed B-cell generation. Such cells do not persist for more than a day, unless the cultures are established from Bcl-2 transgenic mice,[131] suggesting that the short half-life of newly formed cells in these cultures, and possibly also in vivo, is due to their low level of antiapoptotic mediators. Both Bcl-2 and Bcl-X_L mRNA are present at only very low levels in these cells, in contrast with other B-lineage stages where either one or the other predominates. Overexpression of Bcl-X_L from a transgene results in accumulation of a population of pro-B phenotype cells with nonfunctional rearrangements,[291] implicating this protein in the process of pre-BCR selection of cells with functional rearrangements.

Peripheral Maturation Stages and Functional Subsets

Transitional B Cells

Newly formed immature B cells migrate to the spleen where they either die or undergo further maturation to a mature B cell. These maturing B cells can be subdivided based on differential expression of several surface proteins, including CD21, CD23, CD24/HSA, and AA4.1. These subdivisions have been referred to as "transitional B cells."[292] One recent subdivision based on CD21, CD23, AA4.1, and IgM levels has shown progression from an AA4.1+CD21−CD23− T1 stage to an AA4.1+CD21-CD23+ T2 stage, followed by down-regulation of IgM as a T3 stage, and finally loss of AA4.1 with upregulation of CD21 to yield the mature follicular phenotype.[289] As shown in Figure 8.12, this approach also resolves two AA4.1- subsets that lack CD23, the B-1 subset with low CD21 and the MZ subset with very high CD21 (see following). The transitional stage cells are all short-lived as shown by bromodeoxyuridine incorporation.[289,293] They are also not functionally competent, as shown by inability to proliferate after BCR cross-linking.[287,294] Another well-characterized functional distinction is that B-cell tolerance, rather than an immune response, is induced by BCR cross-linking of immature B cells.[295,296,297] More recent studies with transgenic models of self-reactivity have shown that these B cells can be deleted, undergo receptor editing, or rendered functionally unresponsive (anergic) by BCR signaling at the immature stage.[281,285,298,299,300,301,302] One group has suggested that the "T3" stage is not an intermediate in production of follicular B cells, but rather a population of autoreactive anergic cells,[303] so additional work needs to be done to establish the identities of all the B-cell subpopulations present in spleen. For example, careful comparison of T1 and T2 B-cell populations shows that a fraction of T2 cells do respond to certain microenvironmental stimuli and include a portion of cycling cells that appear to constitute a branchpoint in generation of MZ B cells.[304]

It is not simply the inability to receive T-cell help due to differences in microenvironment or receptor expression that makes immature B cells incapable of responding as mature B cells. Different from the result with mature B cells, cross-linking the BCR on immature B cells has been shown to induce apoptosis, suggesting distinctions in the signaling pathways between these two stages.[305,306] Studies with transgenic mice suggest that this apoptosis is not mediated through the Fas/Fas-ligand pathway, as central deletion is intact in Fas-mutant mice.[307,308] Prior to induction of apoptosis, immature B cells have been shown to complete

some of the early events associated with entry into cell cycle while failing to complete this program.[309] Distinct stages in maturation appear more or less capable of responding to BCR cross-linking by reinduction (or maintenance) of Rags to facilitate receptor editing.[286] Furthermore, it appears that immature B cells are more sensitive to smaller changes in intracellular free calcium, compared to mature B cells.[310] It is possible that the capacity to upregulate antiapoptotic molecules, such as A1, may play a critical role in the inability of immature B cells to survive and complete a normal response.[311,312] The characterization of signaling pathways in different immature stages of developing B cells is ongoing and should eventually provide insights into the detailed mechanism for immature B-cell tolerance.

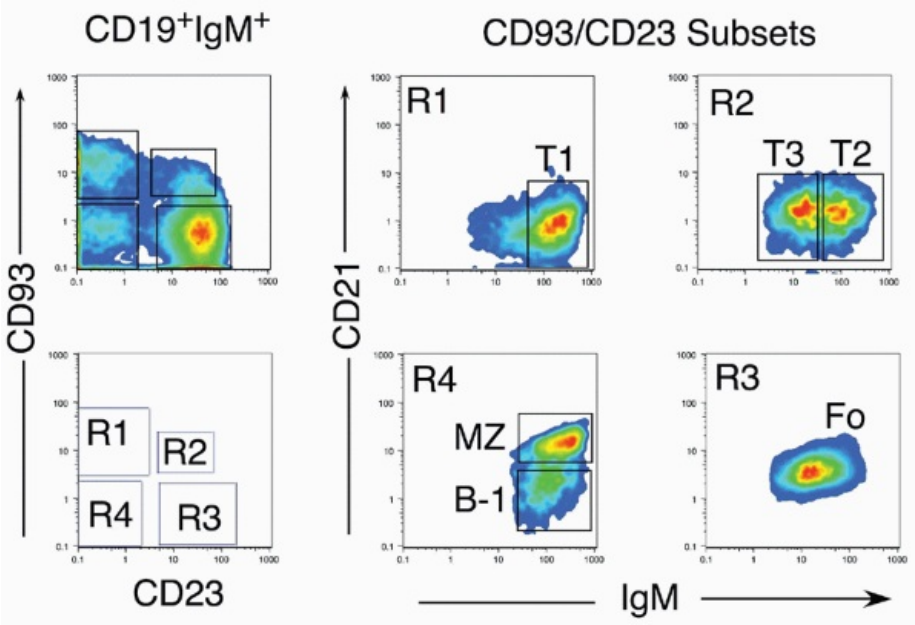

FIG 8.12. Flow Cytometry Approach for Resolution of Transitional (T1-T3) and Mature (Follicular, B1, Marginal Zone) Populations of B Cells in Mouse Spleen. Left panels show the distribution of AA4.1 and cluster of differentiation (CD)23 on B cells (defined as CD19+immunoglobulin [Ig] M+). Cells in the boxed regions are then analyzed for correlated expression of CD21 and IgM, facilitating resolution of three AA4.1+ fractions (T1-T3), the follicular subset (AA4.1−CD23+CD21+), the marginal zone subset (AA4.1−CD23−CD21++), and the B-1 subset (AA4.1−CD23−$CD21^{low}$).

Analyses of various normally occurring or engineered mutant mice have provided approaches

for investigation of the process of progressing from a newly formed B cell to a mature follicular B cell. B-cell populations and B-cell function has been studied for many years in CBA/N mice bearing the xid mutation. This mouse has a mutation in the Btk gene that produces a milder phenotype than the complete absence of peripheral B cells seen in humans. The Btk gene likely plays a role at several stages of B-cell development and activation, which complicates the analysis, but it appears clear that one major consequence is altered BCR signaling that has a profound effect on progression through the various transitional stages in spleen. A likely consequence of diminished strength of BCR signaling is a compensatory requirement for higher surface BCR expression that eventually produces at decreased frequency a type of "mature" B cell that is still functionally handicapped.[313,314] Several groups have produced xid mice on a nu/nu T-cell less background that results in a more profound absence of mature B cells, suggesting a requirement for T cells or T cell-produced factors in the maturation of xid B cells.[315,316] A more recent variation of this type of investigation is the production of xid/CD40-deficient mice that show a similar deficit in mature B cells, suggesting a role for the CD40/CD40L interaction in the generation of mature B cells from transitional B cells, particularly when the BCR is handicapped by defective Btk.[317]

Lyn is a Src family protein kinase that is associated with the BCR and functions in signaling in mature B cells.[318] Lyn-deficient mice exhibit defects in maturation of immature cells,

suggesting a positive role for BCR/Lyn signaling at this stage, but these mice also develop a severe autoimmune condition, suggesting an additional negative regulatory role for Lyn in maintaining tolerance in mature B cells.[319,320] Cross-linking of the BCR on T2 and mature B cells results in c-Rel activation that upregulates antiapoptotic genes and the prosurvival BAFF receptor.[321] Furthermore, migration of early bone marrow emigrants to areas where T1 and T2 cell mature has been shown to depend on chemokine signaling that is disrupted in Rac1/Rac2 double-deficient mice.[322] Finally, progression of transitional cells to the mature B-cell stage is dependent on "tonic" BCR signaling, as mice engineered to express low-surface BCR levels, and, thereby lower tonic BCR signaling showed decreased generation of mature B cells.[323] Analysis of these mice suggested that maturation was dependent on activation of ERK via a signaling pathway requiring Ras. CD72 is a predominantly B-lineage restricted C-type lectin, and ligating this molecule was recognized for many years (when it was known as Lyb2) as having functional consequences.[324] Recent analyses of a CD72 null mouse has clarified its function in B-cell development and activation.[325] CD72 has been shown to recruit SHP-1 to the BCR, supporting a negative regulatory role in BCR activation.[326] Consistent with this model for CD72 function, null gene-targeted mice have been shown to produce B cells that are hyperresponsive.[325] Interestingly, late stages of B-cell development are affected, with fewer mature B cells and relatively normal numbers of immature B cells in spleen.[325] Thus, too intense signaling may also delay maturation of immature B cells.

A number of studies have demonstrated the important role that the phosphoinositide 3-kinase (PI3K) signaling pathway plays in the maturation of transitional cells and generation of mature B cells. Under conditions of normal tonic signaling, PI3K signaling inhibitors block the

normal downregulation of Rags in immature B cells and also decrease progression past this stage.[327] This BCR signal is dependent on CD19 and BCAP, a BCR complex adapter molecule,[328] and acts through AKT to activate PI3K. The survival of B cells at the mature stage has been shown to depend on an intact BCR. More recent work revealed that the survival of BCR-deficient B cells can be rescued by activation of the PI3K signaling pathway alone.[329]

Follicular B Cells

The major population of mature recirculating B cells in the spleen is located in the B-cell follicle region, hence the term follicular B cells. Entry into this anatomic site appears to constitute a final stage in maturation for developing bone marrow B cells, as competition for this site is compromised in several transgenic models of B-cell tolerance.[330,331,332,333] Cells in this compartment do not proliferate but persist in the resting state for several months. A conditional knockout study, eliminating expression of the BCR (by deleting the V region), revealed that expression of the BCR is required for cell survival.[232,233] It is not yet established whether this is due to "tonic signaling" (simple assembly of the BCR signaling complex) or instead reflects signaling by low-affinity binding to cross-reactive self-determinants, a kind of "positive selection." It is interesting to note that ablation of heavy chain from immature B cell, thereby eliminating any possibility of pre-BCR or BCR tonic signaling, results in a "reversion" of cell phenotype to an earlier developmental stage.[334] Finally, the maintenance of follicular B cells has been found to depend on the function of the c-Myb transcription factor due to its role in BAFF signaling[335] (see following section). The repertoire of the follicular B-cell pool appears to differ from the earlier immature splenic B-cell population, as assessed by sequence analysis of the light chain repertoire in heavy chain transgenic mice.[336] The approach of fixing the heavy chain and then examining the light chain repertoire simplifies the analysis, and the results of this study were interpreted to indicate that BCR-mediated antigen selection is indeed operating. However, the resolution of the analysis probably could not have rigorously excluded populations known to show V gene biases, such as B-1 or MZ B cells (see following sections), so further work will be required to provide convincing evidence of antigenic selection in the follicular B-cell pool.

B-Cell Migration and Maintenance

Newly formed B cells migrate from the bone marrow to the spleen, undergo further maturation in the red pulp, and eventually enter the follicle where they constitute the mature B-cell pool that recirculates. Their migration is dependent on chemokines/receptor interactions, notably the SLC(CCL21)/CCR7 interaction, as demonstrated by the inability of mature B cells to be retained normally in spleens of CCR7 null mice.[337] The role of the CXCR5 receptor on B cells in homing to the lymphoid follicle due a gradient of the B-lymphocyte chemoattractant CXCL13 is also well known, and CXCL13 can directly induce Ltα1β2 on the recruited cells.[338] Finally, it is also possible that the SDF1 (CXCL12)/CXCR4 interaction, critical for normal B lymphopoiesis, may also be important at this later stage, although investigation of this issue is complicated by the early defect. This is an ongoing area of investigation and may eventually be clarified by developmentally regulated gene targeting studies.

Recently, considerable interest has focused on the role of a tumor necrosis factor family member cytokine known variously as BAFF, BLyS, TALL-1, zTNF4, or THANK in the process of peripheral B-cell maturation.[339] BAFF is a tumor necrosis factor family member found to enhance survival of B cells or even produce autoimmunity in transgenic mice constitutively expressing it.[340,341] Initially, two receptors defined for BAFF, BCMA and TACI, provided a complex picture, as targeted inactivation of BCMA yielded no B-cell defect and deletion of TACI had increased B cell numbers (suggesting that TACI might be a negative regulator). This puzzle was resolved by identification of a third receptor, BAFF-R/BR3,[342,343] which was mutated in a strain of mice known to lack most mature B cells, A/WySnJ.[344] A second ligand, APRIL, can bind to BCMA and TACI, but not to BAFF/BR3, and this binding is proliferative rather than survival promoting.[345] Thus, the critical interaction for maintenance of follicular B cells is BAFF/BLyS with its receptor. B-1 cells are not deficient in A/WySnJ,[344] suggesting that their maintenance does not depend on this pathway

but instead is more BCR dependent. Interestingly, excessive levels of BAFF promote survival of highly autoreactive BCR transgenic mouse B cells, consistent with the finding of elevated levels in patients with autoimmune diseases such as systemic lupus erythematosus and Sjögren syndrome.[346]

Mechanism of B-Cell Receptor Signaling

Considering that the structure of the Ig molecule was elucidated more than 40 years ago, it is surprising that the details of initiation of B-cell activation following antigen binding are still not firmly established. Recent work using single-molecule imaging demonstrated that antigen binding altered the mobility of BCRs within microclusters on the B cell, resulting in rigid oligomeric structures that generated a signal.[347] Interestingly, this oligomerization required the membrane proximal domain of IgH-μ (Cμ), and such domains in isolation spontaneously formed aggregates in the membrane, leading to the hypothesis that antigen ligation induces a conformational change in Cμ4 that then promotes assembly of signaling clusters. Other work visualizing simple Ig molecules has demonstrated the importance of the membrane cytoskeleton in limiting diffusion of the BCR and revealed the importance of Igb/CD79b in mediating such restriction, showing that alterations in BCR distribution rapidly result in strong intracellular signaling.[348] Finally, an alternative model has been proposed, based on analysis using quantitative bifluorescence complementation, suggesting that preexisting BCR aggregates are less active in signaling, and that the disruption of such complexes upon antigen binding is responsible for generating a signal.[349] Additional work using such advanced imaging techniques will undoubtedly resolve this apparent contradiction and lead to a more complete understanding of BCR signaling.

B-Cell Turnover

It is estimated that 10 to 20 million B cells are produced in bone marrow of the mouse each day,[350] yet it appears that only about 10% of this number reach the periphery.[293] Thus, there is considerable loss at this bone marrow emigration stage/spleen entry stage, possibly due to elimination of autoreactive cells (B-cell tolerance) or to homeostatic regulation. The latter possibility is supported by the observation that depletion of the mature B-cell population

results in a relatively rapid recovery of this pool, suggesting that most of the immature B-cell population can enter the mature follicular subset in this situation.[351]

Once functionally mature B cells are generated, it has been difficult to unambiguously determine their half-life, although accumulating data from several laboratories using bromodeoxyuridine labeling has led to the idea that follicular B cells have a relatively long half-life on the order of months.[293,352] A recent elegant study provided definitive confirmation of this by conditional elimination of Rag-2 expression, allowing termination of B-cell development in adult mice.[353] This study showed that follicular B cells have a half-life of about 4.5 months. This same analysis showed that two other subsets of B cells, B-1 and MZ B cells, did not diminish over time, consistent with their well-known capacity for self-renewal and lifelong persistence.

Germinal Center B Cells

T cell-dependent immune responses usually give rise to anatomically distinctive structures in spleen and lymph nodes that are referred to as germinal centers (GCs) and contain large numbers of rapidly cycling B cells.[354,355] These cells can be recognized in stained sections of spleen by binding of high levels of peanut agglutinin and by the absence of IgD.[356,357] Many of the B cells with this phenotype have downregulated-Bcl-2/upregulated-Fas expression and, in the absence of strong BCR signaling, will likely die by apoptosis.[358,359,360,361] The termination of IgD expression means that surface BCR expression decreases at least 10-fold, and so limiting amounts of antigen will favor the cells with increased affinity for antigen, generated by a process termed *somatic hypermutation*. Recently, a miRNA, miR-155, has been found to be critical for the generation of GCs by regulating cytokine production.[362]

A major advance in understating the GC response has been the discovery that activation-induced cytidine deaminase (AID), an RNA-editing enzyme that can induce class switch recombination in fibroblasts,[363,364,365] is also a key player in the process of hypermutation.[366,367] In fact, a third means for Ig gene diversification used in nonmammalian species, V gene conversion, is also dependent on AID.[368,369] AID is now thought to be responsible for genetic mutations outside the Ig loci in lymphomas that arise from GC B cells.[370]

The precise mechanism of selection for higher affinity B cells generated by hypermutation of the BCR V regions remains to be fully understood, but the regulation of pro-apoptotic and antiapoptotic genes likely plays a major role. B cells able to bind antigen with high affinity can present antigen to specialized CD4+ T cells (follicular helper T cells) that then signal the B cell through a CD40/CD40L interaction, resulting in the upregulation of Bcl-X_L.[358,359,360] Most GC B cells have sharply downregulated levels of Bcl-2 and up-regulated levels of Fas,[360,361] and so in the absence of rescue by expression of the alternative antiapoptotic mediator Bcl-X_L, cell death by apoptosis will be the fate of most B cells in the GC. Careful regulation of selection is critical to affinity maturation, and elimination of self-reactive cells that potentially could be generated during this process must also occur efficiently to avoid the potential of autoimmune disease. Recently, the role of the immunoinhibitory receptor PD-1,

present at high levels on follicular helper T cells, in GC B-cell selection has been described.[371] Furthermore, high-resolution imaging of fluorescent reporters is revealing details of cell dynamics within the GC, indicating that competition for T-cell help is a key limiting factor in the selection of higher affinity B cells.[372]

Memory B Cells

Memory B cells were initially defined functionally as cells that could respond rapidly by production of high-affinity antibody when challenged in a host reconstituted with B cells and T cells from a primed animal.[373,374,375,376,377,378] Subsequently, such cells have been purified based on their antigen-binding properties[379] and shown to consist primarily of isotype-switched (IgG+) B cells that continue to express CD45R/B220 and have distinctively

lower levels of cell surface BCR.[380] They arise during the T cell-dependent immune response, probably only from follicular B cells, in the GC. They are very long-lived or self-regenerating, as cell transfer assays have shown that memory responses can be detected for long periods after the primary immunization.[380,381] More recent work has focused on examining the heterogeneity of secondary B-cell subsets, examining cell surface proteins that may aid in distinguishing them.[382] In terms of genes that distinguish memory B cells, it appears that phospholipase C gamma 2 is important both for the generation and maintenance of memory B cells.[383]

It is not clear whether all B cells are capable of giving rise to memory B cells. Memory or "secondary" B cells were originally described as expressing distinctively low levels of CD24/HSA, recognized by the monoclonal antibody J11d.[384] Some years later, fractionation of naïve spleen B-cell precursors into J11d^{low} and J11d^{high} subsets in a spleen focus assay system showed that while rapid antibody secretion derived from J11d^{high} cells, memory came largely from the J11d^{low} subset.[385] Subsequent experiments demonstrated that GCs (the site where most memory B cells are generated) were only produced in cell transfers of J11d^{low} B cells and not with J11d^{high} or CD5+ B cells.[386] Considering the rapid Ig secretory response of B1 B cells and MZ B cells, both contained in the CD24/HSA^{high} fraction, and the fact that most other CD24/HSA^{high} cells are immature (transitional) B cells, likely to be highly susceptible to apoptosis, it seems quite reasonable that memory B cells would not be a major product of this fraction. Rather, the most likely candidate for the memory B-cell precursor is the follicular B-cell subset (Fo), which has variable but lower expression of CD24/HSA. Whether there is heterogeneity for GC formation or memory B-cell generation within the Fo population remains to be determined.

Whether the maintenance of memory requires periodic restimulation by antigen has been a longstanding controversial issue. On the one hand, transfer of B cells and T cells into irradiated recipients usually required simultaneous challenge with antigen in order to elicit the full response and maintain B-cell memory in recipients.[387] Antigenic fragments can persist for very extended periods on follicular dendritic cells, which are very potent antigen-presenting cells, and, so in this model, the memory B cells are periodically triggered to self-

renew by interaction with antigen on follicular dendritic cells. However, on the other hand, memory B cells can be maintained in the apparent absence of T cells or follicular dendritic cells.[388,389] Furthermore, analysis by bromodeoxyuridine labeling of memory B-cell populations showed that they were nondividing.[381] This issue has been addressed in elegant experiments using inducible Cre recombinase to switch the BCR on memory cells away from the immunizing antigen.[390] Such antigen-negative memory cells still persisted for extended periods, clearly demonstrating that this was a physiologic property of the cell type, independent of the presence of antigen.

B1 B Cells

B1 B cells, initially described as Ly-1/CD5+ B cells, are distinguished from follicular B cells by phenotype, anatomic distribution, and function.[23,391] The B1 B-cell phenotype encompasses both CD5+ and CD5− B cells that are IgM^{high}, $IgD^{low/-}$, CD23−, and CD43+. They constitute a large proportion of the B cells found in the peritoneal and pleural cavities (30% to 50% of B cells, around 10^6 cells), but are also found in spleen where they are present at numerically similar levels, but constitute a much lower proportion of the total B-cell pool (2% of B cells, around 10^6 cells). The B1 B cells in the peritoneal cavity are also CD11b/Mac-1+ unlike those in spleen. They appear early in ontogeny, representing 30% or more of the B cells in spleen of 1-week-old animals. They also have a distinctively higher frequency of λ light chain usage compared to follicular B cells (20% vs 5%). Also unlike follicular B cells, they maintain their population in adult animals largely by self-renewal (possibly dependent on periodic stimulation by self-antigen; see following discussion), rather than by input from precursor cells, as shown in cell transfer studies.[392] There is also a recently described population of IgM-secreting cells in bone marrow with a B1 cell surface phenotype.[393] Perhaps the most distinctive feature of CD5+/B1 B cells is their enrichment of certain self-reactive specificities, notably for branched carbohydrates, glycolipids, and glycoproteins, including phosphorylcholine, phosphatidylcholine (PtC), the Thy-1 glycoprotein, and bacterial cell wall constituents.[394,395,396,397] These antibodies, although autoantibodies, are not pathogenic, but rather referred to as “natural autoantibodies” whose existence has been recognized in serum for several decades.[398,399,400] Their function is still under active investigation, but at least some natural autoantibodies are thought to function in clearance of senescent cells or proteins and to provide an initial immunity to common bacterial or viral pathogens, serving as a kind of “hardwired” memory B-cell population.[401,402,403,404]

Under physiologic conditions in normal mice, most of the CD5+ B cells in the B1 population (so called “B1a B cells”) arise from precursors in fetal liver, as cell transfer studies showed many years ago that B cells with this phenotype were inefficiently generated from bone marrow precursors in adult mice, compared to fetal or neonatal precursors.[405] This is shown by repopulation of SCID mice by pro-B stage cells isolated from fetal liver and adult bone marrow (Fig. 8.13). Part of the reason for this difference may be that novel BCRs are enriched by distinctive mechanisms of fetal B lymphopoiesis, including recombination in the absence of TdT (thereby favoring rearrangement of certain D-J and V-D junctions possessing short regions of homology[16]) and distinctive pre-BCR selection.[263,273,406]

Forced expression of transgenic BCRs cloned from CD5+ B cells can give rise to CD5+ B cells from bone marrow of adult animals, but the physiologic relevance of this remains to be carefully assessed. B1 B cells that lack CD5, termed B1b B cells, can be generated from bone marrow and a distinctive phenotype for a precursor capable of producing such cells has been described,[162] although the developmental relationships between conventional ("B2") B-cell precursors and these cells are complex.[407] Embryonic day 9 YS hematopoietic progenitors have been sown to give rise to B1 and MZ B cells but not conventional follicular B cells. The development of B1/CD5+ B cells is thought to be more dependent on antigenic selection compared to follicular B cells. This idea was first suggested by the finding of particular specificities enriched in this population and

strengthened by the observation of repeated occurrences of particular V_H/V_L pairs.[408] Thus, for example, the anti-PtC specificity is predominantly encoded by V_H11V_K9 and $V_H12V\kappa4$ utilizing two V_H genes rarely found in conventional T-dependent immune responses.[395,409] These cells appear to participate in T-independent responses, but in normal physiology may in fact provide an initial low-affinity "first wave" response to many pathogens that eventually will also elicit a T-dependent response.[404,410]

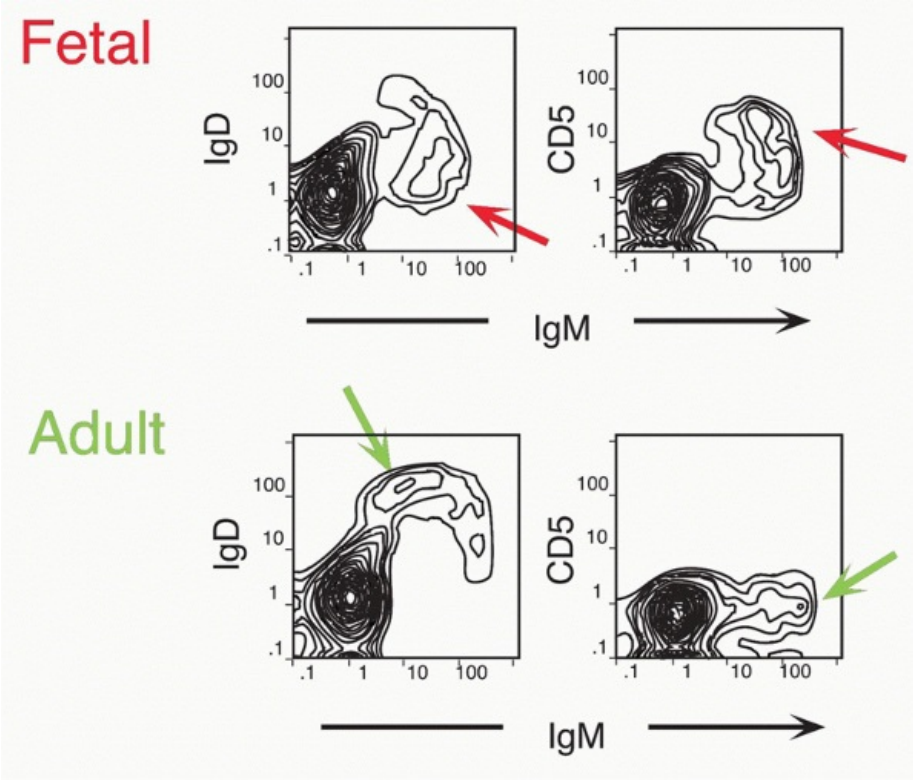

FIG 8.13. Generation of B Cells in Severe Combined Immunodeficiency (SCID) Mice Shows the Distinctive Phenotypes Produced from Fetal and Adult Pro-B Cells. Similar numbers of pro-B cells, isolated as in Figure 8.5, Fr. B/C, were injected intravenously into sublethally irradiated adult SCID mice. Recipients were analyzed 3 weeks later for spleen cell lymphocytes by staining as shown in the figure.

The observation of their self-reactive bias, their capacity for self-renewal, and their restricted repertoire of distinctive BCRs all are consistent with an important role for BCR-antigen interaction in generation and maintenance of this population. This has been formally confirmed recently by studying mice bearing a BCR transgene specific for a glycosylation present only on the Thy-1 membrane protein.[411] In these mice, transgene-encoded serum anti-Thy-1 autoantibody[396] was readily detected, and there was a corresponding accumulation of a population of CD5+ transgene BCR+ B cells in the peritoneal cavity. Importantly, in Thy-1 null mice generated by gene targeting, neither serum autoantibody nor the B-cell population was found, demonstrating the critical role for antigen in selection of this B-cell population.[412]

Consistent with the importance of antigen selection in the generation and/or maintenance of these cells, this population is often severely affected in mice bearing mutations that alter BCR signaling intensity. The loss of negative mediators such as PTP1C/SHP-1 in “moth-eaten” mice,[413] of CD72 that recruits this phosphatase,[413] and of the CD22 coreceptor[414,415] all result in an increased frequency of B1/CD5+ B cells relative to follicular B cells. On the other hand, the loss of critical BCR signaling components or positive mediators in this pathway such as an Ig-α tail mutant,[416] Btk deficiency (in xid or Btk null mice),[417,418] CD19 null mice,[419,420] CD21 null mice,[421] CD45 null mice,[410] and vav null mice[422] all negatively impact this B-cell population. A further indication of a signal-dependent selection model is the accumulation of B1-type B cells with high-level expression of a BCR surrogate, LMP2A, but only B2 B cells and MZ B cells with low-level expression.[423]

The functional role of B1 B cells is still incompletely understood. Whereas it is clear that they produce natural autoantibody, there also is evidence for their participation in immune responses. B1 B cells have also been shown to accumulate in draining lymph nodes of the respiratory tract during influenza infection of mice.[393] Treatment of mice with *Francisella tularensis*-derived lipopolysaccharide protects them from subsequent exposure to the live bacteria (the etiologic agent of tularemia) by activating a small fractions of B1 B cells with this antigen specificity.[424]

IL-10 is a cytokine with anti-inflammatory properties that also enhances B-cell survival and antibody production. Thus, it can function to damping down cellular immune responses and promote humoral immunity. Many years ago, B1 B cells were identified as the B-cell source of this cytokine that is also secreted by monocytes, mast cells, and regulatory T cells.[425] Recently, based on observations of alterations in the level of inflammation in mice lacking or overexpressing CD19, a CD1d(high) subset of B-1 cells expressing IL-10 has been identified.[426] Considering the anti-inflammatory regulatory effects of IL-10, this subset has been referred to as “B Regs” or “B10” B cells. Investigation of this subset in the experimental autoimmune encephalomyelitis mouse

model of multiple sclerosis showed that initiation and progression of disease was influenced reciprocally by depletion of B cells. Because experimental autoimmune encephalomyelitis is considered a T cell-mediated disease, this provides strong evidence for the capacity of B1 cells in modulating T cells during the initiation of disease. Curiously, depleting B cells after

experimental autoimmune encephalomyelitis was already established actually diminished symptoms, indicting that B cells were required for persistence of autoreactive T cells. This suggests that simple depletion of all B cells in certain autoreactive pathologies may have different consequences, depending on the progression of the disease. Finally, examination of B10 function in the autoimmune lupus-prone NZB/W mouse F1 indicated that these cells were responsible for inducing regulatory T cells that could ameliorate the course of disease in these animals.[427]

Marginal Zone B Cells

Another B-cell subset distinct from follicular B cells is the MZ B-cell population. MZ B cells are localized in a distinct anatomic region of the spleen that represents the major antigen filtering and scavenging area (by specialized macrophages resident there). It appears that they are preselected to express a BCR repertoire similar to B1 B cells biased toward bacterial cell wall constituents[428] and senescent self-components (such as oxidized low-density lipoprotein [LDL]).[429,430] Similar to B1 B cells, they respond very rapidly to antigenic challenge, likely independently of T cells, but participating in the early phase of T-dependent responses.[431,432,433] Uniquely, among all populations of B cells, MZ B cells are dependent on Notch2 signaling for their development.[434,435]

There are similarities and differences in the cell surface phenotype of MZ and B-1 B cells. Thus, they both are IgM+++IgD-/+, CD23-, and CD9+.[436] However while B1/CD5+ B cells express CD5 and CD43, MZ B cells do not, and MZ B cells express distinctively high levels of CD21, while B1 cells have distinctively low levels (see Fig. 8.12). Also, MZ B cells have high levels of CD1d, while most B1 B cells do not,[437] the exception being the “B10” subset mentioned previously. Certain mutant mice show similar effects on MZ and B1 B cells, distinct from follicular B cells. For example, most of the mutations described for B1 B cells that alter BCR signaling have similar consequences for MZ B cells,[438] although cells that resemble MZ B cells are present in xid/Btk-deficient mice, leading to some controversy in their origins.[439] Both are decreased by a mutation in the Ig-α tail that weakens overall BCR signaling.[440] They are also both decreased in the Aiolos transcription factor null mouse.[63] Interestingly, deletion of the Pyk-2 tyrosine kinase results in elimination of MZ B cells while B1 cells are still found.[441] MZ B-cell development can be studied in a heavy chain transgenic mouse model system where large numbers of such cells are produced.[428,442] In this V_H81X heavy chain mouse, B cells with a specific light chain accumulate with an MZ phenotype. This MZ population is eliminated by deletion of CD19, Btk, or CD45, all genetic changes that weaken BCR signaling.[438] MZ B cells are generated in mice even when B-cell development is blocked shortly after birth by conditional deletion of Rag-2,[353] showing that like B1 B cells, a significant portion of MZ B cells are produced during fetal development. Consistent with this fetal origin, many MZ B cells have Ig heavy chains with little or no TdT-mediated N-regions at their V-D and D-J junctions.[443] Interestingly, irradiated mice repopulated with bone marrow precursors still accumulate significant numbers of MZ B cells with low levels on N-addition, indicating that the strong BCR-mediated selection guiding entry into this subset can select such cells from adult bone marrow-generated B cells. In recent

years, there has been considerable progress in understating some of the non-BCR-related pathways that regulate the generation of MZ B cells. For example, the can-nabinoid receptor 2 appears important in localization of MZ B-cell precursors to the anatomic region where Notch signals are available.[444] Notch ligand expression for generation of MZ B cells is regulated by the E3 ubiquitin ligase Mind bomb 1, which also is critical for T-cell development.[445] Furthermore, Notch2 activation in MZ B cells is potentiated by expression of the fringe glycosyltransferases lunatic fringe and manic fringe.[446] Notch activation requires its proteolysis, and this is now known to occur through activity of a disintegrin and metalloproteinase 10 (ADAM10).[447]

B-Cell Tolerance and Receptor Editing

A number of transgenic mouse models have been developed for the study of B-cell tolerance (Table 8.2). In two of these systems, high-affinity BCRs are expressed as IgM-IgD transgenes, one specific for the antigen hen egg white lysozyme (HEL), the other for a specific polymorphic determinant on MHC class I.[281,298,299,300,448,449,450] The advantage of these systems is that they utilize antigens that can be regulated: Transgenic B cells can develop in either the presence or absence of antigen, and cell transfer experiments can be employed to alter the B cell's antigenic milieu. These have been used initially to confirm ideas on B-cell tolerance that originated in work with nontransgenic B cells, namely that exposure to antigen is generally deleterious to developing B cells, resulting in their elimination or failure to mature (instead entering an “anergic” state). However, the resolution of these systems, coupled with advances in gene targeting and other molecular technologies, have uncovered important new details regarding the way that self-reactive B cells develop (or fail to develop). For example, studies in the anti-H-2 model uncovered an alternative to deletion in response to immature B-cell encounter with antigen, BCR editing to escape autoreactivity.[281]

In the HEL system, differences have been discovered in immature B-cell responses to soluble versus membrane-bound antigen, suggesting that the extent of BCR cross-linking can influence cell fate.[299] Furthermore, studies with this system on different mutant backgrounds that shift BCR signaling thresholds up or down has shown that such alterations can result in striking alterations in selection outcomes.[451,452] Work in this system has shown that one consequence of B-cell tolerance may be arrest of B-cell migration so that follicular entry is inefficient.[330,331] Presumably, failure to reach such follicular niches contributes to handicapping the autoreactive B cells, resulting in their relatively speedy elimination.

TABLE 8.2 Transgenic Models of B-Cell Tolerance

Ig Transgene	Antigen	Background	Effect	Reference
3-83 μk	MHC class I	H-2Kk	Deletion, receptor	281,300,450

			editing	
	H2-Kk,b	H2-Kd	Normal development	
3-83 μk	MHC class I	H-2Kk lpr autoimmune-prone	Deletion unaffected	308
	H-2Kk,b			
anti-HEL μδ	HEL	sHEL-Tg	Anergy	298,448,449
		Wild-type	Normal development	
anti-HEL μδ	HEL	mHEL-TG	Deletion	299
anti-HEL μδ	HEL	HEL-Tg lpr autoimmune prone	Deletion unaffected	307
anti-HEL μδ	HEL	sHEL-Tg	Self-antigen promoted	452
		CD45 null	development	
anti-HEL μδ	HEL	sHEL-Tg motheaten	Deletion by lower valency autoantigen	451
3H9 μ-only	ssDNA with many light chains	BALB/c	No anti-DNA autoantibodies	301
3H9 μ-only	ssDNA with many light chains	lpr Autoimmune-prone	Anti-DNA autoantibodies	597
3H9 μκ	dsDNA	BALB/c	Deletion, editing	302
3H9 μκ	dsDNA	J_H^-/J_K^-	Deletion	285

3H9 μλ	dsDNA	Wild-type and lpr Autoimmune prone	Anergy	332,333
3H9-R/Vκ4-R	dsDNA	BALB/c	Deletion, editing	598
3H9-R/Vκ8-R	ssDNA		anergy	
3H9-R/Vκ4-R	dsDNA, ssDNA	Rag-2$^-$	Deletion, activation	599
3H9-R/Vκ8-R				
3H9/56R	dsDNA, ssDNA	BALB/c	Deletion, editing	600
3H9/56R76R				
Antierythrocyte	Red blood cell	Wild-type	Tg$^+$ cells only in peritoneal cavity	457,458,459
$V_H11^μ$, $V_H12μ$	PtC, BrMRBC	Wild-type	Increased number of B1 B cells	601,602
6C10μ	ATA determinant (glycosylation of Thy-1)	Wild-type Thy-1 null	ATA B cells ATA serum Normal development; no ATA	412
ATA μκ (6C10μVκ21c)	ATA determinant	Wild-type	Receptor editing or development block in bone marrow; ATA in serum	464,465
		Thy-1 null	Normal development	

ATA, antithymocyte autoantibody; DNA, deoxyribonucleic acid; HEL, hen egg white lysozyme; Ig, immunoglobulin; MHC, major histocompatibility complex.

Another major line of investigation has focused on a more “physiologic” example of pathogenic autoreactivity, the anti-DNA antibodies produced in lpr (Fas-deficient) mice that are generally considered to model the human disease of systemic lupus erythematosus. Analysis of transgenic mice bearing a heavy chain transgene known to be capable of generating anti-dsDNA reactivity with numerous light chains showed that only light chains with ssDNA activity were tolerated in the periphery, and even these did not contribute to the serum antibody pool.[301] Follow-up work uncovered receptor editing in this model[302,453,454,455] and also showed that when such editing was blocked, the B cells were eliminated in the bone marrow at an immature stage.[285]

Receptor editing, due to failure of Rag downregulation, in replacement of the original light chain by a different one, resulted in decrease or elimination of autoreactivity. Although discovered in several BCR transgenic models, it also occurs at some level in the newly formed B-cell stage in bone marrow, as random pairing of heavy and light chains will result in autoreactivity by some of these cells. Studies with a NF-κB-dependent IκB alpha gene reporter mouse revealed that autoreactive BCRs resulted in activation of the reporter and that reporter+ cells have elevated levels of IRF4, indicating that BCR cross-linking by self-antigen signals through a NF-κB pathway and acts through IRF4 to regulate receptor editing.[456]

Later work analyzing transgenic B cells expressing λ light chain (or in kappa null mice) where B cells have dsDNA binding has shown the failure of follicular entry previously described in the HEL system.[332,333] Interestingly, similar analyses on an “autoreactive” (Fas-deficient) background have shown that this follicular exclusion is lost and production of pathogenic autoantibodies ensues, providing a powerful model for the further characterization of the development of autoimmunity due to breakdown of B-cell tolerance.

A different model of a pathogenic antierythrocyte autoantibody has shown another possible mechanism whereby

self-reactive B cells may avoid deletion or receptor editing by sequestration from self-antigen.[457,458] In this system, the transgenic

B cells are largely absent from spleen but instead survive in the peritoneal cavity where exposure to the distinctive microenvironment may also contribute to the persistence of these cells. Eventual activation of the B cells by mitogen or antigen can lead to an autoimmune condition in these mice.[459] B cells may express BCR coreceptors or modulators of signaling pathways that change the response to self-antigen cross-linking. For example, it appears that enzymatic acetylation and deacetylation of a cell surface carbohydrate by sialate: O-acetylesterase alters B-cell responsiveness, by regulating the function of CD22, a sialic acid binding Ig-like lectin (siglec) that inhibits BCR signaling.[460] As mentioned previously, the level of CD19 also modulates BCR signaling and selection, such that altering CD19 expression can alter the frequency of B1 B cells. Recent work has revealed that mutation of

the Wiskott-Aldrich syndrome protein selectively in B cells results in severe autoimmunity due to BCR and TLR hyperresponsiveness.[461]

A common thread in all of the studies described here is the negative impact that the B cell experiences upon interaction with self-antigen, an expected result for systems that model the regulation of pathogenic autoantibodies. However, a class of autoantibodies is produced in healthy individuals, and these "natural autoantibodies" may play a role in early responses to certain classes of pathogens.[401,402,404,462] Such a natural autoantibody has been used to construct a transgenic model system where the self-antigen can be regulated. Most natural self-antigens are common glycosylations or cell constituents such as PtC that cannot be eliminated, but a class of natural autoantibodies binds to thymocytes (antithymocyte autoantibody [ATA]) and many of these recognize a glycosylation that is only present on the abundant thymocyte cell surface glycoprotein CD90/Thy-1.[412] Thy-1-null mice have already been generated,[463] so production of ATA-BCR μ-only transgenic mice enabled the study of the role of antigen in the generation of this natural autoantibody. Interestingly, both production of serum ATA and accumulation of B cells with the appropriate light chain (by rearrangement of endogenous Ig light chain locus) for the ATA-BCR required the presence of Thy-1 self-antigen.[412] Thus, at least some B cells are selected for binding to self-antigen, although these may belong exclusively to specialized B-cell compartments, such as the B1 B-cell subset.

This ATA-BCR/Thy-1 tolerance model has been extended by the production of ATA μκ transgenic mice, where most developing B cells express the natural autoreactive specificity.[464] In such mice, most of B cells developing from bone marrow become arrested in spleen and either cease maturation or else "edit" their BCR by replacing the transgene-encoded light chain by a different light chain as a consequence of rearranging the endogenous light chain locus. This resembles typical "negative selection," as seen with other transgenic models.[281,298,299,300,448,450] Nevertheless, some B1 B cells are detected, and high levels of serum autoantibody are produced, suggesting that some B cells with this BCR are capable of maturing, perhaps in specialized microenvironments or at distinctive (fetal/neonatal) times.

The role of BCR signaling intensity in directing B cells to various specialized subpopulations has been studied using the ATA μκ BCR transgenic model, by altering the levels of Thy-1 self-antigen and then examining B-cell development.[465] This analysis observed follicular B-cell development in the absence of antigen, but MZ B-cell production in the presence of very low levels of autoantigen. As shown previously, normal physiologic levels of the Thy-1 autoantigen results in a block in bone marrow development, but allowed B1 production, with concomitant generation of ATA detected in serum. This work led the authors to propose a model of B-cell development where strong BCR signaling produces B1 B cells (particularly during fetal development), intermediate BCR signaling yields MZ B cells, and weak (or negligible) BCR signaling results in follicular B-cell generation.

Role of Complement, Serum Antibody, and Cluster of Differentiation 5 in B-Cell Tolerance and Response

The importance of complement in the immune system has long been recognized based on

classic experiments showing that cobra toxin decreased responses.[466,467,468] Over the past 10 years, the importance of complement in immune responses, explaining the function of adjuvants, has become clearer.[469] In the context of B-cell development, components of the complement system also play a role in modulating BCR responses and negative selection of B cells. At least in the HEL-Ig/sHEL system, altering the strength of the BCR signal, by CD45R or PTP1C/SHP1 inactivation, could either reduce or enhance B-cell deletion.[452] Similar results could be obtained by altering the BCR-associated chain CD19.[470] Considering the role of complement as a coreceptor for modulating BCR signaling thresholds, the finding that HEL double transgenic mice that also are Cr2 (CD21/CD35) null develop peripheral B cells that are apparently not fully anergized, as they could still respond to antigen challenge.[471] This could be due to an inefficient retention of antigen (sHEL) on stromal/dendritic cells and, therefore, weaker signaling by a monomeric soluble antigen to developing/transitional B cells in the bone marrow or spleen. Potentially, a major role for complement would be to localize self-antigens on bone marrow stromal and dendritic cells facilitating tolerance at the newly formed B-cell stage. An implication of these findings is that complement deficiency could result in accumulation of functional autoreactive cells, possibly leading to autoimmunity. Natural autoantibody, a major component of the serum, may have a role (together with complement) in maintaining tolerance to highly conserved self-antigens.[469] Some such IgM antibodies, highly conserved phylogenetically, have been shown to recognize conserved carbohydrates beta-glucan and chitin structures present on fungi and so serve to enhance host defense.[472] It also appears that it plays a role in amplifying immune responses, as suggested by studies with a mutant mouse lacking the μ heavy chain secretory exon.[473] The deficit due to a lack of serum IgM was particularly severe in an acute peritonitis model, where some restoration of responsiveness could be obtained by injection of a monoclonal IgM antibody derived from CD5+ B cells.[473] Finally, in infection with influenza virus, it appears that early presence

of natural autoantibody was equally as important as antibody induced during the course of infection in mediating viral clearance and survival.[404] This work has led to the proposal of a two-phase description of immune responses, with an early T cell-independent phase dominated by natural autoantibody, likely critically dependent on innate immune recognition and activation of complement, leading to a later T cell-dependent phase culminating in the GC reaction, producing high-affinity antibody and memory B cells.[474]

The presence of CD5 on many of the B1 B cells has led to the question of whether it plays a direct role in maintaining such self-reactive B cells, particularly in light of its role in altering T-cell selection thresholds as demonstrated in the CD5-null mouse.[475] Analysis of B-cell responses to BCR cross-linking in CD5-null mice suggested that the presence of CD5 makes the normal population less likely to respond by secreting IgM,[476] effectively “raising the threshold” for their response, possibly by promoting interaction of the BCR with SHP-1.[477] Analysis of the effect of CD5 expression on tolerance in the HEL-Ig/sHEL system also suggested a modulating role, with absence of CD5 leading to a loss of B-cell tolerance and production of serum anti-HEL autoantibody.[478]

B-CELL DEVELOPMENT IN HUMANS: SIMILARITIES AND DIFFERENCES FROM MOUSE

Fetal Development

As described for mouse, human B-cell development can be broadly divided into that taking place prior to birth and that operating after birth and throughout life. The liver and spleen are major sits of fetal B lymphopoiesis in humans and, as in mouse, the bone marrow is the predominant site in adults, with production continuing over most of an individual's lifespan, with some decrease in the aged. The fetal omentum has also been described as a site for B-cell development similar to mouse.[9,479] There is some indication that VH gene usage is more restrictive in fetal development.[480] TdT, absent from mouse fetal development, is similarly missing very early in human fetal development but is expressed by the eighth or ninth week of gestation based on detection of N-region addition.[480] In general, there is more N-addition (and consequently longer CDR3 regions) in human antibodies compared to mouse at all stages of development, including the adult, although the significance of this is not known. Analysis of human T-cell development indicates that fetal and adult T cells arise from distinct developmental pathways, with fetal cells allowing more self-reactivity to persist[481] similar to mouse.[482] Finally, as recently described in mouse, B cells with the capacity to rapidly secrete IL-10 have also been identified in humans, and such cells appear to be increased in patients with autoimmune disease, such as rheumatoid arthritis.[483]

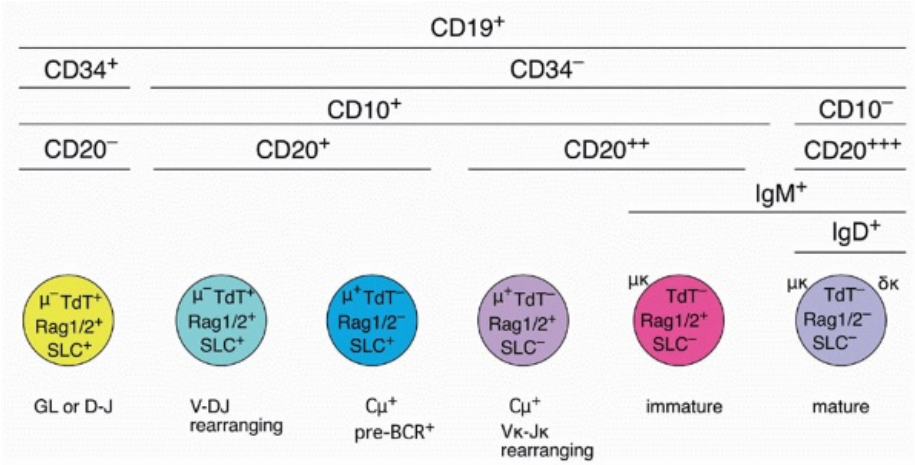

FIG. 8.14. Stages of Developing B-Lineage Cells in Human Bone Marrow can be Delineated, Based on Expression of Combinations of Cell Surface Proteins, Similar to Mouse.

Bone Marrow Development

B-cell development can be detected in human bone marrow from 20 weeks of gestation and continues throughout life.[484,485] Phenotypic subdivisions similar to those in the mouse have been described for developing bone marrow cells[486] (Fig. 8.14). Thus, CD19 identifies B-lineage cells at all stages, with the earliest stages also expressing CD34, a molecule found

on MLPs.[487] These CD19+CD34+ cells have been shown to express TdT, Rag-1 and Rag-2, the SLC orthologs,

and Ig-α/Ig-β.[486] They contain D-J, but not productive VDJ rearrangements. The next stage can be defined by loss of CD34, with a fraction that is still IgM− (pre-B cells) and another subset that is IgM+. The pre-B cells express heavy chain in their cytoplasm and have downregulated expression of TdT.[486] All of the IgM+ cells express a marker lost upon final maturation, CD10, so they are immature B cells. A recent study examined the B-lineage potential of cells expressing different levels of CD10 and found that increasing levels of this molecule were found on more differentiated B-lineage cells, suggesting its potential for identifying distinctive stages in B-cell development in humans.[488] Human early B-lineage cells have proven much more difficult to grow in culture than mouse cells, although conditions have now been defined that allow their expansion in vitro. One of the most surprising differences between mouse and human B precursor growth has been much less dependence on IL-7, suggesting that a different cytokine may substitute in humans.[489,490,491] The finding that lack of the cytokine common gamma chain (λc) in mouse blocks B-cell development but spares T-cell development, whereas the reverse is the case in humans,[154,492] demonstrates clear differences in cytokine dependence between mouse and human lymphopoiesis.

Human B precursors also express orthologs of λ5 and VpreB, although their organization and number differ from the mouse.[493,494,495] Their expression occurs at a corresponding stage and analysis of the human EBF gene has shown that its targets are similar to those of mouse EBF, including Ig-α, Ig-β, and 14.1 (the human ortholog of λ5).[496] Importantly, mutations in components of the pre-BCR lead to immunodeficiency diseases in humans, pointing out the similar and crucial role that pre-BCR signaling plays in human B-cell development.[497,498,499]

Germinal Center Differentiation

As in mouse, GCs are anatomic structures in secondary lymphoid organs where T-dependent immune responses occur, selecting high-affinity clones during the process of somatic hypermutation and eventually generating memory B cells. The GC consists of B cells at a variety of differentiation states, from early activated B cells through the plasmablast stage. Therefore, study of this process has benefited significantly from resolution of intermediate stages using multiparameter flow cytometry and carefully chosen cell surface markers. In one study, this allowed definition of two IgD+ stages, two GC stages, and a memory stage in tonsillar B cells.[500] This work focused on the levels of somatic mutations accumulating as cells passed through this pathway, finding that it was first detected in the initial GC stage (centroblast). Subsequent work has shown that IgD+ somatically mutated cells can be detected in peripheral blood, based on expression of CD27, suggesting that they are IgM+IgD+ memory B cells.[500]

Recent work has found an interesting link between somatic hypermutation and certain Bcls. Bcl-6 is a transcriptional repressor that is linked to both GC B cells and to B lymphomas that likely derive from GC B cells.[501,502] Bcl-6 expression is high in GC B cells and is required

for formation of the GC.[503] Strikingly, its 5′ regulatory region is mutated as a consequence of hypermutation in GC B cells, the first example of hypermutation targeted outside the Ig regions,[501,502,504] and links this process to deregulated cell growth and lymphomagenesis. These findings have focused interest on Bcl-6, and recent work has shown that it binds to the promoter of the antiapoptotic proto-oncogene Bcl-2 by interacting with the transcriptional activator Miz1, also suppressing Miz1 activation of Bcl-2.[505] In lymphomas, Bcl-6-mediated suppression of Bcl-2 is lost, either by chromosomal translocations to Bcl-2, mutation of Bcl-2, or Miz1 deregulation.

Abnormalities of Development

A key discovery in the past decade has been the finding that a well-known immunodeficiency, xid, characterized by inability to respond to bacterial infections and a severe deficit in peripheral B cells,[506] is due to mutations in Btk.[507] Shortly after this finding, the mouse ortholog of Btk was shown to be the cause of murine xid, an extensively studied mutation originally identified in CBA/N mice.[508] Btk deficiency in humans is more severe than xid, with little B-cell development past the early B-cell stage, in contrast with an absence of normal peripheral B-cell development in mouse and inability to respond to certain types of T-independent antigens.[509] This difference is not simply due to specific difference in the mutations, as a complete null mutation in mouse is indistinguishable from xid.[418] Thus, human B-cell development, likely at the pre-BCR signaling stage, is much more dependent on Btk.

While xid is by far the most common B-cell deficiency, amounting to more than 80% of those identified, non-X-linked mutations have also been observed. These correspond to mutations in the pre-BCR signaling complex, and, in most cases, similar effects had been observed in the mouse. For example, deletions or mutations in the μ constant regions accounted for another 5%.[499] Examples have been found of mutations in the 14.1 gene, the human ortholog of the mouse surrogate light chain λ5 protein,[497] and also in Ig-α.[498] In both of these cases, early B-lineage cells, identified as CD19+CD34+ were present in normal numbers in bone marrow, but CD19+CD34- Cμ+ cells (pre-B cells) and all later stages were absent. Finally, a patient with a mutation in BLNK, an adaptor protein that links pre-BCR signaling from syk to the rest of the signaling cascade, showed a similar phenotype.[510]

Very few peripheral B cells are detected in any of these disorders, which suggests that pre-BCR signaling is more critical in human than in the mouse, where mutations in λ5 and BLNK allow the generation of variable numbers of peripheral B cells. The reason for this difference is not yet understood. Interestingly, common variable immunodeficiency has been shown to result from mutations in the common gamma chain[154] or in the JAK3 gene[511] consistent with IL-7 playing a much less critical role in human pre-B-cell growth than in mouse, as had already been determined from culture studies.[489,490] B-cell development is relatively

intact, whereas T cells are ablated. The reverse is true for mouse.[512,513]

Analysis of B cells with mutations in genes required for normal TLR signaling has revealed the importance of such signaling for maintenance of B-cell tolerance.[514] This study

examined the binding specificity of BCRs isolated from B cells of patients with defects in IL-1 receptor-associated kinase 4, myeloid differentiation factor 88, and UNC-93B. As all TLRs but TLR3 depend on IL-1 receptor-associated kinase 4 and because TLR3 depends on UNC-93B, this work assessed the contribution of all TLR signaling. Autoreactive BCRs were found at high frequency in the newly formed and mature B-cell pools in all patients, establishing a critical role for TLR signaling in establishing and maintaining B-cell tolerance. Interestingly, none of these patients had serum autoantibody or autoimmune disease, suggesting that some further signal, possibly TLR mediated, is needed for disease progression.

Insights into B-Cell Malignancies

One of the principle reasons for studying the regulation of B-cell development is that defects in this process may result in lymphoma. Human B-lineage neoplasias can be viewed as transformed counterparts of normal B-cell developmental stages, such as pro-B, pre-B, immature B, mature B, or plasma cell, based on rearrangement status and surface phenotype.[493] In some cases, transformed cells may even retain growth characteristics of the normal counterpart. This type of classification scheme, correlating features of lymphomas and leukemias with their normal counterparts, has been useful in diagnosis and prognosis of B-lineage neoplasias. For example, B-precursor ALL, the most common type of ALL in children (ALL accounts for 25% of childhood cancer), is a clonal expansion of a cell defined by surface phenotype and Ig rearrangement status as representing the pro-B stage.[515,516,517] Recent analyses of B-precursor ALL suggests that they can be subdivided into a pro-B type, predominant in pediatric patients, and a pre-B type, more frequent in adults.[518] A subtype of adult pre-B ALL is Philadelphia chromosome-positive, being defined by the oncogenic BCR-ABL1 kinase and showing deletion of the Ikaros gene in most cases. Pre-BCR signaling mediated cell cycle arrest has been shown to depend on normal Ikaros function,[519] providing an explanation for the high incidence of Ikaros deletion in this disease.

Whereas traditional chemotherapeutic agents typically target proliferating cells without specificity for malignant cells, the identification of molecular abnormalities that result in the abnormal survival and proliferation of leukemic cells may lead to the design of novel therapies that specifically target these cells. For example, the successful treatment of mice carrying human B-precursor leukemias with antisense strategies and tyrosine kinase inhibitors holds promise for efficacy in humans.[520,521] The identification of novel translocations in B-precursor ALL also may identify mechanism(s) responsible for this disease at the molecular level.[522,523,524,525]

Another example is diffuse large B-cell lymphoma (DLBCL) that appears to arise from GC B cells[526] that are undergoing somatic hypermutation of their IgV genes mediated through activity of AID.[366,367] As mentioned previously, aberrant somatic hypermutation in DLBCL has been shown to induce mutations in the Bcl-6 gene.[501,502,504] As mentioned previously, Bcl-6 is often found to be overex-pressed in DLBCL by either hypermutation of its promoter or chromosomal translocation, but investigation of DLBCLs lacking these genetic alterations has revealed that Bcl-6 is targeted for ubiquitylation and proteasomal degradation by a SKP1-CUL1-F-box protein ubiquitin ligase complex that contains the orphan F-box protein

FBXO11.[527] FBXO11 was found to be mutated or deleted in DLBCL cell lines and also in primary leukemias, thus revealing another mechanism for Bcl-6 dysregulation, leading to lymphoma.

AID in DLBCL likely is responsible for widespread genome instability,[528] and using a mouse model system, AID was clearly identified as responsible for c-myc to Ig V-J(H) translocations.[529] Examination of known non-Ig targets of AID also identified a high rate of mutation of PAX5 in both classic and nodular lymphocyte-predominant Hodgkin lymphoma.[530] Microarray gene profiling showed that the NF-κB signaling pathway is constitutively activated in DLBCL and as normal NF-κB activation by BCR cross-linking requires CARD11, a cytoplasmic scaffolding protein, this gene was examined for mutations. Ten percent of DLBCL tested had missense mutations, providing a rationale for development of new therapeutic agents targeting this pathway.[531] Other work has identified a negative regulator of NF-κB signaling, A20, as a frequent target in B-cell lymphomas, by using genome-wide analysis of genetic lesions.[532] Thus, early “whole genome” analyses of leukemias and lymphomas by microarray gene profiling has been followed up by high-resolution determination of gene copy and targeted sequence analysis. Now, the availability of whole exome or even whole genome high-throughput sequence determination will undoubtedly identify as yet unsuspected key driver mutations in these diseases, potentially leading to new and more personalized therapies.

Another therapeutic approach makes use of knowledge of the surface phenotype of the transformed cell, as in the recent development of anti-CD20 therapy for several types of B lymphomas.[533] CD20 is a 33 kDa phosphoprotein expressed highly on the surface of mature B cells.[534,535,536] Antibodies to CD20, originally called B1, were initially characterized by their stimulatory and inhibitory effects on human B cells, indicating the importance of CD20 in regulating B-cell proliferation and differentiation.[537,538,539] Therapeutic anti-CD20, called rituximab, is a chimeric antibody derived by fusing the V regions of a mouse antibody to the human IgG1 constant segment.[540] The precise mechanism of depletion is not yet fully understood but likely include contributions from antibody-dependent cell-mediated cytotoxicity, complement-mediated cytotoxicity, and direct antibody binding effects, including sensitization to apoptosis.[533] As most of the cells in many indolent B-cell neoplasms, such as non-Hodgkin lymphoma or chronic lymphocytic leukemia (CLL), are not predominantly in cycle, the problem may be more a failure to die appropriately, rather than a failure to regulate proliferation. A greater understanding of the

growth and sensitivity to apoptosis of different types of lymphomas and leukemias may allow more specific targeting using this approach.[541] For example, while CLL cells express relatively low levels of CD20, likely requiring higher doses of anti-CD20 for a response, a combination with another antibody recognizing a molecule that is highly expressed on CLL, CD52 has shown promise.[542] Alternatively, treatment with anti-CD20 may render the cells generally more sensitive to apoptosis so that use in combination with more conventional chemotherapeutic agents will be efficacious. These combination therapies are already in clinical trials. Finally, new insights into distinctive growth properties of transformed B cells,

such as the role of Wnt signaling in CLL.[543] may suggest new approaches to treat such disorders.

ALTERNATIVE STRATEGIES FOR B-CELL DEVELOPMENT

The broad outlines and even many of the details of B-cell development are quite similar in mouse and man, but there are striking differences in other species. There is a notable common alternative approach that involves generation of Ig+ cells during fetal/neonatal development with a relatively restricted repertoire that is then diversified by novel approaches (gene conversion or somatic hypermutation) in specialized lymphoid organs that are associated with the gut. In these species, most development from Ig- precursors appears to cease by birth, and the B-cell population is maintained by self-renewal of mature B cells. Here, we consider the development and diversification of B cells in chicken and rabbit.

Chicken

B-cell development in the avian occurs in the bursa of Fabricius.[544,545] In fact, the term "B" cell refers to "bursa derived," reflecting the historic origins of research in lymphocyte development. That is, removal of the bursa just after hatching eliminated the ability to mount an antibody response, demonstrating the importance of this organ in generating cells capable of antibody formation.[544] In contrast with the bone marrow, the bursa, being associated with the gut,[546] facilitates exposure of developing cells to external antigens and bacterial flora. B-cell development in chicken is usually divided into three stages: prebursal, bursal, and postbursal.[547]

During prebursal development, at day E5, early precursors can be identified in the para-aortic foci,[548] likely corresponding with similar precursor stages localized in this anatomic site in mammals.[2] B-lineage commitment, as indicated by D-J rearrangements, is detected in the YS at day E5/6, and V gene rearrangement is found 3 days later.[549] Unlike mammalian ordered development, light chain rearrangement is detected at about the same time as heavy chain, and light chain can precede heavy chain.[550] This means that there is no pre-B stage, per se, and also probably no requirement for SLC. Rearranging B-lineage precursors migrate into the bursal mesenchyme at about day E12, and, thereafter, these cells begin to proliferate in bursal epithelial buds. This proliferation selectively expands cells that have BCRs. These receptors have limited diversity, as the heavy chain is formed by rearrangement of a single VH, several Ds, and a single JH pairing with a light chain generated by rearrangement of a single VL with a single JL.[551,552] As in mouse fetal development, there is no TdT-mediated N-region addition at the junctions.[552]

This "prebursal" receptor is diversified by gene conversion by a set of V pseudogenes during this proliferative phase. At about hatching, these cells become exposed to the contents of the bursal lumen that is connected to the gut lumen via the bursal duct similar to the appendix. Thus, these proliferating B cells are exposed to the contents of the digestive tract, and there is also reverse peristalsis at the end of the gut that transports external antigens into the bursal duct.[546] At about this time, the level of apoptosis increases dramatically, and it is possible that only 5% of the cells generated in the bursa eventually emerge.[553] This death

may be due to generation of nonfunctional receptors during the course of gene conversion or it may reflect antigenic selection. At hatching, emigration of B cells from the bursa increases, but most of these cells constitute a population with a relatively short half-life measured in days.[554] The long-lived pool colonizes the peripheral lymphoid organs over several weeks as the bursa atrophies. By 3 weeks after hatching, bursectomy no longer results in agammaglobulinemia, indicating that the postbursal phase has become established.

Rabbit

B-cell development in rabbit is similar to chicken, in that B cells initially are produced with a limited BCR diversity[555,556,557] during fetal life, with little new production after birth.[558,559] This repertoire is then expanded through gene conversion[555,560] and somatic hypermutation[560,561] in a specialized gut-associated organ, the appendix.[562] However, unlike chicken, this diversification process is dependent on antigen availability.[563,564,565]

Pre-B cells can be found in rabbit before birth in the liver, bone marrow, and omentum,[558,566,567,568] but B lymphopoiesis decreases at birth and is negligible in adult animals.[559] Ig rearrangement during fetal and neonatal times is dominated by usage of the most D-proximal V_H gene paired with multiple V_L genes in the light chain.[556,557,569,570,571,572] In contrast with chicken and mouse, there is significant N-addition.[557,571] From 4 to 8 weeks after birth, there is a striking increase in the diversity of this primary repertoire that occurs during proliferation of B cells in the gut-associated lymphoid tissues (GALTs).[559,560] V genes are diversified by both gene conversion[555] and also by somatic hypermutation.[560,561] Importantly, surgical removal of GALT organs, appendix, sacculus rotundus, and the Peyer patch, from neonatal rabbits led to unresponsiveness to many antigens, suggesting that this diversification was crucial for normal immune function.[573] This finding has been confirmed by sequence analysis, demonstrating that removal of the GALT blocks diversification.[562]

Considering the relatively late diversification in rabbit compared to the chicken, these gut areas will provide a milieu

of microbial antigens, and this appears to be a critical aspect of the diversification process.[574] For example, surgery to prevent access of intestinal flora to the appendix blocked diversification in this organ[575,576] that could be restored by reversing the ligation.[575] Furthermore, analysis of rabbits reared in germfree conditions revealed abnormal cellular development in the GALT[564] and a lack of responsiveness to certain antigens.[563] The dependence of V gene diversification on antigen has been directly demonstrated in animals where the sacculus rotundus and Peyer patches were removed at birth and the appendix ligated. Testing the peripheral blood B cell V gene repertoire showed an absence of diversification in contrast to controls.[565] Although the mechanism for this stimulation remains to be established, possibilities include B-cell activation by a BCR superantigen[577,578,579] or through a B-cell TLR.[580]

Two B-Cell Developmental Pathways

The similarities of chicken and rabbit B-cell development with that in other species such as sheep, swine, and cow suggests that the initial production of a limited BCR repertoire during fetal/neonatal life, diversification at a later time, and maintenance of the B-cell pool in adult life by self-renewal (rather than de novo generation from unrearranged precursors) is a major pattern in the design of the immune system (Fig. 8.15). This pattern contrasts with that described for mouse and man, where ordered heavy and light chain rearrangement, pre-BCR selection, and replacement of senescent B cells by newly generated B cells are major aspects of development. This raises the interesting question of whether there is an analogous B-cell development pathway in humans or mouse, perhaps as a vestige.

The idea of two pathways in bone marrow development was suggested previously based on the observation that heavy and light chain ordering is not absolute: Light chain rearrangements are detected in mice incapable of making heavy chain rearrangements and can be detected in cells early in B-cell development.[581] In one pathway, heavy chains rearrange first and the pre-BCR is assembled and signals downregulation of Rag-1/2 expression (ending heavy chain rearrangement), clonal expansion, and accessibility of the kappa locus. Cessation of proliferation is coincident

with reinduction of Rag-1/2 and light chain rearrangement. Expression of a complete BCR signals a final termination of Rag expression. In this model, pre-BCR and BCR mediates allelic exclusion by a feedback mechanism regulating Rag expression. Thus, most B cells have D-J or even VDJ rearrangements on both alleles. In the alternative pathway, heavy and light chains rearrange stochastically, and allelic exclusion is mediated by relative inaccessibility of the loci (and thereby low frequency of rearrangement). If this is the major pathway in chicken and rabbit, it is consistent with the observation that most chicken B cells have only one allele rearranged.[552,582,583] Furthermore, the careful ordering of TdT expression, being downregulated at the light chain rearranging stage, is apparently not the case for rabbit, where a large percentage of light chains have N-regions.[572]

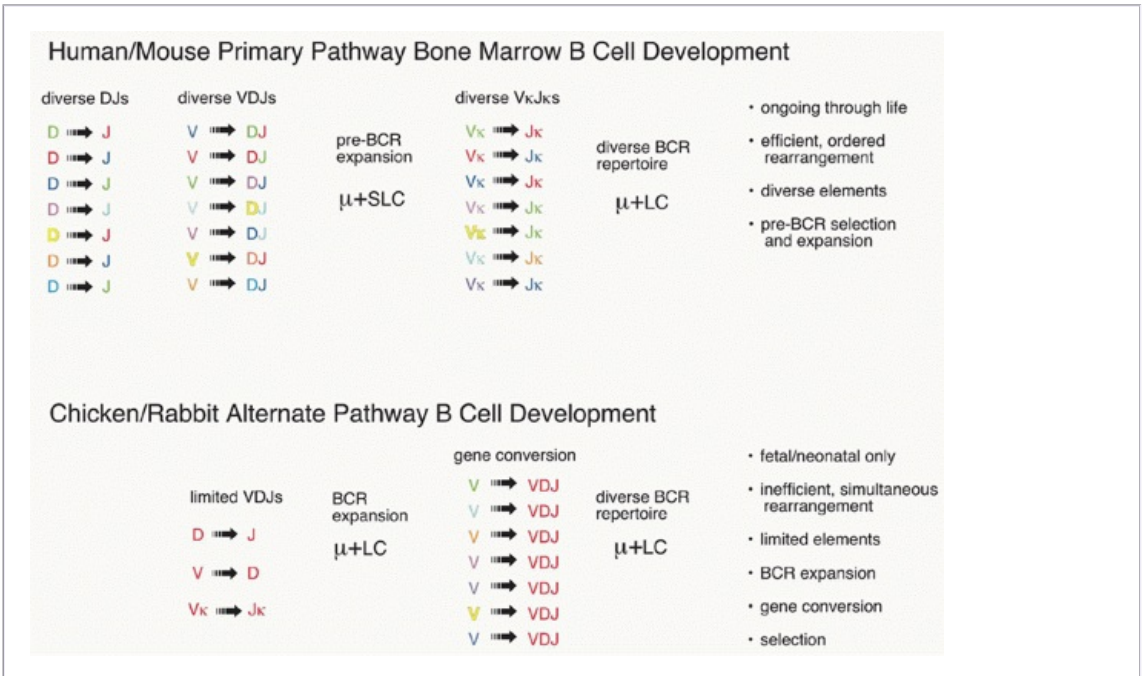

FIG. 8.15. Alternative Strategies of Repertoire Diversification and B-Cell Development. In both mouse and human bone marrow B-cell development, ordered rearrangement predominates, with a pre-B-cell receptor (BCR_ selection phase dividing heavy and light chain rearrangement. The eventual outcome is a population of newly formed B cells with a diverse set of BCRs produced throughout life. In chicken and rabbit, immunoglobulin rearrangement appears less efficient (usually the other allele is germline) and much less diverse, with a single BCR for chicken. However, these cells proliferate and undergo gene conversion during fetal/neonatal life, followed by selection, eventually generating a set of B cells with a more diverse repertoire that persist for the life of the animal.

Most rabbit B cells express CD5.[584] In mouse, much of the CD5+ B-cell population is generated during fetal/neonatal development and persists in the adult through self-renewal.[392] Furthermore, these cells express a relatively restricted BCR repertoire that is dependent on antigen selection.[23] Finally, the pre-BCR selection phase of several heavy chains abundant in CD5 B cells appears to follow different rules than classical pre-BCR-mediated expansion.[263] In fact, the process of development in mouse fetal liver may predominantly follow this alternative pathway.[406] Thus, in mouse, fetal development, culminating in production of B1 B cells, may represent a type of alternative or "primitive" B-cell development, as has been proposed previously.[585] It is less clear whether a similar distinction exists in human B-cell development, as data are lacking. Nevertheless, one can envision a primordial pathway, randomly combining heavy and light chains and simply selecting cells that express BCRs with weak reactivity to self or environmental antigens as an alternative to ordered rearrangement, pre-BCR selection, and BCR selection—an elaborate process fine tuned to generate more variation.

CONCLUSION

There has been considerable progress over the past decade in understanding the mechanisms that regulate B-cell development, with important insights coming from application of the novel genetic approaches of transgenesis and gene targeting. In a sense, this has allowed progression from research with simple model systems using cell lines to analysis of "normal B cells" in whole animals. The use of such mutant mouse "reagents," together with much higher resolution of normal development made possible by multiparameter flow cytometry, has proven a powerful combination for unraveling much of the complexity of this process. It is daunting to attempt to predict where new advances in the field will come, but undoubtedly, the completion of the genome sequence for both mouse and human will provide impetus to large-scale gene profiles of B-cell development, as have already begun.[586,587] A complementary approach to gene targeting based on such profiling will involve characterizing new mutant mice generated by chemical mutagenesis.[588,589] Considering the recent unanticipated discoveries of AID in isotype switching and BAFF in peripheral B-cell development, it seems likely that the coming decade will provide many surprises. A goal will be to eventually understand how the interplay of the innate and adaptive immune systems generates protective responses while avoiding autoimmune

pathologies at the organism level.

REFERENCES

1. Moore MA, Metcalf D. Ontogeny of the haemopoietic system: yolk sacorigin of in vivo and in vitro colony forming cells in the developing mouse embryo. *Br J Haematol* . 1970;18:279-296.

2. Godin IE, Garcia-Porrero JA, Coutinho A, et al. Para-aortic splanchnopleura from early mouse embryos contains B1a cell progenitors. *Nature*. 1993;364:67-70.

3. Medvinsky AL, Samoylina NL, Muller AM, et al. An early pre-liver intraembryonic source of CFU-S in the developing mouse. *Nature.* 1993;364:64-67.

4. Muller AM, Medvinsky A, Strouboulis J, et al. Development of hematopoietic stem cell activity in the mouse embryo. *Immunity*. 1994;1:291-301.

5. Medvinsky A, Dzierzak E. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell*. 1996;86:897-906.

6. Yoder MC, Hiatt K, Dutt P, et al. Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. *Immunity*. 1997;7:335-344.

7. Owen JJ, Cooper MD, Raff MC. In vitro generation of B lymphocytes in mouse foetal liver, a mammalian “bursa equivalent.” *Nature*. 1974;249:361-363.

8. Strasser A, Rolink A, Melchers F. One synchronous wave of B cell development in mouse fetal liver changes at day 16 of gestation from dependence to independence of a stromal cell environment. *J Exp Med*. 1989;170:1973-1986.

9. Solvason N, Lehuen A, Kearney JF. An embryonic source of Ly1 but not conventional B cells. *Int Immunol* . 1991;3:543-550.

10. Gregoire KE, Goldschneider I, Barton RW, et al. Ontogeny of terminal deoxynucleotidyl transferase-positive cells in lymphohemopoietic tissues of rat and mouse. *J Immunol* . 1979;123:1347-1352.

11. Li YS, Hayakawa K, Hardy RR. The regulated expression of B lineage associated genes during B cell differentiation in bone marrow and fetal liver. *J Exp Med*. 1993;178:951-960.

12. Landau NR, Schatz DG, Rosa M, et al. Increased frequency of N-region insertion in a murine pre-B-cell line infected with a terminal deoxynucleotidyl transferase retroviral expression vector. *Mol Cell Biol* . 1987;7:3237-3243.

13. Desiderio SV, Yancopoulos GD, Paskind M, et al. Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. *Nature*. 1984;311:752-755.

14. Gilfillan S, Dierich A, Lemeur M, et al. Mice lacking TdT: mature animals with an immature lymphocyte repertoire. *Science*. 1993;261:1175-1178.

15. Feeney AJ. Lack of N regions in fetal and neonatal mouse immunoglobulin V-D-J junctional sequences. *J Exp Med*. 1990;172:1377-1390.

16. Feeney AJ. Comparison of junctional diversity in the neonatal and adult immunoglobulin repertoires. *Int Rev Immunol* . 1992;8:113-122.

17. Chang Y, Paige CJ, Wu GE. Enumeration and characterization of DJH structures in mouse fetal liver. *EMBO J*. 1992;11:1891-1899.

18. Oltz EM, Yancopoulos GD, Morrow MA, et al. A novel regulatory myosin light chain gene distinguishes pre-B cell subsets and is IL-7 inducible. *EMBO J*. 1992;11:2759-2767.

19. Hayakawa K, Tarlinton D, Hardy RR. Absence of MHC class II expression distinguishes fetal from adult B lymphopoiesis in mice. *J Immunol* 1994;152:4801-4807.

20. Lam KP, Stall AM. Major histocompatibility complex class II expression distinguishes two distinct B cell developmental pathways during ontogeny. *J Exp Med*. 1994;180:507-516.

21. Miller JP, Izon D, DeMuth W, et al. The earliest step in B lineage differentiation from common lymphoid progenitors is critically dependent upon interleukin 7. *J Exp Med*. 2002;196:705-711.

22. Carvalho TL, Mota-Santos T, Cumano A, et al. Arrested B lymphopoiesis and persistence of activated B cells in adult interleukin 7(-/)- mice. *J Exp Med*. 2001;194:1141-1150.

23. Hardy RR, Hayakawa K. B cell development pathways. *Annu Rev Immunol* . 2001;19:595-621.

24. Stephan RP, Lill-Elghanian DA, Witte PL. Development of B cells in aged mice: decline in the ability of pro-B cells to respond to IL-7 but not to other growth factors. *J Immunol*. 1997;158:1598-1609.

25. Stephan RP, Sanders VM, Witte PL. Stage-specific alterations in murine B lymphopoiesis with age. *Int Immunol* . 1996;8:509-518.

26. Ogawa M, Matsuzaki Y, Nishikawa S, et al. Expression and function of c-kit in

hemopoietic progenitor cells. *J Exp Med*. 1991;174:63-71.

27. Ikuta K, Weissman IL. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proc Natl Acad Sci U S A* . 1992;89:1502-1506.

28. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. *Science*. 1988;241:58-62.

29. Morrison SJ, Weissman IL. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity*. 1994;1:661-673.

30. Morrison SJ, Hemmati HD, Wandycz AM, et al. The purification and characterization of fetal liver hematopoietic stem cells. *Proc Natl Acad Sci U S A* . 1995;92:10302-10306.

31. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell*. 1997;91:661-672.

32. Akashi K, Traver D, Miyamoto T, et al. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*. 2000;404: 193-197.

33. Traver D, Miyamoto T, Christensen J, et al. Fetal liver myelopoiesis occurs through distinct, prospectively isolatable progenitor subsets. *Blood*. 2001;98:627-635.

34. Kondo M, Scherer DC, Miyamoto T, et al. Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines. *Nature*. 2000;407:383-386.

35. Miyamoto T, Iwasaki H, Reizis B, et al. Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment. *Dev Cell* . 2002;3:137-147.

36. Rolink A, Kudo A, Karasuyama H, et al. Long-term proliferating early pre B cell lines and clones with the potential to develop to surface Ig-positive, mitogen reactive B cells in vitro and in vivo. *EMBO J*. 1991;10:327-336.

37. Krop I, de Fougerolles AR, Hardy RR, et al. Self-renewal of B-1 lymphocytes is dependent on CD19. *Eur J Immunol* . 1996;26:238-242.

38. Li YS, Wasserman R, Hayakawa K, et al. Identification of the earliest B lineage stage in mouse bone marrow. *Immunity*. 1996;5:527-535.

39. Nutt SL, Heavey B, Rolink AG, et al. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature*. 1999;401: 556-562.

40. Rolink A, ten Boekel E, Melchers F, et al. A subpopulation of B220 + cells in murine bone marrow does not express CD19 and contains natural killer cell progenitors. *J Exp*

Med. 1996;183:187-194.

41. Tudor KS, Payne KJ, Yamashita Y, et al. Functional assessment of precursors from murine bone marrow suggests a sequence of early B lineage differentiation events. *Immunity*. 2000;12:335-345.

42. Nakano H, Yanagita M, Gunn MD. CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *J Exp Med*. 2001;194:1171-1178.

43. Nikolic T, Dingjan GM, Leenen PJ, et al. A subfraction of B220(+) cells in murine bone marrow and spleen does not belong to the B cell lineage but has dendritic cell characteristics. *Eur J Immunol* . 2002;32:686-692.

44. Rumfelt LL, Zhou Y, Rowley BM, et al. Lineage specification and plasticity in CD19-early B cell precursors. *J Exp Med*. 2006;203:675-687.

45. Allman D, Aster JC, Pear WS. Notch signaling in hematopoiesis and early lymphocyte development. *Immunol Rev*. 2002;187:75-86.

46. Borghesi L, Hsu LY, Miller JP, et al. B lineage-specific regulation of V(D)J recombinase activity is established in common lymphoid progenitors. *J Exp Med*. 2004;199:491-502.

47. Allman D, Sambandam A, Kim S, et al. Thymopoiesis independent of common lymphoid progenitors. *Nat Immunol*. 2003;4:168-174.

48. Wada H, Masuda K, Satoh R, et al. Adult T-cell progenitors retain myeloid potential. *Nature*. 2008;452:768-772.

49. Welner RS, Pelayo R, Nagai Y, et al. Lymphoid precursors are directed to produce dendritic cells as a result of TLR9 ligation during herpes infection. *Blood*. 2008;112:3753-3761.

50. Cumano A, Paige CJ, Iscove NN, et al. Bipotential precursors of B cells and macrophages in murine fetal liver. *Nature*. 1992;356:612-615.

51. Montecino-Rodriguez E, Dorshkind K. Identification of B/macrophage progenitors in adult bone marrow. *Semin Immunol*. 2002;14:371-376.

52. Nutt SL, Urbanek P, Rolink A, et al. Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. *Genes Dev*. 1997;11:476-491.

53. Igarashi H, Gregory SC, Yokota T, et al. Transcription from the RAG1 locus marks the

earliest lymphocyte progenitors in bone marrow. *Immunity*. 2002;17:117-130.

54. Tsai FY, Keller G, Kuo FC, et al. An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature*. 1994;371:221-226.

55. Okuda T, van Deursen J, Hiebert SW, et al. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell*. 1996;84:321-330.

56. Niki M, Okada H, Takano H, et al. Hematopoiesis in the fetal liver is impaired by targeted mutagenesis of a gene encoding a non-DNA binding subunit of the transcription factor, polyomavirus enhancer binding protein 2/core binding factor. *Proc Natl Acad Sci U S A*. 1997;94:5697-5702.

57. Wang Q, Stacy T, Binder M, et al. Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc Natl Acad Sci U S A*. 1996;93:3444-3449.

58. Cai Z, de Bruijn M, Ma X, et al. Haploinsufficiency of AML1 affects the temporal and spatial generation of hematopoietic stem cells in the mouse embryo. *Immunity*. 2000;13:423-431.

59. Kuo YH, Gerstein RM, Castilla LH. Cbfbeta-SMMHC impairs differentiation of common lymphoid progenitors and reveals an essential role for RUNX in early B-cell development. *Blood*. 2008;111:1543-1551.

60. Georgopoulos K, Bigby M, Wang JH, et al. The Ikaros gene is required for the development of all lymphoid lineages. *Cell*. 1994;79:143-156.

61. Molnar A, Georgopoulos K. The Ikaros gene encodes a family of functionally diverse zinc finger DNA-binding proteins. *Mol Cell Biol*. 1994;14:8292-8303.

62. Wang JH, Nichogiannopoulou A, Wu L, et al. Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. *Immunity* 5:537-549.

63. Wang JH, Avitahl N, Cariappa A, et al. Aiolos regulates B cell activation and maturation to effector state. *Immunity*. 1998;9:543-553.

64. Reynaud D, Demarco IA, Reddy KL, et al. Regulation of B cell fate commitment and immunoglobulin heavy-chain gene rearrangements by Ikaros. *Nat Immunol*. 2008;9:927-936.

65. Scott EW, Simon MC, Anastasi J, et al. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science*. 1994;265:1573-1577.

66. McKercher SR, Torbett BE, Anderson KL, et al. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO J*. 1996;15:5647-5658.

67. DeKoter RP, Singh H. Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science*. 2000;288:1439-1441.

68. Colucci F, Samson SI, DeKoter RP, et al. Differential requirement for the transcription factor PU.1 in the generation of natural killer cells versus B and T cells. *Blood*. 2001;97:2625-2632.

69. DeKoter RP, Lee HJ, Singh H. PU.1 regulates expression of the interleukin-7 receptor in lymphoid progenitors. *Immunity*. 2002;16:297-309.

70. Ye M, Ermakova O, Graf T. PU.1 is not strictly required for B cell development and its absence induces a B-2 to B-1 cell switch. *J Exp Med*. 2005;202:1411-1422.

71. Polli M, Dakic A, Light A, et al. The development of functional B lymphocytes in conditional PU.1 knock-out mice. *Blood*. 2005;106:2083-2090.

72. Bain G, Maandag EC, Izon DJ, et al. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell*. 1994;79:885-892.

73. Bain G, Robanus Maandag EC, te Riele HP, et al. Both E12 and E47 allow commitment to the B cell lineage. *Immunity*. 1997;6:145-154.

74. Romanow WJ, Langerak AW, Goebel P, et al. E2A and EBF act in synergy with the V(D)J recombinase to generate a diverse immunoglobulin repertoire in nonlymphoid cells. *Mol Cell*. 2000;5:343-353.

75. Espinoza CR, Feeney AJ. The extent of histone acetylation correlates with the differential rearrangement frequency of individual VH genes in pro-B cells. *J Immunol*. 2005;175:6668-6675.

76. Pui JC, Allman D, Xu L, et al. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity*. 1999;11:299-308.

77. Ikawa T, Fujimoto S, Kawamoto H, et al. Commitment to natural killer cells requires the helix-loop-helix inhibitor Id2. *Proc Natl Acad Sci U S A* . 2001;98:5164-5169.

78. Spits H, Couwenberg F, Bakker AQ, et al. Id2 and Id3 inhibit development of CD34(+) stem cells into predendritic cell (pre-DC)2 but not into pre-DC1. Evidence for a lymphoid origin of pre-DC2. *J Exp Med*. 2000;192:1775-1784.

79. Yokota Y, Mansouri A, Mori S, et al. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature*. 1999;397:702-706.

80. Koch U, Lacombe TA, Holland D, et al. Subversion of the T/B lineage decision in the thymus by lunatic fringe-mediated inhibition of Notch-1. *Immunity*. 2001;15:225-236.

81. Izon DJ, Aster JC, He Y, et al. Deltex1 redirects lymphoid progenitors to the B cell lineage by antagonizing Notch1. *Immunity*. 2002;16:231-243.

82. Maillard I, Weng AP, Carpenter AC, et al. Mastermind critically regulates Notch-mediated lymphoid cell fate decisions. *Blood*. 2004;104:1696-1702.

83. Lin H, Grosschedl R. Failure of B-cell differentiation in mice lacking the transcription factor EBF. *Nature*. 1995;376:263-267.

84. Jiang XX, Nguyen Q, Chou Y, et al. Control of B cell development by the histone H2A deubiquitinase MYSM1. *Immunity*. 2011;35:883-896.

85. O'Riordan M, Grosschedl R. Coordinate regulation of B cell differentiation by the transcription factors EBF and E2A. *Immunity*. 1999;11:21-31.

86. Sigvardsson M, O'Riordan M, Grosschedl R. EBF and E47 collaborate to induce expression of the endogenous immunoglobulin surrogate light chain genes. *Immunity*. 1997;7:25-36.

87. Kee BL, Murre C. Induction of early B cell factor (EBF) and multiple B lineage genes by the basic helix-loop-helix transcription factor E12. *J Exp Med*. 1998;188:699-713.

88. Roessler S, Gyory I, Imhof S, et al. Distinct promoters mediate the regulation of Ebf1 gene expression by interleukin-7 and Pax5. *Mol Cell Biol*. 2007;27:579-594.

89. Medina KL, Pongubala JM, Reddy KL, et al. Assembling a gene regulatory network for specification of the B cell fate. *Dev Cell*. 2004;7: 607-617.

90. Singh H, Medina KL, Pongubala JM. Contingent gene regulatory networks and B cell fate specification. *Proc Natl Acad Sci U S A*. 2005;102:4949-4953.

91. Morrison AM, Nutt SL, Thevenin C, et al. Loss- and gain-of-function mutations reveal an important role of BSAP (Pax-5) at the start and end of B cell differentiation. *Semin Immunol*. 1998;10:133-142.

92. Fuxa M, Skok J, Souabni A, et al. Pax5 induces V-to-DJ rearrangements and locus contraction of the immunoglobulin heavy-chain gene. *Genes Dev*. 2004;18:411-422.

93. Urbanek P, Wang ZQ, Fetka I, et al. Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell*. 1994;79:901-912.

94. Rolink AG, Nutt SL, Melchers F, et al. Long-term in vivo reconstitution of T-cell development by Pax5- deficient B-cell progenitors. *Nature*. 1999;401:603-606.

95. Tagoh H, Ingram R, Wilson N, et al. The mechanism of repression of the myeloid-specific c-fms gene by Pax5 during B lineage restriction. *EMBO J*. 2006;25:1070-1080.

96. Souabni A, Cobaleda C, Schebesta M, et al. Pax5 promotes B lymphopoiesis and blocks T cell development by repressing Notch1. *Immunity*. 2002;17:781-793.

97. Wilson A, MacDonald HR, Radtke F. Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. *J Exp Med*. 2001;194:1003-1012.

98. Cobaleda C, Schebesta A, Delogu A, et al. Pax5: the guardian of B cell identity and function. *Nat Immunol*. 2007;8:463-470.

99. Dengler HS, Baracho GV, Omori SA, et al. Distinct functions for the transcription factor Foxo1 at various stages of B cell differentiation. *Nat Immunol*. 2008;9:1388-1398.

100. Amin RH, Schlissel MS. Foxo1 directly regulates the transcription of recombination-activating genes during B cell development. *Nat Immunol*. 2008;9:613-622.

101. Xue HH, Bollenbacher-Reilley J, Wu Z, et al. The transcription factor GABP is a critical regulator of B lymphocyte development. *Immunity*. 2007;26:421-431.

102. Alkhatib A, Werner M, Hug E, et al. FoxO1 induces Ikaros splicing to promote immunoglobulin gene recombination. *J Exp Med*. 2012;209:395-406.

103. Eastman Q, Grosschedl R. Regulation of LEF-1/TCF transcription factors by Wnt and other signals. *Curr Opin Cell Biol* . 1999;11:233-240.

104. Reya T, O'Riordan M, Okamura R, et al. Wnt signaling regulates B lymphocyte proliferation through a LEF-1 dependent mechanism. *Immunity*. 2000;13:15-24.

105. Lee G, Namen AE, Gillis S, et al. Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by transforming growth factor-beta. *J Immunol*. 1989;142:3875-3883.

106. Kee BL, Rivera RR, Murre C. Id3 inhibits B lymphocyte progenitor growth and survival in response to TGF-beta. *Nat Immunol*. 2001;2: 242-247.

107. Schilham MW, Oosterwegel MA, Moerer P, et al. Defects in cardiac outflow tract

formation and pro-B-lymphocyte expansion in mice lacking Sox-4. *Nature*. 1996;380:711-714.

108. Lenardo MJ, Baltimore D. NF-kappa B: a pleiotropic mediator of inducible and tissue-specific gene control. *Cell*. 1989;58:227-229.

109. Horwitz BH, Scott ML, Cherry SR, et al. Failure of lymphopoiesis after adoptive transfer of NF-kappaB-deficient fetal liver cells. *Immunity*. 1997;6:765-772.

110. Franzoso G, Carlson L, Xing L, et al. Requirement for NF-kappaB in osteoclast and B-cell development. *Genes Dev*. 1997;11:3482-3496.

111. Corcoran LM, Karvelas M, Nossal GJ, et al. Oct-2, although not required for early B-cell development, is critical for later B-cell maturation and for postnatal survival. *Genes Dev*. 1993;7:570-582.

112. Corcoran LM, Karvelas M. Oct-2 is required early in T cell-independent B cell activation for G1 progression and for proliferation. *Immunity*. 1994;1:635-645.

113. Humbert PO, Corcoran LM. oct-2 gene disruption eliminates the peritoneal B-1 lymphocyte lineage and attenuates B-2 cell maturation and function. *J Immunol* . 1997;159:5273-5284.

114. Kim U, Qin XF, Gong S, et al. The B-cell-specific transcription coactivator OCA-B/OBF-1/Bob-1 is essential for normal production of immunoglobulin isotypes. *Nature*. 1996;383:542-547.

115. Schubart DB, Rolink A, Kosco-Vilbois MH, et al. B-cell-specific coactivator OBF-1/OCA-B/Bob1 required for immune response and germinal centre formation. *Nature*. 1996;383:538-542.

116. Nielsen PJ, Georgiev O, Lorenz B, et al. B lymphocytes are impaired in mice lacking the transcriptional co-activator Bob1/OCA-B/OBF1. *Eur J Immunol* . 1996;26:3214-3218.

117. Wolf I, Pevzner V, Kaiser E, et al. Downstream activation of a TATAless promoter by Oct-2, Bob1, and NF-kappaB directs expression of the homing receptor BLR1 to mature B cells. *J Biol Chem*. 1998;273: 28831-28836.

118. Schubart DB, Rolink A, Schubart K, et al. Cutting edge: lack of peripheral B cells and severe agammaglobulinemia in mice simultaneously lacking Bruton's tyrosine kinase and the B cell-specific transcriptional coactivator OBF-1. *J Immunol* . 2000;164:18-22.

119. Koralov SB, Muljo SA, Galler GR, et al. Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell*. 2008;132: 860-874.

120. Xiao C, Calado DP, Galler G, et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell*. 2007;131: 146-159.

121. Xiao C, Srinivasan L, Calado DP, et al. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. *Nat Immunol*. 2008;9:405-414.

122. Medina PP, Nolde M, Slack FJ. OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. *Nature*. 2010;467:86-90.

123. Viswanathan SR, Daley GQ. Lin28: a microRNA regulator with a macro role. *Cell*. 2010;140:445-449.

124. Yuan J, Nguyen CK, Liu X, et al. Lin28b reprograms adult bone marrow hematopoietic progenitors to mediate fetal-like lymphopoiesis. *Science*. 2012;335:1195-1200.

125. Hayashi S, Kunisada T, Ogawa M, et al. Stepwise progression of B lineage differentiation supported by interleukin 7 and other stromal cell molecules. *J Exp Med*. 1990;171:1683-1695.

126. Miyake K, Medina K, Ishihara K, et al. A VCAM-like adhesion molecule on murine bone marrow stromal cells mediates binding of lymphocyte precursors in culture. *J Cell Biol*. 1991;114:557-565.

127. Miyake K, Weissman IL, Greenberger JS, et al. Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. *J Exp Med*. 1991;173:599-607.

128. Era T, Ogawa M, Nishikawa S, et al. Differentiation of growth signal requirement of B lymphocyte precursor is directed by expression of immunoglobulin. *EMBO J*. 1991;10:337-342.

129. Hardy RR, Carmack CE, Shinton SA, et al. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J Exp Med*. 1991;173:1213-1225.

130. Reichman-Fried M, Bosma MJ, Hardy RR. B-lineage cells in mutransgenic scid mice proliferate in response to IL-7 but fail to show evidence of immunoglobulin light chain gene rearrangement. *Int Immunol*. 1993;5:303-310.

131. Rolink A, Grawunder U, Haasner D, et al. Immature surface Ig+ B cells can continue to rearrange kappa and lambda L chain gene loci. *J Exp Med*. 1993;178:1263-1270.

132. Owen JJ, Raff MC, Cooper MD. Studies on the generation of B lymphocytes in the mouse embryo. *Eur J Immunol*. 1976;5:468-473.

133. Owen JJ, Wright DE, Habu S, et al. Studies on the generation of B lymphocytes in fetal liver and bone marrow. *J Immunol*. 1977;118: 2067-2072.

134. Johnson P, Greenbaum L, Bottomly K, et al. Identification of the alternatively spliced exons of murine CD45 (T200) required for reactivity with B220 and other T200-restricted antibodies. *J Exp Med*. 1989;169:1179-1184.

135. Johnson P, Maiti A, Ng DHW. CD45: a family of leukocyte-specific cell surface glycoproteins. In: Herzenberg LA, Weir DW, Herzenberg LA, Blackwell C, eds. *Weirs Handbook of Experimental Immunology*. Cambridge, MA: Blackwell Science; 1997:62.61-62.16.

136. Wang J, Walker H, Lin Q, et al. The mouse BP-1 gene: structure, chromosomal localization, and regulation of expression by type I interferons and interleukin-7. *Genomics*. 1996;33:167-176.

137. ten Boekel E, Melchers F, Rolink A. The status of Ig loci rearrangements in single cells from different stages of B cell development. *Int Immunol*. 1995;7:1013-1019.

138. Whitlock CA, Witte ON. Long-term culture of B lymphocytes and their precursors from murine bone marrow. *Proc Natl Acad Sci U S A* . 1982;79:3608-3612.

139. Whitlock CA, Robertson D, Witte ON. Murine B cell lymphopoiesis in long term culture. *J Immunol Methods*. 1984;67:353-369.

140. Whitlock C, Denis K, Robertson D, et al. In vitro analysis of murine B-cell development. *Annu Rev Immunol*. 1985;3:213-235.

141. Grawunder U, Melchers F, Rolink A. Interferon-gamma arrests proliferation and causes apoptosis in stromal cell/interleukin-7-dependent normal murine pre-B cell lines and clones in vitro, but does not induce differentiation to surface immunoglobulin-positive B cells. *Eur J Immunol* . 1993;23:544-551.

142. Reininger L, Radaszkiewicz T, Kosco M, et al. Development of autoimmune disease in SCID mice populated with long-term “in vitro” proliferating (NZB × NZW)F1 pre-B cells. *J Exp Med*. 1992;176: 1343-1353.

143. Pietrangeli CE, Hayashi S, Kincade PW. Stromal cell lines which support lymphocyte growth: characterization, sensitivity to radiation and responsiveness to growth factors. *Eur J Immunol* . 1988;18: 863-872.

144. Miyake K, Underhill CB, Lesley J, et al. Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. *J Exp Med*. 1990;172:69-75.

145. Lesley J, He Q, Miyake K, et al. Requirements for hyaluronic acid binding by CD44: a role for the cytoplasmic domain and activation by antibody. *J Exp Med*. 1992;175:257-266.

146. Miyake K, Medina KL, Hayashi S, et al. Monoclonal antibodies to Pgp-1/CD44 block lympho-hemopoiesis in long-term bone marrow cultures. *J Exp Med*. 1990;171:477-488.

147. Dorshkind K. IL-1 inhibits B cell differentiation in long term bone marrow cultures. *J Immunol*. 1988;141:531-538.

148. Namen AE, Lupton S, Hjerrild K, et al. Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature*. 1988;333:571-573.

149. Namen AE, Schmierer AE, March CJ, et al. B cell precursor growth-promoting activity. Purification and characterization of a growth factor active on lymphocyte precursors. *J Exp Med*. 1988;167:988-1002.

150. Morrissey PJ, Conlon P, Charrier K, et al. Administration of IL-7 to normal mice stimulates B-lymphopoiesis and peripheral lymphadenopathy. *J Immunol*. 1991;147:561-568.

151. Hirayama F, Shih JP, Awgulewitsch A, et al. Clonal proliferation of murine lymphohemopoietic progenitors in culture. *Proc Natl Acad Sci U S A* . 1992;89:5907-5911.

152. Valenzona HO, Pointer R, Ceredig R, et al. Prelymphomatous B cell hyperplasia in the bone marrow of interleukin-7 transgenic mice: precursor B cell dynamics, microenvironmental organization and osteolysis. *Exp Hematol*. 1996;24:1521-1529.

153. Park LS, Friend DJ, Schmierer AE, et al. Murine interleukin 7 (IL-7) receptor. Characterization on an IL-7-dependent cell line. *J Exp Med*. 1990;171:1073-1089.

154. Noguchi M, Yi H, Rosenblatt HM, et al. Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell*. 1993;73:147-157.

155. Peschon JJ, Morrissey PJ, Grabstein KH, et al. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med*. 1994;180:1955-1960.

156. Ray RJ, Furlonger C, Williams DE, et al. Characterization of thymic stromal-derived lymphopoietin (TSLP) in murine B cell development in vitro. *Eur J Immunol* . 1996;26:10-16.

157. Levin SD, Koelling RM, Friend SL, et al. Thymic stromal lymphopoietin: a cytokine that promotes the development of IgM+ B cells in vitro and signals via a novel mechanism. *J Immunol*. 1999;162:677-683.

158. Park LS, Martin U, Garka K, et al. Cloning of the murine thymic stromal lymphopoietin (TSLP) receptor: formation of a functional heteromeric complex requires interleukin 7 receptor. *J Exp Med*. 2000;192:659-670.

159. Blagoev B, Nielsen MM, Angrist M, et al. Cloning of rat thymic stromal lymphopoietin receptor (TSLPR) and characterization of genomic structure of murine Tslpr gene. *Gene*. 2002;284:161-168.

160. Isaksen DE, Baumann H, Trobridge PA, et al. Requirement for stat5 in thymic stromal lymphopoietin-mediated signal transduction. *J Immunol*. 1999;163:5971-5977.

161. Vosshenrich CA, Cumano A, Muller W, et al. Thymic stromal-derived lymphopoietin distinguishes fetal from adult B cell development. *Nat Immunol*. 2003;4:773-779.

162. Montecino-Rodriguez E, Leathers H, Dorshkind K. Identification of a B-1 B cell-specified progenitor. *Nat Immunol*. 2006;7:293-301.

163. Astrakhan A, Omori M, Nguyen T, et al. Local increase in thymic stromal lymphopoietin induces systemic alterations in B cell development. *Nat Immunol*. 2007;8:522-531.

164. Migliaccio G, Migliaccio AR, Valinsky J, et al. Stem cell factor induces proliferation and differentiation of highly enriched murine hematopoietic cells. *Proc Natl Acad Sci U S A*. 1991;88:7420-7424.

165. Metcalf D. Lineage commitment of hemopoietic progenitor cells in developing blast cell colonies: influence of colony-stimulating factors. *Proc Natl Acad Sci U S A*. 1991;88:11310-11314.

166. Tsuji K, Lyman SD, Sudo T, et al. Enhancement of murine hematopoiesis by synergistic interactions between steel factor (ligand for c-kit), interleukin-11, and other early acting factors in culture. *Blood*. 1992;79:2855-2860.

167. Yasunaga M, Wang F, Kunisada T, et al. Cell cycle control of c-kit +IL-7R+ B precursor cells by two distinct signals derived from IL-7 receptor and c-kit in a fully defined medium. *J Exp Med*. 1995; 182:315-323.

168. Mackarehtschian K, Hardin JD, Moore KA, et al. Targeted disruption of the flk2/flt3 gene leads to deficiencies in primitive hematopoietic progenitors. *Immunity*. 1995;3:147-161.

169. Jacobsen SE, Veiby OP, Myklebust J, et al. Ability of flt3 ligand to stimulate the in vitro growth of primitive murine hematopoietic progenitors is potently and directly inhibited by transforming growth factor-beta and tumor necrosis factor-alpha. *Blood*.

1996;87:5016-5026.

170. Ray RJ, Paige CJ, Furlonger C, et al. Flt3 ligand supports the differentiation of early B cell progenitors in the presence of interleukin-11 and interleukin-7. *Eur J Immunol* . 1996;26:1504-1510.

171. Veiby OP, Jacobsen FW, Cui L, et al. The flt3 ligand promotes the survival of primitive hemopoietic progenitor cells with myeloid as well as B lymphoid potential. Suppression of apoptosis and counteraction by TNF-alpha and TGF-beta. *J Immunol* . 1996;157:2953-2960.

172. Veiby OP, Lyman SD, Jacobsen SE. Combined signaling through interleukin-7 receptors and flt3 but not c-kit potently and selectively promotes B-cell commitment and differentiation from uncommitted murine bone marrow progenitor cells. *Blood*. 1996;88:1256-1265.

173. Billips LG, Petitte D, Dorshkind K, et al. Differential roles of stromal cells, interleukin-7, and kit-ligand in the regulation of B lymphopoiesis. *Blood*. 1992;79:1185-1192.

174. Collins LS, Dorshkind K. A stromal cell line from myeloid long-term bone marrow cultures can support myelopoiesis and B lymphopoiesis. *J Immunol* . 1987;138:1082-1087.

175. Cumano A, Dorshkind K, Gillis S, et al. The influence of S17 stromal cells and interleukin 7 on B cell development. *Eur J Immunol* . 1990;20:2183-2189.

176. Winkler TH, Melchers F, Rolink AG. Interleukin-3 and interleukin-7 are alternative growth factors for the same B-cell precursors in the mouse. *Blood*. 1995;85:2045-2051.

177. Kee BL, Cumano A, Iscove NN, et al. Stromal cell independent growth of bipotent B cell—macrophage precursors from murine fetal liver. *Int Immunol* . 1994;6:401-407.

178. Rolink AG, Winkler T, Melchers F, et al. Precursor B cell receptor-dependent B cell proliferation and differentiation does not require the bone marrow or fetal liver environment. *J Exp Med*. 2000;191:23-32.

179. Faust EA, Saffran DC, Toksoz D, et al. Distinctive growth requirements and gene expression patterns distinguish progenitor B cells from pre-B cells. *J Exp Med*. 1993;177:915-923.

180. Spanopoulou E, Roman CA, Corcoran LM, et al. Functional immunoglobulin transgenes guide ordered B-cell differentiation in Rag-1-deficient mice. *Genes Dev*. 1994;8:1030-1042.

181. Gordon MY, Riley GP, Watt SM, et al. Compartmentalization of a haematopoietic

growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. *Nature*. 1987;326:403-405.

182. Borghesi LA, Yamashita Y, Kincade PW. Heparan sulfate proteoglycans mediate interleukin-7-dependent B lymphopoiesis. *Blood*. 1999;93:140-148.

183. Kincade PW, Medina KL, Payne KJ, et al. Early B-lymphocyte precursors and their regulation by sex steroids. *Immunol Rev*. 2000;175:128-137.

184. Igarashi H, Kouro T, Yokota T, et al. Age and stage dependency of estrogen receptor expression by lymphocyte precursors. *Proc Natl Acad Sci U S A* . 2001;98:15131-15136.

185. Landreth KS, Narayanan R, Dorshkind K. Insulin-like growth factor-I regulates pro-B cell differentiation. *Blood*. 1992;80:1207-1212.

186. Jardieu P, Clark R, Mortensen D, et al. In vivo administration of insulin-like growth factor-I stimulates primary B lymphopoiesis and enhances lymphocyte recovery after bone marrow transplantation. *J Immunol* . 1994;152:4320-4327.

187. Zhang Y, Lu L, Furlonger C, et al. Hemokinin is a hematopoietic-specific tachykinin that regulates B lymphopoiesis. *Nat Immunol* . 2000;1:392-397.

188. Montecino-Rodriguez E, Clark RG, Powell-Braxton L, et al. Primary B cell development is impaired in mice with defects of the pituitary/thyroid axis. *J Immunol* . 1997;159:2712-2719.

189. Montecino-Rodriguez E, Clark R, Johnson A, et al. Defective B cell development in Snell dwarf (dw/dw) mice can be corrected by thyroxine treatment. *J Immunol* . 1996;157:3334-3340.

190. Nagai Y, Garrett KP, Ohta S, et al. Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment. *Immunity*. 2006;24:801-812.

191. Allman D, Karnell FG, Punt JA, et al. Separation of Notch1 promoted lineage commitment and expansion/transformation in developing T cells. *J Exp Med*. 2001;194:99-106.

192. Izon DJ, Punt JA, Pear WS. Deciphering the role of Notch signaling in lymphopoiesis. *Curr Opin Immunol* . 2002;14:192-199.

193. Jacobsen K, Osmond DG. Microenvironmental organization and stromal cell associations of B lymphocyte precursor cells in mouse bone marrow. *Eur J Immunol* . 1990;20:2395-2404.

194. Bleul CC, Fuhlbrigge RC, Casasnovas JM, et al. A highly efficacious lymphocyte

chemoattractant, stromal cell-derived factor 1 (SDF-1). *J Exp Med*. 1996;184:1101-1109.

195. Nagasawa T, Kikutani H, Kishimoto T. Molecular cloning and structure of a pre-B-cell growth-stimulating factor. *Proc Natl Acad Sci U S A* . 1994;91:2305-2309.

196. D'Apuzzo M, Rolink A, Loetscher M, et al. The chemokine SDF-1, stromal cell-derived factor 1, attracts early stage B cell precursors via the chemokine receptor CXCR4. *Eur J Immunol* . 1997;27:1788-1793.

197. Nagasawa T, Tachibana K, Kishimoto T. A novel CXC chemokine PBSF/SDF-1 and its receptor CXCR4: their functions in development, hematopoiesis and HIV infection. *Semin Immunol*. 1998;10:179-185.

198. Nagasawa T, Hirota S, Tachibana K, et al. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature*. 1996;382:635-638.

199. Ma Q, Jones D, Borghesani PR, et al. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc Natl Acad Sci U S A*. 1998;95:9448-9453.

200. Zou YR, Kottmann AH, Kuroda M, et al. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature*. 1998;393:595-599.

201. Ma Q, Jones D, Springer TA. The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. *Immunity*. 1999;10:463-471.

202. Lennon GG, Perry RP. The temporal order of appearance of transcripts from unrearranged and rearranged Ig genes in murine fetal liver. *J Immunol* . 1990;144:1983-1987.

203. Lennon GG, Perry RP. C mu-containing transcripts initiate heterogeneously within the IgH enhancer region and contain a novel 5′-nontranslatable exon. *Nature*. 1985;318:475-478.

204. Nelson KJ, Haimovich J, Perry RP. Characterization of productive and sterile transcripts from the immunoglobulin heavy-chain locus: processing of micron and muS mRNA. *Mol Cell Biol* . 1983;3:1317-1332.

205. Oettinger MA, Schatz DG, Gorka C, et al. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science*. 1990;248:1517-1523.

206. Schatz DG, Oettinger MA, Baltimore D. The V(D)J recombination activating gene, RAG-1. *Cell*. 1989;59:1035-1048.

207. McBlane JF, van Gent DC, Ramsden DA, et al. Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. *Cell*. 1995;83:387-395.

208. Ji Y, Resch W, Corbett E, et al. The in vivo pattern of binding of RAG1 and RAG2 to antigen receptor loci. *Cell*. 2010;141:419-431.

209. Ehlich A, Martin V, Muller W, et al. Analysis of the B-cell progenitor compartment at the level of single cells. *Curr Biol* . 1994;4:573-583.

210. Allman D, Li J, Hardy RR. Commitment to the B lymphoid lineage occurs before DH-JH recombination. *J Exp Med*. 1999;189:735-740.

211. Kitamura D, Rajewsky K. Targeted disruption of mu chain membrane exon causes loss of heavy-chain allelic exclusion. *Nature*. 1992;356: 154-156.

212. Bosma GC, Custer RP, Bosma MJ. A severe combined immunodeficiency mutation in the mouse. *Nature*. 1983;301:527-530.

213. Hardy RR, Kemp JD, Hayakawa K. Analysis of lymphoid population in scid mice; detection of a potential B lymphocyte progenitor population present at normal levels in scid mice by three color flow cytometry with B220 and S7. *Curr Top Microbiol Immunol* . 1989;152:19-25.

214. Blunt T, Finnie NJ, Taccioli GE, et al. Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation. *Cell*. 1995;80:813-823.

215. Peterson SR, Kurimasa A, Oshimura M, et al. Loss of the catalytic subunit of the DNA-dependent protein kinase in DNA double-strandbreak-repair mutant mammalian cells. *Proc Natl Acad Sci U S A* . 1995;92:3171-3174.

216. Reichman-Fried M, Hardy RR, Bosma MJ. Development of B-lineage cells in the bone marrow of scid/scid mice following the introduction of functionally rearranged immunoglobulin transgenes. *Proc Natl Acad Sci U S A* . 1990;87:2730-2734.

217. Kitamura D, Roes J, Kuhn R, et al. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature*. 1991;350:423-426.

218. Loffert D, Schaal S, Ehlich A, et al. Early B-cell development in the mouse: insights from mutations introduced by gene targeting. *Immunol Rev*. 1994;137:135-153.

219. Karasuyama H, Kudo A, Melchers F. The proteins encoded by the VpreB and lambda 5 pre-B cell-specific genes can associate with each other and with mu heavy

chain. *J Exp Med*. 1990;172:969-972.

220. Sakaguchi N, Melchers F. Lambda 5, a new light-chain-related locus selectively expressed in pre-B lymphocytes. *Nature*. 1986;324:579-582.

221. Kudo A, Melchers F. A second gene, VpreB in the lambda 5 locus of the mouse, which appears to be selectively expressed in pre-B lymphocytes. *EMBO J*. 1987;6:2267-2272.

222. Kitamura D, Kudo A, Schaal S, et al. A critical role of lambda 5 protein in B cell development. *Cell*. 1992;69:823-831.

223. Papavasiliou F, Jankovic M, Nussenzweig MC. Surrogate or conventional light chains are required for membrane immunoglobulin mu to activate the precursor B cell transition. *J Exp Med*. 1996;184:2025-2030.

224. Sakaguchi N, Kashiwamura S, Kimoto M, et al. B lymphocyte lineage-restricted expression of mb-1, a gene with CD3- like structural properties. *EMBO J*. 1988;7:3457-3464.

225. Hermanson GG, Eisenberg D, Kincade PW, et al. B29: a member of the immunoglobulin gene superfamily exclusively expressed on betalineage cells. *Proc Natl Acad Sci U S A*. 1988;85:6890-6894.

226. Hombach J, Lottspeich F, Reth M. Identification of the genes encoding the IgM-alpha and Ig-beta components of the IgM antigen receptor complex by amino-terminal sequencing. *Eur J Immunol* . 1990;20:2795-2799.

227. Campbell KS, Hager EJ, Friedrich RJ, et al. IgM antigen receptor complex contains phosphoprotein products of B29 and mb-1 genes. *Proc Natl Acad Sci U S A* . 1991;88:3982-3986.

228. Gong S, Nussenzweig MC. Regulation of an early developmental checkpoint in the B cell pathway by Ig beta. *Science*. 1996;272:411-414.

229. Cheng AM, Rowley B, Pao W, et al. Syk tyrosine kinase required for mouse viability and B-cell development. *Nature*. 1995;378:303-306.

230. Turner M, Mee PJ, Costello PS, et al. Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature*. 1995;378:298-302.

231. Wienands J, Larbolette O, Reth M. Evidence for a preformed transducer complex organized by the B cell antigen receptor. *Proc Natl Acad Sci U S A* . 1996;93:7865-7870.

232. Lam KP, Kuhn R, Rajewsky K. In vivo ablation of surface immunoglobulin on mature

B cells by inducible gene targeting results in rapid cell death. *Cell*. 1997;90:1073-1083.

233. Kraus M, Alimzhanov MB, Rajewsky N, et al. Survival of resting mature B lymphocytes depends on BCR signaling via the Igalpha/beta het-erodimer. *Cell*. 2004;117:787-800.

234. Hirabayashi Y, Lecerf JM, Dong Z, et al. Kinetic analysis of the interactions of recombinant human VpreB and Ig V domains. *J Immunol*. 1995;155:1218-1228.

235. Wasserman R, Li YS, Hardy RR. Down-regulation of terminal deoxy-nucleotidyl transferase by Ig heavy chain in B lineage cells. *J Immunol*. 1997;158:1133-1138.

236. Ubelhart R, Bach MP, Eschbach C, et al. N-linked glycosylation selectively regulates autonomous precursor BCR function. *Nat Immunol*. 2010;11:759-765.

237. Aifantis I, Borowski C, Gounari F, et al. A critical role for the cytoplasmic tail of pTalpha in T lymphocyte development. *Nat Immunol*. 2002;3: 483-488.

238. Yamasaki S, Ishikawa E, Sakuma M, et al. Mechanistic basis of pre-T cell receptor-mediated autonomous signaling critical for thymocyte development. *Nat Immunol*. 2006;7:67-75.

239. Pang SS, Berry R, Chen Z, et al. The structural basis for autonomous dimerization of the pre-T-cell antigen receptor. *Nature*. 2010;467:844-848.

240. Middendorp S, Dingjan GM, Hendriks RW. Impaired precursor B cell differentiation in Bruton's tyrosine kinase-deficient mice. *J Immunol*. 2002;168:2695-2703.

241. Hayashi S, Witte PL, Kincade PW. The xid mutation affects hemopoiesis in long term cultures of murine bone marrow. *J Immunol*. 1989;142:444-451.

242. Narendran A, Ramsden D, Cumano A, et al. B cell developmental defects in X-linked immunodeficiency. *Int Immunol*. 1993;5:139-144.

243. Nisitani S, Satterthwaite AB, Akashi K, et al. Posttranscriptional regulation of Bruton's tyrosine kinase expression in antigen receptor-stimulated splenic B cells. *Proc Natl Acad Sci U S A*. 2000;97:2737-2742.

244. Kurosaki T, Johnson SA, Pao L, et al. Role of the Syk autophosphorylation site and SH2 domains in B cell antigen receptor signaling. *J Exp Med*. 1995;182:1815-1823.

245. Kurosaki T. Genetic analysis of B cell antigen receptor signaling. *Annu Rev Immunol*. 1999;17:555-592.

246. Xu S, Tan JE, Wong EP, et al. B cell development and activation defects resulting in

xid-like immunodeficiency in BLNK/SLP-65-deficient mice. *Int Immunol*. 2000;12:397-404.

247. Xu S, Wong SC, Lam KP. Cutting edge: B cell linker protein is dispensable for the allelic exclusion of immunoglobulin heavy chain locus but required for the persistence of CD5+ B cells. *J Immunol*. 2000;165: 4153-4157.

248. Kersseboom R, Middendorp S, Dingjan GM, et al. Bruton's tyrosine kinase cooperates with the B cell linker protein SLP-65 as a tumor suppressor in Pre-B cells. *J Exp Med*. 2003;198:91-98.

249. Ta VB, de Bruijn MJ, ter Brugge PJ, et al. Malignant transformation of Slp65-deficient pre-B cells involves disruption of the Arf-Mdm2-p53 tumor suppressor pathway. *Blood*. 2010;115:1385-1393.

250. Yasuda T, Sanjo H, Pages G, et al. Erk kinases link pre-B cell receptor signaling to transcriptional events required for early B cell expansion. *Immunity*. 2008;28:499-508.

251. Grawunder U, Leu TM, Schatz DG, et al. Down-regulation of RAG1 and RAG2 gene expression in preB cells after functional immunoglobulin heavy chain rearrangement. *Immunity*. 1995;3:601-608.

252. Schlissel MS, Baltimore D. Activation of immunoglobulin kappa gene rearrangement correlates with induction of germline kappa gene transcription. *Cell*. 1989;58:1001-1007.

253. Liang HE, Hsu LY, Cado D, et al. The “dispensable” portion of RAG2 is necessary for efficient V-to-DJ rearrangement during B and T cell development. *Immunity*. 2002;17:639-651.

254. Hsu LY, Lauring J, Liang HE, et al. A conserved transcriptional enhancer regulates RAG gene expression in developing B cells. *Immunity*. 2003;19:105-117.

255. Lin WC, Desiderio S. Regulation of V(D)J recombination activator protein RAG-2 by phosphorylation. *Science*. 1993;260:953-959.

256. Li Z, Dordai DI, Lee J, et al. A conserved degradation signal regulates RAG-2 accumulation during cell division and links V(D)J recombination to the cell cycle. *Immunity*. 1996;5:575-589.

257. Lee J, Desiderio S. Cyclin A/CDK2 regulates V(D)J recombination by coordinating RAG-2 accumulation and DNA repair. *Immunity*. 1999;11: 771-781.

258. Karasuyama H, Nakamura T, Nagata K, et al. The roles of preB cell receptor in early B cell development and its signal transduction. *Immunol Cell Biol*. 1997;75:209-216.

259. Marshall AJ, Fleming HE, Wu GE, et al. Modulation of the IL-7 doseresponse

threshold during pro-B cell differentiation is dependent on pre-B cell receptor expression. *J Immunol*. 1998;161:6038-6045.

260. Keyna U, Beck-Engeser GB, Jongstra J, et al. Surrogate light chain-dependent selection of Ig heavy chain V regions. *J Immunol*. 1995;155: 5536-5542.

261. Kline GH, Hartwell L, Beck-Engeser GB, et al. Pre-B cell receptor-mediated selection of pre-B cells synthesizing functional mu heavy chains. *J Immunol*. 1998;161:1608-1618.

262. ten Boekel E, Melchers F, Rolink AG. Changes in the V(H) gene repertoire of developing precursor B lymphocytes in mouse bone marrow mediated by the pre-B cell receptor. *Immunity*. 1997;7:357-368.

263. Wasserman R, Li YS, Shinton SA, et al. A novel mechanism for B cell repertoire maturation based on response by B cell precursors to pre-B receptor assembly. *J Exp Med*. 1998;187:259-264.

264. Wu GE, Paige CJ. VH gene family utilization in colonies derived from B and pre-B cells detected by the RNA colony blot assay. *EMBO J*. 1986;5:3475-3481.

265. Decker DJ, Boyle NE, Koziol JA, et al. The expression of the Ig H chain repertoire in developing bone marrow B lineage cells. *J Immunol*. 1991;146:350-361.

266. Ebert A, McManus S, Tagoh H, et al. The distal V(H) gene cluster of the Igh locus contains distinct regulatory elements with Pax5 transcription factor-dependent activity in pro-B cells. *Immunity*. 2011;34: 175-187.

267. Guo C, Yoon HS, Franklin A, et al. CTCF-binding elements mediate control of V(D)J recombination. *Nature*. 2011;477:424-430.

268. Decker DJ, Boyle NE, Klinman NR. Predominance of nonproductive rearrangements of VH81X gene segments evidences a dependence of B cell clonal maturation on the structure of nascent H chains. *J Immunol*. 1991;147:1406-1411.

269. Decker DJ, Kline GH, Hayden TA, et al. Heavy chain V gene-specific elimination of B cells during the pre-B cell to B cell transition. *J Immunol*. 1995;154:4924-4935.

270. Keyna U, Applequist SE, Jongstra J, et al. Ig mu heavy chains with VH81X variable regions do not associate with lambda 5. *Ann N Y Acad Sci*. 1995;764:39-42.

271. Bankovich AJ, Raunser S, Juo ZS, et al. Structural insight into pre-B cell receptor function. *Science*. 2007;316:291-294.

272. Marshall AJ, Wu GE, Paige GJ. Frequency of VH81x usage during B cell

development: initial decline in usage is independent of Ig heavy chain cell surface expression. *J Immunol*. 1996;156:2077-2084.

273. Marshall AJ, Paige CJ, Wu GE. V(H) repertoire maturation during B cell development in vitro: differential selection of Ig heavy chains by fetal and adult B cell progenitors. *J Immunol*. 1997;158: 4282-4291.

274. Keenan RA, De Riva A, Corleis B, et al. Censoring of autoreactive B cell development by the pre-B cell receptor. *Science.* 2008;321:696-699.

275. Kohler F, Hug E, Eschbach C, et al. Autoreactive B cell receptors mimic autonomous pre-B cell receptor signaling and induce proliferation of early B cells. *Immunity*. 2008;29:912-921.

276. Schulz D, Vassen L, Chow KT, et al. Gfi1b negatively regulates Rag expression directly and via the repression of FoxO1. *J Exp Med*. 2012;209: 187-199.

277. Duy C, Yu JJ, Nahar R, et al. BCL6 is critical for the development of a diverse primary B cell repertoire. *J Exp Med*. 2010;207:1209-1221.

278. Nahar R, Ramezani-Rad P, Mossner M, et al. Pre-B cell receptor-mediated activation of BCL6 induces pre-B cell quiescence through transcriptional repression of MYC. *Blood*. 2011;118:4174-4178.

279. Mandal M, Powers SE, Ochiai K, et al. Ras orchestrates exit from the cell cycle and light-chain recombination during early B cell development. *Nat Immunol*. 2009;10:1110-1117.

280. Shapiro MA, Weigert M. How immunoglobulin V kappa genes rearrange. *J Immunol*. 1987;139:3834-3839.

281. Tiegs SL, Russell DM, Nemazee D. Receptor editing in self-reactive bone marrow B cells. *J Exp Med*. 1993;177:1009-1020.

282. Lang J, Jackson M, Teyton L, et al. B cells are exquisitely sensitive to central tolerance and receptor editing induced by ultralow affinity, membrane-bound antigen. *J Exp Med*. 1996;184:1685-1697.

283. Yamagami T, ten Boekel E, Andersson J, et al. Frequencies of multiple IgL chain gene rearrangements in single normal or kappaL chain-deficient B lineage cells. *Immunity*. 1999;11:317-327.

284. Derudder E, Cadera EJ, Vahl JC, et al. Development of immuno-globulin lambda-chain-positive B cells, but not editing of immunoglobulin kappa-chain, depends on NF-kappaB signals. *Nat Immunol*. 2009;10:647-654.

285. Chen C, Nagy Z, Radic MZ, et al. The site and stage of anti-DNA B-cell deletion. *Nature*. 1995;373:252-255.

286. Melamed D, Benschop RJ, Cambier JC, et al. Developmental regulation of B lymphocyte immune tolerance compartmentalizes clonal selection from receptor selection. *Cell*. 1998;92:173-182.

287. Allman DM, Ferguson SE, Cancro MP. Peripheral B cell maturation. I. Immature peripheral B cells in adults are heat-stable antigenhi and exhibit unique signaling characteristics. *J Immunol*. 1992;149: 2533-2540.

288. Rolink AG, Andersson J, Melchers F. Characterization of immature B cells by a novel monoclonal antibody, by turnover and by mitogen reactivity. *Eur J Immunol*. 1998;28:3738-3748.

289. Allman D, Lindsley RC, DeMuth W, et al. Resolution of three non-proliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation. *J Immunol*. 2001;167: 6834-6840.

290. Petrenko O, Beavis A, Klaine M, et al. The molecular characterization of the fetal stem cell marker AA4. *Immunity*. 1999;10:691-700.

291. Fang W, Mueller DL, Pennell CA, et al. Frequent aberrant immunoglobulin gene rearrangements in pro-B cells revealed by a bcl-xL transgene. *Immunity*. 1996;4:291-299.

292. Carsetti R, Kohler G, Lamers MC. Transitional B cells are the target of negative selection in the B cell compartment. *J Exp Med*. 1995;181: 2129-2140.

293. Allman DM, Ferguson SE, Lentz VM, et al. Peripheral B cell maturation. II. Heat-stable antigen(hi) splenic B cells are an immature developmental intermediate in the production of long-lived marrow-derived B cells. *J Immunol*. 1993;151:4431-4444.

294. Weiner HL, Moorhead JW, Claman HN. Anti-immunoglobulin stimulation of murine lymphocytes. I. Age dependency of the proliferative response. *J Immunol*. 1976;116:1656-1661.

295. Nossal GJ, Pike BL, Battye FL. Mechanisms of clonal abortion tolerogenesis. II. Clonal behaviour of immature B cells following exposure to anti-mu chain antibody. *Immunology*. 1979;37:203-215.

296. Metcalf ES, Klinman NR. In vitro tolerance induction of neonatal murine B cells. *J Exp Med*. 1976;143:1327-1340.

297. Metcalf ES, Klinman NR. In vitro tolerance induction of bone marrow cells: a marker

for B cell maturation. *J Immunol*. 1977;118:2111-2116.

298. Goodnow CC, Crosbie J, Adelstein S, et al. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature*. 1988;334:676-682.

299. Hartley SB, Crosbie J, Brink R, et al. Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. *Nature*. 1991;353:765-769.

300. Nemazee DA, Burki K. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature*. 1989;337: 562-566.

301. Erikson J, Radic MZ, Camper SA, et al. Expression of anti-DNA immunoglobulin transgenes in non-autoimmune mice. *Nature*. 1991;349: 331-334.

302. Gay D, Saunders T, Camper S, et al. Receptor editing: an approach by autoreactive B cells to escape tolerance. *J Exp Med*. 1993;177:999-1008.

303. Merrell KT, Benschop RJ, Gauld SB, et al. Identification of anergic B cells within a wild-type repertoire. *Immunity*. 2006;25:953-962.

304. Meyer-Bahlburg A, Andrews SF, Yu KO, et al. Characterization of a late transitional B cell population highly sensitive to BAFF-mediated homeostatic proliferation. *J Exp Med*. 2008;205:155-168.

305. Norvell A, Mandik L, Monroe JG. Engagement of the antigen-receptor on immature murine B lymphocytes results in death by apoptosis. *J Immunol*. 1995;154:4404-4413.

306. Norvell A, Monroe JG. Acquisition of surface IgD fails to protect from tolerance-induction. Both surface IgM- and surface IgD-mediated signals induce apoptosis of immature murine B lymphocytes. *J Immunol*. 1996;156:1328-1332.

307. Rathmell JC, Goodnow CC. Effects of the lpr mutation on elimination and inactivation of self-reactive B cells. *J Immunol*. 1994;153: 2831-2842.

308. Rubio CF, Kench J, Russell DM, et al. Analysis of central B cell tolerance in autoimmune-prone MRL/lpr mice bearing autoantibody transgenes. *J Immunol*. 1996;157:65-71.

309. Carman JA, Wechsler-Reya RJ, Monroe JG. Immature stage B cells enter but do not progress beyond the early G1 phase of the cell cycle in response to antigen receptor signaling. *J Immunol*. 1996;156: 4562-4569.

310. Benschop RJ, Melamed D, Nemazee D, et al. Distinct signal thresholds for the

unique antigen receptor-linked gene expression programs in mature and immature B cells. *J Exp Med*. 1999;190:749-756.

311. Tomayko MM, Cancro MP. Long-lived B cells are distinguished by elevated expression of A1. *J Immunol*. 1998;160:107-111.

312. Grumont RJ, Rourke IJ, Gerondakis S. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. *Genes Dev*. 1999;13:400-411.

313. Khan WN. Regulation of B lymphocyte development and activation by Bruton's tyrosine kinase. *Immunol Res*. 2001;23:147-156.

314. Maas A, Hendriks RW. Role of Bruton's tyrosine kinase in B cell development. *Dev Immunol*. 2001;8:171-181.

315. Mond JJ, Scher I, Cossman J, et al. Role of the thymus in directing the development of a subset of B lymphocytes. *J Exp Med*. 1982;155: 924-936.

316. Wortis HH, Burkly L, Hughes D, et al. Lack of mature B cells in nude mice with X-linked immune deficiency. *J Exp Med*. 1982;155:903-913.

317. Oka Y, Rolink AG, Andersson J, et al. Profound reduction of mature B cell numbers, reactivities and serum Ig levels in mice which simultaneously carry the XID and CD40 deficiency genes. *Int Immunol*. 1996;8:1675-1685.

318. Clark MR, Campbell KS, Kazlauskas A, et al. The B cell antigen receptor complex: association of Ig-alpha and Ig-beta with distinct cytoplasmic effectors. *Science*. 1992;258:123-126.

319. Hibbs ML, Tarlinton DM, Armes J, et al. Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. *Cell*. 1995;83:301-311.

320. Chan VW, Meng F, Soriano P, et al. Characterization of the B lymphocyte populations in Lyn-deficient mice and the role of Lyn in signal initiation and down-regulation. *Immunity*. 1997;7:69-81.

321. Castro I, Wright JA, Damdinsuren B, et al. B cell receptor-mediated sustained c-Rel activation facilitates late transitional B cell survival through control of B cell activating factor receptor and NF-kappaB2. *J Immunol*. 2009;182:7729-7737.

322. Henderson RB, Grys K, Vehlow A, et al. A novel Rac-dependent checkpoint in B cell development controls entry into the splenic white pulp and cell survival. *J Exp Med*. 2010;207:837-853.

323. Rowland SL, DePersis CL, Torres RM, et al. Ras activation of Erk restores impaired tonic BCR signaling and rescues immature B cell differentiation. *J Exp Med*. 2010;207:607-621.

324. Subbarao B, Mosier DE. Induction of B lymphocyte proliferation by monoclonal anti-Lyb 2 antibody. *J Immunol*. 1983;130:2033-2037.

325. Pan C, Baumgarth N, Parnes JR. CD72-deficient mice reveal nonredundant roles of CD72 in B cell development and activation. *Immunity*. 1999;11:495-506.

326. Adachi T, Flaswinkel H, Yakura H, et al. The B cell surface protein CD72 recruits the tyrosine phosphatase SHP-1 upon tyrosine phosphorylation. *J Immunol*. 1998;160:4662-4665.

327. Verkoczy L, Duong B, Skog P, et al. Basal B cell receptor-directed phosphatidylinositol 3-kinase signaling turns off RAGs and promotes B cell-positive selection. *J Immunol*. 2007;178:6332-6341.

328. Aiba Y, Kameyama M, Yamazaki T, et al. Regulation of B-cell development by BCAP and CD19 through their binding to phosphoinositide 3-kinase. *Blood*. 2008;111:1497-1503.

329. Srinivasan L, Sasaki Y, Calado DP, et al. PI3 kinase signals BCR-dependent mature B cell survival. *Cell*. 2009;139:573-586.

330. Cyster JG, Hartley SB, Goodnow CC. Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire. *Nature*. 1994;371:389-395.

331. Cyster JG, Goodnow CC. Antigen-induced exclusion from follicles and anergy are separate and complementary processes that influence peripheral B cell fate. *Immunity*. 1995;3:691-701.

332. Mandik-Nayak L, Bui A, Noorchashm H, et al. Regulation of anti-double-stranded DNA B cells in nonautoimmune mice: localization to the T-B interface of the splenic follicle. *J Exp Med*. 1997;186: 1257-1267.

333. Mandik-Nayak L, Seo SJ, Sokol C, et al. MRL-lpr/lpr mice exhibit a defect in maintaining developmental arrest and follicular exclusion of anti-double-stranded DNA B cells. *J Exp Med*. 1999;189:1799-1814.

334. Tze LE, Schram BR, Lam KP, et al. Basal immunoglobulin signaling actively maintains developmental stage in immature B cells. *PLoS Biol*. 2005;3:e82.

335. Thomas MD, Kremer CS, Ravichandran KS, et al. c-Myb is critical for B cell development and maintenance of follicular B cells. *Immunity*. 2005;23:275-286.

336. Levine MH, Haberman AM, Sant'Angelo DB, et al. A B-cell receptor-specific selection step governs immature to mature B cell differentiation. *Proc Natl Acad Sci U S A* . 2000;97:2743-2748.

337. Forster R, Schubel A, Breitfeld D, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell*. 1999;99:23-33.

338. Ansel KM, Ngo VN, Hyman PL, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature*. 2000;406:309-314.

339. Waldschmidt TJ, Noelle RJ. Immunology. Long live the mature B cell—a baffling mystery resolved. *Science*. 2001;293:2012-2013.

340. Khare SD, Sarosi I, Xia XZ, et al. Severe B cell hyperplasia and autoimmune disease in TALL-1 transgenic mice. *Proc Natl Acad Sci U S A* . 2000;97:3370-3375.

341. Khare SD, Hsu H. The role of TALL-1 and APRIL in immune regulation. *Trends Immunol*. 2001;22:61-63.

342. Thompson JS, Bixler SA, Qian F, et al. BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. *Science*. 2001;293: 2108-2111.

343. Yan M, Brady JR, Chan B, et al. Identification of a novel receptor for B lymphocyte stimulator that is mutated in a mouse strain with severe B cell deficiency. *Curr Biol* . 2001;11:1547-1552.

344. Lentz VM, Cancro MP, Nashold FE, et al. Bcmd governs recruitment of new B cells into the stable peripheral B cell pool in the A/WySnJ mouse. *J Immunol* . 1996;157:598-606.

345. Yu G, Boone T, Delaney J, et al. APRIL and TALL-I and receptors BCMA and TACI: system for regulating humoral immunity. *Nat Immunol* . 2000;1:252-256.

346. Ota M, Duong BH, Torkamani A, et al. Regulation of the B cell receptor repertoire and self-reactivity by BAFF. *J Immunol* . 2010;185: 4128-4136.

347. Tolar P, Hanna J, Krueger PD, et al. The constant region of the membrane immunoglobulin mediates B cell-receptor clustering and signaling in response to membrane antigens. *Immunity*. 2009;30:44-55.

348. Treanor B, Depoil D, Gonzalez-Granja A, et al. The membrane skeleton controls diffusion dynamics and signaling through the B cell receptor. *Immunity*. 2010;32:187-199.

349. Yang J, Reth M. Oligomeric organization of the B-cell antigen receptor on resting cells. *Nature*. 2010;467:465-469.

350. Opstelten D, Osmond DG. Pre-B cells in mouse bone marrow: immunofluorescence stathmokinetic studies of the proliferation of cytoplasmic mu-chain-bearing cells in normal mice. *J Immunol* . 1983;131: 2635-2640.

351. Bazin H, Platteau B, Maclennan IC, et al. B-cell production and differentiation in adult rats. *Immunology*. 1985;54:79-88.

352. Forster I, Rajewsky K. The bulk of the peripheral B-cell pool in mice is stable and not rapidly renewed from the bone marrow. *Proc Natl Acad Sci U S A* . 1990;87:4781-4784.

353. Hao Z, Rajewsky K. Homeostasis of peripheral B cells in the absence of B cell influx from the bone marrow. *J Exp Med*. 2001;194:1151-1164.

354. MacLennan IC. Germinal centers. *Annu Rev Immunol* . 1994;12:117-139.

355. Przylepa J, Himes C, Kelsoe G. Lymphocyte development and selection in germinal centers. *Curr Top Microbiol Immunol* . 1998;229:85-104.

356. Butcher EC, Rouse RV, Coffman RL, et al. Surface phenotype of Peyer's patch germinal center cells: implications for the role of germinal centers in B cell differentiation. *J Immunol* . 1982;129:2698-2707.

357. Kraal G, Hardy RR, Gallatin WM, et al. Antigen-induced changes in B cell subsets in lymph nodes: analysis by dual fluorescence flow cyto-fluorometry. *Eur J Immunol* . 1986;16:829-834.

358. Wang Z, Karras JG, Howard RG, et al. Induction of bcl-x by CD40 engagement rescues sIg-induced apoptosis in murine B cells. *J Immunol* . 1995;155:3722-3725.

359. Tuscano JM, Druey KM, Riva A, et al. Bcl-x rather than Bcl-2 mediates CD40-dependent centrocyte survival in the germinal center. *Blood*. 1996;88:1359-1364.

360. Zhang X, Li L, Choe J, et al. Up-regulation of Bcl-xL expression protects CD40-activated human B cells from Fas-mediated apoptosis. *Cell Immunol* . 1996;173:149-154.

361. Takahashi Y, Ohta H, Takemori T. Fas is required for clonal selection in germinal centers and the subsequent establishment of the memory B cell repertoire. *Immunity*. 2001;14:181-192.

362. Thai TH, Calado DP, Casola S, et al. Regulation of the germinal center response by microRNA-155. *Science*. 2007;316:604-608.

363. Muramatsu M, Kinoshita K, Fagarasan S, et al. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell*. 2000;102:553-563.

364. Fagarasan S, Kinoshita K, Muramatsu M, et al. In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. *Nature*. 2001;413:639-643.

365. Okazaki IM, Kinoshita K, Muramatsu M, et al. The AID enzyme induces class switch recombination in fibroblasts. *Nature*. 2002;416: 340-345.

366. Martin A, Bardwell PD, Woo CJ, et al. Activation-induced cytidine deaminase turns on somatic hypermutation in hybridomas. *Nature*. 2002;415:802-806.

367. Nagaoka H, Muramatsu M, Yamamura N, et al. Activation-induced deaminase (AID)-directed hypermutation in the immunoglobulin Smu region: implication of AID involvement in a common step of class switch recombination and somatic hypermutation. *J Exp Med*. 2002;195:529-534.

368. Arakawa H, Hauschild J, Buerstedde JM. Requirement of the activation-induced deaminase (AID) gene for immunoglobulin gene conversion. *Science*. 2002;295:1301-1306.

369. Harris RS, Sale JE, Petersen-Mahrt SK, et al. AID is essential for immunoglobulin V gene conversion in a cultured B cell line. *Curr Biol* . 2002;12:435-438.

370. Staszewski O, Baker RE, Ucher AJ, et al. Activation-induced cytidine deaminase induces reproducible DNA breaks at many non-Ig Loci in activated B cells. *Mol Cell*. 2011;41:232-242.

371. Good-Jacobson KL, Szumilas CG, Chen L, et al. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol*. 2010;11:535-542.

372. Victora GD, Schwickert TA, Fooksman DR, et al. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell*. 2010;143:592-605.

373. Herzenberg LA, Okumura K, Metzler CM. Regulation of immunoglobulin and antibody production by allotype suppressor T cells in mice. *Transplant Rev*. 1975;27:57-83.

374. Jacobson EB, L'Age-Stehr J, Herzenberg LA. Immunological memory in mice. II. Cell interactions in the secondary immune response studies by means of immunoglobulin allotype markers. *J Exp Med*. 1970;131:1109-1120.

375. L'Age-Stehr J, Herzenberg LA. Immunological memory in mice. I. Physical separation and partial characterization of memory cells for different immunoglobulin classes from each other and from antibody-producing cells. *J Exp Med*. 1970;131:1093-1108.

376. Romano TJ, Mond JJ, Thorbecke GJ. Immunological memory function of the T and B cell types: distribution over mouse spleen and lymph nodes. *Eur J Immunol* . 1975;5:211-215.

377. Okumura K, Metzler CM, Tsu TT, et al. Two stages of B-cell memory development with different T-cell requirements. *J Exp Med*. 1976;144: 345-357.

378. Herzenberg LA, Black SJ, Tokuhisa T. Memory B cells at successive stages of differentiation. Affinity maturation and the role of IgD receptors. *J Exp Med*. 1980;151:1071-1087.

379. Yefenof E, Sanders VM, Snow EC, et al. Preparation and analysis of antigen-specific memory B cells. *J Immunol* . 1985;135:3777-3784.

380. Hayakawa K, Ishii R, Yamasaki K, et al. Isolation of high-affinity memory B cells: phycoerythrin as a probe for antigen-binding cells. *Proc Natl Acad Sci U S A* . 1987;84:1379-1383.

381. Schittek B, Rajewsky K. Maintenance of B-cell memory by long-lived cells generated from proliferating precursors. *Nature*. 1990;346:749-751.

382. Anderson SM, Tomayko MM, Ahuja A, et al. New markers for murine memory B cells that define mutated and unmutated subsets. *J Exp Med*. 2007;204:2103-2114.

383. Hikida M, Casola S, Takahashi N, et al. PLC-gamma2 is essential for formation and maintenance of memory B cells. *J Exp Med*. 2009;206: 681-689.

384. Bruce J, Symington FW, McKearn TJ, et al. A monoclonal antibody discriminating between subsets of T and B cells. *J Immunol* . 1981;127: 2496-2501.

385. Linton PL, Decker DJ, Klinman NR. Primary antibody-forming cells and secondary B cells are generated from separate precursor cell subpopulations. *Cell* . 1989;59:1049-1059.

386. Linton PJ, Lo D, Lai L, et al. Among naive precursor cell subpopulations only progenitors of memory B cells originate germinal centers. *Eur J Immunol* . 1992;22:1293-1297.

387. Gray D, Skarvall H. B-cell memory is short-lived in the absence of antigen. *Nature*. 1988;336:70-73.

388. Vieira P, Rajewsky K. Persistence of memory B cells in mice deprived of T cell help. *Int Immunol*. 1990;2:487-494.

389. Karrer U, Lopez-Macias C, Oxenius A, et al. Antiviral B cell memory in the absence of mature follicular dendritic cell networks and classical germinal centers in TNFR1-/- mice. *J Immunol*. 2000;164:768-778.

390. Maruyama M, Lam KP, Rajewsky K. Memory B-cell persistence is independent of persisting immunizing antigen. *Nature*. 2000;407:636-642.

391. Hardy RR. B-1 B cell development. *J Immunol*. 2006;177:2749-2754.

392. Hayakawa K, Hardy RR, Stall AM, et al. Immunoglobulin-bearing B cells reconstitute and maintain the murine Ly- 1 B cell lineage. *Eur J Immunol*. 1986;16:1313-1316.

393. Choi YS, Dieter JA, Rothaeusler K, et al. B-1 cells in the bone marrow are a significant source of natural IgM. *Eur J Immunol*. 2012;42:120-129.

394. Hayakawa K, Hardy RR, Honda M, et al. Ly-1 B cells: functionally distinct lymphocytes that secrete IgM autoantibodies. *Proc Natl Acad Sci U S A*. 1984;81:2494-2498.

395. Hardy RR, Carmack CE, Shinton SA, et al. A single VH gene is utilized predominantly in anti-BrMRBC hybridomas derived from puri-fied Ly-1 B cells. Definition of the VH11 family. *J Immunol*. 1989;142: 3643-3651.

396. Hayakawa K, Carmack CE, Hyman R, et al. Natural autoantibodies to thymocytes: origin, VH genes, fine specificities, and the role of Thy-1 glycoprotein. *J Exp Med*. 1990;172:869-878.

397. Masmoudi H, Mota-Santos T, Huetz F, et al. All T15 Id-positive antibodies (but not the majority of VHT15+ antibodies) are produced by peritoneal CD5+ B lymphocytes. *Int Immunol*. 1990;2:515-520.

398. Boyden S. Autoimmunity and inflammation. *Nature*. 1964;201:200-201.

399. Schlesinger M. Spontaneous occurrence of autoantibodies cytotoxic to thymus cells in the sera of mice of the 129 strain. *Nature*. 1965;207: 429-430.

400. Steele EJ, Cunningham AJ. High proportion of Ig-producing cells making autoantibody in normal mice. *Nature*. 1978;274:483-484.

401. Ochsenbein AF, Fehr T, Lutz C, et al. Control of early viral and bacterial distribution and disease by natural antibodies. *Science*. 1999;286: 2156-2159.

402. Macpherson AJ, Gatto D, Sainsbury E, et al. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science*. 2000;288:2222-2226.

403. Baumgarth N, Herman OC, Jager GC, et al. Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. *Proc Natl Acad Sci U S A*. 1999;96:2250-2255.

404. Baumgarth N, Herman OC, Jager GC, et al. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *J Exp Med*. 2000;192: 271-280.

405. Hardy RR, Hayakawa K. A developmental switch in B lymphopoiesis. *Proc Natl Acad Sci U S A*. 1991;88:11550-11554.

406. Hardy RR, Wasserman R, Li YS, et al. Response by B cell precursors to pre-B receptor assembly: differences between fetal liver and bone marrow. *Curr Top Microbiol Immunol*. 2000;252:25-30.

407. Esplin BL, Welner RS, Zhang Q, et al. A differentiation pathway for B1 cells in adult bone marrow. *Proc Natl Acad Sci U S A* . 2009;106: 5773-5778.

408. Forster I, Rajewsky K. Expansion and functional activity of Ly-1+ B cells upon transfer of peritoneal cells into allotype-congenic, newborn mice. *Eur J Immunol* . 1987;17:521-528.

409. Pennell CA, Sheehan KM, Brodeur PH, et al. Organization and expression of VH gene families preferentially expressed by Ly-1+ (CD5) B cells. *Eur J Immunol* . 1989;19:2115-2121.

410. Martin F, Kearney JF. B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a “natural immune memory.” *Immunol Rev*. 2000;175:70-79.

411. Gui M, Wiest DL, Li J, et al. Peripheral CD4+ T cell maturation recognized by increased expression of Thy-1/CD90 bearing the 6C10 carbohydrate epitope. *J Immunol*. 1999;163:4796-4804.

412. Hayakawa K, Asano M, Shinton SA, et al. Positive selection of natural autoreactive B cells. *Science*. 1999;285:113-116.

413. Sidman CL, Shultz LD, Hardy RR, et al. Production of immunoglobulin isotypes by Ly-1+ B cells in viable motheaten and normal mice. *Science*. 1986;232:1423-1425.

414. O'Keefe TL, Williams GT, Davies SL, et al. Hyperresponsive B cells in CD22-deficient mice. *Science*. 1996;274:798-801.

415. Sato S, Miller AS, Inaoki M, et al. CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. *Immunity*. 1996;5:551-562.

416. Torres RM, Flaswinkel H, Reth M, et al. Aberrant B cell development and immune response in mice with a compromised BCR complex. *Science*. 1996;272:1804-1808.

417. Hayakawa K, Hardy RR, Parks DR, et al. The “Ly-1 B” cell subpopulation in normal immunodefective, and autoimmune mice. *J Exp Med*. 1983;157:202-218.

418. Khan WN, Alt FW, Gerstein RM, et al. Defective B cell development and function in Btk-deficient mice. *Immunity*. 1995;3:283-299.

419. Engel P, Zhou LJ, Ord DC, et al. Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. *Immunity*. 1995;3:39-50.

420. Rickert RC, Rajewsky K, Roes J. Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. *Nature*. 1995;376:352-355.

421. Ahearn JM, Fischer MB, Croix D, et al. Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen. *Immunity*. 1996;4:251-262.

422. Tarakhovsky A, Turner M, Schaal S, et al. Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. *Nature*. 1995;374:467-470.

423. Casola S, Otipoby KL, Alimzhanov M, et al. B cell receptor signal strength determines B cell fate. *Nat Immunol* . 2004;5:317-327.

424. Cole LE, Yang Y, Elkins KL, et al. Antigen-specific B-1a antibodies induced by Francisella tularensis LPS provide long-term protection against F. tularensis LVS challenge. *Proc Natl Acad Sci U S A* . 2009;106:4343-4348.

425. O'Garra A, Chang R, Go N, et al. Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10. *Eur J Immunol* . 1992;22:711-717.

426. Yanaba K, Bouaziz JD, Haas KM, et al. A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. *Immunity*. 2008;28:639-650.

427. Watanabe R, Ishiura N, Nakashima H, et al. Regulatory B cells (B10 cells) have a

suppressive role in murine lupus: CD19 and B10 cell deficiency exacerbates systemic autoimmunity. *J Immunol*. 2010;184:4801-4809.

428. Chen X, Martin F, Forbush KA, et al. Evidence for selection of a population of multi-reactive B cells into the splenic marginal zone. *Int Immunol*. 1997;9:27-41.

429. Shaw PX, Horkko S, Chang MK, et al. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *J Clin Invest*. 2000;105:1731-1740.

430. Silverman GJ, Shaw PX, Luo L, et al. Neo-self antigens and the expansion of B-1 cells: lessons from atherosclerosis-prone mice. *Curr Top Microbiol Immunol* . 2000;252:189-200.

431. Oliver AM, Martin F, Kearney JF. IgMhighCD21high lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells. *J Immunol*. 1999;162:7198-7207.

432. Martin F, Oliver AM, Kearney JF. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity*. 2001;14:617-629.

433. Balazs M, Martin F, Zhou T, et al. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. *Immunity*. 2002;17:341-352.

434. Saito T, Chiba S, Ichikawa M, et al. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. *Immunity*. 2003;18:675-685.

435. Kuroda K, Han H, Tani S, et al. Regulation of marginal zone B cell development by MINT, a suppressor of Notch/RBP-J signaling pathway. *Immunity*. 2003;18:301-312.

436. Martin F, Kearney JF. B1 cells: similarities and differences with other B cell subsets. *Curr Opin Immunol* . 2001;13:195-201.

437. Amano M, Baumgarth N, Dick MD, et al. CD1 expression defines subsets of follicular and marginal zone B cells in the spleen: beta 2-microglobulin-dependent and independent forms. *J Immunol*. 1998;161: 1710-1717.

438. Martin F, Kearney JF. Positive selection from newly formed to marginal zone B cells depends on the rate of clonal production, CD19, and btk. *Immunity*. 2000;12:39-49.

439. Cariappa A, Tang M, Parng C, et al. The follicular versus marginal zone B lymphocyte cell fate decision is regulated by Aiolos, Btk, and CD21. *Immunity*. 2001;14:603-615.

440. Kraus M, Pao LI, Reichlin A, et al. Interference with immunoglobulin (Ig)alpha immunoreceptor tyrosine-based activation motif (ITAM) phosphorylation modulates or blocks B cell development, depending on the availability of an Igbeta cytoplasmic tail. *J Exp Med*. 2001;194:455-469.

441. Guinamard R, Okigaki M, Schlessinger J, et al. Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. *Nat Immunol*. 2000;1:31-36.

442. Martin F, Chen X, Kearney JF. Development of VH81X transgenebearing B cells in fetus and adult: sites for expansion and deletion in conventional and CD5/B1 cells. *Int Immunol*. 1997;9:493-505.

443. Carey JB, Moffatt-Blue CS, Watson LC, et al. Repertoire-based selection into the marginal zone compartment during B cell development. *J Exp Med*. 2008;205:2043-2052.

444. Muppidi JR, Arnon TI, Bronevetsky Y, et al. Cannabinoid receptor 2 positions and retains marginal zone B cells within the splenic marginal zone. *J Exp Med*. 2011;208:1941-1948.

445. Song R, Kim YW, Koo BK, et al. Mind bomb 1 in the lymphopoietic niches is essential for T and marginal zone B cell development. *J Exp Med*. 2008;205:2525-2536.

446. Tan JB, Xu K, Cretegny K, et al. Lunatic and manic fringe cooperatively enhance marginal zone B cell precursor competition for delta-like 1 in splenic endothelial niches. *Immunity*. 2009;30:254-263.

447. Gibb DR, El Shikh M, Kang DJ, et al. ADAM10 is essential for Notch2-dependent marginal zone B cell development and CD23 cleavage in vivo. *J Exp Med*. 2010;207:623-635.

448. Goodnow CC, Crosbie J, Jorgensen H, et al. Induction of self-tolerance in mature peripheral B lymphocytes. *Nature*. 1989;342:385-391.

449. Goodnow CC, Adelstein S, Basten A. The need for central and peripheral tolerance in the B cell repertoire. *Science*. 1990;248:1373-1379.

450. Russell DM, Dembic Z, Morahan G, et al. Peripheral deletion of selfreactive B cells. *Nature*. 1991;354:308-311.

451. Cyster JG, Goodnow CC. PTP1C negatively regulates antigen receptor signaling in B lymphocytes and determines thresholds for negative selection. *Immunity*. 1995;2:13-24.

452. Cyster JG, Healy JI, Kishihara K, et al. Regulation of B-lymphocyte negative and positive selection by tyrosine phosphatase CD45. *Nature*. 1996;381:325-328.

453. Radic MZ, Erikson J, Litwin S, et al. B lymphocytes may escape tolerance by revising their antigen receptors. *J Exp Med*. 1993;177: 1165-1173.

454. Chen C, Radic MZ, Erikson J, et al. Deletion and editing of B cells that express antibodies to DNA. *J Immunol*. 1994;152:1970-1982.

455. Prak EL, Trounstine M, Huszar D, et al. Light chain editing in kappadeficient animals: a potential mechanism of B cell tolerance. *J Exp Med*. 1994;180:1805-1815.

456. Cadera EJ, Wan F, Amin RH, et al. NF-kappaB activity marks cells engaged in receptor editing. *J Exp Med*. 2009;206:1803-1816.

457. Murakami M, Tsubata T, Okamoto M, et al. Antigen-induced apoptotic death of Ly-1 B cells responsible for autoimmune disease in transgenic mice. *Nature*. 1992;357:77-80.

458. Okamoto M, Murakami M, Shimizu A, et al. A transgenic model of autoimmune hemolytic anemia. *J Exp Med*. 1992;175:71-79.

459. Murakami M, Tsubata T, Shinkura R, et al. Oral administration of lipopolysaccharides activates B-1 cells in the peritoneal cavity and lamina propria of the gut and induces autoimmune symptoms in an autoantibody transgenic mouse. *J Exp Med*. 1994;180:111-121.

460. Cariappa A, Takematsu H, Liu H, et al. B cell antigen receptor signal strength and peripheral B cell development are regulated by a 9-O-acetyl sialic acid esterase. *J Exp Med*. 2009;206:125-138.

461. Becker-Herman S, Meyer-Bahlburg A, Schwartz MA, et al. WASpdeficient B cells play a critical, cell-intrinsic role in triggering autoimmunity. *J Exp Med*. 2011;208:2033-2042.

462. Fehr T, Rickert RC, Odermatt B, et al. Antiviral protection and germinal center formation, but impaired B cell memory in the absence of CD19. *J Exp Med*. 1998;188:145-155.

463. Nosten-Bertrand M, Errington ML, Murphy KP, et al. Normal spatial learning despite regional inhibition of LTP in mice lacking Thy-1. *Nature*. 1996;379:826-829.

464. Hayakawa K, Asano M, Shinton SA, et al. Positive selection of antithy-1 autoreactive B-1 cells and natural serum autoantibody production independent from bone marrow B cell development. *J Exp Med*. 2003;197:87-99.

465. Wen L, Brill-Dashoff J, Shinton SA, et al. Evidence of marginal-zone B cell-positive selection in spleen. *Immunity*. 2005;23:297-308.

466. Lay WH, Nussenzweig V. Receptors for complement of leukocytes. *J Exp Med*. 1968;128:991-1009.

467. Pepys MB. Role of complement in induction of the allergic response. *Nat New Biol* . 1972;237:157-159.

468. Pepys MB. Role of complement in the induction of immunological responses. *Transplant Rev*. 1976;32:93-120.

469. Carroll MC. The role of complement in B cell activation and tolerance. *Adv Immunol*. 2000;74:61-88.

470. Inaoki M, Sato S, Weintraub BC, et al. CD19-regulated signaling thresholds control peripheral tolerance and autoantibody production in B lymphocytes. *J Exp Med*. 1997;186:1923-1931.

471. Prodeus AP, Goerg S, Shen LM, et al. A critical role for complement in maintenance of self-tolerance. *Immunity*. 1998;9:721-731.

472. Rapaka RR, Ricks DM, Alcorn JF, et al. Conserved natural IgM antibodies mediate innate and adaptive immunity against the opportunistic fungus Pneumocystis murina. *J Exp Med*. 2010;207:2907-2919.

473. Boes M, Prodeus AP, Schmidt T, et al. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. *J Exp Med*. 1998;188:2381-2386.

474. Baumgarth N. A two-phase model of B-cell activation. *Immunol Rev*. 2000;176:171-180.

475. Tarakhovsky A, Kanner SB, Hombach J, et al. A role for CD5 in TCR-mediated signal transduction and thymocyte selection. *Science*. 1995;269:535-537.

476. Bikah G, Carey J, Ciallella JR, et al. CD5-mediated negative regulation of antigen receptor-induced growth signals in B-1 B cells. *Science*. 1996;274:1906-1909.

477. Bondada S, Bikah G, Robertson DA, et al. Role of CD5 in growth regulation of B-1 cells. *Curr Top Microbiol Immunol* . 2000;252: 141-149.

478. Hippen KL, Tze LE, Behrens TW. CD5 maintains tolerance in anergic B cells. *J Exp Med*. 2000;191:883-890.

479. Solvason N, Kearney JF. The human fetal omentum: a site of B cell generation. *J*

Exp Med. 1992;175:397-404.

480. Pascual V, Verkruyse L, Casey ML, et al. Analysis of Ig H chain gene segment utilization in human fetal liver. Revisiting the “proximal utilization hypothesis.” *J Immunol*. 1993;151:4164-4172.

481. Mold JE, Venkatasubrahmanyam S, Burt TD, et al. Fetal and adult hematopoietic stem cells give rise to distinct T cell lineages in humans. *Science*. 2010;330:1695-1699.

482. Ikuta K, Kina T, MacNeil I, et al. A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. *Cell*. 1990;62:863-874.

483. Iwata Y, Matsushita T, Horikawa M, et al. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. *Blood*. 2011;117:530-541.

484. LeBien TW, Wormann B, Villablanca JG, et al. Multiparameter flow cytometric analysis of human fetal bone marrow B cells. *Leukemia*. 1990;4:354-358.

485. Nunez C, Nishimoto N, Gartland GL, et al. B cells are generated throughout life in humans. *J Immunol*. 1996;156:866-872.

486. Ghia P, ten Boekel E, Sanz E, et al. Ordering of human bone marrow B lymphocyte precursors by single-cell polymerase chain reaction analyses of the rearrangement status of the immunoglobulin H and L chain gene loci. *J Exp Med*. 1996;184:2217-2229.

487. Simmons DL, Satterthwaite AB, Tenen DG, et al. Molecular cloning of a cDNA encoding CD34, a sialomucin of human hematopoietic stem cells. *J Immunol*. 1992;148:267-271.

488. Ichii M, Oritani K, Yokota T, et al. The density of CD10 corresponds to commitment and progression in the human B lymphoid lineage. *PLoS One*. 2010;5:e12954.

489. Dittel BN, LeBien TW. The growth response to IL-7 during normal human B cell ontogeny is restricted to B-lineage cells expressing CD34. *J Immunol*. 1995;154:58-67.

490. Rawlings DJ, Quan SG, Kato RM, et al. Long-term culture system for selective growth of human B-cell progenitors. *Proc Natl Acad Sci U S A* . 1995;92:1570-1574.

491. LeBien TW. Fates of human B-cell precursors. *Blood*. 2000;96:9-23.

492. Gougeon ML, Drean G, Le Deist F, et al. Human severe combined immunodeficiency disease: phenotypic and functional characteristics of peripheral B lymphocytes. *J Immunol*. 1990;145:2873-2879.

493. Bauer SR, Kubagawa H, Maclennan I, et al. VpreB gene expression in hematopoietic malignancies: a lineage- and stage-restricted marker for B-cell precursor leukemias. *Blood*. 1991;78:1581-1588.

494. Bossy D, Milili M, Zucman J, et al. Organization and expression of the lambda-like genes that contribute to the mu-psi light chain complex in human pre-B cells. *Int Immunol* . 1991;3:1081-1090.

495. Bauer SR, Kudo A, Melchers F. Structure and pre-B lymphocyte restricted expression of the VpreB in humans and conservation of its structure in other mammalian species. *EMBO J*. 1998;7:111-116.

496. Gisler R, Jacobsen SE, Sigvardsson M. Cloning of human early B-cell factor and identification of target genes suggest a conserved role in B-cell development in man and mouse. *Blood*. 2000;96:1457-1464.

497. Minegishi Y, Coustan-Smith E, Wang YH, et al. Mutations in the human lambda5/14.1 gene result in B cell deficiency and agammaglobulinemia. *J Exp Med*. 1998;187:71-77.

498. Minegishi Y, Coustan-Smith E, Rapalus L, et al. Mutations in Igalpha (CD79a) result in a complete block in B-cell development. *J Clin Invest*. 1999;104:1115-1121.

499. Yel L, Minegishi Y, Coustan-Smith E, et al. Mutations in the mu heavy-chain gene in patients with agammaglobulinemia. *N Engl J Med* . 1996;335:1486-1493.

500. Pascual V, Liu YJ, Magalski A, et al. Analysis of somatic mutation in five B cell subsets of human tonsil. *J Exp Med*. 1994;180:329-339.

501. Pasqualucci L, Migliazza A, Fracchiolla N, et al. BCL-6 mutations in normal germinal center B cells: evidence of somatic hypermutation acting outside Ig loci. *Proc Natl Acad Sci U S A*. 1998;95:11816-11821.

502. Staudt LM, Dent AL, Shaffer AL, et al. Regulation of lymphocyte cell fate decisions and lymphomagenesis by BCL-6. *Int Rev Immunol* . 1999;18: 381-403.

503. Dalla-Favera R, Ye BH, Lo Coco F, et al. BCL-6 and the molecular pathogenesis of B-cell lymphoma. *Cold Spring Harb Symp Quant Biol* . 1994;59:117-123.

504. Shen HM, Peters A, Baron B, et al. Mutation of BCL-6 gene in normal B cells by the process of somatic hypermutation of Ig genes. *Science*. 1998;280:1750-1752.

505. Saito M, Novak U, Piovan E, et al. BCL6 suppression of BCL2 via Miz1 and its

disruption in diffuse large B cell lymphoma. *Proc Natl Acad Sci U S A* . 2009;106:11294-11299.

506. Conley ME, Parolini O, Rohrer J, et al. X-linked agammaglobulinemia: new approaches to old questions based on the identification of the defective gene. *Immunol Rev*. 1994;138:5-21.

507. Tsukada S, Saffran DC, Rawlings DJ, et al. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell*. 1993;72:279-290.

508. Rawlings DJ, Saffran DC, Tsukada S, et al. Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. *Science*. 1993;261:358-361.

509. Campana D, Farrant J, Inamdar N, et al. Phenotypic features and proliferative activity of B cell progenitors in X-linked agammaglobulinemia. *J Immunol* . 1990;145:1675-1680.

510. Minegishi Y, Rohrer J, Coustan-Smith E, et al. An essential role for BLNK in human B cell development. *Science*. 1999;286:1954-1957.

511. Macchi P, Villa A, Giliani S, et al. Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID). *Nature*. 1995;377:65-68.

512. DiSanto JP, Muller W, Guy-Grand D, et al. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor gamma chain. *Proc Natl Acad Sci U S A* . 1995;92:377-381.

513. Baird AM, Lucas JA, Berg LJ. A profound deficiency in thymic progenitor cells in mice lacking Jak3. *J Immunol* . 2000;165:3680-3688.

514. Isnardi I, Ng YS, Srdanovic I, et al. IRAK-4- and MyD88-dependent pathways are essential for the removal of developing autoreactive B cells in humans. *Immunity*. 2008;29:746-757.

515. Margolin JF, Poplack DG. Acute lymphoblastic leukemia. In Pizzo PA, ed. *Principles and Practice of Pediatric Oncology*. Philadelphia, PA: Lippincott, Williams, and Wilkins; 1997:409-462.

516. Cherepakhin V, Baird SM, Meisenholder GW, et al. Common clonal origin of chronic lymphocytic leukemia and high-grade lymphoma of Richter's syndrome. *Blood*. 1993;82:3141-3147.

517. Felix CA, Poplack DG. Characterization of acute lymphoblastic leukemia of childhood by immunoglobulin and T-cell receptor gene patterns. *Leukemia*. 1991;5:1015-

1025.

518. Lemmers B, Arnoulet C, Fossat C, et al. Fine characterization of childhood and adult acute lymphoblastic leukemia (ALL) by a proB and preB surrogate light chain-specific mAb and a proposal for a new B cell ALL classification. *Leukemia*. 2000;14:2103-2111.

519. Trageser D, Iacobucci I, Nahar R, et al. Pre-B cell receptor-mediated cell cycle arrest in Philadelphia chromosome-positive acute lymphoblastic leukemia requires IKAROS function. *J Exp Med*. 2009;206:1739-1753.

520. Uckun FM, Evans WE, Forsyth CJ, et al. Biotherapy of B-cell precursor leukemia by targeting genistein to CD19-associated tyrosine kinases. *Science*. 1995;267:886-891.

521. Meydan N, Grunberger T, Dadi H, et al. Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor. *Nature*. 1996;379:645-648.

522. Bertrand FE, Vogtenhuber C, Shah N, et al. Pro-B-cell to pre-B-cell development in B-lineage acute lymphoblastic leukemia expressing the MLL/AF4 fusion protein. *Blood*. 2001;98:3398-3405.

523. Brumpt C, Delabesse E, Beldjord K, et al. The incidence of clonal T-cell receptor rearrangements in B-cell precursor acute lymphoblastic leukemia varies with age and genotype. *Blood*. 2000;96:2254-2261.

524. Pui CH. Acute lymphoblastic leukemia in children. *Curr Opin Oncol* . 2000;12:3-12.

525. Domer PH, Fakharzadeh SS, Chen CS, et al. Acute mixed-lineage leukemia t(4;11)(q21;q23) generates an MLL-AF4 fusion product. *Proc Natl Acad Sci U S A* . 1993;90:7884-7888.

526. Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature*. 2000;403:503-511.

527. Duan S, Cermak L, Pagan JK, et al. FBXO11 targets BCL6 for degradation and is inactivated in diffuse large B-cell lymphomas. *Nature*. 2012;481:90-93.

528. Lenz G, Nagel I, Siebert R, et al. Aberrant immunoglobulin class switch recombination and switch translocations in activated B cell-like diffuse large B cell lymphoma. *J Exp Med*. 2007;204:633-643.

529. Dorsett Y, Robbiani DF, Jankovic M, et al. A role for AID in chromosome translocations between c-myc and the IgH variable region. *J Exp Med*. 2007;204:2225-2232.

530. Liso A, Capello D, Marafioti T, et al. Aberrant somatic hypermutation in tumor cells of

nodular-lymphocyte-predominant and classic Hodgkin lymphoma. *Blood*. 2006;108:1013-1020.

531. Lenz G, Davis RE, Ngo VN, et al. Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. *Science*. 2008;319:1676-1679.

532. Kato M, Sanada M, Kato I, et al. Frequent inactivation of A20 in B-cell lymphomas. *Nature*. 2009;459:712-716.

533. Maloney DG, Smith B, Rose A. Rituximab: mechanism of action and resistance. *Semin Oncol*. 2002;29:2-9.

534. Bhan AK, Nadler LM, Stashenko P, et al. Stages of B cell differentiation in human lymphoid tissue. *J Exp Med*. 1981;154:737-749.

535. Nadler LM, Ritz J, Hardy R, et al. A unique cell surface antigen identifying lymphoid malignancies of B cell origin. *J Clin Invest*. 1981;67: 134-140.

536. Tedder TF, Streuli M, Schlossman SF, et al. Isolation and structure of a cDNA encoding the B1 (CD20) cell-surface antigen of human B lymphocytes. *Proc Natl Acad Sci U S A*. 1988;85:208-212.

537. Golay JT, Clark EA, Beverley PC. The CD20 (Bp35) antigen is involved in activation of B cells from the G0 to the G1 phase of the cell cycle. *J Immunol*. 1985;135:3795-3801.

538. Clark EA, Shu G, Ledbetter JA. Role of the Bp35 cell surface polypeptide in human B-cell activation. *Proc Natl Acad Sci U S A* . 1985;82:1766-1770.

539. Ledbetter JA, Clark EA. Surface phenotype and function of tonsillar germinal center and mantle zone B cell subsets. *Hum Immunol*. 1986;15:30-43.

540. Reff ME, Carner K, Chambers KS, et al. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood*. 1994;83:435-445.

541. Reed JC, Kitada S, Kim Y, et al. Modulating apoptosis pathways in lowgrade B-cell malignancies using biological response modifiers. *Semin Oncol*. 2002;29:10-24.

542. Nabhan C, Rosen ST. Conceptual aspects of combining rituximab and Campath-1H in the treatment of chronic lymphocytic leukemia. *Semin Oncol*. 2002;29:75-80.

543. Lu D, Zhao Y, Tawatao R, et al. Activation of the Wnt signaling pathway in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A* . 2004;101:3118-3123.

544. Glick G, Chang TS, Jaap RG. The bursa of Fabricius and antibody production. *Poult Sci.* 1956;35:224-234.

545. Cooper MD, Raymond DA, Peterson RD, et al. The functions of the thymus system and the bursa system in the chicken. *J Exp Med*. 1966;123:75-102.

546. Sorvari T, Sorvari R, Ruotsalainen P, et al. Uptake of environmental antigens by the bursa of Fabricius. *Nature*. 1975;253:217-219.

547. McCormack WT, Tjoelker LW, Thompson CB. Avian B-cell development: generation of an immunoglobulin repertoire by gene conversion. *Annu Rev Immunol*. 1991;9:219-241.

548. Dieterlen-Lievre F, Martin C. Diffuse intraembryonic hemopoiesis in normal and chimeric avian development. *Dev Biol*. 1981;88:180-191.

549. Reynaud CA, Imhof BA, Anquez V, et al. Emergence of committed B lymphoid progenitors in the developing chicken embryo. *EMBO J*. 1992;11:4349-4358.

550. Benatar T, Tkalec L, Ratcliffe MJ. Stochastic rearrangement of immunoglobulin variable-region genes in chicken B-cell development. *Proc Natl Acad Sci U S A*. 1992;89:7615-7619.

551. Reynaud CA, Anquez V, Dahan A, et al. A single rearrangement event generates most of the chicken immunoglobulin light chain diversity. *Cell*. 1985;40:283-291.

552. Reynaud CA, Dahan A, Anquez V, et al. Somatic hyperconversion diversifies the single Vh gene of the chicken with a high incidence in the D region. *Cell*. 1989;59:171-183.

553. Lassila O. Emigration of B cells from chicken bursa of Fabricius. *Eur J Immunol*. 1989;19:955-958.

554. Paramithiotis E, Ratcliffe MJ. Bursa-dependent subpopulations of peripheral B lymphocytes in chicken blood. *Eur J Immunol*. 1993;23:96-102.

555. Becker RS, Knight KL. Somatic diversification of immunoglobulin heavy chain VDJ genes: evidence for somatic gene conversion in rabbits. *Cell*. 1990;63:987-997.

556. Raman C, Spieker-Polet H, Yam PC, et al. Preferential VH gene usage in rabbit Ig-secreting heterohybridomas. *J Immunol*. 1994;152: 3935-3945.

557. Tunyaplin C, Knight KL. Fetal VDJ gene repertoire in rabbit: evidence for preferential rearrangement of VH1. *Eur J Immunol*. 1995;25:2583-2587.

558. McElroy PJ, Willcox N, Catty D. Early precursors of B lymphocytes. I. Rabbit/mouse

species differences in the physical properties and surface phenotype of pre-B cells, and in the maturation sequence of early B cells. *Eur J Immunol* . 1981;11:76-85.

559. Crane MA, Kingzette M, Knight KL. Evidence for limited B-lymphopoiesis in adult rabbits. *J Exp Med*. 1996;183:2119-2121.

560. Weinstein PD, Anderson AO, Mage RG. Rabbit IgH sequences in appendix germinal centers: VH diversification by gene conversion-like and hypermutation mechanisms. *Immunity*. 1994;1:647-659.

561. Short JA, Sethupathi P, Zhai SK, et al. VDJ genes in VHa2 allotype-suppressed rabbits. Limited germline VH gene usage and accumulation of somatic mutations in D regions. *J Immunol*. 1991;147:4014-4018.

562. Vajdy M, Sethupathi P, Knight KL. Dependence of antibody somatic diversification on gut-associated lymphoid tissue in rabbits. *J Immunol*. 1998;160:2725-2729.

563. Tlaskalova-Hogenova H, Stepankova R. Development of antibody formation in germ-free and conventionally reared rabbits: the role of intestinal lymphoid tissue in antibody formation to E. coli antigens. *Folia Biol*. 1980;26:81-93.

564. Stepankova R, Kovaru F, Kruml J. Lymphatic tissue of the intestinal tract of germfree and conventional rabbits. *Folia Microbiol*. 1980;25:491-495.

565. Lanning D, Sethupathi P, Rhee KJ, et al. Intestinal microflora and diversification of the rabbit antibody repertoire. *J Immunol*. 2000;165: 2012-2019.

566. Hayward AR, Simons MA, Lawton AR, et al. Pre-B and B cells in rabbits. Ontogeny and allelic exclusion of kappa light chain genes. *J Exp Med*. 1978;148:1367-1377.

567. Gathings WE, Mage RG, Cooper MD, et al. Immunofluorescence studies on the expression of VH a allotypes by pre-B and B cells of homozygous and heterozygous rabbits. *Eur J Immunol* . 1981;11:200-206.

568. Solvason N, Chen X, Shu F, et al. The fetal omentum in mice and humans. A site enriched for precursors of CD5 B cells early in development. *Ann N Y Acad Sci* . 1992;651:10-20.

569. Becker RS, Suter M, Knight KL. Restricted utilization of VH and DH genes in leukemic rabbit B cells. *Eur J Immunol* . 1990;20:397-402.

570. Friedman ML, Tunyaplin C, Zhai SK, et al. Neonatal VH, D, and JH gene usage in rabbit B lineage cells. *J Immunol*. 1994;152:632-641.

571. Zhu X, Boonthum A, Zhai SK, et al. B lymphocyte selection and agerelated changes

in VH gene usage in mutant Alicia rabbits. *J Immunol*. 1999;163:3313-3320.

572. Sehgal D, Johnson G, Wu TT, et al. Generation of the primary antibody repertoire in rabbits: expression of a diverse set of Igk-V genes may compensate for limited combinatorial diversity at the heavy chain locus. *Immunogenetics*. 1999;50:31-42.

573. Cooper MD, Perey DY, Gabrielsen AE, et al. Production of an antibody deficiency syndrome in rabbits by neonatal removal of organized intestinal lymphoid tissues. *Int Arch Allergy Appl Immunol*. 1968;33: 65-88.

574. Mage RG, Lanning D, Knight KL. B cell and antibody repertoire development in rabbits: the requirement of gut-associated lymphoid tissues. *Dev Comp Immunol*. 2006;30:137-153.

575. Perey DY, Good RA. Experimental arrest and induction of lymphoid development in intestinal lymphoepithelial tissues of rabbits. *Lab Invest*. 1968;18:15-26.

576. Stramignoni A, Mollo F, Rua S, et al. Development of the lymphoid tissue in the rabbit appendix isolated from the intestinal tract. *J Pathol*. 1969;99:265-269.

577. Berberian L, Goodglick L, Kipps TJ, et al. Immunoglobulin VH3 gene products: natural ligands for HIV gp120. *Science*. 1993;261:1588-1591.

578. Pospisil R, Mage RG. B-cell superantigens may play a role in B-cell development and selection in the young rabbit appendix. *Cell Immunol*. 1998;185:93-100.

579. Silverman GJ, Cary SP, Dwyer DC, et al. A B cell superantigen-induced persistent “Hole” in the B-1 repertoire. *J Exp Med*. 2000;192:87-98.

580. Chan VW, Mecklenbrauker I, Su I, et al. The molecular mechanism of B cell activation by toll-like receptor protein RP-105. *J Exp Med*. 1998;188:93-101.

581. Ehlich A, Schaal S, Gu H, et al. Immunoglobulin heavy and light chain genes rearrange independently at early stages of B cell development. *Cell*. 1993;72:695-704.

582. Reynaud CA, Anquez V, Grimal H, et al. A hyperconversion mechanism generates the chicken light chain preimmune repertoire. *Cell*. 1987;48:379-388.

583. McCormack WT, Tjoelker LW, Barth CF, et al. Selection for B cells with productive IgL gene rearrangements occurs in the bursa of Fabricius during chicken embryonic development. *Genes Dev*. 1989;3:838-847.

584. Raman C, Knight KL. CD5+ B cells predominate in peripheral tissues of rabbit. *J Immunol*. 1992;149:3858-3864.

585. Herzenberg LA. Toward a layered immune system. *Cell*. 1989;59:953-954.

586. Glynne R, Akkaraju S, Healy JI, et al. How self-tolerance and the immunosuppressive drug FK506 prevent B-cell mitogenesis. *Nature*. 2000;403:672-676.

587. Hoffmann R, Seidl T, Neeb M, et al. Changes in gene expression profiles in developing B cells of murine bone marrow. *Genome Res*. 2002;12:98-111.

588. Nelms KA, Goodnow CC. Genome-wide ENU mutagenesis to reveal immune regulators. *Immunity*. 2001;15:409-418.

589. Loy AL, Goodnow CC. Novel approaches for identifying genes regulating lymphocyte development and function. *Curr Opin Immunol* . 2002;14:260-265.

590. Lee G, Namen AE, Gillis S, et al. Recombinant interleukin-7 supports the growth of normal B lymphocyte precursors. *Curr Top Microbiol Immunol* . 1988;141:16-18.

591. Hirayama F, Ogawa M. Cytokine regulation of early lymphohematopoietic development. *Stem Cells*. 1996;14:369-375.

592. Baird AM, Gerstein RM, Berg LJ. The role of cytokine receptor signaling in lymphocyte development. *Curr Opin Immunol* . 1999;11:157-166.

593. Funk PE, Kincade PW, Witte PL. Native associations of early hematopoietic stem cells and stromal cells isolated in bone marrow cell aggregates. *Blood*. 1994;83:361-369.

594. Medina KL, Kincade PW. Pregnancy-related steroids are potential negative regulators of B lymphopoiesis. *Proc Natl Acad Sci U S A* . 1994;91: 5382-5386.

595. Smithson G, Couse JF, Lubahn DB, et al. The role of estrogen receptors and androgen receptors in sex steroid regulation of B lymphopoiesis. *J Immunol* . 1998;161:27-34.

596. Foster MP, Montecino-Rodriguez E, Dorshkind K. Proliferation of bone marrow pro-B cells is dependent on stimulation by the pituitary/thyroid axis. *J Immunol* . 1999;163:5883-5890.

597. Roark JH, Kuntz CL, Nguyen KA, et al. Breakdown of B cell tolerance in a mouse model of systemic lupus erythematosus. *J Exp Med*. 1995;181:1157-1167.

598. Chen C, Prak EL, Weigert M. Editing disease-associated autoantibodies. *Immunity*. 1997;6:97-105.

599. Xu H, Li H, Suri-Payer E, et al. Regulation of anti-DNA B cells in recombination-

activating gene-deficient mice. *J Exp Med*. 1998;188: 1247-1254.

600. Li H, Jiang Y, Prak EL, et al. Editors and editing of anti-DNA receptors. *Immunity*. 2001;15:947-957.

601. Chumley MJ, Dal Porto JM, Kawaguchi S, et al. A VH11V kappa 9 B cell antigen receptor drives generation of CD5+ B cells both in vivo and in vitro. *J Immunol*. 2000;164:4586-4593.

602. Arnold LW, Pennell CA, McCray SK, et al. Development of B-1 cells: segregation of phosphatidyl choline-specific B cells to the B-1 population occurs after immunoglobulin gene expression. *J Exp Med*. 1994;179: 1585-1595.

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Chapter 9

B-Lymphocyte Receptors, Signaling Mechanisms, and Activation

Akanksha Chaturvedi

Angel Davey

Wanli Liu

Hae Won Sohn

Susan K. Pierce

INTRODUCTION

The primary function of mature B lymphocytes in response to foreign antigens is to proliferate and differentiate into antibody-producing plasma cells that secrete large quantities of antibodies. Effective antibody responses are highly specific and of high affinity for the inducing antigen and of the appropriate isotype to allow the antibodies to carry out the effector functions, as described in Chapter 5, that best serve to eliminate an antigen-containing pathogen. For B cells to produce the most effective antibodies, they must have mechanisms that allow them to discern their specificity and affinity for antigens and to produce plasma cells that secrete antibodies of the appropriate isotype. In this chapter, we explore what we know and are learning about these mechanisms. Understanding how B cells recognize and respond to antigens will aid in our efforts to develop effective vaccines and to target therapies to block hyper-B-cell responses as in systemic autoimmune diseases and in some B-cell tumors.

B cells are activated by antigen by a process of clonal selection, a fundamental feature of the adaptive immune response. Each B cell expresses a membrane form of a single heavy (H) chain (V_HC_H) and a single light (L) chain (V_LC_L) that are assembled together with a noncovalently bound immunoglobulin (Ig)α-Igβ heterodimer into an antigen receptor, the B-cell receptor (BCR), which is expressed on the B-cell surface (Fig. 9.1). It is conservatively estimated that during a lifetime, an adult human expresses more than 10^{13} unique clonally distributed BCRs. This estimate is based, in part, on the diversity of H and L chain variable (V) regions that can be generated given the germ line encoded V gene segments and the molecular combinational and mutational mechanisms that create the V genes described in Chapter 6. When an antigen enters the immune system, it selects from among this extraordinarily large array those B cells whose receptors fit it best and signal these to proliferate and differentiate into antibody-secreting plasma cells. Even very simple antigens are predicted to find hundreds of B cells that express BCRs with sufficient specificity and affinity to be activated. This process of antigen selecting best fits from among the enormous array of preexisting B cells to proliferate and differentiate into antibody-secreting cells is a complicated process that takes place in highly specialized microenvironments in the secondary lymphoid organs and involves the functions of both T cells and innate immune system cells, as will be described in Chapter 10. Here, we focus on the events that follow antigen binding to the BCR that initiate signaling, an essential, critical first step in B-cell activation.

Antigen binding to the BCR triggers several signaling cascades that lead to the transcriptional activation of a variety of genes associated with B-cell activation (see Fig. 9.1). The BCR is also an internalizing receptor; under the influence of the signaling cascades, the BCR and bound antigen are actively endocytosed into the cell and trafficked to specialized intracellular compartments in which the antigen is processed and presented on major histocompatibility complex (MHC) class II molecules, as described in Chapter 22. The MHC-peptide complexes then move to the B-cell surface where they activate antigen-specific helper T cells to provide T-cell help, a complex process involving both direct interactions between B cells and T cells as well as the release of soluble cytokines by the T cells.

A considerable amount of what we know about the biochemical nature of the signaling

pathways triggered by antigen binding to the BCR comes from studies in which multivalent antigens are provided to the B cell in solution (see Fig. 9.1). Although B cells can respond to antigens in solution, a variety of recent studies suggest that antigens are presented to B cells on the surface of antigen-presenting cells (APCs) including macrophages and dendritic cells present in the local microenvironments of the lymph nodes and spleen in which B cells are activated in vivo (see Fig. 9.1). Antigens may be presented on macrophages and dendritic cells as part of complement-fixed complexes bound to the complement receptors or immune complexes bound to FcRs expressed by these cells. We do not yet know if the molecular mechanisms by which antigens in solution and antigens on cell surfaces initiate signaling are identical, even though it appears in both cases that the engagement of antigen by the BCR results in the triggering of similar signaling cascades.

In this chapter, we describe what we are learning about the events that occur following the binding of the BCR to antigen that trigger signaling cascades that lead to B-cell activation. The question that will be addressed is how is the BCR's specificity and affinity for an antigen read by the BCR and transduced across the B cell's membrane to trigger intracellular signaling cascades that induce the B cell's response. The signaling cascades that are set off upon antigen binding have been described in considerable biochemical detail and will be reviewed in this chapter. However, biochemical analyses do not capture the spatial and temporal dynamics of the events that are triggered by antigen binding to the BCR. The recent applications of live cell imaging technologies are providing the first views of B cells as they encounter antigen with the resolution to follow individual BCRs. These images are showing us

that the activation of B cells by antigen is far more dynamic than originally thought. It has been possible to infer from the behavior of the B cells and BCRs in these images the mechanisms underlying antigen-driven events that lead to B-cell activation. Such images are changing our view of BCR-mediated B-cell activation and are providing the tools to gain an understanding of the mechanisms underlying diseases that result from inappropriate B-cell activation including systemic autoimmune diseases and certain B-cell tumors.

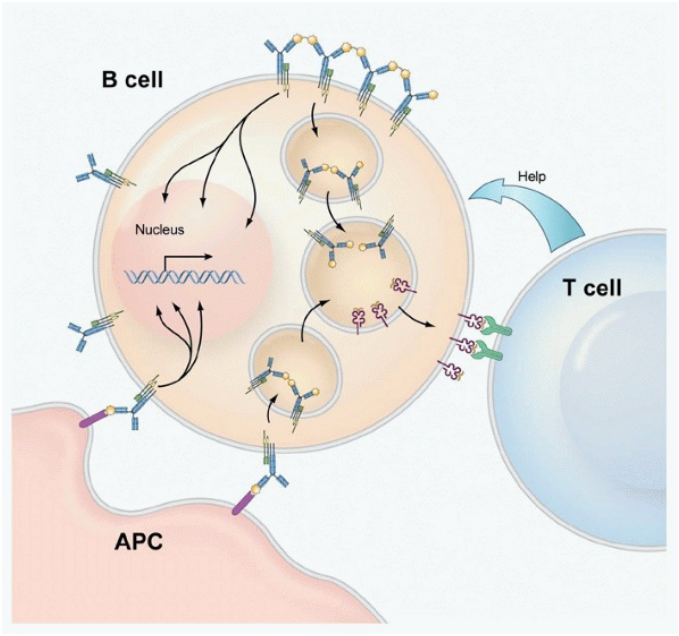

FIG. 9.1. Overview of the Activation of B Cells by Antigen. B-cell activation is initiated by the binding of antigen to the B-cell receptor (BCR). B cells encounter antigen either in solution or on the surface of an antigen-presenting cell (APC), likely as complement coupled antigens or as immune complexes bound to APC complement receptors or FcRs. Antigen binding triggers signaling cascades that lead to the activation of a variety of genes associated with B-cell activation. The BCR is also a trafficking receptor; under the influence of BCR signals, it transports antigens into intracellular compartments where the antigens are processed and presented on major histocompatibility complex class II molecules for recognition by helper T cells.

As we consider the mechanisms by which antigens trigger B cells through BCRs, we need to be aware that B cells encounter antigen in both developmental and environmental contexts, and that these contexts greatly influence the outcome of antigen engagement. Consider, for example, that B cells express BCRs throughout their development from immature B cells to memory B cells. However, engagement of the BCR by antigen at different developmental stages has different outcomes. The binding of self-antigens to the BCRs expressed on immature B cells signals these cells, but the signals do not result in activation. This means that the developmental state of the B cell dictates the outcome of the BCR's engagement of antigen, either driving proliferation and differentiation in mature B cells or triggering mechanisms that lead to elimination or silencing of self-reactive immature B cells, as described in Chapter 8. The basic structure of the BCR does not change from the immature to the mature B-cell stage, but the BCR signals differently in different developmental cellular contexts. The outcome of BCR signaling is also influenced by the environmental context in which the antigen is encountered. Antigens enter the immune system in various contexts as relatively simple vaccines to complex microorganisms including viruses, bacteria, and parasites. Both vaccines and pathogens bring with them materials that can activate B cells through coreceptors other than the BCR as well as activate T cells and cells of the innate immune system to secrete products that bind to B cell coreceptors that influence how the BCR signals in response to the antigen. The molecular form of the antigen itself can influence the outcome of BCR antigen binding, as described in Chapters 10 and 23. Bacteria and some viruses display rigid arrays of antigens on their surface that induce antibody responses in the absence of helper T cells (coined T-independent antigens), as do polysaccharides on bacteria in which the carbohydrate moieties are arrayed as multimers. Thus, in predicting the outcome of BCR-antigen binding, we need to consider not only the antigen's interaction with the BCR but also the developmental state of the B cells, the environment in which the B cell is activated, and the nature of the antigen itself. In other words, BCR signaling always occurs in a context; to predict the outcome of antigen binding to the BCR, we need to be aware of that context.

The BCR also signals during development to provide survival signals to the B cell, often referred to as tonic signaling, and to keep B cells in nonresponsive or tolerant states, termed *anergic signaling*; however, we do not yet know how these signals relate to those that activate mature B cells.

Concerning the impact of the environmental context in which B cells are activated, this chapter will focus on the effect of B-cell coreceptors that directly affect BCR signaling. We will provide a comprehensive list of the coreceptors and what they respond to and describe how two of the best studied coreceptors, the enhancing cluster of differentiation (CD)19/CD21 complex and the inhibitory FcγRIIB receptor, function to influence B-cell responses. We will also comment on the interactions between the innate immune system's toll-like receptors (TLRs) and the BCR that appear to serve to regulate each other's signaling.

We hope that this chapter leaves the reader with a clear view of the early events that follow the engagement of antigen by the BCR and the signaling cascades that are triggered that ultimately activate B cells to proliferate and differentiate into antibody-secreting cells. We also hope that the reader gains an appreciation that B-cell activation occurs in both a developmental and environmental context that dictates the outcome of antigen binding to the BCR. Lastly, we consider the repercussions of uncontrolled BCR signaling that may result in B-cell tumors and in systemic autoimmune diseases.

THE STRUCTURE OF THE B-CELL RECEPTOR

The BCR belongs to the multichain immune recognition receptor (MIRR) family that includes the T-cell receptor for antigen and the high-affinity receptor for IgE. MIRR family members contain ligand-binding chains, which for the BCR is a membrane form of Ig (mIg) (Fig. 9.2). B cells express BCRs composed of Igs of all isotypes that are expressed in a developmentally controlled fashion beginning with IgM-BCRs in immature B cells, IgM- and IgD-BCRs in mature B cells, and then isotype switched BCRs, containing IgGs, IgAs, and IgEs in memory B cells. The mIgs have short cytoplasmic tails of 3 to 28 amino acids that, with the exception of IgG- and IgE-BCRs, do not connect directly with the cell's signaling apparatus.[1,2,3] Rather, the MIRRs ligand binding chains noncovalently associate with membrane proteins that contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tails. For the BCR, the mIg associates with a disulfide linked heterodimer, Igα and Igβ, each chain of which has a single ITAM in its cytoplasmic tail (see Fig. 9.2).[4] The stoichiometry of

the BCR complex is 1mIg:1Igα-Igβ determined by biochemical analysis as well as by live cell imaging (see Fig. 9.2).[5,6]

In considering how the BCR's engagement of antigen triggers signaling, it is helpful to consider what we know about the structure of the BCR. From x-ray crystallographic studies, we know that the antibody Fab does not undergo large conformational changes between the free and antigen-bound states that could transduce the information that the BCR has bound antigen to the BCR's cytoplasmic domains to initiate signaling.[7] However, the BCR is a complicated multichain complex and to date, there is no structure of a complete BCR containing mIg, Igα, and Igβ. Thus, a full understanding of the molecular basis of the antigen-induced initiation of BCR signaling will await the determination of the structure of the BCR. Recently, as an effort toward this goal, the structure of the disulphide-linked homodimer of Igβ was solved, which allowed the modeling of an Igα-Igβ heterodimer with the existing structure of the Cα4 domain.[8] These results predicted an unexpectedly extensive contact surface between the ectodomains of mIg and both Igα and Igβ through multiple charged residues that could potentially be sensitive to antigen-induced changes in the BCR's mIg.

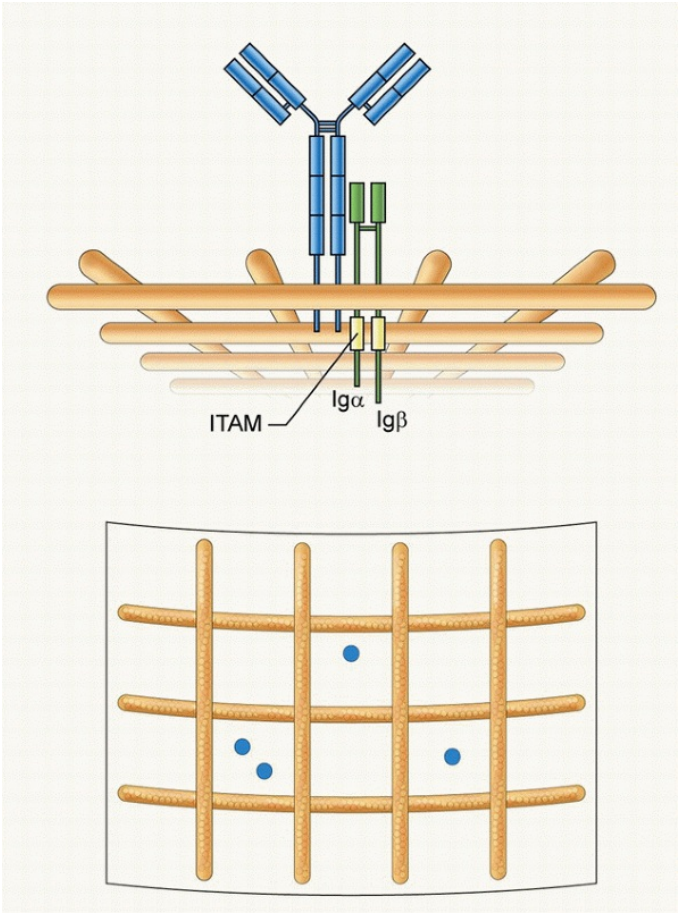

FIG. 9.2. The Structure and Organization of the B-Cell Receptor (BCR). The immunoglobulin (Ig)M BCR is composed of a membrane form of IgM associated with a covalently linked heterodimer of Igα and Igβ that contain in their cytoplasmic domains immunoreceptor tyrosine-based activation motifs. The BCR is depicted compartmentalized on the plasma membrane by actin cytoskeleton "fence"[9] as proposed by Batista et al.[21] Two different views are provided: a side view showing the BCR short cytoplasmic tail and the Igα-Igβ tails in the cytosol and a view from above showing the BCRs compartmentalized by actin "fences."

In considering how the BCR's engagement of antigen triggers signaling, it is also useful to consider the organization of the plasma membrane in which the BCR resides (see Fig. 9.2). Our current understanding of the plasma membrane is that it is not simply a fluid mosaic structure of freely diffusing proteins in a phospholipid bilayer, but rather, the plasma

membrane appears to be partitioned into highly dynamic compartments formed by actin cytoskeleton “fences” and actin-anchored protein “pickets.”[9,10] These compartments serve to organize the plasma membrane to control the diffusion of membrane proteins and concentrate proteins in one compartment or conversely segregate proteins into separate compartments. The fences and pickets are also

dynamic structures that can disassemble and reassemble during B-cell activation.

THE ANTIGEN-INDUCED CLUSTERING OF THE B-CELL RECEPTOR

Like other MIRRs, the BCR has no intrinsic receptor kinase activity, but upon antigen binding, the ITAM tyrosines in the Igα and Igβ chains are phosphorylated by the membrane-tethered kinase Lyn.[11,12] The phosphorylation of the BCR ITAMs leads to the recruitment of the Src homology 2 (SH2) domain-containing kinase Syk and the initiation of a variety of downstream signaling pathways described later in this chapter. The focus of this section is on the early events following antigen binding that lead to the phosphorylation of the BCR.

Until recently, most of what we learned about the responses of B cells to antigens came from biochemical studies of B cells responding to antigens in solution. Such studies showed that BCR signaling can only be initiated by the binding of multivalent antigens. As an example, only the bivalent $F(ab')_2$ fragments, but not the monovalent Fab fragments, of anti-IgM antibodies trigger BCR signaling.[12] By immunofluorescence imaging, following multivalent antigen binding, BCRs were observed to form microscopic clusters or patches on the cell surface that then move to one pole of the B cell to form a cap. Based on these biochemical studies, a widely accepted concept emerged that the physical aggregation of the BCR by multivalent antigens promoted the patching and capping of BCR that initiated signaling.

However, there is growing evidence both from studies in vitro as well as from intravital imaging in live animals that B cells are activated by membrane-bound antigens and not by antigens in solution. B cells were shown to be efficiently activated by antigens expressed on the surfaces of APCs in vitro resulting in the formation of a polarized bull's eye-like structure in which the BCRs are concentrated in the center, surrounded by the adhesion molecule lymphocyte function-associated antigen, which engages intercellular adhesion molecule on the APC surface.[13] Recent intravital imaging studies showed that B cells interacted with antigens on the surfaces of APCs in lymph nodes in vivo.[14,15,16,17,18] Antigens in lymphatic fluid enter lymph nodes through efferent vessels and gain access to B cells through various mechanisms.[19] Small soluble antigens move through follicular conduit networks and are presented to B cells within the follicles. Particle-like antigens, including viruses and immune complexes, are captured by macrophages lining the subcapsular sinuses and are then transported into the cortex of the lymph node where they are presented to B cells. In addition, B cells are also able to engage antigens on dendritic cell surfaces in the lymph nodes.[20] These findings provided a new view of the initiation of antigen-driven BCR signaling in which BCR signaling is initiated at the contact interface between B cells and APCs. Live cell imaging technologies are providing the tools to observe B cells as they first engage antigen on membrane surfaces. To facilitate these studies and gain high-resolution images, B cells are often activated by antigens incorporated into fluid planar lipid bilayers as surrogate APCs. These studies are revealing the B cells' engagement of membrane-bound antigens to be a remarkably dynamic event[21,22] that contrasts with the view of the patching and capping of BCRs that resulted from the simple physical cross-linking of BCRs by multivalent antigens in solution.

B cells first touch the antigen-containing bilayer through finger-like protrusions of their plasma membrane from which the BCRs engage antigen and form microclusters (Fig. 9.3).

These BCR microclusters are enriched in tyrosine phosphorylated proteins and are the site of Lyn and Syk recruitment and thus appear to be the elemental signaling units. B cells subsequently spread over the bilayer allowing the engagement of additional antigens (see Fig. 9.3). Following maximal spreading, the BCR-antigen microclusters are actively moved toward the center of the contact area, and the B cell contracts to form an immunologic synapse. This dynamic process of touching, spreading, and contraction occurs within minutes of the BCR's first contact with the bilayer.[23] These observations bring us back to the fundamental questions: How are BCR microclusters formed, and how do BCR microclusters trigger BCR signaling? Our understanding of these processes is still incomplete. However,

based on current data, several models have been proposed that address these questions.

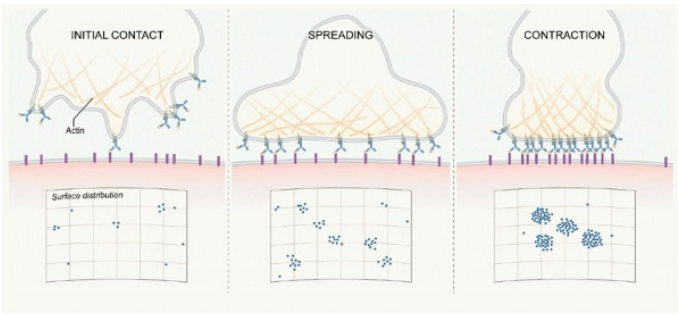

FIG. 9.3. The Activation of B Cells on Antigen-Presenting Cell (APC) Surfaces. The initial contact of the B cell with an antigen-containing APC surface is through finger-like protrusions of the membrane. Antigen binding induces B-cell receptor (BCR) clustering and signaling that triggers the B cell to spread over the APC surface, forming additional BCR clusters as antigens are encountered. After maximal spreading, the B cell contracts, actively moving BCR clusters to the center of the contact area ultimately forming an immune synapse.

One model, the conformation-induced oligomerization model, is based on evidence from live cell imaging at the level of individual BCRs. Single BCR tracking provided evidence that BCRs are dispersed and freely diffusing in the plasma membrane of resting B cells. Upon binding antigens presented on lipid bilayers, BCRs formed immobile oligomers that grew into microclusters by trapping additional antigen-bound BCRs.[24] Remarkably, contrary to soluble antigens, monovalent antigens presented on fluid lipid bilayers, which could not physically cross-link BCRs, activated B cells equivalently to multivalent antigens. This observation led to the conclusion that the physical cross-linking of BCRs by multivalent antigens was not a requirement for BCR oligomerization or clustering. Early oligomerization and microcluster formation that occurred within 60 seconds of antigen binding were shown to be BCR-intrinsic events that did not require the signaling apparatus of the BCR.[25] Mutagenesis studies showed that the membrane-proximal Cμ4 portion of the ectodomain of mIgM (or the Cγ3 membrane-proximal domain of mIgG) was both necessary and sufficient for BCR oligomerization and signaling.[25] Thus, the membrane proximal domain of the mIg appeared to contain an oligomerization domain that was not exposed in the absence of antigen binding. It was hypothesized that the B cell's binding antigen presented on an APC surface exerted a force on the BCR that revealed the oligomerization domain, allowing oligomerization of antigen-bound BCRs as they randomly bumped on the membrane. This model provides a mechanism by which the universe of foreign, structurally distinct antigens can bring BCRs into a precise signaling-active oligomer by the monovalent binding of BCRs to epitopes on APC-presented antigens. This model also suggests how BCRs are able to so exquisitely discriminate their affinity for antigen by monovalent binding, avoiding the affinity obscuring effects of the avidity contributed by multivalent antigen binding to bivalent BCRs.

Another model for the initiation of BCR signaling based on live cell single molecule imaging focuses on the role of the actin cytoskeleton in signaling.[26,27,28,29] In this model, the membrane cytoskeleton fences and pickets restrict BCR mobility and interactions with signaling molecules and coreceptors. BCR signaling results in the disruption of the cytoskeleton barriers and increases the likelihood that the antigen-engaged BCR will encounter activated kinases and coreceptors.[29] The actin cytoskeleton may also segregate BCRs from kinases and phosphatases at steady state. Current evidence suggests that the cytoskeleton fences and pickets confine the BCR and inhibitory phosphatases to the same areas, and that BCR signaling serves to disrupt the cytoskeleton and allow the BCRs and phosphatases to diffuse away, promoting B-cell activation.

A third model, the dissociation activation model, is based on biochemical studies.[6,30] In this model, in resting B cells in the absence of antigen most BCRs exist on the B-cell surface as signaling-inactive, autoinhibited oligomers in equilibrium with a small number of signaling-active BCR monomers.[30] The binding of multivalent antigen disrupts the oligomers shifting the equilibrium toward BCR active monomers, which are then clustered by the multivalent

antigen in a manner that prevents the formation of inactive oligomers within the cluster. This model also accounts for the ability of structurally diverse antigens to activate B cells by proposing that antigens keep BCRs apart rather than bringing them together into well-ordered oligomers.

THE ROLE OF B-CELL RECEPTOR AFFINITY AND ISOTYPE IN SIGNALING

Affinity maturation and class switching are two hallmark features of humoral immune responses.[31] In a typical T-cell-dependent antibody response, antigen-specific antibodies become increasingly higher in affinity and predominantly of the IgG isotype through the linked molecular processes of somatic hypermutation and class switching. Because the B-cell immune response functions through clonal selection, it is presumed that affinity maturation and class switching reflect an advantage of B cells expressing high-affinity, classswitched BCRs in the selection process. Indeed, adoptive transfer studies provided clear evidence that high-affinity B-cell outcompete low-affinity B-cell clones for survival in vivo.[32,33,34,35,36,37,38] Similarly, when comparing IgM and class-switched BCRs, several seminal studies showed that class-switched B cells outcompete IgM B cells for survival in vivo, and that this survival advantage can be attributed to the 28 amino acid cytoplasmic tail of mIgG,[1,2] missing in mIgM and mIgD. The question is at what point in the antigen-selection process does a high-affinity, class-switched BCR gain an advantage?

Biochemical experiments suggested that the signals that are triggered through high-affinity BCRs are qualitatively different from signals through low-affinity BCRs.[39] At an earlier point in B-cell activation, the affinity of the BCR for antigen was shown to determine the degree to which B cells spread over antigen-containing fluid lipid bilayers, allowing increased accumulation of antigen into the immune synapse and subsequent enhanced responses.[23] The IgG-BCR cytoplasmic tail is responsible for the enhanced B-cell proliferative responses to antigen in vitro and signaling cascades that have been shown to be qualitatively different from those triggered by IgM-BCRs.[3,40,41,42] Enhanced signaling through IgG-BCRs is dependent on the phosphorylation of a tyrosine in the IgG tail that serves to recruit growth factor-receptor-bound protein 2 (Grb2), resulting in sustained kinase activation and enhanced B-cell proliferation.[3]

Recent studies using high-resolution imaging to follow single BCRs showed that the earliest events that occur following antigen binding to the BCR are also highly sensitive to both the affinity of the BCR for antigen and the isotype of the BCR. Comparing BCRs that differed 50-fold in their affinity for antigen, it was observed that high-affinity BCRs more readily formed BCR microclusters that grow more rapidly, thereby resulting in larger microclusters that recruit more Syk and signal for more robust calcium responses.[24] These imaging experiments also demonstrate that IgG-BCRs are dramatically enhanced in their ability to oligomerize and to grow microclusters, ultimately leading to increased recruitment of Syk and more robust calcium responses as compared to IgM-BCRs of the same affinity.[43] The enhanced function of IgG-BCRs was mapped to a novel membrane proximal 15 amino acid region of the cytoplasmic tail. These studies place the effects of both affinity maturation and class switching at the earliest steps in the initiation of BCR signaling.

HOW B-CELL RECEPTOR CLUSTERING TRIGGERS SIGNALING

Another fundamental yet still open question related to the initiation of BCR signaling is how BCR clustering in response to antigen recruits Lyn to phosphorylate the antigen-bound BCRs and triggers signaling. One simple explanation is that Lyn is constitutively associated with the cytoplasmic domains of some monomeric BCRs, but only phosphorylates BCRs in trans when the BCRs are clustered. However, recent studies suggest that the mechanism for recruitment of Lyn may be more complicated. Lyn is lipidated and tethered to the inner leaflet of the plasma membrane. BCR clustering has been shown to perturb the local lipid environment leading to the transient coalescence of lipid rafts around the BCR oligomers followed by a more stable association of the BCR microcluster with lipid tethered Lyn.[44] Thus, the BCR's perturbation of the membrane may serve to recruit Lyn to the BCR cluster. BCR clustering has also been shown to alter the way the Igα and Igβ chains' cytoplasmic domains associate. In the absence of antigen, the domains are in close proximity in a “closed” conformation and upon antigen binding, the domains “open”; the opening is simultaneous with Lyn's phosphorylation of the ITAMs.[5] Thus, BCR clustering may serve to reveal the ITAMs for

phosphorylation by Lyn.

B-CELL RECEPTOR-TRIGGERED SIGNALING CASCADES

BCR signaling is a multistep process that involves the initiation of signaling by the activation of protein tyrosine kinases (PTKs), serine-threonine kinases, and lipid kinases; amplification of signaling by recruiting adaptors; generation of second messengers; and finally activation of the transcription of genes involved in B-cell responses.

Initiation of B-Cell Receptor Signaling—Protein Tyrosine Kinase Activation

Following BCR clustering, three different families of PTKs, Src, Syk, and Tec, are activated sequentially. This sequential activation of the members of the three different PTK families is essential to trigger and regulate downstream signaling. The importance of these PTKs in the B-cell signaling is underscored by the fact that deficiencies in any one of the three families result in aberrant B cell development and function (Table 9.1). The first kinases that are activated following BCR cross-linking are the Src family PTKs, primarily Lyn, but also Blk and Fyn followed by the activation of Syk and the Tec family kinase, Btk[45] (Fig. 9.4). ITAM phosphorylation of Igα-Igβ by Lyn generates phosphotyrosine motifs that allow the binding of the SH2 domains (Box 9.1) of the second kinase, Syk, resulting in rapid Syk activation. Upon binding to phosphorylated ITAMs, Syk undergoes autophosphorylation at multiple tyrosines within its linker regions that not only prolongs Syk's activation but also creates SH2 binding sites on Syk for the recruitment of downstream signaling molecules, including PLC-γ2,[46] leading to a positive feedback of BCR signaling and the concomitant influx of calcium.[12] Btk is the third PTK that is activated upon BCR cross-linking.[47] The importance of Btk in BCR signaling for the development, activation, and differentiation of B cells is underscored by the fact that the loss-of-function mutations of the gene encoding Btk lack circulating B lymphocytes, are unable to generate Igs, and cannot mount humoral immune responses.[48] This primary immunodeficiency is named X-linked agammaglobulinemia.[49,50] Similarly, a spontaneous mutation in the mouse Btk gene leads to X-linked immunodeficiency.[51] Btk consists of multiple protein domains including PH, SH2, SH3, and kinase domains (see Box 9.1), which define its subcellular location and regulate its activity. For Btk activation, plasma membrane localization is important, which is governed by the interactions between the PH domain of Btk with phosphatidylinositol (PI)(3,4,5)P_3, the product of PI3K activity and between the SH2 domain of Btk with phosphorylated BLNK, an adaptor protein. Mutation in the PH domain of Btk (R28C) leads to classical X-linked agammaglobulinemia,[52] substantiating the importance of plasma membrane localization for Btk activation. Following BCR cross-linking, Btk translocates from the cytosol to the plasma membrane where it is activated by phosphorylation at Y551 in its catalytic domain by Lyn[53,54,55] followed by an autophosphorylation of its SH3 domain.[54,56] Once activated, Btk triggers a cascade of signaling events that culminate in calcium mobilization through phosphorylation of PLC-γ2, cytoskeletal rearrangements, Vav activation, and transcriptional activation involving NF-κB.

Amplification of B-Cell Receptor Signaling— Recruitment of Adaptors

BCR-mediated signaling is a complex process in which each phosphorylation event is linked with another in a regulated manner, thereby generating a large number of protein-protein interaction networks ultimately resulting in the formation of a large, well-ordered structure often referred to as a signalosome. Interactions between signaling networks are regulated by a number of scaffolding or adaptor proteins, which regulate BCR-mediated signaling cascades not only by recruiting multiple signaling intermediates to the proper location but also by controlling interactions between signaling components. Recent studies have identified the roles of

adaptors in B-cell activation, leading to important insights into how they integrate BCR signaling. Although adaptors generally lack any enzymatic activity, they consist of multiple protein-protein or protein-lipid interaction domains, including SH2, SH3, PH and PX homology domains (see Box 9.1). Each of these domains has the potential to interact with a number of proteins that are critical to amplify BCR signaling by facilitating the coupling of multiple downstream signaling pathways. Essentially, the adaptors define where and when macromolecular complexes are assembled, allowing both spatial and temporal regulation of signaling cascades. To illustrate the importance of adaptors, we describe the functions of three: B-cell linker protein (BLNK); B-cell adaptor for PI3K (BCAP), and B-cell adaptor

molecule of 32 kDa (Bam32).

TABLE 9.1 The Phenotype of Mice Deficient in Key B-Cell Signaling Components

Target Protein	Major Phenotype	Reference
Igα cytoplasmic domain	Normal pre-B-cell development; completely impaired mature B-cell development	184
Igβ	Complete block at the pro-B-cell stage	185
Igμ	Deletion of mature B cells; increased Fas expression	186
Lyn	Normal B-cell development in the bone marrow; reduced number of peripheral B cells; increased proportion of immature B cells; enhanced BCR induced ERK activation and hyperproliferative responses	187
Syk	Block in transition of the pro-B-to the pre-B-cell stage; intact Igα-Igβ ITAM phosphorylation in remaining B cells; abolished BCR-induced calcium influx; failure to transmit downstream signals	188,189
Btk	Reduced number of peripheral B cells; increased immature B cells; complete loss of B1 B cells; fail to respond to TI-II antigens	190,191,192,193
BLNK	Block in transition of the pro-B-to the pre-B-cell stage; incomplete block in B-cell development; fail to respond to both TD and TI antigens	194,195,196
PLC-γ2	Decreased mature B cells; block in pro-B-cell differentiation; B1 B-cell deficiency; block in BCR-induced calcium influx and proliferation	197,198
BCAP	Reduced number of B cells; B1 B-cell deficiency; reduced serum IgM and IgG3 levels; abolished TI antibody response; reduced BCR induced calcium influx and proliferation	82
PI3K, p110 subunit	Reduced numbers of B1 and marginal zone B cells; reduced serum Ig levels; defective primary and secondary response to TD antigens; diminished response to TI-II antigens; reduced BCR, CD40, and LPS induced proliferation	199,200
PI3K p85 subunit	Reduced number of peripheral B cells and B1 cell; reduced serum Ig levels; reduced BCR- and CD40-induced proliferative response; abolished TI antibody response	201,202
PKC-β	Failure to activate IKK and degrade IκB; failure to upregulate NF-κ-dependent survival signals; impaired humoral immune responses and reduced cellular responses	203,204

Bam32	Normal B-cell development; impaired TI-II antibody responses; reduced responses to BCR cross-linking	89
Vav1/2	Reduced number of B cells; defects in formation of germinal centers and class switching; defective immune responses against TD and TI antigens; impaired BCR-induced calcium influx and proliferation	205,206
Rac	Reduced number of mature and marginal zone B cells and B1-a cells and IgM secreting plasma cells; increased number of peripheral B cells in blood; reduced serum IgM and IgA concentration	207
CD19	Defective response to TD antigens; failure to form germinal centers and undergo affinity maturation; lack of B-1, marginal zone B cells; reduced BCR- and CD40-induced proliferative responses	191,208,209
CD22	Decreased surface IgM levels; augmented BCR-induced calcium; compromised marginal zone B-cell compartment	210,211
PIRB	Increased number of peritoneal B1 cells; constitutive activation of follicular B cells; increased BCR-induced proliferation	212
Calcineurin	Reduced number of B1 cells; reduced plasma cell differentiation; decreased TD antibody response; BCR-induced proliferation defects	102
IKKα	Reduced mature B-cell population; impaired basal and Ag-specific Ig production; disrupted splenic architecture including germinal center formation; decreased expression of NF-κB target genes	213,214
IKKβ	Disappearance of mature B cells 215 NEMO Disappearance of mature B cells	215

BCR, B-cell receptor; CD, cluster of differentiation; Ig, immunoglobulin; ITAM, immunoreceptor tyrosine-based activation motif; LPS, lipopolysaccharide; TD, T-dependent; TI, T-independent.

BLNK—Integrating Protein Tyrosine Kinases and PLC-γ2

BCR clustering activates Lyn and Syk that regulate a variety of effectors including PLC-γ2, an essential phospholipase for

calcium responses. Although Syk can directly phosphorylate PLC-γ2 in vitro,[57] expression of a functional BCR, Lyn, and Syk in nonlymphoid cells did not induce PLC-γ2 phosphorylation or calcium mobilization,[58] leading to the discovery of the B-cell-specific adaptor protein, BLNK. BLNK (also known as SLP-65 or BASH) is a cytoplasmic protein consisting of an N-terminal region containing a short leucine zipper motif, a proline-rich region within the middle third of the molecule, and a C-terminal SH2 domain. BLNK contains 13 tyrosine residues, of which 6 are in putative SH2 binding motifs and are phosphorylated upon BCR clustering.

BLNK is recruited to the clustered BCR by binding through its SH2 domain to the phosphorylated non-ITAM tyrosine 204 in Igα. Mice with a Igα Y204 mutation exhibit reduced BLNK phosphorylation and calcium fluxes.[59] Once translocated to the plasma membrane, BLNK is phosphorylated by Syk, which creates docking sites for SH2 domains of multiple effector molecules facilitating interactions among them and allowing them to phosphorylate and activate their respective signaling pathways. One of the best studied examples of how BLNK bridges multiple signaling components is provided by the activation of PLC-γ2. PLC-γ2 activation is completely abolished in Syk-deficient B cells.[60] In addition, BCR-induced PLC-γ2 activation is diminished in Btk-deficient B cells, suggesting that both Syk and Btk are required for PLC-γ2 activation.[61] However, the mechanisms underlying how these two families of PTKs regulate PLC-γ2 activation became clear only after the discovery of BLNK. After phosphorylation by Syk, BLNK creates binding sites for the SH2 domains of both Btk and PLC-γ2, bringing them into close proximity with each other, and hence facilitating PLC-γ2 phosphorylation at Tyr753 and Tyr759 by Btk, which is required for PLC-γ2 activation.[62,63,64,65,66,67,68] Cells expressing a mutant BLNK lacking either Btk or PLC-γ2 binding sites

show significant reduction in calcium activation, further suggesting that BLNK acts as a scaffold for bridging Btk to PLC-γ2.[69] A single BLNK molecule can bind three PLC-γ2 molecules, thus BLNK also functions as an amplifier of BCR signaling.[62] Following BCR clustering, both BLNK and Btk are also translocated to the plasma membrane bringing along PLC-γ2 and allowing it to gain access to its substrate $PI(4,5)P_2$ in the inner leaflet of the plasma membrane.[66,67,68] In addition to phosphorylating PLC-γ2 and recruiting it to the plasma membrane, Btk also recruits PIP5 kinase to the plasma membrane, which catalyzes $PI(4,5)P_2$ synthesis from PIP(4)P to ensure that activated PLC-γ2 does not run out of its substrate.[70] Once activated, PLC-γ2 hydrolyzes PI(4,5) P2 to generate two important second messengers: inositol trisphosphate (IP3) and diacyl glycerol (DAG). BLNK not only couples Btk and PLC-γ2, but it also binds to the SH2 domains of Vav and Nck, resulting in activating the mitogen-activated protein (MAP) kinase pathway, cytoskeletal rearrangements, and BCR internalization.[71]

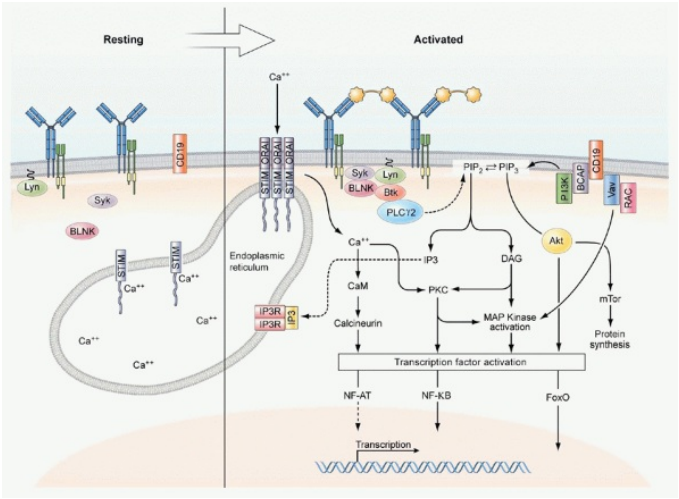

FIG. 9.4. Schematic Depiction of B-Cell Receptor (BCR) Signaling. Presented is a highly simplified version of the signaling pathways triggered by antigen-induced BCR clustering. Antigen binding to the BCR leads to the formation of signalosome consisting of BCR, protein tyrosine kinases, adaptors, and effector molecules. BCR clustering results in PI3K activation, leading to the production of PIP3 from PIP2 and PLC-γ2 activation resulting in the production of IP3 and diacyl glycerol (DAG) from PIP2. Binding of IP3 to the IP3 receptor on the endoplasmic reticulum (ER) opens channels in the ER membrane leading to calcium release and depletion of ER calcium. Calcium depletion is sensed by STIM, a calcium-binding protein, which results in its oligomerization and movement to regions of the ER membrane in close proximity to the plasma membrane where it interacts with Orai opening the calcium release-activated channel, resulting in calcium influx. Calcium, DAG, and PIP3 trigger signaling cascades leading to mitogen-

activated protein kinase and transcription factor activation and activation of the Akt pathways promoting cell survival and growth.

BOX 9.1. PROTEIN MODULES INVOLVED IN SIGNAL TRANSDUCTION

Src homology 2 (SH2) domain. The SH2 domain is a structurally conserved protein domain present in the Src oncoprotein from which its name is derived and in many signaling molecules including those involved in B-cell signaling. SH2 domains bind with high affinity to peptide sequences within target proteins that contain phosphorylated tyrosine residues but have no affinity for the unphosphorylated sequence. This allows tyrosine phosphorylation to act as a molecular switch, recruiting SH2 domain-containing proteins to activated receptors and initiating signaling cascades. Important SH2 domain-containing proteins involved in B-cell signaling include Lyn, Syk, Btk, PLC-γ2, BLNK, and Grb2.

Src homology 3 (SH3) domain. SH3 domains are protein modules that recognize proline-rich sequences, in particular those containing a PxxP motif, which do not require phosphorylation. However, recent studies have suggested that the SH3 domains also bind to nonproline-rich sequences accounting for diverse functions they mediate. Some examples in B cells are Lyn, Btk, and Grb2.

Pleckstrin homology (PH) domain. The PH domain is a lipid binding module that recognizes phosphoinositides phosphorylated at the 3 position of the inositol ring, the most important of which is PI(3,4,5)P_3, the product of PI3K's phosphorylation of PI(4,5)P_2. This property allows PH domain-containing proteins to bind to the inner leaflet of the plasma membrane following PI3K activation where they function. PH domain-containing proteins in B cells include Btk, Vav, Akt, Gab, and Bam32.

Phox homology (PX) domain. The PX domain is another phosphoinositide-binding domain that was originally identified in two cytosolic components of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, p40phox and p47phox. In a majority of cases, PX domains contain a proline-rich motif, PxxP, in the middle that serves as SH3 binding motif, suggesting that PX domains might directly interact with SH3 domains as well. PX domains have been shown to play diverse cellular functions including vesicle docking and fusion, cell signaling, and protein sorting. An example in B cells is phospholipase D.

BCAP—Integrating Protein Tyrosine Kinases and PI3K

PI3K is a heterodimeric enzyme consisting of a catalytic subunit (p110) and a regulatory subunit (p85) that phosphorylates phosphatidylinositol lipids generating lipid derivatives at the inner leaflet of the plasma membrane that serve as second messengers for downstream signaling.[72,73] Of particular interest is the generation of PI(3,4,5)P_3 from PI(4,5)P_2, which serves as a ligand for the proteins containing PH domains and tethers these to the membrane. Although multiple isoforms of PI3K are known, p110δ and p85α are the predominant isoforms in B cells. Gene targeting experiments have established the role of these subunits in the development and activation of B cells (Table 9.2). Deletion or inactivation of either of the two subunits of PI3K demolish BCR-induced phosphorylation of Akt, FoxO, and protein kinase D and results in reduced calcium flux, impaired cell cycle progression, and reduced glucose metabolism.[74,75,76,77,78,79,80] Despite the critical role of PI3K in BCR signaling, the mechanisms by which the BCR-associated PTKs regulate the PI3K pathway is unclear. It has been proposed that tyrosines in the cytoplasmic tail of CD19, which are phosphorylated upon BCR clustering, provide binding sites for the SH2 domains of the p85 subunit of PI3K, recruiting it to the plasma membrane and increasing the specific activity of the enzyme. However, the observation that defects in p85α KO mice are more severe than those in CD19 KO mice suggested that additional adaptor molecules may participate in bridging BCR-associated PTKs and PI3K, leading to the discovery of another B-cell-specific adaptor molecule, BCAP.[81] BCAP contains multiple tyrosines, which are phosphorylated after BCR stimulation through the concerted actions of Syk and Btk, thereby creating binding sites for SH2 domains of the p85 subunit of PI3K. BCAP was shown to regulate PI3K signaling in CD19-deficient B cells, but in BCAP KO mice, PI3K signaling was largely unaffected in B cells,[81,82] suggesting that in the absence of BCAP, CD19 functions as a major adaptor. Indeed, CD19/BCAP double knockout mice show an almost complete block of BCR-mediated Akt activation and severe defects in the generation of immature and mature B cells.[83] Recently a GTPase, TC21, was also identified as an adaptor for PI3K activation by binding directly to Igα-Igβ.[84]

Bam32—Linking B-Cell Receptors to Mitogen-Activated Protein Kinase Activation and Cytoskeletal Rearrangements

Bam32 bridges clustered BCRs to two important pathways, namely, Rac and cdc42, which trigger cytoskeletal rearrangement leading to cell adhesion, polarization, and motility and to activation of the MAP kinase c-Jun N-terminal kinase (Jnk) and extracellular signal-regulated kinases (ERK)

that play central roles in the activation of transcription factors that regulate the expression of genes involved in B-cell proliferation, differentiation, and survival.[85,86,87,88] Following BCR clustering, Bam32 translocates to the plasma membrane in a PI3K-dependent mechanism through its PH domain[88] where it binds to Rac and cdc42 leading to their activation, triggering downstream signaling to Jnk and ERK. Mice deficient in Bam32 show diminished Jnk and ERK phosphorylation.[89] Rac activation is diminished in Bam32-deficient B cells, whereas overexpression of Bam32 results in enhanced Rac activation.[90]

TABLE 9.2 B-Cell Coreceptors that Influence B-Cell Receptor Signaling

Coreceptor	Activating (+) or Inhibitory (−)	Phosphorylated Motif(s)	Ligand(s)	Signaling Molecules Recruited upon Motif Phosphorylation	Expressed in Humans (H) or Mice (M)	References
CD19	+	Tyr residues	C3-coated antigen	PI3K + Vav + Lyn + PLC-γ2	H + M	126,127,216
FcμR (FAIM3/TOSO)	+	Ser + Tyr residues	IgM	PI3K + PLCγ-1	H + M	131,217
FcRL1 (FcRH1)	+	ITAM	Unknown	Unknown Tyr kinase?	H + M	132,218,219
PIRA	+	ITAM on associated FcRγ	MHC-1	Unknown	M	133,134,220,221
CD45R (B220)	+	PTP domain	CD22?	C-terminal Tyr of SFKs	H + M	135,136,222,223
CD148 (HPTPh)	+	PTP domain	Unknown	C-terminal Tyr of SFKs	H + M	137,138
FcγRIIB (CD32B)	-	ITIM	IgG-IC	SHIP	H + M	139,140,141,224
CD22 (BL-CAM)	-	ITIM	Sialic acid	SHP-1	H + M	223,224,225,226,227
PIRB	-	ITIM	MHC-1	SHP-1 (+ SHP-2?)	M	133,220,221,224
ILT-2	-	ITIM	HLA-A, -B + -G (MHC-1)	SHP-1	H	224
CD72 (Lyb-2)	-	ITIM	CD100, CD5	SHP-1	H + M	224,227,228

CD5 (Ly1)	-	ITIM	CD72 (+ others?)	SHP-1	H + M	224,229
PD-1	-	ITIM	IgSF protein	SHP-2	H + M	224
CD66a (BGP-1)	-	ITIM	CD66a	SHP-1 + SHP-2	H + M	224
FcRL4	-	ITIM	Unknown	SHP-1 + SHP-2	H	132,219
FcRL2 (FcRH2)	-	ITAM/ITIM	Unknown	SHP-1	H	132,153
FcRL3 (FcRH3)	-	ITAM/ITIM	Unknown	SHIP + SHP-1 + SHP-2	H	132,154
FcRL5 (FcRH5)	-	ITAM/ITIM	Unknown	SHP-1	H + M	132,155
FcRL6 (FcRH6)[a]	-	ITIM	HLA-DR (MHC-2)	SHP-2	M	132,230,231

CD, cluster of differentiation; HLA, human leukocyte antigen; Ig, immunoglobulin; IC, immune-complex; IgSF, immunoglobulin superfamily; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; MHC, major histocompatibility complex; PTP, protein tyrosine phosphatase; SFK, Src-family kinase; SHIP, SH2-containing inositol 5′-phosphatase; SHP, SH2-domain-containing protein tyrosine phosphatase.

[a] FcRL6 motif, ligand, and signaling molecule information was obtained from studies of human T and natural killer cells.

Generation of Second Messengers

As described previously, binding of the PTKs and various signaling intermediates such as PLC-γ2 and PI3K on various adaptors results in the activation of these enzymes resulting in the generation of second messengers including calcium, DAG, and phosphoinositides. These second messengers play a crucial role in regulating BCR signaling by virtue of their ability to affect downstream signaling initiated by the BCR.

Calcium as a Second Messenger

Calcium mobilization is a multistep process that is initiated with the activation of PLC-γ2 that hydrolyzes $PI(4,5)P_2$ present in the inner leaflet of the plasma membrane to generate IP3 and DAG. A receptor for IP3 (IP3R) is present as a tet-ramer on the ER membrane that forms a calcium channel.[91] The binding of IP3 to the IP3R activates the receptor and opens the channel resulting in the release of calcium from the ER into the cytosol. Depletion of intracellular ER calcium stores results in the oligomerization of the ER calcium sensor, STIM1, an ER membrane protein that contains in its cytoplasmic domain an EF-hand calcium-binding domain.[92,93] STIM1 oligomers rapidly redistribute into clusters and move to junctions of the ER and plasma membrane where STIM1 interacts with Orai, a calcium release-activated channel, and activates Orai to open, triggering the entry of extracellular calcium into the cytosol.[94,95,96] A single missense mutation (R91W) in human Orai or a truncation mutation in STIM1 causes severe combined immune deficiency supporting the functional roles of STIM1 and Orai in calcium signaling in the immune system.[97,98] The increase in cytosolic calcium is sensed by various calcium-binding proteins, ultimately leading to activation of several downstream pathways including activation of protein kinase C (PKC) and transcription factors nuclear factor of activated T cells (NFAT) and NF-κB. One of the major cytosolic calcium-binding proteins, calmodulin, functions as a calcium sensor by binding to calcium with its two EF hands. Calcium-bound calmodulin activates several molecules including calcineurin, which is a phosphatase that dephosphorylates NFAT resulting in NFAT's translocation into the nucleus where it activates the transcription of target

genes. One of the targets of NFAT in B cells is interferon

regulatory factor 4, a critical transcription factor for the differentiation of plasma cells.[99,100,101] Indeed, calcineurin-deficient B cells show reduced plasma cell differentiation and decreased antigen-specific antibody responses to antigens.[102]

Diacylglycerol as Second Messenger

The second product of $PI(4,5)P_2$ hydrolysis by PLC-γ2 is DAG that together with calcium activates PKC-β that regulates the NF-κB pathway. NF-κB in resting B cells is retained in the cytoplasm by binding to its inhibitor, IκB. BCR clustering induces IκB phosphorylation, through a PKC-β-initiated pathway, ubiquitination, and subsequent degradation, allowing translocation of NF-κB into the nucleus.[103,104,105] Aberrant activation of NF-κB has been linked to defective B-cell activation leading to multiple immune disorders. Ablation of PKC-β, which leads to the defective activation of the NF-κB pathway, results in defective B-cell activation and maturation.[106] In humans, mutations in components of the NF-κB pathway are associated with the formation of lymphomas.[107,108,109,110,111,112,113] In addition to activating PKC-β, DAG also recruits the DAG binding Ras guanine nucleotide releasing protein 3 (RasGRP3) to the plasma membrane, thus initiating the Ras to Raf to MEK to ERK pathway.[114] Deletion of the DAG-binding domain from RasGRP blocks the movement of RasGRP3 to the plasma membrane and the activation of Ras. The Ras-Raf-MEK-ERK signaling pathway plays a critical role in antigen-induced proliferation of mature B cells.[115] Ras is also involved in developing B cells, as transgenic expression of dominant negative forms of Ras blocks developmental progression to the pre-B cell and immature B-cell stages.[116,117] Major targets of ERK in B cells are Ets-family transcription factors, resulting in the expression of early response genes including Egr1 that has been shown to promote expression of adhesion molecules.[118]

Phosphoinositides as Signaling Mediators—A Diverse Group of Molecules Regulating Diverse Pathways

PI is unique among membrane lipids as it can undergo reversible phosphorylation at multiple sites on its inositol head group by lipid kinases and phosphatases to generate a variety of phosphorylated PI lipids called phosphoinositides. Phosphoinositides play crucial roles in cell signaling and membrane trafficking. As described previously, BCR clustering leads to the accumulation of the phosphoinositide, $PI(4,5)P_2$, the substrate for PLC-γ2, which generates two crucial second messengers, IP3 and DAG. Another important phosphoinositide is the PI3K-generated $PI(3,4,5)P_3$ that provides a ligand for recruiting important PH domain-containing proteins to the plasma membrane, including Btk, PLC-γ2, and Akt. Akt is activated at the membrane by phosphorylation and in turn phosphorylates several proteins and transcription factors that regulate protein synthesis, cell survival, and proliferation. Some of the targets of Akt in B cells are mammalian target of rapamycin, glycogen synthase kinase 3, forkhead family transcription factors, Caspase-9, and proapoptotic protein, BAD.[119,120,121] Given the crucial role of $PI(3,4,5)P_3$ in B-cell activation, its levels are tightly regulated by two different lipid phosphatases that oppose the activity of PI3K, phosphatase and tensin homolog, which removes a phosphate from the D3 position generating $PI(4,5)P_2$, and SH2-domain-containing inositol 5′ phosphatase (SHIP), which generates $PI(3,4)P_2$ by removing a phosphate from the five position and essentially shuts down signaling. One of the pathways that activates SHIP in B cells is the ligation of the inhibitory FcR FcγRIIB,[122] as described in the next section of this chapter.

The Spatial and Temporal Dynamics of B-Cell Receptor Signaling Cascades

Advanced fluorescence-based imaging techniques are just now providing the first view of the spatial and temporal dynamics of the recruitment of individual signaling molecules to BCR microclusters following BCR oligomerization in response to membrane-bound antigens. Each newly formed BCR cluster recruits the earliest PTKs, Lyn and Syk[24,123,124]; as the BCR moves to form an immune synapse, Syk is lost and PI3K is recruited. It has also recently been possible to use imaging to follow signaling as the BCR is internalized into the B cell. Such studies showed that BCR signaling is initiated at the plasma membrane with the recruitment and phosphorylation of Lyn and Syk and continues after the BCR is endocytosed and trafficked through early endosomes then to late endosomes and multivesicular antigen

processing compartments with the recruitment and phosphorylation of downstream kinases, initially cRaf and subsequently the MAP kinases, ERK, p38, and Jnk.[125] Internalization of the BCR is necessary for proper signaling as when internalization is blocked, phosphorylation of kinases become dysregulated as does gene transcription. The continued application of imaging technologies to describe B-cell activation should lead to an increasingly detailed spatial and temporal view of BCR signaling.

CORECEPTOR REGULATION OF B-CELL RECEPTOR SIGNALING

The outcome of signaling through the BCR is carefully regulated not only by the developmental stage of the B cells but also by a variety of B-cell coreceptors that both promote and attenuate BCR signaling in response to antigen. Ideally, co-receptors ensure that sufficient amounts of antigen-specific antibodies are produced to control an infection, and that once controlled, antibody production is turned off. We have provided a list of B-cell coreceptors and have indicated whether they function to enhance or inhibit BCR signaling and, when it is known, what they recognize and the molecular basis of their effect on BCR signaling (see Table 9.2). Presumably, all B-cell coreceptors respond to clues from the environment as to the course of the immune response and relate this information to the B cells by enhancing or inhibiting BCR signaling. The best understood examples of the role of coreceptors in B-cell responses are the activating CD19/CD21 complex and the inhibitory FcγRIIB. Both coreceptors become physically cross-linked to the BCR through the recognition of antigen-containing complexes. The CD19/CD21 complex binds complement fixed antigens (C3d-modified antigen, as described in Chapter 36) through CD21, a complement receptor that binds to C3d. Complement modification of antigens can be viewed as a sign that the antigen is dangerous, having the ability to

activate the complement pathway, and thus, the CD19/CD21 complex serves to enhance BCR signaling. The FcγRIIB binds to antigen-antibody immune complexes (described in Chapter 24) through the Fc portion of the IgG antibody complexed with the antigen. Immune complexes form when antigen-specific antibodies reach sufficient levels relative to the antigen, an indication that further B-cell activation should be curbed, and thus, the FcγRIIB inhibits BCR signaling.

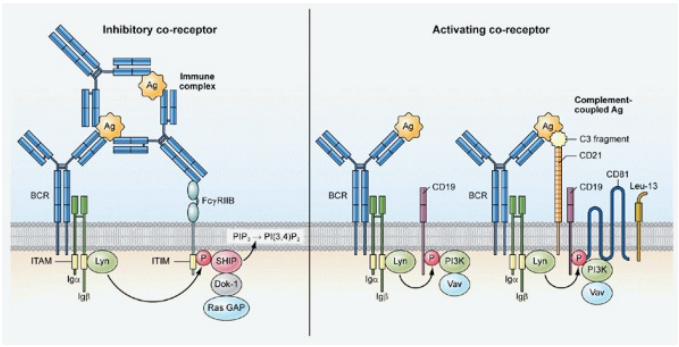

FIG. 9.5. Inhibitory and Activating B-Cell Receptor (BCR) Coreceptors. Antigen-containing immune complexes engage both the BCR and the inhibitory coreceptor FcγRIIB (**Left**). Lyn associated with clustered BCRs phosphorylates the immunoreceptor tyrosine-based inhibitory motif of FcγRIIB, recruiting SH2-domain-containing inositol 5′ phosphatase that dephosphorylates PI(3,4,5)P_3, the product of PI3K activation, to PI(3,4)P_2, blocking downstream signaling. Antigen binding to BCRs induces their clustering and signaling that results in the phosphorylation of CD19 by BCR-associated Lyn, recruiting PI3K and Vav (**Right**) and enhancing BCR signaling. Enhancement of BCR signaling is stronger when complement conjugated antigens engage both the BCR and the complement receptor, CD21, recruiting CD19 to the signaling complex where it is phosphorylated by Lyn recruiting PI3K and Vav (**Right**).

CD19/CD21, a transmembrane glycoprotein complex expressed on the surface of all B cells, is the best understood activating coreceptor for BCR signaling. On mature B cells, the CD19 component can exist alone or as part of a tetra-meric complex with CD21 (complement receptor type 2), the tetraspanin family protein, CD81, and the small interferon-induced transmembrane protein 1, Leu-13[126] (Fig. 9.5). CD21 links this complex to the BCR in

response to complement-coupled antigens and boosts BCR signaling and ultimately antibody production. Upon binding of complement-coupled antigen, the BCR and CD19/CD21 complex are coligated, and several tyrosine residues in the cytoplasmic tail of CD19 are phosphorylated by Lyn, providing binding sites for the SH2 domains of the p85 subunit of PI3K, Vav and Lyn, thus enhancing the signaling triggered by BCR antigen binding.[127] It is hypothesized that the association of Lyn with CD19 may counteract the autoinhibition of Lyn, which is maintained by interaction between its SH2 domain and its carboxy-terminal phosphotyrosine, thus stimulating the enzymatic activity and phosphorylation of Lyn during BCR signaling.[128] CD81 may aid in the stable association of the BCR with saturated lipids that coalesce around the antigen-bound BCRs to sustain antigen-induced signaling when the CD19-CD21 complex is coengaged with the BCR.[129] CD81 and Leu-13 may also be involved in mediating cell-cell interactions during the B-cell response to antigen.[130]

In addition to the CD19-CD21 complex, B cells express other activating coreceptors whose ligands and mechanisms by which they affect BCR signaling are less well characterized (see Table 9.2). These activating coreceptors include the recently identified Fc receptor for IgM (FcμR), which contains several conserved tyrosine and serine residues in its cytoplasmic tail that are targets for phosphorylation after receptor ligation with IgM-containing immune complexes.[131] Although the physiologic role of FcμR in B-cell responses is not known, it has been hypothesized that FcμR may become coligated to the BCR and CD19/CD21 complex through the recognition of complement-coupled IgM-antigen immune complexes and drive the B cell response toward isotype-switched antibodies. Other activating coreceptors are Fc receptor-like 1 (FcRL1)[132] and paired Ig-like receptor A (PIRA)[133,134] that respectively contain or associate with cytoplasmic ITAMs. The tyrosine residues within these ITAMs are phosphorylated by one of the Src-family tyrosine kinases of B cells (Lyn, Fyn, or Blk) upon receptor engagement by their ligands, mediating costimulatory signals by creating docking sites

for other proteins involved in the B-cell signaling pathway. Instead of ITAMs, the cytoplasmic domains of the activating coreceptors CD45R and CD148 contain an active protein tyrosine phosphatase domain that dephosphorylates a key regulatory tyrosine at the C-terminal end of Src kinases during BCR signaling.[135,136,137,138]

While various coreceptors promote BCR signaling, there are also numerous coreceptors that are poised to counter B-cell activation. FcγRIIB, the best characterized inhibitory coreceptor for BCR signaling, is expressed on the surface of all B cells and is composed of two extracellular Ig-like domains: a transmembrane domain and a cytoplasmic domain that contains a single immunoreceptor tyrosine-based inhibitory motif (ITIM)[139] (see Fig. 9.5). When a B cell binds IgG-antigen immune complexes, FcγRIIB and the BCR are coengaged. Serum contains a relatively high concentration of soluble IgG, but the affinity of the FcγRIIB for soluble IgG is low and such that it is only able to stably engage multi-meric IgG-antigen immune complexes. The presence of IgG immune complexes indicates that sufficient antigen-specific antibody has been made to counter any foreign threat, and that antibody production can be attenuated or stopped, depending on the amount of IgG-immune complex present. Coengagement of FcγRIIB and the BCR promotes phosphorylation of the ITIM of FcγRIIB by BCR-associated Lyn, recruiting the lipid phosphatase SH2-containing inositol 5′-phosphatase (SHIP).[140] SHIP dephosphorylates PI(3,4,5) P_3 to PI(3,4)P_2, preventing the recruitment of PH domain-containing kinases (eg, BTK and PLC-γ2) to the cell membrane, thereby downregulating downstream signaling and proliferation.[141] SHIP also recruits RasGAP via the adaptor Dok-1, which inactivates Ras and thus further downregulates proliferation.[142,143]

Besides affecting downstream BCR signaling, it has also been shown that coengagement of FcγRIIB with the BCR greatly destabilizes newly formed BCR oligomers that are essential for the initiation of signaling.[144] This early point of inhibition is not influenced by interactions between the cytoplasmic tails of the two receptors but instead by the perturbation of the local lipid environment by FcγRIIB, which destabilizes BCR association with saturated lipids and Lyn.[144,145] It is of interest that a loss-of-function mutation in the transmembrane domain of FcγRIIB that is associated with the autoimmune disease systemic lupus erythematosus (SLE) prevents the association of ligated FcγRIIB with saturated lipids[146,147] and thus inhibits the ability of FcγRIIB to block BCR oligomerization and signaling. FcγRIIB may also function when

engaged by immune complexes independently of the BCR. Cross-linking FcγRIIB by immune complexes that do not contain the antigen for which the BCR is specific leads to apoptosis through a c-Abl-dependent pathway.[148,149] Recent evidence indicates that this pathway may be important in eliminating preexisting FcγRIIB-expressing plasma cells in the bone marrow after infection or immunization, creating space in the limited niches in the bone marrow for newly formed plasma cells.[150]

In addition to FcγRIIB, B cells express a variety of other coreceptors that inhibit BCR signaling via a similar ITIM-mediated mechanism, such as those shown in Table 9.2. In general, the Lyn-phosphorylated ITIMs recruit lipid phosphatases (eg, SHIP) or protein phosphatases (eg, SH2-domain-containing protein tyrosine phosphatase-1 [SHP-1]) that effectively block BCR signaling.[151] SHP-1 likely downmodulates BCR signaling by dephosphory-lating early signaling components that include BLNK.[152] Coreceptors such as FcRL2, 3, and 5 have both ITAM-like and ITIM sequences in their cytoplasmic tails, but the inhibitory effects of their ITIMs appear to dominate their regulatory function.[153,154,155] Negative modulators of BCR signaling other than FcγRIIB do not require coligation to the BCR by immune complexes to attenuate signaling but are instead regulated functionally by binding various ligands on the B-cell surface.

A recurrent theme throughout the discussion of the mechanism by which the CD19/CD21 complex and FcγRIIB function is the requirement for coligation to the BCR by complement-coupled antigens or immune complexes. The requirement for joint binding of the BCR and coreceptors to antigen-containing complexes ensures that only the antigen-specific B-cell response is regulated. However, some coreceptors that function to regulate BCR signaling do not appear to require physical cross-linking with the BCR to affect BCR signaling. In fact, for several such coreceptors their ligands, if they exist, are not known. These coreceptors raise a number of yet unanswered questions concerning how they sense that a BCR is activated and the mechanism by which they regulate BCR signaling. For example, CD19 regulates BCR signaling independently of CD21. In response to antigen presented on fluid lipid bilayers as surrogate APCs, CD19 was shown by live cell imaging to transiently associate with signaling-active BCR microclusters, mediating the recruitment and activation of associated signaling molecules and, therefore, amplifying signaling within the individual microclusters.[28,124] CD19-deficient B cells are unable to form BCR microclusters and undergo a spreading response or flux calcium in response to membrane-bound antigens, suggesting that the BCR-CD19 complex may be a basic signaling unit, essential for BCR activation. Notably, B-cell spreading is not abrogated in the absence of CD21, suggesting that CD19 can function independently of the CD19/CD21 complex. As evidence toward an independently functioning CD19, mice lacking CD19 exhibited a more severe phenotype than those lacking CD21.[156] It is interesting that a CD19 deficiency in mouse B cells does not impair the BCR response to soluble antigens,[127] suggesting that the process by which BCRs cluster in response to membrane-associated antigens provides a mechanism for recruiting CD19 to the BCR, resulting in enhanced signaling. In addition to affecting early BCR signaling, a CD19 deficiency is also implicated in impaired pre-BCR-dependent development and responses to antigen in vivo.[157,158] Another potential example of ligand-independent coreceptor regulation of BCR signaling is FcRL4, a member of the FcR-like family of proteins, which has no known ligand.[132] FcRL4 is expressed on a subpopulation of atypical memory B cells that are expanded in individuals with chronic infections, including

acquired immunodeficiency syndrome and malaria, and are hyporesponsive to BCR triggering. Antigen binding leads to association of FcRL4 with the BCR and a block in BCR signaling at the point of Syk phosphorylation[159] and eliminating FcRL4 expression in atypical memory B cells from individuals infected with human immunodeficiency virus restores BCR signaling.[160] An important unanswered question is in the absence of coligation, by what mechanism do these coreceptors associate with the BCRs? Possible suggested mechanisms are through intracellular adaptor molecules or through membrane perturbations that facilitate BCR-coreceptor interactions. In the absence of a mechanism that triggers the association of these coreceptors with the BCR, it is also not easy to guess what environmental clues these receptors are responding to.

Another family of innate immune system receptors that receive information from the B cell's environment and can affect signaling through the BCR is the TLR family. TLRs are distinctive in that they recognize highly conserved motifs present in microorganisms, including bacteria,

viruses, fungi, and protozoans, referred to as pathogen-associated molecular patterns, as described in Chapter 15.[161] As for the BCR, TLR-initiated signaling results in the activation of MAP kinases and NF-κB[162]; thus, TLR and BCR signaling have the potential to synergize. Of particular interest to BCR signaling are TLR7 and TLR9, which reside in intracellular compartments of B cells and respectively recognize pathogen-associated molecular patterns in single-stranded ribonucleic acid (ssRNA) derived from RNA viruses and un-methylated CpG-containing deoxyribonucleic acid (DNA) of bacterial and viral origin. The ssRNA-induced TLR7 signaling and CpG-containing DNA-induced TLR9 signaling have been shown to synergize with antigen-induced BCR signaling in NF-κB activation.[162] It was recently shown that synergistic signaling in response to CpG-containing DNA-containing antigens is mediated by a novel mechanism in which, following antigen binding, the BCR signals from the plasma membrane to recruit TLR9 from small endocytic intracellular vesicles to autophagosomal compartments into which the BCR traffics the CpG-containing antigen and from which synergistic signaling occurs.[163] By this mechanism, TLR9 can alert the BCR to the presence of pathogenic DNA in the environment and heighten BCR signaling. Similar mechanisms may be at play for BCR and TLR7 signaling in response to ssRNA-containing antigens.

ABNORMAL B-CELL RECEPTOR SIGNALING IN HUMAN DISEASE

In this chapter, we have provided an overview of our current understanding of the molecular mechanisms underlying the early BCR-intrinsic events that lead to normal BCR oligomerization and subsequent signaling and have emphasized that early events in the initiation of BCR signaling are tightly regulated. It might be predicted that alterations in any step of the initiation of signaling could drive the BCR to hyperactivation. For example, the outcome of antigen engagement could be affected by mutations in the BCR itself, changes in the composition of the membrane lipids that stabilize BCR oligomers, or mutations affecting the activity of coreceptors or the kinases in the BCR signaling pathway. Hyperactivation of the BCR is associated with both B-cell tumors and autoimmune disease. In the future, it may be possible to develop new therapeutic strategies that target spontaneous BCR oligomerization clustering and chronic active signaling to treat B-cell tumors and systemic autoimmune disease.

The BCR is required for the survival of the activated B cell-like (ABC) subtype of diffuse large B-cell lymphomas (DLBCLs).[164] Using live cell imaging, the BCRs on these activated DLBCLs were found to form immobile oligomers within microclusters similar to those observed on normal antigen-stimulated B cells. In contrast, the BCRs on Burkitt's lymphoma, mantle cell lymphoma, and germinal center B cell-like DLBCL tumors, which are not dependent on the BCR for survival, show no clustering. Somatic mutations affecting the cytoplasmic domains of Igα and Igβ are detected frequently in ABC DLBCL biopsy samples but rarely for other DLBCLs and never for Burkitt lymphoma or mucosa-associated lymphoid tissue (MALT) lymphoma, although the contributions of these mutations to chronic BCR clustering is not known.

Interestingly, the spontaneous BCR clustering on ABC DLBCLs is similar to that observed for human H chain disease in which ligand-independent BCR self-aggregation and constitutive activation are a consequence of BCR H chain gene mutations that cause misfolding and disrupt antigen binding.[165] Impaired glycosylation and folding of the μ-H chain are implicated in chronic lymphocytic leukemia, which has symptoms similar to those of μ-H chain disease; however, it has not yet been determined whether this misfolding leads to enhanced BCR ligand-independent, or chronic active, signaling.

In addition to the BCR itself, other BCR signaling molecules that are key regulators of the NF-κB pathway are also known targets of mutations found in B-cell lymphomas. Specifically, MALT1 and Bcl10 are independently associated with chromosomal translocations in MALT lymphoma, whereas CARD11 has activating point mutations in a percentage of ABC DLBCLs that result in constitutive NF-κB activation.[113,165,166,167] The ABC DLBCLs are also dependent for their survival on MyD88, the adaptor that mediates TLR signaling.[168] Remarkably, nearly 30% of ABC DLBCLs have a single gain-of-function mutation in MyD88 that promotes cell survival by activating the NF-κB pathway. The dual dependence on both the BCR and TLR pathways suggest that these work in concert to drive B-cell tumorigenesis.

B cells from patients with systemic autoimmune disease exhibit B-cell hyperactivation that may be due to alterations in the BCR signaling pathway or alterations in coreceptor

function.[169] In SLE, stimulating peripheral blood B cells with BCR ligand leads to increased intracellular calcium flux and increased phosphorylation of various cytosolic proteins as compared to healthy individuals and patients with other rheumatic diseases.[170] In most of patients with SLE analyzed, expression of Lyn is also significantly decreased in resting, and BCR stimulated peripheral blood B cells[171]; the ability of Lyn to associate with saturated membrane lipids is reduced in SLE B cells as compared with healthy donors.[172]

Decreased Lyn expression negatively impacts its ability to inhibit BCR signaling via phosphorylation of FcγRIIB and other inhibitory coreceptors that contain ITIMs. In addition, a loss-of-function mutation in the transmembrane domain of FcγRIIB that is associated with SLE susceptibility prevents BCR-ligated FcγRIIB from associating with saturated membrane lipids[146] and consequently inhibits the ability of FcγRIIB to block BCR oligomerization, clustering, and subsequent signaling. The synergistic engagement of the BCR and TLR7 and TLR9 in response to antigens containing ssRNA or CpG-containing DNA has also been implicated in the activation of autoimmune B cells. Many of the antigens targeted in SLE contain DNA and RNA that are thought to be released from apoptotic cells. Indeed, genetic variations in TLR9 are linked to SLE susceptibility[173]; in a mouse model of SLE, multiple copies of a normal TLR7 gene are sufficient to drive SLE.[174] Overall, it seems that enhanced B-cell signaling as a result of a multiple genetic abnormalities is the defining pathogenic event of SLE and provides a therapeutic target for treating autoimmune disease.

BCR signaling can also be altered in infectious diseases by viruses that commandeer B cells for their own replication. Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis and is implicated in Burkitt's lymphoma, Hodgkin disease, nasopharyngeal carcinoma, and various lymphoproliferative disorders that arise in immunocompromised individuals.[175] EBV infects B cells of the oral epithelium and establishes a lifelong latent infection in a subset of those cells.[176] Latent membrane protein 2A (LMP2A) messenger RNA is readily detected in the peripheral blood B cells of healthy individuals, and LMP2A is often found in tumors from EBV-associated malignancies.[177] To subvert immune responses and consequently maintain viral latency in B cells, EBV blocks signaling and antigen-trafficking functions of the BCR through the activity of the cytoplasmic tail of LMP2A[178,179,180] that, when phosphorylated, binds to Lyn (Y112) and Syk (Y74, Y85),[181,182] thus blocking BCR signaling. LMP2A also activates the PI3K/Akt pathway, which normally provides a survival signal in response to BCR signaling.[183] Overall, LMP2A maintains viral latency by preventing normal BCR activation, which initiates viral replication, while sustaining survival pathways in latently infected B cells.

CONCLUSION

B-cell activation is initiated by the binding of antigen to the BCR resulting in the triggering of a number of signaling pathways that ultimately drive B-cell proliferation and differentiation to antibody-secreting cells. At present, we understand the biochemical nature of the signaling pathway in some detail. What remains less well understood is the nature of the events following antigen binding to the BCR that trigger these signaling cascades. New tools of live cell imaging both in vitro and in vivo are anticipated to provide an increasingly detailed spatial and temporal picture of events that initiate signaling in both time and space. Our increased understanding of B-cell activation is likely to lead to new approaches for developing therapies for diseases caused by hyper-B-cell activation.

REFERENCES

1. Kaisho T, Schwenk F, Rajewsky K. The roles of gamma 1 heavy chain membrane expression and cytoplasmic tail in IgG1 responses. *Science*. 1997;276:412-415.

2. Martin SW, Goodnow CC. Burst-enhancing role of the IgG membrane tail as a molecular determinant of memory. *Nat Immunol*. 2002;3:182-188.

3. Engels N, Konig LM, Heemann C, et al. Recruitment of the cytoplasmic adaptor Grb2 to surface IgG and IgE provides antigen receptor-intrinsic costimulation to class-switched B cells. *Nat Immunol*. 2009;10:1018-1025.

4. Reth M. Antigen receptors on B lymphocytes. *Annu Rev Immunol*. 1992;10:97-121.

5. Tolar P, Sohn HW, Pierce SK. The initiation of antigen-induced B cell antigen receptor signaling viewed in living cells by fluorescence resonance energy transfer. *Nat Immunol*. 2005;6:1168-1176.

6. Schamel WW, Reth M. Monomeric and oligomeric complexes of the B cell antigen receptor. *Immunity*. 2000;13:5-14.

7. Metzger H. Effect of antigen binding on the properties of antibody. *Adv Immunol*. 1974;18:169-207.

8. Radaev S, Zou Z, Tolar P, et al. Structural and functional studies of Igalphabeta and its assembly with the B cell antigen receptor. *Structure*. 2010;18:934-943.

9. Kusumi A, Nakada C, Ritchie K, et al. Paradigm shift of the plasma membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-speed single-molecule tracking of membrane molecules. *Annu Rev Biophys Biomol Struct*. 2005;34:351-378.

10. Kusumi A, Shirai YM, Koyama-Honda I, Suzuki KG, Fujiwara TK. Hierarchical organization of the plasma membrane: investigations by single-molecule tracking vs. fluorescence correlation spectroscopy. *FEBS Lett*. 2010;584:1814-1823.

11. DeFranco AL. The complexity of signaling pathways activated by the BCR. *Curr Opin Immunol*. 1997;9:296-308.

12. Dal Porto JM, Gauld SB, Merrell KT, Mills D, Pugh-Bernard AE, Cambier J. B cell antigen receptor signaling 101. *Mol Immunol*. 2004;41:599-613.

13. Batista FD, Iber D, Neuberger MS. B cells acquire antigen from target cells after synapse formation. *Nature*. 2001;411:489-494.

14. Carrasco YR, Batista FD. B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. *Immunity*. 2007;27:160-171.

15. Junt T, Moseman EA, Iannacone M, et al. Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature*. 2007;450:110-114.

16. Phan TG, Grigorova I, Okada T, Cyster JG. Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells. *Nat Immunol*. 2007;8:992-1000.

17. Qi H, Egen JG, Huang AY, Germain RN. Extrafollicular activation of lymph node B cells by antigen-bearing dendritic cells. *Science*. 2006;312:1672-1676.

18. Schwickert TA, Lindquist RL, Shakhar G, et al. In vivo imaging of germinal centres reveals a dynamic open structure. *Nature*. 2007;446:83-87.

19. Harwood NE, Batista FD. Antigen presentation to B cells. *F1000 Biol Rep*. 2010;2:87.

20. Batista FD, Harwood NE. The who, how and where of antigen presentation to B cells. *Nat Rev Immunol*. 2009;9:15-27.

21. Batista FD, Treanor B, Harwood NE. Visualizing a role for the actin cytoskeleton in the regulation of B-cell activation. *Immunol Rev*. 2010;237:191-204.

22. Pierce SK, Liu W. The tipping points in the initiation of B cell signalling: how small changes make big differences. *Nat Rev Immunol*. 2010;10:767-777.

23. Fleire SJ, Goldman JP, Carrasco YR, Weber M, Bray D, Batista FD. B cell ligand discrimination through a spreading and contraction response. *Science*. 2006;312:738-741.

24. Liu W, Meckel T, Tolar P, Sohn HW, Pierce SK. Antigen affinity discrimination is an intrinsic function of the B cell receptor. *J Exp Med*. 2010;207:1095-1111.

25. Tolar P, Hanna J, Krueger PD, Pierce SK. The constant region of the membrane immunoglobulin mediates B cell-receptor clustering and signaling in response to membrane antigens. *Immunity*. 2009;30:44-55.

26. Arana E, Vehlow A, Harwood NE, et al. Activation of the small GTPase Rac2 via the B cell receptor regulates B cell adhesion and immunological-synapse formation. *Immunity*. 2008;28:88-99.

27. Lin KB, Freeman SA, Cabetian S, et al. The rap GTPases regulate B cell morphology, immune-synapse formation, and signaling by particulate B cell receptor ligands. *Immunity*. 2008;28:75-87.

28. Weber M, Treanor B, Depoil D, et al. Phospholipase C-γ2 and Vav cooperate within signaling microclusters to propagate B cell spreading in response to membrane-bound antigen. *J Exp Med*. 2008;205:853-868.

29. Treanor B, Depoil D, Gonzalez-Granja A, et al. The membrane skeleton controls diffusion dynamics and signaling through the B cell receptor. *Immunity*. 2010;32:187-199.

30. Yang J, Reth M. Oligomeric organization of the B-cell antigen receptor on resting cells. *Nature*. 2010;467:465-469.

31. Gray D. Immunological memory. *Annu Rev Immunol*. 1993;11:49-77.

32. Brink R, Phan TG, Paus D, Chan TD. Visualizing the effects of antigen affinity on T-dependent B-cell differentiation. *Immunol Cell Biol*. 2008;86:31-39.

33. Dal Porto JM, Haberman AM, Kelsoe G, Shlomchik MJ. Very low affinity B cells form germinal centers, become memory B cells, and participate in secondary immune responses when higher affinity competition is reduced. *J Exp Med*. 2002;195:1215-1221.

34. Shih TA, Meffre E, Roederer M, Nussenzweig MC. Role of BCR affinity in T cell dependent antibody responses in vivo. *Nat Immunol*. 2002;3:570-575.

35. Shih TA, Roederer M, Nussenzweig MC. Role of antigen receptor affinity in T cell-independent antibody responses in vivo. *Nat Immunol*. 2002;3:399-406.

36. Takahashi Y, Dutta PR, Cerasoli DM, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. V. Affinity maturation develops in two stages of clonal selection. *J Exp Med*. 1998;187:885-895.

37. Paus D, Phan TG, Chan TD, Gardam S, Basten A, Brink R. Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. *J Exp Med*. 2006;203:1081-1091.

38. Phan TG, Paus D, Chan TD, et al. High affinity germinal center B cells are actively selected into the plasma cell compartment. *J Exp Med*. 2006;203:2419-2424.

39. Kouskoff V, Famiglietti S, Lacaud G, et al. Antigens varying in affinity for the B cell receptor induce differential B lymphocyte responses. *J Exp Med*. 1998;188:1453-1464.

40. Horikawa K, Martin SW, Poque SL, et al. Enhancement and suppression of signaling

by the conserved tail of IgG memory-type B cell antigen receptors. *J Exp Med*. 2007;204:759-769.

41. Waisman A, Kraus M, Seagal J, et al. IgG1 B cell receptor signaling is inhibited by CD22 and promotes the development of B cells whose survival is less dependent on Ig alpha/beta. *J Exp Med*. 2007;204: 747-758.

42. Wakabayashi C, Adachi T, Wienands J, Tsubata T. A distinct signaling pathway used by the IgG-containing B cell antigen receptor. *Science*. 2002;298:2392-2395.

43. Liu W, Meckel T, Tolar P, Sohn HW, Pierce SK. Intrinsic properties of immunoglobulin IgG1 isotype-switched B cell receptors promote microclustering and the initiation of signaling. *Immunity*. 2010;32: 778-789.

44. Sohn HW, Tolar P, Pierce SK. Membrane heterogeneities in the formation of B cell receptor-Lyn kinase microclusters and the immune synapse. *J Cell Biol* . 2008;182:367-379.

45. Saouaf SJ, Mahajan S, Rowley RB, et al. Temporal differences in the activation of three classes of non-transmembrane protein tyrosine kinases following B-cell antigen receptor surface engagement. *Proc Natl Acad Sci U S A* . 1994;91:9524-9528.

46. Weber M, Treanor B, Depoil D, et al. Phospholipase C-γ2 and Vav cooperate within signaling microclusters to propagate B cell spreading in response to membrane-bound antigen. *J Exp Med*. 2008;205:853-868.

47. Smith CI, Baskin B, Humire-Greiff P, et al. Expression of Bruton's agammaglobulinemia tyrosine kinase gene, BTK, is selectively down-regulated in T lymphocytes and plasma cells. *J Immunol* . 1994;152: 557-565.

48. Rawlings DJ, Witte ON. Bruton's tyrosine kinase is a key regulator in B-cell development. *Immunol Rev*. 1994;138:105-119.

49. Tsukada S, Saffran DC, Rawlings DJ, et al. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell*. 1993;72:279.

50. Vetrie D, Vorechovsky I, Sideras P, et al. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. *Nature*. 1993;361:226-233.

51. Rawlings DJ, Saffran DC, Tsukada S, et al. Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. *Science*. 1993;261:358.

52. Valiaho J, Smith CI, Vihinen M. BTKbase: the mutation database for X-linked agammaglobulinemia. *Human Mutation*. 2006;27:1209-1217.

53. Mahajan S, Fargnoli J, Burkhardt AL, Kut SA, Saouaf SJ, Bolen JB. Src family protein tyrosine kinases induce autoactivation of Bruton's tyrosine kinase. *Mol Cell Biol* . 1995;15:5304-5311.

54. Rawlings DJ, Scharenberg AM, Park H, et al. Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. *Science*. 1996;271:822-825.

55. Scharenberg AM, Kinet JP. PtdIns-3,4,5-P3: a regulatory nexus between tyrosine kinases and sustained calcium signals. *Cell*. 1998;94:5-8.

56. Park H, Wahl MI, Afar DE, et al. Regulation of Btk function by a major autophosphorylation site within the SH3 domain. *Immunity*. 1996;4:515-525.

57. Law CL, Chandran KA, Sidorenko SP, Clark EA. Phospholipase C-γ1 interacts with conserved phosphotyrosyl residues in the linker region of Syk and is a substrate for Syk.

Mol Cell Biol. 1996;16:1305-1315.

58. Richards JD, Gold MR, Hourihane SL, DeFranco AL, Matsuuchi L. Reconstitution of B cell antigen receptor-induced signaling events in a nonlymphoid cell line by expressing the Syk protein-tyrosine kinase. *J Biol Chem*. 1996;271:6458-6466.

59. Patterson HC, Kraus M, Kim YM, Ploegh H, Rajewsky K. The B cell receptor promotes B cell activation and proliferation through a non-ITAM tyrosine in the Igalpha cytoplasmic domain. *Immunity*. 2006;25:55-65.

60. Takata M, Sabe H, Hata A, et al. Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca2+ mobilization through distinct pathways. *EMBO J*. 1994;13:1341-1349.

61. Takata M, Kurosaki T. A role for Bruton's tyrosine kinase in B cell antigen receptor-mediated activation of phospholipase C-γ2. *J Exp Med*. 1996;184:31-40.

62. Chiu CW, Dalton M, Ishiai M, Kurosaki T, Chan AC. BLNK: molecular scaffolding through 'cis'-mediated organization of signaling proteins. *EMBO J*. 2002;21:6461-6472.

63. Fu C, Turck CW, Kurosaki T, Chan AC. BLNK: a central linker protein in B cell activation. *Immunity*. 1998;9:93-103.

64. Hashimoto S, Iwamatsu A, Ishiai M, et al. Identification of the SH2 domain binding protein of Bruton's tyrosine kinase as BLNK—functional significance of Btk-SH2 domain in B-cell antigen receptor-coupled calcium signaling. *Blood*. 1999;94:2357-2364.

65. Ishiai M, Sugawara H, Kurosaki M, Kurosaki T. Cutting edge: association of phospholipase C-γ2 Src homology 2 domains with BLNK is critical for B cell antigen receptor signaling. *J Immunol*. 1999;163:1746-1749.

66. Humphries LA, Dangelmaier C, Sommer K, et al. Tec kinases mediate sustained calcium influx via site-specific tyrosine phosphorylation of the phospholipase C-γ Src homology 2-Src homology 3 linker. *J Biol Chem*. 2004;279:37651-37661.

67. Kim YJ, Sekiya F, Poulin B, Bae YS, Rhee SG. Mechanism of B-cell receptor-induced phosphorylation and activation of phospholipase C-γ2. *Mol Cell Biol*. 2004;24:9986-9999.

68. Watanabe D, Hashimoto S, Ishiai M, et al. Four tyrosine residues in phospholipase C-γ2, identified as Btk-dependent phosphorylation sites, are required for B cell antigen receptor-coupled calcium signaling. *J Biol Chem*. 2001;276:38595-38601.

69. Kurosaki T, Tsukada S. BLNK: connecting Syk and Btk to calcium signals. *Immunity*. 2000;12:1-5.

70. Saito K, Tolias KF, Saci A, et al. BTK regulates PtdIns-4,5-P2 synthesis: importance for calcium signaling and PI3K activity. *Immunity*. 2003;19:669-678.

71. Johmura S, Oh-hora M, Inabe K, et al. Regulation of Vav localization in membrane rafts by adaptor molecules Grb2 and BLNK. *Immunity*. 2003;18:777-787.

72. Limon JJ, Fruman DA. B cell receptor signaling: picky about PI3Ks. *Sci Signal*. 2010;3:pe25.

73. Okkenhaug K, Ali K, Vanhaesebroeck B. Antigen receptor signalling: a distinctive role for the p110delta isoform of PI3K. *Trends Immunol*. 2007;28:80-87.

74. Bilancio A, Okkenhaug K, Camps M, et al. Key role of the p110delta isoform of PI3K in B-cell antigen and IL-4 receptor signaling: comparative analysis of genetic and pharmacologic interference with p110delta function in B cells. *Blood*. 2006;107:642-650.

75. Yusuf I, Zhu X, Kharas MG, Chen J, Fruman DA. Optimal B-cell proliferation requires phosphoinositide 3-kinase-dependent inactivation of FOXO transcription factors. *Blood*. 2004;104:784-787.

76. Glassford J, Vigorito E, Soeiro I, et al. Phosphatidylinositol 3-kinase is required for the transcriptional activation of cyclin D2 in BCR activated primary mouse B lymphocytes. *Eur J Immunol* . 2005;35:2748-2761.

77. Donahue AC, Fruman DA. Proliferation and survival of activated B cells requires sustained antigen receptor engagement and phosphoinositide 3-kinase activation. *J Immunol*. 2003;170:5851-5860.

78. Vigorito E, Kovesdi D, Turner M. Synergistic activation of PKD by the B cell antigen receptor and CD19 requires PI3K, Vav1 and PLCgamma. *Cell Signal*. 2006;18:1455-1460.

79. Doughty CA, Bleiman BF, Wagner DJ, et al. Antigen receptor-mediated changes in glucose metabolism in B lymphocytes: role of phos-phatidylinositol 3-kinase signaling in the glycolytic control of growth. *Blood*. 2006;107:4458-4465.

80. Piatelli MJ, Wardle C, Blois J, et al. Phosphatidylinositol 3-kinase-dependent mitogen-activated protein/extracellular signal-regulated kinase kinase 1/2 and NF-kappa B signaling pathways are required for B cell antigen receptor-mediated cyclin D2 induction in mature B cells. *J Immunol*. 2004;172:2753-2762.

81. Okada T, Maeda A, Iwamatsu A, Gotoh K, Kurosaki T. BCAP: the tyrosine kinase substrate that connects B cell receptor to phosphoinositide 3-kinase activation. *Immunity*. 2000;13:817-827.

82. Yamazaki T, Takeda K, Gotoh K, Takeshima H, Akira S, Kurosaki T. Essential immunoregulatory role for BCAP in B cell development and function. *J Exp Med*. 2002;195:535-545.

83. Aiba Y, Kameyama M, Yamazaki T, Tedder TF, Kurosaki T. Regulation of B-cell development by BCAP and CD19 through their binding to phosphoinositide 3-kinase. *Blood*. 2008;111:1497-1503.

84. Delgado P, Cubelos B, Calleja E, et al. Essential function for the GTPase TC21 in homeostatic antigen receptor signaling. *Nat Immunol*. 2009;10:880-888.

85. Dowler S, Currie RA, Downes CP, Alessi DR. DAPP1: a dual adaptor for phosphotyrosine and 3-phosphoinositides. *Biochem J*. 1999; 342(pt 1):7-12.

86. Anderson KE, Lipp P, Bootman M, et al. DAPP1 undergoes a PI 3- kinase-dependent cycle of plasma-membrane recruitment and endocytosis upon cell stimulation. *Curr Biol* . 2000;10:1403-1412.

87. Rao VR, Corradetti MN, Chen J, et al. Expression cloning of protein targets for 3-phosphorylated phosphoinositides. *J Biol Chem*. 1999;274:37893-37900.

88. Marshall AJ, Niiro H, Lerner CG, et al. A novel B lymphocyte-associated adaptor protein, Bam32, regulates antigen receptor signaling downstream of phosphatidylinositol 3-kinase. *J Exp Med*. 2000;191: 1319-1332.

89. Han A, Saijo K, Mecklenbrauker I, Tarakhovsky A, Nussenzweig MC. Bam32 links the B cell receptor to ERK and JNK and mediates B cell proliferation but not survival. *Immunity*. 2003;19:621-632.

90. Allam A, Niiro H, Clark EA, Marshall AJ. The adaptor protein Bam32 regulates Rac1 activation and actin remodeling through a phosphorylation-dependent mechanism. *J Biol*

Chem. 2004;279:39775-39782.

91. Foskett JK, White C, Cheung KH, Mak DO. Inositol trisphosphate receptor Ca2+ release channels. *Physiol Rev*. 2007;87:593-658.

92. Liou J, Kim ML, Heo WD, et al. STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. *Curr Biol* . 2005;15:1235-1241.

93. Roos J, DiGregorio PH, Yeromin AV, et al. STIM1, an essential and conserved component of store-operated Ca2+ channel function. *J Cell Biol* . 2005;169:435-445.

94. Prakriya M, Feske S, Gwack Y, Srikanth S, Rao A, Hogan PG. Orai1 is an essential pore subunit of the CRAC channel. *Nature*. 2006;443: 230-233.

95. Vig M, Beck A, Billingsley JM, et al. CRACM1 multimers form the ion-selective pore of the CRAC channel. *Curr Biol* . 2006;16:2073-2079.

96. Baba Y, Hayashi K, Fujii Y, et al. Coupling of STIM1 to store-operated Ca2+ entry through its constitutive and inducible movement in the endoplasmic reticulum. *Proc Natl Acad Sci U S A*. 2006;103:16704-16709.

97. Feske S, Gwack Y, Prakriya M, et al. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature*. 2006;441:179-185.

98. Picard C, McCarl CA, Papolos A, et al. STIM1 mutation associated with a syndrome of immunodeficiency and autoimmunity. *N Engl J Med* . 2009;360:1971-1980.

99. Klein U, Casola S, Cattoretti G, et al. Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. *Nat Immunol* . 2006;7:773-782.

100. Sciammas R, Shaffer AL, Schatz JH, Zhao H, Staudt LM, Singh H. Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. *Immunity*. 2006;25: 225-236.

101. Kurosaki T, Shinohara H, Baba Y. B cell signaling and fate decision. *Annu Rev Immunol*. 2010;28:21-55.

102. Winslow MM, Gallo EM, Neilson JR, Crabtree GR. The calcineurin phosphatase complex modulates immunogenic B cell responses. *Immunity*. 2006;24:141-152.

103. Hacker H, Karin M. Regulation and function of IKK and IKK-related kinases. *Sci STKE*. 2006:re13.

104. Li Q, Verma IM. NF-kappaB regulation in the immune system. *Nat Rev Immunol* . 2002;2:725-734.

105. Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. *Cell*. 2008;132:344-362.

106. Saijo K, Mecklenbrauker I, Santana A, Leitger M, Schmedt C, Tarakhovsky A. Protein kinase C beta controls nuclear factor kappaB activation in B cells through selective regulation of the IkappaB kinase alpha. *J Exp Med*. 2002;195:1647-1652.

107. Dierlamm J, Baens M, Wlodarska I, et al. The apoptosis inhibitor gene API2 and a novel 18q gene, MLT, are recurrently rearranged in the t(11;18)(q21;q21) associated with mucosa-associated lymphoid tissue lymphomas. *Blood*. 1999;93:3601-3609.

108. Zhang Q, Siebert R, Yan M, et al. Inactivating mutations and overex-pression of BCL10, a caspase recruitment domain-containing gene, in MALT lymphoma with t(1;14)(p22;q32). *Nat Genet*. 1999;22:63-68.

109. Willis TG, Jadayel DM, Du MQ, et al. Bcl10 is involved in t(1;14) (p22;q32) of MALT B cell lymphoma and mutated in multiple tumor types. *Cell*. 1999;96:35-45.

110. Morgan JA, Yin Y, Borowsky AD, et al. Breakpoints of the t(11;18) (q21;q21) in mucosa-associated lymphoid tissue (MALT) lymphoma lie within or near the previously undescribed gene MALT1 in chromosome 18. *Cancer Res*. 1999;59:6205-6213.

111. Uren AG, O'Rourke K, Aravind LA, et al. Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. *Mol Cell*. 2000;6:961-967.

112. Streubel B, Lamprecht A, Dierlamm J, et al. T(14;18)(q32;q21) involving IGH and MALT1 is a frequent chromosomal aberration in MALT lymphoma. *Blood*. 2003;101:2335-2339.

113. Lenz G, Davis RE, Ngo VN, et al. Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. *Science*. 2008;319:1676-1679.

114. Oh-hora M, Johmura S, Hashimoto A, Hikida M, Kurosaki T. Requirement for Ras guanine nucleotide releasing protein 3 in coupling phospholipase C-γ2 to Ras in B cell receptor signaling. *J Exp Med*. 2003;198:1841-1851.

115. Coughlin JJ, Stang SL, Dower NA, Stone JC. RasGRP1 and RasGRP3 regulate B cell proliferation by facilitating B cell receptor-Ras signaling. *J Immunol*. 2005;175:7179-7184.

116. Iritani BM, Forbush KA, Farrar MA, Perlmutter RM. Control of B cell development by Ras-mediated activation of Raf. *EMBO J*. 1997;16:7019-7031.

117. Nagaoka H, Takahashi Y, Hayashi R, et al. Ras mediates effector pathways responsible for pre-B cell survival, which is essential for the developmental progression to the late pre-B cell stage. *J Exp Med*. 2000;192:171-182.

118. Maltzman JS, Carman JA, Monroe JG. Role of EGR1 in regulation of stimulus-dependent CD44 transcription in B lymphocytes. *Mol Cell Biol*. 1996;16:2283-2294.

119. Huang H, Tindall DJ. Dynamic FoxO transcription factors. *J Cell Sci*. 2007;120:2479-2487.

120. Fruman DA. Phosphoinositide 3-kinase and its targets in B-cell and T-cell signaling. *Curr Opin Immunol*. 2004;16:314-320.

121. Fruman DA, Bismuth G. Fine tuning the immune response with PI3K. *Immunol Rev*. 2009;228:253-272.

122. Ono M, Bolland S, Tempst P, Ravetch JV. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc(gamma)RIIB. *Nature*. 1996;383:263-266.

123. Sohn HW, Tolar P, Pierce SK. Membrane heterogeneities in the formation of B cell receptor-Lyn kinase microclusters and the immune synapse. *J Cell Biol*. 2008;182:367-379.

124. Depoil D, Fleire S, Treanor BL, et al. CD19 is essential for B cell activation by promoting B cell receptor-antigen microcluster formation in response to membrane-bound ligand. *Nat Immunol*. 2008;9:63-72.

125. Chaturvedi A, Martz R, Dorward D, Waisberg M, Pierce SK. Endocytosed B cell receptors sequentially regulate MAP kinase and Akt signaling pathways from intracellular compartments. *Nat Immunol*. 2011;12(11):1119-1126.

126. Fearon DT, Carter RH. The CD19/CR2/TAPA-1 complex of B lymphocytes: Linking natural to acquired immunity. *Annu Rev Immunol*. 1995;13:127-149.

127. Sato S, Miller A, Howard M, Tedder T. Regulation of B lymphocyte development and activation by the CD19/CD21/CD81/Leu 13 complex requires the cytoplasmic domain of CD19. *J Immunol*. 1997;159:3278-3287.

128. Fujimoto M, Fujimoto Y, Poe JC, et al. CD19 regulates Src family protein tyrosine kinase activation in B lymphocytes through processive amplification. *Immunity*. 2000;13:47-57.

129. Cherukuri A, Shoham T, Sohn HW, et al. The tetraspanin CD81 is necessary for partitioning of coligated CD19/CD21-B cell antigen receptor complexes into signaling-active lipid rafts. *J Immunol*. 2004;172: 370-380.

130. Levy S, Todd SC, Maecker HT. CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. *Annu Rev Immunol*. 1998;16:89-109.

131. Kubagawa H, Oka S, Kubagawa Y, et al. Identity of the elusive IgM Fc receptor (FcmR) in humans. *J Exp Med*. 2009;206:2779-2793.

132. Davis RS. Fc receptor-like molecules. *Annu Rev Immunol*. 2007;25: 525-560.

133. Kubagawa H, Burrows PD, Cooper MD. A novel pair of immunoglobulin-like receptors expressed by B cells and myeloid cells. *Proc Natl Acad Sci U S A*. 1997;94:5261-5266.

134. Hayami K, Fukuta D, Nishikawa Y, et al. Molecular cloning of a novel murine cell-surface glycoprotein homologous to killer cell inhibitory receptors. *J Biol Chem*. 1997;272:7320-7327.

135. Justement LB, Campbell KS, Chien NC, Cambier JC. Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. *Science*. 1991;252:1839-1842.

136. Trowbridge IS, Thomas ML. CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. *Annu Rev Immunol*. 1994;12:85-116.

137. Hermiston ML, Zikherman J, Zhu JW. CD45, CD148, and Lyp/Pep: critical phosphatases regulating Src family kinase signaling networks in immune cells. *Immunol Rev*. 2009;228:288-311.

138. Zhu JW, Brdicka T, Katsumoto TR, Lin J, Weiss A. Structurally distinct phosphatases CD45 and CD148 both regulate B cell and macrophage immunoreceptor signaling. *Immunity*. 2008;28:183-196.

139. Nimmerjahn F, Ravetch JV. Fcg receptors as regulators of immune responses. *Nat Rev Immunol*. 2008;8:34-47.

140. Cambier JC, Fong D, Tamir I. The unexpected complexity of Fc gamma RIIB signal transduction. *Curr Top Microbiol Immunol*. 1999;244:43-55.

141. Ravetch JV, Bolland S. IgG Fc receptors. *Annu Rev Immunol*. 2001;19: 275-290.

142. Yamanashi Y, Tamura T, Kanamori T, et al. Role of the rasGAP-associated docking protein p62(dok) in negative regulation of B cell receptor-mediated signaling. *Genes Dev*. 2000;14:11-16.

143. Tamir I, Stolpa JC, Helgason CD, et al. The RasGAP-binding protein-p62dok is a mediator of inhibitory FcgammaRIIB signals in B cells. *Immunity*. 2000;12:347-358.

144. Liu W, Won Sohn H, Tolar P, Meckel T, Pierce SK. Antigen-induced oligomerization of the B cell receptor is an early target of FcgRIIB inhibition. *J Immunol*. 2010;184:1977-1989.

145. Sohn HW, Pierce SK, Tzeng SJ. Live cell imaging reveals that the inhibitory FcgammaRIIB destabilizes B cell receptor membrane-lipid interactions and blocks immune synapse formation. *J Immunol*. 2008;180:793-799.

146. Floto RA, Clatworhty MR, Heilbronn KR, et al. Loss of function of a lupus-associated FcgRIIb polymorphism through exclusion from lipid rafts. *Nat Med*. 2005;11:1056-1058.

147. Kono H, Kyogoky C, Suzuki T, et al. FcgRIIB Ile232Thr transmembrane polymorphism associated with human systemic lupus erythematosus decreases affinity to lipid rafts and attenuates inhibitory effects on B cell receptor signaling. *Hum Mol Genet*. 2005;14: 2881-2892.

148. Pearse RN, Kawabe T, Bolland S, Guinamard R, Kurosaki T, Ravetch JV. SHIP recruitment attenuates FcgRIIB-induced B cell apoptosis. *Immunity*. 1999;10:753-760.

149. Tzeng SJ, Bolland S, Inabe K, Kurosaki T, Pierce SK. The B cell inhibitory Fc receptor triggers apoptosis by a novel c-Abl family kinase-dependent pathway. *J Biol Chem*. 2005;280:35247-35254.

150. Xiang Z, Cutler AJ, Brownlie RJ, et al. FcgRIIb controls bone marrow plasma cell persistence and apoptosis. *Nat Immunol*. 2007;8: 419-429.

151. D'Ambrosio D, Hippen KL, Minskoff SA, et al. Recruitment and activation of PTP1C in negative regulation of antigen receptor signaling by FcgRIIB1. *Science*. 1995;268:293-297.

152. Mizuno K, Tagawa Y, Mitomo K, et al. Src homology region 2 (SH2) domain-containing phosphatase-1-dephosphorylates B cell linker protein/SH2 domain leukocyte protein of 65 kDa and selectively regulates c-Jun NH2-terminal kinase activation in B cells. *J Immunol*. 2000; 165:1344-1351.

153. Jackson TA, Haga CL, Ehrhardt GR, Davis RS, Cooper MD. FcR-like 2 inhibition of B cell receptor-mediated activation of B cells. *J Immunol*. 2010;185:7405-7412.

154. Kochi Y, Myouzen K, Yamada R, et al. FCRL3, an autoimmune susceptibility gene, has inhibitory potential on B-cell receptor-mediated signaling. *J Immunol*. 2009;183:5502-5510.

155. Haga CL, Ehrhardt GR, Boohaker RJ, Davis RS, Cooper MD. Fc receptor-like 5 inhibits B cell activation via SHP-1 tyrosine phosphatase recruitment. *Proc Natl Acad Sci U S A*. 2007;104:9770-9775.

156. Fearon DT, Carroll MC. Regulation of B lymphocyte responses to foreign and self-antigens by the CD19/CD21 complex. *Annu Rev Immunol*. 2000;18:393-422.

157. Tedder TF, Inaoki M, Sato S. The CD19-CD21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity. *Immunity*. 1997;6:107-118.

158. Otero DC, Rickert RC. CD19 function in early and late B cell development. II. CD19 facilitates the pro-B/pre-B transition. *J Immunol*. 2003;171:5921-5930.

159. Sohn HW, Krueger PD, Davis RS, Pierce SK. FcRL4 acts as an adaptive to innate molecular switch dampening B cell receptor signaling and enhancing Toll-like receptor signaling. *Blood*. 2011;118(24):6332-6341.

160. Kardava L, Moir S, Wang W, et al. Attenuation of HIV-associated human B cell exhaustion by siRNA downregulation of inhibitory receptors. *J Clin Invest*. 2011;121:2614.

161. Janeway CAJ, Medzhitov R. Innate immune recognition. *Annu Rev Immunol*. 2002;20:197-216.

162. Takeda K, Akira S. TLR signaling pathways. *Semin Immunol*. 2004;16:3-9.

163. Chaturvedi A, Dorward D, Pierce SK. The B cell receptor governs the subcellular location of Toll-like receptor 9 leading to hyperresponses to DNA-containing antigens. *Immunity*. 2008;28:799-809.

164. Davis RE, Ngo VN, Lenz G, et al. Chronic active B cell receptor signaling in diffuse large B cell lymphoma. *Nature*. 2010;463:88-92.

165. Corcos D, Osborn MJ, Matheson LS. B-cell receptors and heavy chain diseases: guilty by association? *Blood*. 2011;117:6991-6998.

166. Lucas PC, Yonezumi M, Inohara N, et al. Bcl10 and MALT1, independent targets of chromosomal translocation in malt lymphoma, cooperate in a novel NF-kappa B signaling pathway. *J Biol Chem*. 2001;276:19012-19019.

167. Thome M. CARMA1, BCL-10 and MALT1 in lymphocyte development and activation. *Nat Rev Immunol*. 2004;4:348-359.

168. Ngo VN, Young RM, Schmitz R, et al. Oncogenically active MYD88 mutations in human lymphoma. *Nature*. 2011;470:115-119.

169. Pugh-Bernard AE, Cambier JC. B cell receptor signaling in human systemic lupus erythematosus. *Curr Opin Rheumatol*. 2006;18:451-5.

170. Liossis SN, Kovacs B, Dennis G, Kammer GM, Tsokos GC. B cells from patients with systemic lupus erythematosus display abnormal antigen receptor-mediated early signal transduction events. *J Clin Invest*. 1996;98:2549-2557.

171. Liossis SN, Solomou EE, Dimopoulos MA, Panayiotidis P, Mavrikakis MM, Sfikakis PP. B-cell kinase lyn deficiency in patients with systemic lupus erythematosus. *J Investig Med*. 2001;49:157-165.

172. Flores-Borja F, Kabouridis PS, Jury EC, Isenberg DA, Mageed RA. Decreased Lyn expression and translocation to lipid raft signaling domains in B lymphocytes from patients with systemic lupus erythematosus. *Arthritis Rheum*. 2005;52:3955-3965.

173. Richez C, Blanco P, Rifkin I, Moreau JF, Schaeverbeke T. Role for tolllike receptors in autoimmune disease: the example of systemic lupus erythematosus. *Joint Bone Spine*. 2011;78:124-130.

174. Deane JA, Pisitkun P, Barrett RS, et al. Control of toll-like receptor 7 expression is essential to restrict autoimmunity and dendritic cell proliferation. *Immunity*. 2007;27:801-810.

175. Portis T, Longnecker R. Epstein-Barr virus (EBV) LMP2A mediates B lymphocyte survival through constitutive activation of the Ras/PI3K/Akt pathway. *Oncogene*. 2004;23:8619-8628.

176. Merchant M, Swart R, Katzman RB, et al. The effects of the Epstein-Barr virus latent membrane protein 2A on B cell function. *Int Rev Immunol*. 2001;20:805-835.

177. Thorley-Lawson DA. Epstein-Barr virus: exploiting the immune system. *Nat Rev*

Immunol. 2001;1:75-82.

178. Snyder MD, Pierce SK. A mutation in Epstein-Barr virus LMP2A reveals a role for phospholipase D in B-cell antigen receptor trafficking. Traffic. 2006;7:993-1006.

179. Miller CL, Burkhardt AL, Le JH, et al. Integral membrane protein 2 of Epstein-Barr virus regulates reactivation from latency through dominant negative effects on protein-tyrosine kinases. Immunity. 1995;2:155-166.

180. Dykstra ML, Longnecker R, Pierce SK. Epstein-Barr virus co-opts lipid rafts to block the signaling and antigen transport functions of the BCR. Immunity. 2001;14:57-67.

181. Fruehling S, Longnecker R. The immunoreceptor tyrosine-based activation motif of Epstein-Barr virus LMP2A is essential for blocking BCR-mediated signal transduction. Virology. 1997;235:241-251.

182. Fruehling S, Swart R, Dolwick KM, Kremmer E, Longnecker R. Tyrosine 112 of latent membrane protein 2A is essential for protein tyrosine kinase loading and regulation of Epstein-Barr virus latency. J Virol. 1998;72:7796-7806.

183. Fukuda M, Longnecker R. Latent membrane protein 2A inhibits transforming growth factor-beta 1-induced apoptosis through the phosphatidylinositol 3-kinase/Akt pathway. J Virol. 2004;78:1697-1705.

184. Torres RM, Flaswinkel H, Reth M, Rajewsky K. Aberrant B cell development and immune response in mice with a compromised BCR complex. Science. 1996;272:1804-1808.

185. Gong S, Nussenzweig MC. Regulation of an early developmental checkpoint in the B cell pathway by Ig beta. Science. 1996;272:411-414.

186. Lam KP, Kuhn R, Rajewsky K. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. Cell. 1997;90:1073-1083.

187. Chan VW, Meng F, Soriano P, DeFranco AL, Lowell CA. Characterization of the B lymphocyte populations in Lyn-deficient mice and the role of Lyn in signal initiation and down-regulation. Immunity. 1997;7:69-81.

188. Turner M, Gulbranson-Judge A, Quinn ME, Walters AE, MacLennan IC, Tybulewicz VL. Syk tyrosine kinase is required for the positive selection of immature B cells into the recirculating B cell pool. J Exp Med. 1997;186:2013-2021.

189. Cornall RJ, Cheng AM, Pawson T, Goodnow CC. Role of Syk in B-cell development and antigen-receptor signaling. Proc Natl Acad Sci U S A. 2000;97:1713-1718.

190. Cariappa A, Tang M, Parng C, et al. The follicular versus marginal zone B lymphocyte cell fate decision is regulated by Aiolos, Btk, and CD21. Immunity. 2001;14:603-615.

191. Martin F, Kearney JF. Positive selection from newly formed to marginal zone B cells depends on the rate of clonal production, CD19, and Btk. Immunity. 2000;12:39-49.

192. Rawlings DJ, Saffran DC, Tsukada S, et al. Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. Science. 1993;261:358-361.

193. Tsukada S, Baba Y, Watanabe D. Btk and BLNK in B cell development. Adv Immunol. 2001;77:123-162.

194. Pappu R, Cheng AM, Li B, et al. Requirement for B cell linker protein (BLNK) in B cell development. Science. 1999;286:1949-1954.

195. Xu S, Tan JE, Wong EP, Manickam A, Ponniah S, Lam KP. B cell development and activation defects resulting in xid-like immunodeficiency in BLNK/SLP-65-deficient mice. Int Immunol. 2000;12:397-404.

196. Hayashi K, Nittono R, Okamoto N, et al. The B cell-restricted adaptor BASH is required for normal development and antigen receptor-mediated activation of B cells. Proc Natl Acad Sci U S A. 2000;97:2755-2760.

197. Hashimoto A, Takeda A, Inaba M, et al. Cutting edge: essential role of phospholipase C-γ2 in B cell development and function. J Immunol. 2000;165:1738-1742.

198. Wang D, Feng J, Wen R, et al. Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. Immunity. 2000;13:25-35.

199. Okkenhaug K, Bilancio A, Farjot G, et al. Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. Science. 2002;297:1031-1034.

200. Clayton E, Bardi G, Bell SE, et al. A crucial role for the p110delta subunit of phosphatidylinositol 3-kinase in B cell development and activation. J Exp Med. 2002;196:753-763.

201. Fruman DA, Snapper SB, Yballe CM, et al. Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha. Science. 1999;283:393-397.

202. Suzuki H, Terauchi Y, Fujiwara M, et al. Xid-like immunodeficiency in mice with disruption of the p85alpha subunit of phosphoinositide 3-kinase. Science. 1999;283:390-392.

203. Leitges M, Schmedt C, Guinamard R, et al. Immunodeficiency in protein kinase cbeta-deficient mice. *Science*. 1996;273:788-791.

204. Su TT, Guo B, Kawakami Y, et al. PKC-beta controls I kappa B kinase lipid raft recruitment and activation in response to BCR signaling. *Nat Immunol*. 2002;3:780-786.

205. Doody GM, Bell SE, Vigorito E, et al. Signal transduction through Vav-2 participates in humoral immune responses and B cell maturation. *Nat Immunol*. 2001;2:542-547.

206. Tedford K, Nitschke L, Girkontaite I, et al. Compensation between Vav-1 and Vav-2 in B cell development and antigen receptor signaling. *Nat Immunol*. 2001;2:548-555.

207. Croker BA, Tarlinton DM, Cluse LA, et al. The Rac2 guanosine triphosphatase regulates B lymphocyte antigen receptor responses and chemotaxis and is required for establishment of B-1a and marginal zone B lymphocytes. *J Immunol*. 2002;168:3376-3386.

208. Engel P, Zhou LJ, Ord DC, Sato S, Koller B, Tedder TF. Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. *Immunity*. 1995;3:39-50.

209. Rickert RC, Rajewsky K, Roes J. Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. *Nature*. 1995;376:352-355.

210. Samardzic T, Marinkovic D, Danzer CP, Gerlach J, Nitschke L, Wirth T. Reduction of marginal zone B cells in CD22-deficient mice. *Eur J Immunol*. 2002;32:561-567.

211. Sato S, Miller AS, Inaoki M, et al. CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. *Immunity*. 1996;5:551-562.

212. Ujike A, Takeda K, Nakamura A, Ebihara S, Akiyama K, Takai T. Impaired dendritic cell maturation and increased T(H)2 responses in PIR-B(-/-) mice. *Nat Immunol*. 2002;3:542-548.

213. Senftleben U, Cao Y, Xiao G, et al. Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science*. 2001;293:1495-1499.

214. Kaisho T, Takeda K, Tsujimara T, et al. IkappaB kinase alpha is essential for mature B cell development and function. *J Exp Med*. 2001;193: 417-426.

215. Pasparakis M, Schmidt-Supprian M, Rajewsky K. IkappaB kinase signaling is essential for maintenance of mature B cells. *J Exp Med*. 2002;196:743-752.

216. Carter RH, Wang Y, Brooks S. Role of CD19 signal transduction in B cell biology. *Immunol Res*. 2002;26:45-54.

217. Boross P, van de Poel K, Van de Winkel JG, Leusen JH. Fc receptors. In: *Encyclopedia of Life Sciences (ELS).* Chichester, England: John Wiley & Sons, Ltd; 2008.

218. Leu CM, Davis RS, Gartland LA, Fine WD, Cooper MD. FcRH1: an activation coreceptor on human B cells. *Blood*. 2005;105:1121-1126.

219. Davis RS, Ehrhardt GRA, Leu C-M, Hirano M, Cooper MD. An extended family of Fc receptor relatives. *Eur J Immunol* . 2005;35:674-680.

220. Kubagawa H, Cooper MD, Chen CC, et al. Paired immunoglobulin-like receptors of activating and inhibitory types. *Curr Top Microbiol Immunol* . 1999;244:137-149.

221. Takai T, Ono M. Activating and inhibitory nature of the murine paired immunoglobulin-like receptor family. *Immunol Rev*. 2001;181: 215-222.

222. Chan AC, Desai DM, Weiss A. The role of protein tyrosine kinases and protein tyrosine phosphatases in T cell antigen receptor signal transduction. *Annu Rev Immunol*. 1994;12:555-592.

223. Leprince C, Draves KE, Geahlen RL, Ledbetter JA, Clark EA. CD22 associates with the human surface IgM-B-cell antigen receptor complex. *Proc Natl Acad Sci U S A* . 1993;90:3236-3240.

224. Pritchard NR, Smith KGC. B cell inhibitory receptors and autoimmunity. *Immunology*. 2003;108:263-273.

225. Nitschke L. The role of CD22 and other inhibitory co-receptors in B-cell activation. *Curr Opin Immunol* . 2005;17:290-297.

226. Nitschke L. CD22 and Siglec-G: B-cell inhibitory receptors with distinct functions. *Immunol Rev*. 2009;230:128-143.

227. Nitschke L, Tsubata T. Molecular interactions regulate BCR signal inhibition by CD22 and CD72. *Trends Immunol*. 2004;25:543-550.

228. Parnes JR, Pan C. CD72, a negative regulator of B-cell responsiveness. *Immunol Rev*. 2000;176:75-85.

229. Ochi H, Watanabe T. Negative regulation of B cell receptor-mediated signaling in B-1 cells through CD5 and Ly49 co-receptors via Lyn kinase activity. *Int Immunol*. 2000;12:1417-1423.

230. Schreeder DM, Cannon JP, Wu J, Li R, Shakhmatov MA, Davis RS. Cutting edge: FcR-like 6 is an MHC class II receptor. *J Immunol*. 2010;185:23-27.

231. Wilson TJ, Presti RM, Tassi I, Overton ET, Cella M, Colonna M. FcRL6, a new ITIM-bearing receptor on cytolytic cells, is broadly expressed by lymphocytes following HIV-1 infection. *Blood*. 2007;109:3786-3793.

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Chapter 10

B-Lymphocyte Responses

Michael McHeyzer-Williams

INTRODUCTION

B-lymphocyte responses provide the effective and longlasting immune protection induced by most vaccines in use today. We measure antibody molecules as the circulating agent of immune protection but now understand much more about the underlying B-lymphocyte response that progressively matures in response to foreign antigen exposure. This chapter will focus on the highly-regulated cellular and molecular development of antigen-specific B-lymphocyte responses.

The three cardinal features of effective B-cell immunity are antigen specificity, antibody class, and antigen binding affinity. To be effective, antibodies must bind vulnerable antigens on the targeted pathogen. During infection, this is a struggle between the evasion mechanisms of the pathogen and preexisting diversity within the adaptive immune system. Different classes of antibodies engage distinct mechanisms of antigen clearance. These various classes of antibody provide either more or less protection depending on the pathogen's portal of entry at infection. Finally, the right antigen-specific antibody still requires induction of sufficiently high binding affinity to provide adequate sensitivity for long-term immune protection. Many promising antigens fail to achieve adequate immunogenicity in contemporary vaccine strategies due to poor affinity maturation. Here, we focus on the sequential mechanisms that program these central attributes of effective antigen-specific B-cell memory.

In the past few years, experimental access to immune response biology has dramatically shifted with the advent of multiphoton laser-based intravital imaging techniques. These studies provide direct access to the mechanics and cell dynamics of antigen-specific cognate regulation in vivo. This information serves to integrate existing knowledge in the field using a real-time scaffold for developmental progression in vivo. Importantly, follicular helper T (T_{FH}) cells have recently emerged as a new class of antigen-specific immune regulator that controls multiple stages of high-affinity B-cell immunity. Understanding antigen-specific T_{FH}-cell development and function remains an active research challenge with great potential for the rational design of future vaccines. Current information regarding the role of antigenspecific T_{FH} cells will be integrated into this chapter to provide a regulatory dimension to B-lymphocyte responses.

Following initial exposure to antigen, T_{FH} -cell-regulated B-cell immunity progresses in three separable stages of antigen-specific development. Each stage is characterized by a B-cell antigen-recognition event followed by contact with cognate T_{FH} cells that determines

subsequent B-cell fate. Naïve B cells recognize foreign antigen and then present antigenic peptides to specific T_{FH} cells to progress in development across two major pathways (pre-germinal center [GC] development). Extrafollicular development permits antibody class switch and plasma cell differentiation while entry into the GC reaction is the major pathway to highaffinity B-cell memory (GC cycle). GC B cells in this pathway can switch antibody class and affinity mature their expressed B-cell receptor (BCR) following access to antigen and presentation to GC T_{FH} cells. Upon antigen reexposure, memory B cells recognize, uptake, and then present antigen to memory T_{FH} cells to promote rapid memory B-cell responses and boost circulating high-affinity antibody (memory B-cell response). Antigen recall is the least studied facet of B-lymphocyte responses but provides an important developmental juncture for vaccine-based prophylactic or therapeutic intervention.

This chapter mainly focuses on what is known of antigen-specific B-lymphocyte responses in mouse models with reference to work conducted in humans. Further, there is an emphasis on the response to model antigens that provides a greater understanding of how to manipulate adaptive immunity for preventative vaccination rather than a focus on the immune response to infection.

PRE-GERMINAL CENTER DEVELOPMENT

The initial antibody response to many infectious agents is largely based on the rapid T-cell independent (TI) expansion of B cells and their subsequent differentiation into plasma cells. B1 and marginal zone B cells are largely responsible for these rapid TI humoral responses suggesting that some level of “natural memory” function resides in these B-cell subsets and may be predetermined in an evolutionarily conserved manner. TI antigens can be separated into two broad categories based on their ability to polyclonally activate B cells (TI-1) or require BCR recognition of multivalent epitopes to induce B-cell differentiation in the absence of T-cell help (TI-2). TI-2 antigens can activate BCR signaling but require accessory signals to promote the development of antigen-specific plasma cells. In contrast, monovalent protein antigens require antigen-specific helper T-cell regulation to promote high titer antibody responses and the development of B-cell memory. These T_{FH}-cell-dependent antibody responses take longer to emerge and display a spectrum of BCR affinities and antibody isotypes as multiple strategies for antigen-specific clearance in vivo. Immune responses to

model protein antigens provide experimental access to this complex cascade of cellular and molecular events that underpin long-term protective immunity.

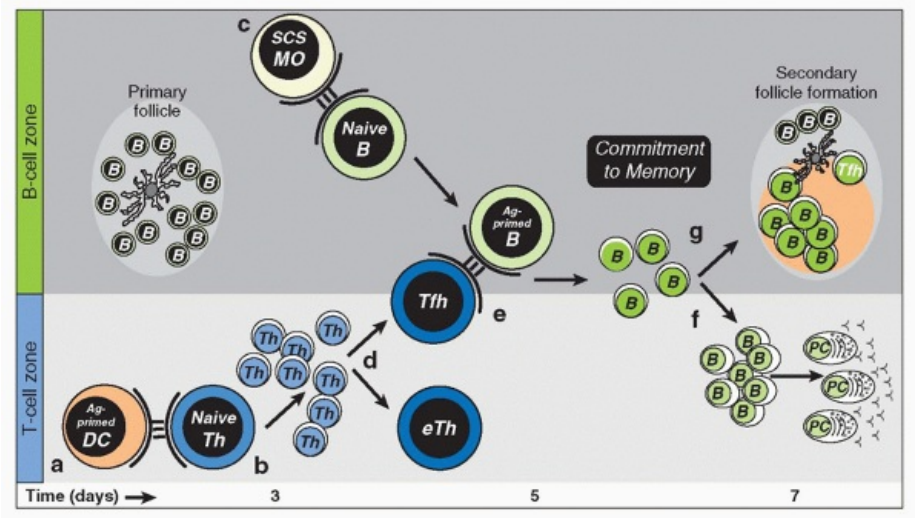

FIG. 10.1. Pre—Germinal Center (GC) Development. A: Local protein vaccination induces dendritic cells (DC) maturation and migration to the T-cell zones of draining lymph nodes (LNs). **B:** Peptide major histocompatibility class II expressing DCs will engage naive antigen-specific helper T (T_H) cells to induce proliferation and effector T_H-cell differentiation. **C:** Whole antigen will be trapped by subcapsular sinus macrophage and presented to naive follicular B cells. Antigen-specific B cells will become activated and uptake, process, and present antigenic peptides and migrate toward the T-B borders of the draining LN. **D:** Effector T_H cells emerge in multiple forms with emigrant T_H cells exiting the LN to function at distal tissue sites and T_{FH} cells relocating to T-B borders and interfollicular regions. **E:** Cognate contact between pre-GC T_{FH} cells and antigen-primed B cells is required for multiple programming events in the pathway to B-cell immunity. **F:** Clonal expansion, antibody class switch, and non-GC plasma cell development proceeds within extrafollicular regions of LNs. **G:** Secondary follicle formation and antibody class switch precede formation of the GC reaction as the dominant pathway to memory B-cell formation.

B-cell immunity is initiated on two fronts. Initially, naïve B cells are activated through cell-associated antigen to uptake antigen, process and present peptide major histocompatibility complex [MHC] class II (pMHCII) complexes to enable cognate contact with pMHCII-specific T_{FH} cells. These antigen-primed B cells relocate to T-B-cell borders in lymphoid tissues to increase the likelihood of contact with antigenprimed T_{FH} cells. On the second front, populations of dendritic cells (DCs) also take up antigen, process and present pMHCII, and migrate to draining lymphoid tissues to initiate T_{FH}-cell responses. One outcome of contact with pMHCII-expressing DCs is differentiation of the T_{FH}-cell lineage and the productive contact between pMHCII-specific T_{FH} cells and antigen-primed B cells. This pre-GC cell contact and bidirectional exchange of molecular programming is central to the development of effective B-cell immunity. Antibody class switch, extrafollicular plasma cell differentiation, and initiation of the GC reaction are major B-cell fates associated with this initial phase in B-cell immunity (Fig. 10.1).

Antigen Presentation to B Cells

B cells can acquire soluble antigen by free diffusion into lymphoid follicles[1] or through the lymphoid system of conduits.[2] However, populations of lymph node (LN) subcapsular sinus (SCS) macrophages appear most effective at presenting cell-associated antigen to follicular B cells.[3] B cells take up noncognate antigen presented by SCS macrophages through complement receptors and transfer it into follicular regions and onto follicular DCs (FDCs), which can serve as a source of antigen to prime naïve B cells.[4] In contrast, priming with the cognate antigen at first contact with SCS macrophages results in movement of antigen-specific B cells to the T-B-cell borders and antigen-specific B-cell responses to captured antigens.[4,5,6] Hence, the SCS macrophages filtering the lymphatic fluid not only protect from systemic infection,[7] but also effectively initiate T helper cell-regulated antigen-specific B-cell immunity.

DCs can also effectively prime B-cell immunity. Injection of DCs pulsed with protein antigen can induce isotype switch and promote efficient B-cell responses. B cells can form synapse-like interactions with antigen-pulsed DCs.[8,9] More recently, two-photon imaging revealed that naïve B cells entering local LNs surveyed protein antigen-pulsed DCs before entering the follicular areas.[10] In this model, engagement of BCR led to calcium flux, migration arrest, and the local accumulation of the antigen-specific B cells. Furthermore, there is a reticular network of collagen fibers that physically connects the subcapsular and paracortical sinuses of LNs to blood vessels and separates these regions from T- and B-cell areas.[11] This organization facilitates the efficient delivery of

soluble antigen toward the lumen of high endothelial venules without entering the LN parenchyma. While resident DCs can access this conduit transport system, follicular B cells may have difficulty accessing soluble antigen.

Nevertheless, in the context of protein antigens, B cells must recognize their cognate antigen and internalize, process, and present peptides from this antigen in the context of MHCII in order to receive pMHCII-specific T-cell help. If the antigen is cell associated, in the presence of adequate costimulation, some aspects of the B-cell response can proceed in a TI manner. B-cell proliferation and plasma cell development can occur in the absence of cluster of differentiation (CD)40-CD40L interactions with some residual isotype switch induced by the action of TACI and BAFF-R.[12] Thus, B cells can respond in a TI manner to protein antigens but do not express the characteristic range of outcomes and the full extent of protective immunity that is found with cognate regulation and T-cell help.

Antigen-Specific B-Cell Activation

Initial activation of naïve B cells through the BCR triggers multiple gene expression programs that enable effective contact with cognate T helper cells. Dynamic contacts with membrane-associated antigens determines the amount of antigen naïve B cells accumulate following antigen exposure.[13] Effective cell contacts require expression of the signaling adaptor dedicator of cytokinesis 8 (DOCK8).[14] Mutations in DOCK8 disrupted integrin ligand accumulation in the immune synapse without altering BCR signaling events. B-cell-specific

conditional ablation of the calcineurin regulatory subunit 1 (CNB1),[15] myocyte enhancer factor 2c,[16,17] and stromal interaction molecule 1 and stromal interaction molecule 2[18] have shown that calcium responsiveness is necessary for cell cycle progression in these early pre-GC stages of B-cell responses. Hydrogen voltage-gated proton channels 1, which are internalized with the BCR, have been recently implicated in early B-cell programming events.[19] Single-pulsed BCR signaling[20] that only partially activated NF-κB increased CC chemokine receptor 7 and MHC class II expression, and responsiveness to CD40, indicating some of the early facets of B-cell activation. Severe defects in early B-cell proliferation have also implicated integrin-binding CD98hc[21] and extracellular signal-regulated kinase activation[22] in preparing the antigen-primed B cells to receive cognate T-cell help in vivo. Hence, initial antigen recognition, uptake, processing, and presentation critically impact the early B-cell developmental fate. High-resolution dynamic imaging has provided substantial insight into the earliest events associated with initial BCR engagement on naïve B cells. The membrane cytoskeleton controls BCR diffusion; disruption of this organization initiates signaling.[23,24] Discrete microclusters form upon antigen binding[25] and recruit multiple components of the intracellular BCR signaling network to initiate signal transduction.[26] One rapid response involves B-cell spreading to increase surface contact with antigen on the presenting cells.[13] Central clustered antigen at the cellular interface is then internalized into antigen-processing lysosomes for presentation with MHCII[8] as the cognate point of T_{FH} contact. Curiously, antigen presentation appears asymmetrically segregated with one daughter retaining larger antigen stores thereby more able to contact pMHCII-specific T_{FH} cells.[27] Whether these developmental outcomes are directed by initial context of antigen presentation or stochastically assorted[28] remains an interesting fundamental issue with an early impact on antigen-specific B-cell development.

Dendritic Cell Maturation

DCs are essential antigen-presenting cells (APCs) for initiating adaptive immunity. Multiple DC subsets exist prior to antigen challenge. Different phenotypic schemes can be used to characterize DC subsets with the origins and developmental relatedness of different DC subsets still subject to debate.[29,30] In the murine system, three main bone marrow-derived DC subsets enter all secondary lymphoid organs via the blood and reside at different levels. These bloodderived DCs all express CD11c and are distinguishable as $CD11b^{hi}$ $CD8a^{neg}$ DCs, $CD8a^{hi}$ DCs, and $6B2^{hi}$ plasmacytoid DCs. In LNs draining the skin, there are at least two further CD11c+ DC subsets, Langerhans cells (LCs) and dermal DCs, that emigrate from the skin, even at homeostasis.[31] Thus, before antigen challenge, multiple subsets of DCs are available to differentially process and present antigen to the adaptive immune compartment.

Protein antigen administration in the absence of inflammation induces immune tolerance. In contrast, coadministration of an immune adjuvant activates facets of innate immunity, induces inflammation, and primes antigenspecific adaptive immunity. Sensing pathogens involves pattern recognition receptors such as the evolutionarily conserved toll-like receptors[32,33] and the more recently described nucleotide oligomerization domain-like receptors.[34] Most

forms of antigen and innate stimulators require DC priming at some level for B-cell immunity, whereas particulate antigen in virus-like particles are more reliant on B-cell innate receptor activation.[35] Nevertheless, both families of innate receptors recognize different types of microbial components initiating programs of DC maturation that promote immediate local inflammation and innate effector clearance mechanisms.

Temporal and spatial constraints on DC maturation provides another layer of regulation for the innate system that can impact adaptive immunity. In the steady state, DCs form dense networks at the T-B borders of LNs. Interestingly, motile lipopolysaccharide-activated DC immigrants will rapidly coalesce with this preexisting network in vivo.[36] In separate studies using genetically tagged LCs, the emigrants of LCs and dermal DCs were shown to emerge separately in time and colonize separate regions of the T-B border.[37] A similar temporal regulation was seen using antibodies to specific pMHCII complexes with resident DCs presenting an early wave of pMHCII and dermal DCs emerging later in a second wave.[38] In each of these studies, the immune stimulus was varied and the coordinated response of the innate system also qualitatively and quantitatively different.

Clonal Selection in Helper T Cells

The initial outcome for pMHCII+ DC interactions with naive T_H cells is T-cell receptor (TCR)-driven clonal selection. T_H- cell responses that focus the specific T_H-cell response to a set of dominant TCRs provide access to the mechanisms that underpin T_H clonal selection.[39,40,41] Earlier studies indicate the importance of TCR-pMHCII affinity in determining T_H cell fate.[39,42,43,44] In this model, selective expansion of clonotypes expressing higher-affinity TCRs would rely on competition for pMHCII complexes on APCs. Alternatively, in vitro studies using altered peptide ligands suggested that the duration of TCR-pMHCII contact was critical to cell fate and therefore defined “best fit” in vivo.[45,46,47,48] In support of this model, T_H cells expressing TCR with fast off rates were lost over time in vivo.[49] Further, there is evidence for different peptides stabilizing the pMHCII to create a hierarchy of dominant peptides.[50] Each and all of these variables would impact what is considered “best fit” and influence the outcome of clonal selection. More recently, we provide evidence for a model of T_H clonal selection that is based on a TCR affinity threshold.[51] Multiple affinity-based thresholds underpin cell fate and T_H clonal expansion[52] with evidence that the highest affinity TCR assort into the T_{FH} compartment.[53] Hence, selection thresholds generate antigen-specific clonal diversity in ways that impact the development of effector T_H cell function in vivo.

Follicular Helper T Cells

There were early reports of effector T_H cells specialized to regulate B-cell responsiveness. CXCR5 expression was first reported on CD4+CD45RO+ cells in the peripheral blood and secondary lymphoid tissue in humans.[54] Gene ablation studies emphasized the role of CXCR5 and CC chemokine receptor 7 in the correct positioning of T and B cells in secondary

lymphoid tissue that was also needed to support effective T_H cell-dependent B-cell immunity.[55,56] Blocking CD28 and OX40 interactions in vivo blocked the development of CXCR5+ T_H cells and the GC reaction.[57] Further, CXCR5 expression was induced in an antigen-specific manner on T_H cells in vivo, and these cells relocated to follicular areas and the GC of responding lymphoid tissue.[58] CXCR5+ T_H cells were sorted from human tonsil and shown to support antibody production in vitro.[59,60] The tonsillar CXCR5+ T_H cells expressed high levels of CD40L and ICOS and were found in both the follicular mantle and GC. Adoptive transfers distinguished CXCR5+ B cell helper activity (T_{FH}) from P-selectin ligand^{hi} tissue homing inflammatory mediation (DTH-promoting T_H) that emerged together from the same set of precursors in vivo.[61] The term T_{FH} cells was coined to categorize this functionally and phenotypically distinct effector T_H cell compartment.[59,60]

As the name implies, the cardinal characteristic of all T_{FH} cells is their repositioning into the follicular regions of secondary lymphoid tissues. Early assessments of cytokine production by in vitro restimulated CXCR5+ T_{FH} cells indicated interleukin (IL)-2, interferon (IFN)-γ, and IL-10 from human peripheral blood[60] with evidence for IL-4 and IFN-γ from TCR transgenic mouse T_{FH} cells.[61] Early microarray analyses suggested separable gene expression programs for T_{FH} cells and other known T_H-cell subsets. CXCL13 was highlighted early[62] with evidence for ICOS, IL-21,[63,64] IL-21R,[65] and the differential expression of Bcl-6[63] being used as the most reliable attributes of T_{FH} function in vivo. Thus, acquisition of special pre-GC T_{FH}-cell functions was associated with the programming of a separate T_H-cell lineage.

Molecules important in the development of normal B-cell immunity were implicated in early studies of T_{FH} development. CD28 deficiency or treatment with blocking CD28 antibodies led to profound defects in B-cell immunity.[66] CD28 was required early to initiate naive T_H-cell responsiveness to pMHCII+ CD80- and CD86-expressing DCs. In contrast, CD40-CD40L interactions were central to the delivery of T-cell help to B cells.[67,68,69] The CD28 family member ICOS[70] was implicated in T_{FH}-cell function in pre-GC interactions with pMHCII-expressing B cells.[71,72,73] ICOS-deficient humans and mice[74,75,76] and ICOS-L-deficient mice had marked deficits in all aspects of B-cell immunity. ICOS deficiency was associated with decreased T_{FH}-cell development and considered an important molecule in the delivery of effector T_{FH} function.[77,78] Conversely, overexpression of ICOS in mice with a regulatory defect in ICOS expression[79] resulted in an overproduction of CXCR5+ T_{FH} cells and breakthrough autoimmune disease.[64] Recent studies indicated that ICOS can substitute for CD28 and rescue the T_{FH} defects and B-cell defects in CD28-deficient mice.[80] Furthermore, the abundant CXCR5+ T_{FH} cells in this model act in a T-cell autonomous manner to promote autoantibody production.[81] PD-1, another CD28 family member that has been implicated in the negative regulation of chronically activated T cells, was also found on GC T_{FH} cells in

human tonsil and mouse.[82] Positive and negative influences of antigen-specific T_{FH}-cell costimulation are balanced in ways that remain poorly understood in vivo and are an active avenue of current research in this field. BCL6 is required for development of the T_{FH} program[83,84,85] and is reinforced within T_{FH} cells upon pre-GC B-cell contact.[86,87] IL-21 also plays a major role in T_{FH} function with substantial loss of B-cell immunity in its absence.[88,89] Recent studies have identified BCL6 in antigen-specific T_{FH} cells and BLIMP1, which has an opposing function, in non-T_{FH} cells.[53] BCL6 and BLIMP1 expression was mutually exclusive across these two T_H-cell subtypes, already evident by the second cell division in vivo.[29] More recently, transcription factors c-Maf and BATF were shown to act with BCL6 to program T_{FH} development.[90,91] Hence, distinct transcriptional programming of unique cellular functions directs the early T_{FH}-cell development that is central to subsequent B-cell immunity (Fig. 10.2).

Pre-Germinal Center Follicular Helper T-B-Cell Contact

First contact between antigen-specific T_{FH} and antigenprimed B cells has also been captured through dynamic imaging.[92] Stable "monogamous" interactions between one antigen-specific T_{FH} cell and one B cell can last for a duration of 10 to 60 minutes in the follicular regions of the LNs. These interactions were accompanied by highly dynamic movements, with the B cells migrating extensively and leading

the T_{FH} cells.[92] A recent study demonstrated that expression of the adaptor molecule signaling lymphocytic activation molecule-associated protein (SAP) was needed to form long duration contacts with antigen-primed B cells.[93] In these studies, there was no role for SAP in early T_{FH}-DC contacts, and the SAP-deficient T_{FH} cells reached the follicular regions and expressed all the hallmarks of pre-GC T_{FH} cells ($CXCR5^{hi}$, CD40L+, $ICOS^{hi}$, and $OX40^{hi}$).[93] Furthermore, in the absence of SAP, T_{FH} cells were still capable of cytokine production[94] but unable to promote GC formation.[95,96] More recently, dynamic imaging places ongoing critical pre-GC contacts in the interfollicular zones of LNs[97] with a requirement for persistent BCL6 expression in B cells to maintain effective cognate contact.[98] Therefore, early T_{FH}-cell developmental programs establish the capacity for cognate contact needed to promote ongoing antigen-specific B-cell immunity.

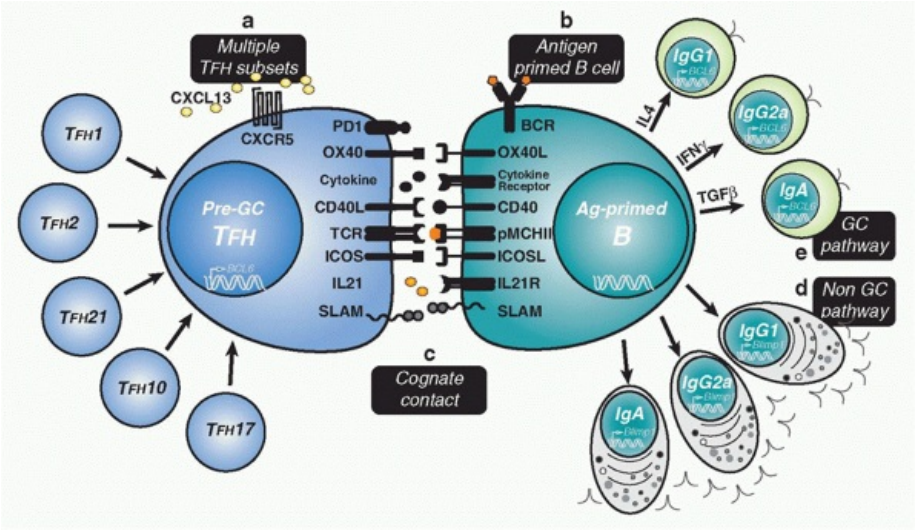

FIG. 10.2. Pre-Germinal Center (GC) Follicular Helper T (TFH)-B-Cell Contact. A: Multiple subsets of antigen-specific pre-GC T_{FH} are produced to regulate B-cell immunity. The organization of function within T_{FH} subsets remains speculative. There is evidence for different cytokine-secreting T_{FH} cells that regulate commitment to separate antibody class as well as different types of T_{FH} cells that regulate non-GC plasma cell differentiation. Bcl-6 and CXCR5 expression is thought to be a common feature of all T_{FH} cell subsets. **B:** Antigen-primed B cells must process and present peptide major histocompatibility class II to receive cognate help from pre-GC T_{FH} cells. Upregulation of molecules involved in helper T cell contact is a poorly resolved component of early antigen-driven B-cell maturation. **C:** Cognate contact between antigen-specific T-cell receptor and peptide major histocompatibility class II complexes focus pre-GC T_{FH}-B cell intercellular exchange of molecular information. Modifying interactions at first contact are known to involve costimulatory molecules (eg, cluster of differentiation [CD]40L-CD40, ICOS-ICOSL), accessory interactions (eg, signaling lymphocytic activation molecule family interactions, OX40-OX40L), cytokine and cytokine receptors (eg, IL4-IL4R, interferon [IFN]γ-IFNγ receptor, IL21-IL21R). Distribution of these functional attributes within pre-GC TFH compartments is not yet well resolved in vivo. **D:** The non-GC pathway to plasma cell development permits antibody class switch recombination (CSR) without somatic hypermutation (SHM) depending largely on the cytokine stimulus provide by pre-GC T_{FH} cells. Blimp-1 expression is required for plasma cell commitment across all antibody classes. **E:** The GC pathway to memory B-cell development begins with extensive B-cell proliferation within secondary follicles that will polarize into dark and light zone to initiate the GC reaction. The GC pathway is associated with Bcl-6 upregulation and activationinduced cytidine deaminase expression to supports both antibody CSR and SHM. These GC features emerge across all antibody classes and require productive and long duration pre-GC TFH contact.

It has been unclear how differential BCR affinity can impact the early fate of antigen-primed B cells. Very-low-affinity B cells are capable of forming GCs[99] but fail to do so in the presence of high-affinity competition.[100] In contrast, there is evidence for the highest-affinity B cells

preferentially entering the non-GC plasma cell pathway, leaving lower-affinity B cells to mature within the GC cycle.[101] This issue has been addressed more recently using intravital imaging to examine the early pre-GC selection events.[102] In this model, access to antigen was not impacted by BCR affinity, but the differential capacity to present antigen to pre-GC T_{FH} cells assorted with BCR affinity. Increased T-cell help promoted greater access to both the plasma cell pathway and the GC reaction. Thus, BCR affinity thresholds regulate B-cell fate at the earliest pre-GC junctures of antigen-specific T_{FH}-B interactions. Overall, the precise function of different costimulatory molecules and their combinatorial impact on antigen-specific B cell fate remains an exciting area of current interest. Quantitative differences in cell surface molecules in combination with mixtures of cytokines will likely synergize in

predetermined ways to skew pMHCII-expressing B cells into separate pathways of B-cell immunity. Unraveling these molecular combinations will help to define the rules of molecular control for antigen-specific B-cell immunity.

Controlling Antibody Class

Pre-GC cognate T_{FH}-cell contact controls multiple antigenspecific B-cell differentiation options. Beyond this early pre-GC developmental juncture, antibody class switch appears in both in the extrafollicular pathway to plasma cell differentiation and the GC pathway to memory development.[103] Class switch also requires multiple rounds of cell division in the target cell before expression of non-immunoglobulin (Ig)M antibody.[104] Hence, it is likely that initial commitment to antibody class occurs at the first pre-GC point of cognate contact and is propagated in a lineal manner across each developmental option (see Fig. 10.2).

Class switch recombination (CSR) is an intrachromosonal deletional process between the switch (S) regions that reside 5′ of each constant region gene in B cells (except Cδ).[105] Signaling through CD40 and cytokine receptors induces germline transcription through the targeted S regions providing activation-induced cytidine deaminase (AID; also known as Aicda) access to deaminate cytosines in the single stranded template. AID is required and sufficient for the initiation of the CSR reaction in the activated locus.[106] AID-deficient animals[107] and humans[108] display no CSR or somatic hypermutation (SHM) of the Ig genes. Recent evidence indicates that following antigen stimulation, AID expression is regulated in B cells by paired box gene/protein 5, E-box proteins,[109] homeobox C4,[110] and forkhead box O1.[111] Removal of the resulting uracils and deoxyribonucleic acid (DNA) cleavage generates double strand breaks that trigger the recruitment of DNA damage machinery, mismatch repair, and nonhomologous end joining to complete the CSR event.[112] The adapter protein 14.3.3 is recruited with AID to switch regions[113] and polymerase ζ has been implicated in the repair process associated with CSR.[114] Peripheral B cells undergoing CSR in the absence of the x-ray-repair crosscomplementing protein 4 component of the double strand break repair machinery are also highly susceptible to translocation events and oncogenic transformation.[115] Antibody class switch is a destabilizing and potentially dangerous cellular event that can proceed without SHM in the extrafollicular pathway.

Induced CD40L on pre-GC T_{FH} cells and the receipt of this signal through CD40 on B cells is required for antibody class switch.[67,116] Animals and humans lacking CD40 display a hyper-IgM syndrome with profound defects in class switch, GC formation, and the development of affinity matured B-cell memory.[116] ICOS expression on activated T_{FH} cells is thought to act upstream of CD40L in this temporally orchestrated set of events.[117] ICOS-deficient animals also have clear defects in antibody class switch, GC formation, and the development of B-cell memory.[74,75,76] Some residual class switch in the absence of CD40/CD40L interactions may be explained by the action of TACI and BAFF-R.[12] OX40/OX40L interactions also quantitatively impact class switch while CD27-CD70 interactions promote plasma cell (PC) production.[118] Thus, the range of molecules expressed at the pre-GC T_{FH} cell surface influences the developmental impact of TCR-pMHCII contact on antigen-primed B cells. Considering the range of antibodies that can be produced, there must be multiple subtypes of pre-GC T_{FH} cells that control antibody class. Different classes of pre-GC T_{FH} would vary in production of T_{FH} cell-derived cytokines to control antibody isotype. IL-4 and IFN-γ are reciprocal regulators of IgG1 and IgG2a production.[119] Animals lacking IL-4 or Stat 6 have decreased IgG1 levels and no IgE.[120,121] IL-4 also acts together with IL-21 to control IgG subtypes and IgE levels.[88] In contrast, transforming growth factor (TGF)β is implicated in the induction IgA, while IL-2 and IL-5 augment IgA production.[122] Similarly, IL-6 may selectively support IgG2a and IgG2b expressing B cells in vivo.[123] Each of these factors can exert their effects in vitro or in a bystander manner in vivo. However, it is thought that the directed delivery of these soluble molecules toward points of TCR-pMHCII contact allows soluble signals to focus locally in an antigen-specific cognate manner.

Within antigen-responsive B cells, the molecular machinery that regulates CSR is deployed in an antibody class-specific manner. The global CSR machinery is targeted by transcription factors downstream of the cytokine receptors that control specific antibody classes. For example, IFNγ activates signal transducer and activator of transcription downstream of the IFNγ receptor to induce T-bet and promote IgG2a class switching.[124,125] Similarly, TGFβ signals through TGFβ-receptor to activate SMAD and RUNX transcription factors to promote IgA class switch.[126] Furthermore, the transcriptional regulator BATF required for T_{FH} development is also required in B cells to generate germline switch transcripts and promote AID expression.[91] Finally, Ikaros regulates antibody class decisions by differentially controlling transcriptional accessibility of constant region genes.[127] How the initial commitment to antibody class is maintained and propagated during clonal expansion, BCR diversification, and affinity-based selection within the GC reaction remains an important but unresolved issue. Therefore, it remains plausible that functional reprogramming accompanies CSR creating separable lineages of class-specific memory B cells in vivo.

Extrafollicular Plasma Cell Development

Under the cognate regulation of pre-GC T_{FH} cells, a cohort of antigen-primed B cells clonally expand within the T-cell zones of secondary lymphoid organs and rapidly give rise to PCs. Within the first few days after antigen exposure, small foci of B-cell blasts can be seen within

the T-cell zones.[103] This plasmablast stage appears transitional and defines pre-PCs that may secrete antibody but also retain the capacity to proliferate. In contrast, PCs are typically considered terminally differentiated and in a postmitotic state.[128,129] PCs display a marked increase in IgH and IgL messenger ribonucleic acid and prominent amounts of rough endoplasmic reticulum to accommodate translation and secretion of abundant Ig. They have reduced or lost numerous cell surface molecules

including B220, CD19, CD21, and CD22 with an increase in the proteoglycan syndecan-1 (CD138),[130,131] often used as a distinguishing marker for PCs. We recently demonstrated that class-switched plasma cells retained robust antigenpresentation capacity as a negative feedback loop for regulation of T_{FH} cells.[132] These studies further highlight the bidirectional programming between antigen-primed B cells and pMHCII-specific T_{FH} cells during developing immune responses.

In mice deficient for SAP, the extrafollicular pathway to PC development, remains largely intact but the GC pathway is blocked.[95] Recent studies indicate that SAP-deficient T_{FH} cells exhibit heightened CD40L expression but decreased ICOS induction that alone can account for this defect.[94] More recent studies indicate that SAP-deficient T_{FH} cells can only form short duration cognate contact with antigen-primed B cells, which are insufficient for GC entry but adequate for PC formation. It will be important to dissect the separable signals and cellular subsets of pre-GC T_{FH} cells that regulate the rapid but short-lived effector B-cell response in vivo. Extrafollicular PCs have half-lives of 3 to 5 days[133] and express germline-encoded antigen-specific antibodies.[131,134] Newly formed PCs migrate via the marginal zone bridging channels into the red pulp of the spleen and into the medullary cords of LNs. Migration patterns in vivo appear controlled by increased responsiveness to the CXCR4 ligand CXCL12 and decreased expression of CXCR5 and CC chemokine receptor 7.[135] There is evidence for BCR affinity-based selection even at this early stage in the response.[131] In some studies, B cells expressing low affinity BCR remain non-GC and high-affinity B cells preferentially enter the GC pathway. 100 The converse can also be seen with high-affinity B cells preferentially entering the non-GC short-lived PC pathway.[101] While seemingly contradictory, these different outcomes in vivo may reflect the plasticity of intercellular control at the T_{FH}-B point of contact.

The transcriptional control of PC development also exists in multiple layers controlling a cascade of developmental change. The transcriptional repressor prdm-1 (encoding Blimp-1) plays a central role in the regulation of PC development.[136] B cells lacking Blimp-1 do not differentiate into extrafollicular PC or post-GC PC, with TI and TD antibody responses profoundly diminished in vivo.[137] These Blimp-1-deficient animals also display defective levels of serum antibody, suggesting that spontaneous production of antibody by B-1-B cells also requires Blimp-1 expression. Structurefunction analysis indicates the modular action of Blimp-1 integrating a variety of environmental cues that lead to PC development.[138,139] Blimp-1 represses proliferation through c-myc as one direct target among many others involved in cell cycle control. Blimp-1 also induces antibody secretion by repressing the

transcription factor Pax-5, thereby derepressing Xbp-1. The transcription factor Xbp-1 controls the unfolded protein response, and many facets of the cellular secretory mechanism that are critical to PC function and survival.[140,141,142]

The transcriptional coactivator OBF-1 also appears important for B cells to complete the PC program.[143] In the absence of OBF-1, Bcl-6, Pax-5, and AID are not repressed, blocking the induction of Blimp-1 and PC development. In contrast, ablation of the transcription factor MitF leads to the spontaneous development of PC that appears independent of antigen stimuli.[144] Interestingly, reexpression of bcl-6 and its cofactor MTA3 re-activates the B-cell program and increased CD19 and MHC II, and decreased CD138 in plasma cell lines.[145] This remarkable study suggests that PC fate is not as terminal and passive as it has been thought to be, and that the PC fate remains subject to dynamic gene expression programs. Regulation of the unfolded-protein response by X-box-binding protein 1 is not needed for plasma cell development but is necessary for antibody secretion.[146,147] Epstein-Barr virus-induced molecule 2 also appears essential for B-cell movement to extrafollicular sites and the non-GC plasma cell response.[148,149] In addition, Epstein-Barr virus-induced molecule 2 guides recently activated B cells to interfollicular LN regions and then to outer follicular areas as a prelude to GC formation. Furthermore, there appears to be an early pre-GC proliferative phase at the perimeter of follicles that also precedes GC formation and BCR diversification.[150] Interestingly, recent dynamic imaging studies indicate migration of T_{FH} cells to the follicle interior, even before accumulation of GC B cells.[97]

THE GERMINAL CENTER CYCLE

Movement of antigen-primed B cells into the follicular regions of lymphoid organs after effector T_{FH}-cell contact initiates the GC pathway to memory B-cell development. These pre-GC B cells expand rapidly within the follicular region to form areas of B220+IgD^{low} antigen-specific B cells referred to as secondary follicles. Secondary follicles polarize into T-cell proximal dark zones of cycling centroblasts and opposing light zones of largely noncycling centrocytes among the dense processes of FDC and sparse presence of GC T_{FH} cells.[151,152] This broad anatomical distribution of the GC reaction orients the activities of clonal expansion, BCR diversification, and clonal variant selection that underpin the evolution of high-affinity memory B cells and post-GC plasma cells in this pathway (Fig. 10.3). Under normal physiologic conditions, the GC reaction emerges as the most efficient means to control affinity maturation and memory B-cell development. However, in the disorganized or absent preimmune lymphoid subcompartments of mice lacking LTa, LTb, tumor necrosis factor receptor I, and LTbR, the activities of the GC reaction remain disorganized but largely intact.[153,154,155,156] Vestiges of the GC or small cell aggregates manage the expansion, diversification, and selection steps required for affinity maturation and memory B-cell development.

Germinal Center Cellular Dynamics

Dynamic imaging of the GC reaction in situ has provided outstanding clarity to the kinetics of GC B cell[157,158] and GC T_{FH}-cell movements and interactions.[159] There was evidence for

interzonal movement of GC B cells indicating bidirectional migration between light and dark zones,[157,158,159,160] with one study emphasizing the majority of movement to be

intrazonal.[157,160] Surprisingly, cell division appeared in both zones of the GC in contrast to classical models.[157,158,159] There was also evidence for the ability of naive B cells to traverse the GC environment with evidence that high-affinity B cells could also enter and dominate existing GCs.[158] All studies identified highly motile GC B-cell movements with evidence for continuous uninterrupted movement over FDC processes. This movement contrasted with the capacity of GC B cells to form frequent short-term contacts but infrequent stable contacts with GC T_{FH} cells.[159] The latter group suggested that these infrequent cognate contacts play a dominant role in the selection of high-affinity BCR expressing GC B cells.[161] Hence, a unique model for affinity-based selection emerges with uninterrupted access of GC B cells to antigen-coated FDCs followed by the ability of "reprimed" B cells to express pMHCII complexes to amounts that gain competitive access to pMHCII-specific GC T_{FH} cells.

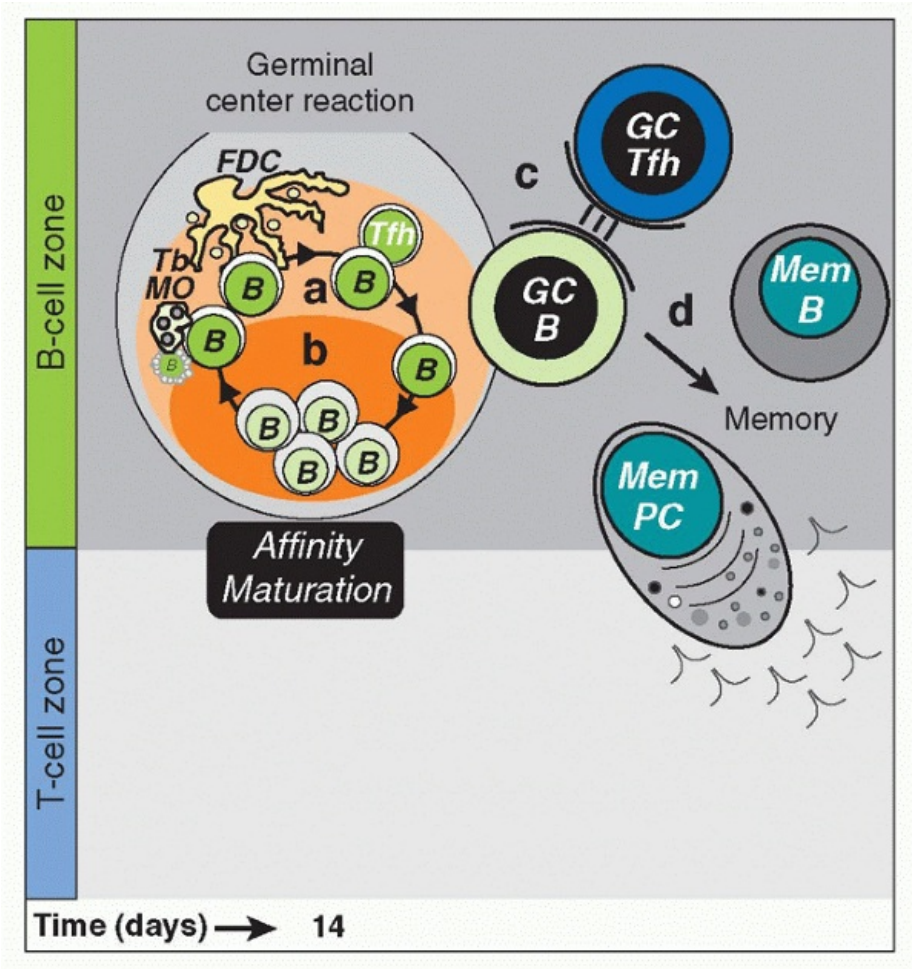

FIG. 10.3. The Germinal Center (GC) Reaction. A: Polarization of the secondary follicle anatomically signifies initiation of the GC cycle with the dark zone supporting GC centroblast expansion, class switch recombination and B-cell receptor (BCR)

diversification through somatic hypermutation. **B:** Noncycling GC centrocytes move to the light zone and continually scan follicular dendritic cell networks. Centrocytes that lose antigen binding will undergo apoptosis while those that express variant BCR with higher affinity can compete for binding to antigen-specific GC TFH cells. **C:** Cognate contact with GC TFH cells requires peptide major histocompatibility class II expression by GC centrocytes that can promote reentry into the dark zones and the GC cycle or exit from the GC. **D:** Entry into the affinity-matured memory B cell compartments of nonsecreting memory B cells and post-GC plasma cells.

More recently, labeling of B cells based on GC zonal location with a photo-activatable green fluorescence protein tag provided more conclusive evidence for these activities in vivo.[162] These elegant studies indicated that proliferation was largely restricted to the dark zone followed by a net movement to the light zone. Importantly, movement back into the dark zone and reinitiation of proliferation was controlled by antigen presentation to GC T_{FH} cells.[162] These studies provide experimental evidence for the reiterative cycles of BCR diversification and positive selection as central events during affinity maturation driving clonal evolution in the antigen-specific memory B cell compartment (Fig. 10.4).

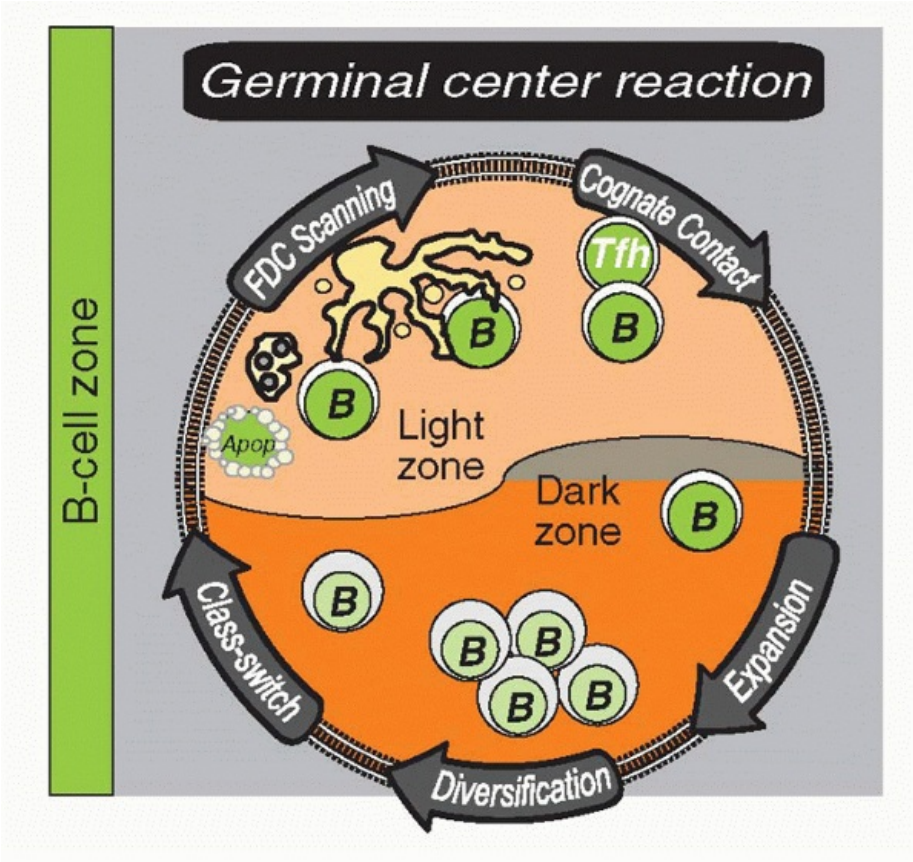

FIG. 10.4. The Germinal Center (GC) Cycle. The GC cycle is initiated through pre-GC contact with cognate follicular helper T (T_{FH}) cells that promotes extensive proliferation of antigen-primed B cells. The GC cycle is thought to begin when immunoglobulin D-secondary follicles polarize into micro-anatomically distinguishable dark zones (DZ)

(containing centroblasts) and light zones (LZ) (containing centrocytes, antigen-laden follicular dendritic cell [FDC] networks, and antigen-specific GC T_{FH} cells). Antigen-specific GC B cells' clonal expansion in the DZ is accompanied by B-cell receptor (BCR) diversification through SHM and antibody class switch. Both SHM and CSR are associated with transcriptionally active gene loci, are associated with deoxyribonucleic acid replicative and repair machinery, and occur during cell cycle. Hence, we associated these activities with the DZ phase of the GC cycle. Exit from cell cycle coincides with relocation of noncycling GC B cells to the LZ. Continual scanning of immune complex-coated FDC is observed in the LZ and has been associated with the potential for GC B cells to test variant BCR for antigen binding. Loss of antigen binding can lead to death by apoptosis and clearance of dead cells by tingible body macrophages in the LZ. Positive signals through the BCR during FDC scanning program GC B cells to compete for contact with cognate GC TFH cells. Productive contact with GC TFH cells can induce reentry into the GC cycle through movement back into the DZ and induction of cell cycle and BCR rediversification. Alternatively, affinitymatured GC B cells can exit the GC as either a nonsecreting memory B cell precursors for the memory response, or secreting long-lived “memory” plasma cells that contribute to serologic memory.

Memory B-Cell Evolution

The GC reaction requires T-cell help as it is absent in athymic nude mice, CD40- and CD40L-deficient mice, and is diminished using reagents that block or deplete T_H-cell function such as anti-CD4, anti-CD40, and anti-CD28.[163,164,165] Mice deficient in ICOS also display profound defects in all aspects of T-cell-dependent B-cell responses.[74,75,76] Conversely, mice deficient in Roquin, an inhibitor of ICOS expression, produce excessive numbers of T_{FH} cells and GCs and are prone to autoimmunity.[64] However, TI antigens can also promote GC

reactions, but they collapse within the first week after priming with no evidence of BCR diversification.[166] In contrast, marginal zone B cells can be recruited into T-cell-dependent responses and form GCs that diversify and affinity mature.[167] Interestingly, in these studies, the marginal zone B cells responding to antigen challenge expressed a preimmune BCR repertoire distinct from that of the follicular B cells responding in the same animals. Hence, there is some redundancy to the control of GC formation and flexibility to the origins of the B cells recruited into the GC reaction.

The transcriptional repressor Bcl6 is highly expressed in the GC and is necessary for GC formation.[168] However, IgM and IgG1 antigen-specific memory B cells can develop in the absence of Bcl6.[169] These studies were based on direct labeling with antigen and their capacity to respond to soluble low-dose antigen recall. However, there were no GC formed, and the memory B cells expressed no SHM or evidence for affinity maturation. Bcl6 has been shown to repress p53, the tumor suppressor gene that controls DNA-damage-induced apoptosis.[170] Regulation of p53 in this manner may protect GC B cells to allow for the DNA breaks that are necessary intermediates in SHM and CSR. Bcl6 also directly represses Blimp-1[171] and hence must be lost at some point during the GC reaction to allow

subsequent development of memory B cells and post-GC PCs in vivo.[137]

The GC cycle of activity regulates clonal evolution within antigen-primed B-cell responders. All pre-GC B cells express some measure of antigen specificity and appear preselected into the memory B-cell pathway based on germline BCR expression. Massive and rapid clonal expansion with doubling times of 6 to 8 hours underpins secondary follicle formation and drives clonal expansion in the GC reaction. It is now clear that GC B cells in the light zone also have the capacity to proliferate.[172] Intravital studies emphasize the more open nature of the GC itself demonstrating the capacity of naive B cells to traverse the follicular region occupied by the antigen-responsive GC.[173] Importantly, antigen-specific B cells could also be recruited into ongoing GCs if they expressed sufficiently high-affinity BCR. While these studies use high frequencies of BCR transgenic B cells pulsed into the ongoing response, it is intriguing to consider the ramifications of such an open network of affinity-based selection and its impact on the composition of the memory B-cell compartment.

Somatic Hypermutation

BCR diversification is dependent on DNA replication and largely restricted to GC B cells in the pathway to memory. Upon expansion in the GC reaction, antigen-specific B cells downregulate their germline BCR and diversify their variable region genes through SHM. Single base substitutions, rare insertions, and deletions are introduced into a region spanning 1.5 to 2.0 kb downstream of the transcription initiation site; however, activity peaks within the V(D)J region and decreases within the J-C intronic region of IgH and IgL variable genes.[174] The mutation rate approaches 10-3 per base pair per generation, which is approximately six orders of magnitude higher than spontaneous mutation frequencies. Thus, approximately one mutation is introduced with each cell division. Analysis of mutation in “passenger” Ig transgenes that are not under selection pressure indicate intrinsic sequence hot spots for the mutator mechanism.[175] Hence, BCR diversification accompanies extensive clonal expansion within the GC reaction generating progeny that express variant antigen-binding BCR (Fig. 10.5).

AID is the central component of the SHM mechanism. Originally discovered through complementary DNA subtraction focused on novel genes in GC B cells,[176] it was then found to be defective in an autosomal-recessive form of hyper-IgM syndrome.[108] Mice deficient in AID were able to form the GC reaction but were unable to undergo CSR or SHM.[107] AID deaminates cytosine to uracil in singlestranded DNA that can be processed by a mutagenic repair pathway.[105,177] The initial changes target sequence-dependent hotspots within rearranged variable region genes of antibodies. Uracil excision by uracil DNA glycosylase is then processed by error-prone DNA replication to introduce point mutations within the actively transcribed Ig locus. Errorprone processing using mismatch repair and base excision repair factors is selectively offset with high-fidelity processing to protect genome stability.[178] The targeting spectrum of AID associated with its active site can be altered to modify variable region gene SHM[179] and rate of antibody diversification.[180] AID stability within the cytoplasm of Ramos B-cell lines can be regulated by heat shock protein 90 with specific inhibition leading to destabilized AID[181] providing a means to modify the rate of antibody diversification. The details of this mutating complex, as well as its action and regulation within

the GC reaction, are active areas of research that have been reviewed in detail elsewhere.[182]

Cell Cycle Arrest and Apoptosis

The control of cell cycle in the GC environment is of fundamental importance to the evolution of high-affinity B-cell memory. Variant GC B cells exit the cell cycle and move toward the light zone to undergo selection. Dysregulated cell cycle arrest promotes enlarged GCs in situ. This phenotype is found in multiple genetic knockout models that also influence the composition of the memory B-cell compartment. Large GCs form in the absence of AID and are thought to indicate a negative feedback mechanism that follows SHM and/or CSR.[107] The absence of the CDK inhibitor $p18^{INK4c}$ more directly impacts GC size through blocking cell cycle arrest and also decreases the formation of long-lived PCs.[183] The large GCs in the absence of Blimp-1[137] may also be related to the lack of cell cycle control that may contribute to the post-GC PC defect in these mice. The absence of the main regulatory subunit of calcineurin, CnB1, results in the large GC phenotype, the loss of late stage antibody production, and the diminution of the memory B-cell response to antigen recall.[184] It will be important to assess whether these putative cell cycle defects directly impact high-affinity memory B-cell evolution or indirectly modify GC B-cell fate and the onset of apoptosis.

It is generally believed that the majority of GC B cells expressing variant BCR will die in situ. Extensive local apoptosis

is characteristic of the GC reaction. Furthermore, overexpression of antiapoptotic molecules such as Bcl-2 or Bcl-xL prolongs survival of GC B cells without improving selection efficiency.[185] In the absence of CD95 (Fas), there is also a dilution of high-affinity clonotypes in the memory B-cell compartment.[186] Cell death is a prevalent outcome of the GC cycle.[187] Fas receptor expression on B cells regulates negative selection in the GC.[188] Moreover, myeloid cell leukemia sequence 1 has emerged as a major antiapoptotic factor controlling GC B-cell formation and survival.[189] Positive selection of variant GC B cells must be a major driving force within the GC and is based on the increased capacity of the mutated BCR to bind antigen. Mutations in the comodifiers of BCR signal also impact GC B-cell dynamics. Unlike the CnB1 mutation, defects in CD45 and CD19 reduce proliferation and survival of GC B cells arguing for a more global impact of these molecules on BCR signal integration.[172,190,191] Initiation of the GC development program may also be altered to differing degrees in these animal models in ways that are difficult to dissect. Driving the expression of Cre recombinase at a stage in development when germline Cγ1 has been transcribed has helped to overcome these problems.[192] This model allows the conditional deletion of alleles at a late stage in antigen-driven B-cell development that occurs after the first T_H -B-cell checkpoint in development.

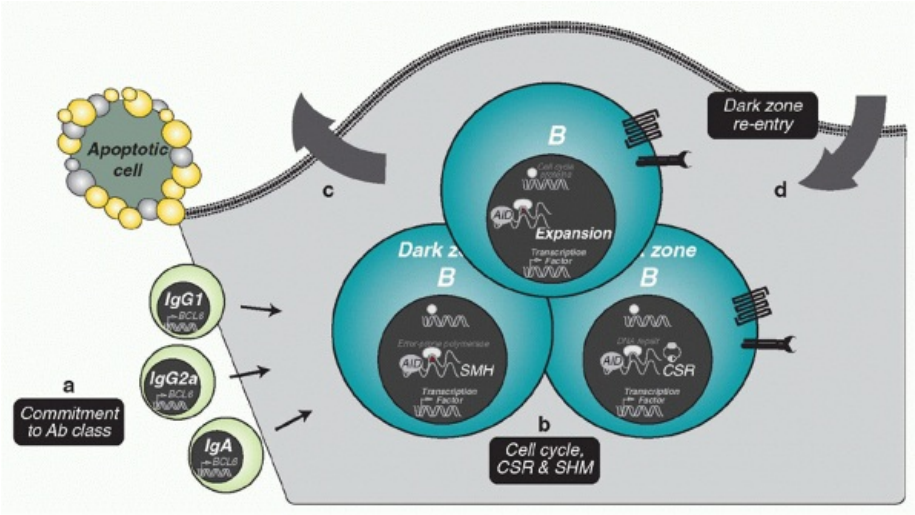

FIG. 10.5. The Germinal Center (GC) Dark Zone (DZ). A: Pre-GC cognate follicular helper T cues instruct antigen-primed B cells to initiate the GC reaction. It is likely that commitment to antibody class is preprogrammed at this initial juncture and all classes of B cells can seed the primary GC response. **B:** Molecular control of the cell cycle is an integral component of DZ B-cell dynamics and Bcl6 expression involved in ways that remain poorly resolved. Activation-induced cytidine deaminase (AID) expression, cytosine deaminase activity, and uracil deoxyribonucleic acid (DNA) glycosylase initiate the somatic hypermutation (SHM) machinery targeted to single-stranded DNA. Uracil excision is then processed by error-prone DNA replication to introduce point mutations into the rearranged antibody variable genes. Class switch recombination (CSR) can also occur during this DZ phase using AID to target DNA cleavage to antibody switch regions, generate double strand DNA breaks that trigger the DNA damage machinery to complete the CSR event. The interrelatedness of cell cycle control, SHM, and CSR is not clearly resolved in vivo. **C:** DZ GC B cells will reexpress diversified B-cell receptors and enter the light zone (LZ) of the GC reaction to undergo antigen-based selection. **D:** Following antigen-based selection and cognate contact with GC follicular helper T cells, LZ GC B cells can reenter the DZ regions and rediversify the BCR to initiate secondary rounds of affinity maturation.

Affinity Maturation

Affinity maturation requires the positive selection of GC B cells expressing high-affinity variant BCRs. The details of this process are still poorly understood. Receipt and integration of signals through the BCR are clearly involved in positive selection and must be based on the affinity for antigen. Ablation of BCR signals such as calcineurin-dependent signals, mutations in the CD19 signaling pathway, and loss of CD45 interferes with positive selection and memory B-cell development.[172,184,190,191] However, as discussed previously, it is difficult to dissect the GC BCR interactions with antigen from the initial BCR triggers that recruited naive B cells into the primary response. Most models suggest immune complex (IC) trapping on FDCs as the most likely means for variant BCRs to receive a rescuing signal from native antigen. Antigen appears rapidly in this location and can persist for extended periods of time focused to FDC in lymphoid tissue draining the site of antigen administration.[193] In support

of this notion, complement receptors CD21/CD35 on FDCs are needed to generate long-term serum antibody responses.[194] However, animals that

do not secrete antibody and therefore cannot form ICs can still support affinity maturation.[195] In the absence of inhibitor of NF-κB kinase 2-dependent activation of the NF-κB pathway, FDCs can still capture ICs but are not stimulated to upregulate vascular cellular adhesion molecule-1 and intercellular adhesion molecule-1.[196] In the absence of these adhesion molecules, GC B cells appear more susceptible to apoptosis with evidence for altered gene expression and decreased affinity maturation.

Evidence connecting BCR signal strength within the GC B-cell compartment and affinity maturation has been lacking. BCR signaling and antigen presentation are required to initiate the GC reaction and hence are difficult to manipulate specifically within the GC. In the absence of DOCK8, which causes compromised early immune synapse formation, early GCs still develop.[14] However, without DOCK8, these GCs do not persist and GC B cells do not undergo affinity maturation. Calcium influx as a consequence of BCR signaling also appears dispensable for affinity maturation under multiple T cell-dependent priming conditions in vivo. Although B cells deficient for stromal interaction molecule 1 and stromal interaction molecule 2 or for CNB1 exhibit profound defects in proliferation in vitro,[15,18] these signaling molecules are dispensable for the maturation of antibody responses in vivo. Downstream of BCR signals, the transcription factor myocyte enhancer factor 2c is necessary for early B-cell proliferation and GC formation,[16,17] but dissecting the pre-GC versus GC function remains unresolved. B cell-specific deletion of nuclear factor of activated T cells, cytoplasmic 1 also compromises B-cell responses in vivo[197] but the level of the defect remains unclear. Nevertheless, as BCR signal strength must drive affinity maturation at some level, it remains important to resolve the B-cell intrinsic mechanisms that also help to shape the affinity of the memory B-cell compartment.

Antigen Scanning on Follicular Dendritic Cell Networks

Polarity in the GC microenvironment is partly controlled by the differential expression of chemokines and their receptors.[198] Higher expression of CXCL12 in the dark zone assorts CXCR4-expressing centroblasts, while higher CXCL13 in the light zone attracts CXCR5-expressing centrocytes. This study used flow cytometry and cell cycle status to identify GC B-cell subsets and demonstrate the aberrant behavior of GC B cells from various genetically modified host animals. Recent two-photon analysis revealed the movement of GC B cells along FDC processes within the GC.[159] This study emphasized the lack of dwell time for GC B cells on FDC processes with little change in GC B-cell velocity upon FDC contact. Therefore, if antigen binding is associated with GC B cell-FDC contact, there appears to be no evidence for interclonal competition between different GC B cells. These studies describe when variant GC B cells are most likely to contact antigen with the opportunity to test the binding properties of their expressed mutated BCR (Fig. 10.6).

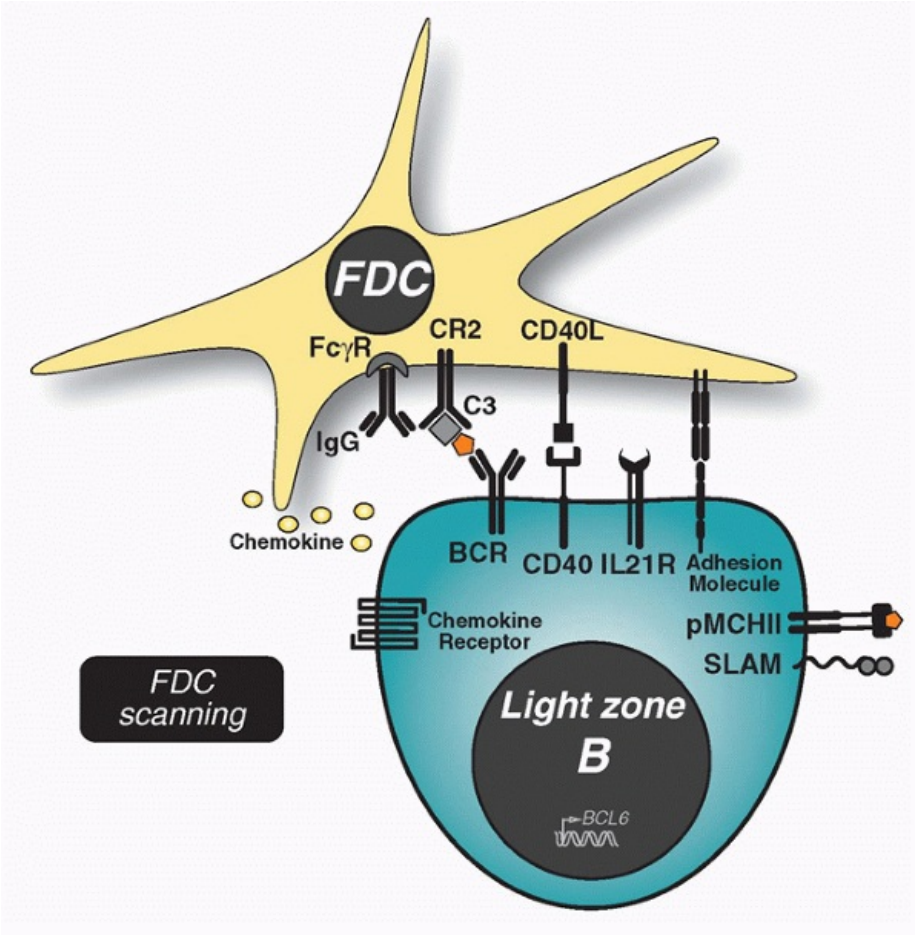

FIG. 10.6. Antigen Scanning on Follicular Dendritic Cell (FDC) Networks. FDC scanning appears as continuous movement of germinal center B cells along mature immune complex-laden FDC processes. These contacts are more similar to stromal cell-associated trafficking behavior than stable immune synapse-like interactions. Differential B-cell receptor (BCR) affinity for antigen may influence antigen-uptake and peptide major histocompatibility class II presentation at this juncture of development. Programs of gene expression for molecules able to modify cognate contact may also be differentially induced due to BCR signal strength through FDC scanning.

As with all in vivo studies, there are substantial influences associated with the choice of models, affinities of BCR, type of antigen, and method of immunization that will impact the observations presented in the studies. The issues of antigen receptor monoclonality are self-evident in BCR transgenic models in the context of repertoire studies, but are less clearly understood in association with the expression of diverse effector functions in vivo. More importantly, in the context of T-cell responses, it is now apparent that elevated precursor frequency deviates the dynamics of clonal expansion[199,200] and can alter the development of T-cell memory in vivo.[201] Changing the balance or affinity of regulator populations will impact cell fate of the target populations in ways that remain difficult to assess. Nevertheless, new insights offered by dynamic imaging continue to buttress our appreciation for the workings of the immune system and serve to challenge existing dogma in powerful and

productive ways.

Germinal Center Follicular Helper T Cells

In contrast to pre-GC effector T_{FH} cells discussed previously, CXCR5+ T_H in GC will be referred to as GC T_{FH} cells. Many CXCR5+ T_{FH} cells were found very early in the immune response (day 3), at the T-B borders and within follicular regions of LNs.[58] These early studies also provided evidence for a circulating compartment of CXCR5+ T_H cells. Hence, it is clear that CXCR5 expression is not a reliable marker of GC T_{FH} cells only. Further, the expected function of GC T_{FH} cells would be broadly different from those described for pre-GC effector T_{FH} cells. The presence of GC T_{FH} cells among the dense FDC networks of the GC light zone predict a role in the propagation of high-affinity variant GC B cells into the long-lived memory B-cell compartment[198] (Fig. 10.7). Subsequent to FDC scanning, some GC B cells were shown to make more stable, immune synapse-like contacts with GC T_{FH} cells, as determined by two-photon imaging.[159] These early images gave rise to the notion that competition for GC T_{FH} cells may be the limiting factor in GC B cell selection of variant high-affinity BCRs.[161] More recently, antigen presentation by GC B cells without engaging the BCR was shown to dominate the selection apparatus within GCs.[162] GC B cells capable of presenting more antigen rapidly exited the GC reaction and produced more post-GC plasma cells. These studies indicated similar mechanisms to the pre-GC selection event[102] and argued strongly that antigen presentation to GC T_{FH} cells was the rate-limiting event during affinity maturation in the GC cycle.

It remains technically difficult to manipulate cellular and molecular activities within the GC cycle without interfering with the developmental programs that initiate the GC reaction in the first place. Many of the molecules associated with pre-GC T_{FH} function may also function within the GC. BCL6 expression itself is reinforced within T_{FH} cells upon contact with pre-GC B cells.[98] ICOS-ICOSL interactions are important throughout this pathway at early DC contact,[202] pre-GC contact,[78] and likely during the GC reaction itself. IL-21 and its receptor appear of continued importance at the pre-GC stage and during the GC reaction.[86,87] Sphingosine-1 phosphate receptor 2 has an important role in confining GC B cells to the GC niche in vivo.[203] Elevated programmed cell death 1 expression also correlates with GC localization of the T_{FH} compartment[82] and the absence of programmed cell death ligand 2 on B cells impacts PC production and affinity maturation.[204] Most interestingly, cytokine production and class-specific GC B cells appear to be associated in the GC well beyond the original CSR event.[205] This surprising functional pairing between GC T_{FH} cells and switched GC B cells (eg, IL-4+ T_{FH} cells with IgG1+ GC B cells; IFNγT_{FH} cells with IgG2a+ GC B cells) hints at the extended level of heterogeneity that exits within the GC cycle of memory B-cell development. Hence, it is likely that each separable class-specific GC B-cell compartment requires cognate contact with separate class-specific GC T_{FH} cells. During antiprotein immune responses, the T_{FH} cells within the light zone of the GC express pMHCII-specific TCRs. There appears to be sequential movement of antigenspecific T_{FH} cells from

the T-cell zones into the GC microenvironment 40,[206] with evidence that suggests GC T_{FH} cells can also move between different GCs.[207] Interestingly, all antigen-specific T_{FH} cells responding to a protein antigen are not represented within the GC reaction, indicating pre-GC functional differentiation for responders T_{FH} cells.[208] Furthermore, non-GC T_H cells can reemerge in a memory response, indicating that the GC is not required for memory T_H-cell development.[40] Downregulation of CD90 (Thy-1) has been used as a marker of GC T_{FH} cells in the mouse, although when this occurs and how well it discriminates only GC T_{FH} cells is not clear.[207] Interfering with CD40L-CD40 interactions disrupts the GC reaction, while blocking B7-2 interaction impairs memory B-cell development.[209] Hence, the cognate control of GC B-cell fate appears to be modified by the cellular and molecular context of pMHCII presentation.

The work on human T_{FH} cells use expression of CD57, as well as CXCR5, to define T_{FH} cells that are contained within the GC of tonsils.[63,210] Microarray analysis highlights the distantly related functional programs of CD57+ GC T_{FH} cells as compared to naive T_H cells, central memory T_H cells, and effector memory T_H cells from the peripheral blood.[210] Differences in adhesion molecules, chemokine receptors, cytokines, and transcription factors appear as distant as different lineages. Interestingly, the CXCR5 ligand, CXCL13, is highly expressed in GC T_{FH} cells. In vitro-derived T_H1 and T_H2 effector T_H cells also appear distant in gene expression program to GC T_{FH} cells.[63] This analysis associates CD84, CD200, IL-21, and BCL6 with CD57+ GC T_{FH} cells. A separate analysis of T_{FH} cells by function and gene expression demonstrates that T_{FH} activity in human tonsil is independent of CD57 expression.[65] These studies highlight $ICOS^{hi}$ $CXCR5^{hi}$ T_{FH} as CXCL13 secretors and the most potent inducers of antibody production in vitro. Therefore, it is clear in situ that all GC T_{FH} cells express CXCR5 and many express CD57; however, these molecules are not exclusively expressed on GC T_{FH} cells nor expressed on all T_{FH} cells in the tonsil. Nevertheless, it remains important to clarify GC T_{FH} phenotype so that the details of their function can be pursued in vivo.

MEMORY B-CELL RESPONSE

It is now clear that centrocytes can undergo proliferation in the light zone,[191] and there is direct evidence for movement

of centrocytes back into the dark zone of GCs.[159,173] Thus, it appears that one outcome of GC B cell-GC T_{FH} interactions is reentry into the GC cycle permitting reiterative rounds of expansion, diversification, and selection. In this model, each subsequent round of clonal expansion and BCR diversification is applied to GC B-cell variants that have been positively selected based on increased BCR affinity. Hence, the introduction of a few mutations in each clonal progeny of selected variants is less likely to destroy BCR specificity and leaves room for further increases in an already high-affinity variant BCR.

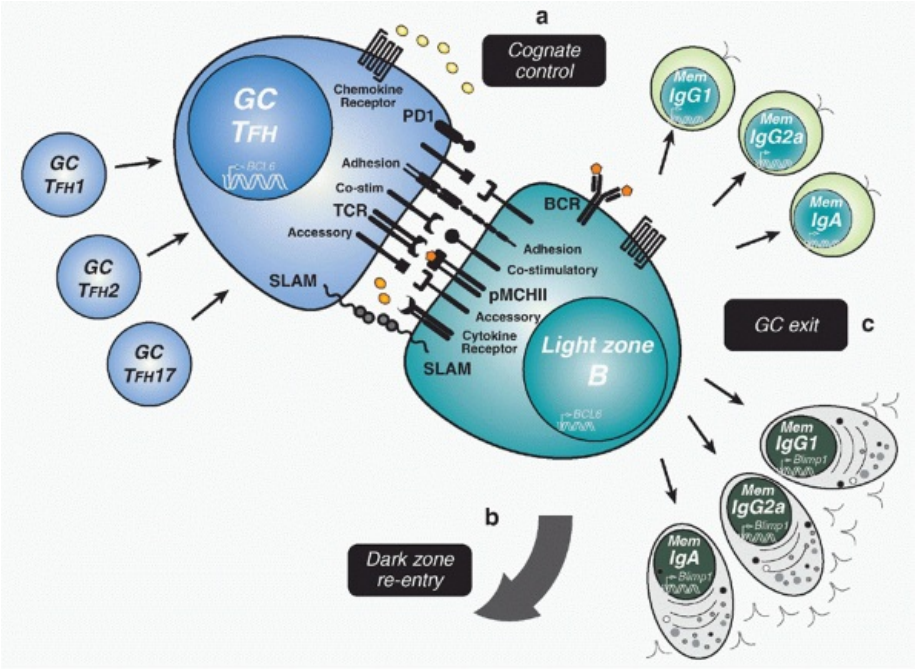

FIG. 10.7. Germinal Center (GC) Follicular Helper T (TFH)-B-Cell Contact. A: Longer duration cognate contact with GC TFH cells in the light zone can be visualized directly in vivo. Similar to earlier pre-GC events these contacts must focus around T-cell receptor-peptide major histocompatibility class II interactions and can be modified by a multitude of intercellular exchanges of molecular information. There is still little detailed analysis of these interactions in vivo. We depict the class of molecules that can be associated with this critical programming event but do not appreciate the organization of these interactions or their precise developmental imprint as yet. **B:** Antigen presentation by B cells can influence dark zone reentry and reinitiation of B-cell receptor diversification with cell proliferation, somatic hypermutation, and class switch recombination. **C:** GC cognate contact can also initiate GC exit into separable nonsecreting memory B cell and post-GC longlived memory plasma cell compartments.

The second major developmental outcome of cognate contact in the GC is exit from the GC cycle and entry into the memory B-cell compartment. The GC reaction produces at least two broad categories of affinity-matured memory B cells.[187] The most typical memory B cells are the precursors for a memory response to antigen recall. These memory B cells are easily distinguished functionally from the second cellular compartment of memory, the post-GC PCs.[211] The post-GC PCs are terminally differentiated cells that continually produce high-affinity antibody and will not be drawn into a secondary response. In both categories of memory B cells, the expression of antibody isotype distinguishes separable memory B-cell compartments. By definition, these subsets differ in the antibody they can produce and hence their developmental program and cellular function is distinct. However, these “class-specific” memory B-cell subsets may also differ in migration patterns, survival needs, and the requirements for reactivation at the time of antigen recall. These issues define a level of heterogeneity that has received

very little attention at the current time and remain important features in the organization of B-

cell memory in vivo (Fig. 10.8).

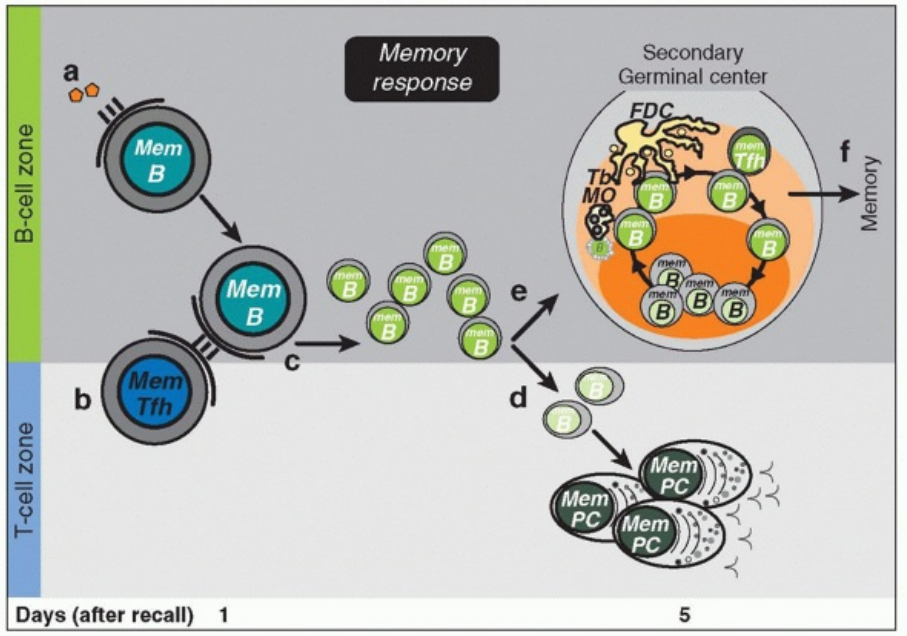

FIG. 10.8. Memory Response to Antigen Recall. A: Memory B-cell responses can emerge in the absence of innate inflammatory stimuli. In this case, the central antigen presenting cells are the affinity-matured memory B cells themselves. **B:** Memory B-cell response to T-cell-dependent antigens still require helper T (T_H)-cell regulation upon antigen recall. When priming and recall antigens are identical, memory T_H cells are the rapid responders and are thought to emerge preferentially over their low-frequency naïve counterparts. Regarding the regulation of memory B-cell responses, antigen-specific memory T_{FH} cells are the most likely candidate for rapid cognate regulation. **C:** Cognate contact at this developmental juncture occurs across sets of memory B cells and memory T_{FH} cells with an organization and kinetics that remain poorly resolved in vivo. There is rapid and vigorous local clonal expansion within the first 2 to 3 days in both memory B and memory T_{FH} compartments. **D:** Expansion of affinity-matured memory B response plasma cells occurs very quickly with evidence that most memory-response plasma cells already appear affinity-matured. **E:** There is evidence for memory B-cell subsets that express a germinal center (GC) phenotype and create GC-like structures upon antigen recall. Whether these structures are residual from the primary response GC or reemerge with GC activities upon recall has not been resolved. **F:** Increased numbers of memory B cells and memory-response plasma cells persist after antigen recall. It remains unclear whether these cells are the product of memory GC reactions or extrafollicular non-GC memory response outcomes.

Memory Response Precursors

There are reports of non-GC early memory B-cell development,[86,169] although how well these germline BCR-expressing memory B cells compete with post-GC memory B cells in the response to antigen recall remains to be evaluated. Affinity-matured IgM+ memory B cells can

emerge from the GC reaction and persist for long periods in vivo.[212] These nonswitched memory cells appear more active in secondary responses in the absence of circulating antibodies. Genetic labeling of AID-expressing cells with yellow fluorescent protein allowed memory B cells to be monitored over long periods.[213] Surprisingly, primary response GC reactions were persistent for extended periods of time (over 8 months after priming) depending on the form of antigen used to immunize. In these studies, class-switched memory B cells rapidly promoted plasma cell differentiation, whereas their IgM+ counterparts promoted secondary GC reactions. Depending on the form of antigen delivery and the combination of innate stimuli, B-cell responses can be skewed toward memory formation with extended GC reactions, which can last over 1.5 years.[214] Hence, it is possible that persistent GCs can continuously produce nonsecreting memory B cells well after the initial priming event. Memory response precursors are more typically affinity-matured post-GC B cells that have switched to non-IgM antibody isotypes and can respond, in a T_H -cell-dependent manner, to low-dose soluble antigen recall. The long-term persistence in vivo of antigen-binding B cells, after the decline of the primary response, provides the single best indicator for all memory response precursors. Beyond this particular attribute, antibody isotype and

stage of development within the memory B-cell compartment introduces a level of cellular heterogeneity that is only recently being appreciated.

As soluble antigen is the ligand for the BCR, most experimental approaches use variations of labeled antigen to identify antigen-specific B cells. While panning techniques used gel-associated antigen to detect antigen-specific B cells,[215,216] flow cytometry provided the most reliable access to antigenspecific B cells.[217,218] These earliest studies[217,218] coupled cell sorting technology and direct labeling to enrich antigen-specific B cells for adoptive transfer. These early studies helped to demonstrate that B cells with receptors for antigen were the precursors for antibody-forming cells. This approach has been adopted by many groups,[219,220,221,222] with the subsequent evaluation of specificity and purity demonstrated by the frequency for antibody forming cells or the enrichment for production of antigen-specific antibodies in vitro. Early studies indicated antigen-binding B-cell populations of long-lived cells with slow or no turnover in vivo.[223] These memory B cells can survive independent of their expressed BCR specificity and hence do not require persistent antigen depots in vivo.[224] Loss of surface IgM/IgD and expression of downstream isotype are also indicative of antigen experience but are not required for memory-cell development.[218,220,225] Further, the expression of mutated BCRs with evidence for affinity increasing changes is the most useful molecular marker for the memory B-cell compartment.[131,226,227,228] However, there are also abundant examples of germline-encoded BCRs expressed by memory response precursors. Location has also been a reliable means for isolating memory B cells from the blood very soon after intentional priming.[226] The combination of location, phenotype, genotype, and time after intentional priming has many elements of a comprehensive definition for memory B cells.

Post-Germinal Center Plasma Cells

High-affinity antibody-producing PCs that emerge from the GC reaction can also be considered an integral part of antigen-specific B-cell memory. High-affinity GC B cells preferentially assort into the PC compartment giving rise to highaffinity circulating antibodies.[229] In LNs, affinity-matured PCs dwell in paracortical areas to mature[230] then migrate toward the medullary regions prior to export.[231] CD93 is expressed at this early stage and is required for PC survival in the bone marrow.[232] Clearly, the circulating antibody that is produced by post-GC PCs contributes to ongoing serologic immune protection.[233] Post-GC PCs do not self-replenish through turnover, secrete isotype-switched antibody, and display evidence of SHM with affinity increasing mutation patterns.[211,234,235] This post-GC antigen-specific B-cell compartment appears during the second week after initial antigen exposure[131] and preferentially homes to the bone marrow for growth factor support of stromal cells.[236] Based on gene ablation studies, these cells use a variety of redistribution mechanisms such as upregulation of CXCR4, α4β1 integrin binding to its ligand vascular cellular adhesion molecule-1[135] to get to the bone marrow, where they can persist for the life of the animal.[133,237,238,239,240,241] In the bone marrow, long-lived PC need signals through the tumor necrosis factor receptor family member, BCMA, for survival.[242] It has also been proposed that a pre-PC precursor[243] or memory cells themselves[233] produce PCs in a non-antigen-dependent manner as a means of maintaining serum antibody levels for extended periods.

The extended longevity of the post-GC PC can be demonstrated using BrdU incorporation and adoptive transfer.[238,239,240,241] The extinguished gene programs associated with PC development[142,244,245] support a terminally differentiated end-stage cell that needs to arrest cell cycle progression[183] and will not be reactivated on antigen recall. Nevertheless, based on the evidence of a GC phase in development, the extended longevity of these PCs and the continued production of high-affinity antibody, it is reasonable to consider that these end-stage B cells belong to the memory B-cell compartment. We have recently demonstrated that post-GC antibody-secreting B cells not only express BCR, but also present antigen and can modulate cognate T_{FH}-cell responses.[132] These surprising studies further demonstrate that PCs negatively regulate BCL6 and IL-21 expression in antigen-specific T_{FH} cells.[132] Thus, PCs are not only the producers of antibody, but can also engage in antigen-specific immune regulation. Signals through the BCR or MHC class II on post-GC PCs may serve to regulate the ongoing production of high-affinity antibodies in the serum. The longterm antigen presenting or regulatory function of post-GC PCs has not yet been elucidated.

Based on variability in memory B-cell formation across different antigen models, it appears likely that different sets of initiating stimuli modify the balance of cells within the different memory B-cell compartments. The memory cell balance may be a quantitative “interpretation” of the quality of the initial immunizing signals. Ultimately, memory B-cell fate is likely to be controlled by interactions in the GC itself. The strength of BCR signal and costimulatory context serve to select variant BCR and initiate changes in memory B-cell development. Cognate interactions with GC T_{FH} cells may consolidate these functional outcomes. Understanding the role of the different memory B-cell subsets in long-term protection and the

rules that govern their development in vivo remain important unresolved areas in this field.

Memory Response to Antigen Recall

Persistent high-affinity serum antibody provides the first layer of protection against antigen recall. While binding to antigen is a clearance mechanism, it also serves to increase the efficiency of antigen presentation to memory B cells through rapid IC formation and binding to FcR or complement receptors on cells of the innate system. In this manner, antibody may amplify the sensitivity of the memory B-cell response to antigen recall. However, the boost with antigen rechallenge also seems necessary to establish adequate longterm protection following protein vaccination. The cognate cellular dynamics and molecular regulation of the memory response boost are important factors in the consolidation of B-cell memory that remain poorly understood and inadequately optimized in most vaccine regimes.

Primary response GCs persist for about 3 weeks after initial priming, but this timing may vary substantially depending on the immune stimuli.[151,152] There is evidence for continued selection in the memory B-cell compartment even after the demise of the GC reaction.[235] This selection process represents more typical interclonal competition without further BCR diversification.[246] Where this secondary selection occurs and how it relates to the selection mechanisms in the GC remains to be determined. Nevertheless, secondary selection events appear capable of reshaping the memory compartment toward higher-affinity clonotypes that can substantially influence the quality of the secondary response and consolidation of the memory compartment.[233] Recent evidence indicates that memory B cells can reinstitute a GC reaction upon antigen recall. The form of antigen appears to have a role in primary response GC persistence with particulate antigen more likely to promote GC longevity.[213] Moreover, innate system stimuli differentially impact persistent GC structures with combinations of toll-like receptor-4 and toll-like receptor-7 more effective than single stimuli.[214] Whether the secondary GC is a continuation and reexpansion of a primary GC activity remains unclear. More importantly, whether these secondary or persistent GC-like structures support the rediversification of affinity-matured BCR and the selection of even higher-affinity clonotypes remains to be determined. These issues are central to the future management of prime-boost vaccination protocols with substantial practical impact in this field.

Using priming doses of antigen and adjuvant, antigenspecific memory T_H-cell responses[40,41,247] and memory B-cell response[248] emerge more rapidly than their naive response counterparts. Memory T_H-cell responses reach peak levels more rapidly but to similar levels as the primary response. In contrast, memory B cells display accelerated kinetics and reach substantially higher maximal levels compared to the primary response. Even in the presence of priming doses of antigen and adjuvant, the memory responders dominate the recall response,[40,130] outcompeting naive lymphocytes that may express specific Ag-R.

Antigen recall promotes accelerated clonal expansion and rapid differentiation to high-affinity PCs. Initiation and microclustering of IgG1 BCRs is enhanced at the single cell level due to membrane proximal regions in the cytoplasmic tail of IgG1.[249] The cytoplasmic tails of class-

switched memory B cells can contribute substantially to the expanded clonal burst associated with retriggering by antigen.[250] There is evidence for distinct changes in BCR signaling pathways.[251,252,253,254] Increased affinity of the BCR on memory cells must also contribute to memory B-cell sensitivity to low-dose soluble antigen that does not induce a primary immune response. In addition to these intrinsic attributes, circulating highaffinity antibody contributes to differential management of antigen in vivo. Rapid presentation of immune complexes to the memory B cells maybe enhanced. Furthermore, memory B cells require antigen-specific T_H-cell regulation to initiate secondary immune responses.[255] These issues have not been well studied but remain central to the capacity of memory B cells to expand, self-replenish, and boost high-affinity PCs and circulating antibody levels that provide long-term immune protection.

Antigen-Specific Memory Follicular Helper T Cells

Memory B-cell response to protein antigens require antigen-specific T-cell help.[187] Under normal physiologic circumstances, this secondary T-cell help must be antigen-specific and is most likely delivered by memory T_H cells that are specific to the immunizing antigen. Under typical low-dose antigen rechallenge regime in the absence of immune adjuvant, antigen-specific B cells are likely to be the predominant APC for the memory response. Hence, the cognate interactions of pMHCII+ memory B cells and antigen-specific memory T_H cells define a major developmental checkpoint in the evolution of long-term immune protection. Alternately, circulating antibody and innate immune activation induce an accelerated memory response due to increased frequencies and heightened responsiveness of memory response precursors. In this scenario, the memory T_H cell-memory B-cell checkpoint may be preceded by an independent series of antigen presentation events prior to cognate memory T_{FH}-memory B-cell contact.

The major cellular outcome of the secondary boost is rapid and exaggerated memory B-cell expansion and the production of large numbers of high-affinity PCs. While high-affinity B cells can be drawn into GC reactions,[158] it is not clear that the GC pathway is operative at the time of the secondary boost. Regardless, there is clear evidence for secondary selection events upon antigen rechallenge that are more likely to be driven by cellular selection without somatic BCR diversification.[187] The nature of the selection mechanism for B cells at this stage of the response is also poorly understood. In contrast, the accelerated local reexpansion of antigen-specific memory T_H cells occurs with little change in the expressed TCR repertoire[256] or cytokinesecreting potential.[247] Hence, the antigen-specific memory T_H-cell compartment appears to conserve and reexpress many of the functions associated with the initial primary immune response.

There is evidence for a division of labor in the memory T-cell compartment that involves the migratory capacity of memory T_H-cell subsets.[257] Recirculating central memory T cells and tissue-homing effector memory T cells further divide on their readiness to reexpress effector function upon reexposure to antigen. It is reasonable to propose that each antigen-specific effector T_H-cell subset will produce a memory T_H-cell counterpart.[247] However, the range of T_H memory effector attributes required to control antigenspecific memory B-cell responses is

not well understood. Compartments of tissue-localized memory T_H cells provide rapid "reactive" immune protection that is antigen-specific.[257] For example, the focal placement of effector memory T-cell subsets at sites of original antigen entry preempts the behavior of the pathogen. In this manner, location provides an opportunity to accelerate the recall response enhancing the capacity of the immune system to block overt infection. As the precursors of the memory response to antigen recall,

high-affinity memory B cells belong to a similar "reactive" set of memory cells. However, in contrast to local hypersensitivity responses by T_H cells, antigen reexposure promotes rapid and exaggerated memory B-cell responses in the lymphoid tissue that drains the site of rechallenge. In this context, we can refer to the memory T_H-cell compartment that controls memory B cell responses as memory T_{FH} cells.

We have provided evidence for the local persistence of an antigen-specific memory T_{FH} cell compartment. CXCR5+ T_{FH} cells bind pMHCII tetramers, express lower levels of ICOS, and have lost the capacity to express messenger ribonucleic acid for a series of cytokines.[53] These putative memory T_{FH} cells rapidly upregulated cytokine signals following specific peptide restimulation in vivo. We propose that these locally confined memory T_{FH} cells are the cognate regulators of the memory B-cell response. Many of the antigen-specific memory T_{FH} cells retain expression of CD69, which suggests recent contact with pMHCII complexes and provides a plausible mechanism for local retention. It is likely that memory B cells with high-affinity BCR will rapidly capture minute levels of secondary antigen and present this antigen to the cognate memory T_{FH} cells. Hence, memory B cells may be the primary APC in the memory response, further accelerating the memory B-cell response to recall.

It is not clear whether memory T_{FH} cells would necessarily express CXCR5+ like their primary response T_{FH} compartment. Furthermore, it is not clear whether both effector T_{FH} and GC T_{FH} produce memory T_{FH} counterparts. Nevertheless, it will be important to unravel the programs of cognate control used by antigen-specific memory T_{FH} to regulate the memory B-cell response to antigen rechallenge. It is plausible that manipulating cellular and molecular interactions at this developmental juncture can alter the shape of antigen-specific B-cell memory.

Antigen Persistence In Vivo

Tonic signaling through the BCR and the downstream activation of phosphoinositide 3-kinase, together with B cell-activating factor signaling through the B cell-activating factor receptor are required for survival of naïve B cells in the periphery.[224,258] Similarly, inducible deletion of phospholipase Cγ2 after the formation of antigen-specific B-cell memory substantially depleted the memory B-cell compartment and suggested a BCR signaling requirement in memory.[259] Nevertheless, earlier genetic studies indicated that cognate BCR specificity was not required after formation of B-cell memory to provide the tonic survival signal.[224] Thus, persistent antigen appears not to be required for the survival of antigen-specific memory B cells, although memory B-cell function has not been addressed in this model. More recently, there has been evidence of persistent pMHCII complexes in the context

of antiviral responses in vivo,[260] leading to local activation of naïve T_H cells even after clearance of virus. We recently demonstrated a similar persistence of pMHCII complexes for longer than 100 days following protein antigen vaccination in a nondepot adjuvant.[261] The depots of pMHCII complexes were restricted to the LNs draining initial vaccination site and persistent antigen presentation induced naïve T_H-cell proliferation.[261] We proposed that pMHCII complexes on immunocompetent APCs had a role in confining the antigenspecific memory T_{FH}-cell compartment to LNs draining the site of initial priming.[262] Although it has been known for some time that FDC networks are capable of trapping whole antigen as immune complexes for extended periods of time,[161] the nature of the long-lived local APC remains unresolved. Unlike CD8 T cells, CD4 T_H cells appear to require continued presence of antigen to reach maximal clonal expansion in vivo. The conditional induction and abrogation of pMHCII molecules demonstrate that as soon as a lower threshold level of antigen is breached, T_H cells cease to divide.[263] Surprisingly, this model induces no form of inflammation and would otherwise be considered a model of tolerance induction. Recent intravital imaging studies also indicate that antigen-responsive T_H cells engage multiple DCs at successive early stages after priming.[264] These multiple DC-T_H-cell contacts also impact the development of effector T_H-cell function in vivo. Most surprisingly, there is evidence for persistent depots of pMHCII complexes up to 3 weeks after viral infection.[265] These pMHCII depots are present at times after viral clearance in vivo. The same persistent depots can be demonstrated for pMHCI with the capacity for local activation of viralspecific naive CD8 cells.[266] While persistent antigen is not required for the maintenance of antigen-specific T-cell memory,[267,268] there may be a role for persistent depots of pMHC complexes as local guidance cues for antigen-specific memory T cells.

CONCLUSION

Understanding the cellular and molecular control of antigen-specific memory B-cell development remains a high priority for basic research with important application to future vaccine design. The largest growing class of pharmaceutical agents today is the antigen-targeting antibodies with an endless spectrum of biologic impact. Producing potent antibodies with high affinity is an important facet of this growing new industry.

There has been substantial progress in our understanding of this complex developmental cascade in vivo with many of the cellular processes being more carefully defined in their most relevant in vivo context. The sophisticated manipulation of mouse genetics provides a powerful set of tools that begin to unravel the regulatory mechanisms operative across each developmental checkpoint controlling cell fate in vivo. Real-time intravital imaging provides a major advance to our appreciation of cell dynamics and intercellular communication as it proceeds in the crowded confines of secondary lymphoid tissue. Most importantly, a surge in research activity has revealed many of the mechanisms controlling T_{FH}-cell development and function in ways that have changed the conceptual landscape surrounding our appreciation of cognate regulation in B-cell memory.

Beyond antigen recognition, antibody class determines immune function and binding affinity

controls sensitivity within memory B cells. Antigen contact initiates presentation by B cells and a program of events, which is consolidated following B-cell contact with antigen-specific T_{FH} cells. We have outlined these events as a progressive developmental program across three related but distinct phases of antigen encounter. We remain cautious of manipulating critical adaptive immune functions but quietly optimistic. The molecular regulation of antigen-specific cellular events initiates a complex but finite set of regulatory programs that can be modified both indirectly, following vaccination with innate stimuli, and perhaps directly, during the acquisition of high-affinity B-cell memory. The vaccine boost is the most readily accessible phase of this strategy that can directly target antigen-specific adaptive responses. Unraveling the molecules and programs that control each phase of memory B-cell development provides a plethora of new targets for vaccine-based modification in vivo.

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REFERENCES

1. Pape KA, Catron DM, Itano AA, Jenkins MK. The humoral immune response is initiated in lymph nodes by B cells that acquire soluble antigen directly in the follicles. *Immunity*. 2007;26:491-502.

2. Roozendaal R, Mempel TR, Pitcher LA, et al. Conduits mediate transport of low-molecular-weight antigen to lymph node follicles. *Immunity*. 2009;30:264-276.

3. Batista FD, Harwood NE. The who, how and where of antigen presentation to B cells. *Nat Rev Immunol* . 2009;9:15-27.

4. Carrasco YR, Batista FD. B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. *Immunity*. 2007;27:160-171.

5. Junt T, Moseman EA, Iannacone M, et al. Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature*. 2007;450:110-114.

6. Phan TG, Green JA, Gray EE, Xu Y, Cyster JG. Immune complex relay by subcapsular sinus macrophages and noncognate B cells drives antibody affinity maturation. *Nat Immunol*. 2009;10:786-793.

7. Iannacone M, Moseman EA, Tonti E, et al. Subcapsular sinus macrophages prevent CNS invasion on peripheral infection with a neurotropic virus. *Nature*. 2010;465:1079-1083.

8. Batista FD, Iber D, Neuberger MS. B cells acquire antigen from target cells after synapse formation. *Nature*. 2001;411:489-494.

9. Batista FD, Neuberger MS. Affinity dependence of the B cell response to antigen: a threshold, a ceiling, and the importance of off-rate. *Immunity*. 1998;8:751-759.

10. Qi H, Egen JG, Huang AY, Germain RN. Extrafollicular activation of lymph node B cells by antigen-bearing dendritic cells. *Science*. 2006;312:1672-1676.

11. Sixt M, Kanazawa N, Selg M, et al. The conduit system transports soluble antigens from the afferent lymph to resident dendritic cells in the T cell area of the lymph node. *Immunity*. 2005;22:19-29.

12. Castigli E, Wilson SA, Scott S, et al. TACI and BAFF-R mediate isotype switching in B cells. *J Exp Med*. 2005201:35-39.

13. Fleire SJ, Goldman JP, Carrasco YR, Weber M, Bray D, Batista FD. B cell ligand discrimination through a spreading and contraction response. *Science*. 2006;312:738-741.

14. Randall KL, Lambe T, Goodnow CC, Cornall RJ. The essential role of DOCK8 in humoral immunity. *Dis Markers*. 2010;29:141-150.

15. Winslow MM, Gallo EM, Neilson JR, Crabtree GR. The calcineurin phosphatase complex modulates immunogenic B cell responses. *Immunity*. 2006;24:141-152.

16. Khiem D, Cyster JG, Schwarz JJ, Black BL. A p38 MAPK-MEF2C pathway regulates B-cell proliferation. *Proc Natl Acad Sci U S A* . 2008;105:17067-17072.

17. Wilker PR, Kohyama M, Sandau MM, et al. Transcription factor Mef2c is required for B cell proliferation and survival after antigen receptor stimulation. *Nat Immunol* . 2008;9:603-612.

18. Matsumoto M, Fujii Y, Baba A, Hikida M, Kurosaki T, Baba Y. The calcium sensors STIM1 and STIM2 control B cell regulatory function through interleukin-10 production. *Immunity*. 2011;34:703-714.

19. Capasso M, Bhamrah MK, Henley T, et al. HVCN1 modulates BCR signal strength via regulation of BCR-dependent generation of reactive oxygen species. *Nat Immunol* . 2010;11:265-272.

20. Damdinsuren B, Zhang Y, Khalil A, et al. Single round of antigen receptor signaling programs naive B cells to receive T cell help. *Immunity*. 2010;32:355-366.

21. Cantor J, Browne CD, Ruppert R, et al. CD98hc facilitates B cell proliferation and adaptive humoral immunity. *Nat Immunol* 2009;10: 412-419.

22. Yasuda T, Kometani K, Takahashi N, Imai Y, Aiba Y, Kurosaki T. ERKs induce expression of the transcriptional repressor Blimp-1 and subsequent plasma cell differentiation. *Sci Signal*. 2011;4:ra25.

23. Schnyder T, Castello A, Feest C, et al. B cell receptor-mediated antigen gathering requires ubiquitin ligase Cbl and adaptors Grb2 and Dok-3 to recruit dynein to the signaling microcluster. *Immunity*. 2011;34: 905-918.

24. Treanor B, Depoil D, Gonzalez-Granja A, et al. The membrane skeleton controls diffusion dynamics and signaling through the B cell receptor. *Immunity*. 2010;32:187-199.

25. Depoil D, Fleire S, Treanor BL, et al. CD19 is essential for B cell activation by promoting B cell receptor-antigen microcluster formation in response to membrane-bound ligand. *Nat Immunol*. 2008;9:63-72.

26. Weber M, Treanor B, Depoil D, et al. Phospholipase C-gamma2 and Vav cooperate within signaling microclusters to propagate B cell spreading in response to membrane-bound antigen. *J Exp Med*. 2008;205:853-868.

27. Thaunat O, Granja AG, Barral P, et al. Asymmetric segregation of polarized antigen on B cell division shapes presentation capacity. *Science*. 2012;335:475-479.

28. Duffy KR, Wellard CJ, Markham JF, et al. Activation-induced B cell fates are selected by intracellular stochastic competition. *Science*. 2012;335:338-341.

29. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature*. 1998;392:245-252.

30. Shortman K, Liu YJ. Mouse and human dendritic cell subtypes. *Nat Rev Immunol*. 2002;2:151-161.

31. Itano AA, Jenkins MK. Antigen presentation to naive CD4 T cells in the lymph node. *Nat Immunol*. 2003;4:733-739.

32. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. *Nature*. 2000;406:782-787.

33. Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol*. 2002;20:197-216.

34. Inohara N, Nunez G. NODs: intracellular proteins involved in inflammation and

apoptosis. *Nat Rev Immunol* . 2003;3:371-382.

35. Hou B, Saudan P, Ott G, et al. Selective utilization of toll-like receptor and MyD88 signaling in B cells for enhancement of the antiviral germinal center response. *Immunity*. 2011;34:375-384.

36. Lindquist RL, Shakhar G, Dudziak D, et al. Visualizing dendritic cell networks in vivo. *Nat Immunol* . 2004;5:1243-1250.

37. Kissenpfennig A, Henri S, Dubois B, et al. Dynamics and function of Langerhans cells in vivo dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. *Immunity*. 2005;22:643-654.

38. Itano AA, McSorley SJ, Reinhardt RL, et al. Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity. *Immunity*. 2003;19:47-57.

39. Malherbe L, Filippi C, Julia V, et al. Selective activation and expansion of high-affinity CD4+ T cells in resistant mice upon infection with Leishmania major. *Immunity*. 2000;13:771-782.

40. McHeyzer-Williams LJ, Panus JF, Mikszta JA, McHeyzer-Williams MG. Evolution of antigen-specific T cell receptors in vivo: preimmune and antigen-driven selection of preferred complementarity-determining region 3 (CDR3) motifs. *J Exp Med*. 1999;189:1823-1837.

41. McHeyzer-Williams MG, Davis MM. Antigen-specific development of primary and memory T cells in vivo. *Science*. 1995;268:106-111.

42. Alam SM, Travers PJ, Wung JL, et al. T-cell-receptor affinity and thymocyte positive selection. *Nature*. 1996;381:616-620.

43. Busch DH, Pamer EG. T cell affinity maturation by selective expansion during infection. *J Exp Med*. 1999;189:701-710.

44. Fasso M, Anandasabapathy N, Crawford F, Kappler J, Fathman CG, Ridgway WM. T cell receptor (TCR)-mediated repertoire selection and loss of TCR vbeta diversity during the initiation of a CD4(+) T cell response in vivo. *J Exp Med*. 2000;192:1719-1730.

45. Kedl RM, Rees WA, Hildeman DA, et al. T cells compete for access to antigen-bearing antigen-presenting cells. *J Exp Med*. 2000;192:1105-1113.

46. Kedl RM, Schaefer BC, Kappler JW, Marrack P. T cells down-modulate peptide-MHC complexes on APCs in vivo. *Nat Immunol* . 2002;3: 27-32.

47. Lyons DS, Lieberman SA, Hampl J, et al. A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. *Immunity*. 1996;5:53-61.

48. Matsui K, Boniface JJ, Steffner P, Reay PA, Davis MM. Kinetics of T-cell receptor binding to peptide/I-Ek complexes: correlation of the dissociation rate with T-cell responsiveness. *Proc Natl Acad Sci U S A* . 1994;91:12862-12866.

49. Savage PA, Boniface JJ, Davis MM. A kinetic basis for T cell receptor repertoire selection during an immune response. *Immunity*. 1999;10:485-492.

50. Lazarski CA, Chaves FA, Jenks SA, et al. The kinetic stability of MHC class II:peptide complexes is a key parameter that dictates immunodominance. *Immunity*. 2005;23:29-40.

51. Malherbe L, Hausl C, Teyton L, McHeyzer-Williams MG. Clonal selection of helper T cells is determined by an affinity threshold with no further skewing of TCR binding properties. *Immunity*. 2004;21: 669-679.

52. Malherbe L, Mark L, Fazilleau N, McHeyzer-Williams LJ, McHeyzer-Williams MG. Vaccine adjuvants alter TCR-based selection thresholds. *Immunity*. 2008;28:698-709.

53. Fazilleau N, McHeyzer-Williams LJ, Rosen H, McHeyzer-Williams MG. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. *Nat Immunol*. 2009;10:375-384.

54. Forster R, Emrich T, Kremmer E, Lipp M. Expression of the G-protein—coupled receptor BLR1 defines mature, recirculating B cells and a subset of T-helper memory cells. *Blood*. 1994;84:830-840.

55. Forster R, Mattis AE, Kremmer E, Wolf E, Brem G, Lipp M. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell*. 1996;87:1037-1047.

56. Forster R, Schubel A, Breitfeld D, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell*. 1999;99:23-33.

57. Walker LS, Gulbranson-Judge A, Flynn S, et al. Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXC chemokine receptor 5-positive CD4 cells and germinal centers. *J Exp Med*. 1999;190:1115-1122.

58. Ansel KM, McHeyzer-Williams LJ, Ngo VN, McHeyzer-Williams MG, Cyster JG. In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. *J Exp Med*. 1999;190:1123-1134.

59. Breitfeld D, Ohl L, Kremmer E, et al. Follicular B helper T cells express CXC

chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med*. 2000;192:1545-1552.

60. Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med* 2000;192:1553-1562.

61. Campbell DJ, Kim CH, Butcher EC. Separable effector T cell populations specialized for B cell help or tissue inflammation. *Nat Immunol* . 2001;2:876-881.

62. Kim CH, Rott LS, Clark-Lewis I, Campbell DJ, Wu L, Butcher EC. Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. *J Exp Med*. 2001;193:1373-1381.

63. Chtanova T, Tangye SG, Newton R, et al. T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. *J Immunol* . 2004;173:68-78.

64. Vinuesa CG, Cook MC, Angelucci C, et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature*. 2005;435:452-458.

65. Rasheed AU, Rahn HP, Sallusto F, Lipp M, Muller G. Follicular B helper T cell activity is confined to CXCR5(hi)ICOS(hi) CD4 T cells and is independent of CD57 expression. *Eur J Immunol* . 2006;36:1892-1903.

66. Shahinian A, Pfeffer K, Lee KP, et al. Differential T cell costimulatory requirements in CD28-deficient mice. *Science*. 1993;261:609-612.

67. Armitage RJ, Fanslow WC, Strockbine L, et al. Molecular and biological characterization of a murine ligand for CD40. *Nature*. 1992;357: 80-82.

68. Lederman S, Yellin MJ, Inghirami G, Lee JJ, Knowles DM, Chess L. Molecular interactions mediating T-B lymphocyte collaboration in human lymphoid follicles. Roles of T cell-B-cell-activating molecule (5c8 antigen) and CD40 in contact-dependent help. *J Immunol* . 1992;149:3817-3826.

69. Noelle RJ, Roy M, Shepherd DM, Stamenkovic I, Ledbetter JA, Aruffo A. A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proc Natl Acad Sci U S A* . 1992;89:6550-6554.

70. Hutloff A, Dittrich AM, Beier KC, et al. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature*. 1999;397:263-266.

71. Fazilleau N, McHeyzer-Williams LJ, McHeyzer-Williams MG. Local development of effector and memory T helper cells. *Curr Opin Immunol* . 2007;19:259-267.

72. King C, Tangye SG, Mackay CR. T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annu Rev Immunol* . 2008;26: 741-766.

73. Vinuesa CG, Tangye SG, Moser B, Mackay CR. Follicular B helper T cells in antibody responses and autoimmunity. *Nat Rev Immunol* . 2005; 5:853-865.

74. Dong C, Juedes AE, Temann UA, et al. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature*. 2001;409:97-101.

75. McAdam AJ, Greenwald RJ, Levin MA, et al. ICOS is critical for CD40-mediated antibody class switching. *Nature*. 2001;409:102-105.

76. Tafuri A, Shahinian A, Bladt F, et al. ICOS is essential for effective T-helper-cell responses. *Nature*. 2001;409:105-109.

77. Nurieva RI, Chung Y, Hwang D, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity*. 2008;29:138-149.

78. Vogelzang A, McGuire HM, Yu D, Sprent J, Mackay CR, King C. A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity*. 2008;29:127-137.

79. Yu D, Tan AH, Hu X, et al. Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. *Nature*. 2007;450: 299-303.

80. Linterman MA, Rigby RJ, Wong R, et al. Roquin differentiates the specialized functions of duplicated T cell costimulatory receptor genes cd28 and Icos. *Immunity*. 2009;30(2):228-241.

81. Linterman MA, Rigby RJ, Wong R, et al. Follicular helper T cells are required for systemic autoimmunity. *J Exp Med.* 2009;206(3): 561-576.

82. Haynes NM, Allen CD, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. *J Immunol* . 2007;179:5099-5108.

83. Johnston RJ, Poholek AC, DiToro D, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science.* 2009;325:1006-1010.

84. Nurieva RI, Chung Y, Martinez GJ, et al. Bcl6 mediates the development of T follicular helper cells. *Science*. 2009;325:1001-1005.

85. Yu D, Rao S, Tsai LM, et al. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity*. 2009;31:457-468.

86. Linterman MA, Beaton L, Yu D, et al. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J Exp Med*. 2010;207:353-363.

87. Zotos D, Coquet JM, Zhang Y, et al. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J Exp Med*. 2010;207:365-378.

88. Ozaki K, Spolski R, Feng CG, et al. A critical role for IL-21 in regulating immunoglobulin production. *Science*. 2002;298:1630-1634.

89. Spolski R, Leonard WJ. The yin and yang of interleukin-21 in allergy, autoimmunity and cancer. *Curr Opin Immunol* . 2008;20: 295-301.

90. Betz BC, Jordan-Williams KL, Wang C, et al. Batf coordinates multiple aspects of B and T cell function required for normal antibody responses. *J Exp Med*. 2010;207:933-942.

91. Ise W, Kohyama M, Schraml BU, et al. The transcription factor BATF controls the global regulators of class-switch recombination in both B cells and T cells. *Nat Immunol*. 2011;12:536-543.

92. Okada T, Miller MJ, Parker I, et al. Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS Biol*. 2005;3:e150.

93. Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAPcontrolled T-B cell interactions underlie germinal center formation. *Nature*. 2008;455:764-769.

94. Cannons JL, Yu LJ, Jankovic D, et al. SAP regulates T cell-mediated help for humoral immunity by a mechanism distinct from cytokine regulation. *J Exp Med*. 2006;203:1551-1565.

95. Crotty S, Kersh EN, Cannons J, Schwartzberg PL, Ahmed R. SAP is required for generating long-term humoral immunity. *Nature*. 2003; 421:282-287.

96. Schwartzberg PL, Mueller KL, Qi H, Cannons JL. SLAM receptors and SAP influence lymphocyte interactions, development and function. *Nat Rev Immunol* . 2009;9:39-46.

97. Kerfoot SM, Yaari G, Patel JR, et al. Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. *Immunity*. 2011;34(6):947-960.

98. Kitano M, Moriyama S, Ando Y, et al. Bcl6 protein expression shapes pre-germinal

center B cell dynamics and follicular helper T cell heterogeneity. *Immunity*. 2011;34(6):961-972.

99. Dal Porto JM, Haberman AM, Kelsoe G, Shlomchik MJ. Very low affinity B cells form germinal centers, become memory B cells, and participate in secondary immune responses when higher affinity competition is reduced. *J Exp Med*. 2002;195:1215-1221.

100. Shih TA, Meffre E, Roederer M, Nussenzweig MC. Role of BCR affinity in T cell dependent antibody responses in vivo. *Nat Immunol*. 2002;3:570-575.

101. Paus D, Phan TG, Chan TD, Gardam S, Basten A, Brink R. Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. *J Exp Med*. 2006;203:1081-1091.

102. Schwickert TA, Victora GD, Fooksman DR, et al. A dynamic T celllimited checkpoint regulates affinity-dependent B cell entry into the germinal center. *J Exp Med*. 2011;208:1243-1252.

103. Jacob J, Kassir R, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. I. The architecture and dynamics of responding cell populations. *J Exp Med*. 1991;173:1165-1175.

104. Hasbold J, Corcoran LM, Tarlinton DM, Tangye SG, Hodgkin PD. Evidence from the generation of immunoglobulin G-secreting cells that stochastic mechanisms regulate lymphocyte differentiation. *Nat Immunol*. 2004;5:55-63.

105. Honjo T, Kinoshita K, Muramatsu M. Molecular mechanism of class switch recombination: linkage with somatic hypermutation. *Annu Rev Immunol*. 2002;20:165-196.

106. Okazaki IM, Kinoshita K, Muramatsu M, Yoshikawa K, Honjo T. The AID enzyme induces class switch recombination in fibroblasts. *Nature*. 2002;416:340-345.

107. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activationinduced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell*. 2000;102:553-563.

108. Revy P, Muto T, Levy Y, et al. Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell*. 2000;102:565-575.

109. Tran TH, Nakata M, Suzuki K, et al. B cell-specific and stimulationresponsive enhancers derepress Aicda by overcoming the effects of silencers. *Nat Immunol*. 2010;11:148-154.

110. Park SR, Zan H, Pal Z, et al. HoxC4 binds to the promoter of the cytidine deaminase AID gene to induce AID expression, class-switch DNA recombination and somatic hypermutation. *Nat Immunol*. 2009;10:540-550.

111. Dengler HS, Baracho GV, Omori SA, et al. Distinct functions for the transcription factor Foxo1 at various stages of B cell differentiation. *Nat Immunol*. 2008;9:1388-1398.

112. Stavnezer J, Guikema JE, Schrader CE. Mechanism and regulation of class switch recombination. *Annu Rev Immunol*. 2008;26:261-292.

113. Xu Z, Fulop Z, Wu G, et al. 14-3-3 adaptor proteins recruit AID to 5′-AGCT-3′ switch regions for class switch recombination. *Nat Struct Mol Biol*. 2010;17:1124-1135.

114. Schenten D, Kracker S, Esposito G, et al. Pol zeta ablation in B cells impairs the germinal center reaction, class switch recombination, DNA break repair, and genome stability. *J Exp Med*. 2009;206:477-490.

115. Wang JH, Gostissa M, Yan CT, et al. Mechanisms promoting translocations in editing and switching peripheral B cells. *Nature*. 2009;460: 231-236.

116. Banchereau J, Bazan F, Blanchard D, et al. The CD40 antigen and its ligand. *Annu Rev Immunol*. 1994;12:881-922.

117. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. *Nat Rev Immunol*. 2002;2:116-126.

118. Bishop GA, Hostager BS. B lymphocyte activation by contact-mediated interactions with T lymphocytes. *Curr Opin Immunol*. 2001;13: 278-285.

119. Snapper CM, Paul WE. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science*. 1987;236: 944-947.

120. Kuhn R, Rajewsky K, Muller W. Generation and analysis of interleukin-4 deficient mice. *Science*. 1991;254:707-710.

121. Takeda K, Tanaka T, Shi W, et al. Essential role of Stat6 in IL-4 signalling. *Nature*. 1996;380:627-630.

122. Cazac BB, Roes J. TGF-beta receptor controls B cell responsiveness and induction of IgA in vivo. *Immunity*. 2000;13:443-451.

123. Kopf M, Herren S, Wiles MV, Pepys MB, Kosco-Vilbois MH. Interleukin 6 influences germinal center development and antibody production via a contribution of C3 complement component. *J Exp Med*. 1998;188:1895-1906.

124. Mohr E, Cunningham AF, Toellner KM, et al. IFN-γ produced by CD8 T cells induces T-bet-dependent and -independent class switching in B cells in responses to alum-precipitated protein vaccine. *Proc Natl Acad Sci U S A* . 2010;107:17292-17297.

125. Peng SL, Szabo SJ, Glimcher LH. T-bet regulates IgG class switching and pathogenic autoantibody production. *Proc Natl Acad Sci U S A* . 2002;99:5545-5550.

126. Watanabe K, Sugai M, Nambu Y, et al. Requirement for Runx proteins in IgA class switching acting downstream of TGF-beta 1 and retinoic acid signaling. *J Immunol* . 2010;184:2785-2792.

127. Sellars M, Reina-San-Martin B, Kastner P, Chan S. Ikaros controls isotype selection during immunoglobulin class switch recombination. *J Exp Med*. 2009;206:1073-1087.

128. Chen-Kiang S. Cell-cycle control of plasma cell differentiation and tumorigenesis. *Immunol Rev*. 2003;194:39-47.

129. Morse L, Chen D, Franklin D, Xiong Y, Chen-Kiang S. Induction of cell cycle arrest and B cell terminal differentiation by CDK inhibitor p18(INK4c) and IL-6. *Immunity*. 1997;6:47-56.

130. McHeyzer-Williams LJ, Cool M, McHeyzer-Williams MG. Antigenspecific B cell memory: expression and replenishment of a novel B220 - memory b cell compartment. *J Exp Med*. 2000;191:1149-1166.

131. McHeyzer-Williams MG, McLean MJ, Lalor PA, Nossal GJV. Antigendriven B cell differentiation in vivo. *J Exp Med*. 1993;178:295-307.

132. Pelletier N, McHeyzer-Williams LJ, Wong KA, et al. Plasma cells negatively regulate the follicular helper T cell program. *Nat Immunol* . 2010;11:1110-1118.

133. Ho F, Lortan JE, MacLennan ICM, Khan M. Distinct short-lived and long-lived antibody-producing cell populations. *Eur J Immunol*. 1986;16:1297-1301.

134. Jacob J, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. II. A common clonal origin for periarteriolar lymphoid sheath-associated foci and germinal centers. *J Exp Med*. 1992;176:679-687.

135. Hargreaves DC, Hyman PL, Lu TT, et al. A coordinated change in chemokine responsiveness guides plasma cell movements. *J Exp Med*. 2001;194:45-56.

136. Turner CA Jr, Mack DH, Davis MM. Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. *Cell*. 1994;77:297-306.

137. Shapiro-Shelef M, Lin KI, McHeyzer-Williams LJ, Liao J, McHeyzer-Williams MG, Calame K. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity*. 2003;19:607-620.

138. Calame K. Transcription factors that regulate memory in humoral responses. *Immunol Rev*. 2006;211:269-279.

139. Calame KL, Lin KI, Tunyaplin C. Regulatory mechanisms that determine the development and function of plasma cells. *Annu Rev Immunol*. 2003;21:205-230.

140. Iwakoshi NN, Lee AH, Vallabhajosyula P, Otipoby KL, Rajewsky K, Glimcher LH. Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. *Nat Immunol*. 2003;4:321-329.

141. Reimold AM, Iwakoshi NN, Manis J, et al. Plasma cell differentiation requires the transcription factor XBP-1. *Nature*. 2001;412:300-307.

142. Shaffer AL, Shapiro-Shelef M, Iwakoshi NN, et al. XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. *Immunity*. 2004;21:81-93.

143. Corcoran LM, Hasbold J, Dietrich W, et al. Differential requirement for OBF-1 during antibody-secreting cell differentiation. *J Exp Med*. 2005;201:1385-1396.

144. Lin L, Gerth AJ, Peng SL. Active inhibition of plasma cell development in resting B cells by microphthalmia-associated transcription factor. *J Exp Med*. 2004;200:115-122.

145. Fujita N, Jaye DL, Geigerman C, et al. MTA3 and the Mi-2/NuRD complex regulate cell fate during B lymphocyte differentiation. *Cell*. 2004;119:75-86.

146. Hu CC, Dougan SK, McGehee AM, Love JC, Ploegh HL. XBP-1 regulates signal transduction, transcription factors and bone marrow colonization in B cells. *EMBO J*. 2009;28:1624-1636.

147. Todd DJ, McHeyzer-Williams LJ, Kowal C, et al. XBP1 governs late events in plasma cell differentiation and is not required for antigen-specific memory B cell development. *J Exp Med*. 2009;206: 2151-2159.

148. Gatto D, Paus D, Basten A, Mackay CR, Brink R. Guidance of B cells by the orphan G protein-coupled receptor EBI2 shapes humoral immune responses. *Immunity*. 2009;31:259-269.

149. Pereira JP, Kelly LM, Xu Y, Cyster JG. EBI2 mediates B cell segregation between the outer and centre follicle. *Nature*. 2009;460:1122-1126.

150. Coffey F, Alabyev B, Manser T. Initial clonal expansion of germinal center B cells takes place at the perimeter of follicles. *Immunity*. 2009;30:599-609.

151. MacLennan IC. Germinal centers. *Annu Rev Immunol* . 1994;12:117-139.

152. MacLennan ICM, Gray D. Antigen-driven selection of virgin and memory B cells. *Immunol Rev.* 1986;91:61-85.

153. Matsumoto M, Mariathasan S, Nahm MH, Baranyay F, Peschon JJ, Chaplin DD. Role of lymphotoxin and the type I TNF receptor in the formation of germinal centers. *Science*. 1996;271:1289-1291.

154. Matsumoto M, Lo SF, Carruthers CJ, et al. Affinity maturation without germinal centres in lymphotoxin-alpha-deficient mice. *Nature*. 1996;382:462-466.

155. Koni PA, Sacca R, Lawton P, Browning JL, Ruddle NH, Flavell RA. Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin beta-deficient mice. *Immunity*. 1997;6:491-500.

156. Futterer A, Mink K, Luz A, Kosco-Vilbois MH, Pfeffer K. The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity*. 1998;9:59-70.

157. Hauser AE, Junt T, Mempel TR, et al. Definition of germinal-center B cell migration in vivo reveals predominant intrazonal circulation patterns. *Immunity*. 2007;26:655-667.

158. Schwickert TA, Lindquist RL, Shakar G, et al. In vivo imaging of germinal center reveals a dynamic open structure. *Nature*. 2007;446(7131): 83-87.

159. Allen CD, Okada T, Tang HL, Cyster JG. Imaging of germinal center selection events during affinity maturation. *Science*. 2007;315: 528-531.

160. Hauser AE, Shlomchik MJ, Haberman AM. In vivo imaging studies shed light on germinal-centre development. *Nat Rev Immunol* . 2007;7:499-504.

161. Allen CD, Okada T, Cyster JG. Germinal-center organization and cellular dynamics. *Immunity*. 2007;27:190-202.

162. Victora GD, Schwickert TA, Fooksman DR, et al. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell*. 2010;143:592-605.

163. Jacobson EB, Caporale LH, Thorbecke GJ. Effect of thymus cell injections on germinal center formation in lymphoid tissues of nude (thymusless) mice. *Cell Immunol* . 1974;13:416-430.

164. Kawabe T, Naka T, Yoshida K, et al. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity*. 1994;1:167-178.

165. Renshaw BR, Fanslow WC 3rd, Armitage RJ, et al. Humoral immune responses in CD40 ligand-deficient mice. *J Exp Med*. 1994;180: 1889-1900.

166. de Vinuesa CG, Cook MC, Ball J, et al. Germinal centers without T cells. *J Exp Med*. 2000;191:485-494.

167. Song H, Cerny J. Functional heterogeneity of marginal zone B cells revealed by their ability to generate both early antibody-forming cells and germinal centers with hypermutation and memory in response to a T-dependent antigen. *J Exp Med*. 2003;198:1923-1935.

168. Dent AL, Shaffer AL, Yu X, Allman D, Staudt LM. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science*. 1997;276:589-592.

169. Toyama H, Okada S, Hatano M, et al. Memory B cells without somatic hypermutation are generated from Bcl6-deficient B cells. *Immunity*. 2002;17:329-339.

170. Phan RT, Dalla-Favera R. The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. *Nature*. 2004;432:635-639.

171. Tunyaplin C, Shaffer AL, Angelin-Duclos CD, Yu X, Staudt LM, Calame KL. Direct repression of prdm1 by Bcl-6 inhibits plasmacytic differentiation. *J Immunol*. 2004;173:1158-1165.

172. Wang Y, Carter RH. CD19 regulates B cell maturation, proliferation, and positive selection in the FDC zone of murine splenic germinal centers. *Immunity*. 2005;22:749-761.

173. Schwickert TA, Lindquist RL, Shakar G, et al. In vivo imaging of germinal center reveals a dynamic open structure. *Nature*. 2007;446:83-87.

174. Neuberger MS, Milstein C. Somatic hypermutation. *Curr Opin Immunol* . 1995;7:248-254.

175. Jolly CJ, Wagner SD, Rada C, Klix N, Milstein C, Neuberger MS. The targeting of somatic hypermutation. *Semin Immunol*. 1996;8:159-168.

176. Muramatsu M, Sankaranand VS, Anant S, et al. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family

in germinal center B cells. *J Biol Chem*. 1999;274:18470-18476.

177. Larson ED, Cummings WJ, Bednarski DW, Maizels N. MRE11/RAD50 cleaves DNA in the AID/UNG-dependent pathway of immunoglobulin gene diversification. *Mol Cell*. 2005;20:367-375.

178. Liu M, Duke JL, Richter DJ, et al. Two levels of protection for the B cell genome during somatic hypermutation. *Nature*. 2008;451:841-845.

179. Wang M, Rada C, Neuberger MS. Altering the spectrum of immunoglobulin V gene somatic hypermutation by modifying the active site of AID. *J Exp Med*. 2010;207:141-153.

180. Wang M, Yang Z, Rada C, Neuberger MS. AID upmutants isolated using a high-throughput screen highlight the immunity/ cancer balance limiting DNA deaminase activity. *Nat Struct Mol Biol* . 2009;16: 769-776.

181. Orthwein A, Patenaude AM, Affar el B, Lamarre A, Young JC, Di Noia JM. Regulation of activation-induced deaminase stability and antibody gene diversification by Hsp90. *J Exp Med*. 2010;207:2751-2765.

182. Di Noia JM, Neuberger MS. Molecular mechanisms of antibody somatic hypermutation. *Annu Rev Biochem*. 2007;76:1-22.

183. Tourigny MR, Ursini-Siegel J, Lee H, et al. CDK inhibitor p18(INK4c) is required for the generation of functional plasma cells. *Immunity*. 2002;17:179-189.

184. Winslow MM, Gallo EM, Neilson JR, Crabtree GR. The calcineurin phosphatase complex modulates immunogenic B cell responses. *Immunity*. 2006;24:141-152.

185. Smith KG, Light A, O'Reilly LA, Ang SM, Strasser A, Tarlinton D. bcl-2 transgene expression inhibits apoptosis in the germinal center and reveals differences in the selection of memory B cells and bone marrow antibody-forming cells. *J Exp Med*. 2000;191:475-484.

186. Takahashi Y, Cerasoli DM, Dal Porto JM, et al. Relaxed negative selection in germinal centers and impaired affinity maturation in bcl-xL transgenic mice. *J Exp Med*. 1999;190:399-410.

187. McHeyzer-Williams LJ, McHeyzer-Williams MG. Antigen-specific memory B cell development. *Annu Rev Immunol* . 2005;23:487-513.

188. Hao Z, Duncan GS, Seagal J, et al. Fas receptor expression in germinal-center B cells is essential for T and B lymphocyte homeostasis. *Immunity*. 2008;29:615-627.

189. Vikstrom I, Carotta S, Luthje K, et al. Mcl-1 is essential for germinal center formation

and B cell memory. *Science*. 2010;330:1095-1099.

190. Huntington ND, Xu Y, Puthalakath H, et al. CD45 links the B cell receptor with cell survival and is required for the persistence of germinal centers. *Nat Immunol*. 2006;7:190-198.

191. Wang Y, Brooks SR, Li X, Anzelon AN, Rickert RC, Carter RH. The physiologic role of CD19 cytoplasmic tyrosines. *Immunity*. 2002;17:501-514.

192. Casola S, Cattoretti G, Uyttersprot N, et al. Tracking germinal center B cells expressing germ-line immunoglobulin gamma1 transcripts by conditional gene targeting. *Proc Natl Acad Sci U S A* . 2006;103: 7396-7401.

193. Mitchell J, Abbot A. Ultrastructure of the antigen-retaining reticulum of lymph node follicles as shown by high-resolution autoradiography. *Nature*. 1965;208:500-502.

194. Fearon DT, Carroll MC. Regulation of B lymphocyte responses to foreign and self-antigens by the CD19/CD21 complex. *Annu Rev Immunol*. 2000;18:393-422.

195. Hannum LG, Haberman AM, Anderson SM, Shlomchik MJ. Germinal center initiation, variable gene region hypermutation, and mutant B cell selection without detectable immune complexes on follicular dendritic cells. *J Exp Med*. 2000;192:931-942.

196. Victoratos P, Lagnel J, Tzima S, et al. FDC-specific functions of p55T-NFR and IKK2 in the development of FDC networks and of antibody responses. *Immunity*. 2006;24:65-77.

197. Bhattacharyya S, Deb J, Patra AK, et al. NFATc1 affects mouse splenic B cell function by controlling the calcineurin—NFAT signaling network. *J Exp Med*. 2011;208:823-839.

198. Allen CD, Ansel KM, Low C, et al. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol*. 2004;5:943-952.

199. Ford ML, Koehn BH, Wagener ME, et al. Antigen-specific precursor frequency impacts T cell proliferation, differentiation, and requirement for costimulation. *J Exp Med*. 2007;204(2):299-309.

200. Hataye J, Moon JJ, Khoruts A, Reilly C, Jenkins MK. Naive and memory CD4+ T cell survival controlled by clonal abundance. *Science*. 2006;312:114-116.

201. Marzo AL, Klonowski KD, Le Bon A, Borrow P, Tough DF, Lefrancois L. Initial T cell frequency dictates memory CD8+ T cell lineage commitment. *Nat Immunol*. 2005;6:793-799.

202. Choi YS, Kageyama R, Eto D, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity*. 2011;34(6):932-946.

203. Green JA, Suzuki K, Cho B, et al. The sphingosine 1-phosphate receptor S1P(2) maintains the homeostasis of germinal center B cells and promotes niche confinement. *Nat Immunol*. 2011;12:672-680.

204. Good-Jacobson KL, Szumilas CG, Chen L, Sharpe AH, Tomayko MM, Shlomchik MJ. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol*. 2010;11:535-542.

205. Reinhardt RL, Liang HE, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol*. 2009;10:385-393.

206. Gulbranson-Judge A, MacLennan I. Sequential antigen-specific growth of T cells in the T zones and follicles in response to pigeon cytochrome c. *Eur J Immunol* . 1996;26:1830-1837.

207. Zheng B, Han S, Kelsoe G. T helper cells in murine germinal centers are antigen-specific emigrants that downregulate Thy-1. *J Exp Med*. 1996;184:1083-1091.

208. Mikszta JA, McHeyzer-Williams LJ, McHeyzer-Williams MG. Antigendriven selection of TCR In vivo: related TCR alpha-chains pair with diverse TCR beta-chains. *J Immuno*l. 1999;163:5978-5988.

209. Han S, Hathcock K, Zheng B, Kepler TB, Hodes R, Kelsoe G. Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. *J Immunol*. 1995;155:556-567.

210. Kim CH, Lim HW, Kim JR, Rott L, Hillsamer P, Butcher EC. Unique gene expression program of human germinal center T helper cells. *Blood*. 2004;104:1952-1960.

211. McHeyzer-Williams MG, Ahmed R. B cell memory and the long-lived plasma cell. *Curr Opin Immunol* . 1999;11:172-179.

212. Pape KA, Taylor JJ, Maul RW, Gearhart PJ, Jenkins MK. Different B cell populations mediate early and late memory during an endogenous immune response. *Science*. 2011;331:1203-1207.

213. Dogan I, Bertocci B, Vilmont V, et al. Multiple layers of B cell memory with different effector functions. *Nat Immunol*. 2009;10:1292-1299.

214. Kasturi SP, Skountzou I, Albrecht RA, et al. Programming the magnitude and persistence of antibody responses with innate immunity. *Nature*. 2011;470:543-547.

215. Nossal GJ, Pike BL, Battye FL. Sequential use of hapten-gelatin fractionation and fluorescence-activated cell sorting in the enrichment of hapten-specific B lymphocytes. *Eur J Immunol* . 1978;8:151-157.

216. Noelle RJ, Snow EC, Uhr JW, Vitetta ES. Activation of antigen-specific B cells: role of T cells, cytokines, and antigen in induction of growth and differentiation. *Proc Natl Acad Sci U S A*. 1983;80: 6628-6631.

217. Bonner WA, Hulett HR, Sweet RG, Herzenberg LA. Fluorescence activated cell sorting. *Rev Sci Instrum*. 1972;43:404-409.

218. Julius MH, Masuda T, Herzenberg LA. Demonstration that antigenbinding cells are precursors of antibody-producing cells after purification with a fluorescence-activated cell sorter. *Proc Natl Acad Sci U S A* . 1972;69:1934-1938.

219. Greenstein JL, Leary J, Horan P, Kappler JW, Marrack P. Flow sorting of antigen-binding B cell subsets. *J Immunol* . 1980;124:1472-1481.

220. Hayakawa K, Ishii R, Yamasaki K, Kishimoto T, Hardy RR. Isolation of high-affinity memory B cells: phycoerythrin as a probe for antigenbinding cells. *Proc Natl Acad Sci U S A*. 1987;84:1379-1383.

221. Gray D, Skarvall H. B-cell memory is short-lived in the absence of antigen. *Nature*. 1988;336:70-73.

222. Kodituwakku AP, Jessup C, Zola H, Roberton DM. Isolation of antigen-specific B cells. *Immunol Cell Biol* . 2003;81:163-170.

223. Schittek B, Rajewsky K. Maintenance of B-cell memory by longlived cells generated from proliferating precursors. *Nature*. 1990;346: 749-751.

224. Maruyama M, Lam KP, Rajewsky K. Memory B-cell persistence is independent of persisting immunizing antigen. *Nature*. 2000;407: 636-642.

225. McHeyzer-Williams MG, Nossal GJ, Lalor PA. Molecular characterization of single memory B cells. *Nature*. 1991;350:502-505.

226. Blink EJ, Light A, Kallies A, Nutt SL, Hodgkin PD, Tarlinton DM. Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization. *J Exp Med*. 2005;201: 545-554.

227. Jacob J, Kelsoe G, Rajewsky K, Weiss U. Intraclonal generation of antibody mutants in germinal centres. *Nature*. 1991;354:389-392.

228. McHeyzer-Williams MG, Nossal GJV, Lalor PA. Molecular characterization of single memory B cells. *Nature*. 1991;350:502-505.

229. Phan TG, Paus D, Chan TD, et al. High affinity germinal center B cells are actively selected into the plasma cell compartment. *J Exp Med*. 2006;203:2419-2424.

230. Mohr E, Serre K, Manz RA, et al. Dendritic cells and monocyte/macrophages that create the IL-6/APRIL-rich lymph node microenvironments where plasmablasts mature. *J Immunol*. 2009;182:2113-2123.

231. Fooksman DR, Schwickert TA, Victora GD, Dustin ML, Nussenzweig MC, Skokos D. Development and migration of plasma cells in the mouse lymph node. *Immunity*. 2010;33:118-127.

232. Chevrier S, Genton C, Kallies A, et al. CD93 is required for maintenance of antibody secretion and persistence of plasma cells in the bone marrow niche. *Proc Natl Acad Sci U S A*. 2009;106:3895-3900.

233. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science*. 2002;298:2199-2202.

234. Smith KG, Light A, Nossal GJV, Tarlinton DM. The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response. *Embo Journal*. 1997;16:2996-3006.

235. Takahashi Y, Dutta PR, Cerasoli DM, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. V. Affinity maturation develops in two stages of clonal selection. *J Exp Med*. 1998;187:885-895.

236. Minges Wols HA, Underhill GH, Kansas GS, Witte PL. The role of bone marrow-derived stromal cells in the maintenance of plasma cell longevity. *J Immunol*. 2002;169:4213-4221.

237. Benner R, Hijmans W, Haaijman JJ. The bone marrow: the major source of serum immunoglobulins, but still a neglected site of antibody formation. *Clin Exp Immunol*. 1981;46:1-8.

238. Manz RA, Thiel A, Radbruch A. Lifetime of plasma cells in the bone marrow. *Nature*. 1997;388:133-134.

239. Manz RA, Lohning M, Cassese G, Thiel A, Radbruch A. Survival of long-lived plasma cells is independent of antigen. *International Immunol*. 1998;11:1703-1711.

240. Slifka MK, Matloubian M, Ahmed R. Bone marrow is a major site of long-term antibody production after acute viral infection. *J Virol*. 1995;69:1895-1902.

241. Slifka MK, Antia R, Whitmire JK, Ahmed R. Humoral immunity due to long-lived plasma cells. *Immunity*. 1998;8:363-372.

242. O'Connor BP, Raman VS, Erickson LD, et al. BCMA is essential for the survival of long-lived bone marrow plasma cells. *J Exp Med*. 2004;199:91-98.

243. O'Connor BP, Cascalho M, Noelle RJ. Short-lived and long-lived bone marrow plasma cells are derived from a novel precursor population. *J Exp Med*. 2002;195:737-745.

244. Shaffer AL, Lin KI, Kuo TC, et al. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity*. 2002;17:51-62.

245. Underhill GH, George D, Bremer EG, Kansas GS. Gene expression profiling reveals a highly specialized genetic program of plasma cells. *Blood*. 2003;101:4013-4021.

246. Furukawa K, Akasako-Furukawa A, Shirai H, Nakamura H, Azuma T. Junctional amino acids determine the maturation pathway of an antibody. *Immunity*. 1999;11:329-338.

247. Panus JF, McHeyzer-Williams LJ, McHeyzer-Williams MG. Antigenspecific T helper cell function: differential cytokine expression in primary and memory responses. *J Exp Med*. 2000;192:1301-1316.

248. McHeyzer-Williams LJ, McHeyzer-Williams MG. Analysis of antigen-specific B-cell memory directly ex vivo. *Methods Mol Biol*. 2004;271:173-188.

249. Liu W, Meckel T, Tolar P, Sohn HW, Pierce SK. Intrinsic properties of immunoglobulin IgG1 isotype-switched B cell receptors promote microclustering and the initiation of signaling. *Immunity*. 2010;32:778-789.

250. Martin SW, Goodnow CC. Burst-enhancing role of the IgG membrane tail as a molecular determinant of memory. *Nat Immunol*. 2002;3:182-188.

251. Engels N, Konig LM, Heemann C, et al. Recruitment of the cytoplasmic adaptor Grb2 to surface IgG and IgE provides antigen receptor-intrinsic costimulation to class-switched B cells. *Nat Immunol*. 2009;10:1018-1025.

252. Horikawa K, Martin SW, Pogue SL, et al. Enhancement and suppression of signaling by the conserved tail of IgG memory-type B cell antigen receptors. *J Exp Med*. 2007;204:759-769.

253. Waisman A, Kraus M, Seagal J, et al. IgG1 B cell receptor signaling is inhibited by CD22 and promotes the development of B cells whose survival is less dependent on Ig

alpha/beta. *J Exp Med*. 2007;204:747-758.

254. Wakabayashi C, Adachi T, Wienands J, Tsubata T. A distinct signaling pathway used by the IgG-containing B cell antigen receptor. *Science*. 2002;298:2392-2395.

255. Aiba Y, Kometani K, Hamadate M, et al. Preferential localization of IgG memory B cells adjacent to contracted germinal centers. *Proc Natl Acad Sci U S A* . 2010;107:12192-12197.

256. McHeyzer-Williams LJ, Panus JF, Mikszta JA, McHeyzer-Williams MG. Evolution of antigen-specific T cell receptors in vivo: preimmune and antigen-driven selection of preferred complementarity-determining region 3 (CDR3) motifs. *J Exp Med*. 1999;189:1823-1838.

257. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* . 2004;22:745-763.

258. Srinivasan L, Sasaki Y, Calado DP, et al. PI3 kinase signals BCR-dependent mature B cell survival. *Cell*. 2009;139:573-586.

259. Hikida M, Casola S, Takahashi N, et al. PLC-gamma2 is essential for formation and maintenance of memory B cells. *J Exp Med*. 2009;206:681-689.

260. Jelley-Gibbs DM, Dibble JP, Brown DM, Strutt TM, McKinstry KK, Swain SL. Persistent depots of influenza antigen fail to induce a cytotoxic CD8 T cell response. *J Immunol*. 2007;178:7563-7570.

261. Fazilleau N, Eisenbraun MD, Malherbe L, et al. Lymphoid reservoirs of antigen-specific memory T helper cells. *Nat Immunol* . 2007;8:753-761.

262. Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG. Follicular helper T cells: lineage and location. *Immunity* 2009;30:324-335.

263. Obst R, van Santen HM, Mathis D, Benoist C. Antigen persistence is required throughout the expansion phase of a CD4(+) T cell response. *J Exp Med*. 2005;201:1555-1565.

264. Celli S, Garcia Z, Bousso P. CD4 T cells integrate signals delivered during successive DC encounters in vivo. *J Exp Med*. 2005;202: 1271-1278.

265. Jelley-Gibbs DM, Brown DM, Dibble JP, Haynes L, Eaton SM, Swain SL. Unexpected prolonged presentation of influenza antigens promotes CD4 T cell memory generation. *J Exp Med*. 2005;202: 697-706.

266. Zammit DJ, Turner DL, Klonowski KD, Lefrancois L, Cauley LS. Residual antigen

presentation after influenza virus infection affects CD8 T cell activation and migration. *Immunity*. 2006;24: 439-449.

267. Lau LL, Jamieson BD, Somasundaram T, Ahmed R. Cytotoxic T-cell memory without antigen. *Nature*. 1994;369:648-652.

268. Swain SL, Hu H, Huston G. Class II-independent generation of CD4 memory T cells from effectors. *Science*. 1999;286:1381-1383.

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Chapter 11

T-Cell Antigen Receptors

Mark M. Davis

Yueh-Hsiu Chien

T-lymphocytes expressing αβ or γδ T-cell antigen receptors (TCRs) are found together with B-lymphocytes in all but the most primitive vertebrate animals. These three cell types are the only ones that use random variable (V), diversity (D) in the case of TCR-β and -δ, joining (J), gene rearrangement to generate diverse antigen receptors. During the last two decades, there has been a great deal of progress in identifying the molecules and genes of TCRs, and there is considerable information on their biochemistry and structure. While TCRs share structural and genetic similarities with B-cell antigen receptors (immunoglobulins [Igs]), they also possess a number of unique features pertinent to their specific functions. The first major difference was suggested by the experiments of Zinkernagel and Doherty, who found that cytotoxic cells specific for a viral antigen could only lyse infected cells that expressed a particular major histocompatibility complex (MHC) molecule.[1,2] This phenomenon of "MHC-restricted recognition" is in marked contrast to the recognition of intact antigens by Igs.[3,4] Later work demonstrated that what was being recognized by these T cells, which were of the αβ TCR type, were fragments of antigens or peptides bound to a characteristic groove in MHC molecules.[5] These αβ TCRs are expressed on classical helper and cytotoxic T cells, which predominate in most lymphoid compartments (90% to 95%) of humans and mice.[6] They are also expressed on natural killer (NK)T cells,[7] regulatory T cells,[8] and T cells in the mucosal sites such as the intestinal epithelial compartment (IEL).[9] In most cases, the αβ TCR ligand is a peptide antigen bound to a class I or class II MHC molecule; but in the case of NKT cells, the antigen is a glycolipid bound to a nonclassical class I MHC molecule, cluster of differentiation (CD)Id.[7]

T cells bearing γδ TCRs are less numerous than the αβ type in most cellular compartments of humans and mice (<5%). However, they make up a substantial fraction of T-lymphocytes in cows, sheep, and chickens.[10] γδ T cells coexist with αβ T cells but seem to be better represented in the mucosal compartments.[10,11] Although αβ T cells perform most of the functions classically attributed to T cells, mice lacking γδ T cells are clearly have a compromised immune defense indicating that γδ and αβ T cells contribute to host immune defense differently.[11,12] γδ TCRs also recognize antigens directly, like antibodies, with no apparent need for antigen processing,[13] at least in the most thoroughly studies cases. During the past few years, there have been considerable advances in our understanding of antigen recognition by γδ T cells. This should lead to a better understanding of how γδ T cells contribute to immune competence.

T-CELL ANTIGEN RECEPTOR POLYPEPTIDES

The search for the molecules responsible for T-cell recognition first focused on deriving antisera or monoclonal antibodies specific for molecules on T-cell surfaces. Ultimately, a number of groups identified "clonotypic" sera[14] or monoclonal antibodies.[15,16,17,18,19] Several of these antibodies were able to block antigen-specific responses by the T cells they were raised against or, when coated on a surface, could activate the T cells they are specific for. They were also able to immunoprecipitate 85,000 to 90,000 molecular weight (MW) disulfide-bonded heterodimers from different T-cell clones or hybridomas consisting of two 40,000 to 50,000 MW glycosylated subunits referred to as α and β. Peptide mapping studies showed that there was a striking degree of polymorphism between heterodimers isolated from T cells of differing specificity, thus suggesting that these antigen recognition molecules

might be akin to Igs.[20,21]

Work in parallel to these serologic studies exploited the small differences (approximately 2%) observed between B- and T-cell gene expression,[22] and isolated both a mouse[23,24] and a human[25] T cell-specific gene that had antibodylike V, J, and C region sequences and could rearrange in T-lymphocytes.[24] This molecule was identified as TCR-β by partial sequence analysis of immunoprecipitated materials.[26]

Subsequent subtractive cloning work rapidly identified two other candidate TCR complementary deoxyribonucleic acids (DNAs) identified as TCR-α[27,28] and TCR-γ.[29] It was quickly established that all antigen-specific helper or cytotoxic T cells expressed TCR-αβ heterodimers. Where TCR-γ fit in remained a puzzle until work by Brenner et al.[30] showed that it was expressed on a small (5% to 10%) subset of peripheral T cells together with another polypeptide, TCR-δ. The nature of TCR-δ remained unknown until it was discovered within the TCR-α locus, between the Vα and Jα regions.[31] Formal proof that the TCR-α and -β subunits were sufficient to transfer antigen/MHC recognition from one T cell to another came from gene transfection experiments,[32,33] and equivalent experiments have also been done with γδ TCRs.[34]

As shown in Figure 11.1, all TCR polypeptides have a similar primary structure, with distinct V, D in the case of TCR-β and -δ, J, and constant (C) regions exactly analogous to their Ig counterparts. They also share many of the amino acid residues thought to be important for the characteristic variable and constant domains of Igs.[35] The Cβ region is particularly homologous, sharing 40% of its amino acid sequences with C_K and C_λ. The TCR polypeptides all contain a single C region domain (versus up to four for Igs) followed by a connecting peptide. These usually contain the cysteine for the disulfide linkage that joins the two chains of the heterodimer (some human TCR-γδ isoforms lack this cysteine and consequently are not disulfide-linked[36]). *N*-linked glycosylation sites vary from two to four for each polypeptide with no indications of O-linked sugar addition. C-terminal to the connecting peptide sequences are the hydrophobic transmembrane regions. These have no similarity to those of the *IgH* genes but instead have one (TCR-β and -γ) or two (TCR-α and -δ) positively charged residues. As discussed later, these charged residues are critical for the association of the ligand-binding TCR polypeptides with the CD3 signaling polypeptides. This is important because the TCR polypeptides have very short cytoplasmic regions with no known role in signaling.

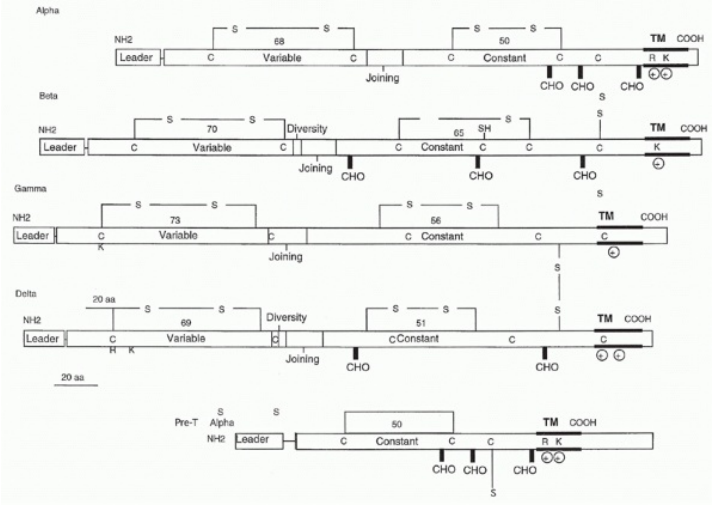

FIG. 11.1. Structural Features of T-Cell Receptors and Pre-T α Polypeptides. Leader (L), variable (V), diversity (D), joining (J), and constant region (C) gene segments are indicated. *Transmembrane* and *bold horizontal lines* delineate the putative transmembrane regions; *CHO* indicates potential carbohydrate addition sites; *C* and *S*

refer to cysteine residues that form interchain and intrachain disulfide bonds; *R* and *K* indicate the positively charged amino acids (arginine and lysine, respectively) that are found in the transmembrane regions.

A more recent member of the TCR polypeptide family is the pre-Tα chain, which serves as a chaperone for TCR-β in early thymocytes, similar to the role of λ5 in pre-B cells.[37] It was first identified and cloned by von Boehmer and colleagues.[38] It has an interesting structure that consists of a single Ig constant region-like domain followed by a cysteinecontaining connecting peptide, a transmembrane region

containing two charged residues: an arginine and a lysine spaced identically to the TCR-α transmembrane region. The cysteine in the connecting peptide is presumably what allows heterodimer formation with TCR-β and the similarity to TCR-α in the transmembrane region is most likely to accommodate the CD3 polypeptides. Recently, Rossjohn and colleagues have presented a structure of the pre-Tα chain that is comprised of a single Ig-like domain that is distinct from the C domain of the TCR-α chain; nevertheless, the mode of association between pre-Tα and TCR-β mirrored that mediated by the Cα-Cβ domains of the αβTCR.[39] The pre-TCR has a propensity to dimerize in solution, and the molecular envelope of the pre-TCR dimer correlated well with the observed head-to-tail pre-TCR dimer. This mode of pre-TCR dimerization enables the pre-Tα domain to interact with the Vβ domain through residues that are highly conserved across the Vβ and Jβ gene families, thus mimicking the interactions at the core of the αβ TCR's Vα-Vβ interface. Disruption of this pre-Tα-Vβ dimer interface abrogates pre-TCR dimerization in solution and impaired pre-TCR expression on the cell surface. Their work suggests a mechanism of pre-TCR self-association that allows the pre-Tα chain to simultaneously "sample" the correct folding of both the V and C domains of any TCR β-chain, regardless of its ultimate specificity,[39] which likely represents a critical checkpoint in T-cell development.

In both the mouse and humans, the cytoplasmic tail of pre-Tα is much longer than any of the TCR chains (37 and 120 amino acids, respectively), and the murine sequence contains two likely phosphorylation sites and sequences homologous to an SH3 domain binding region. These are not present in the human sequence, however, and so their functional significance is unclear.[38] Thus, at least in principal, the murine pre-Tα molecule could function as signaling intermediate independent of the CD3 polypeptides, and it has recently been shown that at least one CD3 component (δε, see the following) is not required for it to function normally in early thymocyte differentiation.[40]

CLUSTER OF DIFFERENTIATION 3 POLYPEPTIDES

Immunoprecipitation of the human TCR with anti-idiotypic antibodies after solubilization with the nonionic detergent, noniodet P-40 (NP-40), initially revealed only the α- and β-chain heterodimer. However, the use of other detergents, such as digitonin or Triton-X100, revealed four other proteins.[41,42,43,44] These are known as the CD3γ, δ, ε, and ζ. γ and δ form distinct heterodimers with ε within the TCR/CD3 complex (γε and δε), and ζ usually occurs as a disulfide-linked homodimer. In mouse T cells, NP-40 does not dissociate TCR heterodimers from CD3 molecules.[44,45] In some cases, the ζ-chain can be part of a heterodimer in at least two forms. In mouse T cells, the ζ-chain can disulfide bond with a minor variant called the η (eta) chain.[46,47] This latter chain is an alternate splicing variant of the ζ-chain gene.[48] This alternatively spliced species of the ζ-chain is not found in significant quantities in human T cells.[48] The second type of ζ-chain containing heterodimer contains the γ-chain associated with the $F_C\varepsilon RI$ ($F_C\varepsilon RI$ γ) and $F_C\gamma RIII$ (CD16) receptors.[49,50] These CD3 subunits, in their various forms, are an integral part of TCR-mediated T-cell recognition because only they possess the immunoreceptor tyrosine-based activation motifs (ITAMs) that are necessary for cellular activation when the TCR engages ligand.

Characterization and Structural Features of the Cluster of Differentiation 3 Polypeptides

Figure 11.2 illustrates the principal structural features of the CD3 γ, δ, ε, and ζ polypeptides

as derived from gene cloning and sequencing,[42,51] and more recently by protein crystal structures of the extracellular domains of γ, δ, and ε.[52,53,54,55] The extracellular domains of the γ, δ, and ε chains show a significant degree of similarity to one another. These domains retain the cysteines that have been shown to form intrachain disulfide bonds and each consists of a single Ig superfamily domain. The spacing of the cysteines in these domains produces a compact Ig-fold, similar to a constant region domain. The γ and δ subunits form distinct heterodimers with ε via highly conserved residues at the dimerizing interface.[52,53,54,55] The connecting peptides of the CD3 γ, δ, and ε chains all contain highly conserved, closely spaced cysteines just before the membrane-spanning regions. These residues are likely candidates for the formation of interchain disulfide bonds and appear to play a role in the assembly of the CD3 and TCR polypeptides.[52,56] The extracellular domain of the ζ chain consists of only nine amino acids and contains the only cysteine, which is responsible for the disulfide linkage of the ζζ homodimer or the ζ $F_C\varepsilon RI\gamma$ heterodimer. Each of the γ, δ, ε, and ζ polypeptides contain a conserved, negatively charged amino acids in their transmembrane region complementary to the positive charges seen in the TCR transmembrane regions.[57,58,59,60]

The cytoplasmic regions of the γ, δ, ε, and ζ chains are the intracellular signaling "domains" of the TCR heterodimer. Each of these molecules contains one or more amino acid sequence motifs that can mediate cellular activation.[61] The intracellular sequences responsible for this activation are contained within an 18 amino acid conserved ITAM[62] with the sequence $X_2YX_2L/IX_7YX_2L/I$. Both of the tyrosines in this motif are absolutely required to mediate signal transduction as mutation of either completely prevents the mobilization of free calcium or cytolytic activity.[63] This sequence occurs three times in the ζ chain and once in each of the CD3 γ, δ, ε, and $F_C\varepsilon RI$ γ chains. There are also pairs of tyrosines present in the cytoplasmic domains of the γ, δ, ε, and ζ chains. This sequence motif is also present in the mβ-1 and B29 chains associated with the Ig β-cell receptor and in the $F_C\varepsilon RI$ β-chain but there are many more[10] in TCR/CD3 than in any other receptors which use ITAMs. The tyrosines in these cytoplasmic sequences are substrates for the tyrosine phosphorylation that is one of earliest steps in T-cell signaling[61] and is thought to occur aberrantly in nonproductive T-cell responses (eg, antagonism; see following). Serine phosphorylation of the CD3γ also occurs upon

antigen or mitogenic stimulation of T cells[64] and may play a role in T-cell activation as well.

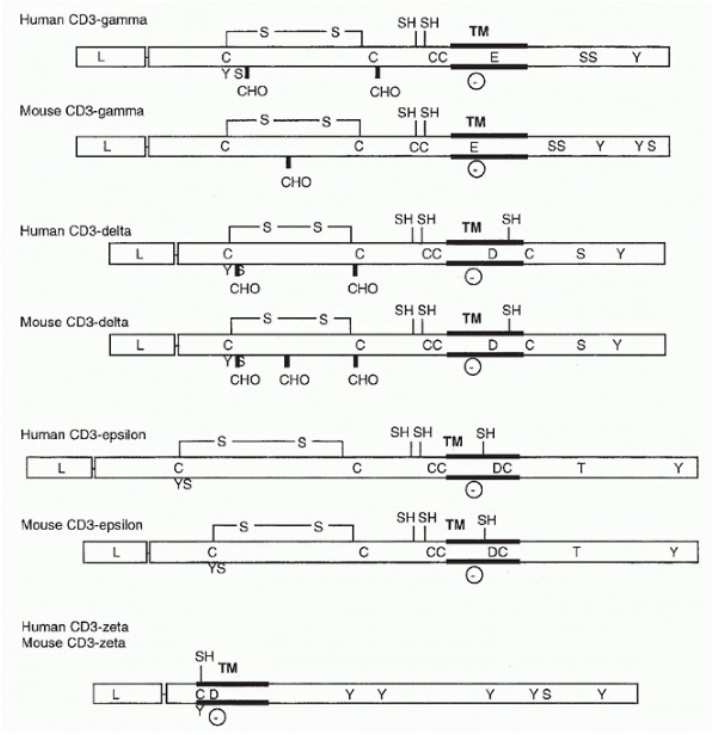

FIG. 11.2. Structural Features of the CD3 Molecules. As in Figure 11.1, transmembrane regions, carbohydrate addition sites, and cysteine residues are indicated. In addition, negatively charged transmembrane residues (*D* for aspartic acid and *E* for glutamic acid) and putative phosphorylation sites are shown.

Recently, live-cell imaging studies have shown a close interaction of the CD3epsilon cytoplasmic domain of the TCR with the plasma membrane, using fluorescence resonance energy transfer (FRET) between a C-terminal fluorescent protein and a membrane fluorophore.[65] Electrostatic interactions between basic CD3epsilon residues and acidic phospholipids enriched in the inner leaflet of the plasma membrane were required for binding. Nuclear magnetic resonance studies of the lipid-bound state of this cytoplasmic domain revealed a deep insertion of the two key tyrosines of the ITAM into the hydrophobic core of the lipid bilayer, likely preventing their phosphorylation.[65] Similar studies of CD3 zeta have confirmed that this is also the case for that component of the TCR/CD3 complex as well.[66] This then likely defines the “off” state of the TCR/CD3 complex and is a novel explanation for how it can prevent tyrosine phosphorylation by Ick. What is still mysterious is how ligand binding by the TCR disengages these signaling modules from the plasma membrane and triggers the kinase cascade needed to activate T cells.

Assembly and Organization of the T-Cell Receptor/Cluster of Differentiation 3 Complex

The assembly of newly formed TCR-α and -β chains with the CD3 γ, δ, ε, and ζ chains and their intracellular fate have been studied in detail.[39,40,41,61,63] Early studies have focused on mutant hybridoma lines, which fail to express TCR on their cell surface, and on transfection studies using complementary DNA for the different chains in the receptor; but recently, Wucherpfennig and colleagues have developed an elegant in vitro translation and assembly system that has clarified a number of important issues.[54,55]

Experiments in a nonlymphoid cell system[67] have shown that TCR-α can assemble with CD3 δ and ε but not CD3 γ

and ζ. In contrast, the TCR-β chain can assemble with any of the CD3 chains except the ζ chain. When the ζ chain was transfected with either α or β chain genes, or any of the three CD3 chains, no pairwise interaction occurred.

Only when all six complementary DNAs were cotransfected was it shown that the ζ chain could be coprecipitated with the other chains.[67] Based on these data, a model has been proposed that suggests that TCR-α pairs with CD3 δ and ε chains and that TCR-β pairs with the CD3 γ and ε chains in the completed molecule. The ζ chain is thought to join the TCR and other CD3 polypeptides in that last stage of assembly.

Pulse-chase experiments have shown that all six chains are assembled in the endoplasmic reticulum (ER), transported to the Golgi apparatus, and then transferred to the plasma membrane. It also appears that the amount of ζ chain is rate limiting, as it is synthesized at only 10% the level of the other chains. This results in the vast majority of newly synthesized α, β, or CD3 components being degraded within 4 hours of their synthesis. The remaining nondegraded chains are long lived due to the formation of complete TCR/CD3 complexes with the limiting ζ chain.[68] TCR/CD3 lacking CD3 ζ chains migrate through the ER and Golgi intact but then are transported to and degraded in the lysosomes. The immunologic significance of this pre-Golgi degradation pathway is most evident in CD4+CD8+thymocytes where, despite high levels of synthesis of both messenger ribonucleic acid and protein for all the TCR, CD3, and ζ chains, surface expression is relatively low. The TCR chains in immature thymocytes seem to be selectively degraded.[68] Thus posttranslation regulation appears to be an important means of controlling the cell surface expression of TCR heterodimers.

The TCR and CD3 γ, δ, and ε chains contain ER retention signals.[68,69] If the γ and δ signals are removed, then the chains are transported through the Golgi and rapidly degraded in the lysosomes. In contrast, removal of the CD3 ε ER retention signal allows this chain, and any associated chains, to be transported to the cell surface. Thus, association of the TCR and other CD3 chains with ε renders their ER retention signals inoperative. However, the ε ER retention signal remains functional. This prevents the surface expression of partial complex intermediaries until CD3 ζ is incorporated into the complex, which then masks the ε ER retention signal and allows the transport of mature complexes to the cell surface.

The overall stoichiometry of the αβ TCR/CD3 complex is controversial. The work of Call and colleagues[57] has shown the relationships between different CD3 dimers (γε, δε, and ζ ζ) and a single TCR αβ heterodimer (as shown in Fig. 11.3). Using mutagenesis, they found very specific interactions based on the positive charges in each TCR transmembrane domain with complementary negative charges in the transmembrane domains of the different CD3 components. The two positively charged residues of TCR-α mediate interactions with the negatively charged residues of the of CD3δε and CD3ζζ dimers, while the single positively charged residue of TCR-β mediates interactions with the negatively charged residues of CD3γε. In addition, their data suggest a highly ordered assembly process as they found that the TCR/CD3δε association facilitates the assembly of CD3γε into the complex and that the association of the TCR and CD3 heterodimers was a prerequisite for incorporation of CD3ζζ into the complex. Importantly, the data show that there is only one TCR heterodimer per nascent TCR/CD3 complex in their in vitro expression system.

In contrast, a number of groups have found evidence that there can be two TCR heterodimers in a given TCR/CD3 cluster on T-cell surfaces. In particular, Terhorst and colleagues[42] showed that in a T-T hybridoma, a monoclonal antibody against one TCR-αβ pair could comodulate a second αβ heterodimer. In addition, sucrose gradient centrifugation of TCR/CD3 showed a predicted molecular weight of 300 kDa, more than 100 kDa larger than expected from a minimal δ subunit complex (α, β, γ, δ, ε_2, ζ_2).[70] Another study suggesting that there are least two TCRs in a given CD3 complex is the Scatchard analysis indicating that the number of CD3ε molecules on a T-cell surface equals the number of αβ TCRs.[71,72,73] Finally, there is the work of Fernandez-Miguel et al.,[74] who showed that in T cells which have two transgenic TCR-β chains, antibodies to one Vβ can immunoprecipitate the other. It was also found that they are often close enough to allow fluorescence energy transfer, meaning that the two TCR-βs in a cluster are within 50 Angstroms of each other.[74] Interestingly, it appears that the TCR complexes with CD3 either have CD3 γ or CD3 δ, but not both, and these two

receptor types are expressed in different ratios in different cells. These data may not be

irreconcilable, because while the initial TCR/CD3 assembly may involve only one TCR, these may dimerize or multimerize later on the cell surface.[50,75]

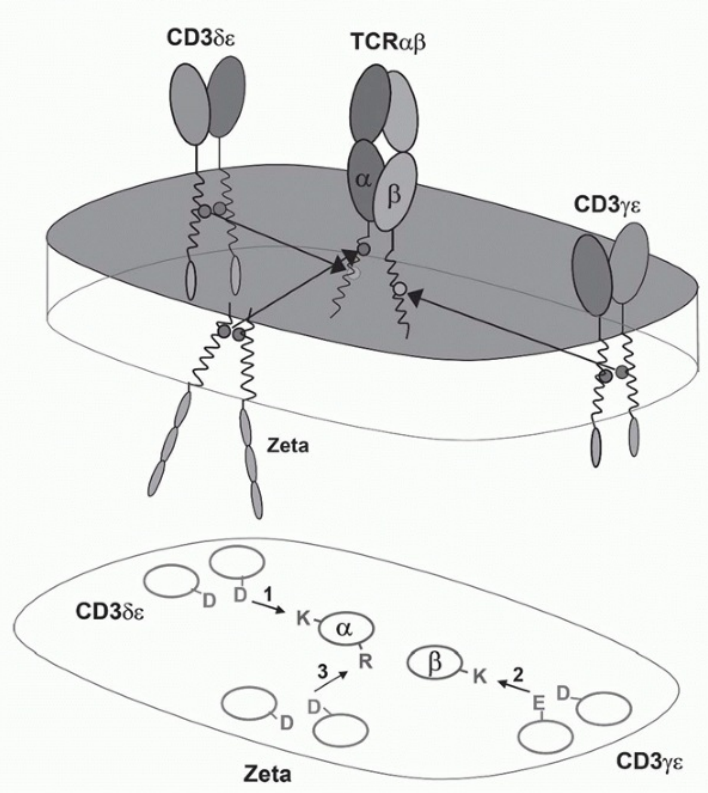

FIG. 11.3. A Model of the αβ T-Cell Receptor (TCR)-Cluster of Differentiation (CD)3 Complex. This shows the approximate positions of the αβ TCR chains and the CD3α, δ, ε, and ζ chains based on the membrane reconstitution and mutagenesis experiments of Call and Wucherpfennig.[55] More recent experiments suggest that the CD3 polypeptides may be somewhat more clustered on the side of the TCR heterodimer.[299]

The composition of TCR/CD3 complexes on γδ T cells is distinct from that of αβ T cells and changes with the activation state of the cell. Biochemical analysis showed that most murine γδ TCRs contain only CD3 γε dimers. Interestingly, a differentially glycosylated form of CD3 γ was found to associated with γδ TCRs dependent on the activation state of the cells.[76] In addition, while C3ζζ is incorporated into the complexes of naïve cells, activation results in the expression and incorporation of $F_c\varepsilon RI$ γ into the γδTCR complex.[76] Using quantitative immunofluorescence, Hayes and Love have derived data and proposed a model of murine γδTCR stoichiometry in which there are two CD3γε dimers, as well as one CD3ζ dimer in each TCR complex.[77] Taken together, these findings strongly suggest that signal transduction through the TCR will occur differently in γδ versus αβ T cells.

T-CELL RECEPTOR GENES

As shown in Figure 11.4, TCR gene segments are organized similarly to those of Igs and the same recombination machinery is responsible for joining separate V and D segments to a particular J and C. This was initially indicated by the fact that the characteristic seven and nine nucleotide conserved sequences adjacent to the V, D, and J regions with the 12 or 23 nucleotide spacing between them, first described for Ig genes, are also present in TCRs.[78] The most conclusive evidence of this common rearrangement mechanism is that both a naturally occurring recombination-deficient mouse strain (severe combined immune deficiency[79]) and mice engineered to lack recombinase activating genes 1[80] or 2[81] are unable to rearrange either *TCR* or Ig gene segments properly. Many of the other molecules

involved in Ig gene rearrangement serve the same function in TCRs as well.[82] As with Igs, if the V region and J region gene segments are in the same transcriptional orientation, the intervening DNA is deleted during recombination. DNA circles of such material can be observed in the thymus,[83,84] the principal site of TCR recombination (see the following). In the case of *TCR-β* and *TCR-δ*, there is a single V region 3' to the C in the opposite transcriptional orientation to J and C. Thus, rearrangement to these gene segments occurs via an inversion. Variable points of joining are seen along the *V, D*, and *J* gene segments as well as random nucleotide addition (N regions) in postnatal TCRs. The addition of several nucleotides in an inverted repeat pattern, referred to a P element insertion, at the V-J junction of the TCR-γ chains has also been observed.[85]

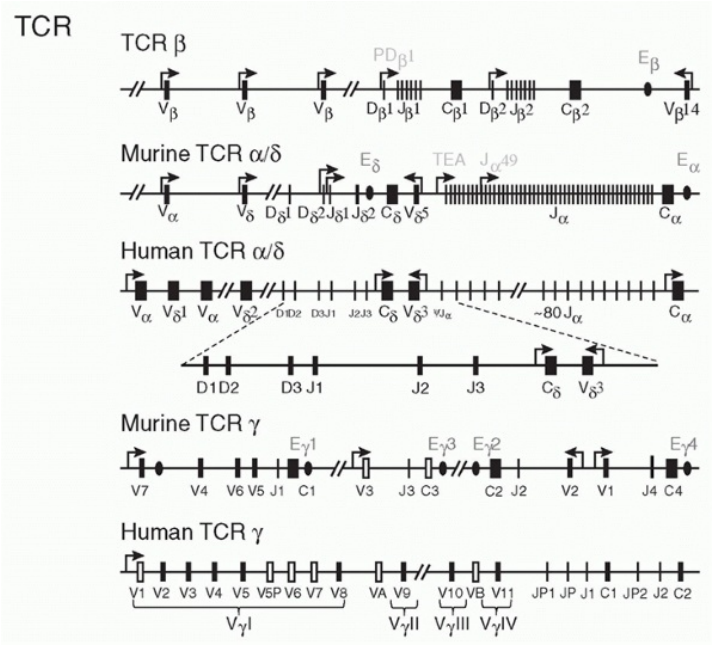

FIG. 11.4. T-Cell Receptor Gene Organization in Mice and Humans. Schematic of V, D, J, and C elements of the T-cell receptor genes. Transcriptional orientation is from *left to right*, except where noted. *E* designates enhancer elements, and *S* are silencer elements.

Organization of the T-Cell Receptor α/δ Locus

In humans and in mice, there is a single α-chain C-region gene that is composed of four exons encoding: 1) the constant region domain, 2) 16 amino acids including the cysteine that forms the interchain disulfide bond, 3) the transmembrane and intracytoplasmic domains, and 4) the 3' untranslated region (see Fig. 11.4). The entire α/δ locus spans about 1.1 MB in both mice and humans. There are 50 different J-region gene segments upstream of the C-region in the murine locus. At least eight of these J-regions are nonfunctional because of in-frame stop codons or rearrangement and splicing signals that are likely to be defective. A similar number of α-chain J-regions are present in the human locus. This very large number of J-regions compared to the Ig loci may indicate that

the functional diversity contributed by the J segment of the TCR (which constitutes a major portion of the complementarity-determining region [CDR]3 loop) makes an important contribution to antigen recognition (see the following).

In both the murine and human loci, the Cδ, Jδ, and two Dδ gene segments are located between the Vα and Jα gene segments. In the murine system, there are two Jδ and two Dδ gene segments on the 5' side of Cδ and the Cδ exons are approximately 75 kb upstream of the Cα gene and approximately 8 kb upstream of the most 5' known Jα gene segments. The human organization is similar, with three Dα gene segments and three Jδs. Surprisingly, in both species all of the D elements can be used in one rearranged gene rather than

alternating as is the case with TCR-β or IgH. That is, in mice one frequently finds Vδ D_1, D_2, and Jδ rearrangements,[86] and in humans Vδ, D_1, D_2, D_3, and Jδ.[87] This greatly increases the junctional or CDR3 diversity that is available, especially because of the potential for N-region addition in between each gene segment. This property makes TCR-δ the most diverse of any of the antigens receptors known, with approximately 10^{12} to 10^{13} different amino acid sequences in a relatively small (10 to 15 amino acid) region.[86] The implications for this and comparisons with other antigen receptor genes are discussed subsequently.

The location of Dδ, Jδ, and Cδ genes between Vα and Jα gene segments suggests that TCR-δ and -α could share the same pool of V gene segments. While there is some overlap in V gene usage, in the murine system, four of the commonly used Vδ genes (Vδ1, Vδ2, Vδ4, Vδ5) are very different than known Vα sequences and they have not been found to associate with Cα.[88] The other four Vδ gene families overlap with or are identical to Vα subfamilies (Vδ3, Vδ6, Vδ7, and Vδ8, with Vα6, Vα7, Vα4, and Vα11, respectively).

The mechanisms that account for the preferential usage of certain gene segments to produce δ versus α chain are not known. While some Vδ genes are located closer to the Dδ and Jδ fragments than Vα genes (such as Vδ1), other Vδs (such as Vδ6) are rarely deleted by Vα Jα rearrangements and thus seem likely to be located 5' of many Vα gene segments.

One of the Vδ gene segments, Vδ5, is located approximately 2.5 kb to the 3' of Cδ in the opposite transcriptional orientation and rearranges by inversion. Despite its close proximity to Dδ Jδ gene segments, Vδ5 is not often found in fetal γδ T cells. Instead, the Vδ5 → DJδ rearrangement predominates in adult γδ T cells.

An implicit characteristic of the α/δ gene locus is that a rearrangement of Vα to Jα deletes the entire D-J-C core of the δ-chain locus. In many αβ T cells, the α-chain locus is rearranged on both chromosomes and thus no TCR δ could be made. In most cases, this is due to Vα → Jα rearrangement, but evidence suggesting an intermediate step in the deletion of TCR-δ has been reported.[89] This involves rearrangements of an element termed T early alpha (TEA) to a pseudo-Jα 3' of Cδ. The rearrangement of TEA to this psuedo-Jα would eliminate the δ-chain locus in αβ T cells. Gene targeting of the TEA element resulted in normal levels of αβ and γδ T cells, but usage of the most Jαs was severely restricted,[90] suggesting that its function is to govern the accessibility of the most proximal 5' Jαs for recombination.

Organization of the T-Cell Receptor β Locus

The entire human 685 kb β chain gene locus was originally sequenced by Hood and coworkers[91] (Fig. 11.5). One interesting feature is the tandem nature of *Jβ-Cβ* in the *TCR-β* locus. This arrangement is preserved in all higher vertebrate species that have been characterized thus far (mouse, human, chicken, frog). The two *Cβ* coding sequences are identical in the mouse and nearly so in humans and other species. Thus it is unlikely that they represent two functionally distinct forms of *Cβ*. However, the *Jβ* clusters have relatively unique sequences, and thus this may be a mechanism for increasing the number of *Jβ* gene segments. Together with the large number of Jα gene segments, there is far more combinatorial diversity (Jα × Jβ = 50× 12=600) provided by J regions in αβ TCRs than in Igs.

Most of the V-regions are located upstream of the joining and constant regions and in the same transcriptional orientation as the D and J gene element, and rearrange to DβJβ gene via deletion. Similar to the case of Vδ5, a single Vβ gene, Vβ14 is located 3' to C-regions and in the opposite transcriptional orientation, thus rearrangements involving Vβ14 occur via inversion.

In the NZW strain of mouse, there is a deletion in the β chain locus that spans from Cβ1, up to and including the Jβ 2 cluster.[92] In SJL, C57BR and C57L mice, there is a large deletion[93] in the V-region locus from Vδ5-Vβ9. These mice

also express a V gene, Vβ17, that is not expressed in other strains of mice. Deletion of about half of the V genes (in SJL, C57BR and C57L mice) does not seem to have any particular effect on the ability of these mice to mount immune responses whereas mice that have deleted the *Jβ*2 cluster show impaired responses.[94]

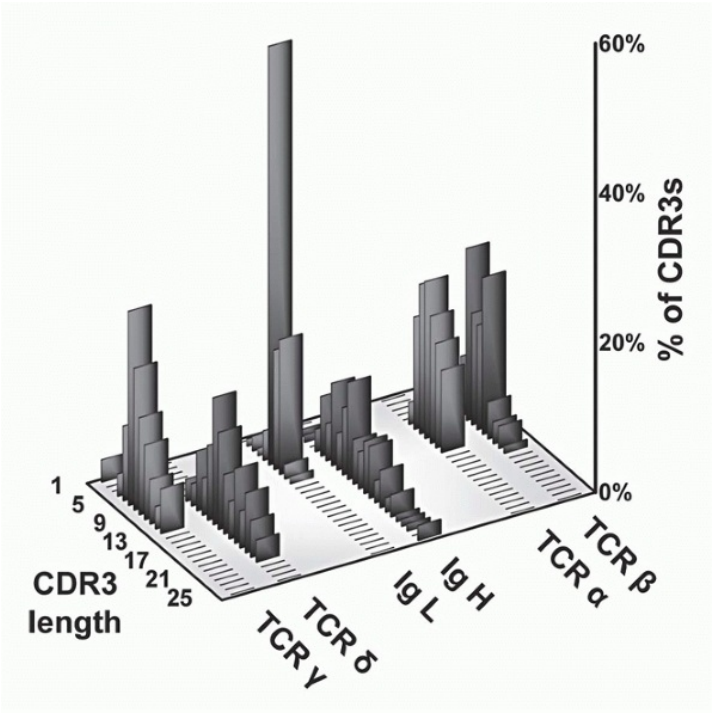

FIG. 11.5. Complementarity-Determining Region (CDR)3 Length Analysis of T-Cell Receptor (TCR) Polypeptides versus Immunoglobulin (Ig) Heavy and Light Chains. These data, modified from Rock et al.,[129] show that whereas TCR-α and -β CDR3 regions are relatively uniform with respect to each other, the other antigen receptor pairs show a marked asymmetry. Specifically, both Ig light chains (κ and λ) show very short CDR3s, as do TCR-γ chains. In contrast, both IgH and TCR *S* TCRs are quite heterogeneous and tend to be longer. These data suggests that γδ TCRs have a more antibody-like structure and binding properties. This has been borne out by subsequent analysis (see text).

Organization of the T-Cell Receptor γ Locus

The organization of the mouse and human γ-chain loci are shown in Figure 11.4. The human γ genes span about 150 kb[85] and are organized in a fashion similar to that of the β chain locus with two JγCγ regions. There is more than one nomenclature commonly used to describe the γ chain genes.[85,96,97,98] Here, we use that of Lefranc and Rabbitts[99] and Tonegawa and colleagues[85] for the human and mouse γ chains, respectively. The organization of the human γ chain genes consists an array of Vγs in which at least six of the V-regions are pseudogenes is located 5' to these JγCγ clusters, and each of the V genes are potentially capable of rearranging to any of the five J-regions. The sequences of the two human Cγ regions are very similar overall and only differ significantly in the second exon. In Cγ2, this exon is duplicated two or three times and the cysteine that forms in the interchain disulfide bond is absent. Thus, Cγ2-bearing human T cells have an extra large γ-chain (55,000 MW) that is not disulfide bonded to its δ-chain partner.

The organization of the murine γ chain genes is very different than that of the human genes in that there are three separate rearranging loci that span about 205 kb.[100,101] Of four murine Cγ genes, Cγ3 is apparently a pseudogene in BALB/c mice, and the Jγ3 Cγ3 region is deleted in several mouse strains including C57 Bl/10. Cγl and Cγ2 are very similar in coding sequences. The major differences between these two genes is in the five amino acid deletion in the Cγ2 gene that is located in the C ll exon at the amino acid terminal of the cysteine residue used for the disulfide formation with the δ chain. The Cγ4 gene differs significantly in sequences from the other Cγ genes (in 66% overall amino acid identity). In addition, the Cγ4 sequences contains a 17 amino acid insertion (compared to Cγ1) in the C ll exon located at

similar position to the five amino acid deletions in the Cγ2 gene.[101a] Each Cγ gene is associated with a single Jγ gene segment. The sequences of Jγ1 and Jγ2 are identical at the amino acid level, whereas Jγ4 differs from Jγ1 and Jγ2 at 9 out of 19 amino acid residues.

The murine Vγ genes usually rearrange to the Jγ Cγ gene that is most proximal and in the same transcriptional orientation. Thus Vγ1 rearranges to Jγ4; Vγ2 to Jγ2; and Vγ4, Vγ5, Vγ6, and Vγ7 to Jγ1. Interestingly, some Vγ genes are rearranged and expressed preferentially during γδ T-cell ontogeny and in different adult tissues as well.[101] In particular, Vγ5+ and Vγ6+ T cells are generated in the fetal thymus with very limited/no junctional diversity. Instead, the adult thymus produces γδ T cells expressing Vγ1, Vγ2, Vγ4, and Vγ7 gene segments with highly diverse junctional sequences. Moreover, γδ T cells that localize to the secondary lymphoid organs tend to express Vγ4, Vγ1, and Vγ2, whereas those that localize to the intestinal epithelium express Vγ7.[88,102,103,104,105,106] There are also reports suggesting that some intestinal epithelial γδ T cells develop extrathymically.[107] Regardless, it has been suggested that the Vγ gene rearrangement is a programmed process.[108,109]

Control of Transcription and Rearrangement

It has become increasingly apparent that transcriptional accessibility and rearrangement of TCR and Igloci are closely linked, following the early work of Alt and Yancopoulous.[110] Factors governing accessibility and rearrangement include histone methylation,[111,112,113,114] DNA methlyation, and the presence of enhancer and specific promoter elements.[115] Even specific variations in the recombination signal sequences have been shown to elicit specific biases in V(D)J joining.[86] With respect to enhancer elements in the TCR loci, these were first identified in the TCR-β locus, 3' of Cβ2,[116,117] and subsequently for the other TCR loci as well,[115] as indicated in Figure 11.4. These TCR enhancers all share sequence similarities with each other. Some of the transcriptional factors that bind to the TCR genes are also found to regulate Ig gene expressions. It has been shown that TCR-α enhancer (Eα) is not only important for normal rearrangement and expression for the α chain locus but also is required for the normal expression level of mature TCR-δ transcripts.[118] Also interesting is the work of Lauzurica and Krangel,[119,120] who have shown that a human TCR-δ enhancer-containing minilocus in transgenic mice is able to rearrange equally well in αβ T cells as in αδ T cells but that an Eα-containing construct was only active in αβ lineage T cells. Similar to Ig genes, promoter sequences are located 5' to the V gene segments. Although D → J_β rearrangement and transcription occur fairly often in B cells and in B-cell tumors,[121] V_β rearrangement and/or transcription appears highly specific to T cells. In addition to enhancers, there also appear to be "silencer" sequences 3' of C α[122,123] and in the Cγ1 locus.[124] It has been suggested that these "repressor sites" could turn off the expression of either of these genes, influencing T-cell differentiation toward either the αβ or the γδ T-cell lineage.

The murine TCR Cγ1 gene cluster comprises four closely linked Vγ gene segments, in the order Vγ7, 4, 6, and 5, which rearrange to a single common downstream J gene segment, Jγ1 (see Fig. 11.4). In early fetal thymocytes, rearrangements of Vγ5 and Vγ6 genes predominate, and the resulting Vγ3+ and Vg4+ cells migrate to the skin or reproductive tissue, respectively. Later in ontogeny, Vγ4 and Vγ7 rearrangements predominate, and cells expressing these V regions migrate from the adult thymus to the secondary lymphoid organs and the intestinal epithelium.[100,101] At least two *cis*-acting, enhancer/locus control region (LCR) elements are present in the Cγ1 cluster. One is a T cell-specific transcriptional enhancer, 3γEγ, located 3 kb downstream of the Cγ1 gene segment.[125] A second element, "has," was found between the Vγ7 and Vγ4 genes, based on DNAse I hypersensitivity.[126] Similar enhancers have also been found to be associated with the Cγ2 and Cγ3 genes.[95] Experiments suggest that simultaneous deletion of both enhancer elements in Cγ1 cluster severely diminishes TCR-γ transcription, but only modestly reduces TCR-γ gene rearrangement, while deletion of each element

separately has little effect.[127] In contrast to these results in thymocytes, deletion of "has"

alone reduces transcription of one Vγ gene specifically in peripheral γδ T cells. Thus, the two elements not only exhibit functional redundancy in thymocytes but also have unique functions in other settings.

Allelic Exclusion

In Igs, normally only one allele of the heavy chain locus and one of the light chain alleles is productively rearranged and expressed, a phenomenon termed "allelic exclusion." With respect to αβ TCR expression, while TCR-β exhibits allelic exclusion,[128] TCR-α seems much less constrained,[129,130] and many mature T cells express two functional TCR-α chains. As the chances of forming an in-frame joint with any antigen receptor is only one in three, the probability that a T cell would have two productively rearranged TCRαs is only 1/3 × 1/3 = 1/9, or 11%. However, even when this happens, the two TCR-α chains may not form heterodimers equally well with the single TCR-β that is expressed; thus, only one heterodimer may be expressed. But this simple calculation is complicated by the likelihood that only thymocytes that make at least one productive TCR rearrangement of each type will have a chance at maturation, which would eliminate almost half of the T cells (four-ninths, which is the product of a two-thirds chance of failure on one chromosome, followed by the same failure rate on the second). Secondly, it has been found that there is a mechanism called receptor editing, which means that a given rearrangement that is not productive, either because of an out-of-frame joint or for reasons of self reactivity, can induce a V region further 5' or "upstream" of the initial VJ joint to rearrange to one of the remaining Jas.[131] In any event, it has been reported that one-third of human T cells express two TCRs,[132] which is slightly higher than what is expected from the probabilities discussed previously (approximately 20%), perhaps reflecting the effect of receptor editing.

There also appears to be an important role for the pre-TCR heterodimer (eg, pre-Tα:TCR-β) in blocking further TCR-β rearrangement and thus ensuring allelic exclusion at that locus.[133,134] In particular, pre-Tα-deficient mice had a significant increase in the number of cells with two productive TCR=β rearrangements, compared with wildtype mice.[133]

T-Cell Receptor Diversity

Although the basic organization and V(D)J recombination machinery are shared between TCR loci and Igs, there are a number of striking differences. One of these is somatic hypermutation. In antibodies, this form of mutation typically raises the affinities of antigen specific Igs several order of magnitude, typically from the micromolar (10^{-6} M) to the nanomolar (10^{-9} M) range.[135,136] We now know that most cellsurface receptors that bind ligands on other cell surfaces, including TCRs, typically have affinities in the micromolar range (see later section) but that they compensate for this relatively low affinity by engaging multiple receptors simultaneously (eg, increasing the valency) and by functioning in a confined, largely two-dimensional volume (eg, between two cells). Cells employing such receptors require weak, but highly specific, interactions so that they can disengage quickly.[137,138] The rapid off-rates seen with TCRs (see later section) may even amplify the effects of small numbers of ligands.[139,140]

There has also been no enduring evidence for a naturally secreted form of either an αβ or γδ TCR. Here again, it can be argued that such a molecule would have no obvious use as the affinities are too low to be very useful in solution. Thus, for most TCRs, the concentration of protein would have to be extremely high in order to achieve an effect similar to soluble antibodies (in the milligram/milliliter range).

A third mechanism seen in antibodies but not TCRs is C_H switching, which allows different Ig isotypes to maintain a given V region specificity and associate it with different constant regions that have different properties in solution (such as complement fixation, basophil binding, etc.). As there is no secreted form of the TCR, this feature would also lack any obvious utility.

Where TCRs are equal—and in fact generally superior— to Igs is in the sheer number of possible receptors that can be generated through recombination alone. Table 11.1 summarizes the potential V region diversity that TCRs are capable of when the number of V region gene segments is multiplied by D, J, and N region diversity. It can be seen from this

table that while the V region number is generally lower in murine TCRs, particularly TCR-δ and TCR-γ, this is more than compensated for by the degree of junctional diversity (where V and J or V, D, and J come together) and chain combinations, such that overall TCRs have orders of magnitude greater potential diversity than Igs. This junctional region corresponds to CDR3 as originally defined by Kabat and Wu for Igs.[141] With respect to αβ TCRs, the concentration of diversity in this region (in both chains) can be explained by the key role that these sequences play in recognizing diverse peptides in MHC molecules (see later section), as supported by mutagenesis and structural studies. For γδ TCRs and Igs, however, the diversity is almost all in just one chain (TCRδ and IgH, respectively), and the implications of this are discussed subsequently. Recent work using high throughput sequencing techniques are in remarkable agreement with these crude early estimates of TCR-β diversity.[142]

The Complementarity-Determining Region 3 Length Distributions of γδ T-Cell Receptors are More Similar to Those of Immunoglobulin than to Those of αβ T-Cell Receptors

Because CDR regions are loops between different β strands of an Ig or TCR V region (see later section), the configurations they adopt are generally very sensitive to their length, such that a difference of even one amino acid may produce a significant change in the overall structure.[3,143] A comparison of CDR3 length distributions between the αβ TCRs, γδ TCRs, and Igs (see Fig. 11.5)[144,145] showed that those of TCR-α and -β have a very constrained distribution of lengths and that these are nearly identical in size. These length constraints may reflect a requirement for both the α

and β chains of TCRs to contact both the MHC molecules and bound peptides on the same plane, as borne out by structural studies (see later section). In contrast, the CDR3s of Ig heavy chains are long and variable, whereas those of Ig light chains are short and constrained. This may reflect the fact that Igs recognize both very small molecules (eg, haptens) as well as very large ones (eg, proteins). Surprisingly, γδ TCR CDR3 length distributions are similar to those of Igs in that the CDR3 lengths of TCR-δ chains are long and variable, whereas those of the TCR γ chains are short and constrained. Thus, on the basis of this measure of ligand recognition, one might expect γδ TCRs to be more similar to Igs than to αβ TCRs. This has been validated in subsequent biochemical and structural studies (see later sections).

TABLE 11.1 Sequence Diversity in T-Cell Receptor and Immunoglobin Genes

	Immunoglobulin		**TCR-α/β**		**TCR-γ/δ**	
	H	**κ**	**α**	**β**	**γ**	**δ**
Variable segments	250-1,000	250	100	25	7	10
Diversity segments	10	0	0	2	0	2
Ds read in all frames	Rarely	—	—	Often	—	Often
N-region addition	V-D, V-J	none	V-J	V-D, V-J	V-J	V-D1, D1-D2, D1-J
Joining segments	4	4	50	12	2	2
Variable region combinations	62,500-250,000		2,500		70	
Junctional						

combinations	~10^{11}	~10^{15}	~10^{18}

Calculated potential amino acid sequence diversity in TCR and immunoglobulin genes without allowance for somatic mutation. The approximate number of V gene segments are listed for the four TCR polypeptides and contrasted with immunoglobulin heavy and light chains. CDR1 and CDR2 are encoded within the V gene segments. The pairing of random V regions generates the combinatorial diversity listed as "variable region combinations." Because there are fewer TCR V gene segments than immunoglobulin V gene segments, the combinatorial diversity is lower in TCRs than in immunoglobulins. Estimates for the number of unique sequences possible within the junctional region are contrasted for TCRs and immunoglobulins. Amino acids within CDR3 are encoded almost entirely within the D and/or J region gene segments. (The last few amino acids encoded by a TCR V gene segment can contribute to diversity within the TCR CDR3-equivalent region, but the effects of these residues on junctional diversity are not included in these calculations.) The mechanisms for generation of diversity within the junctional region that are used for this calculation include usage of different D and J gene segments. N region addition up to six nucleotides at each junction, variability in the 3' joining position in V and J gene segments, and translation of D region in different reading frames. Numbers are corrected for out-of-frame joining codon redundancy and N-region mimicry of germ-line sequences. Modified from Elliott et al.[86]

CDR, complementarity-determining region; TCR, T-cell receptor.

Chromosomal Translocations and Disease

The chromosomal locations of the different TCR loci have been delineated in both mouse and humans, and the results are summarized in Table 11.2. One significant factor in cancers of hematopoietic cells are chromosomal translocations that result in the activation of genes normally turned off or the inactivation of genes that are normally turned on. Thus, B- or T-lymphocyte neoplasia is frequently associated with inter- or intrachromosomal rearrangements of Ig or TCR loci or in some cases both.[146,147]

These translocations seem to mediated by the V(D)J recombinase machinery, indicating the inherent danger and need for tight regulation of this pathway. Such rearrangements are particularly common in the α/δ locus, perhaps because this locus spans the longest developmental window in terms of gene expression, with *TCR-δ* being the first and *TCR-α* the last gene to rearrange during T-cell ontogeny (as discussed in more detail in the following). In addition, the α/δ locus is in excess of 1 mb in size, and this provides a larger target for rearrangement than either TCR-β or TCR-γ. Interestingly, in humans, *TCR-α/δ* is on the same chromosome as the *IgH* locus and $V_H \rightarrow J_\alpha$ rearrangements (by inversion) have been observed in some human tumor material.[148,149] The functional significance of this is not known.

Particularly frequent is the chromosome 8-14 translocation [t(8;14) (q24;q11)] that joins the α/δ locus to the c-myc gene, analogous to the c-myc → IgH translocation in many mouse myeloma tumors and in Burkitt lymphomas in humans. In one cell line, a rearrangement occurred between the Jα-region coding sequences, and a region 3' of c-myc.[150] In both B- and T-cell malignancies, the translocation of c-myc into IgH or TCR-α/β appears to increase the expression of c-myc and may be a major factor in the unregulated cell growth that characterizes cancerous cells. Other

putative proto-oncogenes that have been found translocated into the TCR-α/β locus are the LIM domain-containing transcription factors Ttg-1[151] and Ttg-2,[152,153] which are involved in neural development; the helix-loop-helix proteins Lyl-1[154] and Scl,[155] which are involved in early hematopoietic development; and the homeobox gene Hox 11,[156] which is normally active in the liver. How these particular translocations contribute to malignancy is unknown, but they presumably causes aberrations in gene expression that contribute to cell growth or escape from normal regulation. In patients with T-cell leukemia infected with the human T-cell

lymphotrophic-I virus, there are large numbers of similar translocations; it is thought that human T-cell lymphotrophic-I itself is not directly leukemogenic but acts by causing aberrant rearrangements in the T cell that it infects, some of which become malignant.

TABLE 11.2 Chromosomal Locations of T-Cell Receptor, Immunoglobulin, and Related Loci in Mouse and Human

	Mouse Chromosome	Human Chromosome
TCR-α	14	14(q11-q12)
TCR-δ	14	14(q11-q12)
IgH	12	14(qter)
TCR-β	6	7(q35)
CD4	6	12
CD8	6	2(p11)
Igκ	6	62(p12)
TCR-γ	13	7(p14)
CD3-γ	9	11(q23)
CD3-δ	9	11(q23)
CD3-ε	9	11(q23)
CD3-ζ	1	1
Thy-1	9	11(q23)
Ig-λ	16	22(q11.2)
MHC	17	6(p21)
Pre-Tα	17	6

CDR, complementarity-determining region; Ig, immunoglobulin; MHC, major histocompatibility complex; TCR, T-cell receptor.

Another disorder, which frequently associates with TCR and Ig locus translocation, is ataxia telangiectasia, an autosomal recessive disorder characterized by ataxia, vascular telangiectasis, immunodeficiency, increased incidence of neoplasia, and an increased sensitivity to ionizing radiation. Peripheral blood lymphocytes from patients with ataxia telangiectasia have an especially high frequency of translocations involving chromosomes 7 and 14.[157] These sites correspond to the TCR-γ, -β, and -α loci, and the Ig heavy chain locus. Thus, it appears as though one of the characteristics of patients with ataxia telangiectasia is a relatively error-prone rearrangement process that indiscriminately recombines genes that have the TCR and Ig rearrangement signals.[158]

The Structure of αβ and γ δ T-Cell Receptors

As discussed previously, the sequences of TCR polypeptides show many similarities to Igs, and thus it has long been suggested that both αβ and γδ heterodimers would be antibodylike in structure.[24,25,159] The similarities between TCRs and Igs include the number and spacing of specific cysteine residues within domains, which in antibodies form intrachain disulfide bonds. Also conserved are many of the inter- and intradomain contact residues and, in addition, secondary structure predictions are largely consistent with an Ig-like "β barrel" structure. This consists of three to four antiparallel β strands on one side of the "barrel" facing a similar number on the other side, with a disulfide bridge (usually) connecting the two β "sheets" (sets of β strands in the same plane). All Ig variable and constant region domains have this structure, with slight variations in the number of β strands in variable region domains (by convention including V, D, and J sequences) compared with constant domains.

α β T-Cell Receptor Structure

Efforts to derive x-ray crystal structures of TCR heterodimers and fragments of heterodimers presented many technical hurdles.[160] One difficulty is that structure determination required engineering the molecules into a soluble form. A second problem is that many of the TCRs are heavily glycosylated, and it was necessary to eliminate most or all of the carbohydrates on each chain to achieve highquality crystals. An alternative is to express soluble TCRs in insect cells, where they have compact N-linked sugars, or in *Escherichia coli*, where they are unglycosylated. The first successes in TCR crystallization come from the laboratory of Mariuzza and collaborators who solved the structure of first a Vβ Cβ polypeptide[161] and then a Vα fragment.[162] In the following year, the first complete αβ TCR structures were solved.[163,164] The structure of the 2C TCR, by Garcia and colleagues, is shown in Figure 11.6.[163] In general, as predicted from sequence homologies, these domains are all Iglike, with the classical β-barrel structure in evidence in all three domains. At each end of the barrel in each V-region domain there are four loops between the β sheets, three of which form the CDRs of Igs, which are numbered in Figure 11.6. The fourth loop, between the D and E strands, has been implicated in superantigen binding. The six CDR loops from the two variable domains form the antigenbinding surface in both Igs and TCRs. The major anomaly in terms of similarity of TCRs to Igs is the structure of the Cα.[165] Cα consists of one-half of the classical β-barrel, that is, one set (or "sheet") of β strands while the rest of the partially truncated domain exhibits random coils. This type of structure is unprecedented in the Ig gene family. The functional significance of such a variant structure in unknown, but it has been suggested that this incompletely formed Iglike domain may be responsible for the observed lability of TCR-α, and this may allow greater flexibility in the regulation of its expression. Another possible explanation is that this configuration is designed to accommodate one or more of the CD3 molecules.

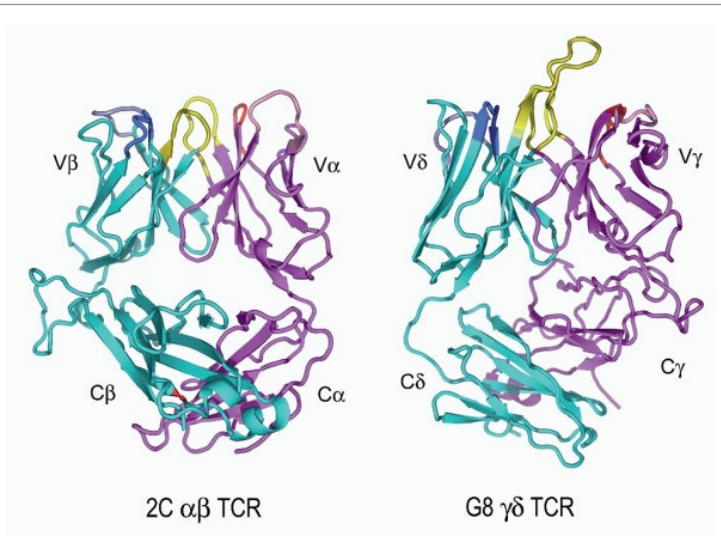

FIG. 11.6. Ribbon Diagrams of the T-Cell Receptor (TCR) Structures. This shows

the structures of the 2Cαβ TCR[150] versus the G8γδ TCR.[153] The TCR-β and the -γ chains are in *cyan*, and the TCR-α and -γ chains are in *vermillion*. The complementarity-determining regions (CDRs) of both are in *yellow*. The very long TCR-δ CDR3 in G8, which binds the T10/T22 ligands, is very apparent here but is shorter in most other γδ TCRs. Note the different C-region interactions with these TCRs and the deviations from the classic "β barrel" structure in both Cα and Cδ. The prominent Cβ loop to the left is also unusual and may mediate interactions with CD3 or other molecules on the T cell surface. (Figure courtesy of Dr. K.C. Garcia)

The now large number of solved αβ TCR structures can be compared to the three γδ heterodimers (discussed in more detail in the following), and while these also resemble the Fab fragment of an antibody, there are several features that are unique to the αβ molecules, which may be significant. These include the following:

1. In one structure,[165] four out of seven N-linked sugars diffracted to high resolution, indicating that they are not free to move very much and thus are likely to play a structural role, particularly in Cα:Cβ interactions. This correlates with mutagenesis data indicating that certain Cα sugars cannot be eliminated without abolishing protein expression[166] and the disordered state of a Cα domain in the structure of a TCR lacking glycosylation.[164]
2. There is significantly more contact between Vβ and Cβ and between Vα and Cα than in the equivalent regions of antibodies.
3. The geometry of the interaction of Vα and Vβ more closely resembles that of the C_H3 domains of antibodies than V_HV_L.
4. Between the CDR3 loops of Vα and Vβ, there is a pocket that can (and does in at least one case[165]) accommodate a large side chain from the peptide bound to an MHC.

γδ T-Cell Receptor Structure

There are now three γδ heterodimer structures: a γδ TCR from a human T-cell clone G115,[167] which can be activated by natural or synthetic pyrophosphomonoesters, a γδ TCR from the murine T cell clone G8 together with its ligand, the nonclassical MHC class I molecule T22,[168] and a human MHC class I chain-related-reactive γδ TCR (δ1A/B-3),[169a] which was determined as a single-chain Fv construct. The G8 structure is shown in Figure 11.6, alongside the 2C αβ TCR. The structure of a single human Vδ domain also has been determined.[169] The Vδ domain of the G115 structure is similar to the isolated Vδ domain and the quaternary structure of G8 is similar to that of G115.[168]

The most distinctive feature of both the G115 and the G8 TCR, when compared with αβ TCRs and Igs, is that the C domains "swing out" from under the V domains. This unusual shape is highlighted by both a small elbow angle of 110 degrees, defined as the angle between the pseudo twofold symmetry axes that relate V to V and C to C, and a small V-C interdomain angle. This contrasts with an average of 149 degrees for αβ TCR structures. The small angle between the Vγ and Cγ domains shifts both Cδ and Cγ to one side. Moreover, the molecular surfaces of the constant domains are different than those of αβ TCRs with no clear similarities either in the shape or the nature of the CαCβ and CγCδ surfaces; there are only a few solvent-exposed residues that are conserved in both Cβ and Cγ domains as well. Thus, it is unclear where or how the extracellular domains of the CD3 subunits interact with the extracellular portions of γδ TCRs compared with αβ TCRs. This may explain why the CD3 components of αβ TCRs are so different from those of γδ TCRs.

In terms of ligand binding surfaces, we note that the Vδ CDR3 of G8 protrudes significantly away from the other CDRs, as shown in Figure 11.6. This has significance in that this is the major region of contact with the T22 ligand (see later section). In the case of G115, both Vδ and Vγ CDR3 loops protrude from the rest of the putative binding surface and create a cleft between them. Portions of the CDR1γ and δ and CDR2γ combine with the clefts between the CDR3 loops to form a pocket, which is surrounded by positively charged amino acid residues contributed by CDR2γ and δ, and CDR3γ. The jagged surface of this TCR resembles the

surface of an antibody that binds a small-molecule antigen. Although this would be consistent with the supposition that this TCR binds the negatively charged phosphate compounds,[170] direct binding between the TCR and phosphoantigen including crystal-soaking and cocrystallization experiments have not been successful. Instead, a soluble G115 was found to bind a soluble form of adenotriphosphate (ATP) synthase F1 and apolipoprotein A-1.[171]

While the δ1A/B-3 TCR maintains an overall fold similar to the other γδ TCR structures, it was noted that unlike the G115 and G8 CDR3 regions, which are protruding out, the δ1A/B-3 CDR loops together generate a nearly flat surface on the combining site. This difference is anticipated, as the CDR3 length distribution of the TCRδ chains is quite variable as discussed previously, and like antibodies should have a broad range of binding site shapes.

αβ T-CELL RECEPTOR-LIGAND RECOGNITION

Binding Characteristics

Although it has long been established that this type of T cell generally recognizes a peptide bound to an MHC molecule, a formal biochemical demonstration that this was due to TCR binding to a peptide/MHC complex took many years to establish. Part of the difficulty in obtaining measurements of this type has been the intrinsically membrane-bound nature of MHC and TCR molecules. Another major problem is that the affinities are relatively low, in the micromolar range, which is too unstable to measure by conventional means.

To some extent, the problem of measuring the interactions of membrane-bound molecules can be circumvented by expressing soluble forms of TCR and MHC, which is also essential for structural studies (see previous discussion). For TCRs, many successful strategies have been described, including replacing the transmembrane regions with signal sequences for glycolipid linkage,[172] expressing chains without transmembrane regions in either insect or mammalian cells,[173] or a combination of cysteine mutagenesis and *E. coli* expression.[164] Unfortunately, no one method seems to work for all TCR heterodimers, although the combination of insect cell expression and leucine zippers at the c-terminus to stabilize heterodimer expression has been successful in many cases.[174] The production of soluble forms of MHC molecule has a much longer history, starting with the enzymatic cleavage of detergent solubilized native molecules[175] as well as some of the same methods employed for TCR such as glycophosphatidylinositol (GPI) linkage,[176] *E. coli* expression and refolding,[177,178] and insect cell expression of truncated

(or leucine zippered) molecules.[179] One interesting variant that seems necessary for the stable expression of some class II MHC molecules in insect cells has been the addition of a covalent peptide to the N-terminus of the β chain.[180]

The first measurements of TCR affinities binding to peptide/MHC complexes were performed by Matsui et al.[181] and Weber et al.[182] Matsui and colleagues used a high concentration of soluble peptide/MHC complexes to block the binding of a labeled anti-TCR Fab fragment to T cells specific for those complexes, obtaining an equilibrium binding affinity (K_d) value of approximately 50μM for several different T cells and two different cytochrome peptide/I-E^k complexes (as shown in Table 11.3). Weber and colleagues used a soluble TCR to inhibit the recognition of a flu peptide/I-E^d complex by a T cell and obtained a K_D value of approximately 10 μM. While these measurements were an important start in TCR biochemistry, they gave no direct information about the kinetics of TCR-ligand interactions. Fortunately, the development of surface plasmon resonance instruments, particularly the BIAcore™ (Pharmacia Biosensor, Uppsala, Sweden) with its remarkable sensitivity to weak macromolecular interactions,[183] has allowed rapid progress in this area. In this technique, one component is covalently crosslinked to a surface and then buffer containing the ligand is passed in solution over it. The binding of even approximately 5% of the surface-bound material is sufficient to cause a detectable change in the resonance state of gold electrons on the surface. This method allows the direct measurement of association and dissociation rates, that is, kinetic parameters, and also has the advantage of requiring neither cells nor radioactive labels. Recently, microcalorimetry has also been used to measure some TCR

ligand affinities; and these analyses have confirmed the surface plasmon resonance (SPR) values,[184] but do not allow kinetic measurements. These and other data[138,160] showed definitively that TCR and peptideloaded MHC molecules alone are able to interact and also that expression in a soluble form has not altered their ability to bind to each other. As shown in Table 11.3, SPR measurements show that while the on-rates of TCRs binding to peptide/MHC molecules vary from very slow (1,000 M sec) to moderately fast (200,000 M sec), their off-rates fall in a relatively narrow range (0.5 to 0.01 sec^{-1}) or a $t_{1/2}$ of 12 to 30 seconds at 25°C. This is in the general range of other membrane bound receptors that recognize membrane molecules on other cells,[137] but it has been noted that most TCRs have very slow on-rates,[138] which reflects a flexibility in the binding site that might help to foster cross-reactivity (see the following). In the case of a class I MHC-restricted TCR, 2C, this relatively fast off-rate may be stabilized (10-fold) if soluble CD8 is introduced,[165] but this result is controversial.[185] CD8 stabilization of TCR binding has been seen by Luescher et al. in cell-based TCR labeling assay[186]; however, no enhancement of TCR binding has been seen using soluble CD4[187] (see the following for more discussion of CD4 and CD8).

TABLE 11.3 T-Cell Receptor-Ligand Binding

T Cell	Ligand	K_D (mM)	$k_{on}(M^{-1}s^{-1})$	$K_{off}(s^{-1})$	Method	Reference
T_H Cells						
5C.C7	MCC/E^k	50	—	—	Anti-TCR comp.	166
2B4	MCC/E^k	50	—	—	Anti-TCR comp.	166
2B4	MCC/E^k	30	—	—	Anti-P/MHC comp.	166
2B4	MCC/E^k	90	600	0.057	BIA1	166
228.5	MCC 99E/E^k	50	—	—	Anti-TCR comp.	166
14.3d	Flu H1N1/E^d	~10	—	—	Sol. TCR	167
14.3d	SEC 1,2,3	5.4-18.2	>100,000	>0.1	BIA1	251
HA1.7	HA/DR1	>25	—	—	BIA1	300
HA1.7	SEB	0.82	13,000	0.001	BIA1	300
Tc						

cells						
2C	p2Ca/L^{d}	0.5	11,000	0.0055	Anti-TCR comp.	176
2C	p2/Ca/L^{d}	0.1	21,000	0.026	BIA1	301
2C	OL9/L^{d}	0.065	53,000	0.003	Labeled MHC	176
4G3	pOV/L^{d}	0.65	22,000	0.02	Labeled MHC	176
42.12	OVA/Kb	6.5	3,135	0.02	BIA4	179
2C	p2Ca/L^{d}	3.3	8,300	0.027	BIA1	208
HY	M80/D^{b}	23.4	6,200	0.145	BIA1	208
HY	CD8 α/β+M8/D/^{b}	2.0	5,100	0.01	BIA1	208
2/C	CD8 α/β+p2Ca/L^{d}	0.32	1,200	0.0038	BIA1	208
Tγδ cells						
G8	T10/T22	0.13	65,000	0.0081	BIA1	274

BIA1, TCR amine coupled; BIA2, TCR cysteine coupled; BIA3, MHC-peptide amine coupled in competition experiment; BIA4, TCR coupled by using H57 antibody and MHC coupled via amine chemistry; MHC, major histocompatibility complex; P, peptide; sol., soluble; TCR, T-cell receptor.

More recently, new methodologies have been utilized to measure TCR binding in its native state, that is, on a T-cell surface and interacting with peptide-MHC ligands on either an artificial bilayer[188] or on the surface of a red blood cell[189] "reporter." These types of measurements are important because studies of TCRs and peptide/MHCs binding in

solution only partially reflect what is happening between two membranes. This is because of the very severe constraints on binding that are present in this specialized environment. For one, it is likely that TCRs on T cells and peptide/MHCs on antigen-presenting cells are fixed into a fairly rigid alignment that maximizes their ability to bind to each other, as opposed to the same molecules in solution that are diffusing in all directions and for which only a fraction of collisions will result in a productive binding event. Secondly, binding is happening in a very small space/volume of liquid, making the effective concentration very high. While this environment is not strictly two-dimensional, Dustin and others have used this as a simplifying assumption with relative success. But a direct measurement of TCR affinity and kinetics has been made by Huppa and colleagues,[188] who used a fluorescent probe on an anti-TCR antibody to create a system in which this fluorophore could form a FRET pair with a complementary label on the c-terminus of the peptide that could emit photons of the appropriate wavelength only when the TCR bound its peptide-MHC ligand. This enabled precise measurements of the dissociation rate (koff) and affinity (KD) within an immunologic

synapse, and these measurements could also be used to estimate an average association rate (kon). Another advantage of these measurements is that they can be done at 37°C, whereas most of the SPR measurements cited previously were performed at 25°C due to instrument limitations. Dissociation rates at higher temperatures are significantly faster. As expected from the previous discussion, the association rate was greatly accelerated approximately 100× over the solution rate, presumably because of the ideal orientation of the TCR and peptide/MHC with respect to each other. Not expected was an accelerated dissociation rate of 4 to 10 times faster. This turns out to be a function of actin polymerization and depolymerization activity within the T cell, as shown dramatically in the actin dynamics studies of Vale and colleagues.[190] Once this was inhibited, the dissociation rates within the synapse were quite close to the solution values.[191] Still, it is worth noting that TCR binding to peptide/MHC is even more unstable within an active synapse than the solution measurements have suggested, with half lives of less than 1 second in many cases.

A second experimental system that has been applied to the question of TCR binding in the context of cell-cell contact has been that of Chung and colleagues,[189] in which red blood cells coated with peptide-MHC complexes are brought into repeated contact with a T cell of the appropriate specificity. By visualizing the response of the peptide/MHC-coated red blood cell to different frequencies and durations of T-cell contact, one can infer parameters of TCR affinity, at least in the two-dimensional aspect.[192] While this system has worked well in describing integrin binding, it is indirect and it some ways contradicts results obtained with the FRET system of Huppa et al.[188] and even some of the basic properties determined in solution measurements. Nonetheless, it may be telling us important properties of cell-cell contact that are more difficult to discern with more directly molecular methods.

To what extent are we now able to predict a T-cell response based on the binding characteristic of its TCR to a ligand? One of the most intriguing discoveries concerning T-cell reactivity has been the phenomenon of altered peptide ligands. These are single amino acid variants of antigenic peptides that either change the nature or degree of the T-cell response (partial agonists) or prevent a response to a normally stimulating ligand (antagonists).[193,194] Discussions concerning the mechanism of these “altered peptide” responses have centered on whether they are due to some conformational phenomenon involving TCRs and/or CD3 molecules or to affinity or kinetic characteristics. With the data now available, we can now say that most, but not all, T-cell responses correlate well with the binding characteristics of their TCRs. In particular, Sykulev et al.[195] first noted that higher-affinity peptide variants elicited more robust T-cell responses. Subsequently, Matsui et al.[196] found that in a series of three agonist peptides increasing dissociation rates correlated with decreasing agonist activity. Lyons et al.[197] found that this correlation extended to antagonist peptides in the same antigen system (moth cytochrome c/I-E^k). They also showed that while an antagonist peptide might differ only slightly in affinity compared with the weakest agonist, its dissociation rate differed by 10-fold or more. This data in a class II MHC-restricted system is largely supported by the studies of Alam et al. in a class I MHC system,[198] who also saw a drop-off in affinities and an increase in off-rates (with one exception as noted in Table 11.2) with antagonist versus agonist ligands. In the cell-based TCR labeling system of Luescher and colleagues, a survey of related peptide ligands of varying potency also found a general, but not absolute, correlation between receptor occupancy and stimulatory ability.[199] Thus, while there is a general trend toward weaker T-cell responses and faster off-rates and lower affinities, this does not seem to be an absolute rule, and thus other factors may be important in some cases. Alternatively, Holler[200] has suggested that some or all of the discrepancies may derive from differences in peptide stability (in the MHC) between the relatively short (minutes) time scale of BIAcore analysis at 25°C compared with the much longer (days) cellular assays at 37°C. But this explanation probably only applies to a fraction of the anomalous cases in which TCR ligands fail to adhere to the “$t_{1/2}$ rule.” In particular, Krogsgaard et al.[201] performed a very comprehensive survey of both known and newly derived cytochromic peptide antigens and found that almost half of the peptide-MHC ligands analyzed failed to exhibit a linear relationship between $t_{1/2}$ and T-cell stimulatory ability (Fig. 11.7). Extensive thermodynamic analyses of these ligands showed that one particular

parameter, the change in heat capacity (-ΔCp), which reflects changes in conformation or flexibility upon binding, seems to be synergistic with $t_{1/2}$ in enhancing a ligand's stimulatory capacity. In fact, as shown in Figure 11.7, when ΔCp values are combined with $t_{1/2}$, the x axis values for the range of ligands correlate much better with T-cell stimulation. This suggests that ΔCp may be the "missing" variable in correlating ligand binding to stimulation.

How could a negative change in heat capacity synergize with the stability of binding? One possibility is that large conformational changes at the binding surface of a TCR that can occur when it engages peptide/MHC ligands (as

shown by structural studies; see the following) can translocate the TCR deeper into the membrane—as suggested by the "piston"[52,202] and "twist cap"[184] models—and trigger conformational changes in the CD3 signaling domains. Chakraborty and colleagues recently suggested another possibility for how conformational changes at the binding surface might exert their effect.[203] They found that in the context of membrane-membrane interactions, conformational changes in the surfaces of relatively rigid proteins (such as all but the CDR3 regions of TCRs appear to be) would act to increase the effective half-life of TCR-peptide/MHC interactions. Thus $t_{1/2}$ and ΔCp may be equivalent in the unique environment between two cell surfaces.

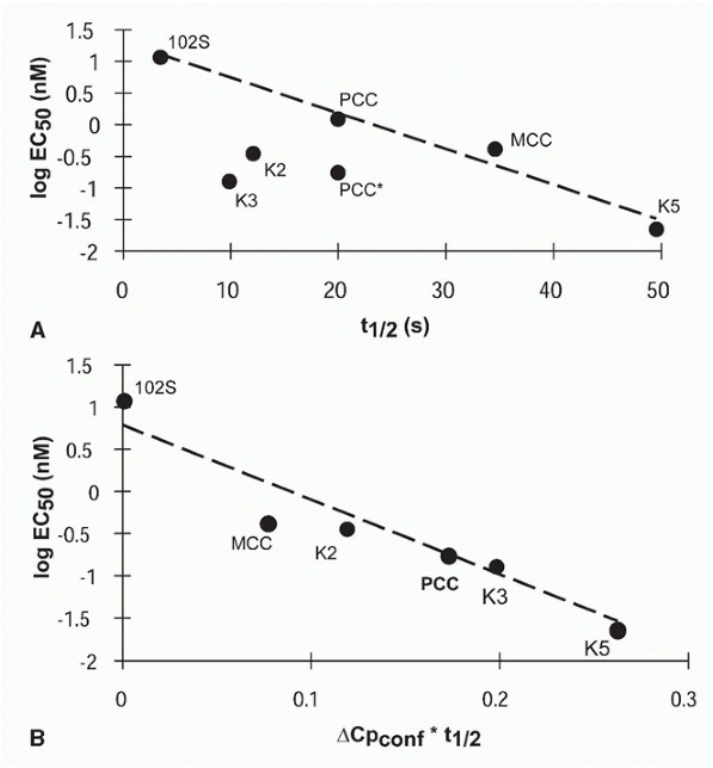

FIG. 11.7. Dissociation Rate and Heat Capacity with Peptide-Major Histocompatibility Complex (MHC) Ligands Both Influence T-Cell Activation. Data from Krogsgaard et al. show that where deviations from the general dependence of T-cell activation on dissociation rate occur, they may be compensated for by another factor, namely the heat capacity (ΔC_P) of the T-cell receptor (TCR)-peptide/MHC interaction. This is a measure of changes in mobility or conformation during the binding interaction and suggests that other binding parameters can influence TCR-mediated activation besides half-life.[184] **A:** The relationship between the half-maximal peptide concentration needed for T-cell activation (EC50) and the half-life ($t_{1/2}$) of the TCR binding to that particular ligand. In this series, three of the seven peptides tested (K2, K3, and PPC) do not seem as dependent on $t_{1/2}$ as the others (PCC* represents a correction for a lack of stability when bound to the MHC at 37°C). **B:** When ΔC_P is factored in, all of the peptides can be plotted on a line.

How might the relatively small differences in the binding characteristics of the ligands cause such different T-cell signaling outcomes as agonism or antagonism? As McKeithan[204] and Rabinowitz[205] have noted, a multistep system such as T-cell recognition has an inherent ability to amplify small differences in signals that are received on the cell surface to much larger differences at the end of the pathway, in this case gene transcription in the nucleus. Thus antagonism may occur at one threshold and an agonist response at another. Alternatively, an antagonist ligand may traverse the activation pathway just far enough to use up some critical substrate, as proposed by Lyons et al.[197] Yet another possibility that has also been suggested is that some antagonists may act even earlier by blocking TCR clustering at the cell surface.[206] Lastly, Germain and colleagues have found evidence that a feedback loop involving the phosphatase SHP-1 may act as an alternative pathway to inactivate TCR signaling of insufficient strength.[207,208]

Another controversy that relates to TCR binding characteristics is the "serial engagement" model of Valittutti and Lanzavecchia and colleagues,[139,209] which proposed that one way in which a small number of peptide/MHC complexes can initiate T-cell activation is by transiently binding many TCRs in a sequential fashion. While the dissociation rates reviewed here show that TCR binding is likely to be very transient, they do not in fact support the statement that more interactions are better. This is because, in most cases, improvements in TCR-peptide/MHC stability within any one system result in a more robust T-cell response. This has been shown by the work of Kranz and colleagues,[210] who selected a nanomolar affinity TCR from a mutagenized library expressed in yeast. With an approximately 100-fold slower off-rate than the original, this TCR should have been only poorly stimulatory based on the serial engagement model. Instead, T cells bearing it are considerably more sensitive to antigen, which casts considerable doubt on this aspect of the model. Similar work of Allen and colleagues on another TCR-ligand interaction produced similar results.[211] Furthermore, recently Xie et al. cross-linked peptide-MHCs to a cognate TCR and found that when aggregated they can still signal efficiently.[212] But the concept that the rapid dissociation rates of TCRs for peptide-MHC ligands could serve to amplify signaling under limiting conditions is still very likely and has been cited in the context of the "pseudodimer" model,[140,213] as discussed in the following.

Topology and Cross-Reactivity

As discussed previously, TCR sequence diversity resides largely in the region between the V and J region gene segments, which corresponds to the CDR3 regions of antibodies.[214] This has led to models in which the CDR3 loops of Vα and Vβ make the principal contacts with the antigenic peptide bound to the MHC.[214] Support for this model has come from many studies in which it has been shown that the CDR3 sequences of TCRs are important predictors of specificity,[214] as well as elegant mutagenesis studies which showed that a single CDR3 point mutation could alter the specificity of a TCR[215] and also a CDR3 "transplant" could confer the specificity of the donor TCR onto the recipient.[216] In addition, a novel approach to TCR-ligand interactions was developed by Jorgensen et al.,[217] who made single amino acid changes in an antigenic peptide at positions that affect T-cell recognition but not MHC binding. These variant peptides are then used to immunize mice that express either α or β chain of a TCR that recognizes the original

peptide and the responding T cells are analyzed. Using these "hemitransgenic" mice allows the resulting T cells to keep one-half of the receptor constant, while allowing considerable variation in the chain that pairs with it. The results from this study and work in another system by Sant'Angelo et al.[218] are very similar in that every mutation at a TCR-sensitive residue triggered a change in the CDR3 sequence of Vα, Vβ, or both, and in some cases, changed the Vα or Vβ gene segment as well (as summarized in Fig. 11.8). One of the more striking examples of a CDR3-peptide interaction occurred in the cytochrome c system where a Lys → Glu change in the central TCR determinant on the peptide triggered a Glu → Lys charge reversal in the Vα CDR3 loop, suggesting a direct Lys → Glu contact between the two

molecules.[217] This prediction has recently been confirmed in the structural work of Newell et al.[219] and extended to a closely related TCR, 2B4, which contacts the central lysine on the peptide with an oppositely charged residue in CDR3β.

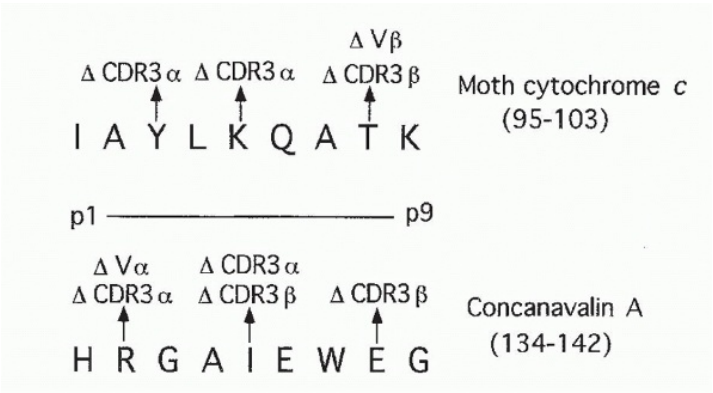

FIG. 11.8. Sensitivity of T-Cell Receptor (TCR) Complementarity-Determining Region 3 (CDR3) Sequences and Vα/Vβ Usage to Changes in the Antigen Peptide. This figure summarizes the data of Jorgensen et al.[196,198] and Sant'Angelo et al.,[197] who immunized single-chain transgenic mice (TCR-α or TCR-β) with antigenic peptides (MCC or CVA) altered at residues that influence T-cell recognition but not major histocompatibility complex binding. These data show that such changes invariably affect the CDR3 sequences of Vα or Vβ or both, and that there appears to be a definite topology in which Vα governs the N-terminal region and Vβ seems more responsible for the C-terminal portion of the peptide.

Another interesting finding was the order of Vα → Vβ preference going from the N-terminal to the C-terminal residues of the peptides. This led Jorgensen to a proposed "linear" topology of TCR-peptide/MHC interaction in which the CDR3 loops of Vα and Vβ line up directly over the peptide.[217,220] Sant'Angelo et al.[218] proposed an orientation of the TCR in which the CDR3 loops are perpendicular to the peptide. This was partially based on intriguing data they found suggesting an interaction between the CDR1 of Vα and an N-terminal residue of the peptide.

This controversy regarding orientation has been largely resolved by the numerous crystal structures of TCR-peptide/MHC complexes.[160,221] These studies show that TCRs bind in roughly diagonal to 90-degree configurations, ranging over 30 degrees. In these structures, one of which is shown in Figure 11.9,[165] the CDR3 loops are centrally located over the peptide, but the Vα CDR1 and the Vβ CDR1 are also in a position to contact the N-terminal and C-terminal peptide residues, respectively. The confined nature of TCR recognition constitutes a major departure from antibodyantigen interactions and may reflect a need to accommodate other molecules into a particular configuration that is optimal for signaling, such as CD4, CD8, and/or CD3 components. Particularly relevant to this possibility are the recent observations by Garcia and colleagues[222] suggesting that TCR signaling can be limited by the docking geometry of the TCR to the peptide/MHC complex. They used a yeast library in which millions of different peptides bound to a class I MHC can be interrogated by a single TCR. Structural analysis showed that three of the peptides isolated bound to the TCR with the typical orientation and induced TCR signaling. But a fourth peptide bound the TCR not in the typical diagonal mode but in a parallel fashion, and failed to induce signaling.[222] This suggests that the typical TCRMHC docking mode is selected for in the thymus and may be critical for signaling.

As αβ TCR heterodimers are first selected in the thymus for reactivity to self-peptides bound to MHC molecules, all foreign-peptide reactive TCRs could be considered to be inherently cross-reactive. Indeed, a number of T cells have reactivity to very different peptide sequences, as shown by Nanda and Sercarz.[223] It has also been argued by Mason[224] that

the universe of peptides is so large that each T cell must on average be cross-reactive to approximately 10^6 different peptides (although many of the differences in peptide sequence in this calculation would not be accessible to the TCR, being buried in the MHC binding groove). Several large-scale screens of a random 9-mer peptide library with different T cells did turn up a great many stimulatory

peptides, but very few have changes in the two to four key TCR-sensitive residues.[225] This led Garcia and colleagues to conclude that cross-reactivity to different agonist peptides may not be a general feature of T-cell specificity.[226] Instead, the major requirement for cross-reactivity may be in thymic selection[227] and/or in the use of endogenous peptide-MHCs to augment T-cell sensitivity to agonist ligands.[207,213,228] Direct evidence of TCR plasticity was first obtained by Garcia et al.,[229] who in comparing the x-ray crystal structures of the same TCR bound to two different peptide-MHC ligands found a large conformational change in the CDR3 loop and a smaller one in the CDR1α loop. An even larger conformational change (15 Å) has been found in the CDR3β residue of another TCR as it binds to a peptide/MHC complex,[230] as shown in Figure 11.10. That each TCR may have many different conformations of its CDR3 loops is suggested by the two-dimensional nuclear magnetic resonance studies of Reinherz and colleagues, who found that the CDR3 regions of a TCR in solution were significantly more mobile than the rest of the structure.[231] That this may be a general feature of most TCRs is supported by thermodynamic analyses of various TCRs binding to their peptide/MHC ligands, both class I and class II. Here, the binding is often accompanied by a substantial loss of entropy (Table 11.4) and, in most cases, an "induced fit" mechanism.[184,192,232] This seems to be a situation where an inherently flexible binding site achieves greater order upon binding. This is a mechanism which is also employed by DNA recognition proteins, and Boniface et al.[192] have suggested that it might represent a common mechanism of "scanning" an array of very similar molecular structures (MHCs or DNA) rapidly for those few that "fit" properly. We have seen previously that the association rates are often remarkably slow, with K_as ranging from 1,000 to 10,000 $M^{-1}s^{-1}$ (see Table 11.3). This indicates that either a multistep process is occurring before stable binding can be achieved or that only a fraction of the TCRs in

solution have the correct conformation. Just how such a scanning mechanism might work for TCRs is seen in the analysis of Wu et al.,[233] who found that a cytochrome c/MHC II-specific TCR derived most of its stability of binding from antigenic peptide residues, but very little of its initial activation energy from these residues. In contrast, MHC residues contributed by far the most of the initial binding, but had relatively modest effects on stability. This indicates that "scanning" may be a process (as shown in Figure 11.11) that first involves contact with (and orientation by) the α-helices of the MHC and then a "fitting" process with and stabilization by peptide residues that involves a substantial loss of entropy. This model of TCR binding might help to explain the striking efficiency and sensitivity of T-cell recognition with the MHC helices guiding the TCR into the correct orientation. It might also be the structural basis for cross-reactivity in which structurally very different peptides bind to the same TCR, as the CDR3 regions of TCR could "fold" into the peptide in many possible configurations. While attractive, there remain caveats about this "two-step binding" model; one is the existence of some important human class I MHC antigens that "bulge" out of the binding groove, creating a barrier to "scanning" as a mechanism of binding to those antigens.[234] A second issue is that one would expect a reproducible "footprint" for MHC binding of particular TCR V regions, which until recently has been elusive. But Garcia and colleagues,[235] as well as Kappler and colleagues,[236] have documented such specific footprints that correlate to particular Vβ usage, although it does seem that there are multiple ways way in which a particular V region can contact a particular MHC.

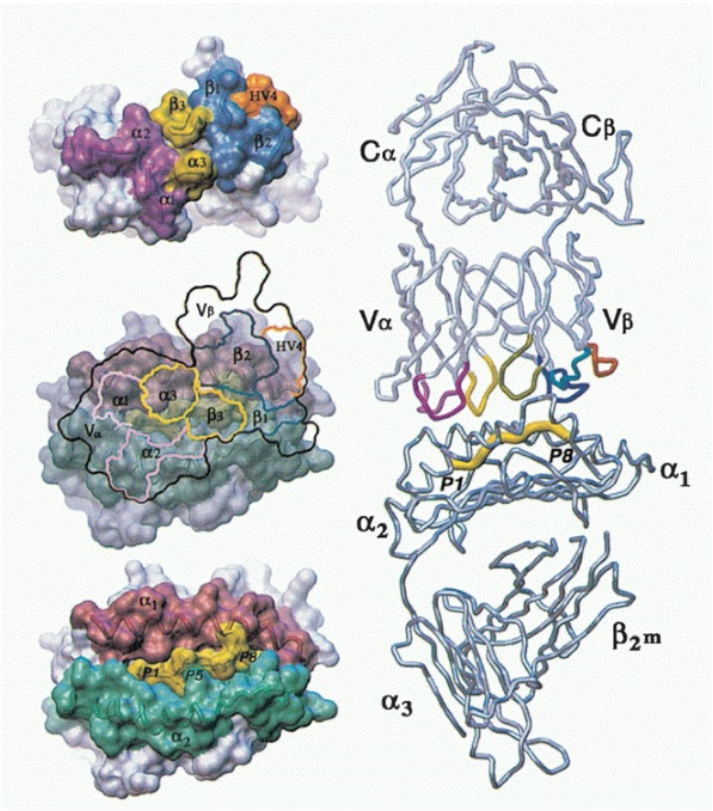

FIG. 11.9. T-Cell Receptor-Peptide/Major Histocompatibility Complex Crystal Structure of a T-Cell Receptor-Peptide/Major Histocompatibility Complex Complex. Peptide and complementarydetermining regions are portrayed in different colors.[150]

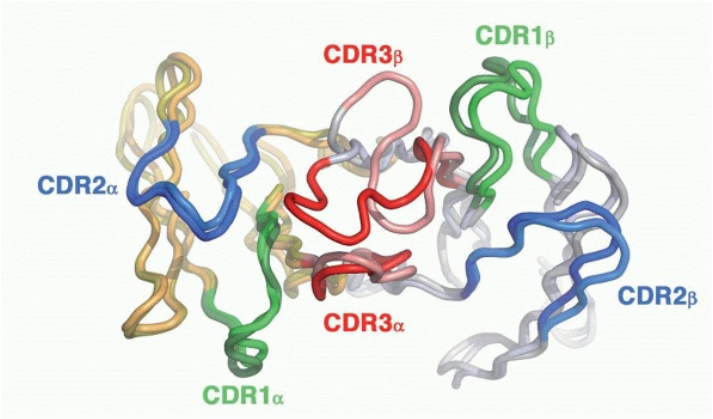

FIG. 11.10. Complementarity-Determining Region (CDR)3 Movement in αβ T-Cell Receptor (TCR) Binding. In most cases where there is a structure for an αβ TCR as well as for one (or more) for that TCR in complex with a peptide-major histocompatibility complex, there is a marked movement of one or the other of the Vα or Vβ CDR3s. This example from Mallissen et al.[209] shows a particularly large (14 A) movement of CDR3β with binding. This meshes well with thermodynamic data showing an “induced fit” binding mechanism for most TCRs.[173,211]

TABLE 11.4 Thermodynamic and Structural Parameters for T-Cell Receptor Peptide-Major Histocompatibility

Complex Interactions							
TCR	**pMHC**	**ΔG° (kcal/mol)**	**ΔH° (kcal/mol)**	**ΔCp (kcal/mol)**	**TCR Conformational Change**	**pMHC Conformational Change**	**Reference**
2C	dEV8-H2-K^{b}	-6.3	-22.7	-1.1	CDR3α(6 Å)	None[a]	169,208
2C	p2Ca-H2-K^{b}	-6.1	-29	-1.5	ND	ND	169
2C	SIYR-H2-K^{b}	-7.2	-8.4	-1.1	CDR3α(3.9 Å)	None	169,208
2C	dEV8-H2-K^{bm3}	-5.8	ND	ND	CDR3α(6 Å)	None	305,308
JM22z	MP(58-66)-HLA-A2	-7.1	-23	ND	—	Q155-A2(2.4Å)	211,307
KB5-C20	PKB1-H2-K^{b}	ND	ND	ND	CDR3β(15 Å)	None	209
LC13	EBNA3A-HLA-B8	-6.8	ND	ND	CDR3α(2.5 Å); CDR1α(1.9 Å):Cα	(Q155)-HLA-B8	304
A6	Tax-HLA-A2	-8.2	ND	ND	CDR3β(4.4 Å)	A2-α2(1.4Å)	149,209
BM3.3	VSV8-H2-K^{b}	-5.4	ND	ND	CDR3α(5.3 Å)	None	303
D10	CA-I-A^{k}	-7.0	ND	ND	CDR3β	None	172,210
F5z	AM9-H2-K^{b}	-6.7	-19	ND	ND	ND	211
2B4	MCC-I-E^{k}	-6.9	-13	-0.6	ND	ND	169,173
2B4	K2-I-E^{k}	-6.9	-9.4	-2.1	ND	ND	169
2B4	K3-I-E^{k}	-6.1	-30.5	-4.0	ND	ND	169
2B4	K5-I-E^{k}	-7.5	-8.0	-1.2	ND	ND	169
2B4	102S-I-E^{k}	-5.6	-13.2	-0.3	ND	ND	169

2B4	PCC-I-E^k	-6.1	13.3	-1.8	ND	ND	169
2B4	PCC-103K-I-E^k	-7.0	-8.4	-1.0	ND	ND	169
172.10	MBP(1-11)-I-A^u	-6.9	-21.2	-0.16	ND	None	305
1934.4	MBP(1-11)-I-A^u	-6.0	-15.7	-1.2	ND	ND	305
D3	SL9-HLA-A2	-7.5	-10.4	-0.4	ND	ND	309

The ΔG values were derived from the equation $\Delta G=RT \ln(K_A)$ where R=0.001987 kcal/mol/K. CDR, complementarity-determining region; ND, not determined; p/MHC, peptide/major histocompatibility complex; TCR, T-cell receptor.

[a] No major conformational change observed after ligand recognition.

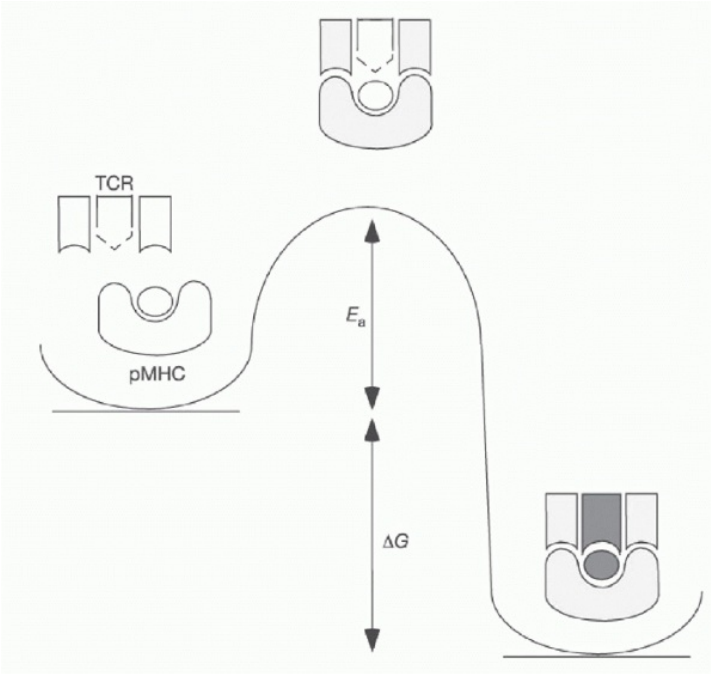

FIG. 11.11. As Shown by Wu etal.,[212] Mutational Analysis of T-Cell Receptor (TCR)-Peptide/Major Histocompatibility Complex (MHC) Binding Indicates that the TCR First Contacts MHC Residues (in the Transition State), and the Peptide has Very Little Influence. Subsequently, however, the peptide residues contribute greatly to the stability of the complex. Thus, we have proposed that the transition state largely involves TCR-MHC contact followed by stabilization of mobile complementarity-determining region 3 residues into a stable state, usually involving significant conformational change and loss of entropy.

The rules for the conserved reaction of αβ TCRs with MHC proteins plus peptides are addressed by evaluating the contact residues between TCR and MHC in cocrystal structures

of TCR/peptide/MHC complexes. Thus it has been suggested that each TCR variable-region gene product engages each type of MHC through a “menu” of structurally coded recognition motifs that have arisen through coevolution.[235,236]

Role of Cluster of Differentiation 4 and Cluster of Differentiation 8

What is the role of CD4 and CD8 with respect to the T-cell response to agonist and antagonist peptides? Clearly, their expression greatly augments activation, and in some cases, determines whether there is a response at all.[237] In addition, the results of Irvine et al.[238] and Purbhoo et al.,[239] using a single-peptide labeling technique, found an appreciable T-cell response to even one agonist peptide in all four T cells analyzed, resulting in a “stop” signal for the T cell and a small, but detectable rise in intracellular calcium. In CD4+ T cells, these effects are attenuated by antibody blockade of CD4, such that many more (25 to 30) peptides

are required in order to elicit a stop signal and a calcium flux.[238] Much of this effect likely comes from the recruitment of lck to the TCR/CD3 complexes via the cytoplasmic tails of either molecule. But in addition, there is also a significant positive effect even with CD4 molecules that are unable to bind lck and thus there appears to be an effect on TCR-ligand interaction as well. Nonetheless, while a weak binding of CD4 to class II MHC has been observed,[187] most recently in a complete crystal structure of CD4-TCRpeptide/MHC,[240] there was no apparent stabilizing effect on TCR binding to peptide-MHC in measurements of binding within a synapse by Huppa et al.[188] This is in contrast to the earlier data of Luescher and colleagues,[241] who found that CD8 had a measureable stabilizing effect on TCR binding to ligand on T cells expressing that molecule. This later result is consistent with measurements of CD8 affinity for class I MHCs that are very similar, or even superior, to that of many TCRs for peptide/MHC.[242] So, are CD4 and CD8 playing very different roles with respect to TCR binding to peptide/MHC? Chakraborty and colleagues suggest not in a model they have developed that indicates that the differences in CD4 and CD8 affinity for MHCs contribute at best marginally to activation and that delivering lck to productive TCR-peptide/MHC complexes is their most important property.[243] In this view, the specificity of these molecules for their respective MHC molecules is more of a targeting mechanism than the stabilization function implicit in the ubiquitous illustrations originally proposed by Janeway[116] and featured in many subsequent models.

How could CD4 be facilitating the recognition of small numbers of peptides? Irvine et al. [238] proposed a “psuedodimer” model, which suggests that a CD4 molecule associated with a TCR binding to an agonist peptide-MHC could bind laterally to an endogenous peptide-MHC complex that is also being bound by an adjacent TCR. This takes advantage of the apparent abundance of endogenous peptide/MHCs that can be bound by a given TCR[244] and uses two weak interactions (CD4 → MHC II and TCR → endogenous peptide-MHC) to help create a dimeric “trigger” for activation. This is supported by the work of Krogsgaard et al.,[213] who also showed that disabling CD4 binding to the endogenous peptide/MHC in a heterodimer had a much a greater effect on activation than the same mutation on the agonist peptide/MHC.

What about the timing of CD4 or CD8 binding with respect to TCR-peptide/MHC? The first hints of this came from the work of Hampl et al.,[245] who found that antagonist peptide/MHCs showed no apparent influence of CD4 versus agonists, and because these interactions are much briefer, suggested that CD4 binding was subsequent to TCR engagement with a ligand. That this is likely to be a general effect was shown recently by Jiang et al.,[246] who found that whereas very brief T-cell contacts with peptide/MHC loaded red blood cells involved TCR but not CD8, longer contact periods involved both molecules. Thus it seems that the TCR must first bind to a high quality (eg, agonist) ligand and then CD4 or CD8 comes in to deliver lck and dissociates (based on the lack of stabilization, at least in the case of CD4). But more direct measurements are needed, as well as further definition of the role of endogenous ligands.

Superantigens

One of the most interesting and unexpected areas to emerge from the study of αβ T-cell reactivities is the discovery of "superantigens." Whereas a particular antigenic peptide might only be recognized by 1 in 100,000 or fewer T cells in a naive organism, a given superantigen might stimulate 1% to 20% of the T cells.[247,248] As will be discussed in more detailed in the following, the physical basis for this is that the superantigen binds to a Vβ domain of the TCR on T cells while simultaneously binding to an MHC class II molecule on an antigen-presenting cell (although not in the peptide-binding groove). This allows a single superantigen, such as staphylococcal enterotoxin A (SEA) in Table 11.5, to stimulate virtually every murine T cell bearing Vβ 1, 3, 10, 11, 12, or 17 (about 15% of all αβ T cells), in most cases regardless of what Vα it is paired with or what CDR3 sequence is expressed. Clearly, this is a unique class of T-cell stimulatory molecules.

The first indication of a superantigen effect was the discovery of minor lymphocyte stimulating determinants by Festenstein in the early 1970s.[249] Many years later, Kappler and colleagues characterized a mouse strain-specific deletion of T cells expressing a specific TCR Vβs that were attributable to these loci.[250] It emerged that these effects were due to endogenous retroviruses of the mouse mammary tumor virus family.[251,252,253,254,255] Different family members bind different TCR Vβ domains (as shown in Table 11.5) and stimulate T cells expressing them. Meanwhile, Janeway and colleagues[256] had shown earlier that staphylococcus enterotoxins could polyclonally active naive T cells in a Vβ-specific manner without a requirement for antigen processing. Many of these enterotoxins have been characterized extensively.[247,248,257,258] Unlike the MMTV proteins, which are type II membrane proteins, the enterotoxins are secreted. Subsequently, proteins having similar properties have been isolated from other bacteria (*Yersinia pseudotuberculosis*,[259,260] streptococcus,[261] and from mycoplasma[262,263]). There is also evidence of superantigen-like activities in other mammalian viruses such as rabies,[264] cytomegalovirus,[265] herpes virus,[266] Epstein-Barr virus,[267] and also in *Toxoplasma gondii*.[268] As so many pathogenic or parasitic organisms possess these molecules, apparently by convergent evolution, there must be some selective advantage, but in most cases there is no conclusive evidence as to what this might be. The one exception is the case of the MMTV superantigens, where it has been shown that polyclonal T-cell stimulation allows the virus to more efficiently infect the B-lymphocytes that are activated by the T cells.[269,270] This may be a special case, however, and most authors have suggested that superantigens primarily serve to confuse and occupy the immune system while the pathogen escapes specific targeting and elimination. Large doses of superantigens have also been implicated in various "shock" syndromes, such as food poisoning or "toxic shock,"[247] but this is probably not their everyday purpose, as it would violate the general rule that the host and parasite should coexist.

It has also been suggested by Stauffer et al.[271] that superantigens might be involved in triggering autoimmune diseases. Here the hypothesis is that a large number of some Vβ-bearing T cells are activated by a pathogenic superantigen

and that subsequently self-reactive T cells within those activated cells are more easily stimulated by a particular tissue antigen. That this may occur in some cases is supported by their work on a human endogenous retrovirus which specifically stimulates Vβ7 T cells and is implicated in the initiation of type I diabetes.[271] Another report implicates a superantigen in Crohn disease, another autoimmune disorder.[272]

TABLE 11.5 Vβ Specificity of Exogenous and Endogenous Superantigens

Bacterial Superantigen	Human Vβ Specificity	Reference	Murine Vβ Specificity	Reference
				(as

				referenced in 235,314)
SEA	ND		1,3,10,11,12,17	310,311
SEB	3,12,14,15,17,20	315,316	(3), 7,8.1,8.3, (11), (17)	310,311,312
SEC_1	12	316	7,8.2,8.3,11	310
SEC_2	12,13,14,15,17,20	315,316	8.2,10	310
SEC_3	5,12	316	(3), 7,8.2	310
SED	5,12	316	3,7,(8.2),8.3,11,17	310
SEE	5.1,6.1-6.3,8,18	315,316	11,15,17	310
TSST-1	2	316	15,16	310
ExFT	2	315	10,11,15	310
Strep M	2,4,8	239	ND	
Endogenous Proviruses	**Vβ Specificity**	**Mls Type**[a]	**Chromosome**	**Reference**
				(as referenced in 235,236)
Mtv-1	3	c, 4a	7	
Mtv-2	14	NA	18	
Mtv-3	3,17	c	11	
Mtv-6	3,17	c,3a	16	
Mtv-7	6,7,8.1,9	a, 1a	1	
Mtv-8	11,12	f, Dvbll.1	6	
Mtv-9	5,11,12	f, Etc-1	12	
Mtv-11	11,12	f, Dvbll.3	14	
Mtv-13	3	c, 2a	4	
Mtv-43	6,7,8.9,9	Mls-like	ND	
Exogenous Viruses	**Vβ Specificity**	**Mls Type**	**Chromosome**	**Reference**
MMTV-C3H	14,15	NA	231	

MMTV-SW	6,7,8.1,9	Mls-like	247	
Rabies	ND		242	
EBV	ND	HERV-K18	245	
CMV	ND		243	
Herpesvirus				244
Other Pathogens	**Vβ Specificity**	**Name**	**Chromosome**	**Reference**
Mycoplasma arthritidis	h17,6,8.1,8.3	MAM		240,241
Toxoplasma gondii	5			246
Yersinia enterocolitica	ND			237
Yersinia pseudotuberculosis	ND			238

Vβ in parentheses are reactive with commercial but not recombinant enterotoxins.

CMV, cytomegalovirus; EBV, Epstein-Barr virus; MMTV, mouse mammary tumor virus; NA, not applicable; ND, not determined.

[a] The nomenclature in use before the discovery that the phenotype resulted from endogenous retroviruses.

While the biochemistry of superantigen binding to TCR and MHC is similar to that of TCR peptide/MHC interactions,[273] mutagenesis, and particularly x-ray structural data, has shown that the topology is both quite different and variable.[248] In particular, it has been found that Mls-la presentation to T cells is most affected by mutations on the "outside" surface of the Vβ domain, which do not affect peptide/MHC recognition.[162] In contrast, CDR1 and CDR2 of regions of Vβs are involved in bacterial superantigen reactivity.[274]

An example of the structural data is shown in Figure 11.12, which shows how a model TCR-SAg-MHC complex (derived from separate structures) would displace the TCR somewhat (but not entirely) away from the MHC binding groove,[275] thus making the interaction largely insensitive to the TCR/peptide specificity. Other TCR-SAg-MHC complexes have very different geometries.[276,277]

Why do all the many independently derived superantigens interact only with the TCR β-chain? One possibility is that the β-chain offers the only accessible "face" of the TCR, perhaps because the CD4 molecules hinders access to the Vα side, as suggested by the antibody blocking studies of Janeway and colleagues.[278,279]

ANTIGEN RECOGNITION BY γδ T CELLS

γδ T cells together with αβ T cells are present together in all but the most primitive vertebrates. This finding argues that γδ T cells functions are different from those of αβ T cells. Yet, most γδ T cells produce cytokines that are similar to

those produced by αβ T cells. γδ T cells also can mount cytotoxic responses upon activation

like CD8+ αβ T cells. These results, together with the fact that αβ T cells are almost always found alongside and usually in excess of γδ T cells, suggest that the difference in how γδ T cells and αβ T cells contribute to host immune competence is less likely because of differences in effector functions or in tissue distribution but rather because of differences in how these two types of cells are triggered. Thus, to understand how γδ T cells function, it is essential to know the antigens and the target cells recognized by γδ T cells.

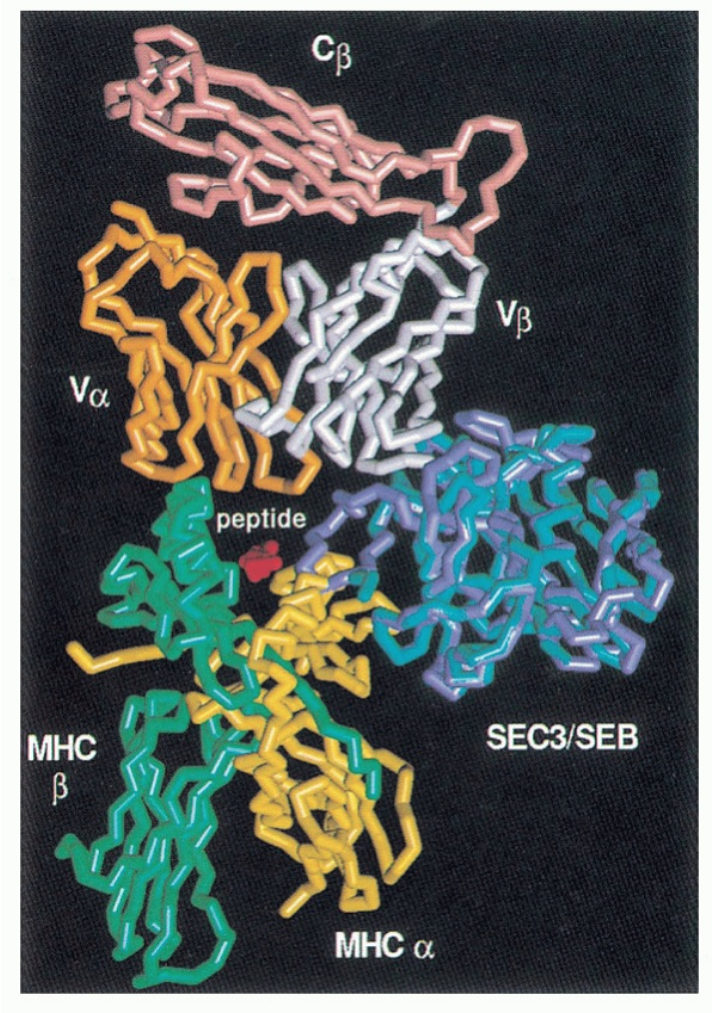

FIG. 11.12. Crystal Structure of a T-Cell Receptor (TCR) β/Superantigen (SAg) Complex. Fields et al.[253] crystallized TCR-SAg complexes and from the structure of the same superantigens with a class II major histocompatibility complex (MHC) molecule and were able to deduce the relative spatial arrangement of the three molecules. This model suggests that TCR does not contact the MHC very strongly, which is consistent with the relative peptide insensitivity of SAg activation.

Antigen Recognition Requirements and γδ T-Cell Ligands

Because the study of γδ T cells is relatively recent and does not stem from any knowledge of their biologic function, experiments designed to characterize their specificity and function had drawn heavily on our knowledge of αβ T cells. Because αβ T-cell recognition requires antigen presentation by MHC and related molecules, it was assumed that γδ T-cell recognition would also follow the same rules. Even in cases where classical MHCs are clearly not involved, it has been suggested that nonclassical MHC molecules or some as yet to be identified surface molecules might play a similar role. These possibilities are difficult to test experimentally. Thus, analysis of γδ T cells specific for MHC molecules were carried out to determine the antigen recognition requirement of γδ TCRs. This approach asks the following questions: When γδ T cells recognize MHC molecules, what kind of antigen processing is required, is

any of the specificity conferred by bound peptide, and which part of the MHC molecule is recognized? This approach took advantage of the detailed knowledge of the molecular structure of MHC class I[280,281] and MHC class II molecules,[282] thus making T-cell epitope mapping feasible and interpretable. In addition, and more importantly, the biosynthetic pathways and antigen-processing requirements for both MHC class I and MHC class II molecules had been extensively studied. Mutant cell lines defective in either pathway were readily available and could be transfected with various MHC class I and class II genes. Therefore, potential antigen processing and presentation requirements for alloreactive γδ T cells could be studied with precision and compared with those of αβ T cells.

The γδ T cell LBK5[283,284] recognizes I-$E^{b,k,s}$ but not I-E^{d}. An analysis of the fine specificities of LBK5 showed that the peptide bound to the I-E molecules does not confer specificity and that no known antigen-processing pathways were required in the recognition of I-E by LBK5. All variations in the ability of different stimulator cells to activate LBK5 can be attributed solely to their level of surface I-E expression and are independent of their species origin (mouse, hamster, human) and cell type (B cells, T cells, fibroblasts). Modifications of the repertoire of peptides loaded onto MHC molecules also showed no effect because native I-E^{k}, GPI-linked I-E^{k}, I-E^{k} expressed with or without invariant chains, and the presence or absence of functional MHC class I or class II antigen-processing pathways all stimulated LBK5 similarly.[285] Thus, LBK5 recognizes native I-E^{k} molecules with a variety of different bound peptides or in the case of GPI-linked I-E^{k}, which most likely does not complex with a peptide. In addition, LBK5 recognizes *E. coli*-produced I-E^{k} α and β chains folded with a single peptide.[285]

The functional epitope on I-E for LBK5 recognition maps to the β67 and β70 residues.[285] This explains why LBK5 recognizes I-$E^{b,k,s}$ but not I-E^{d} and why peptide bound to I-E^{k} does not confer the specificity. Surprisingly, I-E^{k} mutants with an altered carbohydrate structure on the α84 position are not recognized by LBK5.[286] Although the binding interface of the LBK5 γδ TCR and I-E has yet to be determined, it is reasonable to assume that it will not deviate much from those reported for antibodies, αβ TCRs and the G8 γδ TCR. From the coordinates of a published crystal structure of I-E^{k},[287] the distance between β67-70 (the LBK5 epitope) and the carbohydrate attachment site at position α82 was estimated to be around 28-33Å. Hence, it is likely that the carbohydrate structure is peripheral to the core of the LBK5/I-E interaction. This type of interaction may be similar to that described for human growth hormone and the extracellular

domain of its receptor[288] where a central hydrophobic region at the contact site, which is dominated by two tryptophan residues, accounts for more than three-quarters of the binding free energy and where peripheral electrostatic contacts contribute substantially to the specificity of binding but not to the net binding energy. This type of protein-protein interaction has been postulated to ensure the specificity of an interaction without requiring a high affinity.[289]

Two independently derived γδ T-cell clones, KN6[290] and G8,[291] are found to recognize T10 and T22, two closely related, nonclassical MHC class I molecules that have 94% amino acid identity. T22 appears to be expressed constitutively on a variety different cell types, whereas the expression of T10 is inducible on cells of the immune system. Among strains of mice tested so far, all express T22. However, mice of the H-2^{d} or H-2^{k} MHC haplotypes (eg, BALB/c and C3H, respectively) lack functional T10 molecules.[291,291a] T10 and T22 have also been identified as natural ligands for murine γδ T cells. Approximately 0.2% to 1% of the γδ T cells in normal, unimmunized mice are T10/T22-specific (Fig. 11.13).[292]

The primary sequences of T10 and T22 suggest that the necessary structural features that enable classical MHC class I molecules to bind peptides are absent. Indeed, x-ray crystallography has shown that T10 and T22 adopt a severely modified MHC-like fold that lacks a classical peptide- binding groove and exposes part of the β sheet “floor” of the α1/α2 platform.[293,294] Consistent with the structural data, no endogenous peptides can be eluted

from chimeric T10/L^d molecules that are expressed by transfected cells.[295,295a] This indicates that these molecules can reach the cell surface devoid of peptide. Importantly, the *E. coli*-produced, in vitro-folded T10/β_2m and T22/β_2m molecules can stimulate the G8 γδ T cell,[295,296] which provides unequivocal evidence that these peptide-free molecules retain their immunologic function.

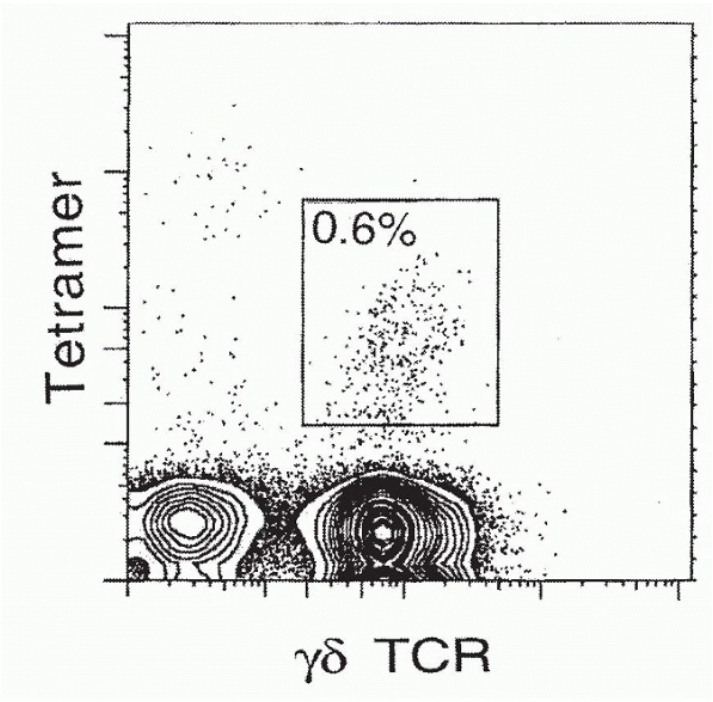

FIG. 11.13. T10/T22-Specific γδ T Cells can be Detected in Normal Mice Through Use of a Tetrameric T22 Staining Reagent. As shown by Crowley et al.,[274] a T22 reagent that was generated by similar methods as tetrameric peptide/major histocompatibility complex reagents stained approximately 0.6% of splenic γδ T cells in normal animals. More than 90% of these cells are cluster of differentiation (CD)4-CD8-; the rest are either CD4 or CD8 single positive (about 3% to 4% each). A similar frequency of tetramer-positive γδ T cells was also found in the intestinal intraepithelial lymphocyte population (data not shown).

Direct binding between soluble G8 γδ TCRs and T10/β_2m and T22/β_2m complexes has been measured.[297] Surface plasmon resonance showed that the dissociation rates for the interaction between G8 and $T10^b$ and $T22^b$ were similar ($k_d = 8.1 \pm 2.3 \times 10^3 s^{-1}$) and slower than those that had been observed for most interactions between αβ TCRs and peptide/MHC complexes. The association rates ($k_a = 6.53 \pm 1.73 \times 10^4\ M^{-1}s^{-1}$) are among the fastest that had been reported for αβ TCRs and their ligands. Therefore, compared to αβ TCRs, the affinity between G8 γδ TCRs and their ligand is rather high ($K_D = 0.13 \pm 0.05$ μM). A Scatchard analysis of equilibrium binding generated a similar affinity of 0.11 ± 0.07 μM for this interaction.[297]

There are only two other pairs of ligand-TCR interaction have been reported. The binding between the soluble form of G115 and its ligand F1-ATPase and the apolipoprotein A-I complex showed a K_D of 1.5 μM and 0.8 μM, respectively.[171] These affinities are within the range of soluble αβ TCRs and their peptide/MHC ligands. Surprisingly, a K_D of 110 to 900 μM was reported for δ1A/B-3:MICA interactions.[299] This affinity is one to two orders of magnitude weaker than the NKG2D:MICA interaction, more on a par with MHC class I:CD8 interactions (from 11 to ≥ 1,000 μM).

It should be noted that the same human γδ T-cell clones that recognize AS/ApoA-I complexes

are activated by a set of nonpeptidic pyrophosphomonoesters that are collectively referred to as phosphoantigens (phosphoAgs).[170] It has been observed for well over a decade that some human peripheral blood Vγ9Vδ2 cells can be stimulated by phosphoAgs such as isopentenyl pyrophosphate and dimethylallyl pyrophosphate. These cells show in vitro responses to tumors, phosphoAgs produced by eukaryotes and prokaryotes, natural and synthetic alkylamines, and aminobisphosphonates. However, repeated attempts to show interactions between phosphoAgs and Vγ9Vδ2 TCRs have failed. The identification of AS/ApoA-I complexes as antigens of some of these T cells further challenges this notion.

As indicated in Table 11.6,[145] other than toward phosphoAgs, the reactivities of Vγ9Vδ2-expressing T cells to other challenges such as tumors and AS/ApoA-I are far from homogeneous. Further, these cells mount a much more robust response to phosphoAgs than to CD3 cross-linking. Thus, it is possible that isopentenyl pyrophosphate and other isoprenoid intermediates enhance antigen-specific responses of Vγ9Vδ2 cells without being TCR antigens themselves. Isopentenyl pyrophosphate and dimethylallyl pyrophosphate are metabolites of the mevalonate pathway that regulates the biosynthesis of cholesterol as well as of isoprenoids that mediate the membrane association of certain GTPases. The addition of isoprenoid intermediates has been shown to augment antigen-specific αβ T-cell responses and to alter their cytokine profiles.[300]

These experimental observations together with the CDR3 length distribution analysis described previously make it clear that the molecular nature of γδ T-cell antigen recognition is fundamentally different than that of αβ T cells. While MHC and MHC-related molecules are recognized by

γδ T cells, γδ T-cell antigens need not be MHC or MHCrelated molecules. Moreover, in all cases, the antigens are recognized directly. This suggests that pathogens and damaged tissues can be recognized directly and cellular immune responses can be initiated by γδ T cells without a requirement for antigen degradation and specialized antigen-presenting cells, such as B cells, macrophages, and dendritic cells. This would allow for greater flexibility than is present in classical αβ T-cell responses. T10/T22, I-E, as well as MICA and MICB expression can be induced under certain physiologic/pathologic conditions.[301] ATP synthase F1 is normally localized in the membranes of mitochondria, but it has also been found on the surfaces of tumors, hepatocytes, and endothelial cells.[302,303] Thus, γδ T-cell activation may be regulated through the level of protein expression. In addition, the recognition of I-E by LBK5 is acutely sensitive to changes in the glycosylation of the I-E molecule. This suggests a novel way by which antigen recognition by γδ T cells can be regulated. Changes in the posttranslational modifications of surface glycoproteins often indicate that tissues have become infected, have undergone neoplastic transformations, or have experienced other types of cellular stress. For example, it has been shown that the infection of mice with *Listeria monocytogenes* impairs the addition of sialic acid to host cell glycoproteins that include MHC molecules.[304] Additionally, whereas the surface glycoprotein mucin is heavily glycosylated in normal cells, it is underglycosylated in breast, ovarian, and pancreatic carcinomas such that the peptide backbone is unmasked.[305] Thus, both the quantity and the quality of the ligand could contribute significantly to the specificity of γδ TCR recognition.

TABLE 11.6 Partial List of γδ T-Cell Reactivities[a]

Name (referred to as)	Source	Reported Reactivities	Comments	Reference
Murine				
DGT3	Lymph node cells from DBA2	Qa-1/($Glu^{50}Tyr^{50}$)		317

	mouse primed with poly ($Glu^{50}Tyr^{50}$)			
KN6	Double-negative thymocytes from C57Bl/6	$T10/T22^{b,k}$ not d		318
Tgl4.4	Lymph node of HSV-infected C3H mouse and restimulated with L cells transfected with HSV-gl	HSV-gl	Can be stimulated by gl protein alone	319,320
G8	BALB/c nu/nu immunized with B10. BR APCs	$T10/T22^{b,k}$ not d	Direct binding and cocrystal structure have been shown	153,274,323
LBK5	C57Bl/10 nu/nu immunized with B10. BR splenocytes	$I\text{-}E^{b,k,s}$ not d	Can be stimulated by I-E proteins alone; reactivity is not peptide-specific	322,323
LKD1	B10.BR immunized with B10.D2 splenocytes	$I\text{-}A^{d}$		322
69BAS-122	C57Bl/10, adult splenocyte	HSP-60 peptide, cardiolipin, β2-glycoprotein 1	Transferring TCR transfers reactivity	324,325
BNT-19.8.12	C57Bl/10, newborn thymocyte	Mycobacterium PPD, cardiolipin, β2-glycoprotein 1	Transferring TCR transfers reactivity	325,326
7-17 and other dendritic epidermal T cells	γδ T cells from murine epidermis	Keratinocytes	Transferring TCR transfers reactivity	33
Human				
Panels of T cell clones expressing Vγ 9Vδ 2	PBMC stimulated with irradiated PBMCs and PHA	Tumor cells (eg, Molt-4), MT, metabolites in the mevalonate pathway	20% of Vγ9Vδ2 clones do not react to MT; only slightly	327,328

			more than 50% of MT- or Molt-4-specific clones recognize the other specificities	
Panels of T-cell clones expressing Vγ9Vδ2 including G42 and G115	γδ T cells from PBM cultured with irradiated PBLs and lymphoblastoid cells	phosphoAg, tumor cells (eg, Daudi), AS/ApoA-I (G115TCR)	Close correlation between Daudi and mycobacterial reactivity; direct binding between AS/ApoA-I; heterogenous reactivity to ApoA-I	156,329,330
DG.SF13 (Vγ9Vδ2)	γδT cells isolated from RA synovial fluid stimulated with sonicate of *Mycobacterium tuberculosis*	Daudi cells, *Mycobacterium tuberculosis*, MEP	Transferring TCR transfers reactivity	331
Panels of T-cell clones expressing Vγ 9Vδ 2 including CP1.15 and DG.SF68	PBMC γδ T cells stimulated with *Mycobacterium tuberculosis* extract	phosphoAg, alkylamines, aminobisphosphonates	Reactivities require cell-cell contact	332,333
Clones 1,2,3,4, 5 (Vδ1)	Lymphocytes extracted from human intestinal epithelial tumors cultured with irradiated ClRMICA and ClR-MICB cells	MICA, MICB	Transferring TCR transfers reactivity; Vδ1-Jδ1 with diverse CDR3 are used; not all Vδ1-Jδ1 cells are MIC-specific	334,335
JR.2 and XV.1 (Vδ1)	PBL stimulated with autologous $CD1^+$ DC and M. tuberculosis extract	CD1c expressing cells	Transferring TCR transfers reactivity; many Vδ1 positive γδ T cells are not	335

			CD1c-reactive	
Vγ1.3Vδ2 expressing BW5147	γδ TCR chains from muscleinfiltrating T cells of a polymyositis patient and transfected into TCR-deficient BW5147	Muscle cell extract and *Escherichia coli* extract		336,337
4-29 and 5-3 (Vδ2 negative)	PBMC from CMV-infected transplant recipients stimulated with irradiated PBMC and PHA	CMV-infected fibroblasts, Hela, HT-29, Caco-2		338

[a]The names of the T cells, the way they are generated, and their reported reactivities.

CMV, cytomegalovirus; HSA, herpes simplex virus; MEP, monoethylphosphate; PBMC, peripheral blood mononuclear cell; PHA, phytohaemagglutinin; PPD, microbacterium purified protein derivative; RA, rheumatoid arthritis.

Thymic Ligand Recognition and γδ T-Cell Repertoire and Function Development

γδ T cells, like αβ T cells, develop in the thymus before entering the periphery. In the case of αβ T cells, thymic development entails endogenous ligand-driven positive and negative selection, which determine what αβ T cells can recognize and whether these T cells will develop into CD4+ helper or CD8+ cytolytic T cells. However, the role of ligandmediated selection in γδ T-cell development and function has been less clear. Recently, this issue has been reexamined and the emerging picture is fundamentally different from what we know about αβ T-cell development and differentiation. These work suggest that thymic development does little to constrain γδ T cell antigen specificities, but determines γδ T cell effector fate.[306] In particular, γδ thymocyte development and exit into the periphery is not contingent on encountering cognate antigen in the thymus and TCR dimerization may be sufficient to induce signaling for γδ T cells to develop in the thymus.[306a] Thus, once the antigen specificity repertoire is generated by V(D)J rearrangement, it is only marginally modified by thymic selection. In fact, a large fraction of peripheral γδ T cells have not encountered ligand either in the thymus or in the periphery, and this antigen-naïve population is actively maintained by rapidly turning over, and cells that have encountered self-ligands do not accumulate.[306a]

While ligand expression does little to constrain antigen specificities of the γδ T cell repertoire, it does play a role in endowing γδ T cells with different functional programs, in that TCR signaling strength experienced by γδ thymocytes seem to determines the functional specification of γδ T cells: γδ thymocytes that have not encountered cognate ligands make IL-17, γδ thymocytes that have encountered thymic ligands make IFN-γ,[306a] and those that are strongly self-reactive make IL-4. Importantly, regardless of ligand experience, some γδ T cells in normal mice are able to make cytokines immediately upon TCR engagement. IL-17 is a cytokine, which regulates the expansion and recruitment of neutrophils and monocytes to initiate the inflammatory response.[306b] In acute inflammation, a swift IL-17 response must be elicited without prior antigen exposure. Therefore, γδ T cells may be uniquely suited to produce IL-17 at the onset of the inflammatory response to initiate an acute inflammatory

response to pathogens and to host antigens revealed by injury. In addition, by acting early in the inflammatory response, γδ T cells may modulate and shape the subsequent αβ T cell and B cell responses that develop during the inflammatory process and thus may play a much larger role in the adaptive immune response than previously recognized.

Antigen Recognition Determinants of γδ T-Cell Receptors: V Genes Versus Complementarity-Determining Region 3 Regions in γδ T-Cell Receptor Ligand Recognition

In part because γδ T cells from different anatomic sites show preferential V gene expression, γδ T cells are commonly been divided into subsets based on V gene usage.[309,310] Numerous studies have also reported that γδ T-cell functions segregate with Vγ or VγVδ usage.[311,312,313,314] These observations have led to suggestions that the bias in V gene usage enables γδ T cells to respond to antigens that are specific to their resident tissues.[11,312,315] If this were the case, then γδ TCRs would function like innate immune receptors even though VDJ recombination of the TCR δ chain leads to orders of magnitude higher potential junctional (CDR3 region) diversity than is found in Ig and αβ TCRs (as discussed previously).

To resolve this issue, it is essential to understand the basis of γδ TCR antigen recognition. Based on the analysis of T22-specific γδ T cells isolated from normal mice, the majority of T22-specific γδ TCRs use Vγ1 and Vγ4 in the spleen and a sizeable number use Vγ7 in the IEL compartment.[316] Thus, at least for T22 specificity, Vγ usage reflects tissue origin and not antigen specificity. Consistent with this, it has been demonstrated that the preferential usage of Vγ7 by γδ T cells that can migrate into the IEL compartment primarily results from interleukin-15-driven control of Vγ7 accessibility during thymic VJ rearrangement.[317]

While different Vγs and Vδs were associated with T22-specific TCR sequences, there is one defining feature that is common among them.[316] This is a prominent CDR3δ motif shown in Figure 11.14 that consists of a Vδ or Dδ1-encoded Trp (W); a Dδ2-encoded sequence of Ser, Glu, Gly, Tyr, and Glu (SEGYE); and a P-nucleotide-encoded Leu (L). Gene transfer experiments established that TCRs with the W-(S)

EGYEL motif bound T22 while those lacking the motif did not[316] (see Table 11.6).

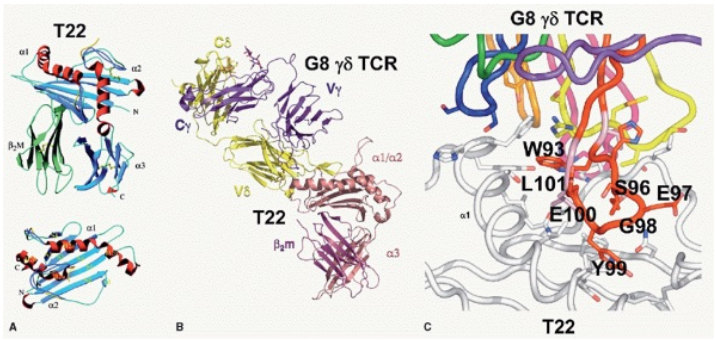

FIG. 11.14. The T22 Structure and the Interaction with the G8 γδ T-Cell Receptor (TCR). A: The structure of a T22 molecule, a nonclassical class I MHC that is missing half of an alpha helix and does not bind peptides.[270] **B,C:** The intersection with the G8 TCR, where the long TCR δ CDR3 accounts for almost all of the contact residues.[153]

Proof that the W- (S)EGYEL motif is the antigen contact site came from the crystal structure of the G8 γδ TCR bound to T22.[168] As discussed in the previous section, T22 has an MHC class I-like fold, but one side of what would normally be a peptide-binding groove is severely truncated and exposes the β-sheet "floor."[296] G8 binds T22 at a tilted angle that contrasts with the essentially parallel alignments of the long axes of the αβ TCR and the peptide/MHC when in complex (Fig. 11.15). The majority of the contact residues are contributed by the β-

sheet floor and the α1 helix of T22, and the fully extended CDR3d loop with Trp anchoring at the N-terminal end and residues Gly, Tyr, Glu, and Leu together with a Thr residue encoded in the J region anchoring the loop at its C-terminal end. This is similar to Ig, where antigen specificity in nonsomatically mutated antibodies resides predominantly in the CDR3 of the heavy chain.[318] This mode of antigen recognition

also fits well with analyses of CDR3 length distributions of all immune receptor chains that first suggested that γδ TCRs bind antigens more similarly to Igs than to αβ TCRs.[144]

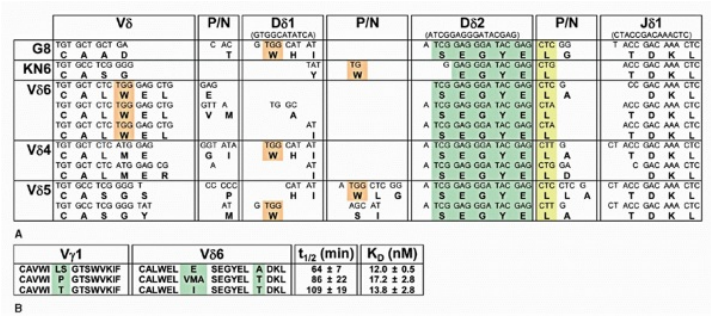

FIG. 11.15. Conserved T-Cell Receptor (TCR) δ Complementarity-Determining Region (CDR)3 Sequences Correlate with T10/T22 Specificity in γδ T Cells.

Sequence data from Shin et al.[290] showing that both established cell lines (G8 and KNG) and γδ T cells from clonal cultures that are specific for the T10/T22 share highly conserved TCRδCDR3 sequences. These are largely derived from the Dδ2 gene segment and from the structural data.Adams et al., color plate 4, [153] These conserved sequences constitute the main interaction between these γδ TCRs and their ligands.

There are two G8/T22 complexes in the asymmetric unit.[168] While the contact residues are similar at the interface of the CDR3/T22 β sheet, the two complexes differ by a relative rotation between the G8 TCRs. This shift alters the contacts formed between the CDR1, CDR2, HV4, and CDR3 loops and T22 in each TCR, suggesting that the CDR3 loop acts as a pivot point for G8 binding, with some flexibility in the interaction between the other CDR loops and T22. The hinge-like flexibility around this pivot point stands in stark contrast to interactions seen in antibody/antigen and αβ TCR-peptide/MHC complexes. In those cases, the relatively straight-on docking mode results in multipoint (ie, multi-CDR) attachment of the receptor to the ligand, essentially rigidifying the intermolecular orientations between the two binding partners. In most cases, the αβ TCR CDR1 and CDR2 loops provide a perimeter of contacts with the MHC helices surrounding the CDR3 loops, and so far, no variation has been seen in the docking angle of TCR to MHC in cases where multiple complexes exist in the asymmetric unit. Thus, the CDR3 motif of G8 and other γδ TCRs may be thought of as a somewhat autonomous binding entity that is presented by a variety of germline-encoded variable domain scaffolds without strong preference for particular CDR1 and 2 sequences. It would be interesting to see other examples of γδ TCR/ligand binding. CDR3 regions were found to be important for γδ T-cell recognition of MICA/MICB. In this case, the reactivity correlates only with a junction between Vδ1 and Jδ1. Aside from the W-(S)EGYEL motif, the T22-specific CDR3γδ sequences were diverse and were encoded by various Vδs, N- and P-nucleotides, and Dδ1s of different lengths and reading frames. Importantly, it was shown that sequence variations in the CDR3 regions around this motif modulated the affinity and the kinetics of T22 binding.[313] In fact, the T22-specific repertoire in normal mice covers a range of affinities, as is evident by the large range of T22 tetramer staining intensities.[300,316] This allows for the selection of T cells with the “most optimal” antigen-binding capabilities during an immune response, which is a hallmark of the adaptive immune response.

Nonetheless, such a repertoire that is created mainly by V, D, and J region-derived germline-encoded nucleotides, despite requiring VDJ recombination, would be “innate” in character, because the antigen specificities would be predetermined and the repertoire would be much

less variable among individuals of the same species.

Analysis of the formation of T10/T22-reactive repertoire indicates that biases linked to the recombination machinery influence the generation of a γδT-cell repertoire toward certain specificities. A repertoire that is generated by recombination but conferred by a limited set of germline or germline-like residues at the CDR3 region will be created at a much higher frequency than one whose specificity is conferred primarily by N-nucleotide additions, as is the case with that of αβ TCRs. Indeed, the 1 in 100 frequency of T22-specific γδ T cells in normal mice is much higher than the estimated 1 in 10^5 to 10^6 frequency of naïve peptide/MHC-specific αβ T cells.[315,316,317] This could provide a solution to the apparent paucity of γδ T cells and could allow for a significant response without an initial need for clonal expansion as is required for most αβ T-cell responses. It was shown that rearrangements at the TCR δ locus are biased towards full-length Dδ2 sequences rather than extensive D region nucleotide deletion, as is the case for the TCR-β locus.[313] Thus, different reading frames of Dδ2 may contribute to the recognition of other ligands by γδ TCRs in a manner similar to that of T22-specific γδ TCRs and would lead to a repertoire that is biased toward a relatively small number of ligands, more on the order of hundreds to thousands versus millions as estimated for αβ T cells, but with highly variable antigen-binding affinities. A repertoire of this type would allow more flexible and efficient responses to changes in ligand expression. These are testable hypotheses, especially once more γδ TCR ligands have been identified.

GENERAL FEATURES OF T-CELL RECEPTOR AND IMMUNOGLOBULIN DIVERSITY

A Dominant Role for Diverse Complementarity-Determining Region 3 Regions in Antigen Specificity

One interesting observation that emerges from a detailed analysis of the gene rearrangements that create both TCR and Igs is how the diversity of the CDR3 loop region in one or both of the chains in a given TCR is so much greater than that available to the other CDRs. A schematic of this skewing of diversity is shown in Figure 11.16 for human Igs and for αβ and γδ TCR heterodimers. In the case of αβ TCRs, this concentration of diversity occurs in both Vα and Vβ CDR3 loops, and numerous TCR-peptide/MHC structures[160] have confirmed that these loops sit largely over the center of the antigenic peptide (see previous section). While this concentration of diversity in αβ TCRs in the regions of principal contact with the many possible antigenic peptides seems reasonable, it is much harder to explain for Ig or γδ TCRs. Clearly, there must be some chemical or structural "logic" behind this phenomenon. A clue as to what this might be comes from the elegant work of Shin et al.[313] in the demonstration that for at least one γδ T-cell specificity, the antigen recognition determinants are encoded by germline V or D region residues, with remaining sequence diversity modulating the affinity. While this may be a feature of some or many γδ TCRs, it could not explain the much broader repertoire of Igs. Instead, one possible explanation comes from the studies of Wells and colleagues,[288] who systematically mutated all of the amino acids (to alanine) at the interface of human growth hormone and its receptor as determined by x-ray crystallography. Interestingly, only a quarter of the 30 or so mutations on either side had any effect on the binding affinity, even in cases where the x-ray structural analysis showed that the amino acid side chains of most of the residues were "buried" in the other. This study illustrates an important caveat to the interpretation of protein crystal structures, which is that while they are invaluable for identifying which amino acids could be important in a given interaction, they do not indicate which ones are the most important. This is presumably because the "fit" at that many positions is not "exact" enough to add significant binding energy to the interaction.

In this context, we have proposed a new model[319,320] in which the principle antigen specificity of an Ig or TCR is derived from its most diverse CDR3 loops. In the case of antibodies, we imagine that most of the specific contacts (and hence the free energy) with antigen are made by the V_H CDR3 and that the other CDRs provide "opportunistic" contacts that make generally only minor contributions to the energy of binding and specificity. Once

antigen has been encountered and clonal selection activates a particular cell, somatic mutation would then "improve" the binding of the CDR1s and 2s to convert the typically low-affinity antibodies to the higheraffinity models as observed by Berek and Milstein,[135] and also by Patten et al.[136] As a test of this model, Xu et al.[315] analyzed mice that have a severely limited Ig V region repertoire, consisting of one V_H and effectively two V_Ls (Vλ1 and Vλ2). These mice are able to respond to a wide variety of protein and haptenic antigens, even with this very limited complement of V regions. In several cases, hybridomas specific for very different antigens (ovalbumin versus 2,4-dinitrophenol [DNP], for example) differ only in the V_H CDR3. A limited V region repertoire also seemed no barrier to deriving high-affinity antibodies with somatic mutation, as repeated immunizations produced IgG monoclonals with very high affinities (10^9 to 10^{-10} M). The major immune deficit in these mice was in their inability to produce antibodies to carbohydrates, which may require a special type of binding site or specific V region. Thus, while these experiments only involved one V^H, the results are highly suggestive about the inherent malleability of V^HV^L in general, at least with respect to protein and haptenic epitopes. With respect to αβ TCRs, we expect that most of the energy of the interaction with a typical ligand will reside in the CDR3-peptide contacts and here again the CDR1 and 2 regions will make less energetically important contacts. For γδ TCRs, it is not yet clear whether the T10/T22 specificity[313] is an isolated case or the general rule. From the hypothesis discussed here, if there are γδ TCRs that use the very large inherent diversity in the Vδ CDR3 directly for antigen recognition, it may be that the lack of somatic mutation forces it to provide more diversity in the initial repertoire (versus Igs).

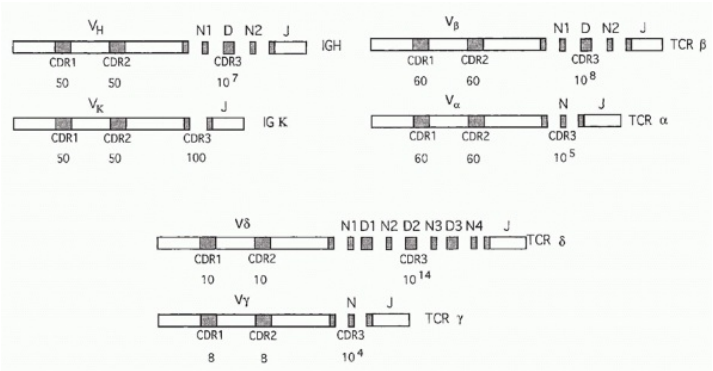

FIG. 11.16. Diversity "Map" of Immunoglobulins and T-Cell Receptors, Calculated Potential for Sequence Diversity in Human Antigen Receptor Molecules.[Adams et al.,153] The N region addition is assumed to contribute zero to six nucleotides to the junction of each gene segment, except for immunoglobulin K chains, in which this form of diversity is seldom used.

CONCLUSION

Because TCR genes were first identified in the early 1980s, information about their genetics, biochemistry, structure, and function has accumulated to become almost a field unto itself. Despite this very real progress, many issues still remain unsolved such as: What do γδ T cells normally "see," and what function do they serve? What do superantigens actually do during the course of a normal response and how is this of benefit to the pathogen/parasite? What is the structural/chemical basis of TCR specificity? What sort of rearrangements or conformational charges occur in the TCR/CD3 molecular ensemble upon ligand engagement? These and other questions will require many more years of effort.

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REFERENCES

1. Zinkernagel RM, Doherty PC. Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis. *Nature*. 1974;251(5475):547-548.

2. Zinkernagel RM, Doherty PC. H-2 compatability requirement for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. Different cytotoxic T-cell specificities are associated with structures coded for in H-2K or H-2D. *J Exp Med*. 1975;141(6): 1427-1436.

3. Davies DR, Metzger H. Structural basis of antibody function. *Annu Rev Immunol*. 1983;1:87-117.

4. Sundberg EJ, Mariuzza RA. Molecular recognition in antibody-antigen complexes. *Adv Protein Chem*. 2002;61:119-160.

5. Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature*. 1987;329(6139):506-512.

6. Davis MM, Reich Z, Lyons D, Hampl J, Arden B, Chien Y. Ligand recognition by ab T cell receptors. *Annu Rev Immunol*. 1998;16:523-544.

7. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. 2007;25: 297-336.

8. Sakaguchi S, Ono M, Setoguchi R, et al. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev*. 2006;212:8-27.

9. Cheroutre H. IELs: enforcing law and order in the court of the intestinal epithelium. *Immunol Rev*. 2005;206:114-131.

10. Haas W, Pereira P, Tonegawa S. Gamma/delta cells. *Annu Rev Immunol*. 1993;11:637-685.

11. Hayday AC. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu Rev Immunol*. 2000;18: 975-1026.

12. Havran WL, Jameson JM, Witherden DA. Epithelial cells and their neighbors. III. Interactions between intraepithelial lymphocytes and neighboring epithelial cells. *Am J Physiol Gastrointest Liver Physiol*. 2005;289(4):G627-G630.

13. Chien YH, Jores R, Crowley MP. Recognition by gamma/delta T cells. *Annu Rev Immunol*. 1996;14:511-532.

14. Infante AJ, Infante PD, Gillis S, Fathman CG. Definition of T cell idiotypes using anti-idiotypic antisera produced by immunization with T cell clones. *J Exp Med*. 1982;155(4):1100-1107.

15. Allison JP, McIntyre BW, Bloch D. Tumor-specific antigen of murine T-lymphoma defined with monoclonal antibody. *J Immunol*. 1982; 129(5):2293-2300.

16. Meuer SC, Fitzgerald KA, Hussey RE, Hodgdon JC, Schlossman SF, Reinherz EL. Clonotypic structures involved in antigen-specific human T cell function. Relationship to the T3 molecular complex. *J Exp Med*. 1983;157(2):705-719.

17. Haskins K, Kubo R, White J, Pigeon M, Kappler J, Marrack P. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. *J Exp Med*. 1983;157(4): 1149-1169.

18. Kaye J, Porcelli S, Tite J, Jones B, Janeway CA Jr. Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen-presenting cells in the activation of T cells. *J Exp Med*. 1983;158(3):836-856.

19. Samelson LE, Germain RN, Schwartz RH. Monoclonal antibodies against the antigen receptor on a cloned T-cell hybrid. *Proc Natl Acad Sci U S A* . 1983;80(22):6972-6976.

20. McIntyre BW, Allison JP. Biosynthesis and processing of murine T-cell antigen receptor. *Cell*. 1984;38(3):659-665.

21. Kappler J, Kubo R, Haskins K, et al. The major histocompatibility complex-restricted antigen receptor on T cells in mouse and man: identification of constant and variable peptides. *Cell*. 1983;35(1):295-302.

22. Davis M, Cohen EA, Nielsen AL, DeFranco AL, Paul WE. *The Isolation of B and T Cell-Specific Genes*. New York: Academic Press; 1982.

23. Hedrick SM, Cohen DI, Nielsen EA, Davis MM. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature*. 1984;308(5955):149-153.

24. Hedrick SM, Nielsen EA, Kavaler J, Cohen DI, Davis MM. Sequence relationships between putative T-cell receptor polypeptides and immunoglobulins. *Nature*. 1984;308(5955):153-158.

25. Yanagi Y, Yoshikai Y, Leggett K, Clark SP, Aleksander I, Mak TW. A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. *Nature*. 1984;308(5955): 145-149.

26. Acuto O, Fabbi M, Smart J, et al. Purification and NH2-terminal amino acid sequencing of the beta subunit of a human T-cell antigen receptor. *Proc Natl Acad Sci U S A*. 1984;81(12):3851-3855.

27. Chien Y, Becker DM, Lindsten T, Okamura M, Cohen DI, Davis MM. A third type of murine T-cell receptor gene. *Nature*. 1984;312(5989):31-35.

28. Saito H, Kranz DM, Takagaki Y, Hayday AC, Eisen HN, Tonegawa S. A third rearranged and expressed gene in a clone of cytotoxic T lymphocytes. *Nature*. 1984;312(5989):36-40.

29. Saito H, Kranz DM, Takagaki Y, Hayday AC, Eisen HN, Tonegawa S. Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. *Nature*. 1984;309(5971):757-762.

30. Brenner MB, McLean J, Dialynas DP, et al. Identification of a putative second T-cell receptor. *Nature*. 1986;322(6075):145-149.

31. Chien YH, Iwashima M, Kaplan KB, Elliott JF, Davis MM. A new T-cell receptor gene located within the alpha locus and expressed early in T-cell differentiation. *Nature*. 1987;327(6124):677-682.

32. Dembic Z, Haas W, Weiss S, et al. Transfer of specificity by murine alpha and beta T-cell receptor genes. *Nature*. 1986;320(6059):232-238.

33. Saito T, Weiss A, Miller J, Norcross MA, Germain RN. Specific antigen-Ia activation of transfected human T cells expressing murine Ti alpha beta-human T3 receptor complexes. *Nature*. 1987;325(7000):125-130.

34. Havran WL, Chien YH, Allison JP. Recognition of self antigens by skinderived T cells with invariant gamma delta antigen receptors. *Science*. 1991;252(5011):1430-1432.

35. Lefranc MP, Lefranc G. *The T Cell Receptor: Facts Book*. London: Academic Press, Ltd. Harcourt Science and Technology Company; 2001.

36. Brenner MB, McLean J, Scheft H, et al. Two forms of the T-cell receptor gamma protein found on peripheral blood cytotoxic T lymphocytes. *Nature*. 1987;325(6106):689-694.

37. Groettrup M, Ungewiss K, Azogui O, et al. A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor beta chain and a 33 kd glycoprotein. *Cell*. 1993;75(2):283-294.

38. Saint-Ruf C, Ungewiss K, Groettrup M, Bruno L, Fehling HJ, von Boehmer H. Analysis and expression of a cloned pre-T cell receptor gene. *Science*. 1994;266(5188):1208-1212.

39. Pang SS, Berry R, Chen Z, et al. The structural basis for autonomous dimerization of the pre-T-cell antigen receptor. *Nature*. 2010;467(7317): 844-848.

40. Dave VP, Cao Z, Browne C, et al. CD3 delta deficiency arrests development of the alpha beta but not the gamma delta T cell lineage. *EMBO J*. 1997;16(6):1360-1370.

41. Call ME, Wucherpfennig KW. The T cell receptor: critical role of the membrane environment in receptor assembly and function. *Annu Rev Immunol*. 2005;23:101-125.

42. Terhorst C, Spits H, Stall F, Exley M. *T Lymphocyte Signal Transduction*. 2nd ed. Oxford, UK: IRL Press; 1996.

43. Klausner RD, Lippincott-Schwartz J, Bonifacino JS. The T cell antigen receptor: insights into organelle biology. *Annu Rev Cell Biol*. 1990; 6:403-431.

44. Allison JP, Lanier LL. Identification of antigen receptor-associated structures on murine T cells. *Nature*. 1985;314(6006):107-109.

45. Lew AM, Maloy WL, Koning F, Valas R, Coligan JE. Expression of the human T cell receptor as defined by anti-isotypic antibodies. *J Immunol*. 1987;138(3):807-814.

46. Orloff DG, Frank SJ, Robey FA, Weissman AM, Klausner RD. Biochemical characterization of the eta chain of the T-cell receptor. A unique subunit related to zeta. *J Biol Chem*. 1989;264(25):14812-14817.

47. Jin YJ, Clayton LK, Howard FD, et al. Molecular cloning of the CD3 eta subunit identifies a CD3 zeta-related product in thymus-derived cells. *Proc Natl Acad Sci U S A*. 1990;87(9):3319-3323.

48. Clayton LK, D'Adamio L, Howard FD, et al. CD3 eta and CD3 zeta are alternatively spliced products of a common genetic locus and are transcriptionally and/or post-transcriptionally regulated during T-cell development. *Proc Natl Acad Sci U S A*. 1991;88(12):5202-5206.

49. Rodewald HR, Arulanandam AR, Koyasu S, Reinherz EL. The high affinity Fc epsilon receptor gamma subunit (Fc epsilon RI gamma) facilitates T cell receptor expression and

antigen/major histocompatibility complex-driven signaling in the absence of CD3 zeta and CD3 eta. *J Biol Chem*. 1991;266(24):15974-15978.

50. Orloff DG, Ra CS, Frank SJ, Klausner RD, Kinet JP. Family of disulphidelinked dimers containing the zeta and eta chains of the T-cell receptor and the gamma chain of Fc receptors. *Nature*. 1990;347(6289):189-191.

51. Clevers H, Alarcon B, Wileman T, Terhorst C. The T cell receptor/CD3 complex: a dynamic protein ensemble. *Annu Rev Immunol*. 1988;6: 629-662.

52. Sun ZJ, Kim KS, Wagner G, Reinherz ELp. Mechanisms contributing to T cell receptor signaling and assembly revealed by the solution structure of an ectodomain fragment of the CD3 epsilon gamma heterodimer. *Cell*. 2001;105(7):913-923.

53. Sun ZY, Kim ST, Kim IC, Fahmy A, Reinherz EL, Wagner G. Solution structure of the CD3epsilondelta ectodomain and comparison with CD3epsilongamma as a basis for modeling T cell receptor topology and signaling. *Proc Natl Acad Sci U S A*. 2004;101(48):16867-16872.

54. Kjer-Nielsen L, Dunstone MA, Kostenko L, et al. Crystal structure of the human T cell receptor CD3 epsilon gamma heterodimer complexed to the therapeutic mAb OKT3. *Proc Natl Acad Sci U S A*. 2004; 101(20):7675-7680.

55. Arnett KL, Harrison SC, Wiley DC. Crystal structure of a human CD3-epsilon/delta dimer in complex with a UCHT1 single-chain antibody fragment. *Proc Natl Acad Sci U S A*. 2004;101(46):16268-16273.

56. Xu C, Call ME, Wucherpfennig KW. A membrane-proximal tetracysteine motif contributes to assembly of CD3{delta} {epsilon} and CD3{gamma} {epsilon} dimers with the T cell receptor. *J Biol Chem*. 2006;281(48):36977-36984.

57. Call ME, Pyrdol J, Wiedmann M, Wucherpfennig KW. The organizing principle in the formation of the T cell receptor-CD3 complex. *Cell*. 2002;111(7):967-979.

58. Cosson P, Lankford SP, Bonifacino JS, Klausner RD. Membrane protein association by potential intramembrane charge pairs. *Nature*. 1991;351(6325):414-416.

59. Hall C, Berkhout B, Alarcon B, Sancho J, Wileman T, Terhorst C. Requirements for cell surface expression of the human TCR/CD3 complex in non-T cells. *Int Immunol*.1991;3(4):359-368.

60. Alarcon B, Ley SC, Sanchez-Madrid F, et al. The CD3-gamma and CD3-delta subunits of the T cell antigen receptor can be expressed within distinct functional TCR/CD3 complexes. *EMBO J*.1991;10(4): 903-912.

61. Klausner RD, Samelson LE. T cell antigen receptor activation pathways: the tyrosine kinase connection. *Cell*. 1991;64(5):875-878.

62. Reth M. Antigen receptor tail clue. *Nature*. 1989;338(6214):383-384.

63. Ashwell JD, Klusner RD. Genetic and mutational analysis of the T-cell antigen receptor. *Annu Rev Immunol*. 1990;8(5):139-167.

64. Cantrell DA, Davies AA, Crumpton MJ. Activators of protein kinase C down-regulate and phosphorylate the T3/T-cell antigen receptor complex of human T lymphocytes. *Proc Natl Acad Sci U S A*. 1985;82(23): 8158-8162.

65. Xu C, Gagnon E, Call ME, et al. Regulation of T cell receptor activation by dynamic

membrane binding of the CD3epsilon cytoplasmic tyrosine-based motif. *Cell*. 2008;135(4):702-713.

66. Zhang H, Cordoba S-P, Dushek O, van der Merwe PA. Basic residues in the T cell receptor zeta cytoplasmic domain mediate membrane association and signaling. *Proc Natl Acad Sci U S A*. 2011;48:19323-19328.

67. Kuhns MS, Davis MM, Garcia KC. Deconstructing the form and function of the TCR/CD3 complex. *Immunity*. 2006;24(2):133-139.

68. Minami Y, Weissman AM, Samelson LE, Klausner RD. Building a multichain receptor: synthesis, degradation, and assembly of the T-cell antigen receptor. *Proc Natl Acad Sci U S A*. 1987;84(9):2688-2692.

69. Bonifacino JS, Suzuki CK, Klausner RD. A peptide sequence confers retention and rapid degradation in the endoplasmic reticulum. *Science*. 1990;247(4938):79-82.

70. Delgado P, Alarcon B. An orderly inactivation of intracellular retention signals controls surface expression of the T cell antigen receptor. *J Exp Med*. 2005;201(4):555-566.

71. Koyasu S, D'Adamio L, Arulanandam AR, Abraham S, Clayton LK, Reinherz EL. T cell receptor complexes containing Fc epsilon RI gamma homodimers in lieu of CD3 zeta and CD3 eta components: a novel isoform expressed on large granular lymphocytes. *J Exp Med*. 1992;175(1):203-209.

72. Exley M, Wileman T, Mueller B, Terhorst C. Evidence for multivalent structure of T-cell antigen receptor complex. *Mol Immunol*. 1995; 32(11):829-839.

73. Blumberg RS, Ley S, Sancho J, et al. Structure of the T-cell antigen receptor: evidence for two CD3 epsilon subunits in the T-cell receptor-CD3 complex. *Proc Natl Acad Sci U S A*. 1990;87(18):7220-7224.

74. Fernández-Miguel G, Alarcón B, Iglesias A, et al. Multivalent structure of an alphabetaT cell receptor. *Proc Natl Acad Sci U S A* . 1999;96(4): 1547-1552.

75. Schamel WW, Risueno RM, Minguet S, Ortiz AR, Alarcon B. A conformation-and avidity-based proofreading mechanism for the TCR-CD3 complex. *Trends Immunol*. 2006;27(4):176-182.

76. Hayes SM, Laky K, El-Khoury D, Kappes DJ, Fowlkes BJ, Love PE. Activation-induced modification in the CD3 complex of the gammadelta T cell receptor. *J Exp Med*. 2002;196(10):1355-1361.

77. Hayes SM, Love PE. Stoichiometry of the murine {gamma} {delta} T cell receptor. *J Exp Med*. 2006;203(1):47-52.

78. Chien YH, Gascoigne NR, Kavaler J, Lee NE, Davis MM. Somatic recombination in a murine T-cell receptor gene. *Nature*. 1984;309(5966): 322-326.

79. Bosma M. B and T cell leakiness in the scid mouse mutant. *Immunodef Rev*. 1992;3:261.

80. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*. 1992;68(5):869-877.

81. Shinkai Y, Rathbun G, Lam KP, et al. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell*. 1992;68(5):855-867.

82. Krangel MS. Gene segment selection in V(D)J recombination: accessibility and beyond. *Nat Immunol*. 2003;4(7):624-630.

83. Fujimoto S, Yamagishi H. Isolation of an excision product of T-cell receptor alpha-chain gene rearrangements. *Nature*. 1987;327(6119): 242-243.

84. Okazaki K, Davis DD, Sakano H. T cell receptor beta gene sequences in the circular DNA of thymocyte nuclei: direct evidence for intramolecular DNA deletion in V-D-J joining. *Cell*. 1987;49(4):477-485.

85. Heilig JS, Tonegawa S. Diversity of murine gamma genes and expression in fetal and adult T lymphocytes. *Nature*. 1986;322(6082):836-840.

86. Elliott JF, Rock EP, Patten PA, Davis MM, Chien YH. The adult T-cell receptor delta-chain is diverse and distinct from that of fetal thymocytes. *Nature*. 1988;331(6157):627-631.

87. Hata S, Satyanarayana K, Devlin P, et al. Extensive junctional diversity of rearranged human T cell receptor delta genes. *Science*. 1988; 240(4858):1541-1544.

88. Xiong N, Raulet DH. Development and selction of gamma/delta T cell receptor. *Immunol Rev*. 2007;215:15-31.

89. de Villartay JP, Lewis D, Hockett R, Waldmann TA, Korsmeyer SJ, Cohen DI. Deletional rearrangement in the human T-cell receptor alpha-chain locus. *Proc Natl Acad Sci U S A*. 1987;84(23):8608-8612.

90. Villey I, Caillol D, Selz F, Ferrier P, de Villartay JP. Defect in rearrangement of the most 5' TCR-J alpha following targeted deletion of T early alpha (TEA): implications for TCR alpha locus accessibility. *Immunity*. 1996;5(4):331-342.

91. Rowen L, Koop BF, Hood L. The complete 685-kilobase DNA sequence of the human beta T cell receptor locus. *Science*. 1996;272(5269): 1755-1762.

92. Noonan DJ, Kofler R, Singer PA, Cardenas G, Dixon FJ, Theofilopoulos AN. Delineation of a defect in T cell receptor beta genes of NZW mice predisposed to autoimmunity. *J Exp Med*. 1986;163(3):644-6453.

93. Chou HS, Nelson CA, Godambe SA, Chaplin DD, Loh DY. Germline organization of the murine T cell receptor beta-chain genes. *Science*. 1987;238(4826):545-548.

94. Woodland DL, Kotzin BL, Palmer E. Functional consequences of a T cell receptor D beta 2 and J beta 2 gene segment deletion. *J Immunol*. 1990;144(1):379-385.

95. Vernooij BT, Lenstra JA, Wang K, Hood L. Organization of the murine T-cell receptor gamma locus. *Genomics*. 1993;17(3):566-574.

96. Garman RD, Doherty PJ, Raulet DH. Diversity, rearrangement, and expression of murine T cell gamma genes. *Cell*. 1986;45(5):733-742.

97. LeFranc MP, Forster A, Baer R, Stinson MA, Rabbitts TH. Diversity and rearrangement of the human T cell rearranging gamma genes: nine germ-line variable genes belonging to two subgroups. *Cell*. 1986;45(2):237-246.

98. Concannon P, Robinson MA. Human T-cell receptor gene nomenclature. *Ann N Y Acad Sci*. 1995;756:124-129.

99. Lefranc MP, Forster A, Rabbitts TH. Genetic polymorphism and exon changes of the constant regions of the human T-cell rearranging gene gamma. *Proc Natl Acad Sci U S A*. 1986;83(24):9596-9600.

100. Raulet DH. The structure, function, and molecular genetics of the gamma/delta T cell receptor. *Annu Rev Immunol*. 1989;7(1):175-207.

101. Havran WL, Allison JP. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. *Nature*. 1988;335(6189):443-445.

101a. G. Kershard, S.M. Hedrick, unpublished results

102. Korman A, Marusic-Galesic S, Spencer D, Kruisbeek A, Raulet DH. Predominant variable region gene usage by γ/δ T cell receptor-bearing cells in the adult thymus. *J Exp Med*. 1988;168:1021-1040.

103. Heilig JS, Tonegawa S. Diversity of murine gamma genes and expression in fetal and adult T lymphocytes. *Nature*. 1986;322: 836-840.

104. Asarnow DM, Goodman T, LeFrancois L, Allison JP. Distinct antigen receptor repertoires of two classes of murine epithelium-associated T cells. *Nature*. 1989;341:60-62.

105. Sperling AI, Cron RQ, Decker DC, Stern DA, Bluestone JA. Peripheral T cell receptor gamma delta variable gene repertoire maps to the T cell receptor loci and is influenced by positive selection. *J Immunol*. 1992;149(10):3200-3207.

106. Pereira P, Gerber D, Huang SY, Tonegawa S. Ontogenic development and tissue distribution of V gamma 1-expressing gamma/delta T lymphocytes in normal mice. *J Exp Med*. 1995 182(6):1921-1930.

107. Lefrancois L, Puddington L. Extrathymic intestinal T-cell development: virtual reality? *Immunol Today*. 1995;16:16-21.

108. Raulet D, Spencer DM, Hsiang YH, et al. Control of γδ T cell development. *Immunol Rev*. 1991;120:185-204.

109. Goldman J, Spencer D, Raulet D. Ordered rearrangement of variable region genes of the T cell receptor gamma locus correlates with transcription of the unrearranged genes. *J Exp Med*. 1993;177:729-739.

110. Yancopoulos GD, Alt FW. Regulation of the assembly and expression of variable-region genes. *Annu Rev Immunol*. 1986;4:339-368.

111. Osipovich O, Milley R, Meade A, et al. Targeted inhibition of V(D) J recombination by a histone methyltransferase. *Nat Immunol*. 2004; 5(3):309-316.

112. Jackson AM, Krangel MS. Allele-specific regulation of TCR beta variable gene segment chromatin structure. *J Immunol*. 2005;175(8): 5186-5191.

113. Hawwari A, Krangel MS. Regulation of TCR delta and alpha repertoires by local and long-distance control of variable gene segment chromatin structure. *J Exp Med*. 2005;202(4):467-472.

114. Hawwari A, Bock C, Krangel MS. Regulation of T cell receptor alpha gene assembly by a complex hierarchy of germline Jalpha promoters. *Nat Immunol*. 2005;6(5):481-489.

115. Abarrategui I, Krangel MS. Regulation of T cell receptor-alpha gene recombination

by transcription. *Nat Immunol*. 2006;7(10):1109-1115.

116. McDougall S, Peterson CL, Calame K. A transcriptional enhancer 3' of C beta 2 in the T cell receptor beta locus. *Science*. 1988;241(4862): 205-208.

117. Krimpenfort P, de Jong R, Uematsu Y, et al. Transcription of T cell receptor beta-chain genes is controlled by a downstream regulatory element. *EMBO J*. 1988;7(3):745-750.

118. Sleckman BP, Bardon CG, Ferrini R, Davidson L, Alt FW. Function of the TCR alpha enhancer in alphabeta and gammadelta T cells. *Immunity*. 1997;7(4):505-515.

119. Lauzurica P, Krangel MS. Temporal and lineage-specific control of T cell receptor alpha/delta gene rearrangement by T cell receptor alpha and delta enhancers. *J Exp Med*. 1994;179(6):1913-1921.

120. Lauzurica P, Krangel MS. Enhancer-dependent and -independent steps in the rearrangement of a human T cell receptor delta transgene. *J Exp Med*. 1994;179(1):43-55.

121. Waldmann TA, Davis MM, Bongiovanni KF, Korsmeyer SJ. Rearrangements of genes for the antigen receptor on T cells as markers of lineage and clonality in human lymphoid neoplasms. *N Engl J Med*. 1985; 313(13):776-783.

122. Winoto A, Baltimore D. Alpha beta lineage-specific expression of the alpha T cell receptor gene by nearby silencers. *Cell*. 1989;59(4): 649-655.

123. Diaz P, Cado D, Winoto A. A locus control region in the T cell receptor alpha/delta locus. *Immunity*. 1994;1(3):207-217.

124. Ishida I, Verbeek S, Bonneville M, Itohara S, Berns A, Tonegawa S. T-cell receptor gamma delta and gamma transgenic mice suggest a role of a gamma gene silencer in the generation of alpha beta T cells. *Proc Natl Acad Sci U S A*. 1990;87(8):3067-3071.

125. Spencer DM, Hsiang YH, Goldman JP, Raulet DH. Identification of a T-cell-specific transcriptional enhancer located 3' of C gamma 1 in the murine T-cell receptor gamma locus. *Proc Natl Acad Sci U S A*. 1991; 88(3):800-804.

126. Baker JE, Kang J, Xiong N, Chen T, Cado D, Raulet DH. A novel element upstream of the Vgamma2 gene in the murine T cell receptor gamma locus cooperates with the 3' enhancer to act as a locus control region. *J Exp Med*. 1999;190(5):669-679.

127. Xiong N, Kang C, Raulet DH. Redundant and unique roles of two enhancer elements in the TCRgamma locus in gene regulation and gammadelta T cell development. *Immunity*. 2002;16(3):453-463.

128. Uematsu Y, Ryser S, Dembic Z, et al. In transgenic mice the introduced functional T cell receptor beta gene prevents expression of endogenous beta genes. *Cell*. 1988;52(6):831-841.

129. Padovan E, Casorati G, Dellabona P, Meyer S, Brockhaus M, Lanzavecchia A. Expression of two T cell receptor alpha chains: dual receptor T cells. *Science*. 1993;262(5132):422-424.

130. Malissen M, Trucy J, Jouvin-Marche E, Cazenave PA, Scollay R, Malissen B. Regulation of TCR alpha and beta gene allelic exclusion during T-cell development. *Immunol Today*. 1992;13(8):315-322.

131. Wagner DH Jr. Re-shaping the T cell repertoire: TCR editing and TCR revision for good and for bad. *Clin Immunol*. 2007;123(1):1-6.

132. Padovan E, Casorati G, Dellabona P, Meyer S, Brockhaus M, Lanzavecchia A. Expression of two T cell receptor alpha chains: dual receptor T cells. *Science*. 1993;262(5132):422-424.

133. Aifantis I, Buer J, von Boehmer H, Azogui O. Essential role of the pre-T cell receptor in allelic exclusion of the T cell receptor beta locus. *Immunity*. 1997;7(5):601-607. Erratum. *Immunity*. 1997;7(6): following 895.

134. O'Shea CC, Thornell AP, Rosewell IR, Hayes B, Owen MJ. Exit of the pre-TCR from the ER/cis-Golgi is necessary for signaling differentiation, proliferation, and allelic exclusion in immature thymocytes. *Immunity*. 1997;7(5):591-599.

135. Berek C, Milstein C. The dynamic nature of the antibody repertoire. *Immunol Rev*. 1988;105:5-26.

136. Patten PA, Gray NS, Yang PL, et al. The immunological evolution of catalysis. *Science*. 1996;271(5252):1086-1091.

137. van der Merwe PA, Barclay AN. Transient intercellular adhesion: the importance of weak protein-protein interactions. *Trends Biocheml Sci*. 1994;19(9):354-358.

138. Davis MM, Boniface JJ, Reich Z, et al. Ligand recognition by alpha beta T cell receptors. *Annu Rev Immunol*. 1998;16:523-544.

139. Valitutti S, Muller S, Cella M, Padovan E, Lanzavecchia A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature*. 1995;375(6527):148-151.

140. Davis MM, Huse M, Lillemeier BF. T cells as a self-referential sensory organ. *Annu Rev Immunol*. 2007;25:681-695.

141. Wu TT, Kabat EA. An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. *J Exp Med*. 1970;132(2):211-250.

142. Robins HS, Campregher PV, Srivastava SK, et al. Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. *Blood*. 2009;114(19):4099-4107.

143. Chothia C, Lesk AM, Tramontano A, et al. Conformations of immunoglobulin hypervariable regions. *Nature*. 1989;342(6252):877-883.

144. Rock EP, Sibbald PR, Davis MM, Chien YH. CDR3 length in antigenspecific immune receptors. *J Exp Med*. 1994;179(1):323-328.

145. Konigshofer Y, Chien YH. Gammadelta T cells - innate immune lymphocytes? *Curr Opin Immunol*. 2006;18(5):527-533.

146. Korsmeyer SJ. Chromosomal translocations in lymphoid malignancies reveal novel proto-oncogenes. *Annu Rev Immunol*. 1992;10(8): 785-807.

147. Finger LR, Harvey RC, Moore RC, Showe LC, Croce CM. A common mechanism of chromosomal translocation in T- and B-cell neoplasia. *Science*. 1986;234(4779):982-985.

148. Baer R, Chen KC, Smith SD, Rabbitts TH. Fusion of an immunoglobulin variable gene and a T cell receptor constant gene in the chromosome 14 inversion associated

with T cell tumors. *Cell*. 1985;43(3, pt 2): 705-713.

149. Denny CT, Yoshikai Y, Mak TW, Smith SD, Hollis GF, Kirsch IR. A chromosome 14 inversion in a T-cell lymphoma is caused by site-specific recombination between immunoglobulin and T-cell receptor loci. *Nature*. 1986;320(6062):549-551.

150. Boehm T, Greenberg JM, Buluwela L, Lavenir I, Forster A, Rabbitts TH. An unusual structure of a putative T cell oncogene which allows production of similar proteins from distinct mRNAs. *EMBO J*. 1990; 9(3):857-868.

151. McGuire EA, Hockett RD, Pollock KM, Bartholdi MF, O'Brien SJ, Korsmeyer SJ. The t(11;14)(p15;q11) in a T-cell acute lymphoblastic leukemia cell line activates multiple transcripts, including Ttg-1, a gene encoding a potential zinc finger protein. *Mol Cell Biol*. 1989;9(5): 2124-2132.

152. Royer-Pokora B, Loos U, Ludwig WD. TTG-2, a new gene encoding a cysteine-rich protein with the LIM motif, is overexpressed in acute T-cell leukaemia with the t(11;14)(p13;q11). *Oncogene*. 1991;6(10): 1887-1893.

153. Boehm T, Foroni L, Kaneko Y, Perutz MF, Rabbitts TH. The rhombotin family of cysteine-rich LIM-domain oncogenes: distinct members are involved in T-cell translocations to human chromosomes 11p15 and 11p13. *Proc Natl Acad Sci U S A* . 1991;88(10):4367-4371.

154. Visvader J, Begley CG, Adams JM. Differential expression of the LYL, SCL and E2A helix-loop-helix genes within the hemopoietic system. *Oncogene*. 1991;6(2):187-194.

155. Finger LR, Kagan J, Christopher G, et al. Involvement of the TCL5 gene on human chromosome 1 in T-cell leukemia and melanoma. *Proc Natl Acad Sci U S A* . 1989;86(13):5039-5043.

156. Hatano M, Roberts CW, Minden M, Crist WM, Korsmeyer SJ. Deregulation of a homeobox gene, HOX11, by the t(10;14) in T cell leukemia. *Science*. 1991;253(5015):79-82.

157. O'Conner rD, Brown MG, Francke U. *Immunologic and Karyotypic Studies in Ataxia-Telangiectasia: Specificity of Break Points on Chromosomes 7 and 14 in Lymphocytes from Patients and Relatives*. New York, NY: Wiley; 1982.

158. Lipkowitz S, Stern MH, Kirsch IR. Hybrid T cell receptor genes formed by interlocus recombination in normal and ataxia-telangiectasis lymphocytes. *J Exp Med*. 1990;172(2):409-418.

159. Novotny J, Tonegawa S, Saito H, Kranz DM, Eisen HN. Secondary, tertiary, and quaternary structure of T-cell-specific immunoglobulinlike polypeptide chains. *Proc Natl Acad Sci U S A*. 1986;83(3):742-746.

160. Rudolph MG, Stanfield RL, Wilson IA. How TCRs bind MHCs, peptides, and coreceptors. *Annu Rev Immunol* . 2006;24:419-466.

161. Bentley GA, Boulot G, Karjalainen K, Mariuzza RA. Crystal structure of the beta chain of a T cell antigen receptor [see comments]. *Science*. 1995;267(5206):1984-1987.

162. Fields BA, Ober B, Malchiodi EL, et al. Crystal structure of the V alpha domain of a T cell antigen receptor. *Science*. 1995;270(5243):1821-1824.

163. Garcia KC, Scott CA, Brunmark A, et al. CD8 enhances formation of stable T-cell receptor/MHC class I molecule complexes. *Nature*. 1996; 384(6609):577-581.

164. Garboczi DN, Ghosh P, Utz U, Fan QR, Biddison WE, Wiley DC. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature*. 1996;384(6605):134-141.

165. Garcia KC, Degano M, Stanfield RL, et al. An alphabeta T cell receptor structure at 2.5 A and its orientation in the TCR-MHC complex [see comments]. *Science*. 1996;274(5285):209-219.

166. Strong RK, Penny DM, Feldman RM, et al. Engineering and expression of a secreted murine TCR with reduced N-linked glycosylation. *J Immunol* . 1994;153(9):4111-4121.

167. Allison TJ, Winter CC, Fournie JJ, Bonneville M, Garboczi DN. Structure of a human gammadelta T-cell antigen receptor. *Nature*. 2001;411(6839):820-824.

168. Adams EJ, Chien YH, Garcia KC. Structure of a gammadelta T cell receptor in complex with the nonclassical MHC T22. *Science*. 2005; 308(5719):227-231.

169. Li H, Lebedeva MI, Llera AS, Fields BA, Brenner MB, Mariuzza RA. Structure of the Vdelta domain of a human gammadelta T-cell antigen receptor. *Nature*. 1998;391(6666):502-506.

169a. Xu B, Pizarro JC, Holmes MA, et al. Crystal structure of a gammadelta T-cell receptor specific for human MHC class I homolog MICA. *Proc Natl Acad Sci U S A* . 2011;108:2414-2419.

170. Bonneville M, Fournie JJ. Sensing cell stress and transformation through Vgamma9Vdelta2 T cell-mediated recognition of the isoprenoid pathway metabolites. *Microbes Infect*. 2005;7(3):503-509.

171. Scotet E, Martinez LO, Grant E, et al. Tumor recognition following Vgamma9Vdelta2 T cell receptor interactions with a surface F1-ATPaserelated structure and apolipoprotein A-I. *Immunity*. 2005;22(1):71-80.

172. Lin AY, Devaux B, Green A, Sagerstrom C, Elliott JF, Davis MM. Expression of T cell antigen receptor heterodimers in a lipid-linked form. *Science*. 1990;249(4969):677-679.

173. Gregoire C, Lin SY, Mazza G, Rebai N, Luescher IF, Malissen B. Covalent assembly of a soluble T cell receptor-peptide-major histocompatibility class I complex. *Proc Natl Acad Sci U S A*. 1996;93(14):7184-7189.

174. Teyton L. Assembly and transport of major histocompatibility complex molecules. *Nouv Rev Fr Hematol* . 1994;36(suppl 1):S33-S36.

175. Parham P, Terhorst C, Herrmann H, Humphreys RE, Waterfield MD, Strominger JL. Immunological and chemical purity of papain-solubilized HL-A antigens. *Proc Natl Acad Sci U S A*. 1975;72(4):1594-1598.

176. Wettstein DA, Boniface JJ, Reay PA, Schild H, Davis MM. Expression of a class II major histocompatibility complex (MHC) heterodimer in a lipid-linked form with enhanced peptide/soluble MHC complex formation at low pH. *J Exp Med*. 1991;174(1):219-228.

177. Garboczi DN, Hung DT, Wiley DC. HLA-A2-peptide complexes: refolding and crystallization of molecules expressed in Escherichia coli and complexed with single antigenic peptides. *Proc Natl Acad Sci U S A* . 1992;89(8):3429-3433.

178. Altman JD, Reay PA, Davis MM. Formation of functional peptide complexes of class

II major histocompatibility complex proteins from subunits produced in Escherichia coli. *Proc Natl Acad Sci U S A* . 1993;90(21):10330-10334.

179. Jackson MR, Song ES, Yang Y, Peterson PA. Empty and peptide-containing conformers of class I major histocompatibility complex molecules expressed in Drosophila melanogaster cells. *Proc Natl Acad Sci U S A* . 1992;89(24):12117-12121.

180. Kozono H, White J, Clements J, Marrack P, Kappler J. Production of soluble MHC class II proteins with covalently bound single peptides. *Nature*. 1994;369(6476):151-154.

181. Matsui K, Boniface JJ, Reay PA, Schild H, Fazekas de St. Groth B, Davis MM. Low affinity interaction of peptide-MHC complexes with T cell receptors. *Science*. 1991;254(5039):1788-1791.

182. Weber S, Traunecker A, Oliveri F, Gerhard W, Karjalainen K. Specific low-affinity recognition of major histocompatibility complex plus peptide by soluble T-cell receptor. *Nature*. 1992;356(6372): 793-796.

183. Malmqvist M. Biospecific interaction analysis using biosensor technology. *Nature*. 1993;361(6408):186-187.

184. Krogsgaard M, Prado N, Adams EJ, et al. Evidence that structural rearrangements and/or flexibility during TCR binding can contribute to T cell activation. *Mol Cell* . 2003;12(6):1367-1378.

185. Wyer JR, Willcox BE, Gao GF, et al. T cell receptor and coreceptor CD8 alphaalpha bind peptide-MHC independently and with distinct kinetics. *Immunity*. 1999;10(2):219-225.

186. Renard V, Romero P, Vivier E, Malissen B, Luescher IF. CD8 beta increases CD8 coreceptor function and participation in TCR-ligand binding. *J Exp Med*. 1996;184(6):2439-2444.

187. Xiong Y, Kern P, Chang H, Reinherz E. T cell receptor binding to a pMHCII ligand is kinetically distinct from and independent of CD4. *J Biol Chem*. 2001;27(8):5659-5667.

188. Huppa JB, Axmann M, Mörtelmaier MA, et al. TCR-peptide-MHC interactions in situ show accelerated kinetics and increased affinity. *Nature*. 2010;463(7283):963-967.

189. Huang J, Zarnitsyna VI, Liu B, et al. The kinetics of two-dimensional TCR and pMHC interactions determine T-cell responsiveness. *Nature*. 2010;464(7290):932-936.

190. Kaizuka Y, Douglass AD, Varma R, Dustin ML, Vale RD. Mechanisms for segregating T cell receptor and adhesion molecules during immunological synapse formation in Jurkat T cells. *Proc Natl Acad Sci U S A* . 2007;104:20296-20301.

191. Dustin ML, Ferguson LM, Chan PY, Springer TA, Golan DE. Visualization of CD2 interaction with LFA-3 and determination of the two-dimensional dissociation constant for adhesion receptors in a contact area. *J Cell Biol* . 1996;132(3):465-474.

192. Boniface JJ, Reich Z, Lyons DS, Davis MM. Thermodynamics of T cell receptor binding to peptide-MHC: evidence for a general mechanism of molecular scanning. *Proc Natl Acad Sci U S A*. 1999;96(20): 11446-11451.

193. Kersh GJ, Allen PM. Essential flexibility in the T-cell recognition of antigen. *Nature*. 1996;380(6574):495-498.

194. Jameson SC, Bevan MJ. T cell receptor antagonists and partial agonists. *Immunity*.

1995;2(1):1-11.

195. Sykulev Y, Brunmark A, Jackson M, Cohen RJ, Peterson PA, Eisen HN. Kinetics and affinity of reactions between an antigen-specific T cell receptor and peptide-MHC complexes. *Immunity*. 1994;1(1):15-22.

196. Matsui K, Boniface JJ, Steffner P, Reay PA, Davis MM. Kinetics of T-cell receptor binding to peptide/I-Ek complexes: correlation of the dissociation rate with T-cell responsiveness. *Proc Natl Acad Sci U S A* . 1994;91(26):12862-12866.

197. Lyons DS, Lieberman SA, Hampl J, et al. A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. *Immunity*. 1996;5(1):53-61.

198. Alam SM, Travers PJ, Wung JL, et al. T-cell-receptor affinity and thymocyte positive selection. *Nature*. 1996;381(6583):616-620.

199. Kessler BM, Bassanini P, Cerottini JC, Luescher IF. Effects of epitope modification on T cell receptor-ligand binding and antigen recognition by seven H-2Kd-restricted cytotoxic T lymphocyte clones specific for a photoreactive peptide derivative. *J Exp Med*. 1997;185(4): 629-640.

200. Holler PD. CD8(-) T cell transfectants that express a high affinity T cell receptor exhibit enhanced peptide-dependent activation. *J Exp Med*. 2001;194(8):1043-1052.

201. Krogsgaard M, Huppa JB, Purbhoo MA, Davis MM. Linking molecular and cellular events in T-cell activation and synapse formation. *Semin Immunol*. 2003;15(6):307-315.

202. Aivazian D, Stern LJ. Phosphorylation of T cell receptor zeta is regulated by a lipid dependent folding transition. *Nat Struct Biol* . 2000; 7(11):1023-1026.

203. Qi S, Krogsgaard M, Davis MM, Chakraborty AK. Molecular flexibility can influence the stimulatory ability of receptor-ligand interactions at cell-cell junctions. *Proc Natl Acad Sci U S A*. 2006;103(12):4416-4421.

204. McKeithan TW. Kinetic proofreading in T-cell receptor signal transduction. *Proc Natl Acad Sci U S A*. 1995;92(11):5042-5046.

205. Rabinowitz JD, Beeson C, Lyons DS, Davis MM, McConnell HM. Kinetic discrimination in T-cell activation. *Proc Natl Acad Sci U S A* . 1996;93(4):1401-1405.

206. Reich Z, Boniface JJ, Lyons DS, Borochov N, Wachtel EJ, Davis MM. Ligand-specific oligomerization of T-cell receptor molecules. *Nature*. 1997;387(6633):617-620.

207. Stefanova I, Hemmer B, Vergelli M, Martin R, Biddison WE, Germain RN. TCR ligand discrimination is enforced by competing ERK positive and SHP-1 negative feedback pathways. *Nat Immunol*. 2003;4(3): 248-254.

208. Altan-Bonnet G, Germain RN. Modeling T cell antigen discrimination based on feedback control of digital ERK responses. *PLoS Biol*. 2005; 3(11):e356.

209. Viola A, Lanzavecchia A. T cell activation determined by T cell receptor number and tunable thresholds. *Science*. 1996;273(5271):104-106.

210. Holler PD, Lim AR, Cho BK, Rund LA, Kranz DM. CD8(-) T cell transfectants that express a high affinity T cell receptor exhibit enhanced peptide-dependent activation. *J Exp Med*. 2001;194(8):1043-1052.

211. Weber KS, Donermeyer DL, Allen PM, Kranz DM. Class II-restricted T cell receptor engineered in vitro for higher affinity retains peptide specificity and function. *Proc Natl Acad Sci U S A*. 2005;52:19033-19038.

212. Xie, J, Huppa JB, Newell EW, et al. Photocrosslinkable pMHC monomers stain T cells specifically and cause ligand-bound TCRs to be 'preferentially' transported to the cSMAC. *Nat Immunol*. 2012;7:674-680.

213. Krogsgaard M, Li QJ, Sumen C, Huppa JB, Huse M, Davis MM. Agonist/endogenous peptide-MHC heterodimers drive T cell activation and sensitivity. *Nature*. 2005;434(7030):238-243.

214. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. *Nature*. 1988;334(6181):395-402. Erratum. *Nature*. 1988; 335(6192):744.

215. Engel I, Hedrick SM. Site-directed mutations in the VDJ junctional region of a T cell receptor beta chain cause changes in antigenic peptide recognition. *Cell*. 1988;54(4):473-484.

216. Katayama CD, Eidelman FJ, Duncan A, Hooshmand F, Hedrick SM. Predicted complementarity determining regions of the T cell antigen receptor determine antigen specificity. *EMBO J*. 1995;14(5):927-938.

217. Jorgensen JL, Esser U, Fazekas de St Groth B, Reay PA, Davis MM. Mapping T-cell receptor-peptide contacts by variant peptide immunization of single-chain transgenics. *Nature*. 1992;355(6357):224-230.

218. Sant'Angelo DB, Waterbury G, Preston-Hurlburt P, et al. The specificity and orientation of a TCR to its peptide-MHC class II ligands. *Immunity*. 1996;4(4):367-376.

219. Newell EW, Ely LK, Kruse AC, et al. Structural basis of specificity and cross-reactivity in T cell receptors specific for cytochrome c-I-E(k). *J Immunol*. 2011;186(10):5823-5832.

220. Jorgensen JL, Reay PA, Ehrich EW, Davis MM. Molecular components of T-cell recognition. *Annu Rev Immunol*. 1992;10(6357):835-873.

221. Hennecke J, Wiley DC. T cell receptor-MHC interactions up close. *Cell*. 2001;104(1):1-4.

222. Adams JJ, Narayanan S, Liu B, et al. T cell receptor signaling is limited by docking geometry to peptide-major histocompatibility complex. *Immunity*. 2011;11:681-693.

223. Nanda NK, Arzoo KK, Geysen HM, Sette A, Sercarz EE. Recognition of multiple peptide cores by a single T cell receptor. *J Exp Med*. 1995; 182(2):531-539.

224. Mason D. A very high level of crossreactivity is an essential feature of the T-cell receptor. *Immunol Today*. 1998;19(9):395-404.

225. Wilson DB, Pinilla C, Wilson DH, et al. Immunogenicity. I. Use of peptide libraries to identify epitopes that activate clonotypic CD4+ T cells and induce T cell responses to native peptide ligands. *J Immunol*. 1999;163(12):6424-6434.

226. Maynard J, Petersson K, Wilson DH, et al. Structure of an autoimmune T cell receptor complexed with class II peptide-MHC: insights into MHC bias and antigen specificity. *Immunity*. 2005;22(1):81-92.

227. Huseby ES, Crawford F, White J, Marrack P, Kappler JW. Interfacedisrupting amino

acids establish specificity between T cell receptors and complexes of major histocompatibility complex and peptide. *Nat Immunol*. 2006;7(11):1191-1199.

228. Yachi PP, Ampudia J, Gascoigne NR, Zal T. Nonstimulatory peptides contribute to antigen-induced CD8-T cell receptor interaction at the immunological synapse. *Nat Immunol*. 2005;6(8):785-792.

229. Garcia KC, Degano M, Pease LR, et al. Structural basis of plasticity in T cell receptor recognition of a self peptide-MHC antigen. *Science*. 1998;279(5354):1166-1172.

230. Reiser JB, Gregoire C, Darnault C, et al. A T cell receptor CDR3beta loop undergoes conformational changes of unprecedented magnitude upon binding to a peptide/MHC class I complex. *Immunity*. 2002;16(3): 345-354.

231. Reinherz EL, Tan K, Tang L, et al. The crystal structure of a T cell receptor in complex with peptide and MHC class II. *Science*. 1999; 286(5446):1913-1921.

232. Willcox BE, Gao GF, Wyer JR, et al. TCR binding to peptide-MHC stabilizes a flexible recognition interface. *Immunity*. 1999;10(3): 357-365.

233. Wu LC, Tuot DS, Lyons DS, Garcia KC, Davis MM. Two-step binding mechanism for T-cell receptor recognition of peptide MHC. *Nature*. 2002;418(6897):552-556.

234. Tynan FE, Burrows SR, Buckle AM, et al. T cell receptor recognition of a "super-bulged" major histocompatibility complex class I-bound peptide. *Nat Immunol*. 2005;6(11):1114-1122.

235. Feng D, Bond CJ, Ely LK, Maynard J, Garcia KC. Structural evidence for a germline-encoded T cell receptor-major histocompatibility complex interaction "codon." *Nat Immunol*. 2007;8:975-983.

236. Dai S, Huseby ES, Rubtsova K, et al. Crossreactive T cells spotlight the germline rules for alphabeta T cell-receptor interactions with MHC molecules. *Immunity*. 2008;28:324-334.

237. Janeway CA Jr. The T cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation. *Annu Rev Immunol*. 1992;10:645-674.

238. Irvine DJ, Purbhoo MA, Krogsgaard M, Davis MM. Direct observation of ligand recognition by T cells. *Nature*. 2002;419(6909):845-849.

239. Purbhoo MA, Irvine DJ, Huppa JB, Davis MM. T cell killing does not require the formation of a stable mature immunological synapse. *Nat Immunol*. 2004;5(5):524-530.

240. Yin Y, Wang XX, Mariuzza RA. Crystal structure of a complete ternary complex of T-cell receptor, peptide-MHC, and CD4. *Proc Natl Acad Sci U S A*. 2012;109(14):5405-5410.

241. Luescher IF, Vivier E, Layer A, et al. CD8 modulation of T-cell antigen receptor-ligand interactions on living cytotoxic T lymphocytes. *Nature*. 1995;373:353-358.

242. Wyer JR, Willcox BE, Gao GF, et al. T cell receptor and coreceptor CD8αα bind peptide-MHC independently and with distinct kinetics. *Immunity*. 1999;10(2):219-225.

243. Artyomov MN, Lis M, Devadas S, Davis MM, Chakraborty AK. CD4 and CD8 binding to MHC molecules primarily acts to enhance Lck delivery. *Proc Natl Acad Sci U S A*. 2010;107(39):16916-16921.

244. Wulfing C, Sumen C, Sjaastad MD, Wu LC, Dustin ML, Davis MM. Costimulation and endogenous MHC ligands contribute to T cell recognition. *Nat Immunol*. 2002;3(1):42-47.

245. Hampl J, Chien YH, Davis MM. CD4 augments the response of a T cell to agonist but not to antagonist ligands. *Immunity*. 1997;7(3):379-385.

246. Jiang N, Huang J, Edwards LJ, et al. Two-stage cooperative T cell receptor-peptide major histocompatibility complex-CD8 trimolecular interactions amplify antigen discrimination. *Immunity*. 2011;34(1): 13-23.

247. Marrack P, Kappler J. The staphylococcal enterotoxins and their relatives. *Science*. 1990;248(4959):1066.

248. Sundberg EJ, Li Y, Mariuzza RA. So many ways of getting in the way: diversity in the molecular architecture of superantigen-dependent T-cell signaling complexes. *Curr Opin Immunol*. 2002;14(1):36-44.

249. Festenstein H. Immunogenetic and biological aspects of in vitro lymphocyte allotransformation (MLR) in the mouse. *Transplant Rev*. 1973; 15:62-88.

250. Kappler JW, Staerz U, White J, Marrack PC. Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. *Nature*. 1988;332(6159):35-40.

251. Woodland DL, Happ MP, Gollob KJ, Palmer E. An endogenous retrovirus mediating deletion of alpha beta T cells? *Nature*. 1991; 349(6309):529-530.

252. Marrack P, Kushnir E, Kappler J. A maternally inherited superantigen encoded by a mammary tumour virus. *Nature*. 1991;349(6309): 524-526.

253. Choi Y, Kappler JW, Marrack P. A superantigen encoded in the open reading frame of the 3' long terminal repeat of mouse mammary tumour virus. *Nature*. 1991;350(6315):203-207.

254. Dyson PJ, Knight AM, Fairchild S, Simpson E, Tomonari K. Genes encoding ligands for deletion of V beta 11 T cells cosegregate with mammary tumour virus genomes. *Nature*. 1991;349(6309):531-532.

255. Frankel WN, Rudy C, Coffin JM, Huber BT. Linkage of Mls genes to endogenous mammary tumour viruses of inbred mice. *Nature*. 1991;349(6309):526-528.

256. Janeway CA Jr, Yagi J, Conrad PJ, et al. T-cell responses to Mls and to bacterial proteins that mimic its behavior. *Immunol Rev*. 1989;107: 61-88.

257. McDonald KR, Acha-Orbea H. Superantigens of mouse mammary tumor virus. *Annu Rev Immunol*. 1995;13:459.

258. Li H, Llera A, Malchiodi EL, Mariuzza RA. The structural basis of T cell activation by superantigens. *Annu Rev Immunol*. 1999;17:435-466.

259. Stuart PM, Woodward JG. Yersinia enterocolitica produces superantigenic activity. *J Immunol*. 1992;148(1):225-233.

260. Abe J, Takeda T, Watanabe Y, et al. Evidence for superantigen production by Yersinia pseudotuberculosis. *J Immunol*. 1993;151(8):4183-4188.

261. Tomai M, Kotb M, Majumdar G, Beachey EH. Superantigenicity of streptococcal M protein. *J Exp Med*. 1990;172(1):359-362.

262. Cole BC, Kartchner DR, Wells DJ. Stimulation of mouse lymphocytes by a mitogen derived from Mycoplasma arthritidis. VII. Responsiveness is associated with expression of a product(s) of the V beta 8 gene family present on the T cell receptor alpha/beta for antigen. *J Immunol*. 1989;142(12):4131-4137.

263. Friedman SM, Crow MK, Tumang JR, et al. Characterization of human T cells reactive with the Mycoplasma arthritidis-derived superantigen (MAM): generation of a monoclonal antibody against V beta 17, the T cell receptor gene product expressed by a large fraction of MAM-reactive human T cells. *J Exp Med*. 1991;174(4):891-8900.

264. Lafon M, Scott-Algara D, Marche PN, Cazenave PA, Jouvin-Marche E. Neonatal deletion and selective expansion of mouse T cells by exposure to rabies virus nucleocapsid superantigen. *J Exp Med*. 1994;180(4): 1207-1215.

265. Dobrescu D, Ursea B, Pope M, Asch AS, Posnett DN. Enhanced HIV-1 replication in V beta 12 T cells due to human cytomegalovirus in monocytes: evidence for a putative herpesvirus superantigen. *Cell*. 1995;82(5):753-763.

266. Yao Z, Maraskovsky E, Spriggs MK, Cohen JI, Armitage RJ, Alderson MR. Herpesvirus saimiri open reading frame 14, a protein encoded by T lymphotropic herpesvirus, binds to MHC class II molecules and stimulates T cell proliferation. *J Immunol*. 1996;156(9):3260-3266.

267. Sutkowski N CB, Thorley-Lawson DA, Huber BT. Epstein-Barr virus transactivates the human endogenous retrovirus HERV-K18 that encodes a superantigen. *Immunity*. 2001;15:579-589.

268. Denkers EY, Caspar P, Sher A. Toxoplasma gondii possesses a superantigen activity that selectively expands murine T cell receptor V beta 5-bearing CD8+ lymphocytes. *J Exp Med*. 1994;180(3):985-994.

269. Held W, Waanders GA, Shakhov AN, Scarpellino L, Acha-Orbea H, MacDonald HR. Superantigen-induced immune stimulation amplifies mouse mammary tumor virus infection and allows virus transmission. *Cell*. 1993;74(3):529-540.

270. Golovkina TV, Chervonsky A, Dudley JP, Ross SR. Transgenic mouse mammary tumor virus superantigen expression prevents viral infection. *Cell*. 1992;69(4):637-645.

271. Stauffer Y, Marquerat S, Meylan F, et al. Interferon-induced endogenous superantigen: a model linking environment and autoimmunity. *Immunity*. 2001;15:591-601.

272. Dalwadi H, Wei B, Kronenberg M, Sutton CL, Braun J. The Crohn's disease-associated bacterial protein I2 is a novel enteric t cell superantigen. *Immunity*. 2001;15(1):149-158.

273. Malchiodi EL, Eisenstein E, Fields BA, et al. Superantigen binding to a T cell receptor of known three-dimensional structure. *J Exp Med*. 1995;182:1833.

274. Patten PA RE, Sonoda T, Fazekas de St. Groth B, Davis MM. Transfer of putative CD$ loops of T cell receptor V domains confers toxin reactivity, but not peptide specificity. *J Immunol*. 1993;150:2281-2294.

275. Fields BA, Malchiodi EL, Li H, et al. Crystal structure of a T-cell receptor beta-chain

complexed with a superantigen. *Nature*. 1996; 384(6605):188-192.

276. Li Y, Li H, Dimasi N, et al. Crystal structure of a superantigen bound to the high-affinity, zinc-dependent site on MHC class II. *Immunity*. 2001;14(1):93-104.

277. Peterson K, Håkansson M, Nilsson H, et al. Crystal structure of a superantigen bound to MHC class II displays zinc and peptide dependence. *EMBO J*. 2001;20:3306-3312.

278. Felds BA, Malchiodi EL, Li HM, et al. Crystal structure of the chain of a T-cell receptor complexed with a superantigen. *Nature*. 1996;384:188.

279. Rojo JM, Janeway CA Jr. The biologic activity of anti-T cell receptor V region monoclonal antibodies is determined by the epitope recognized. *J Immunol*. 1988;140(4):1081-1088.

280. Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature*. 1987;329(6139):506-512.

281. Madden DR, Gorga JC, Strominger JL, Wiley DC. The structure of HLA-B27 reveals nonamer self-peptides bound in an extended conformation. *Nature*. 1991;353(6342):321-325.

282. Brown JH, Jardetzky TS, Gorga JC, et al. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature*. 1993;364(6432):33-39.

283. Bluestone JA, Cron RQ, Cotterman M, Houlden BA, Matis LA. Structure and specificity of T cell receptor gamma/delta on major histocompatibility complex antigen-specific CD3+, CD4-, CD8- T lymphocytes. *J Exp Med*. 1988;168(5):1899-1916.

284. Matis LA, Fry AM, Cron RQ, Cotterman MM, Dick RF, Bluestone JA. Structure and specificity of a class II MHC alloreactive gamma delta T cell receptor heterodimer. *Science*. 1989;245(4919):746-749.

285. Schild H, Mavaddat N, Litzenberger C, et al. The nature of major histocompatibility complex recognition by gamma delta T cells. *Cell*. 1994;76(1):29-37.

286. Hampl J, Schild H, Litzenberger C, Baron M, Crowley MP, Chien YH. The specificity of a weak gamma delta TCR interaction can be modulated by the glycosylation of the ligand. *J Immunol*. 1999;163(1):288-294.

287. Fremont DH, Dai S, Chiang H, Crawford F, Marrack P, Kappler J. Structural basis of cytochrome c presentation by IE(k). *J Exp Med*. 2002;195(8):1043-1052.

288. Clackson T, Wells JA. A hot spot of binding energy in a hormone-receptor interface. *Science*. 1995;267(5196):383-386.

289. Honig B, Nicholls A. Classical electrostatics in biology and chemistry. *Science*. 1995;268(5214):1144-1149.

290. Ito K, Van Kaer L, Bonneville M, Hsu S, Murphy DB, Tonegawa S. Recognition of the product of a novel MHC TL region gene (27b) by a mouse gamma delta T cell receptor. *Cell*. 1990;62(3):549-561.

291. Houlden BA, Matis LA, Cron RQ, et al. A TCR gamma delta cell recognizing a novel TL-encoded gene product. *Cold Spring Harb Symp Quant Biol*. 1989;54(pt 1):45-55.

291a. Crowley MP, thesis

292. Crowley MP, Reich Z, Mavaddat N, Altman JD, Chien Y. The recognition of the nonclassical major histocompatibility complex (MHC) class I molecule, T10, by the gammadelta T cell, G8. *J Exp Med*. 1997;185(7): 1223-1230.

293. Wingren C, Crowley MP, Degano M, Chien Y, Wilson IA. Crystal structure of a gammadelta T cell receptor ligand T22: a truncated MHC-like fold. *Science*. 2000;287(5451):310-314.

294. Rudolph MG, Wingren C, Crowley MP, Chien YH, Wilson IA. Combined pseudo-merohedral twinning, non-crystallographic symmetry and pseudo-translation in a monoclinic crystal form of the gammadelta T-cell ligand T10. *Acta Crystallogr D Biol Crystallogr*. 2004; 60(pt 4):656-664.

295. Kaliyaperumal A, Falchetto R, Cox A, et al. Functional expression and recognition of nonclassical MHC class I T10b is not peptide-dependent. *J Immunol*. 1995;155(5):2379-2386.

295a Schild H, Chien YH, Rammensee H. unpublished results

296. Weintraub BC, Jackson MR, Hedrick SM. Gamma delta T cells can recognize nonclassical MHC in the absence of conventional antigenic peptides. *J Immunol*. 1994;153(7):3051-3058.

297. Crowley MP, Fahrer AM, Baumgarth N, et al. A population of murine gammadelta T cells that recognize an inducible MHC class Ib molecule. *Science*. 2000;287(5451):314-316.

298. Sciammas R, Bluestone JA. HSV-1 glycoprotein I-reactive TCR gamma delta cells directly recognize the peptide backbone in a conformationally dependent manner. *J Immunol*. 1998;161(10):5187-5192.

299. Wu J, Groh V, Spies T. T cell antigen receptor engagement and specificity in the recognition of stress-inducible MHC class I-related chains by human epithelial gamma delta T cells. *J Immunol*. 2002;169(3): 1236-1240.

300. Groh V, Steinle A, Bauer S, Spies T. Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells. *Science*. 1998;279(5357):1737-1740.

301. Das B, Mondragon MO, Sadeghian M, Hatcher VB, Norin AJ. A novel ligand in lymphocyte-mediated cytotoxicity: expression of the beta subunit of H+ transporting ATP synthase on the surface of tumor cell lines. *J Exp Med*. 1994;180(1):273-281.

302. Bie W, Squire JA, Fraser M, Paterson MC, Godbout R. Mitochondrial ATP synthase alpha-subunit gene amplified in a retinoblastoma cell line maps to chromosome 18. *Genes Chromosomes Cancer*. 1995;14(1):63-67.

303. Moser TL, Stack MS, Asplin I, et al. Angiostatin binds ATP synthase on the surface of human endothelial cells. *Proc Natl Acad Sci U S A.* 1999; 96(6):2811-2816.

304. Villanueva MS, Beckers CJ, Pamer EG. Infection with Listeria monocytogenes impairs sialic acid addition to host cell glycoproteins. *J Exp Med*. 1994;180(6):2137-2145.

305. Wittel UA, Goel A, Varshney GC, Batra SK. Mucin antibodies - new tools in diagnosis and therapy of cancer. *Front Biosci*. 2001;6: D1296-D1310.

306. Meyer C, Zeng X, Chien YH. Ligand recognition during thymic development and gammadelta T cell function specification. *Semin Immunol*. 2010;22(4):207-213.

306a. Jensen KD, Su X, Shin S, et al. Thymic selection determines gammadelta T cell effector fate: antigen-naive cells make interleukin-17 and antigenexperienced cells make interferon gamma. *Immunity.* 2008;29:90-100.

306b. Stark MA, Huo Y, Burcin TL, et al. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity*. 2005;22: 285-294.

307. Dunn SE, Youssef S, Goldstein MJ, et al. Isoprenoids determine Th1/Th2 fate in pathogenic T cells, providing a mechanism of modulation of autoimmunity by atorvastatin. *J Exp Med*. 2006;203(2):401-412.

308. Carding SR, Egan PJ. Gammadelta T cells: functional plasticity and heterogeneity. *Nat Rev Immunol* . 2002;2(5):336-345.

309. O'Brien RL, Lahn M, Born WK, Huber SA. T cell receptor and function cosegregate in gamma-delta T cell subsets. *Chem Immunol*. 2001; 79:1-28.

310. Hahn YS, Taube C, Jin N, et al. Different potentials of gamma delta T cell subsets in regulating airway responsiveness: V gamma 1+ cells, but not V gamma 4+ cells, promote airway hyperreactivity, Th2 cytokines, and airway inflammation. *J Immunol* . 2004;172(5):2894-2902.

311. Andrew EM, Newton DJ, Dalton JE, et al. Delineation of the function of a major gamma delta T cell subset during infection. *J Immunol*. 2005;175(3):1741-1750.

312. Janeway CA Jr, Jones B, Hayday A. Specificity and function of T cells bearing gamma delta receptors. *Immunol Today*. 1988;9(3):73-76.

313. Shin S, El-Diwany R, Schaffert S, et al. Antigen recognition determinants of gammadelta T cell receptors. *Science*. 2005;308(5719):252-255.

314. Zhao H, Nguyen H, Kang J. Interleukin 15 controls the generation of the restricted T cell receptor repertoire of gamma delta intestinal intraepithelial lymphocytes. *Nat Immunol*. 2005;6(12):1263-1271.

315. Xu JL, Davis MM. Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. *Immunity*. 2000;13(1):37-45.

316. Klinman NR, Pickard AR, Sigal NH, Gearhart PJ, Metcalf ES, Pierce SK. Assessing B cell diversification by antigen receptor and precursor cell analysis. *Ann Immunol (Paris)*. 1976;127(3-4):489-502.

317. McHeyzer-Williams MG, Altman JD, Davis MM. Enumeration and characterization of memory cells in the TH compartment. *Immunol Rev*. 1996;150:5-21.

318. Arstila TP, Casrouge A, Baron V, Even J, Kanellopoulos J, Kourilsky P. A direct estimate of the human alphabeta T cell receptor diversity. *Science*. 1999;286(5441):958-961.

319. Davis MM, Lyons DS, Altman JD, et al. T cell receptor biochemistry, repertoire selection and general features of TCR and Ig structure. *Ciba Found Symp*. 1997;204:94-100, discussion 104.

320. Davis MM. The evolutionary and structural “logic” of antigen receptor diversity. *Semin Immunol*. 2004;16(4):239-243.

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Chapter 12

Mechanisms of T-Lymphocyte Signaling and Activation

Takashi Saito

INTRODUCTION

The immune system protects us against attack by pathogens through two separate but interacting systems: innate immunity and acquired (or adaptive) immunity. Whereas innate immunity exhibits rapid and transient responses by recognition of conserved molecular patterns of pathogens, adaptive immunity distinguishes any small difference/heterogeneity of a huge number of pathogens and antigens and mediates a rather slow but long-lasting response. For this purpose, as is discussed elsewhere (see Chapter 6) in this volume, our body prepares a vast variety of receptors on T cells and B cells (immunoglobulins [Igs]) to be able to recognize virtually all potential antigens. Because each lymphocyte possesses single antigen specificity, antigen-specific responses begin with the recognition by and activation of a single cell and consequently the cells proliferate, a process called clonal expansion and selection many years ago by Macfarlane Burnet.[1] To understand the mechanism of antigen-specific activation of lymphocytes, it is necessary to study how a single cell of relevant specificity recognizes the antigen and induces its activation to mediate various functions and protection against pathogens. Recognition of antigen only on the cell surface by the T-cell receptor (TCR) of a T cell is unique and totally different from that of Ig on a B cell. Such differences in T-cell antigen recognition force the establishment of extensive cell-cell interactions/communications in the body to achieve protective immunity and to maintain immune homeostasis. In this chapter, we describe the unique feature of antigen recognition of T cells through their unique antigen receptor complex and the mechanisms that trigger T-cell activation.

T-CELL RECEPTOR COMPLEX

Antigen specific recognition is mediated by the TCR, which is composed of a heterodimer of either αβ or γδ polypeptides. This chapter will focus on signaling mediated through the αβ TCR. As described in Chapter 11, both TCRα and β chains contain variable regions, which together are responsible for binding to the complex of antigen peptide-major histocompatibility complex (MHC). After a decades-long search for the biochemical nature of TCR, the TCR genes were finally cloned and found to be quite similar to those encoding Ig genes.[2,3,4,5,6] TCRα and β chains both possess very short cytoplasmic domains without any particular protein-binding motif, and thus cannot transduce antigen-recognition signals into the cell. It is necessary, therefore, for the TCR to assemble with signaltransducing components, the cluster of differentiation (CD)3 complex, to transduce antigen-recognition

signals. The initial finding that CD3 chains are responsible for transducing activation signals into T cells came from the observation that treatment of T cells with a crosslinking anti-CD3ε antibody (OKT3) induces strong T-cell polyclonal activation[7] similar to that induced upon stimulation with mitogens such as concanavalin A. It was also shown that there is a physical but noncovalent association between TCR dimers and the CD3 complex. It is now known that the association of the TCR dimer and the CD3 complex is required first for cell surface expression and then to transduce antigen-recognition signals into T cells.[8,9] The CD3 complex is composed of four different chains. Three of them, γ, δ, and e, are closely related in their protein structure, gene structure, biosynthesis, and chromosome localization. While the γ, δ, and ε are genes are located within a neighboring region of the chromosome (chromosome 11 in human and 9 in mouse), ζ is located on chromosome 1 in both species (Fig. 12.1). The CD3 complex is composed of three distinct dimers, γε, δε, and ζζ, which associate with the TCRαβ dimer. Whereas γε and δε are noncovalently assembled, ζ-ζ is a covalently linked dimmer with a disulfide bond. The ζ chain has an isoform termed η, which is derived from alternative splicing of the ζ transcript and is found only in rodents but not in humans. The cell surface expression of the TCRαβ dimer requires its assembly with the CD3 complex due to a stringent quality control checkpoints in the endoplasmic reticulum (ER) that prevent the release of incompletely assembled TCR-CD3 complexes. Studies of the biosynthesis of these components of the this complex showed that CD3γ, δ, and ε as well as TCRα and β chains are synthesized in great excess and readily degraded in the ER in the absence of assembly with the other chains. The assembly with the partner chain protects the complex from ER degradation. CD3ζ is synthesized at limiting levels, and even the TCRαβ-CD3γδε complex cannot be transferred to the cell surface without assembly with CD3ζζ and is degraded in lysosome.[10] Therefore, the assembly with ζ-ζ defines the fully functional TCR-CD3 complex, and the entire TCRαβ-CD3γδεζζ complex is now allowed to transport to the plasma membrane where it is expressed as the complete and functional TCR-CD3 complex.

THE IMMUNORECEPTOR TYROSINE-BASED ACTIVATION MOTIF AND INITIAL SIGNALING

The cytoplasmic tails of all CD3 chains contain a common signaling motif, immunoreceptor tyrosine-based activation motif (ITAM), which has the consensus sequence YxxL/I x6-8 YxxL; composed of two repeats of YxxL/I motif (Y; tyrosine, L/I; leucine/isoleucine) with a spacer of six to eight

amino acids. CD3δ, γ, and ε have a single and CD3ζ has three tandem ITAMs within the intracellular region. The ITAM was first identified as a signature sequence present in several cell surface receptors on immune cells.[11] ITAM-containing molecules have since been found to be widely distributed among various important immune receptors, not only TCR (CD3s) but also in B-cell antigen receptors (Igα and Igβ), various types of Fc receptors (FcRγ), adaptor proteins associated with MHC-recognizing receptors, or paired receptors on natural killer (NK) cells (DAP12 and DAP10) (Fig. 12.2) and further in various pattern-recognizing receptors of innate cells such as macrophages and dendritic cells (DCs).[12] The ITAM turns out to be specific motif capable of transducing receptor-mediated recognition signals into cellular activation.[13] The function of the ITAM in transducing T-cell activation signals has been shown first by analyzing the capacity of a chimeric protein composed of the extracellular

domain of CD8 and the intracellular domain of CD3ζ to

induce T-cell activation.[14] Antibody crosslinking of the CD8 domain showed that the cytoplasmic tail of CD3ζ can induce almost all the events observed during normal T-cell activation, including tyrosine phosphorylation of various proteins, induction of intracellular calcium mobilization and inositol phosphate metabolism, induction of activation markers, cytokine production, and cell proliferation. Mutational analyses of the ITAM sequences within the CD3ζ cytoplasmic region revealed that both tyrosine and leucine (isoleucine) residues are critical for transducing a T-cell activation signal.[15,16]

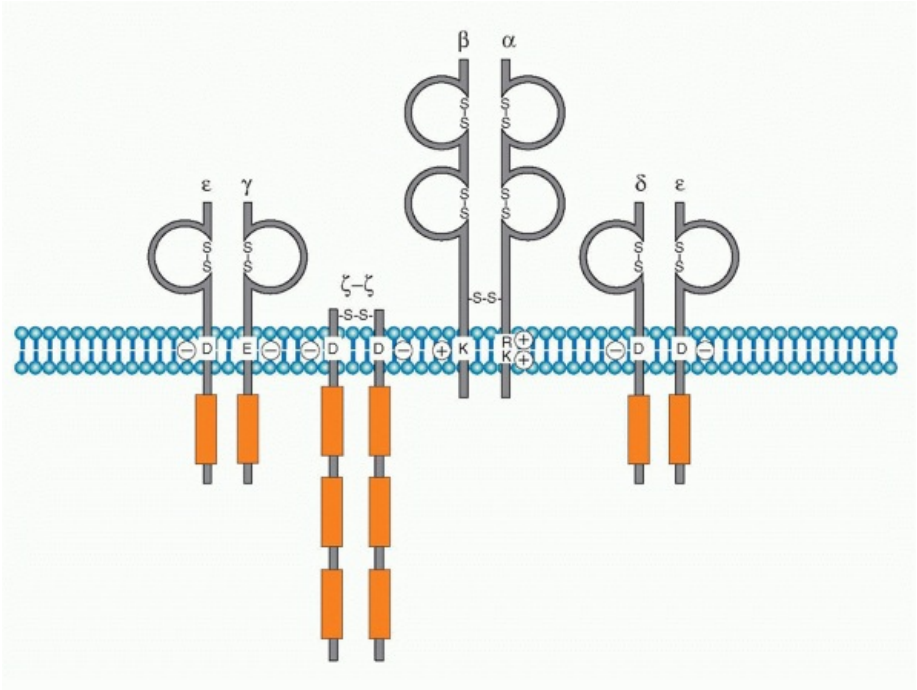

FIG. 12.1. Structure of the T-Cell Receptor (TCR)-Cluster of Differentiation (CD)3 Complex. The TCRαβ dimer is associated with three dimers of CD3 chains: CD3εγ, CD3εδ, and CD3ζζ. Whereas TCRα and β possess positively charged amino acids within the transmembrane region, all CD3 chains contain negatively charged amino acids, which are necessary to be assembled as the complex. CD3 chains possess immunoreceptortyrosinebased activation motif (ITAM) within their cytoplasmic tails as shown in *red boxes* (γ, δ, and ε have one; ζ has three).

		Amino acid sequence
TCR	CD3γ	EQLYQPLKD–REYDQYSHL
	CD3δ	EQLYQPLRD–REDTQYSRL
	CD3ε	NPDYEPIRK–GQRDLYSGL
	CD3ζa	NQLYNELNL–GRREEYDVL
	CD3ζb	EGVYNALQKDKMAEAYSEI
	CD3ζc	DGLYQGLST–ATKDTYDAL
BCR	Igα	ENLYEGLNL–DDCSMYEDI
	Igβ	DHTYEGLNI–DQTATYEDI
FcR	FcRγ	DAVYTGLNT–RSQETYETL
	FcεRIβ	DRLYEELNH–VYSPIYSEL
NKR	DAP12	ESPYQELQG–QRPEVYSDL
Virus	BLV gp30	DSDYQALLP–SAPEIYSHL
	EBV LMP2A	HSDYQPLGT–QDQSLYLGL
	consensus	Dxx Yxx Lxx–x xxxx Yxx L E I I

FIG. 12.2. Structure and Signaling Function of Immunoreceptor Tyrosine-based Activation Motif (ITAM). Cluster of differentiation 3s (T cells), Ig-α/β (B cells), FcR (myeloid cells; macrophages, dendritic cells, neutrophils, etc.), NK cells, as well as some viruses possess ITAM within the cytoplasmic domain. There are increasing numbers of ITAM-bearing or ITAM+ adaptor-associated receptors in innate systems. The consensus sequence of the ITAM is composed of YxxL/I- (seven or eight spacers)- YxxL/I where x is any amino acid. The tyrosine residues within ITAM are phosphorylated by src family kinase Lck upon TCR engagement, followed by the binding of the tandem two SH2 domains of ZAP-70 to both phosphorylated tyrosines in T cells.

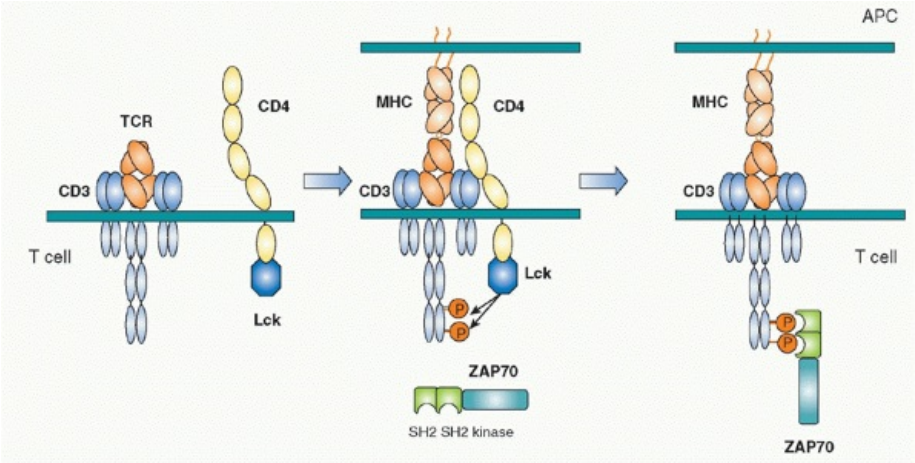

FIG. 12.3. Initial Activation of T Cells by Sequential Two Tyrosine Kinases. Left: The T-cell receptor (TCR)-cluster of differentiation (CD)3 complex and the complex of CD4 and the Src kinase Lck are located separately in resting T cells. **Middle:** Upon antigen peptide-major histocompatibility complex (MHC) recognition by TCR, the TCR complex and the CD4-Lck complex get near through the CD4-MHC binding, which induces Lck close to the CD3 chains. The immunoreceptortyrosine-based activation motif (ITAMs) of CD3 chains are tyrosine-phosphorylated by Lck. **Right:** The phosphorylated ITAM of the CD3 chains became the binding sites for the tandem two Src-homology 2 domains of the second tyrosine kinase ZAP-70. ZAP-70 is phosphorylated by Lck and by itself and activated, and then transduces activation signals by phosphorylating downstream signaling molecules.

Biochemical analyses to define the downstream signaling molecules recruited upon TCR stimulation resulted in the discovery of several important proteins that are involved in tyrosine phosphorylation and activation of several adaptor molecules including a Src family tyrosine kinase, Lck, and a Syk family kinase, Zeta-associated protein-70 (ZAP-70).[17] The ITAM is phosphorylated by Src kinase Lck upon TCR engagement by antigen. Once tyrosines are phosphorylated, they become susceptible to binding in general by proteins possessing Src-homology 2 (SH2) domains. The phosphorylated ITAMs recruit the second tyrosine kinase ZAP-70, one of Syk family kinases. ZAP-70 is contains two tandem SH2 domains in its N terminus and a tyrosine kinase region in the C-terminus.[18,19] The tandem SH2 domains with exactly the right spacing can bind the two phosphorylated tyrosines within an ITAM[20] (Fig. 12.3). Because TCR and CD3 chains do not have any intrinsic effector function, unlike tyrosine kinase-containing receptors such as the epidermal growth factor receptor or the insulin receptor, the binding of ZAP-70 to the phosphorylated CD3ζ ITAM changes the TCR-CD3 complex, making it competent for signal transduction. ZAP-70 assembled with the CD3 phospho-ITAMs must then be enzymatically activated by phosphorylation of otherwise inhibitory tyrosines in its juxtamembrane region by Lck as well as by itself. The binding and activation of ZAP-70 may be associated with the structural changes of CD3 chains induced by phosphorylation, a topic that will be described under the section Triggering Mechanism. Activated ZAP-70 induces phosphorylation of tyrosine residues of various downstream target molecules.[21] These include adaptor molecules LAT and SLP-76. These phosphorylation events lead to further activation of several adaptor and effector molecules and subcellular assembly as multimolecular signaling complex.

REGULATION OF PROXIMAL SIGNALING

Regulation of Tyrosine Kinases

Src family tyrosine kinases (Lck, Fyn in T cells) are composed of an N-terminus unique region, SH2, SH3, and a kinase domain at the C-terminus.[22] The N-terminus unique region contains palmitoylation sites by which Lck is localized in lipid rafts on the inner leaflet of the plasma membrane. Because Lck specifically associates with the cytoplasmic regions of CD4 and CD8 coreceptors, Lck accumulates at the site of TCR engagement because CD4 or CD8 are also present within the engaged site through binding to MHC.[23,24] As a result, Lck is recruited to the vicinity of the CD3 chains where it is able to phosphorylate them.

The activity of Lck is regulated allosterically by phosphorylation of a tyrosine in the carboxyl terminus by the

C-terminal kinase (Csk).[25] This phosphotyrosine interacts with the SH2 domain of Lck, and this induces a conformational change to maintain Lck in a catalytically inactive status. Csk normally and continuously acts to reduce Lck activity and thereby attenuate TCR signaling. Indeed, in the absence of Csk, peripheral T cells become autonomously activated. Dephosphorylation of the Cterminus tyrosine releases Lck from its inactive status. In addition, in order to fully activate Lck, the tyrosine phosphorylation in the catalytic domain is also required. Because the tyrosine phosphatase CD45 induces the dephosphorylation of both of these regulatory tyrosines, CD45 functions as both a positive and negative regulator of T-cell activation[26] through the C-terminal and kinase-domain tyrosines, respectively. The balance between Csk and CD45 regulates the status of T-cell activation[27] (Fig. 12.4). A considerable fraction of Lck in naïve T cells is catalytically active because of this dynamic balance. Such active Lck may be responsible for constitutive activation signals including phosphorylation of CD3ζ and help in initiating T-cell activation.[28] A related src-family kinase Fyn is also expressed in T cells and is weakly associated with the TCR complex. Fyn appears to have some signaling role because T cells deficient in both Lck and Fyn have a more complete block in T-cell development than Lck-deficient mice.

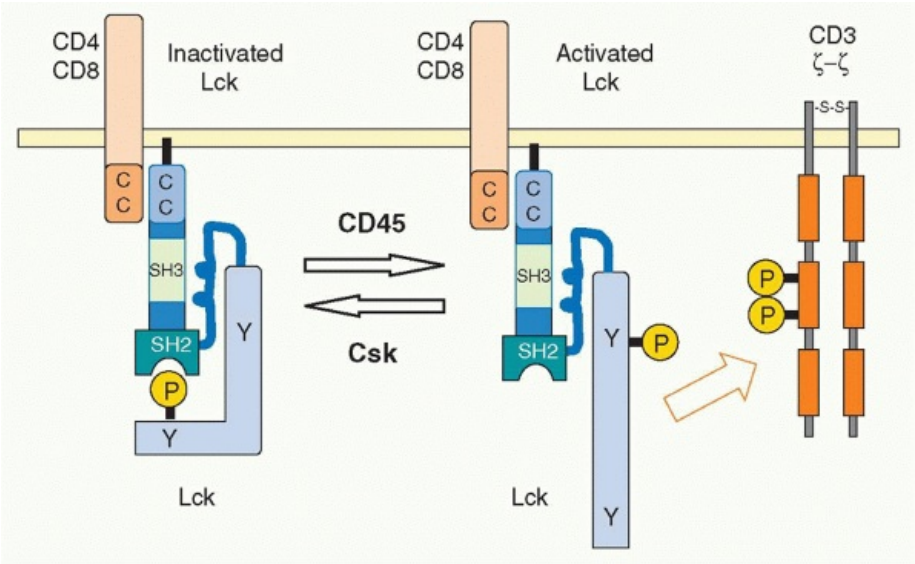

FIG. 12.4. Regulation of the Src Kinase Lck Activation by the Tyrosine Kinase Csk and the Phosphatase Cluster of Differentiation (CD)45. In resting T cells, Lck has a closed configuration through the intra-association between the phosphorylated tyrosine at the C terminus and the Src-homology 2 domain. C-terminus src kinase (Csk) phosphorylates this regulatory tyrosine residue. The association between CD4 and Lck is mediated by the interaction of the cysteine-containing regions of both molecules. CD45 dephosphorylates the C-terminus tyrosine, which alters the configuration from the closed structure as the “inactivated Lck” to an open shape as the “activated Lck” with active kinase function.

Adaptor-Mediated Signaling

Among the targets of ZAP-70-mediated phosphorylation, a transmembrane adaptor protein, linker for the activation of T cells (LAT),[29] and an intracellular adaptor protein, SH2 domain-containing leukocyte phophoprotein of 76 kDa (SLP-76),[30] are the most important substrates (Fig. 12.5). The discovery of these two adaptor proteins revealed the connection between the ZAP-70 tyrosine kinase and PLCγ activation. These adaptor proteins create an important signal assembly to induce downstream activation signals. The critical roles of these two adaptors are evidenced by the observation that T cells deficient in either LAT or SLP-76 exhibit complete defects in TCR activation signaling.

LAT is a transmembrane protein with a short extracellular region and resides in lipid rafts, membrane microdomains enriched in cholesterol and sphingolipids, via two palmitoylation sites within the juxtamembrane region. Mutation of these palmitoylation sites results in the failure of LAT to be expressed on the plasma membrane or to transduce T-cell activation, indicating that the association within lipid rafts regulates trafficking to the membrane. Upon TCR stimulation, LAT becomes tyrosine-phosphorylated on multiple tyrosine residues (five tyrosines among nine conserved residues), and these serve as the docking sites for several SH2-containing molecules. Important effector molecules containing SH2 domains that bind to phosphorylated LAT include PLCγ, growth factor receptor binding protein 2 family members, Grb2 and Gads, and a Tec family kinase, inducible T-cell kinase Itk.[31] Because Grb2 constitutively binds to son of sevenless (Sos), which is a guanine exchange factor (GEF) that mediates guanine triphosphate (GTP) binding of Ras, this LAT-Grb2-Sos complex induces Ras activation; however, RasGRP plays a more critical role for Ras activation than the Grb2-Sos pathway in T cells. Gads binds to phosphor-LAT upon T-cell activation, and Gads associates with SLP-76. Although all tyrosine mutations of LAT lost the function for T-cell activation, the LAT136 mutant mouse exhibits a lymphoproliferative disorder and induction of strong Th2-type cytokine production, suggesting the possibility of a LAT-independent pathway to activate T cells.[32,33]

SLP-76 is a cytosolic adaptor with three domains: an amino terminal region containing three major tyrosines that are phosphorylated upon TCR ligation and become binding sites for various SH2-containing proteins, a central proline-rich domain that binds to SH3-containing proteins, and a carboxy-terminal SH2 domain by which SLP-76 can bind to phosphorylated tyrosines of other proteins.[34] SLP-76 constitutively associates with Gads through its SH3 region, and the SLP-76/Gads complex is recruited to the phosphorylated LAT upon TCR engagement (Fig. 12.6). Both LAT and SLP-76/Gads bind to PLCγ1 independently, and formation of this tetramolecular assembly stabilizes the complex. Because LAT also binds to the Itk Tec family kinase,

which is responsible for phosphorylating PLCγ1, the stable complex of Itk-LAT-SLP-76-PLCγ1 induces PLCγ1 activation. Besides PLCγ1 activation, SLP-76 induces other functions through its association with several other important molecules. At its N-terminus, SLP-76 binds to Vav, a GEF, and Nck, an adaptor protein, both of which are critical for TCR-mediated cytoskeletal changes, and at its C-terminus it binds adhesion- and degranulation-promoting adapter

protein (ADAP),[35,36] an adapter protein coordinating TCR signals with integrin activation (see Fig. 12.12).

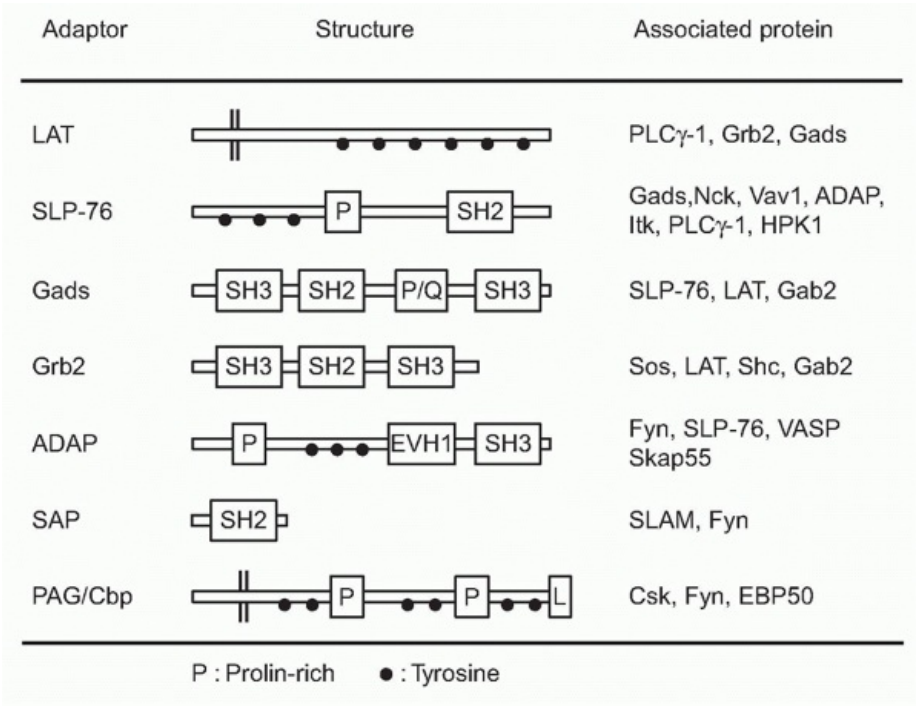

FIG. 12.5. Adapter Molecules Functioning Downstream of T-Cell Receptor Signals. The structure of several adapters described in the text are depicted. These adapter molecules are composed of several modules critical for signal transduction such as Src-homology 2(SH2) and Src-homology 3 (SH3). Whereas tyrosine residues (*black dot*) functions as the docking sites for SH2 upon phosphorylation, the prolin-rich regions (*P*) interact with SH3. Some of these adaptors with the transmembrane regions are localized on the plasma membrane as "membrane adaptors" such as LAT and PAG/Cbp, whereas others within the cytosol as "intracellular adaptors." ADAP, adhesion- and degranulation-promoting adapter protein; Gads, Grb2-related adapter downstream of Shc; LAT, linker for activated T cells; PAG/Cbp, phosphoprotein associated with glycosphingolipid-enriched domains/csk-binding protein; SAP, signal lymphocyte activation molecule-associated protein; SLP-76, SH2 domain-containing leukocyte-specific phosphoprotein of 76 kDa.

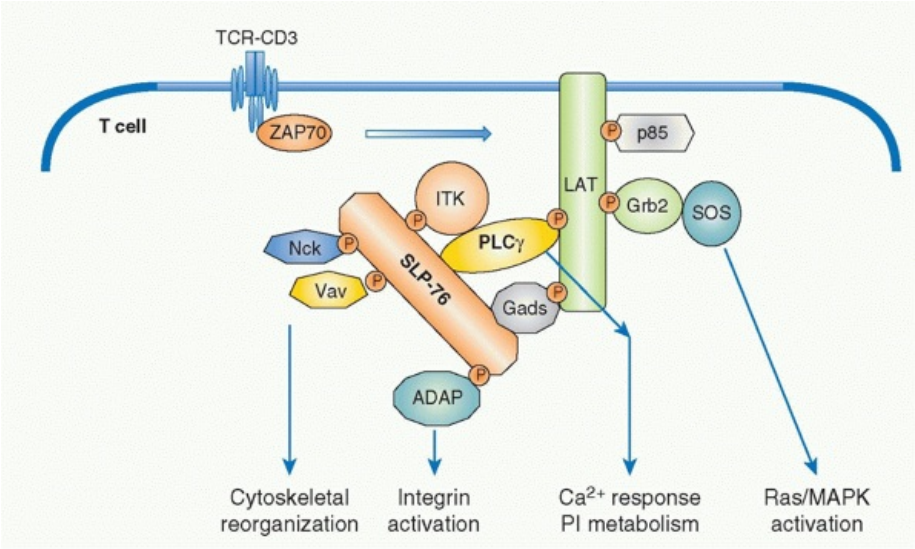

FIG. 12.6. Formation of Multimolecular Signaling Complex Critical for Initial T-Cell Receptor (TCR) Activation Signaling Pathways. Sequential activation of two tyrosine kinases Lck and ZAP-70 upon antigen recognition by TCR induces phosphorylation of two critical adapter proteins LAT and SLP76. Gads constitutively associates with SLP76 and is phosphorylated by TCR activation and recruited to LAT as the Gads-SLP76 complex. Thereafter, several adapter proteins form a physical complex centralized by LAT and SLP-76. Further recruitment of various adapters and effector molecules to the complex leads to the activation of differential signaling pathways. Phospholipase γ1 (PLC-γ1) and Gads link between LAT and SLP76 and a Tec-kinase family ITK, which binds to SLP76, is responsible to phosphorylate/activate PLC γ1. Association of these molecules generates a stable complex of LAT-Gads-SLP76-PLCγ1-ITK critical for further calcium and phosphatidylinositol (PI) responses (as in Fig. 12.7). SLP76 also binds Nck, Vav1, and ADAP, which links to cytoskeletal reorganization (as in Fig. 12.11). LAT binds Grb2/Sos, which induces Ras/MAPK activation (as in Fig. 12.8).

DOWNSTREAM SIGNALING PATHWAYS

The next step of the T-cell activation pathway after ZAP-70-induced phosphorylation of the adaptor LAT and SLP-76 upon TCR ligation is the activation of the key signaling enzyme PLCγ1. PLCγ1 is recruited to the plasma membrane through the binding of its PH domain to PIP3, which is generated within the membrane by the phosphorylation of PIP2 by PI3-kinase. PLCγ1 is activated upon phosphorylation by Itk,[37,38] which is composed of PH, SH2, SH3, and kinase domains, and is also recruited to the plasma membrane through its PH domain by interacting with PIP3. Phosphorylated and activated PLCγ1 then cleaves the membrane bound PIP2 to generate two critical products: the membranebound lipid diacylglycerol (DAG) and the diffusible inositol 1,4,5-triphosphate (IP3), both of which function as second messengers for further inducing downstream signaling.

Calcium-Nuclear Factor of Activated T Cells

Upon TCR stimulation, the levels of intracellular free calcium are regulated in two phases: there is an initial and transient induction of calcium release from storage in the ER, and this is

followed by the induction of a strong influx of high levels of calcium from outside of the cells by opening a calcium channel called the calcium release-activated calcium channel (CRAC) in the plasma membrane. IP3 generated by PLCγ1 catalysis diffuses into the cytoplasm and binds to IP3 receptors on the ER membrane. IP3 receptors are calcium channels and, after IP3 binding, they open and allow the release of the calcium stored within ER into the cytosol. The released low level of calcium induces cluster formation by the calcium-binding transmembrane protein, stromal interaction molecule-1 (STIM1), within the ER.[39] STIM1 is an ERresidential protein that contains an N-terminal sterile motif and paired EF hands that function as a calcium-binding motif and a coiled-coil motif at the C terminus. A critical CRAC called Orai1 has been identified from the analysis of a patient with severe combined immunodeficiency who had a major defect in lymphocyte activation.[40] Clustered STIM1 on the ER membrane is colocalized and assembled with Orai1 on the nearby plasma membrane to open the channel and introduce extracellular calcium into the cytosol (Fig. 12.7). Recent analysis has shown that oligomerization of STIM1 is sufficient to induce CRAC activation independent of ER calcium store depletion, but the mechanism by which STIM1 induces Orai1 oligomerization remains unknown.

Surprisingly, STIM1, STIM2 (another family member), and Orai1 are dispensable for T-cell development as deficiency of these molecules does not affect this process. Because calcium flux is also induced and is important in thymocytes, it is assumed that there must be other functionally redundant and critical calcium channels in thymocytes.[41]

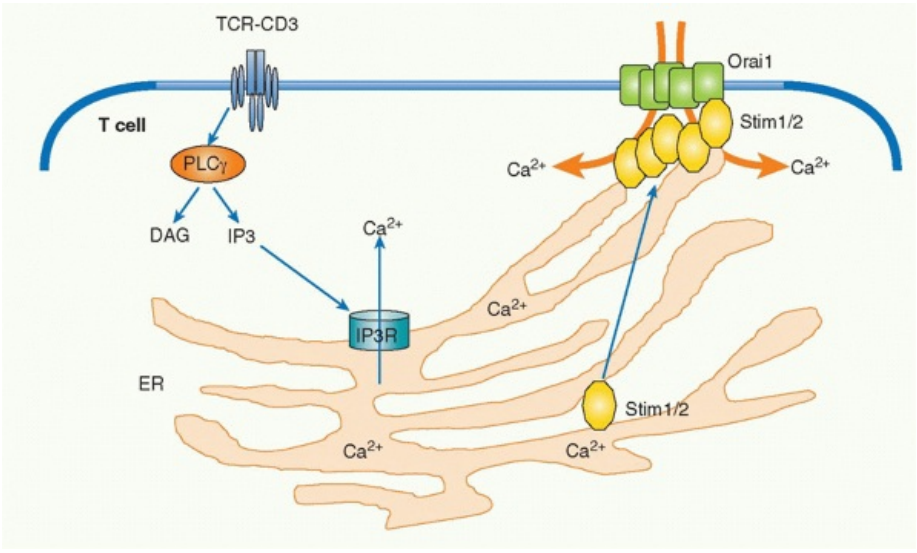

FIG. 12.7. The Mechanism of Calcium Response in T-Cell Activation. T-cell receptor engagement induces activation of PLCγ1 and generates inositol trisphosphate (IP_3), which binds to the IP_3 receptors (IP3R) present on the endoplasmic reticulum (ER) and stimulates the calcium release from intracellular calcium pool within the ER. Increase of intracellular calcium level activates a calcium-binding paired EF hands-bearing protein Stim1/2, which then translocates to the vicinity of the plasma membrane. Stim1 localizes with and induces dimerization of ORAI calcium release-activated calcium modulator 1 (Orai1), resulting in opening functional calcium release-activated calcium channel. The assembly between Orai1 and Stim1/2 induces the activation pathway dependent on

intracellular calcium concentration.

Upon T-cell engagement, intracellular calcium binds to a protein, calmodulin, and induces a conformational change that allows the protein to bind to various target proteins. An increase of cytosolic calcium induces activation of signaling molecules including calcium calmodulin-dependent kinase and a phosphatase calcineurin. Activated calcineurin acts on a critical transcription factor, nuclear factor of activated T cells (NFAT), to dephosphorylate the protein, which induces its translocation into the nucleus.[42] There are five NFAT family members (NFATc1, c2, c3, c4, and NFAT5), and they are expressed in many different tissues.[43] NFAT is present in the cytoplasm in the resting state in T cells. Cytosolic serine/threonine kinases such as glycogen synthase kinase 3 and casein kinase 2 phosphorylate the nuclear localization signal on NFAT to prevent its translocation into nucleus. Upon antigen stimulation, activated calcineurin dephosphorylates this critical phosphorylation site, which allows NFAT to enter into the nucleus, where it triggers various gene expression programs including various cytokine genes by cooperating with other transcription factors. The most well-characterized gene among NFAT targets is the interleukin (IL)-2 gene. NFAT binds to the IL-2 promoter by forming a cooperative complex with activator protein (AP)-1, which is a heterodimer composed of members of the Jun and Fos family, to induce IL-2 transcription. NFAT and AP-1 represent two signaling pathways of calcium and Ras/MAPK, respectively. It has been shown that T-cell activation in the absence of the induction of AP-1 results in anergic or unresponsive T cells.[44]

Ras-Mitogen-Activated Protein Kinase

In parallel with IP3, DAG is generated by PLCγ and remains localized within the plasma membrane where it recruits various signaling molecules that contain a specific DAG-binding motif (called the C1 domain), including members of the protein kinase C (PKC) family[45] and RasGRP,[46] a guaninenucleotide exchange factor for Ras. DAG binding activates PKC and promotes RasGRP to activate Ras by exchange from the guanosine diphosphate (GDP) to GTP-binding form.[47] Ras is active in the GTP-bound form, and this activation step is mediated by GEFs and suppressed by GTPaseactivating proteins (GAPs). RasGRP and Sos are two major GEFs in the TCR activation pathway (Fig. 12.8). In T cells, RasGRP functions dominantly for early activation of Ras. RasGRP is inducibly associated with the plasma membrane through binding of its C1 domain to DAG and is phosphorylated by PKCθ. On the other hand, Sos constitutively associates with an adaptor protein Grb2 and the Grb2 SH2 domain binds to phosphorylated LAT upon T-cell activation; Sos-Grb2 binds to LAT-SLP76, which results in its recruitment to

the vicinity of the TCR. The relationship between RasGRP and Sos appears to be cooperative rather than competitive as RasGRP-mediated Ras activation enhances Sos activity as a positive feedback to induce strong Ras activation.[48]

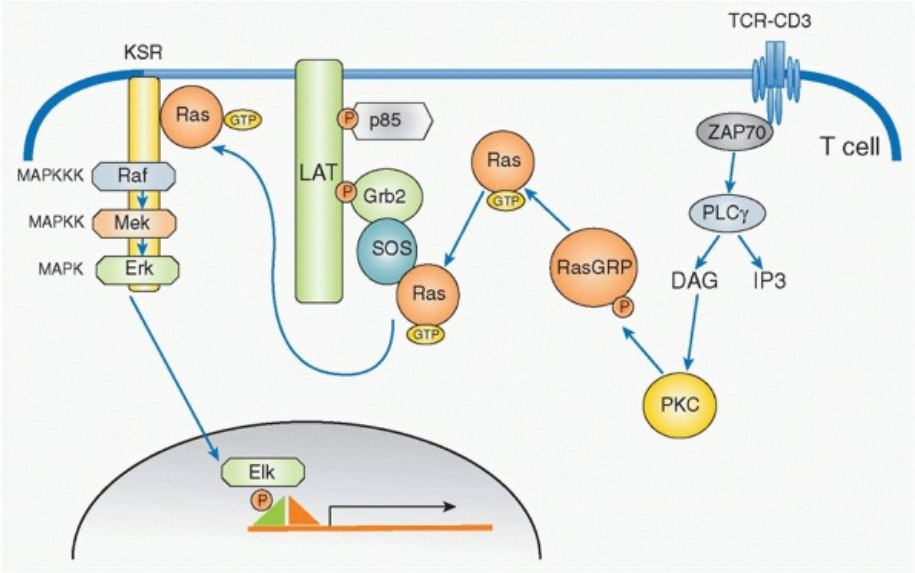

FIG. 12.8. Ras Activation Pathways by T-Cell Receptor (TCR) Stimulation. Activation of Ras in T cells following antigen engagement involves activation of two different guanine triphosphate (GTP) exchanging factors: one is Ras-GRP, and the other is son of sevenless (Sos). TCR activation induces diacylglycerol generation by PLCγ1, which recruits Ras-GRP to the membrane where Ras-GRP is activated by PKCθ. Activated Ras-GRP induces Ras activation by exchanging guanosine diphosphate (GDP) to GTP, which then binds to Sos, because Sos constitutively assembles with Grb2 and the Grb2-SOS complex is recruited to LAT upon TCR activation. Activated Ras then induces the activation cascade of mitogen-activated protein kinase which sequentially contains MAPK kinase kinase (Raf), MAPK kinase (Mek), and MAPK (Erk).

Ras activation triggers a cascade of kinase activation, which finishes by activating a serine/threonine kinase known as a mitogen-activated protein kinase (MAPK). The MAPK cascade is composed of three kinases: MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K), and the MAPK itself. In the case of the TCR signaling pathway, the first MAP3K is Raf, which is a serine/threonine kinase that phosphorylates the next MAP2K, which in T cells is MEK1. MEK1 is called a dual-specificity kinase because it can phosphorylate both a tyrosine and a threonine of the last member of the cascade, the MAPK extracellular signal-regulated kinase 1 (Erk1) and Erk2 in T cells and B cells, respectively. Erk1/2 induces the activation of Elk1, which in turn activates the AP-1 transcription factor complex composed of Fos and Jun.

PKC-CARMA1/Bcl-10/Malt1-Nuclear Factor of KappaB

Historically, it has long been known that phorbol ester (phorbol 12-myristate 13-acetate) plus a calcium ionophore, a molecule that allows calcium ions to cross cell membranes, induce strong activation signals similar to TCR-induced signals.[49] Phorbol ester is known to activate PKC; thus, PKC is a critical component in T-cell activation. T cells predominantly express the PKCθ isoform of protein kinase C. PKCθ has a DAG-binding domain, is recruited to the plasma membrane through binding to DAG, and is activated upon generation of DAG by PLCγ1. One of the main pathways activated by PKCθ is NF-κB. PKCθ plays a critical role in

initiating several cascades of the classical pathway of NF-κB activation downstream of the TCR. NF-κB is a family of transcription factors composed of homo- and heterodimers of five members of the Rel family including NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel (Rel).[50] In resting T cells, NF-κB is found in the cytosol associated with one of a family of inhibitors of NF-κB (IκB) that prevents NF-κB from translocating into the nucleus. Upon T-cell activation, IκB is phosphorylated by the IκB kinase (IKK) complex, ubiquitinylated and degraded, which allows NF-κB to translocate into nucleus, where it activates various genes involved in survival, homeostasis, and activation of T cells and inflammatory responses.

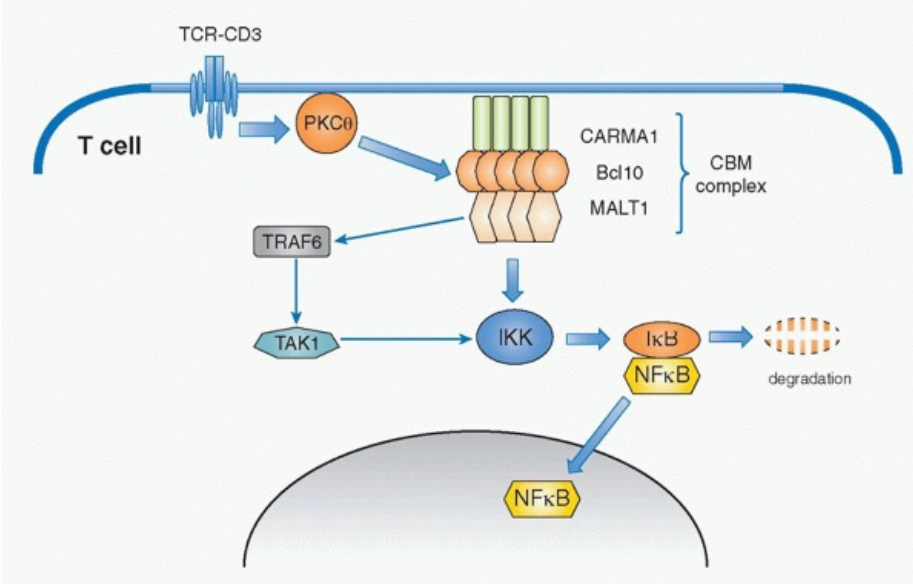

FIG. 12.9. Signaling Pathway for T-Cell Receptor (TCR)-Mediated NF-κB Activation. Upon TCR stimulation, PKCθ is activated and induces phosphorylation and aggregation of caspase-recruitment domaincontaining membrane-associated guanylate kinase protein-1 (CARMA1). Aggregated CARMA1 forms a complex (as the CBM [CARMA1-Bcl-10-Malt1] complex) with two other proteins: B cell lymphoma 10 (Bcl-10) and mucosal-associated lymphoid-tissue lymphoma-translocation gene 1 (Malt1). Malt1 induces the degradation of the regulatory component of IκB kinase (IKK) complex (IKKγ) by ubiquitination via activation of tumor necrosis factor receptor-associated factor-6. IKKγ degradation induces phosphorylation and subsequently degradation of the inhibitor of NF-κB (IκB), which results in activation and nuclear translocation of NF-κB.

Whereas this signaling pathway to activate NF-κB is common among many cell types and has been known for quite some time, the specific pathway by which PKCθ induces NF-κB activation has only been elucidated in the past decade. The critical molecule responsible for mediating the activation signal that connects PKCθ and NF-κB is now known as the CBM complex (Fig. 12.9). It consists of three proteins; a scaffold protein, CARMA1 (caspase recruitment domain [CARD] and membrane-associated guanylate kinase -containing scaffold protein), a CARD-containing

adaptor protein, B-cell lymphoma 10 (Bcl10), and mucosaassociated lymphoid tissue

lymphoma translocation gene 1 (Malt1).[51,52,53] Upon TCR stimulation, this trimolecular complex is induced by PKCθ-mediated phosphorylation of CARMA1, which is required for CARMA1 oligomerization and association with Bcl10. Malt1 binds to Bcl10 and mediates the degradation of the IKKγ subunit (also called NEMO) by inducing polyubiquitination through activation of an E3 ubiquitin-ligase, tumor necrosis factor receptor-associated factor 6 (TRAF6).[55,56] TAK1 recruited by TRAF6 phosphorylates IKK, which in turn induces IκB phosphorylation. This event, together with degradation of NEMO, induces consequently IκB degradation and release and translocation of NF-κB to the nucleus, and thereby induces gene activation.

Overexpression of the CBM complex induces spontaneous NF-κB activation without T-cell (or B-cell) activation. Recently, it has been shown that a high proportion of diffuse large B-cell lymphomas are induced by specific oncogenic mutations of CARMA1, which induce its spontaneous oligomerization of CARMA1, formation of the CBM complex, and subsequent continuous chronic activation of NF-κB, ultimately resulting in tumor induction.[57] This illustrates the importance of maintaining appropriate control of the CBM complex-mediated NF-κB activation for appropriate immune responses without inducing excess inflammation and diseases.

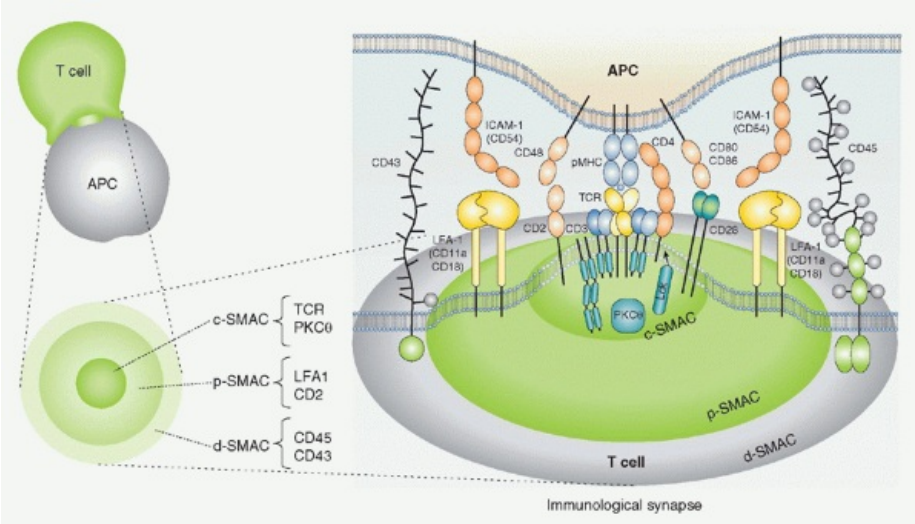

FIG. 12.10. Schematic Structure of the Immunologic Synapse. Antigen recognition and activation of T cells starts by the specific interaction between T cells and antigen-presenting cells (APCs) (**left**). In the interface of two cells, special structure with segregated arrangement of receptors and signaling molecules is induced where T-cell receptros (TCRs) and major histocompatibility complexes (MHCs) are accumulated in the central region termed as the central supramolecular activation cluster (SMAC), the adhesion molecule leukocyte function-associated antigen (LFA)-1-intercellular adhesion molecule 1 is localized at the surrounding area around the TCR-MHC (as the peripheral SMAC), and further large molecules such as cluster of differentiation (CD)45 are located outside peripheral region SMAC as distal SMAC (**right**). This “bulls eye-like” structure is dynamically induced after T cell-APC contact; LFA-1 moves outside and the TCR-CD3 moves into the center within 10 minutes. Activation signals are induced not at the synapse but at TCR microclusters, which are generated at the interface between T cell

and APCs prior to central SMAC formation, which is composed of TCR, kinases, and adaptors.

THE IMMUNOLOGIC SYNAPSE

T-cell activation is induced upon recognition of antigen peptide/MHC on antigen-presenting cells (APCs) such as DCs or B cells by the TCR on T cells upon the antigen-specific interaction between these two cell types. The interaction induces a specific structure with molecular segregation at the interface between the two cells, which is called the immunologic synapse (IS) or supramolecular activation cluster (SMAC).[58,59] In the IS, several surface receptors and intracellular signaling molecules are accumulated and segregated to form the concentric "bulls-eye"-like structure composed of three discrete regions (Fig. 12.10); there is enriched accumulation of molecules such as TCR, CD28, CD2, and PKCθ in the central region (cSMAC), adhesion molecules such as integrin LFA1 in the peripheral region (pSMAC), and large molecules such as CD43 and CD44 in the distal region (d-SMAC). The IS, particularly the cSMAC, was initially thought to be the structure responsible for transducing signals that lead to

T-cell activation. However, the formation of IS takes as long as 10 minutes after cellular conjugate formation, whereas the initial activation events such as intracellular calcium mobilization and protein phosphorylation occur within 1 minute, indicating that the activation signal is initiated much earlier than the IS is formed.[60] Recent studies revealed that the real signal transducing structure, called the TCR microcluster, is generated immediately after the T cell-APC contact and prior to IS formation.[61,62,63] TCR microclusters contain the TCR, kinases such as Lck and ZAP70, adaptor proteins such as LAT and SLP76, and several effector molecules such as PLCγ and PI3K. TCR microclusters function as the signalsome to induce T-cell activation because all TCR microclusters induce tyrosine phosphorylation of various proteins including CD3ζ, ZAP-70, and PLCγ, and their generation occurs in parallel to the initiation of the intracellular calcium flux. Therefore, the TCR microcluster is thought to be the minimal functional unit for TCR signaling. TCR microclusters are generated initially in the periphery of the interface between T cells and APCs, and are then translocated into the central region of the interface to make the cSMAC.[64]

Contrary to the initial idea that the cSMAC is the site for signal transduction, it is now thought that the cSMAC is the place where the accumulated TCR complex is internalized and degraded. In contrast to the negative function of the cSMAC for TCR degradation, costimulation receptors such as CD28 and cytotoxic T-lymphocyte antigen (CTLA)-4 accumulate in the cSMAC, suggesting that the cSMAC also serves a signaling function for costimulation.[65] In addition, T-cell activation results in the polarization of the T cells and movement of the microtubule organizing center toward the TCR-MHC/peptide contact sites. The microtubule organizing center movement appears to be driven by localized accumulation of DAG[66] and also involves ADAP, both of which recruit the microtubule motor protein dynein. cSMAC formation is closely regulated by cytoskeletal arrangement and leads to the polarization of T cells for polarized secretion of secretory vesicles including cytokines, chemokines, and lytic factors such as granzyme B. Various cytokine-containing vesicles are

accumulated at the TCR engaged site and are released directionally and locally. During the T cell-APC interaction, whereas some of cytokines such as interferon γ, IL-2, and IL-4 are secreted directionally toward the interface, some as tumor necrosis factor (TNF)α are randomly secreted.[67]

TRIGGERING MECHANISM

Signaling mechanisms for T-cell activation have been extensively analyzed and are now well understood, as described previously. It has been known for a long time that the antigen recognition event by the TCRαβ dimer is transmitted to the ITAM phosphorylation of the CD3 chains, which induce signal transduction for T-cell activation. However, the actual mechanism by which antigen recognition by the TCR initiates the initial activation event of the signal transduction cascade is still widely debated and controversial. There are several different models to explain the first events of T-cell triggering, and the proposed models can be divided into two main conceptual categories. One is through the receptor clustering by ligand binding, and the other is through the induction of conformational changes within the TCR-CD3 complex. None of these models or their variations (see the following discussion) can satisfactorily account for the diverse experimental observations regarding TCR triggering.

1. Kinetic proofreading model. This is an early quantitative model that attempted to explain a T-cell response by the half-life of the TCR-MHC/peptide interaction.[68] The length of time between the initial ligand binding and receptor signaling was proposed to induce qualitatively differential signaling. Longer and shorter half-life would induce a stronger and weaker response by either strong or weak agonist, respectively.[69]
2. Serial triggering model. T-cell activation requires a sustained signal that lasts for several hours. However, TCR affinity for MHC/peptide is very low, and activation of TCR induces only a brief spike of intracellular signals. This model attempts to resolve this paradoxical requirement for T-cell activation. The model states that sustained signaling is accomplished by the concerted action of multiple TCRs that are sequentially engaged with and triggered by the MHC/peptide complex.[70] A single complex could serially engage and trigger up to approximately 200 TCRs.
3. Kinetic segregation model. Signal initiation takes place by excluding inhibitory molecules such as the phosphatase CD45 from the tight contact area (cSMAC of the IS) between T cell and the APC, thereby shifting the enzymatic steady state toward an activating status. Such segregation is induced by sizes of the ectodomains of the excluded proteins.[71] This model is supported by the structure and kinetics of IS, as shown by the segregated regions.
4. Permissive geometry model. Binding between TCR dimers and MHC/peptide dimers induces rotational scissor-like conformational changes in the CD3 chains that reveal previously hidden intracellular activation motifs. This model integrates receptor clustering and conformational change models, together with the existence of preformed oligomeric receptors, providing a mechanism to explain TCR signal.[72]
5. Multimerization model. This model proposes that the minimum unit of T-cell activation is a dimer and is based on antibody-induced dimerization of B-cell receptors and MHC structure studies. A single MHC/peptide could induce initial and transient calcium signaling,

but monomeric MHC/peptide in solution did not induce stimulation of T cells. Obviously, more multioligomeric ligands induce more intense T-cell activation.[73]

6. Conformational change model. Extensive analyses of the crystal structures of TCR-MHC/peptide have not revealed any significant structural changes upon MHC/peptide binding by the ectodomains of TCRαβ dimers. However, biochemical analyses demonstrated structural changes of TCR complex, particularly in CD3ε. A

proline-rich region of CD3ε is a hidden determinant in the resting state but is exposed and recruits Nck upon stimulation to induce downstream activation signals.[74]

7. Safety model. This model is based on recent findings that the cytoplasmic domains of CD3ε and CD3ζ have affinity for the acidic lipids present on the plasma membrane, which may result in prevention of ITAM phosphorylation. Conversely, these chains in free aqueous solution may be readily phosphorylated by Lck. These results have led to the following model: whereas CD3εζ tails are tightly associated with the lipid-rich inner membrane of plasma membrane in resting T cells, they are released from this membranebound configuration and can be phosphorylated upon TCR ligation.[75]
8. Pseudodimer model. Only a few MHCs carry foreign cognate peptides for the specific TCRs, whereas a great majority of MHCs carry endogenous self-peptides. This model proposes that endogenous MHC/peptide amplify signals produced by agonist MHC/peptide by promoting TCR aggregation. Agonist MHC/peptide-TCR recruits a second TCR in a CD4-dependent manner, which binds endogenous MHC/peptide, stably forming a pseudodimer that triggers T-cell activation.[76]

The clustering models have been claimed to be incompatible with the presence of preformed oligomeric receptors on the surface of resting cells. However, the recent finding that TCR microclusters are the functional unit to transduce activation signals shows that aggregation of preformed TCR “nanoclusters” may form microclusters to induce signals.[77,78,79] On the other hand, models based on CD3 conformational changes that induced as a direct effect of ligand binding are not consistent with the requirement for multivalent ligand to initiate TCR signaling. Structural analyses of the complete molecular complex, including transmembrane regions and cytoplasmic tails, will provide a definite answer to this still confusing issue.

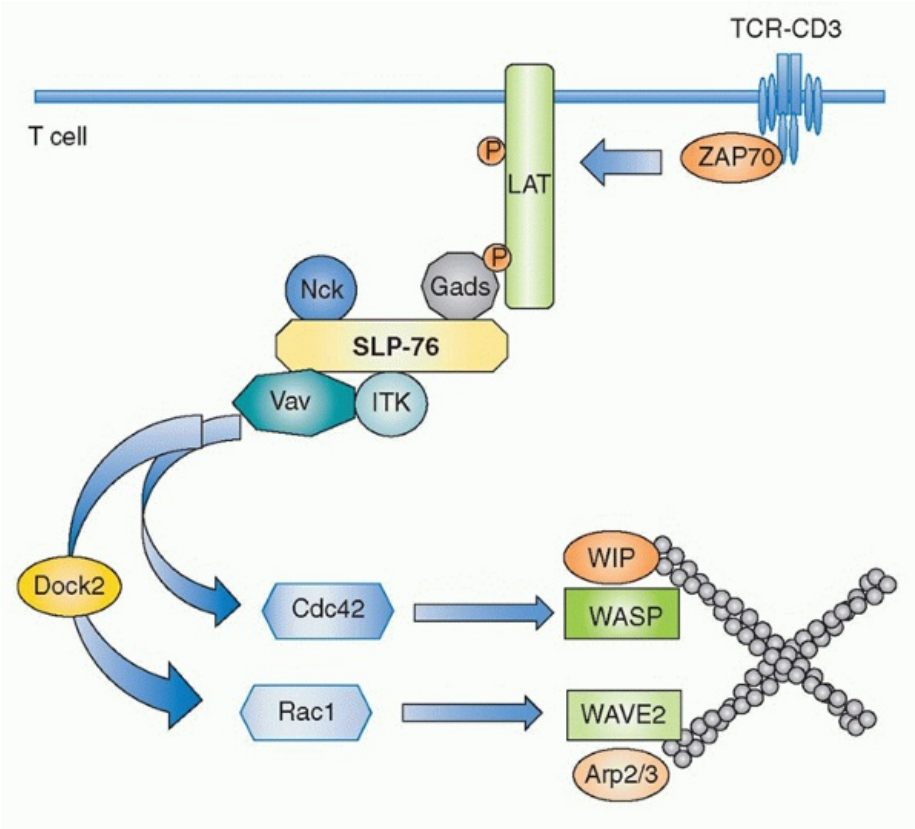

FIG. 12.11. Cytoskeletal Regulation by T-Cell Receptor (TCR) Signaling. TCR stimulation induces actin reorganization, which is required for the alteration of cell shapes, cell movement, and receptor clustering. Actin-related proteins such as Arp2/3, WASp, and WAVE2 regulate accumulation of F-actin. The LAT-SLP-76 signaling cluster induced upon TCR engagement recruits Nck, Vav1, and Itk. Whereas WASp binds to the adapter protein Nck and is activated by the Rhofamily GTPase Cdc42, WAVE2 binds to Vav1 and is activated by another Rho-family GTPase Rac1 through Dock2.

CYTOSKELETAL REGULATION

T-cell stimulation also induces a program for alteration of the cytoskeleton: actin polymerization, a microtubule rearrangement, which induces polarization and activation of T cells, and consequently changes the shape and regulates adhesion and movement of T cells. T cell-APC interaction results in morphologic changes: the cell becomes round and accumulates actin filaments at the stimulatory interface. These changes reflect the increase of fluidity of plasma membrane and a decrease of cellular motility. Accumulation of filamentous actin at the T cell-APC interface is the results of TCR-induced localized activation of multiple actin regulatory and polymerizing pathways. T-cell activation induces SLP76 phosphorylation, which recruits actin cytoskeleton regulatory proteins, Itk, Vav-1, Nck, and Cdc42 as the regulatory proteins. Localized activation of Cdc42 stimulates the actin-related protein 2/3 (Arp2/3)-regulating protein WASp, Wiskott-Aldrich syndrome protein, which interacts with Arp2/3 and activates actin polymerization.[80] WASp is recruited to the TCR activation complex by association with Nck and is activated by assembly with Vav-1, a GEF for the Rho family of GTP binding proteins and Vav-1, a central regulator of cytoskeleton,

migration, and adhesion (Fig. 12.11). WASp functions as a regulator of the Arp2/3 complex that is critical for actin polymerization. Itk recruits Vav-1 to the immune synapse. Cdc42 regulated by the Itk-Vav-1 complex directly stimulates WASp actively, and the Nck-WASP complex regulates actin dynamics. Vav-1-mediated activation of a second Rho family, GTPase Rac1, results in the activation of WAVE2.

CELL ADHESION REGULATION—INSIDE-OUT SIGNALING

Antigen stimulation of T cells induces their strong adhesion to APCs. This adhesion is mediated mainly by activation of integrin on T cells such as leukocyte function-associated antigen (LFA)-1, increasing its affinity and avidity for the ligand on APCs. Integrin activation is induced by a signaling cascade initiated by the TCR engagement and is a process termed inside-out signaling (Fig. 12.12). The inside-out signaling by TCR engagement results in integrin clustering and an increase in affinity and avidity for the ligands, which then induces high-affinity ligand binding.[81] It has been shown that the integrin LFA-1 goes through three conformational changes to achieve high affinity ligand: resting state, transitional state, and active conformation.[82] The integrins predominantly expressed on T cells are LFA-1 and very late antigen-4, which bind to their ligands, intercellular adhesion molecule (ICAM)-1 and 2, and vascular cell adhesion molecule and fibronectin, respectively. The process of inside-out signaling from TCR to integrin activation has been shown to include downstream signaling and actin-cytoskeletal rearrangement. Several critical adaptor molecules, particularly Rap1, ADAP, SKAP55, and RIAM, are necessary to translate TCR engagement to integrin activation.

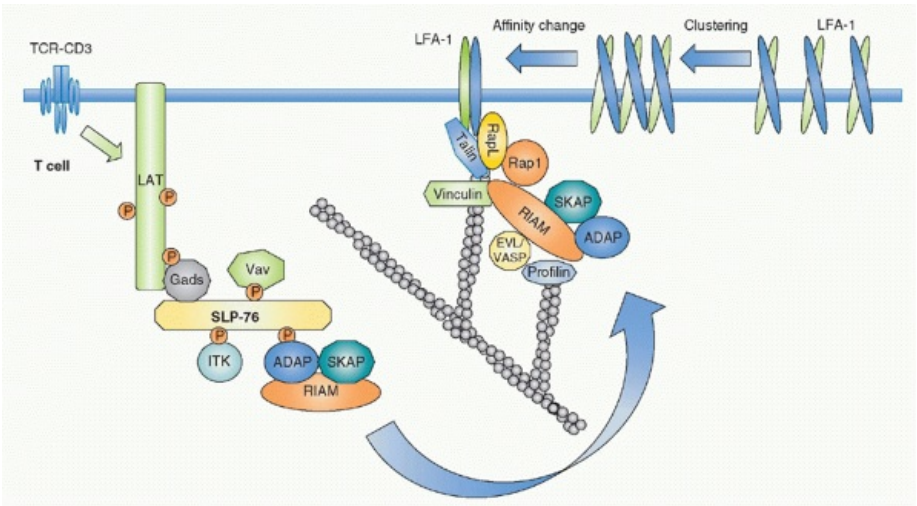

FIG. 12.12. Inside-out Signaling for T-Cell Receptor (TCR)-Mediated Integrin Activation as the Mechanism of TCR-Induced Cell Adhesion. TCR engagement induces signaling complex of linker for the activation of T cells and SLP-76. SLP-76 recruits Vav1 and Itk important for actin reorganization, as in Figure 12.11, and also adhesion- and degranulation-promoting adapter protein (ADAP), which constitutively associates with Src kinase-associated phosphoprotein of 55 kDa (SKAP55) and Rap1-GTP-interacting adapter molecule (RIAM). The complex of ADAPSKAP55-RIAM associates with and activates Rap1 upon TCR activation and then translocates Rap1 to

the membrane. The Rap1-RIAM/SKAP/ADAP complex induces the association of talin with the tail of integrin β. The local accumulation of talin is required for high-affinity binding by integrin. Integrin changes the status by clustering and then affinity maturation through structural changes. Integrin clustering involves Rap1 and Rap1-binding molecule RAPL that also associates with RIAM. RIAM associates with F-actin through the association with EVL/VASP proteins and the actin-binding protein profilin. PKD binds to the β1 integrin tails and recruits and activates Rap1 by Rap1-GEF C3G.

Ras proximity 1 (Rap1), which belongs to the Ras superfamily of GTPases, plays a critical role in the inside-out signaling for integrin activation. Active Rap1 induces increased cell adhesion through integrin activation without changing their expression levels on T cells.[83] TCR stimulation and chemokines induce Rap1 activation, which then induces clustering of high-affinity LFA-1 on the leading edge of the membrane.

Following TCR ligation, SLP-76 inducibly interacts with ADAP, which then forms a complex with SKAP55. The SLP-76-ADAP-SKAP55 pathway leads to regulation of T-cell adhesion.[84] Both ADAP- and SKAP55-deficient T cells showed defective LFA-1 clustering and adhesion. ADAP-induced T-cell adhesion involves Rap1-GTP-interacting adaptor molecule (RIAM), which is required for Rap1 localization at the membrane.[85] Because RIAM associates with integrin, the complex with ADAP-SKAP55-RIAM makes a complex with LFA-1, resulting in adhesion to ICAM-1 upon TCR ligation. RIAM also binds to a cytoskeletal binding protein, Talin, and the Talin-RIAM-Rap1 complex may induce Talin to bind LFA-1 with high affinity. RIAM is also the ligand of Ena/VASP and profiling, which bind to actin cytoskeleton. In addition, similar to Talin, other cytoskeletal proteins such as Vinculin, WAVE2, and the Arp2/3 complex are also involved in the TCR-induced integrin activation.

As to the effector mechanism of Rap1 signaling, Rap-1 binds to RAPL, the effector regulator of cell adhesion and polarization enriched in lymphoid tissues.[86] The interaction induces the membrane localization of RAPL and the binding to the αL subunit of LFA-1, which is critical for LFA-1 clustering and increasing its affinity. RAPL associates with a kinase Mst-1 as an effector molecule. Inside-out signaling induced by TCR engagement results in integrin clustering and increased affinity and avidity for the ligand, which then induces high-affinity binding to the ligand. Further analysis of the precise signaling cascade for inside-out signaling downstream of TCR is required.

COSTIMULATION

Cluster of Differentiation 28-Mediated Costimulation

T-cell activation is influenced by signals through several surface receptors including costimulation receptors (see following discussion), cytokine receptors, and adhesion molecules. T cells cannot be fully activated to proliferate and mediate cytokine secretion in the absence of costimulation signals through these costimulation receptors. TCR engagement induces various early activation signals such as an increase in the levels of intracellular calcium, tyrosine phosphorylation, and inositol metabolism, but these are not sufficient to induce full activation including cytokine production, cell proliferation, and effector

functions. Instead, in the absence of costimulation, T cells fall into a state of unresponsiveness or anergy where they do not respond to antigen stimulation. Therefore, two signals are required for efficient T-cell activation: “signal 1” is defined as the antigen-specific TCR signal by the binding of peptide/MHC complex, and “signal 2” refers to the additional costimulation signal through a costimulation receptor. This “two signal model” for T-cell activation, which was proposed in the early study of T cells,[87,88] has been proved by various observations, particularly of the peripheral tolerance induction in the absence of “signal 2.”[89,90] Although there are a number of receptors that can induce a costimulatory signal, the most physiologic, critical and well characterized is CD28[91] (Fig. 12.13A).

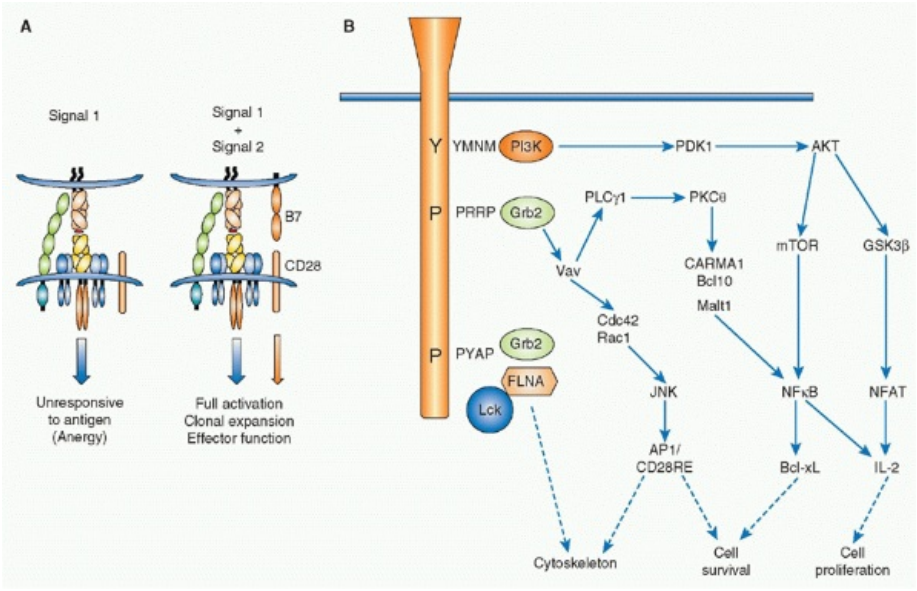

FIG. 12.13. Costimulation and Cluster of Differentiation (CD)28-Mediated Signals in T Cells. A: Full T-cell activation such as cell proliferation and cytokine secretion requires two signals: one (signal 1) through the T-cell receptor-CD3 complex as antigen-recognition signal, and the other (signal 2) through costimulation receptors such as CD28 as costimulatory signals. In the absence of costimulation signal, T cells become unresponsive to antigen stimulation (called anergy). **B:** Detailed signal pathway of CD28-mediated costimulation. The cytoplasmic tail of CD28 has major three motifs: N-terminus tyrosine motif YMNM associates with PI3K leading to the activation of Akt, which induces cell survival through mammalian target of rapamycin-mediated NFκB activation and cell proliferation, the upper Prolin-rich motif binds to Grb2 leading to Vav1 activation, which mediates cytoskeletal regulation and NFκB activation, and the last proline region associates with Lck and Filamin and Lck, which leads to actin-cytoskeletal organization.

CD28 is constitutively expressed on the surface of all T cells as a homodimer. The ligands for CD28 are the B7 family molecules, CD80 (or B7-1) and CD86 (B7-2), which are expressed on APCs. While CD80 is constitutively expressed, CD86 is expressed at low level on APCs in the resting state but is highly induced upon activation by innate signals, such as those

derived from pathogens, in parallel with the induction of high level expression of MHC class II.[92] The induction of MHC class II and B7 molecules on activated APCs such as mature DCs are critical for T-cell stimulation because they can deliver a strong signal 1 and signal 2, respectively.

CD28 induces a profound increase in IL-2 production by both transcriptional and posttranscriptional regulatory mechanisms, as well as increased proliferation and cell survival, which is partly due to the induction of the antiapoptotic gene Bcl-xL (Fig. 12.13B). Whether CD28 induces costimulation totally independently of TCR engagement has been a controversial question for a long time. CD28-specific signals responsible for the costimulation signal have been extensively analyzed. CD28 has a short cytoplasmic tail (41 amino acids in human, 38 amino acids in mouse) and no intrinsic enzymatic function. It contains several tyrosines including an YxxM motif and two PxxP motifs. The YxxM motif, which is conserved in CD28, CTLA-4, and inducible costimulator (ICOS), is a consensus motif for the binding of the p85 subunit of the lipid kinase phosphatidyl-inositol 3-kinase (PI3K). Functional relevance of the YxxM motif is suggested by the fact that PI3K activation is enhanced by CD28 signaling.[93,94] PI3K activation generates PIP2, which recruits various molecules containing pleckstrin homology domains such as Tec family kinases, a serine/threonine kinase Akt, phospoinositide-dependent kinase 1, Vav, and WASP to the plasma membrane. Akt can be phosphorylated by phospoinositide-dependent kinase 1 and plays an important role in cell survival, which is one of the benefits of CD28-mediated costimulation.[95] The PxxP motif, which ICOS and CTLA-4 do not have, also has binding specificity for Grb2/Gads. Grb2 recruits Sos and Vav, which in turn phosphorylates and activates Rac1 and Cdc42, which then activates the MEKK1 cascade to ultimately activate the Jun kinase JNK.[96] Whereas the TCR signal predominantly activates ERK as an MAPK family member, CD28 engagement induces activation of the other member of the MAPK family, JNK, and the balance between the activity of ERK and JNK as a consequence of CD28-mediated costimulation may regulate cell survival.

Early studies showed that the distal PxxP (PYAP) motif in CD28 may recruit Lck, and it was recently shown that filamin A also binds to this motif to connect to the cytoskeleton. Although in vitro analysis and an overexpression system indicated a critical function for these motifs in costimulation, in vivo analysis with specific knock-in mutations revealed that mice with mutated YMNM motifs that failed to bind PI3K and activate Akt had no overt phenotype. By contrast, mice with the mutant distal motif had evidence of impaired CD28-mediated costimulation, such as reduced proliferation and IL-2 production.[97,98]

Enhancement of IL-2 production by CD28 engagement is regulated at both transcriptional and posttranscriptional levels.[99] A specific region responsible for CD28-mediated transcriptional activation (termed a CD28-response element, CD28RE) was found in the IL-2 promoter.[100] The binding of transcription factors to CD28RE is dependent on CD28 signal.

In addition to enhancement of the transcription of the IL-2 gene, CD28-mediated costimulation augments IL-2 production by increasing the stability of IL-2 mRNA.[101] Many cytokine transcripts including IL-2 contain adenylate-uridylate (AU)-rich elements within the 3' untranslated region that stabilizes the message. Upon TCR stimulation, an AU-binding protein, TTP, binds to the AU-rich elements within the 3' untranslated region and induces

degradation of the mRNA. By contrast, CD28 costimulation induces other proteins, such as NF90, a transcription factor associated with NFAT, which may compete with TTP for binding at the AU-rich elements and enhance IL-2 mRNA stability.

CD28 signaling activates I-κB and consequently NF-κB. CD28 regulates the IKK activation step, and IKK activation results in I-κB activation/degradation and induces NF-κB activation. Extensive analysis to define the connection between CD28 signaling and IKK activation resulted in the discovery of CARMA1 as caspase recruitment domain-containing membrane-associated guanylate kinase protein-1, followed by identification of its associated proteins, Bcl-10 and Malt-1, which together form the CBM complex.[102,103] CARMA1 is phosphorylated by PKCθ and binds to Bcl10 through a CARD-CARD domain interaction.[104] The CBM complex is responsible for activation of NF-κB, thus T cells defective in any of the CBM components show impaired NF-κB activation and impaired T-cell proliferation. CD28-mediated activation of the CBM complex and NF-κB dramatically enhances IL-2 production.

Given its powerful activity as a second signal for T-cell activation, CD28 agonists and antagonists have been seriously considered for clinical application in humans. CD28 superagonist monoclonal antibodies were shown to polyclonally activate T cells in vivo, but such treatment ultimately led to the expansion of regulatory T cells, and several studies showed therapeutic benefit in autoimmune and inflammatory disease models in mice and rats. After preclinical safety testing in nonhuman primates, six healthy human volunteers were injected with the TGN1412 CD28 superagonist monoclonal antibodies, and the results were disastrous.[105] All six individuals suffered an immediate and life-threatening release of systemic proinflammatory cytokines, a “cytokine storm” that is now termed cytokine release syndrome. Recent studies suggest that the source of these cytokines was the effector memory population of T cells and have clarified why no such adverse effects were seen in rodents and nonhuman primates. Rodents do not accumulate a large population of effector memory population of T cells because the generation of these cells requires repeated exposure to infections; these experimental animals are kept in extremely clean conditions. The CD4 T

cells in macaques, the nonhuman primate used in the preclinical studies, unlike human T cells, lose CD28 expression when they differentiate into effector memory population of T cells.

COSTIMULATION THROUGH RECEPTORS OTHER THAN CLUSTER OF DIFFERENTIATION 28

CD28 is most prominent costimulation receptor and thus CD28-deficient mice have a general impairment in immune responses. However, not all immune response are dampened by CD28 deficiency, which indicates that other costimulation molecules can compensate for some of the remaining functions. These costimulation receptors include CD2, CD5, CD30, the Ig gene superfamily member ICOS, the TNF receptor family members CD137 (4-1BB) and CD134 (OX40), and LFA-1. These receptors are expressed on the T-cell surface to induce costimulation signals upon crosslinking with their ligands (Fig. 12.14).

In contrast to CD28, which is constitutively expressed on T cells, ICOS is expressed at a very low level on resting T cells and is inducibly expressed on activated T cells upon TCR

stimulation, which is the major difference in its function from CD28 on resting T cells.[106] As ICOS-deficient mice have impaired immune responses similar to that seen with CD28 deficiency, ICOS must play an important role in immune regulation.[107] ICOS binds to a novel B7 family member, ICOS ligand, which is expressed rather broadly as compared to CD80/86 including nonhematopoietic tissues upon stimulation with inflammatory cytokines. ICOS is expressed on the cell surface as a dimer similar to CD28 and shares some structural features with CD28, including the YMXM motif in the cytoplasmic tail that binds p85 of PI3K. ICOS-mediated PI3K activation is stronger than that mediated by CD28, whereas other signals through CD28 that are not triggered through ICOS include Grb2 binding.[108] Such differences in signaling between ICOS and CD28 lead to differential function. Most notably, unlike CD28, ICOS does not induce IL-2 gene transcription. Instead, ICOS-mediated costimulation is critical for B-cell help for Ig production and germinal center formation. ICOS deficiency causes one of the multiple forms of common variable immunodeficiency, and these patients suffered from impaired B-cell function and germinal center formation and from hypogammaglobulinemia including IgA deficiency.[109]

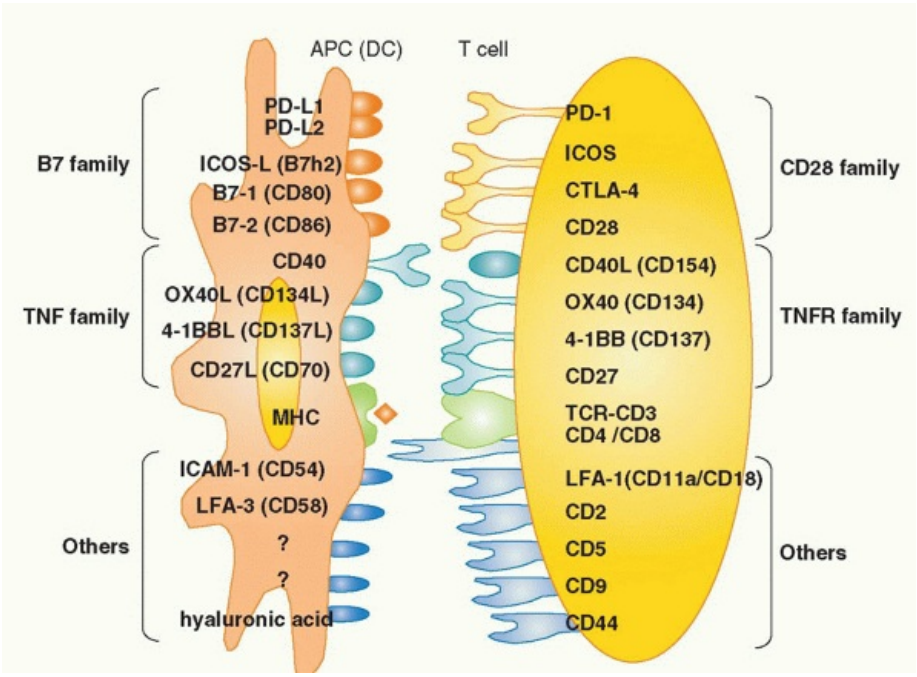

FIG. 12.14. Various Costimulation Receptors and Their Ligands. Various adhesion molecules between T cells and antigenpresenting cells (APCs) function to mediate costimulation signals for T-cell activation. These molecules are divided into three groups: cluster of differentiation (CD)28 family, tumor necrosis factor receptor family, and others including integrin leukocyte function-associated antigen-1. CD28 family molecules consist of two positive (CD28, inducible costimulator) and two negative (cytotoxic T-lymphocyte antigen [CTLA]-4, programmed death-1) receptors and the individually corresponding ligands. Whereas some receptors are constitutively expressed such as CD28, others are expressed upon T-cell stimulation such as CTLA-4 and OX-40. Whereas some of the ligands are expressed exclusively on APCs, others are widely expressed on variety cell types.

Among TNF receptor family molecules, the molecules with costimulatory function represent CD27, OX40 (CD134) and 4-1BB (CD137), CD30, herpes virus entry mediator A (HVEM), and glucocorticoid-induced TNF-receptor (GITR). Among them, OX40 and 4-1BB mediate the most prominent functions. These molecules augment T-cell activation upon engagement of their ligands, OX40L and 4-1BBL, respectively. Engagement of OX40 and 4-1BB with their ligand induces activation of signaling pathways similar to CD28: PI3K and Akt, and NF-κB and MAPKs (JNK and p38), but this is through activation of the TNF receptor-associated factor (TRAF) family of adaptor molecules, which is different from CD28.[110]

One of the most important functional differences between CD28 and others such as ICOS, OX40, or 4-1BB is that CD28 is constitutively expressed on naïve T cells and plays a critical role to stimulate naïve T cells for proliferation, effector function, and functional differentiation, whereas all the others are inducibly expressed upon T-cell activation and therefore play roles to induce costimulation on activated

effector T cells and memory T cells. Thus, there is a big difference in the timing of their function during immune responses. In addition, the distribution of the ligands for these two groups is different: stimulation through CD28 has to be induced by professional APCs such as DCs and macrophages, whereas costimulation through ICOS, OX40, and 4-1BB can even be induced by ICOS-L, OX40L, and 4-1BB-L expressed on nonhematopoietic cells in the periphery. The latter is critical for regulation of autoimmunity and infectious diseases.

Signal Lymphocyte Activation Molecule Family for T-Cell Activation

Signal lymphocyte activation molecule (SLAM; CD150) is a receptor of a family expressed on various cell types including T cells, B cells, NK cells, macrophages, and other cells, which mediate homotypic cell adhesion. This homotypic interaction induces costimulation in T cells. However, the signaling mechanism for SLAM-mediated costimulation has been unclear. Studies of the human immunodeficiency disease X-linked lymphoproliferative syndrome were quite informative when mutations in the SLAM-associated protein (SAP; SH2D1A) gene were identified as the cause of X-linked lymphoproliferative syndrome.[111] SAP was found to associate with SLAM through homotypic interaction upon activation. SAP is a small intracellular adaptor protein consisting of only single SH2 domain. SAP was found to associate with the src kinase Fyn and appears to function as a bridge between SLAM and Fyn (Fig. 12.15). This binding mode is quite unique in that the phosphotyrosine of SLAM binds SAP through its SH2 domain, whereas the SH3 domain of Fyn binds through an atypical proline-rich domain in the SH2 domain of SAP.[112] This unusual binding configuration has been confirmed by crystal structure analysis of the complex. The SAP-Fyn interaction is critical for optimal activation of PKCθ and NF-κB upon T-cell activation. In addition, SAP-induced signaling is important to communicate with B cells for delivering T-cell help for Ig production. Follicular T cells express ICOS and SLAM family members and may provide help for B-cell function in germinal center and humoral responses.[113,114]

The SLAM family is composed of six different members, SLAM (CD150), 2B4 (CD244), NTBA (Ly108), Ly9 (CD229), CD84, and CRACC (CD319), all of which associate with SAP. Mice deficient in each of these molecules exhibit a weak phenotype, probably indicating that the family members have overlapping and redundant functions.

NEGATIVE REGULATION OF ACTIVATION

T-cell activation is induced to trigger appropriate immune responses and must be regulated to have the right timing, to be at the right location, and to have the proper strength. To appropriately regulate these responses, it is necessary to impose negative regulation in order to promote a balanced response, to prevent an excessive response, and to terminate the response properly. As described previously, TCR stimulation induces several activation pathways, but simultaneously also induces negative regulatory pathways.

Inhibitory Effector Enzymes

T-cell activation is initiated by phosphorylation by the src family kinase Lck, and this most proximal signaling event is actively regulated by the balance between the kinase Csk and the phosphatase CD45, as described previously (Fig. 12.4). Positive and negative regulation is actively and in parallel induced downstream of TCR signaling. Therefore, Csk is the dominant negative regulator of TCR proximal signal by phosphorylating the inhibitory tyrosine on Lck, thus main-

taining it in the inactive state. CD45 has an opposing function by dephosphorylating this negative regulatory tyrosine and activating Lck.

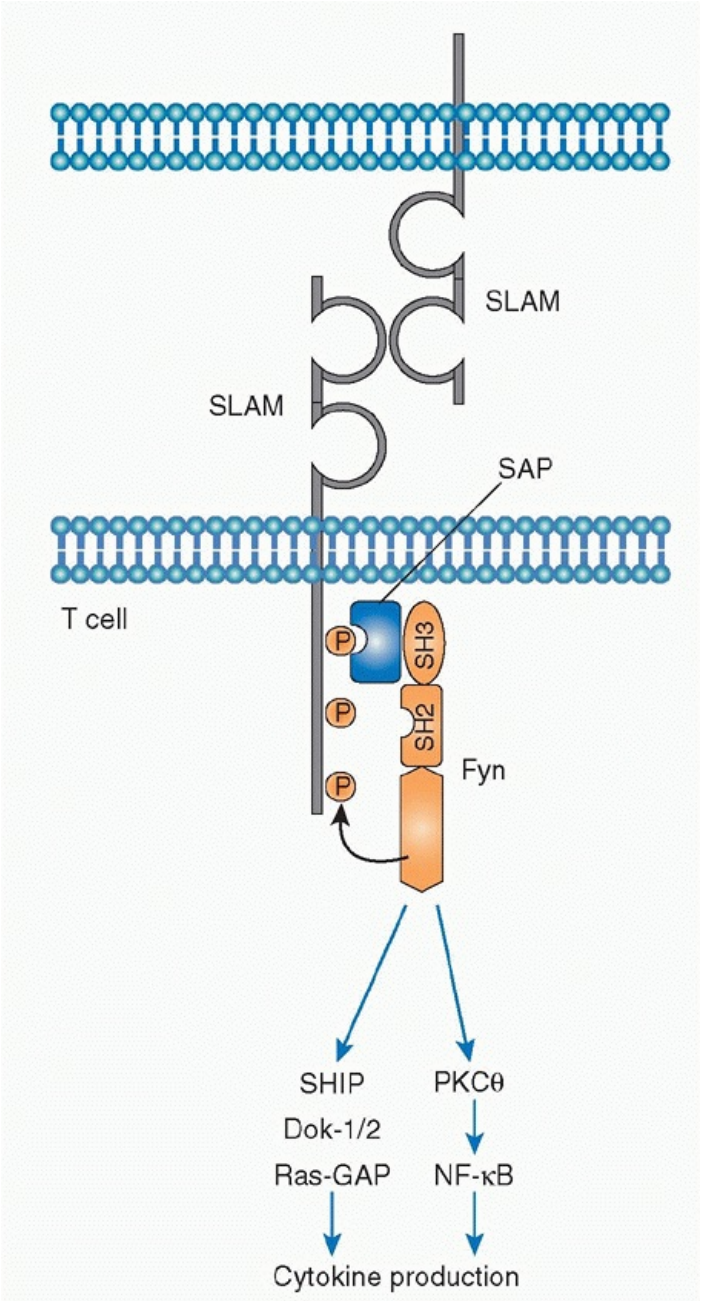

FIG. 12.15. Signal Transduction by Signal Lymphocyte Activation Molecule (SLAM) Family Members Through the Association with the Adaptor Protein SLAM-Associated Protein (SAP). The arginine 78 of SAP binds the SH3 domain of Fyn, which recruits Fyn to the SLAM/SAP complex. Fyn subsequently phosphorylates tyrosine residues in the cytoplasmic domain of SLAM, which serve as the docking sites for SH2 domain-containing inositol phosphatase, which then phosphorylates the adaptor proteins Dok1/2 and Ras-GAP. SAP also binds to PKCθ and activates NF-κB through CARMA1/Bcl-10/Malt1 complex, which cooperates with T-cell receptor signals.

Another set of inhibitory effector enzymes is a family of E3 ubiquitin ligases, which are responsible for ubiquitination of proteins with certain specificity to lead to degradation. These are represented by Cbl and Cbl-b,[115] and also include others such as GRAIL, Itch, and Nedd4. Cbl proteins mediate ubiquitination and targets for degradation of early signaling

proteins include ZAP-70, effectively inducing downregulation of T-cell activation. Evidence of the important negative regulatory role of these proteins in vivo comes from the analysis of Cbl- and Cbl-b-deficient mice, which display hypercellularity and spontaneous T-cell activation.[116,117] It has been shown that the anergic/unresponsive status of T cells is induced by these E3 ligases, particularly Cbl and Itch, by degradation of various signaling molecules.

Immunoreceptor Tyrosine-Based Inhibitory Motif-Mediated Negative Regulation by Phosphatases

Whereas the ITAM recruits the SH2-bearing tyrosine kinase ZAP-70/Syk to induce activation signals in T cells, the immunoreceptor tyrosine-based inhibitory motif (ITIM) recruits SH2-bearing phosphatases to induce negative signals.[118] The ITIM consensus amino acid sequence is very similar to the ITAM: S/I/V/Lx Y xx I/V/L. Receptors containing ITIM include killer inhibitory receptors, which mainly function in NK cells to inhibit activation upon recognition of MHC and also inhibit killing by cytotoxic T cells, the inhibitory receptors of various paired receptors expressed on NK cells, DCs and macrophages such as PIR-B and PILR, and the inhibitory IgG Fc receptor FcγRIIB on B cells, macrophages, and mast cells. The tyrosine residue of an ITIM in T cells is phosphorylated by the src family kinase Lck, similar to an ITAM, which then recruit phosphatases such as the SH2 domain-containing phosphatase (SHP)-1 and SHP-2 and the SH2 domain-containing inositol phosphatase (SHIP).

SHP-1/2 is cytosolic protein phosphatase containing twotandem SH2 domains at the N terminus and a phosphatase domain at the C terminus. The phosphatase is in an inactive closed configuration in the resting stage and becomes active upon tyrosine phosphorylation of the ITIM. These enzymes are recruited to the vicinity of the TCR complex by association with Lck and then dephosphorylate kinases themselves (Lck and ZAP-70), and upstream signaling molecules close to the TCR complex including Vav, PLCγ1, and SLP-76. The functional importance of SHP-1 is evident by the phenotype of SHP-1-deficient mice, which have severe autoimmunity.[119]

Positive versus negative regulation of Lck kinase activity appears to correlate with the capacity of T cells to discriminate between strong and weak stimuli.[120] It has been shown that weak antigens or antagonists induce rapid phosphorylation of SHP-1 by Lck prior to activation of a positive signaling cascade. SHP-1 then dephosphorylates the active site of Lck, resulting in inhibition of T-cell activation. On the other hand, strong agonistic stimuli induce Erk, which is rapidly activated and phosphorylates Lck. This prevents the phosphorylation and recruitment of SHP-1, resulting in T-cell activation. Whether such a dynamic regulation of the kinase-phosphatase regulatory loop is actually operating in vivo remains to be determined. Despite the similar structure and phosphatase activity, SHP-2 appears to have different specificity for SH2 binding receptors than SHP-1, such as programmed death (PD)-1 (see following discussion).

Another phosphatase critical for negative regulation of T cells is the lymphoid tyrosine phosphatase (Lyp), encoded by the PTPN22 gene. Lyp is a cytosolic protein phosphatase belonging to the prolin-, glutamic acid-, serine- and threonine domain (PEST)-rich family of protein tyrosine phosphatase, and its mouse homolog is the PEST domain-rich phosphatase

(Pep).[121] Lyp dephosphorylates various TCR upstream signaling molecules including CD3, Lck, ZAP-70, and Vav. The negative regulation of Lyp/Pep is mediated by its association with Csk. The Lyp-Csk complex is formed upon T-cell activation and exhibits synergistic negative regulation. Deletion of Pep in mice caused expansion of the memory T cells and increased TCR signaling in effector T cells as well as increased positive selection in the thymus, indicating that Lyp/Pep is a key negative regulator of TCR signaling.[122] Another critical finding is that a single-nucleotide polymorphism in the PTPN22 gene correlates strongly with the incidence of type 1 diabetes, rheumatoid arthritis, juvenile rheumatoid arthritis, systemic lupus erythematosus, Graves disease, generalized vitiligo, and various other autoimmune disease.[123] How such single-nucleotide polymorphism results in increased susceptibility to various autoimmune diseases remains to be determined.

Inhibitory Adaptors

There are several adaptor molecules that function to mediate negative regulation of T-cell activation by recruiting phosphatases.[124] These include SLAP, TSAd, Dok, and Gab2. Dok1,2 and Gab2 recruit rasGAP and SHP-2, respectively. Although the Gab and Dok family are known to be positive regulators of cytokine or growth factor signaling in nonhematopoietic cells, they show opposite inhibitory function in antigen receptor signaling. Gab2- and Dok-1/2-deficiency results in enhanced phosphorylation of upstream signaling molecules and increased T-cell activation including cytokine secretion and proliferation. Dok-1/2 expressed in T cells associates with the phosphorylated ITAM of the CD3ζ chain and therefore inhibits CD3ζ binding with ZAP-70. Dok-1/2 then functions to bridge the TCR complex and rasGAP to inhibit T-cell activation.[125] Gab2 is an adaptor protein and also negatively regulates TCR signaling by recruiting the phosphatase SHP-2. Gab2 associates with LAT through binding with Gads upon T-cell activation. Because Gads constitutively associate with SLP-76 and the Gads-SLP-76 complex binds to phosphorylated LAT, Gab2 is competing with the Gads binding with SLP-76. Upon TCR stimulation, ZAP-70 phosphorylates Gab2, which serves as a bridge between LAT and SHP-2 to mediate inhibitory signals, or

phosphorylates SLP-76, which serves to induce downstream activation signals.[126] It is important to note that expression of these inhibitory adaptor proteins is induced upon T-cell activation, indicating that these inhibitors function in feedback regulation of T-cell activation.

Whereas Dok targets the ITAM of CD3ζ and Gab2-SHP-2 targets LAT, the serine/threonine kinase HPK1 inhibits SLP-76 function by inducible association with its SH2 domain and recruitment of 14-3-3.[127] Deficiency of HPK1 in T cells results in enhanced phosphorylation of early TCR signaling molecules, indicating that HPK1 serves an inhibitory function for T-cell activation in vivo.

Inhibitory Costimulation

In contrast to the positive costimulation receptors leading to activation signals, there also exist sets of costimulation receptors that mediate negative signals for T-cell activation. These include CTLA-4 and PD-1. Neither of these inhibitory costimulatory receptors is expressed in resting T cells, but both are inducibly expressed on activated and specialized T cells, indicating that these inhibitory molecules are involved in the feedback regulation of T-cell

activation to prevent excess activation during immune responses. The physiologic importance of both receptors for inhibition of T-cell activation is evident by the strong phenotype of the respective gene targeted mice. CTLA-4-deficient mice have an excess of activated peripheral T cells, splenomegaly, and infiltration of lymphocytes in nonlymphoid tissues, and exhibit a lymphoproliferative disease in all immune tissues and early death.[128,129] On the other hand, deletion of PD-1 results in less dramatic and slow-onset autoimmune diseases, including the development of a lupus-like disease and cardiomyopathy in old mice.[130]

CTLA-4 and CD28 have common ligands, CD80 and CD86, on APCs (Fig. 12.14). However, as CTLA-4 has much higher affinity for these ligands than CD28, even low expression of CTLA-4 on the T-cell surface upon stimulation can effectively compete for ligand binding with CD28. This is the main mechanism by which CTLA-4 inhibits T-cell activation.[131] Except for regulatory T cells, naïve resting T cells do not express CTLA-4. CTLA-4 is transcribed upon T-cell activation and usually accumulates in the lysosome and is not expressed on the cell surface, probably because such strong inhibitory molecules should be transiently expressed only when needed upon T-cell activation. Upon TCR stimulation, the CTLA-4-containing lysosomes are translocated and fuse with the plasma membrane, and CTLA-4 is then expressed on the cell surface.[132] Once on the cell surface, CTLA-4 can compete for ligand binding with CD28 and thereby push CD28 and the associated PKCθ away from the central region of the interface between T cells and APCs, where the CD28-mediated costimulation signal is mediated.[133] The competition for ligand binding with CD28 by the ectodomain of CTLA-4 is a widely accepted phenomenon, but it has been controversial whether CTLA-4 can also exhibit inhibitory function by recruiting a phosphatase to its cytoplasmic tail in a manner independent of ligand binding. Although it has been shown that CTLA-4 is phosphorylated upon stimulation and several phosphatases including SHP-1/2 and PP2A have been suggested to be involved in CTLA-4-mediated inhibitory function,[131] further analysis is required to clarify this point.

PD-1 is also inducibly expressed on T cells upon TCR stimulation; its ligands are PD-L1 and PD-L2 (Fig. 12.14). Whereas PD-L1 is mainly expressed by professional APCs such as DCs and macrophages, PD-L2 is expressed by a relatively wide range of cells including in nonlymphoid tissues. PD-1 possesses two tyrosine motifs within its cytoplasmic domain: ITIM and immunoreceptor tyrosine-based switch motif, and the immunoreceptor tyrosine-based switch motif (ITSM) is the site for recruitment of the phosphatase SHP-2 upon PD-1 ligand engagement. PD-L1/2 ligation induces SHP-2 phosphorylation and dephosphorylation of various TCR upstream signaling molecules including CD3ζ, ZAP-70, Vav, and Erk, which results in inhibition of T-cell activation.[134,135] There is an interesting contrast between the mechanisms of PD-1 and CTLA-4 inhibition of T-cell activation. Whereas PD-1 inhibits the very proximal signaling just downstream of TCR complex, CTLA-4 mainly suppresses CD28-mediated costimulation signals targeting NF-κB activation. Recent studies indicate that T cells from chronic viral infection and immune senescence are in a nonfunctional or unresponsive/suppressive state that is mediated by PD-1.[136] Because the suppressive state can be reversed by interruption of the interaction between PD-1 and PD-ligand by anti-PD-1 or PD-L1 antibody, PD-1 appears to induce an ongoing active suppression of T-cell activation during the course of chronic infection.

CONCLUSION

We describe here the current knowledge of the mechanisms of T-cell recognition and activation as the central regulator of immune responses. The unique features of the antigen recognition by TCR are twofold. One is gene rearrangement to generate enormous diversity together with the unique T-cell selection processes as positive and negative selection during thymic development. The second is the result of MHC restriction, whereby T-cell recognition of antigen can only occur on the cell surface of APCs in the complex with MHC. That is why T cells communicate with many cell types of APCs.

The TCR genes and the TCR-CD3 complex were cloned and characterized nearly three decades ago, and the peptide-MHC binding structure and TCR recognition of a single MHC/peptide complex were clarified two decades ago. During the ensuing two decades, new insight regarding T-cell recognition and activation was obtained by the discovery of the IS, and more recently TCR microclusters, as the machinery for T-cell activation. The discovery of various T-cell subsets such as Th17 and regulatory T cells during the following decade brings closer to reality the possibility of regulating T-cell function by analyzing the detailed mechanism of their differentiation and function. Further analysis on T-cell recognition, differentiation, and activation can be addressed by using the combination of current technologies of conditional knockout mice with tissue or temporal

specificity, of imaging analysis of the dynamic features of various molecules within a cell at single molecule level, and of in vivo dynamics of lymphocytes, and also system biologic approaches including quantitative analysis of the expression of genes and proteins by proteome analysis, together with the pathway analyses of signaling molecules and modeling of regulatory networks.

Further elucidation of the mechanism of T-cell activation and function will provide benefits for overcoming immunologic diseases. The immunologically important questions, such as repertoire selection, induction, and maintenance of self-tolerance and long-term memory, are still elusive and all are related to the regulation of autoimmune diseases. Therefore, the future development of research in this area should be focused on overcoming these immunologic disorders by clarifying the principles of this regulation. In this regard, analysis should move more toward human diseases. Analysis of patients with immune disorders have revealed the identity of critical genes and regulatory mechanisms such as the calcium channel Orai1 and its activation and regulation, Foxp3 and its regulatory function in regulatory T cells, and SAP and SLAM family receptors. All of these insights came from the analysis of primary immunodeficiency patients. For the cure of such diseases, the development of suitable drugs is also critical in the near future. During the past decade, several immunologic reagents including monoclonal antibodies and inhibitors have been successfully developed and applied, such as FK506 as a potent inhibitor of T-cell activation and critical for organ transplantation, monoclonal antibodies against TNF, IL-6 receptors for various autoimmune diseases, and against JAK3 for therapy of tumors and immune disorders. These therapeutic reagents should be further developed on the basis of our understanding of the fundamental mechanisms of immune regulation. Such new developments should be targeted not only for simple inhibitors of kinases and phosphatases, but also to target the dynamic movement of lymphocytes, transcriptional and epigenetic regulation of gene expression, and the development of antibodies against functional surface molecules of several critical T-cell subsets.

REFERENCES

1. Burnet FM. *The Clonal Selection Theory of Acquired Immunity* . Cambridge: Cambridge University Press; 1959.

2. Yanagi Y, Yoshikai Y, Leggett K, Clark SP, Aleksander I, Mak TW. T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. *Nature*. 1984;308:145-149.

3. Hedrick SM, Cohen DI, Nielsen EA, Davis MM. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins.*Nature*. 1984;308:149-153.

4. Chien Y, Becker DM, Lindsten T, Okamura M, Cohen DI, Davis MM. A third type of murine T-cell receptor gene. *Nature*. 1984;312:31-35.

5. Saito H, Kranz DM, Takagaki Y, Hayday AC, Eisen HN, Tonegawa S. A third rearranged and expressed gene in a clone of cytotoxic T lymphocytes. *Nature*. 1984;312:36-40.

6. Chien YH, Iwashima M, Kaplan KB, Elliott JF, Davis MM. A new T-cell receptor gene located within the alpha locus and expressed early in T-cell differentiation. *Nature*. 1987;327:677-682.

7. Meuer SC, Fitzgerald KA, Hussey RE, Hodgdon JC, Schlossman SF, Reinherz EL. Clonotypic structures involved in antigen-specific human T cell function. Relationship to the T3 molecular complex. *J Exp Med*. 1983;157:705-719.

8. Weiss A, Stobo J. Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. *J Exp Med*. 1984;160:1284-1299.

9. Saito T, Weiss A, Gunter K, Shevach EM, Germain RN. Cell surface T3 expression requires the presence of both α- and β- chains of T cell receptor. *J Immunol* . 1987;139:625-628.

10. Klausner RD, Lippincott-Schwartz J, Bonifacino JS. The T cell antigen receptor: insights into organelle biology. *Annu Rev Cell Biol* . 1990; 6:403-431.

11. Reth M. Antigen receptor tail clue. *Nature*. 1989;338:383-384.

12. Hamerman JA, Ni M, Killebrew JR, Chu CL, Lowell CA. The expanding roles of ITAM adapters FcRgamma and DAP12 in myeloid cells. *Immunol Rev*. 2009;232:42-58.

13. Flaswinkel H, Barner M, Reth M. The tyrosine activation motif as a target of protein tyrosine kinases and SH2 domains. *Semin Immunol*. 1995;7:21-27.

14. Irving BA, Weiss A. The cytoplasmic domain of the T cell receptor z chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell*. 1991;64:891-901.

15. Romeo C, Amior M, Seed B. Sequence requirements for induction of cytolysis by the T cell antigen/Fc receptor z chain. *Cell*. 1992;68: 889-897.

16. Letourneur F, Klausner RD. Activation of T cells by a tyrosine kinase activation domain in the cytoplasmic tail of CD3e. *Science*. 1992;255:79-82.

17. Weiss A, Littman DR. Signal transduction by lymphocyte antigen receptors. *Cell*. 1994;76:263-274.

18. Chan AC, Iwashima M, Turck CW, Weiss A. ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCRz chain. *Cell*. 1992;71: 649-662.

19. Iwashima M, Irving BA, van Oers NS, Chan AC, Weiss A. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science*. 1994;263:1136-1139.

20. Aivazian D, Stern LJ. Phosphorylation of T cell receptor z is regulated by a lipid dependent folding transition. *Nat Struct Biol* . 2000;7: 1023-1026.

21. Samelson LE. Signal transduction mediated by the T cell antigen receptor: the role of adapter proteins. *Annu Rev Immunol*. 2001;20:371-394.

22. Salmond RJ, Filby A, Qureshi I, Caserta S, Zamoyska R. T-cell receptor proximal signaling via the Src-family kinases, Lck and Fyn, influences T cell activation, differentiation and tolerance. *Immunol Rev*. 2009;228:9-22.

23. Veilette A, Bookman MA, Horak EM, Bolen JB. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine kinase p56lck. *Cell*. 1988;55:301-308.

24. Barber EK, Dasgupta JD, Schlossman SF, Trevillyan JM, Rudd CE. The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase (p56lck) that phosphorylates the CD3 complex. *Proc Natl Acad Sci U S A* . 1989;86:3277-3281.

25. Chow LM, Veilette A. The Src and Csk families of tyrosine protein kinases in hemopoietic cells. *Semin Immunol*. 1995;7:207-226.

26. Trowbridge IS, Thomas ML. An emerging role as a protein rytosine phosphatase required for lymphocyte activation and development. *Annu Rev Immunol*. 1994;12:85-116.

27. Schoenborn JR, Tan YX, Zhang C, Shokat KM, Weiss A. Feedback circuits monitor and adjust basal Lck-dependent events in T cell receptor signaling. *Sci Signal*.

2011;13:59.

28. Naka K, Soldani C, Salek M, et al. Constitutively active Lck kinase in T cells drives antigen receptor signal transduction. *Immunity*. 2010;32:766-777.

29. Zhang W, Sloan-Lancaster J, Kitchen J, Trible RP, Samelson LE. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell*. 1998;92:83-92.

30. Jackman J, Motto D, Sun Q. Molecular cloning of SLP-76, a 76kDa tyrosine phosphoprotein associated with Grb2 in T cells. *J Biol Chem*. 1995;270:7029-7032.

31. Samelson LE, Bunnell SC, Trible RP, Yamazaki T, Zhang W. Studies on the adaptor molecule LAT. *Cold Spring Harv Symp Quant Biol* . 1999;64:259-263.

32. Aguado E, Richelme S, Nunez-Cruz S, et al. Induction of T helper type 2 immunity be a point mutation in the LAT adaptor. *Science*. 2002;296:2036-2-40.

33. Sommers CL, Park CS, Lee J, et al. A LAT mutation that inhibits T cell development yet induces lymphoproliferation. *Science*. 2002;296: 2040-2043.

34. Koretzky GA, Abtahian F, Silverman MA. SLP76 and SLP65: complex regulation of signaling in lymphocytes and beyond. *Nat Rev Immunol* . 2006;6:67-78.

35. Peterson EJ, Woods ML, Dmowski SA, et al. Coupling of the TCR to integrin activation by Slap-130/Fyb. *Science*. 2001;293:2263-2265.

36. Griffiths EK, Krawczyk C, Kong YY, et al. Positive regulation of T cell activation and integrin adhesion by the adapter Fyb/Slap. *Science*. 2001; 293:2260-2263.

37. Lewis CM, Broussard C, Czar MJ, Schwartzberg PL. Tec kinases: modulators of lymphocyte signaling and development. *Curr Opin Immunol* . 2001;13:317-325.

38. Berg LJ, Finkelstein LD, Lucas JA, Schwartzberg PL. Tec family kinases in T lymphocyte development and function. *Annu Rev Immunol* . 2005; 23:549-600.

39. Liou J, Kim ML, Heo WD, Jones JW, Myers T. STIM is a Ca^{2+} sensor essential for Ca^{2+}-store-depletion-triggered Ca^{2+} influx. *Curr Biol* . 2005;15:1235-1241.

40. Feske S, Gwack Y, Prakriya M, et al. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature*. 2006;441:179-185.

41. Oh-Hora M, Yamashita M, Hogan PG, Sharma S, Lamperti E. Dual function for the endoplasmic reticulum calcium sensors STIM1 and STIM2 in T cell activation and

tolerance. *Nat Immunol*. 2008;9:432-443.

42. Rao A, Luo C, Hogan PG. Transcriptional factors of the NFAT family: regulation and function. *Annu Rev Immunol*. 1997;15:707-747.

43. Crabtree GR, Olson EN. NFAT signaling: choreographing the social lives of cells. *Cell*. 2002;109:67-79.

44. Macian F, Garcia-Cozar F, Im SH, Horton HF, Byrne MC, Rao A. Transcriptional mechanisms underlying lymphocyte tolerance. *Cell*. 2002;109:719-731.

45. Sedwick CE, Altman A. Perspectives on PKCtheta in T cell activation. *Mol Immunol*. 2004;41:675-686.

46. Dower NA, Stang SL, Bottorff DA, et al. RasGRP is essential for mouse thymocyte differentiation and TCR signaling. *Nat Immunol*. 2000;1:317-321.

47. Downward J, Graves JD, Warne PH, Rayter S, Cantrell DA. Stimulation of p21ras upon T-cell activation. *Nature*. 1990;346:719-723.

48. Roose JP, Mollenauer M, Ho M, Kurosaki T, Weiss A. Unusual interplay of two rypes of Ras activators, RasGRP and SOS, establishes sensitive and robust Ras activation in lymphocytes. *Mol Cell Biol*. 2007;27: 2732-2745.

49. Truneh A, Albert F, Golstein P, Schmitt-Verhulst AM. Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. *Nature*. 1985;313:318-320.

50. Vallabhapurapu S, Karin M. Regulation and function of NFkB transcription factors in the immune system. *Annu Rev Immunol*. 2009;27:693-733.

51. Gaide O, Favier B, Legler DF, et al. CARMA1 is a critical lipid raftassociated regulator of TCR-induced NF-kappa B activation. *Nat Immunol*. 2002;3:836-843.

52. Hara H, Wada T, Bakal C, et al. The MAGUK family protein CARD11 is essential for lymphocyte activation. *Immunity*. 2003;18:763-775.

53. Matsumoto R, Wang D, Blonska M, et al. Phosphorylation of CARMA1 plays a critical role in T cell receptor-mediated NF-kappaB activation. *Immunity*. 2005;23:575-585.

54. Thome M. CARMA1, BCL-10 and MALT1 in lymphocyte development and activation. *Nat Rev Immunol*. 2004;4:348-359.

55. Sun L, Deng L, Ea CK, Xia ZP, Chen ZJ. The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes. *Mol Cell*.

2004;427:289-301.

56. Zhou H, Wertz I, O'Rourke K, et al. Bcl10 activates the NF-kB pathway through ubiquitination of NEMO. *Nature*. 2004;427:167-171.

57. Lenz G, Davis RE, Ngo VN, et al. Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. *Science*. 2008;319:1676-1679.

58. Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Threedimentional segregation of supramolecular activation clusters in T cells. *Nature*. 1998;395:82-86.

59. Grakoui A, Bromley SK, Sumen C, et al. The immunological synapse: a molecular machine controlling T cell activation. *Science*. 1999;285: 221-227.

60. Lee KH, Dinner AR, Tu C, et al. The immunological synapse balances T cell receptor signaling and degradation. *Science*. 2003;302:1218-1222.

61. Yokosuka T, Sakata-Sogawa K, Kobayashi W, et al. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of ZAP70 and SLP-76. *Nat Immunol* . 2005;6: 1253-1262.

62. Bunnell SC, Hong DI, Kardon JR, et al. T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *J Cell Biol* . 2002;158:1263-1275.

63. Campi G, Varma R, Dustin ML. Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling. *J Exp Med*. 2005;202:1031-1036.

64. Saito T, Yokosuka T. Immunological synapse and microclusters: the site for recognition and activation of T cells. *Curr Opin Immunol* . 2006;18:305-313.

65. Yokosuka T, Kobayashi W, Sakata-Sogawa K, et al T. Spatiotemporal regulation of T cell costimulation by TCR-CD28 microclusters and protein kinase C theta translocation. *Immunity*. 2008;29:589-601.

66. Quann EJ, Merino E, Furuta T, Huse M. Localized diacylglycerol drives the polarization of the microtubule-organizing center in T cells. *Nat Immunol* . 2009;10:627-635.

67. Huse M, Lillemeier BF, Kuhns MS, Chen DS, Cavis MM. T cells use two directionally distinct pathways for cytokine secretion. *Nat Immunol* . 2006;7:247-255.

68. McKeithan TW. Kinetic proofreading in T-cell receptor signal transduction. *Proc Natl Acad Sci U S A*. 1995;92:5042-5046.

69. Davis MM, Krogsgaard M, Huse M, Huppa J, Lillemeier BF, Li QJ. T cells as a self-referential, sensory organ. *Annu Rev Immunol*. 2007;25:681-695.

70. Valitutti S, Muller S, Cella M, Padovan E, Lanzavecchia A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature*. 1995;375:148-151.

71. Davis MM, van der Merwe PA. The structure and ligand interactions of CD2: implications for T-cell function. *Immunol Today*. 1996;17:177-187.

72. Minguet S, Schamel WW. A permissive geometry model for TCR-CD3 activation. *Trends Biochem Sci*. 2008;33:51-57.

73. Boniface JJ, Rabinowitz JD, Wülfing C, et al. Initiation of signal transduction through the T cell receptor requires the multivalent engagement of peptide/MHC ligands. *Immunity*. 1998;9:891.

74. Gil D, Schamel WW, Montoya M, Sanchez-Madrid F, Alarcon B. Recruitment of Nck by CD3 epsilon reveals a ligand-induced conformational change essential for T cell receptor signaling and synapse formation. *Cell*. 2002;109:901-912.

75. Kuhns MS, Davis MM. The safety on the TCR trigger. *Cell*. 2008;135:594-596.

76. Krogsgaard M, Li QJ, Sumen C, Huppa JB, Huse M, Davis MM. Agonist/endogenous peptide-MHC heterodimers drive T cell activation and sensitivity. *Nature*. 2005;434:238-243.

77. Kumar R, Ferez M, Samy M, et al. Increased sensitivity of antigen-experience T cells through the enrichment of oligometric T cell receptor complexes. *Immunity*. 2011;35:373-385.

78. Lillemeier BF, Mortelmaier M, Forstner MB, Huppa JB, Groves JT, Davis MM. TCR and Lat are expressed on separate protein islands on T cell membranes and concatenate during activation. *Nat Immunol*. 2009;11:90-96.

79. Saito T. Nanocluster formation: more with memory. *Immunity*. 2011;35:318-320.

80. Billadeau DD, Nolz JC, Gomez TS. Regulation of T cell activation by the cytoskeleton. *Nat Rev Immunol*. 2007;7:131-143.

81. Springer TA, Dustin ML. Integrin inside-out signaling and the immunological synapse. *Curr Opin Cell Biol*. 2012;24:107-115.

82. Luo BH,Carman CV, Springer TA. Structural basis of integrin regulation and signaling. *Annu Rev Immunol*. 2007;25:619-647.

83. Katagiri K, Hattori M, Minato N, Irie S, Takatsu K, Kinashi T. Rap1 is a potent activation signal for leukocyte function-associated antigen 1 distinct from protein kinase C and phosphatidylinositol-3-OH kinase. *Mol Cell Biol* . 2000;20:1956-1969.

84. Kliche S, Breitling D, Togni M, et al. The ADAP/SKAP55 signaling module regulates T-cell receptor-mediated integrin activation through plasma membrane targeting of Rap1. *Mol Cell Biol* . 2006;26: 7130-7144.

85. Lafuente EM, van Puijenbroek AA, Krause M, et al. RIAM, an Ena/VASP and profiling ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion. *Dev Cell* . 2004;7:585-595.

86. Katagiri K, Maeda A, Shimonaka M, Kinashi T. RAPL, a Rap1-binding molecule that mediates Rap1-induced adhesion through spatial regulation of LFA-1. *Nat Immunol* . 2003;4:741-748.

87. Bretscher P, Cohn M. A theory of self-nonself discrimination. *Science*. 1970;169:1042-1049.

88. Lafferty KJ, Cunningham AJ. A new analysis of allogeneic interactions. *Aust J Exp Biol Med Sci* . 1975;53:27-42.

89. Quill H, Schwartz RH. Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. *J Immunol* . 1987;138:3704-3712.

90. Schwartz RH, Mueller DL, Jenkins MK, Quill H. T-cell clonal anergy. *Cold Spring Harb Symp Quant Biol* . 1989;54:605-610.

91. Acuto O, Michel F. CD28-mediated co-stimulation: a quantitative support for TCR signaling. *Nat Rev Immunol* . 2003;3:939-951.

92. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. *Nat Rev Immunol* . 2002;2:116-126.

93. Pages F, Ragueneau M, Rottapel R. Binding of phosphatidylinositol-3-OH kinase to CD28 is required for T-cell signaling. *Nature*. 1994;369:327-329.

94. Rudd CE, Schnaider H. Unifying concepts in CD28, ICOS and CTLA4 co-receptor signaling. *Nat Rev Immunol* . 2003;3:544-556.

95. Kane LP, Andres PG, Howland KC, Abbas AK, Weiss A. Akt provides the CD28 costimulatory signal for up-regulation of IL-2 and IFN-gamma but not Th2 cytokines. *Nat Immunol* . 2001;2:37-44.

96. Su B, Jacinto E, Hibi M, Kallunki T, Karin M, Ben-Neriah Y. JNK is involved in signal integration during costimulation of T lymphocytes. *Cell*. 1994;77:727-736.

97. Friend LD, Shah DD, Deppong C, et al. A dose-dependent requirement for the proline motif of CD28 in cellular and humoral immunity revealed by a targeted knockin mutant. *J Exp Med*. 2006;203: 2121-2133.

98. Dodson LE, Boomer JS, Deppong CM, et al. Targeted knockin mice expressing mutations of CD28 reveal an essential pathway for costimulation. *Mol Cell Biol*. 2009;29:3710-3721.

99. Fraser JD, Irving BA, Crabtee GR, Weiss A. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science*. 1991;251:313-316.

100. Shapiro VS, Truitt KE, Imboden JB, Weiss A. CD28 mediates transcriptional upregulation of the interleukin-2 (IL-2) promoter through a composite element containing the CD28RE and NF-IL-2B AP-1 sites. *Mol Cell Biol*. 1997;17:4051-4058.

101. Lindstein T, June CH, Ledbetter JA, Stella G, Thompson CB. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science*. 1989;244:339-343.

102. Jun JE, Wilson LE, Vinuesa CG, et al. Identifying the MAGUK protein Carma-1 as a central regulator of humoral immune responses and atopy by genome-wide mouse mutagenesis. *Immunity*. 2003;18: 751-762.

103. Gaide O, Favier B, Legler DF, et al. CARMA1 is a critical lipid raftassociated regulator of TCR-induced NF-kB activation. *Nat Immunol*. 2002;3:836-843.

104. Sun Z, Arendt CW, Ellmeier W, et al. PKC-theta is required for TCRinduced NF-kB activation in mature but not immature T lymphocytes. *Nature*. 2000;404:402-407.

105. Hunig T. The storm has cleared: lessons from the CD28 superagonist TGN1412 trial. *Nat Rev Immunol*. 2012;12:317-318.

106. Hutloff A, Dittrich AM, Beier KC, et al. ICOS is an inducible T cell co-stimulator structurally and functionally related to CD28. *Nature*. 1999;397:263-266.

107. Clyle AJ, Lehar S, Lloyd C, et al. The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. *Immunity*. 2000;13:95-105.

108. Harada Y, Ohgai D, Watanage R, et al. A single amino acid alteration in cytoplasmic domain determines IL-2 promoter activation by ligation of CD28 but not inducible costimulator (ICOS). *J Exp Med*. 2003;197:257-262.

109. Grimbacher B, Hutloff A, Schlesier M, et al. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. *Nat Immunol*. 2003;4:261-268.

110. Watts TH. TNF/TNFR family members in costimulation of T cell responses. *Annu Rev Immunol*. 2005;23:23-68.

111. Sayos J, Wu C, Morra M, et al. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through co-receptor SLAM. *Nature*. 1998;395:462-469.

112. Latour S, Roncagalli R, Chen R, et al. Binding of SAP SH2 domain to FynT SH3 domain reveals a novel mechanism of receptor signaling in immune regulation. *Nat Cell Biol*. 2003;5:149-154.

113. Cannons JL, Yu LJ, Hill B, et al. SAP regulates T(H)2 differentiation and PKC-theta-mediated activation of NF-kB1. *Immunity*. 2004;21:693-706.

114. Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature*. 2008;455:764-769.

115. Liu YC. Ubiquitin ligases and the immune response. *Annu Rev Immunol*. 2004;22:81-127.

116. Naramura M, Jang IK, Kole H, Huang F, Haines D, Gu H. c-Cbl and Cbl-b regulate T cell responsiveness by promoting ligand-induced TCR down-modulation. *Nat Immunol*. 2002;3:1192-1199.

117. Bachmaier K, Krawczyk C, Kozieradzki I, et al. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature*. 2000;403:211-216.

118. Unkeless JC, Jin J. Inhibitory receptors, ITIM sequences and phosphatases. *Curr Opin Immunol*. 1997;9:338-343.

119. Tsui HW, Siminovitch KA, de Souza L, Tsui FW. Motheaten and viable motheaten mice have mutations in the haematopoietic cell phosphatase gene. *Nat Genet*. 1993;4:124-129.

120. Stefanova I, Hemmer B, Vergelli M, Martin R, Biddison WE, Germain RN. TCR ligand discrimination is enforced by competing ERK positive and SHP-1 negative feedback pathways. *Nat Immunol*. 2003;4: 248-254.

121. Veillette A, Rhee I, Souza CM, Davidson D. PEST family phosphatases in immunity, autoimmunity, and autoinflammatory disorders. *Immunol Rev*. 2009;228:288-311.

122. Hasegawa K, Martin F, Huang G, Tumas D, Diehl L, Chan AC. PEST domain-

enriched tyrosine phosphatase (PEP) regulation of effector/memory T cells. *Science*. 2004;303:685-689.

123. Bottini N, Musuneci L, Alonso A, et al. A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. *Nat Genet*. 2004;36:337-338.

124. Yamasaki S, Saito T. Inhibitory adaptors in lymphocytes. *Semin Immunol*. 2004;16:421-427.

125. Dong S, Corre B, Foulon E, et al. T cell receptor for antigen induces linker for activation of T cell-dependent activation of a negative signaling complex involving Dok-2, SHIP-1, and Grb-2. *J Exp Med*. 2006;203:2509-2518.

126. Yamasaki S, Nishida K, Sakuma M, et al. Gads/Grb2-mediated association with LAT is critical for the inhibitory function of Gab2 in T cells. *Mol Cell Biol* . 2003;23:2515-2529.

127. Shui JW, Boomer JS, Han J, et al. Hematopoietic progenitor kinase 1 negatively regulates T cell receptor signaling and T cell-mediated immune responses. *Nat Immunol*. 2007;8:84-91.

128. Waterhouse P, Penninger JM, Timms E, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science*. 1995;3:541-547.

129. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity*. 1995;3:541-547.

130. Nishimura H, Okazaki T, Tanaka Y, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science*. 2001;20:337-347.

131. Rudd CE, Taylor A, Schneider H. CD28 and CTLA-4 coreceptor expression and signal transduction. *Immunol Rev*. 2009;229:12-26.

132. Saito T, Yamasaki S. Negative feedback of T cell activation through inhibitory adapters and costimulatory receptors. *Immunol Rev*. 2003;192:143-160.

133. Yokosuka T, Kobayashi W, Takamatsu M, et al. Spatiotemporal basis of CTLA-4 costimulatory molecule-mediated negative regulation of T cell activation. *Immunity*. 2010;33:326-339.

134. Riley JL. PD-1 signaling in primary T cells. *Immunol Rev*. 2009;229: 113-125.

135. Barber DL, Wherry EJ, Masopust D, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*. 2006;439:682-687.

136. Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, et al. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J Exp Med*. 2012;209:1201-1217.

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Chapter 14

Peripheral T Lymphocyte Responses and Function

Marc K. Jenkins

INTRODUCTION

This chapter focuses on the processes by which conventional thymus-derived (T)-lymphocytes that express αβ T-cell antigen receptors (TCRs) become activated by their cognate peptide (p): major histocompatibility complex (MHC) ligand. It will cover how TCR ligation by foreign antigen-derived p:MHC ligands causes rare naïve T cells from the preimmune repertoire to proliferate in the secondary lymphoid organs (sometimes referred to by immunologists as the periphery) to become effector cells capable of producing microbicidal cytokines and eliminating foreign antigens from nonlymphoid tissues. It will also describe how the innate immune response and signals from costimulatory and cytokine receptors shape the functional and migratory properties of effector T cells, and ends with a discussion of how effector cells give rise to diverse memory cell subsets. An emphasis is placed on the dynamics and anatomy of naïve to effector to memory-cell conversion.

The chapter will focus on conventional T cells that express αβ TCRs. The reader is directed to other chapters in the book for details concerning natural killer T cells, γδ T cells, and regulatory T cells.

NAÏVE T CELLS

To understand effector T-cell generation, it is important to consider the naïve T cells from which they are derived. The capacity of the host to make effector and memory cells in response to new foreign proteins depends on the existence of naïve T cells that express αβ TCRs specific for MHC-bound peptides derived from that protein.[1] Naïve T cells with this particular p:MHC specificity are rare members of a vast repertoire also containing naïve T cells specific for other p:MHC ligands. Each naïve T cell expresses a randomly generated TCR, which is produced regardless of whether the relevant foreign antigen is in the host's body. Thus, naïve T cells are defined as T cells that have not yet been stimulated by a p:MHC ligand for which their TCR has a high affinity.

Generation

A naïve T cell is the end result of a complex developmental process that occurs in the thymus.[2] To complete development, each thymocyte must produce an αβ TCR by randomly recombining one of multiple variable (V), diversity (D), and joining (J) deoxyribonucleic acid segments at the *Tcrb* locus and one of multiple V and J segments at the *Tcra* locus.[3] Once an in-frame rearrangement at a locus has occurred on one chromosome, further rearrangement

on the other chromosome is suppressed, although not completely. These processes lead to the situation where most developing thymocytes express a single unique type of αβ TCR.

Thymocytes that successfully express a TCR must then pass positive and negative selection based on the p:MHC specificity of that TCR.[4] Positive selection occurs just after completion of TCR gene rearrangement at the time when thymocytes first express TCRs and coexpress both cluster of differentiation (CD)4 and CD8 coreceptors. To pass positive selection, a thymocyte must receive a TCR signal transduced by low affinity binding to a self p:MHC ligand on radio-resistant thymic epithelial cells in the cortex. It was thought that this process was inefficient due to the improbability of VDJ segment rearrangement by chance producing TCRs with any affinity for a self p:MHC ligand.[5] However, recent work from Marrack and colleagues has shown that all TCRs have germ-line encoded complementarity determining region (CDR) 1 and CDR2 domains within their V segments that bind MHC molecules.[6] This binding has been postulated to cause most thymocytes to undergo negative selection,[7] which is a desirable outcome for the host because these cells could cause autoimmunity if allowed to mature. In this model, the only thymocytes that receive the weak positive selection signal are those that produce a CDR3 through VDJ recombination that partially impairs CDR1 and 2 binding to MHC and allows weak binding to a self-p:MHC complex. Cells that undergo positive selection by recognition of a selfp: MHCI ligand lose CD4 and retain CD8, whereas cells that undergo positive selection by recognition of a self-p:MHCII ligand lose CD8 and retain CD4, in a process controlled by ThPOK and Runx3 transcription factors.[8] In contrast, thymocytes that undergo negative selection either die by apoptosis or differentiate into Foxp3+ regulatory T cells.[9]

Recirculation

CD4+ and CD8+ T cells that complete positive selection exit the thymus and enter the secondary lymphoid organs (lymph nodes, spleen, and mucosal lymphoid organs). Just before leaving the thymus, these cells turn on the KLF2 transcription factor, which promotes expression of several gene products that control thymus exit, such as S1P1, and subsequent circulation through secondary lymphoid organs, such as CD62L and CC chemokine receptor (CCR) 7.[10] T cells also express low levels of CD44 and CD45RO and high levels of CD45RB and CD45RA as they leave the thymus. Over the first 2 weeks after leaving the thymus in adults,

T cells increase the levels of expression of CD28 and the interleukin (IL)-7 receptor α chain, and become better able to proliferate and differentiate if confronted with the relevant foreign p:MHC ligand.[11,12] After completing this maturation process, T cells express a $CD44^{low}$ CD45RO- $CD45RB^{high}$ CD45RA+ CCR7+ CD62L+ $CD28^{high}$ IL-$7R\alpha^{high}$ phenotype, which is used to identify naïve T cells.

Naïve T cells spend their lives (T½ of 2 to 5 years in humans,[13] and 50 to 100 days in mice[14]) recirculating through secondary lymphoid organs. Expression of trafficking molecules that bind ligands that are only expressed on the specialized blood vessels and sinuses of these organs explain this behavior. For example, the extravasation of naïve T cells through the high endothelial venules (HEVs) of lymph nodes and mucosal lymphoid organs depends

on CD62L and CCR7, the ligands for which are only expressed on HEVs.[15] After passing through the HEVs, naïve T cells enter the T-cell-rich paracortical areas of the lymph nodes and mucosal lymphoid organs and are constrained in these regions by sensing CCL19 and CCL21 with CCR7.[16] Similarly, naïve T cells migrate from the marginal zone sinuses of the spleen into the T-cell-rich periarteriolar lymphoid sheathes (PALSs) via an unknown G-protein-coupled receptor-dependent mechanism. Naïve T cells again migrate within the PALSs through CCR7 sensing of CCL19 and CCL21. In all secondary lymphoid organs, naïve T cells are excluded from the B-cell-rich follicles due to lack of expression of CXC chemokine receptor (CXCR) 5, which binds to CXCL13 produced in follicles and guides cells to this location.

Survival

The T-cell areas within lymph nodes contain a network of thin collagen tubes called conduits, which carry lymphborne antigens and chemokines from the subcapsular sinus, where lymph enters the lymph nodes through afferent lymphatic vessels, to sinuses surrounding HEVs.[17] The conduits are wrapped with fibroblastic reticular cells, which produce IL-7. Naïve T cells migrate along the conduits, placing themselves in a good position to receive IL-7, on which they depend for survival.[18]

To survive for a normal lifespan, naïve T cells must also receive TCR signals through weak recognition of self p:MHC ligands, perhaps the ones that caused the T cells to undergo positive selection in the thymus.[1] Dendritic cells are probably the important antigen-presenting cell (APC) for this process because they are constantly in contact with T cells in the secondary lymphoid organs, and expression of MHCII molecules under the control of the dendritic cell-specific CD11c (*Itgax*) promoter is sufficient to maintain the survival of naïve CD4+ T cells. On the other hand, mice lacking dendritic cells by *Itgax* promoter-directed expression of diphtheria toxin have normal numbers of naïve T cells.[19] This result does not rule out a role for dendritic cells in naïve T-cell maintenance, however, because it is possible that the naïve T cells die at a higher than normal rate without dendritic cells but are also produced at a higher rate. Studies on the turnover rate of naïve T cells in dendritic cell-deficient mice will be needed to address this question.

Self p:MHC ligand presentation by dendritic cells has different consequences for naïve T cells than foreign p:MHC ligand presentation. For example, self p:MHC ligand presentation results in only a subset of the signals that emanate from the TCR when bound by a high affinity foreign p:MHC ligand, including partial phosphorylation of the TCR-associated CD3-zeta chain.[20] In addition, although signals through the TCR and IL-7 receptor are required for the survival of naïve T cells,[21] these signals do not cause the T cells to proliferate in hosts containing normal numbers of T cells due to competition for IL-7. The Tsc1[22,23] and Foxp1[24] transcription factors, the second of which regulates IL-7Rα levels, enforce the quiescent survival of naïve T cells in this situation. In contrast, naïve T cells proliferate when the number of T cells is very low and IL-7 becomes more available, for example, early in life or after radiation or chemotherapy. This “homeostatic” proliferation also depends on IL-7 and low-affinity TCR recognition of self p:MHC complexes,[21] but differs from proliferation in response to foreign p:MHC ligands by being independent of the CD28 costimulatory

receptor.[25] Thus, the same signaling events that cause naïve T cells to survive in interphase in T-cell-sufficient hosts cause these cells to proliferate in T-cell-deficient hosts, but using a program different from that engaged during the T-cell response to high affinity TCR ligands.

Survival and proliferation could both contribute to control of the number of naïve T cells in normal hosts. In adults, new naïve T cells are exported from the thymus into the secondary lymphoid organs that are already full of other T cells. Naïve T cells survive in interphase under these conditions. However, in neonates[26] and perhaps the very aged, new naïve T cells enter relatively lymphopenic secondary lymphoid organs and undergo homeostatic proliferation to fill the space. Notably, this proliferation causes the T cells to lose many of the markers that define the naïve phenotype and express surface molecules that are characteristic of memory cells.[21] Thus, although cells with the naïve phenotype dominate the preimmune repertoire, it can contain some cells with a memory cell phenotype.

Abundance

An adult mouse has about 10^8 αβ TCR+ T cells in the secondary lymphoid organs and another 5×10^6 in the blood.[1] The naïve T-cell population is split about 60:40 in favor of CD4+ T cells over CD8+ T cells. About 70% of the cells in both subsets are naïve phenotype cells, at least in young mice housed under specific pathogen-free conditions. Thus, an adult mouse has about 7×10^7 naïve phenotype T cells in its body, which extrapolates to about 3×10^{11} naïve phenotype T cells in an adult human. Because the number of potential TCR amino acid sequences that could be produced by VDJ recombination ($> 10^{15}$)[27] is greater than the number of naïve cells in the body, it is possible that each naïve T cell has a different TCR. Thus, it is possible that each of the 7×10^7 naïve T cells in a mouse has a TCR capable of binding to one of 7×10^7 different foreign p:MHC ligands. This would mean that the frequency of naïve cells expressing TCRs specific for a single p:MHC ligand could be as low as $1/7 \times 10^7$ naïve T cells.

The actual number of naïve T cells specific for single foreign p:MHC-specific ligands has been measured by flow cytometry following labeling with fluorochrome-labeled pMHC tetramers and enrichment with anti-fluorochrome antibody-coated magnetic beads. C57BL/6 mice contain about 200 naïve CD4+ T cells specific for an immunogenic peptide called 2W bound to I-A^b but only about 20 cells specific for peptide 427-441 from the FliC protein of *Salmonella typhimurium* bound to I-A^b.[28] Thus, the frequencies of cells capable of binding to these foreign p:MHCII ligands ranges from about 1:200,000 to 1:2,000,000 of the naïve phenotype CD4+ T cells in mice that were never exposed to these peptides. Similar analyses have been done for foreign p:MHCIspecific CD8+ murine T-cell populations.[29,30,31] The number of naïve CD8+ T cells in C57BL/6 mice ranged from 15 lymphocytic choriomeningitis virus (LCMV) $L_{338-346}$:D^b-specific cells to 1,100 vaccinia virus B8R:K^b- specific cells per mouse.[31]

An important conclusion from these studies is that naïve populations specific for different foreign p:MHC ligands vary in size in a predictable fashion. There is evidence that size

differences between foreign pMHC-specific populations in the preimmune repertoire are caused by negative selection.[32] In other words, naïve foreign pMHC-specific populations can be small because their TCRs by chance bind with high affinity to self p:MHC complexes. Another possibility is that peptides that are recognized by larger naïve populations have structural features that are conducive to recognition by a more diverse set of TCRs (eg, amino acids with prominent side chains).[32,33]

Another important conclusion from these experiments is that the size of naïve p:MHC-specific populations can predict the magnitude of the effector cell response after certain forms of antigen administration. For example, the presence of about 200 2W:I-A^b- and 20 FliC:I-A^b-specific naïve CD4+ T cells correlates with the 100,000 2W:I-A^b- and 15,000 FliC:I-A^b-specific effector T cells induced by injection of the relevant peptides.[28] Similarly, the size of naïve LCMV p:MHCI-specific T-cell populations plays a role the magnitude of the primary CD8+ T-cell response to the virus. LCMV infection of B6 mice activates CD8+ T cells specific for at least 28 different p:MHCI.[31] However, about one-third of the total response is directed against three p:MHCI complexes. The feature of these dominant p:MHCI ligands that correlates best with their potent immunogenicity is the large size of their naïve populations.[31] Therefore, although antigen abundance, efficiency of peptide generation by antigen processing, and MHC binding affinity are important factors in immunodominance,[34] so is naïve T-cell population size.

GENERAL ASPECTS OF EFFECTOR T-CELL FORMATION

Unlike the presentation of low-affinity self p:MHC ligands that maintains the survival of naïve T cells in interphase under nonlymphopenic conditions, the presentation of highaffinity foreign p:MHC ligands induces the specific naïve T cells to produce lymphokines, proliferate, and differentiate into effector T cells that aid in elimination of the antigen from the body. These more dramatic biological effects occur because the responding T cells receive much stronger or more durable signals through the TCR upon recognition of high-affinity foreign p:MHC ligands than they do when recognizing low-affinity self p:MHC ligands.[35] In addition, foreign antigens naturally enter the body during infection or tissue damage, which triggers dendritic cells and other innate immune cells to stabilize foreign p:MHC ligands and induce costimulatory receptors and cytokines.[36,37] The nature of the signals from the TCR, costimulatory receptors, and cytokine receptors then influences the type of effector cells that naïve T cells differentiate into. These three signals and how they vary depending on the nature of the antigen will be described.

Dendritic Cells as Initiating Antigen-Presenting Cells

Arguably, the most important of the three signals is transduced by the TCR pursuant to bind to a foreign p:MHC ligand on an APC. It is clear from ablation experiments that dendritic cells are the only APCs capable of initiating the TCR signal in CD8+ and CD4+ T cells in the spleen.[38] Dendritic cells are also important for initiating APCs for CD4+ T cells in lymph nodes, although other MHCII+ cells can do this job.[38] It has also become clear that many dendritic cell subsets exist.[38,39] A brief synopsis is presented here to give the reader a sense of which dendritic cell types function as APCs for different antigens. Refer to Chapter

16 on dendritic cells for more detailed information on their biology. Dendritic cells are defined in the mouse as cells that express the CD11c integrin, have large amounts of MHCI and MHCII molecules, and reside in or have the capacity to migrate to the T-cell zones of secondary lymphoid organs.[40] The spleen and lymph nodes contain two types of dendritic cells that develop from monocyte-dendritic cell precursors in these organs. One type expresses the myeloid marker CD11b and is often referred to as the myeloid dendritic cell. These dendritic cells are found mainly in the red pulp or marginal zones of the spleen and outer T-cell-rich regions of the lymph nodes. A second type depends on the Batf3 transcription factor, expresses CD8α, and is referred to as the CD8α+ dendritic cell.[41] These dendritic cells are located primarily in the PALSs of the spleen and the T-cell-rich regions of the lymph nodes, and are the major IL-12-producing cells in these locations. Myeloid and CD8α+ dendritic cells turnover rapidly with the latter population having only a 3-day half-life.

Another type of dendritic cell is called the plasmacytoid dendritic cell because of its morphology.[42] These cells develop in the bone marrow and then seed the spleen and lymph nodes from the blood. Plasmacytoid dendritic cells in mice express low amounts of CD11c and the B220 and Gr-1 molecules normally expressed by B cells and granulocytes, and are the most potent producers of type 1 interferons (IFNs)[43] when stimulated through pattern recognition receptors (PRRs).[44] These cells therefore play a key role in inducing an antiviral state in many cell types. The lymph nodes contain several additional migratory CD11c+ dendritic populations that move to this location

from tissues through afferent lymphatic vessels.[39] All lymph nodes contain CD11b+ dendritic cells that also express F4/80 and SIRPα, distinguishing them from myeloid dendritic cells and other CD103+ dendritic cells that often express an intracellular protein called langerin. In the case of the skin, these two types of dendritic cells migrate to lymph nodes from the dermis and are called dermal dendritic cells and dermal langerin-positive dendritic cells. The skin-draining lymph nodes also contain epidermal Langerhans cells, which express langerin and high levels of CD11b and EpCAM. Epidermal Langerhans cells are generated from a local radio-resistant precursor in the skin and are more long-lived than myeloid and CD8α+ dendritic cells.

The dendritic cell type that is most important for the presentation of p:MHC ligands depends on the nature of the antigen. Resident CD8α+ dendritic cells and CD103+ dendritic cell migrants are critical for p:MHCI ligand production from exogenous antigens because these are the only cells in the body that are capable of a process called cross presentation.[41,45] These dendritic cells express receptors such as CD36 that mediate uptake of apoptotic cells. After taking up apoptotic or other extracellular material, these dendritic cells have the unique capacity to move proteins from the phagosome directly into the cytoplasm. Once in the cytosol, the translocated proteins can be cleaved by the proteosome into peptides, which are then pumped by the transporter associated with antigen processing into the endoplasmic reticulum where binding to MHCI occurs. Other cells of the body cannot translocate ingested proteins into the cytosol and thus are only capable of producing p:MHCI complexes from proteins that are translated in their cytosols (eg, their own proteins or proteins from cytosolic microbes that infect them). Thus, the cross presentation pathway is important for initiating the CD8+ T-cell response to viruses that do not infect dendritic cells but kill their host cells. In

contrast, any dendritic cell including plasmacytoid dendritic cells can likely serve as an initiating APC for CD8+ T cells specific for MHCI-binding peptides derived from proteins from viruses that directly infect it.

Early activation of CD4+ T cells specific for p:MHCII ligands derived from soluble antigens can occur in two waves in lymph nodes.[46] Soluble antigens (eg, toxins secreted by bacteria in a subcutaneous infection site) rapidly flow through lymphatic vessels to the draining lymph nodes and into the conduit network. Resident dendritic cells associate with the conduits, then take up the antigen, perhaps from small gaps between the fibroblastic reticular cells that encircle these tubes.[47] These dendritic cells process the antigen, produce p:MHCII complexes, and display them for recognition by naïve CD4+ T cells. This process occurs within several hours of antigen deposition in the skin and results in CD69 induction and proliferation in the T cells.

The initial wave of p:MHCII presentation is followed by a second wave mediated by dermal dendritic cells that take up the antigen at the site of deposition and then migrate to the draining lymph node.[46] These dendritic cells arrive in the T-cell areas 12 to 24 hours after antigen enters the tissue. p:MHCII presentation by these dendritic cells prolongs induction of the IL-2 receptor and is required for T-cell acquisition of the capacity to cause a later delayed-type hypersensitivity reaction.

Other types of antigens are accessed and processed by different dendritic cells. Given their location in the spleen, myeloid dendritic cells probably play a key role as initiating APCs for CD4+ T cells in the case of antigens that are present in the blood.[48] Dendritic cells that migrate from the relevant tissue (eg, submucosal dendritic cells during vaginal infection are likely the only cells capable of producing p:MHCII ligands from microbes that are too large to enter the conduits.[43] For unknown reasons, dermal Langerin-positive dendritic cells are the most important APCs for induction of contact hypersensitivity-causing CD8+ T cells by protein-modifying chemicals applied to the skin surface.[49] Surprisingly, epidermal Langerhans cells are not required for contact hypersensitivity, although the sensitizing chemicals are applied to the skin surface. Rather, p:MHCII presentation by Langerhans cells inhibits the induction of delayed-type hypersensitivity by suppressing the priming of CD4+ Th1 cells and promoting the priming of Th17 cells.[50]

T-Cell Antigen Receptor Signaling

Once dendritic cells displaying foreign p:MHC ligands appear in the T-cell area of a secondary lymphoid organ, they can be recognized by naïve T cells expressing complementary TCRs. In vitro experiments have shown that highaffinity TCR ligation by p:MHC ligands causes the TCR to concentrate in a stable central supramolecular activating cluster (cSMAC) structure at the point of contact between the T cell and the APC.[51] cSMAC formation is often preceded by the formation of TCR microclusters at the periphery of the T cell-APC contact zone, which are capable of the transducing signals.[52] TCR clustering then activates protein tyrosine kinases such as lck, which stimulate signaling cascades that trigger protein kinase c theta, elevate intracellular calcium, convert ras into its active form, and activate the extracellular signal-regulated kinases (ERK1 and ERK2) and stress-activated protein kinases (jun kinase and p38 mitogen-activated protein kinase).[53] These pathways

culminate in the nuclear translocation and binding of transcription factors such as NFAT and NF-κB to deoxyribonucleic acid sequences that regulate lymphokine gene expression.[54]

Very little is known about early TCR signaling events in naïve T cells in vivo because the assays used to measure most of these events rely on cell lines and in vitro culture methods. Interestingly, in vivo imaging of naïve T cells showed rapid, p:MHC-dependent TCR internalization that was not contingent on prolonged contacts between T cells and APCs or cSMACs.[55] These transient interactions must be sufficient for TCR signaling, however, because intracellular staining with antibodies that recognize the active forms of the c-jun transcription factor and the p38 mitogen-activated protein kinase showed that both of these molecules are phosphorylated in p:MHC-specific naïve T cells in the spleen within minutes of intravenous injection of the relevant peptide.[56]

This rapid response is likely explained by the fact that the majority of naïve T cells are constantly contacting dendritic cells in the T-cell areas.[56]

Proliferation and Costimulation

Initial TCR binding to p:MHC ligands on dendritic cells causes naïve T cells to begin proliferating in vivo about 2 days later.[57] In vitro experiments indicate that this cell division is driven by production of IL-2[58] and induction of the IL-2 receptor alpha chain (CD25).[59] Surprisingly, however, p:MHC-driven proliferation of naïve T cells is minimally dependent on IL-2 in vivo.[60,61] Therefore, other signals or growth factors must be capable of driving T-cell proliferation in vivo, although IL-2 may contribute. The role of IL-2 as a T-cell growth factor may be difficult to reveal because IL-2 is also required to maintain regulatory T cells[62] and can promote death of activated T cells.[60] Thus, a reduction in IL-2-dependent effector T-cell proliferation could be masked by a removal of regulatory T-cell-mediated suppression and activation-induced cell death. In addition, although IL-2 is not essential for initial p:MHCI ligand-driven T-cell proliferation, it does appear to be essential for memory cell differentiation.[63]

The initial proliferation of naïve T cells is followed by an exponential increase in the number of p:MHC-specific T cells over the next several days.[64,65] Depending on the stimulus, the number of p:MHC-specific T cells reaches its highest level in the relevant secondary lymphoid organs, 5 to 7 days after antigen enters the body. As mentioned previously, naïve mice contain about 200 CD8+ T cells specific for a given p:MHC I complex. Because p:MHCI-specific CD8+ T cells can increase to 10^7 cells at the peak of the primary response,[64,65] it follows that CD8+ T cells can expand 500,000-fold in vivo. Although naïve CD4+ T cells are also capable of dramatic clonal expansion when stimulated appropriately, their burst size appears to be less than CD8-positive T cells.[64] For both CD4+ and CD8+ T cells, the amount of cell division is inversely proportional to the number of naïve precursors,[14,66,67] indicating that in vivo proliferation is limited by competition between p:MHC-specific T cells.

In vivo T-cell proliferation is regulated by signals from the costimulatory CD28 molecule, which is triggered by binding to CD80 and CD86 on APCs.[68] The proliferation of antigen-stimulated CD4+ or CD8+ T cells is greatly reduced in mice in which CD28 cannot interact

with its ligands. CD40 ligand (CD154) deficiency also affects T-cell expansion,[69] which may be related to the fact that CD40 signaling in APC induces CD80 and CD86.[70] Although costimulatory signals enhance TCR-driven IL-2 production, the in vivo significance of this effect for T-cell proliferation is unclear, as described above. Although it has been proposed that CD28 acts by promoting TCR aggregation in the cSMAC,[71] enhancing lymphokine messenger ribonucleic acid production[72] and stability,[73] and/or promoting T-cell survival by augmenting Bcl-X_L production,[74] the bulk of recent evidence indicates that it acts by NF-κB signaling.[75] Members of the TNF receptor family, such as OX40, CD27, and 4-1BB are induced in T cells by CD28 signaling several days into the primary response.[76,77] These molecules bind ligands of the TNF family on the surface of APC and transduce signals that sustain the proliferation or survival of p:MHC-stimulated T cells.

Enhancement of costimulatory signals may underlie the observation that in vivo T-cell proliferation is also influenced by inflammation at the time of initial p:MHC presentation. This effect is observed in the case of soluble antigens, where the magnitude of T-cell proliferation is several-fold greater if antigen is administered with a microbial substance[78,79] containing a pathogen-associated molecular pattern[43] such as lipopolysaccharide, which is recognized by a PRR such as toll-like receptor 4.[80] PRR signaling stimulates tissue macrophages to produce tumor necrosis factor-α,[81] which in turn stimulates dendritic cells to migrate from nonlymphoid tissues into the T-cell areas and express higher levels of CD80 and CD86.[82] In addition, these signals result in a maturation process that changes the antigen processing and presentation potential of all dendritic cell types.[83] When inflammation is not present, dendritic cells efficiently engulf extracellular fluid and produce p:MHC complexes from the ingested proteins. However, these p:MHC molecules turn over rapidly on the dendritic cell surface and are presented in the context of low amounts of CD80 and CD86. These factors are thought to be part of the explanation for why p:MHCII presentation by dendritic cells in uninflamed secondary lymphoid organs leads to poor T-cell priming and can result in tolerance.[84] In contrast, inflammatory signals cause dendritic cells to reduce antigen uptake and processing, stabilize p:MHCII molecules, and induce expression of CD80 and CD86. Therefore, inflammatory signals enhance proliferation by driving more dendritic cells into the T-cell areas to present p:MHC ligands and by increasing the costimulatory capacity of these dendritic cells.

PRR signaling in dendritic cells or macrophages can also trigger the production of proinflammatory cytokines, which enhance effector cell proliferation. For example, IL-1 from several sources enhances the proliferation of CD4+ T cells through an early indirect effect on APCs,[85] and a later more potent direct effect on the T cells themselves.[86] PRR signaling causes CD8α+ dendritic cells to produce IL-12[41] and plasmacytoid dendritic cells to produce type 1 IFNs,[42] both of which enhance the proliferation of CD8+ effector T cells.[87]

EFFECTOR T-CELL DIFFERENTIATION

The proliferation of p:MHC-specific effector T cells during the primary response is linked with the acquisition of functions that affect the elimination of antigen such as microbicidal cytokine

production and cytolysis. The functional properties that effector cells acquire are influenced by the presence of inflammatory cytokines and costimulatory ligands on APCs present at the time of initial p:MHC presentation in secondary lymphoid organs.

CD4+ T Cells

Many variations on this theme exist in the case of CD4+ T cells. Effector cells that are generated in the presence of IL-12, IL-4, IL-6 and TGF-β, or IL-6 and IL-21 become

IFN-γ-producing Th1 cells, IL-4-producing Th2 cells, IL-17-producing Th17 cells, or IL-21-producing follicular helper cells (Tfh), respectively. These effector T cells play specialized roles in the elimination of intracellular microbes, worms, and extracellular microbes. Polarization of Th cells is covered in detail elsewhere in this volume.

Signaling through the IL-2 receptor plays an essential and early role in the formation of Th1, Th2, and Th17 effector cells. IL-2 receptor alpha chain (CD25) expression is induced in naïve T cells by TCR signaling, which then pairs with IL-2/IL-15 receptor β (CD122) and IL-2 receptor γ(CD132) chains, to produce the high-affinity IL-2 receptor.[88] Under Th1 priming conditions, the IL-2-bound receptor recruits Jak1 and Jak3 that phosphorylate the STAT5 transcription factor, which enters the nucleus and enhances expression of the IL-12 receptor β2 chain and the T-bet and Blimp-1 transcription factors.[89] IL-12 receptor signaling through STAT4 then enhances T-bet expression and enforces the Th1 fate,[90] while Blimp-1 suppresses another transcription factor Bcl6 that is needed for other fates.[91] Under Th2 priming conditions, IL-2 receptor signaling through STAT5 regulates the Th2 cytokine gene cluster and expression of the IL-4 receptor α-chain.[89] In contrast, IL-2 receptor signaling inhibits Th17 differentiation by suppressing components of the IL-6 receptor.[89]

A variation on this theme was recently described for CD4+ T cells during acute systemic *Listeria monocytogenes* or LCMV infections.[92,93,94] In this case, CD25 is induced by TCR signaling in microbe p:MHCII-specific CD4+ T cells 1 day into the infection. However, by day 3, about half of the responding T cells lose CD25 expression and begin to express CXCR5, while half retain CD25 and do not express CXCR5.[92,94] The mechanism underlying this bifurcation is unknown, although asymmetric division of CD25+ mother cells into CD25+ and CD25- daughter cells is a possibility.[95] Another possibility is that very strong TCR signaling favors CD25 expression.[96] In any case, the CD25+ cells go on to form T-bet^{high} Th1 effector cells in the T cell areas, red pulp, and probably nonlymphoid organs where they could produce IFN-γ in response to p:MHCII presentation by macrophages. It is likely that early CD25+ effector cells become Th2 cells during infections that promote IL-4 production by innate immune cells.[97]

The CD25- CXCR5+ cells formed early during acute infection yield two T-bet^{low} effector cell populations,[92,93] both expressing the B-cell-follicle guiding CXCR5 receptor but only one expressing the Tfh marker PD-1.[98] Both of these CXCR5+ effector cell populations depend on the Bcl6 transcription factor for their formation.[92] The CXCR5+ PD-1- and CXCR5+ PD-1+ effector cell populations probably differentiate depending on quantitative differences in

signals from the TCR and the inducible costimulator (ICOS) receptor.[68,94,99,100] Following stimulation by p:MHCII ligands on dendritic cells, the naïve T cells that quickly lose CD25 and express Bcl6 migrate toward the follicles under the guidance of CXCR5 to interact with, and provide helper signals to, antigen-specific B cells that display the relevant p:MHCII complexes and ICOS ligand.[101,102,103] In return, the T cells receive signals from the TCR and ICOS. If these signals are relatively weak, then Bcl6 expression may not be maintained and the cells acquire a CCR7+ $CXCR5^{intermediate}$ P-selectin ligand (PSGL-1)+ PD-1- phenotype and live in the outer T-cell area. As discussed in more detail below, some of these effector cells yield multipotent central memory cells.

In contrast, if early CD25- CXCR5+ effector cells receive strong TCR and ICOS signals when interacting with B cells, perhaps because of expression of TCRs with very high affinity for the inducing p:MHCII ligand,[91,104] then they become $CCR7^{low}$ $CXCR5^{high}$ PSGL-1- PD-1+ Tfh cells. The cytokines IL-6 and IL-21 are also required for this process,[105,106] with IL-21 probably coming from the developing Tfh cells themselves. The $CCR7^{low}$ $CXCR5^{high}$ phenotype causes Tfh cells to localize in germinal centers, which are rich sources of CXCR5 but not CCR7 ligands.[16] Here, Tfh cells produce IL-21 and CD40 ligand that promote germinal center B-cell differentiation, antibody isotype switching, and plasma cell formation.[98]

CD8+ T Cells

As in the case of CD4+ T cells, IL-2 receptor signaling is not essential for the initial proliferation of p:MHCI-stimulated naïve CD8+ T cells.[63] However, IL-2 receptor signaling plays a key role in effector cell differentiation and memory cell formation. Naïve CD8+ T cells that are stimulated by p:MHCI ligands on dendritic cells in secondary lymphoid organs during acute LCMV infection rapidly express CD25 to produce highaffinity IL-2 receptors.[96] However, as in the case of CD4+ T cells, a subset of the CD8+ T cells sustains CD25 expression longer than the other cells in the population. The $CD25^{low}$ cells preferentially upregulate the IL-7 receptor and the central memory T-cell marker CD62L and produce long-lived memory cells. In contrast, the $CD25^{high}$ cells proliferate more rapidly and become apoptosis-prone terminally differentiated effector cells, although it is likely that some of these cells produce effector memory cells. Thus, for both CD4+ and CD8+ T cells, a lack of IL-2 receptor signaling early in the primary response produces effector cell precursors of central memory cells, whereas IL-2 receptor signaling promotes highly differentiated effector cells and like effector memory cells.

Again like CD4+ T cells, costimulatory signals and cytokines produced by innate immune cells are needed for optimal differentiation of CD8+ effector cells. Naïve CD8+ T cells that are stimulated by p:MHCI ligands also require CD28 signals and either IL-12 or type I IFN (IFNα/β) for maximal proliferation and development of cytolytic activity.[87] The effects of IL-12 and type 1 IFN are mediated directly on the CD8+ T cells. When either IL-12 or type 1 IFN are delivered with antigen, the responding CD8+ T cells form a memory cell population, while immunization with antigen alone induces some clonal expansion, but very few cells survive long-term and those that do are anergic. Thus, IL-12 and type 1 IFN provide a “third signal”

that is necessary, along with TCR and CD28 signals, for development of optimal effector functions and formation of a responsive memory population. Dendritic cells activated by PRRs, or CD40 signals pursuant to interaction with CD40 ligand (CD154)+ CD4+ T cells, produce IL-12 and type I IFNs, and are thus equipped to provide all three signals needed by a naïve CD8+ T cell to become a cytotoxic effector cell.

IL-12 and type I IFN are largely redundant in their capacity to support a productive CD8+ effector T-cell response, and which cytokine is critical depends on the infection. Studies using CD8+ T cells lacking receptors for one or both of the cytokines have shown that type I IFN is essential for response to LCMV,[107] while IL-12 signaling plays the major role in responses to vaccinia virus and *Listeria monocytogenes*.[108] In a minor histocompatibility antigen transplant model, rapid graft rejection depends on CD4+ T cells stimulating dendritic cells in a CD40-dependent manner to produce IL-12 needed for generation of cytotoxic CD8+ effector T cells.[109] Thus, it appears that one way in which CD4+ T cells can provide help for CD8+ T-cell responses is by stimulating dendritic cells to produce third signal cytokines.

CD8+ effector T cells differ from their naïve precursors regarding surface markers, function, and trafficking properties. CD8+ effector T cells that are generated in secondary lymphoid organs during microbial infections express slightly lower levels of CD8 and more surface O-glycans than naïve cells,[110] and in the human some CD8+ effector T cells lose CD27 and CD28 but retain CD45RA.[111] Unlike naïve cells, these cells express perforin and granzymes,[110] which are required for efficient cytolytic function. Expression of perforin and granzymes contributes to the defining feature of cytotoxic T cells,[110] that is, the ability to directly kill target cells that display the appropriate p:MHC I complexes. Interestingly, although large numbers of antigen-specific CD8+ T cells accumulate in mice injected with heat-killed *Listeria monocytogenes* bacteria, these T cells do not acquire cytolytic function.[112] This situation may come about because heat-killed *Listeria monocytogenes* bacteria are poor inducers of IL-12 by innate immune cells compared to live organisms.

EFFECTOR T-CELL FUNCTION IN NONLYMPHOID ORGANS

Some CD4+ effector T cells migrate into nonlymphoid tissues at the peak of the primary response.[113] Separate homing pathways to the intestines and skin have been characterized in detail,[114] although other pathways probably exist. Injection of antigen and cholera toxin into the skin induces two populations of CD4+ effector T cells in the draining lymph nodes that do or do not express the fucosylated form of P-selectin glycoprotein-1 (fPSGL-1), which binds to CD62P on inflamed blood vessels.[115] The fPSGL-1- cells are potent helpers of antibody production by B cells and are likely one of the CXCR5+ effector cell populations described above. In contrast, the fPSGL-1+ cells are poor helpers of antibody production by B cells but are capable of IFN-γ production and are likely similar to the T-bet^{high} Th1 effector cells induced during acute infections. After transfer into naïve recipients, these cells migrate to the skin as evidenced by a capacity to cause delayed-type hypersensitivity reactions. Injection of antigen into the skin with complete Freund's adjuvant also induces Th1 effector cells in draining lymph nodes, which migrate to the skin injection site in a CD62E- and

CD62P-dependent fashion.[116] Interestingly, in this case and another involving lung migration,[117] the CD4+ T cells in the lymph nodes that migrate to nonlymphoid tissues are those that undergo the most cell divisions in the secondary lymphoid organs. Thus, only the most-divided subset of Th1 effector cells in lymph nodes acquire the appropriate trafficking receptors and have the capacity to migrate to nonlymphoid organs.

The induction of skin-homing capacity is controlled by several factors produced by dendritic cells that migrate from the skin and present p:MHCII ligands to naïve T cells in the draining lymph nodes. One of these factors is IL-12, which induces the expression of enzymes that fucosylate PSGL-1 and convert it into the CD62P-binding form.[118] Dendritic cells from the skin also produce 1,25 dihydroxy-vitamin D3, which induces the putative skin-homing chemokine receptor CCR10 on T cells.[119] It should be noted, however, that regulation of CCR4, which is probably the most important skin-homing chemokine receptor on T cells,[120] is not understood in this context.

The generation of gut-homing CD4+ effector T cells is also controlled by factors produced by dendritic cells at the time of T-cell priming. Presentation of p:MHCII ligands to CD4+ T cells in mucosal lymphoid organs induces effector cells that express large amounts of α4β7 integrin, which facilitates T-cell migration into tissues containing mucosal addressin cell adhesion molecule-1+ blood vessels such as the intestines.[15] CD103+ dendritic cells in the mucosal lymphoid organs produce all-trans retinoic acid, which enhances the expression of α4β7 integrin and CCR9 on CD4+ T cells.[121]

Mycobacterium tuberculosis (TB) infection by inhalation provides another example of CD4+ effector T-cell migration to nonlymphoid tissue.[122] Inhaled TB organisms are taken up in the lungs by CD11b+ dendritic cells, which carry the bacteria to the mediastinal lymph nodes and present TB p:MHCII ligands to naïve CD4+ T cells expressing relevant TCRs. This presentation occurs in an IL-12-rich context, and Th1 effector cells are produced, many of which traffic back to the lungs. The molecules that control this migration are unknown although CCL27, MIP-1α, IP-10, CCR4, CCR5, and CXCR3 may be involved. Once in the lungs, effector T cells produce IFN-γ in response to TB p:MHCII presentation by infected dendritic cells and macrophages, which triggers the microbicidal functions of these cells. During this process, the lung effector T cells start out as PD-1^{high} $CD69^{high}$ cells that convert to $KLRG1^{high}$ $CD69^{low}$ short-lived, terminally differentiated cells as they become activated by local TB p:MHCII presentation. Because TB organisms are not completely eliminated by this process, it goes on for protracted periods of time as new effector cells migrate to the lungs from the draining lymph nodes, eventually damaging the lung tissue. Thus, TB p:MHCII-specific effector T cells and their effects on infected phagocytes knock the TB organisms down but not out, and in the process protect the host in the relative short-term and damage it in the long-term.

CD8+ effector T cells also migrate out of the T-cell areas and into many nonlymphoid tissues, particularly inflamed sites of antigen deposition (eg, the lungs during influenza infection[123,124] and the gut during vesicular stomatitis virus infection[125]). Strong or prolonged IL-2 receptor signaling is an important factor in the generation of highly differentiated nonlymphoid tissue-homing effector CD8+ T cells.[96] The migratory capacities

of effector CD8-positive T cells correlate with loss of receptors involved in lymph node

migration (CCR7 and CD62L) and acquisition of receptors such as α4β7 integrin,[126] following instructions from dendritic cells as described above for CD4-positive T cells. The migration of CD8+ effector T cells with cytotoxic potential into nonlymphoid organs is an effective way of eliminating cells that display p:MHC I complexes from all parts of the body.

Several CD8+ effector T-cell-specific homing phenomenon are worth mentioning. One is that CD8+ T-cell recruitment to nonlymphoid tissue depends on prior entry of CD4+ T cells in certain situations. Recognition of p:MHCI complexes on dendritic cells in the secondary lymphoid organs induces the expression of CXCR3 on effector CD8+ T cells, which facilitates entry into nonlymphoid tissues through blood vessels displaying CXCL9 and CXCL10.[127] During certain viral infections, CD4+ effector T cells first enter the relevant nonlymphoid tissue and are stimulated by their p:MHCII ligands to produce IFN-γ, causing local epithelial cells to secrete CXCL9 and CXCL10, which then recruit the CD8+ effector T cells.[128]

Another special homing property of some CD8+ effector T cells relates to the capacity to enter a nonlymphoid tissue and never leave.[129] Viral infections in the skin, brain, and intestinal mucosa result in expansion of viral p:MHCI-specific CD8+ effector T cells in the draining lymph nodes and then migration of some effector cells to the site of infection. Some of these cells can be found at this site long after the infection is cleared. Parabiosis experiments indicate that these T cells are not constantly leaving and entering the site from the blood as expected, but rather remain in the original site without leaving. These resident CD8+ T cells express CD103, which may tether them in the site by binding to E-cadherin on local epithelial cells. Tissue-resident CD8+ effector T cells also express CD69, a marker of acute activation, even under conditions where local relevant p:MHCI complexes cannot be detected. Thus, CD69 expression may be driven in these cells by cytokine receptor rather than TCR signaling. It should be noted that not all CD8+ effector T cells in nonlymphoid tissues are noncirculating residents as evidenced by the presence of p:MHCI-experienced CD8+ T cells in efferent lymphatic vessels, which carry cells from tissues to the blood.[130] The factors that determine whether naïve CD8+ T cells will become nonrecirculating CD103+ tissue-resident effector cells or effectors cells that recirculate through nonlymphoid tissues are not known.

GENERATION OF MEMORY T CELLS FROM EFFECTOR CELLS

Another key function of effector T cells is the production of memory cells.[131] The number of antigen-derived p:MHC-specific effector T cells in the body peaks about a week into the primary response, and then falls rapidly over a 2-week contraction period to about 10% of the peak value.[132] This decline must be due to cell death because the total number of cells in all parts of the body declines shortly after the peak.[113] The T cells that survive the contraction phase can persist stably for the life of the host even when the antigen is cleared from the body due to the expression and function of IL-7 and IL-15 receptors.[133] These long-lived cells are known as memory cells, which are capable of rapid secondary responses that can produce protective immunity to a later challenge from a microbe.[134] Memory cells can be distinguished from effector cells in that most memory cells are not blasts, are not in the cell

cycle, and many are not directly cytolytic or producing lymphokines.[135]

Memory cells are heterogenous, however, and exist in at least two subsets: effector memory (Tem) cells and central memory (Tcm) cells.[136] Tem cells express homing receptors that facilitate migration to nonlymphoid sites of inflammation[113] and produce microbicidal cytokines such as IFN-γ within several hours of TCR stimulation. In many ways, these cells resemble effector cells (eg, Th1 or Th2 cells), with the exceptions that they are no longer blasts and express IL-7 receptors. Tcm cells do not produce any of the prototypic effector cell lineage cytokines immediately after stimulation through the TCR, although they secrete IL-2 and proliferate extensively and acquire effector lymphokine production later. These cells express CD62L and CCR7, which are involved in migration through lymph nodes and mucosal lymphoid organs and positioning in the T-cell areas of these organs,[16] and IL-7 receptors, which are critical for their survival.[133]

The Tem and Tcm model fits well for CD8+ memory T cells. A subset of human CD8+ memory cells lacks the naïve cell marker CD45RA but expresses CCR7.[111,137] These memory cells also express CD62L and lack perforin and thus would not be expected to be directly cytotoxic. Mice contain a comparable $CD44^{high}$ memory cell population after clearance of acute viral infection.[125] These similar populations in mice and humans fit the description of Tcm cells. In mice, the CD62L+ Tcm cells undergo slow IL-15-dependent, MHCI-independent homeostatic proliferation, which is thought to account for their numerical stability.[138]

Mice and humans also contain CD45RA- CCR7- CD62L- CD8+ memory T cells. These cells express high levels of β1 and β7 integrins, fPSGL-1, and CCR5, which are predicted to facilitate migration into nonlymphoid tissues,[139] and produce IFN-γ rapidly after TCR stimulation.[125,137] These cells therefore fit the description of Tem cells. In mice, CD8+ Tem cells undergo much less IL-15-dependent, MHCI-independent homeostatic proliferation than Tcm cells,[140] and convert into Tcm cells over long periods of time in some[141] but not all[142] situations. Murine CD8+ Tem cells can recirculate through nonlymphoid organs or reside permanently in nonlymphoid organs, as described above.

Humans also contain a CD45RA+ CCR7- CD8+ T-cell subset that contains especially high levels of perforin and direct ex vivo cytotoxic activity.[111,137] These cells are likely effector cells that were recently stimulated by p:MHCI ligands.

Memory CD4+ T-cell populations also contain Tcm and Tem subsets. The microbial p:MHCII-specific CD4+ T cells that survive in secondary lymphoid organs long after clearance of *Listeria monocytogenes* or LCMV infections consist of two subsets.[92,93] One subset has the characteristics of Tem cells including a $T\text{-}bet^{high}$ CCR7- phenotype, expression of nonlymphoid tissue homing receptors, and immediate IFN-γ production after stimulation with the relevant p:MHCII ligand. These cells correspond to human CD45RA- CCR7- CD4+ Tem cells, which express low or

variable levels of CD62L and high levels of fPSGL-1, and/or β1 and β7 integrins and produce IFN-γ or IL-4 rapidly when stimulated with anti-CD3 antibody in vitro.[137] In the mouse, CD4+

Tem cells are also found in liver, lungs, and gut long after intravenous injection of antigen plus adjuvant.[113] Because CD4+ memory T cells are constantly coming out of tissues and into afferent lymphatic vessels,[130,143] it is likely that CD4+ Tem cells are not fixed in nonlymphoid organs but recirculate through these sites.

The other microbial p:MHCII-specific CD4+ T-cell population that survives after clearance of acute infections in mice has the characteristics of Tcm cells. This population has a T-bet^{low} CCR7+ CXCR5+ Ly6C^{-} PD-1- Tfh-like phenotype and produces IL-2 but not IFN-γ immediately after stimulation with the relevant p:MHCII ligand.[92,93] These cells have been found to be potent helpers of antibody production by B cells.[143,144,145] In humans, this population can give rise to Tfh cells when stimulated in vitro.[146] In mice, these cells also generate Th1 cells and themselves during secondary immune responses and thus have the capacity of Tcm cells to generate diverse effector cells.[92]

Evidence suggests that Tem and Tcm memory cells derive from like effector cells. IFN-γ-producing CD62L- CD4+ Tem cells that survive after clearance of acute LCMV infection are derived from similar IFN-γ-producing CD62L- effector cells present at the peak of infection.[147,148] Similarly, the T-bet^{low} CXCR5+ CCR7+ Tfh-like Tcm cells that survive after clearance of *Listeria monocytogenes* infection are derived from similar T-bet^{low} CXCR5+ CCR7+ effector cells present at the peak of infection.[92]

Even though the various types of memory cells are derived from effector cells, they must be subsets of their effector cell precursor populations because they are 10 times smaller than those populations. In other words, even though Th1 effector memory cells are derived from similar Th1 effector cells, the former cells are 10 times less abundant than the latter cells. Thus, to understand how memory cells emerge at the end of the primary response, it is useful to consider the factors that cause 90% of the effector cells to die. Effector cells produce reactive oxygen species, which can cause cell death by damaging mitochondrial membranes. Bcl-2 family proteins regulate this type of death. Naïve and memory T cells express anti-apoptotic Bcl-2 molecules in their mitochondria, which inhibit the formation of apoptosis-inducing complexes consisting of Bim and Bak. In mice overexpressing Bcl-2 or lacking Bim, a greater fraction of the effector cell population survive contraction and differentiate into memory cells.[149] Thus, it is conceivable that under normal circumstances, the effector cells that are destined to become memory cells produce less reactive oxygen species or protect themselves better than other effector cells in the population.

Another possibility relates to expression of the IL-7 receptor. The survival of naïve T cells depends on IL-7 receptor signaling, which maintains Bcl-2 expression and limits Bim expression.[149] TCR-driven activation causes effector cells to lose the IL-7 receptor, leading to the fatal situation where Bcl-2 is reduced and Bim is increased. It has been reported that a subset of CD8+ effector cells retains IL-7 receptor expression and survives the contraction phase to become memory cells,[150] although some evidence against this idea has accumulated.[151,152] Thus, it is possible that 10% of the effector cells present at the peak of the T-cell response retain the IL-7 receptor or some other survival factor, which allows these cells to become memory cells.

Another possibility is that metabolic rate determines whether an effector cell will survive to become a memory cell. Quiescent naïve and memory T cells produce adenosine-5′-triphosphate by mitochondrial oxidative phosphorylation.[153] In contrast, activation causes effector cells to shift to glycolytic adenosine-5′-triphosphate production by a pathway that depends on mammalian target of rapamycin. Inhibition of mammalian target of rapamycin with rapamycin early during the primary response promotes the formation of CD8+ memory T cells by inhibiting T-bet expression in favor of another transcription factor, eomesodermin.[154,155,156] This result fits with a model in which most effector cells are killed by products of their own high rate of glycolysis through a T-bet-dependent program, which when blocked allows a return to oxidative phosphorylation and memory cell formation. In this model, memory cells arise from effector cells with the lowest glycolytic rates in the population. Again, increased glycolytic rate and the favoring of effector cell death over memory cell formation could involve IL-2 receptor signaling.

Tumor necrosis factor receptor family proteins also promote CD4+ T memory cell survival by inducing antiapoptotic factors by TRAF-dependent triggering of NFκB.[77] For example, effector T cells that do not express CD27 survive poorly in vivo.[157,158] Other work indicates that OX40 is dispensable for effector cell generation but required for memory cell formation.[159]

Recent work indicates that Id transcription factors also regulate effector-memory cell conversion. Id2 is required for the maximal generation of CD8+ effector T cells and perhaps the formation of Tem cells.[160,161,162] Conversely, Id3 appears to be critical for CD8+ Tcm cell formation.[162]

CONCLUSION AND MODELS

What follows is an attempt to unify the information presented in this chapter into a theoretical sequence of events that occur after naïve CD4+ or CD8+ T cells encounter the relevant p:MHC ligands during skin or mucosal infections, respectively. Because these processes are not completely understood, certain aspects of what follows are speculative.

Naïve T-cell populations that are specific for single p:MHC epitopes exist at a frequency of about 1:200,000 in the preimmune repertoire.[1] These T cells, like other naïve T cells, spend their life spans (˜2 months in mice, ˜2 years in humans) in a series of 1-day stops in the T-cell areas of different secondary lymphoid organs with intervening trips through the blood. While in the T-cell area, these naïve T cells receive survival signals through the IL-7 receptor as it binds to IL-7 made by stromal cells and through the TCR as it binds to the relevant selecting self p:MHC ligand on the surface of an APC, probably a dendritic cell.

Consider first the case of the CD4+ T-cell response to a bacterial infection in the dermis of the skin (Fig. 14.1). In this case, local dermal dendritic cells take up the microbe,

produce microbe p:MHCII complexes,[45] upregulate CD28 ligands,[163] begin producing IL-12[164] and vitamin D3,[119] and migrate through an afferent lymphatic vessel to the draining

lymph node. Naïve T cells interact with these dendritic cells[165] and those CD4+ T cells that express bacterial p:MHCII-specific TCRs receive TCR and CD28 signals[56] and induce the translocation of NFAT and NF-κB to the nucleus,[54] which in turn drive expression of CD25, IL-2, Bcl6, and the IL-12 receptor β2 chain, and loss of the IL-7 receptor.[61,89,166] The T cells then divide in response to IL-2 and unknown growth factors,[57] perhaps asymmetrically,[95] to produce CD25+ and CD25- effector cell progeny.[92,94] The CD25+ cells activate STAT5 through IL-2 receptor signaling, [167] which further increases expression of the IL-12 receptor β2 chain,[89] which combines with the IL-12 receptor β1 chain, binds IL-12, and activates STAT4[168] to amplify T-bet expression.[169] IL-2 receptor signaling and STAT5 activation also stimulates Blimp-1 expression,[170] which represses Bcl6,[171] thereby enforcing the Th1 fate and preventing Bcl6-dependent cell fates. The Th1 effector cells also express CCR10, CCR4, and fucosylated PSGL-1 due to activation in the presence of IL-12 and vitamin D3.[114,118] Some of these effector cells then leave the lymph nodes through efferent lymphatic vessels, enter the bloodstream, and migrate into the infection site through skin blood vessels displaying CD62P, CCL17, and CCL27.[116,119,172] After entering this site, the Th1 effector cells interact with infected macrophages displaying bacterial p:MHCII ligands.[173] TCR signaling then causes the T cells to secrete IFN-γ, which triggers production of inducible nitric oxide synthase and nitric oxide as well as killing of intracellular bacteria.[173] Some of the Th1 effector cells in the lymph nodes survive the contraction phase, re-express the IL-7 receptor, and become Th1 effector memory cells,[147] which recirculate through the skin.

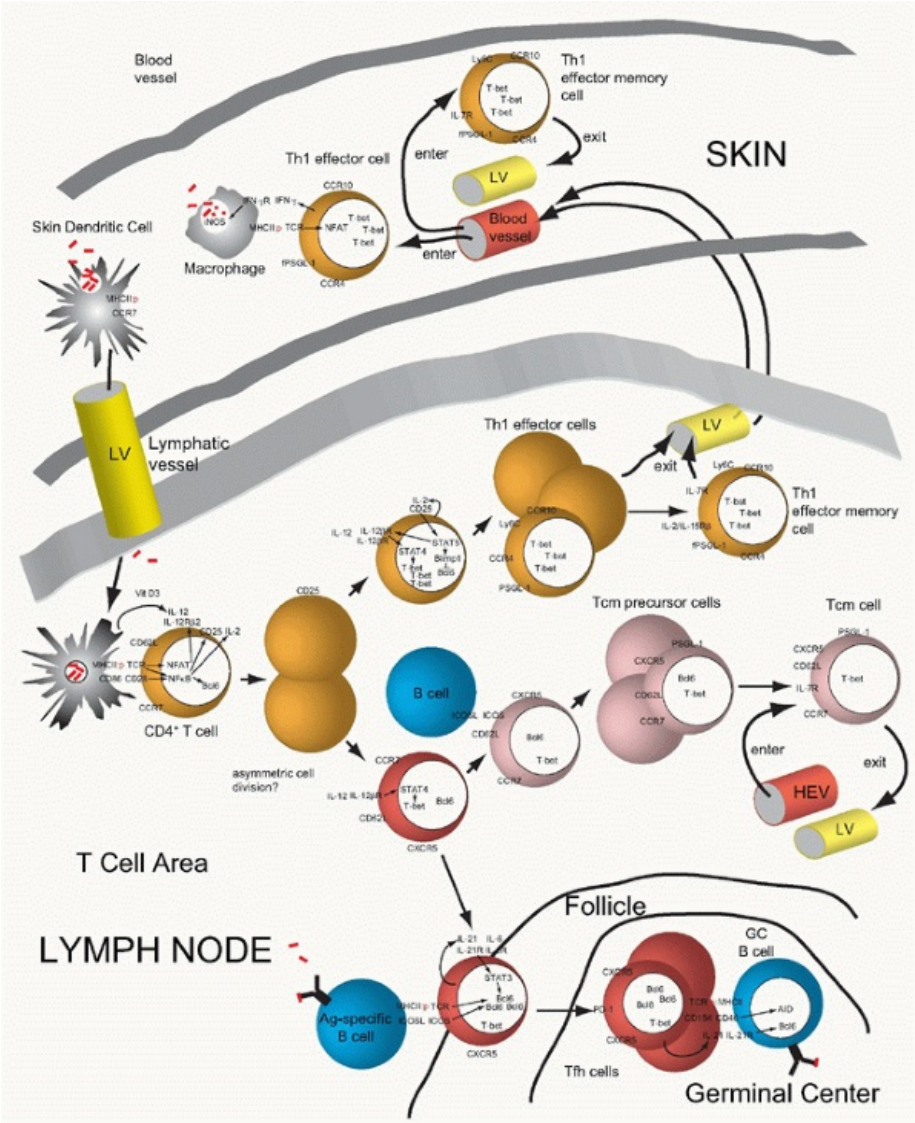

FIG. 14.1. CD4+ T-Cell Response to Bacterial Infection of the Skin. See the text for description. Bacterial antigen is indicated with red rectangles. Surface receptors are shown attached to the cell surface. Transcription factors are shown in the nucleus. Signals are shown with the *smallest arrows*. Sequential cell fates are shown with the *next largest arrows*. Migration routes are shown with the *largest arrows*. LV, lymphatic vessel; R, receptor.

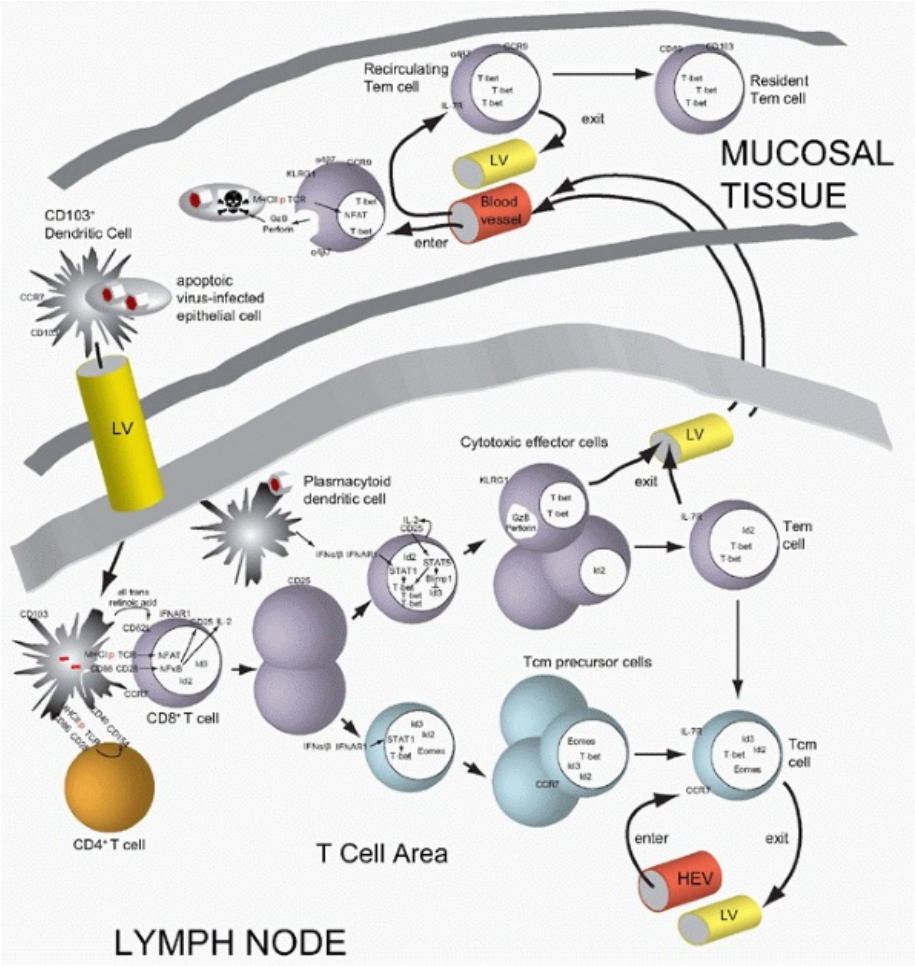

FIG. 14.2. CD8+ T-Cell Response to Viral Infection of a Mucosal Tissue. See the text for description. Virus particles are indicated with hexagons. Surface receptors are shown attached to the cell surface. Transcription factors are shown in the nucleus. Signals are shown with the *smallest arrows*. Sequential cell fates are shown with the *next largest arrows*. A virally infected cell killed by a cytotoxic clust of differentiation 8-positive T cell is indicated with a *skull and cross bones*. Migration routes are shown with the *largest arrows*. LV, lymphatic vessel; R, receptor.

The early CD25- progeny do not experience IL-2 receptor signaling and STAT5 activation, do not upregulate the IL-12 receptor β2 chain, and are left with a low amount of T-bet. Blimp-1 is also not turned on, allowing the cells to retain Bcl6 and express CXCR5. The CXCR5+ T cells then migrate toward the follicles.[103] If they interact with an antigen-specific B cell, then they receive TCR and ICOS ligand signals[98] and produce IL-21, which binds the IL-21 receptor and activates STAT3.[174] The combination of TCR, ICOS, and STAT3 signals then amplifies Bcl6 expression, stimulating the cells to express PD-1 and more CXCR5, and to commit to the Tfh lineage. The Tfh cells then migrate into germinal centers, interact with antigen-specific germinal center B cells, induce expression of CD154, which engages CD40 on the B cells and stimulates them to express activation induced cytidine deaminase, and undergo antibody isotype switching and somatic hypermutation.[98] If the early CXCR5+ T cells interact with B cells that are not specific for bacterial antigens, then they receive an

ICOS signal that maintains their survival.[92] However, without additional TCR signaling, the T cells do not further upregulate CXCR5, do not lose CCR7 or CD62L, and some of these cell become Tcm cells, eventually losing Bcl6.[92] These Tcm cells recirculate through secondary lymphoid organs like naïve cells.

Now consider the CD8+ T-cell response to viral infection of a mucosal tissue (Fig. 14.2). Local CD103+ dendritic cells with cross-presentation capacity take up apoptotic infected cells,[41] produce viral p:MHCI and p:MHCII complexes, upregulate CD28 ligands, produce all-trans retinoic acid,[121] and migrate through an afferent lymphatic vessel to the draining lymph node. Plasmacytoid dendritic cells are recruited to the lymph node and produce type I IFNs in response to PRR recognition of viral pathogen-associated molecular patterns.[42] Interaction with CD154+ virus p:MHCIIspecific CD4+ T cells increases the activation state of the CD103+ dendritic cells through CD40 signaling.[175,176] Naïve virus p:MHCI-specific CD8+ T cells interact with these dendritic cells and produce IL-2 and induce expression of CD25. Unknown T-cell growth factors and perhaps IL-2 then stimulate the CD8+ T cells to divide to produce progeny that express small or large amounts of CD25.[96] The $CD25^{high}$ cells experience IL-2 receptor signaling and STAT5 activation, which stimulates Blimp-1 expression, which cooperates with STAT1 activation from the type I IFN receptor to upregulate T-bet.[177] T-bet and Blimp-1 drive the cells to become terminally differentiated KLRG1+ cytotoxic effector cells loaded with granzyme B and perforin-containing granules.[170,178] Blimp-1 represses another transcription factor Id3, which is required for Tcm cell formation,[162] thereby reinforcing effector cell formation. The effector cells also express α4β7 integrin and CCR9 due to activation in the presence of all-trans retinoic acid,[121] and migrate to the site of infection through mucosal blood vessels displaying mucosal addressin cell adhesion molecule-1 and CCL25.[179] After extravasating into this site, the cytotoxic effector cells interact with infected cells displaying virus p:MHCI ligands. TCR signaling then causes the T cells to expel granzyme B and perforin, which enter the target cell and kill it.[180] Some of the KLRG1+ effector cells in the lymph node survive the contraction phase, perhaps through the action of Id2,[161] and then either become CD103- Tem cells that recirculate through the mucosal tissue or CD103+ Tem cells that enter the tissue and never leave.[129] The recirculating Tem cells may convert to Tcm cells over long periods of time.[141]

The early CD25- progeny do not experience IL-2 receptor signaling and STAT5 activation and do not turn on Blimp-1.[178] Without Blimp-1, the cells retain Id3 expression[162] and are prevented from becoming terminally differentiated $Tbet^{high}$ cytotoxic effector cells. Instead, type I IFN receptor signaling facilitates expression of the Eomes transcription factor,[156] retention of CCR7 expression, and survival as Tcm cells, which recirculate through the secondary lymphoid organs like naïve cells.

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REFERENCES

1. Jenkins MK, Chu HH, McLachlan JB, et al. On the composition of the preimmune repertoire of T cells specific for peptide-major histocompatibility complex ligands. *Annu Rev Immunol*. 2010;28:275-294.

2. Thompson PK, Zuniga-Pflucker JC. On becoming a T cell, a convergence of factors kick it up a Notch along the way. *Semin Immunol*. 2011;23(5):350-359.

3. Thomas LR, Cobb RM, Oltz EM. Dynamic regulation of antigen receptor gene assembly. *Adv Exp Med Biol*. 2009;650:103-115.

4. Starr TK, Jameson SC, Hogquist KA. Positive and negative selection of T cells. *Annu Rev Immunol*. 2003;21:139-176.

5. Surh CD, Sprent J. T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature*. 1994;372:100-103.

6. Marrack P, Scott-Browne JP, Dai S, et al. Evolutionarily conserved amino acids that control TCR-MHC interaction. *Annu Rev Immunol*. 2008;26:171-203.

7. Huseby ES, White J, Crawford F, et al. How the T cell repertoire becomes peptide and MHC specific. *Cell*. 2005;122:247-260.

8. Wang L, Bosselut R. CD4-CD8 lineage differentiation: Thpok-ing into the nucleus. *J Immunol*. 2009;183:2903-2910.

9. Hogquist KA, Moran AE. Treg cells meet their limit. *Nat Immunol*. 2009;10:565-566.

10. Weinreich MA, Hogquist KA. Thymic emigration: when and how T cells leave home. *J Immunol*. 2008;181:2265-2270.

11. Makaroff LE, Hendricks DW, Niec RE, et al. Postthymic maturation influences the CD8 T cell response to antigen. *Proc Natl Acad Sci U S A*. 2009;106:4799-4804.

12. Boursalian TE, Golob J, Soper DM, et al. Continued maturation of thymic emigrants in the periphery. *Nat Immunol*. 2004;5:418-425.

13. Hellerstein MK, Hoh RA, Hanley MB, et al. Subpopulations of longlived and short-lived T cells in advanced HIV-1 infection. *J Clin Invest*. 2003;112:956-966.

14. Hataye J, Moon JJ, Khoruts A, et al. Naïve and memory CD4+ T cell survival controlled by clonal abundance. *Science*. 2006;312:114-116.

15. Denucci CC, Mitchell JS, Shimizu Y. Integrin function in T-cell homing to lymphoid and nonlymphoid sites: getting there and staying there. *Crit Rev Immunol* . 2009;29:87-109.

16. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu Rev Immunol* . 2005;23:127-159.

17. Anderson AO, Shaw S. Conduit for privileged communications in the lymph node. *Immunity*. 2005;22:3-5.

18. Link A, Vogt TK, Favre S, et al. Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naïve T cells. *Nat Immunol* . 2007;8: 1255-1265.

19. Birnberg T, Bar-On L, Sapoznikov A, et al. Lack of conventional dendritic cells is compatible with normal development and T cell homeostasis, but causes myeloid proliferative syndrome. *Immunity*. 2008;29:986-997.

20. Dorfman JR, Stefanova I, Yasutomo K, et al. CD4+ T cell survival is not directly linked to self-MHC-induced TCR signaling. *Nat Immunol* . 2000;1:329-335.

21. Surh CD, Sprent J. Homeostasis of naïve and memory T cells. *Immunity*. 2008;29:848-862.

22. Yang K, Neale G, Green DR, et al. The tumor suppressor Tsc1 enforces quiescence of naïve T cells to promote immune homeostasis and function. *Nat Immunol* . 2011;12:888-897.

23. Wu Q, Liu Y, Chen C, et al. The tuberous sclerosis complex-mammalian target of rapamycin pathway maintains the quiescence and survival of naïve T cells. *J Immunol* . 2011;187:1106-1112.

24. Feng X, Wang H, Takata H, et al. Transcription factor Foxp1 exerts essential cell-intrinsic regulation of the quiescence of naïve T cells. *Nat Immunol* . 2011;12:544-550.

25. Prlic M, Blazar BR, Khoruts A, et al. Homeostatic expansion occurs independently of costimulatory signals. *J Immunol* . 2001;167:5664-5668.

26. Min B, McHugh R, Sempowski GD, et al. Neonates support lymphopenia-induced proliferation. *Immunity*. 2003;18:131-140.

27. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. *Nature*. 1988;334:395-402.

28. Moon JJ, Chu HH, Pepper M, et al. Naïve CD4+ T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity*. 2007;27:203-213.

29. Obar JJ, Khanna KM, Lefrancois L. Endogenous naïve CD8+ T cell precursor frequency regulates primary and memory responses to infection. *Immunity*. 2008;28:859-869.

30. Haluszczak C, Akue AD, Hamilton SE, et al. The antigen-specific CD8+ T cell repertoire in unimmunized mice includes memory phenotype cells bearing markers of homeostatic expansion. *J Exp Med*. 2009;206:435-448.

31. Kotturi MF, Scott I, Wolfe T, et al. Naïve precursor frequencies and MHC binding rather than the degree of epitope diversity shape CD8+ T cell immunodominance. *J Immunol*. 2008;181:2124-2133.

32. Chu HH, Moon JJ, Kruse AC, et al. Negative selection and peptide chemistry determine the size of naïve foreign peptide-MHC class IIspecific CD4+ T cell populations. *J Immunol*. 2010;185:4705-4713.

33. Turner SJ, Kedzierska K, Komodromou H, et al. Lack of prominent peptide-major histocompatibility complex features limits repertoire diversity in virus-specific CD8+ T cell populations. *Nat Immunol*. 2005;6:382-389.

34. Yewdell JW. Confronting complexity: real-world immunodominance in antiviral CD8+ T cell responses. *Immunity*. 2006;25:533-543.

35. Rosette C, Werlen G, Daniels MA, et al. The impact of duration versus extent of TCR occupancy on T cell activation: a revision of the kinetic proofreading model. *Immunity*. 2001;15:59-70.

36. Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* . 1989;54:1-13.

37. Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol*. 1994;12:991-1045.

38. Bar-On L, Jung S. Defining dendritic cells by conditional and constitutive cell ablation. *Immunol Rev*. 2010;234:76-89.

39. Helft J, Ginhoux F, Bogunovic M, et al. Origin and functional heterogeneity of non-lymphoid tissue dendritic cells in mice. *Immunol Rev*. 2010;234:55-75.

40. Steinman RM, Idoyaga J. Features of the dendritic cell lineage. *Immunol Rev*. 2010;234:5-17.

41. Shortman K, Heath WR. The CD8+ dendritic cell subset. *Immunol Rev*. 2010;234:18-31.

42. Swiecki M, Colonna M. Unraveling the functions of plasmacytoid dendritic cells during viral infections, autoimmunity, and tolerance. *Immunol Rev*. 2010;234:142-162.

43. Zhao X, Deak E, Soderberg K, et al. Vaginal submucosal dendritic cells, but not Langerhans cells, induce protective Th1 responses to herpes simplex virus-2. *J Exp Med*. 2003;197:153-162.

44. Janeway CA Jr. The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today*. 1992;13:11-16.

45. Brossart P, Bevan MJ. Presentation of exogenous protein antigens on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. *Blood*. 1997;90:1594-1599.

46. Itano AA, Jenkins MK. Antigen presentation to naïve CD4 T cells in the lymph node. *Nat Immunol*. 2003;4:733-739.

47. Sixt M, Kanazawa N, Selg M, et al. The conduit system transports soluble antigens from the afferent lymph to resident dendritic cells in the T cell area of the lymph node. *Immunity*. 2005;22:19-29.

48. Pooley JL, Heath WR, Shortman K. Intravenous soluble antigen is presented to CD4 T cells by CD8(-) dendritic cells, but cross-presented to CD8 T cells by CD8(+) dendritic cells. *J Immunol*. 2001;166: 5327-5330.

49. Bursch LS, Wang L, Igyarto B, et al. Identification of a novel population of Langerin+ dendritic cells. *J Exp Med*. 2007;204:3147-3156.

50. Igyarto BZ, Haley K, Ortner D, et al. Skin-resident murine dendritic cell subsets promote distinct and opposing antigen-specific T helper cell responses. *Immunity*. 2011;35:260-272.

51. Monks CR, Freiberg BA, Kupfer H, et al. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature*. 1998;395: 82-86.

52. Campi G, Varma R, Dustin ML. Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling. *J Exp Med*. 2005;202:1031-1036.

53. Yokosuka T, Sakata-Sogawa K, Kobayashi W, et al. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nat Immunol*. 2005;6:1253-1262.

54. Kane LP, Lin J, Weiss A. Signal transduction by the TCR for antigen. *Curr Opin Immunol*. 2000;12:242-249.

55. Friedman RS, Beemiller P, Sorensen CM, et al. Real-time analysis of T cell receptors in naïve cells in vitro and in vivo reveals flexibility in synapse and signaling dynamics. *J Exp Med*. 2010;207:2733-2749.

56. Zell T, Khoruts A, Ingulli E, et al. Single-cell analysis of signal transduction in CD4 T cells stimulated by antigen in vivo. *Proc Natl Acad Sci U S A* . 2001;98:10805-10810.

57. Gudmundsdottir H, Wells AD, Turka LA. Dynamics and requirements of T cell clonal expansion in vivo at the single-cell level: effector function is linked to proliferative capacity. *J Immunol* . 1999;162:5212-5223.

58. Schorle H, Holtschke T, Hunig T, et al. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature*. 1991;352:621-624.

59. Willerford DM, Chen J, Ferry JA, et al. Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. *Immunity*. 1995;3:521-530.

60. Kneitz B, Herrmann T, Yonehara S, et al. Normal clonal expansion but impaired Fas-mediated cell death and anergy induction in interleukin-2-deficient mice. *Eur J Immunol* . 1995;25:2572-2577.

61. Khoruts A, Mondino A, Pape KA, et al. A natural immunological adjuvant enhances T cell clonal expansion through a CD28-dependent, interleukin (IL)-2-independent mechanism. *J Exp Med*. 1998;187: 225-236.

62. Furtado GC, Curotto de Lafaille MA, Kutchukhidze N, et al. Interleukin 2 signaling is required for CD4(+) regulatory T cell function. *J Exp Med*. 2002;196:851-857.

63. Obar JJ, Molloy MJ, Jellison ER, et al. CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses. *Proc Natl Acad Sci U S A* . 2010;107:193-198.

64. Homann D, Teyton L, Oldstone MB. Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. *Nat Med*. 2001;7:913-919.

65. Murali-Krishna K, Altman JD, Suresh M, et al. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity*. 1998;8:177-187.

66. Smith AL, Wikstrom ME, Fazekas de St Groth B. Visualizing T cell competition for peptide/MHC complexes: a specific mechanism to minimize the effect of precursor frequency. *Immunity*. 2000;13:783-794.

67. Badovinac VP, Haring JS, Harty JT. Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8(+) T cell response to infection. *Immunity*.

2007;179:53-63.

68. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol*. 2005;23:515-548.

69. Grewal IS, Xu J, Flavell RA. Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand. *Nature*. 1995;378:617-620.

70. Ranheim EA, Kipps TJ. Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. *J Exp Med*. 1993;177:925-935.

71. Tavano R, Contento RL, Baranda SJ, et al. CD28 interaction with filamin-A controls lipid raft accumulation at the T-cell immunological synapse. *Nat Cell Biol*. 2006;8:1270-1276.

72. Sanchez-Lockhart M, Marin E, Graf B, et al. Cutting edge: CD28-mediated transcriptional and posttranscriptional regulation of IL-2 expression are controlled through different signaling pathways. *J Immunol*. 2004;173:7120-7124.

73. Lindsten T, June CH, Ledbetter JA, et al. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pahtway. *Science*. 1989;144:339-343.

74. Boise LH, Minn AJ, Noel PJ, et al. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity*. 1995;3:87-98.

75. Riha P, Rudd CE. CD28 co-signaling in the adaptive immune response. *Self Nonself*. 2010;1:231-240.

76. Watts TH, DeBenedette MA. T cell co-stimulatory molecules other than CD28. *Curr Opin Immunol*. 1999;11:286-293.

77. Croft M. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? *Nat Rev Immunol*. 2003;3:609-620.

78. Vella AT, McCormack JE, Linsley PS, et al. Lipopolysaccharide interferes with the induction of peripheral T cell death. *Immunity*. 1995;2:261-270.

79. Pape KA, Khoruts A, Mondino A, et al. Inflammatory cytokines enhance the in vivo clonal expansion and differentiation of antigenactivated CD4+ T cells. *J Immunol*. 1997;159:591-598.

80. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*. 1998;282:2085-2088.

81. Jones BW, Means TK, Heldwein KA, et al. Different Toll-like receptor agonists induce distinct macrophage responses. *J Leukoc Biol*. 2001;69:1036-1044.

82. Kaisho T, Takeuchi O, Kawai T, et al. Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J Immunol*. 2001;166:5688-5694.

83. Banchereau J, Briere F, Caux C, et al. Immunobiology of dendritic cells. *Annu Rev Immunol*. 2000;18:767-811.

84. Hawiger D, Inaba K, Dorsett Y, et al. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med*. 2001;194:769-780.

85. Khoruts A, Osness RE, Jenkins MK. IL-1 acts on antigen-presenting cells to enhance the in vivo proliferation of antigen-stimulated naïve CD4 T cells via a CD28-dependent mechanism that does not involve increased expression of CD28 ligands. *Eur J Immunol* . 2004;34: 1085-1090.

86. Ben-Sasson SZ, Hu-Li J, Quiel J, et al. IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. *Proc Natl Acad Sci U S A* . 2009;106:7119-7124.

87. Curtsinger JM, Mescher MF. Inflammatory cytokines as a third signal for T cell activation. *Curr Opin Immunol* . 2010;22:333-340.

88. Malek TR. The biology of interleukin-2. *Annu Rev Immunol*. 2008;26:453-479.

89. Liao W, Lin JX, Wang L, et al. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. *Nat Immunol*. 2011;12:551-559.

90. Schulz EG, Mariani L, Radbruch A, et al. Sequential polarization and imprinting of type 1 T helper lymphocytes by interferon-gamma and interleukin-12. *Immunity*. 2009;30:673-683.

91. Johnston RJ, Poholek AC, DiToro D, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science*. 2009;325:1006-1010.

92. Pepper M, Pagan AJ, Igyarto BZ, et al. Opposing signals from the Bcl6 transcription factor and the interleukin-2 receptor generate T helper 1 central and effector memory cells. *Immunity*. 2011;35:583-595.

93. Marshall HD, Chandele A, Jung YW, et al. Differential expression of Ly6C and T-bet distinguish effector and memory Th1 CD4(+) cell properties during viral infection. *Immunity*. 2011;35:633-646.

94. Choi YS, Kageyama R, Eto D, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity*. 2011;34:932-946.

95. Chang JT, Palanivel VR, Kinjyo I, et al. Asymmetric T lymphocyte division in the initiation of adaptive immune responses. *Science*. 2007;315:1687-1691.

96. Kalia V, Sarkar S, Subramaniam S, et al. Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo. *Immunity*. 2010;32:91-103.

97. Seder RA, Paul WE. Acquisition of lymphokine-producing phenotype by CD4+ T cells. *Annu Rev Immunol*. 1994;12:635-673.

98. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol*. 2011;29:621-663.

99. Nurieva RI, Chung Y, Hwang D, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity*. 2008;29:138-149.

100. Vinuesa CG, Cook MC, Angelucci C, et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature*. 2005;435:452-458.

101. Garside P, Ingulli E, Merica RR, et al. Visualization of specific B and T lymphocyte interactions in the lymph node. *Science*. 1998;281:96-99.

102. Fulcher DA, Lyons AB, Korn SL, et al. The fate of self-reactive B cells depends primarily on the degree of antigen receptor engagement and availability of T cell help. *J Exp Med*. 1996;183:2313-2328.

103. Ansel KM, McHeyzer-Williams LJ, Ngo VN, et al. In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. *J Exp Med*. 1999;190:1123-1134.

104. Fazilleau N, McHeyzer-Williams LJ, Rosen H, et al. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. *Nat Immunol*. 2009;10:375-384.

105. Lu KT, Kanno Y, Cannons JL, et al. Functional and epigenetic studies reveal multistep differentiation and plasticity of in vitro-generated and in vivo-derived follicular T helper cells. *Immunity*. 2011;35:622-632.

106. Eto D, Lao C, DiToro D, et al. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation.

PLoS One. 2011;6:e17739.

107. Kolumam GA, Thomas S, Thompson LJ, et al. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *J Exp Med*. 2005;202:637-650.

108. Xiao Z, Casey KA, Jameson SC, et al. Programming for CD8 T cell memory development requires IL-12 or type I IFN. *J Immunol* . 2009;182:2786-2794.

109. Filatenkov AA, Jacovetty EL, Fischer UB, et al. CD4 T cell-dependent conditioning of dendritic cells to produce IL-12 results in CD8-mediated graft rejection and avoidance of tolerance. *J Immunol* . 2005; 174:6909-6917.

110. Harrington LE, Galvan M, Baum LG, et al. Differentiating between memory and effector CD8 T cells by altered expression of cell surface O-glycans. *J Exp Med*. 2000;191:1241-1246.

111. Hamann D, Baars PA, Rep MH, et al. Phenotypic and functional separation of memory and effector human CD8+ T cells. *J Exp Med*. 1997;186:1407-1418.

112. Lauvau G, Vijh S, Kong P, et al. Priming of memory but not effector CD8 T cells by a killed bacterial vaccine. *Science*. 2001;294:1735-1739.

113. Reinhardt RL, Khoruts A, Merica R, et al. Visualizing the generation of memory CD4 T cells in the whole body. *Nature*. 2001;410:101-105.

114. Sigmundsdottir H, Butcher EC. Environmental cues, dendritic cells and the programming of tissue-selective lymphocyte trafficking. *Nat Immunol* . 2008;9:981-987.

115. Campbell DJ, Kim CH, Butcher EC. Separable effector T cell populations specialized for B cell help or tissue inflammation. *Nat Immunol* . 2001;2:876-881.

116. Reinhardt RL, Bullard DC, Weaver CT, et al. Preferential accumulation of antigen-specific effector CD4 T cells at an antigen injection site involves CD62E-dependent migration but not local proliferation. *J Exp Med*. 2003;197:751-762.

117. Roman E, Miller E, Harmsen A, et al. CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function. *J Exp Med*. 2002;196:957-968.

118. Lim YC, Xie H, Come CE, et al. IL-12, STAT4-dependent up-regulation of CD4(+) T cell core 2 beta-1,6-n- acetylglucosaminyltransferase, an enzyme essential for biosynthesis of P-selectin ligands. *J Immunol* . 2001;167:4476-4484.

119. Sigmundsdottir H, Pan J, Debes GF, et al. DCs metabolize sunlightinduced vitamin D3 to “program” T cell attraction to the epidermal chemokine CCL27. *Nat Immunol* .

2007;8:285-293.

120. Tubo NJ, McLachlan JB, Campbell JJ. Chemokine receptor requirements for epidermal T-cell trafficking. *Am J Pathol*. 2011;178:2496-2503.

121. Hall JA, Grainger JR, Spencer SP, et al. The role of retinoic acid in tolerance and immunity. *Immunity*. 2011;35:13-22.

122. Urdahl KB, Shafiani S, Ernst JD. Initiation and regulation of T-cell responses in tuberculosis. *Mucosal Immunol*. 2011;4:288-293.

123. Flynn KJ, Belz GT, Altman JD, et al. Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. *Immunity*. 1998;8:683-691.

124. Hendriks J, Gravestein LA, Tesselaar K, et al. CD27 is required for generation and long-term maintenance of T cell immunity. *Nat Immunol*. 2000;1:433-440.

125. Masopust D, Vezys V, Marzo AL, et al. Preferential localization of effector memory cells in nonlymphoid tissue. *Science*. 2001;291:2413-2417.

126. Lefrancois L, Parker CM, Olson S, et al. The role of beta7 integrins in CD8 T cell trafficking during an antiviral immune response. *J Exp Med*. 1999;189:1631-1638.

127. Groom JR, Luster AD. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol Cell Biol*. 2011;89:207-215.

128. Nakanishi Y, Lu B, Gerard C, et al. CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help. *Nature*. 2009;462:510-513.

129. Bevan MJ. Memory T cells as an occupying force. *Eur J Immunol*. 2011; 41:1192-1195.

130. Mackay C, Marston W, Dudler L. Naïve and memory T cells show distinct pathways of lymphocyte recirculation. *J Exp Med*. 1990;171:801-817.

131. Cui W, Kaech SM. Generation of effector CD8+ T cells and their conversion to memory T cells. *Immunol Rev*. 2010;236:151-166.

132. Seder RA, Ahmed R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. *Nat Immunol*. 2003;4:835-842.

133. Surh CD, Boyman O, Purton JF, et al. Homeostasis of memory T cells. *Immunol Rev*. 2006;211:154-163.

134. Ahmed R, Gray D. Immunological memory and protective immunity: understanding their relation. *Science*. 1996;272:54-60.

135. Dutton RW, Bradley LM, Swain SL. T cell memory. *Annu Rev Immunol*. 1998;16:201-223.

136. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol*. 2004;22:745-763.

137. Sallusto F, Lenig D, Forster R, et al. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*. 1999;401:708-712.

138. Becker TC, Wherry EJ, Boone D, et al. Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. *J Exp Med*. 2002;195:1541-1548.

139. Sallusto F, Mackay CR, Lanzavecchia A. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu Rev Immunol*. 2000;18:593-620.

140. Wherry EJ, Becker TC, Boone D, et al. Homeostatic proliferation but not the generation of virus specific memory CD8 T cells is impaired in the absence of IL-15 or IL-15Ralpha. *Adv Exp Med Biol*. 2002;512:165-175.

141. Wherry EJ, Teichgraber V, Becker TC, et al. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol*. 2003;4:225-234.

142. Marzo AL, Klonowski KD, Le Bon A, et al. Initial T cell frequency dictates memory CD8+ T cell lineage commitment. *Nat Immunol*. 2005;6:793-799.

143. Gebhardt T, Whitney PG, Zaid A, et al. Different patterns of peripheral migration by memory CD4+ and CD8+ T cells. *Nature*. 2011;477: 216-219.

144. MacLeod MK, David A, McKee AS, et al. Memory CD4 T cells that express CXCR5 provide accelerated help to B cells. *J Immunol*. 2011; 186:2889-2896.

145. Chevalier N, Jarrossay D, Ho E, et al. CXCR5 expressing human central memory CD4 T cells and their relevance for humoral immune responses. *J Immunol*. 2011;186:5556-5568.

146. Morita R, Schmitt N, Bentebibel SE, et al. Human blood CXCR5(+) CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity*. 2011;34:108-121.

147. Harrington LE, Janowski KM, Oliver JR, et al. Memory CD4 T cells emerge from effector T-cell progenitors. *Nature*. 2008;452:356-360.

148. Lohning M, Hegazy AN, Pinschewer DD, et al. Long-lived virusreactive memory T cells generated from purified cytokine-secreting T helper type 1 and type 2 effectors. *J Exp Med*. 2008;205:53-61.

149. Kurtulus S, Tripathi P, Opferman JT, et al. Contracting the “mus cells”—does down-sizing suit us for diving into the memory pool? *Immunol Rev*. 2010;236:54-67.

150. Kaech SM, Tan JT, Wherry EJ, et al. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to longlived memory cells. *Nat Immunol*. 2003;4:1191-1198.

151. Hand TW, Morre M, Kaech SM. Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. *Proc Natl Acad Sci U S A*. 2007;104:11730-11735.

152. Klonowski KD, Williams KJ, Marzo AL, et al. Cutting edge: IL-7-independent regulation of IL-7 receptor alpha expression and memory CD8 T cell development. *J Immunol*. 2006;177:4247-4251.

153. Prlic M, Bevan MJ. Immunology: a metabolic switch to memory. *Nature*. 2009;460:41-42.

154. Araki K, Turner AP, Shaffer VO, et al. mTOR regulates memory CD8 T-cell differentiation. *Nature*. 2009;460:108-112.

155. Pearce EL, Walsh MC, Cejas PJ, et al. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. *Nature*. 2009;460:103-107.

156. Rao RR, Li Q, Odunsi K, et al. The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin. *Immunity*. 2010;32: 67-78.

157. Hendriks J, Xiao Y, Borst J. CD27 promotes survival of activated T cells and complements CD28 in generation and establishment of the effector T cell pool. *J Exp Med*. 2003;198:1369-1380.

158. Pepper M, Linehan JL, Pagan AJ, et al. Different routes of bacterial infection induce long-lived TH1 memory cells and short-lived TH17 cells. *Nat Immunol*. 2010;11:83-89.

159. Gramaglia I, Jember A, Pippig SD, et al. The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. *J Immunol*. 2000;165:3043-3050.

160. Yang CY, Best JA, Knell J, et al. The transcriptional regulators Id2 and Id3 control the formation of distinct memory CD8(+) T cell subsets. *Nat Immunol*. 2011;12(12):1221-

1229.

161. Cannarile MA, Lind NA, Rivera R, et al. Transcriptional regulator Id2 mediates CD8+ T cell immunity. *Nat Immunol*. 2006;7:1317-1325.

162. Ji Y, Pos Z, Rao M, et al. Repression of the DNA-binding inhibitor Id3 by Blimp-1 limits the formation of memory CD8(+) T cells. *Nat Immunol*. 2011;12(12):1230-1237.

163. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature*. 1997;388:394-397.

164. Reis e Sousa C, Hieny S, Scharton-Kersten T, et al. In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. *J Exp Med*. 1997;186:1819-1829.

165. Ingulli E, Mondino A, Khoruts A, et al. In vivo detection of dendritic cell antigen presentation to CD4+ T cells. *J Exp Med*. 1997;185:2133-2141.

166. Lund R, Ahlfors H, Kainonen E, et al. Identification of genes involved in the initiation of human Th1 or Th2 cell commitment. *Eur J Immunol*. 2005;35:3307-3319.

167. Hou J, Schindler U, Henzel WJ, et al. Identification and purification of human Stat proteins activated in response to interleukin-2. *Immunity*. 1995;2:321-329.

168. Jacobson NG, Szabo SJ, Weber-Nordt RM, et al. Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4. *J Exp Med*. 1995;181:1755-1762.

169. Grogan JL, Mohrs M, Harmon B, et al. Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. *Immunity*. 2001;14:205-215.

170. Gong D, Malek TR. Cytokine-dependent Blimp-1 expression in activated T cells inhibits IL-2 production. *J Immunol*. 2007;178:242-252.

171. Crotty S, Johnston RJ, Schoenberger SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat Immunol*. 2010;11:114-120.

172. Chong BF, Murphy JE, Kupper TS, et al. E-selectin, thymus- and activation-regulated chemokine/CCL17, and intercellular adhesion molecule-1 are constitutively coexpressed in dermal microvessels: a foundation for a cutaneous immunosurveillance system. *J Immunol*. 2004;172:1575-1581.

173. Mogues T, Goodrich ME, Ryan L, et al. The relative importance of T cell subsets in immunity and immunopathology of airborne Mycobacterium tuberculosis infection in mice.

J Exp Med. 2001;193:271-280.

174. Wei L, Laurence A, Elias KM, et al. IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J Biol Chem*. 2007;282:34605-34610.

175. Schoenberger SP, Toes RE, van der Voort EI, et al. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature*. 1998;393:480-483.

176. Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T- helper and a T-killer cell. *Nature*. 1998;393:474-478.

177. Agarwal P, Raghavan A, Nandiwada SL, et al. Gene regulation and chromatin remodeling by IL-12 and type I IFN in programming for CD8 T cell effector function and memory. *J Immunol*. 2009;183:1695-1704.

178. Pipkin ME, Sacks JA, Cruz-Guilloty F, et al. Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity*. 2010;32:79-90.

179. Iwata M, Hirakiyama A, Eshima Y, et al. Retinoic acid imprints guthoming specificity on T cells. *Immunity*. 2004;21:527-538.

180. Zhang N, Bevan MJ. CD8(+) T cells: foot soldiers of the immune system. *Immunity*. 2011;35:161-168.

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Chapter 15

The Innate Immune System

Luke A. J. O'Neill

INTRODUCTION

Every organism has to contend with infection by microbes. Infectious agents such as bacteria, viruses, and fungi threaten health and viability by competing for nutrients or more dangerously by killing the infected host as a consequence of infection or in order to spread to another host. This threat occurs largely because microorganisms divide at a faster rate than the host and because of the production of toxins that the microorganism uses for its own viability. The job of the immune system is firstly to sense the danger posed and secondly to mount an appropriate response. This response can be to tolerate the microorganism or to resist it such that at best sterilizing immunity occurs.

The immune system is traditionally split into two categories: innate and acquired (or adaptive) immunity. The term “innate” refers to the feature that the sensors involved are encoded by genes that do not undergo any rearrangement to generate variants. Adaptive immunity, on the other hand, refers to host defense proteins encoded by genes that undergo rearrangement to generate great diversity. This rearrangement process is not driven by the infectious agent, but happens by genetic programming. Antigens then lead to an expansion of antigen-specific B cells or T cells that help clear the infection and importantly persist in the form of memory cells. This means that when the host is re-infected, there are substantially greater numbers of the specific B- and T-cell populations that lead to clearance of the infectious agent. There are therefore insufficient numbers of microbes to trigger substantial systemic symptoms.

It is estimated that more than 99% of life on earth only has innate immunity.[1] What advantage would the evolution of adaptive immunity bestow on an organism? The answer lies in part in the nature of innate immunity. As will be discussed, the effector mechanisms here involve the inflammatory process, which when out of control can harm the host. In mammals, a “sickness behavior” also occurs, which includes processes such as fever, aches and pains, and drowsiness, which are thought to allow the infected host to rest and recuperate.[2] They also present a disadvantage, however, because they leave the host vulnerable to predators and decrease reproductive potential. Adaptive immunity occurs later in the cycle of infection, typically becoming evident several days after infection, and probably only after innate immune defenses have been breached. It is needed for sterilizing immunity. The fact that memory is a key feature of adaptive immunity, however (which is evident because of the expansion of specific B and T cells), means that upon re-infection, the innate response is limited because the pathogen is more rapidly cleared and there are therefore fewer microbial products present to activate innate immunity. In addition, there is evidence that adaptive immune cells

actually inhibit the innate immune response, which in the first infection acts in a negative feedback manner.[3] In subsequent infections, the memory cells probably inhibit innate immunity from the start of the infection. In essence, therefore, it appears that adaptive immunity evolved to promote sterilizing immunity and also to prevent innate immunity happening upon reexposure to pathogen, given its potential disadvantages in terms of systemic inflammation. Figure 15.1 illustrates this principal.

Previously thought to be crude and nonspecific, innate immunity is now known to involve a range of receptor families that recognize diverse microbial products. Importantly, these receptors program gene expression changes in target cells. The products of these genes drive inflammation and are also required for adaptive immunity. These proteins include cytokines that promote expansion of B and T cells. Other innate immune proteins have direct antimicrobial effects or act as opsonins, coating bacteria and promoting phagocytosis. Innate immunity has therefore gone from being viewed as a process lacking sophistication, to one determining for both inflammation and adaptive immunity.[4]

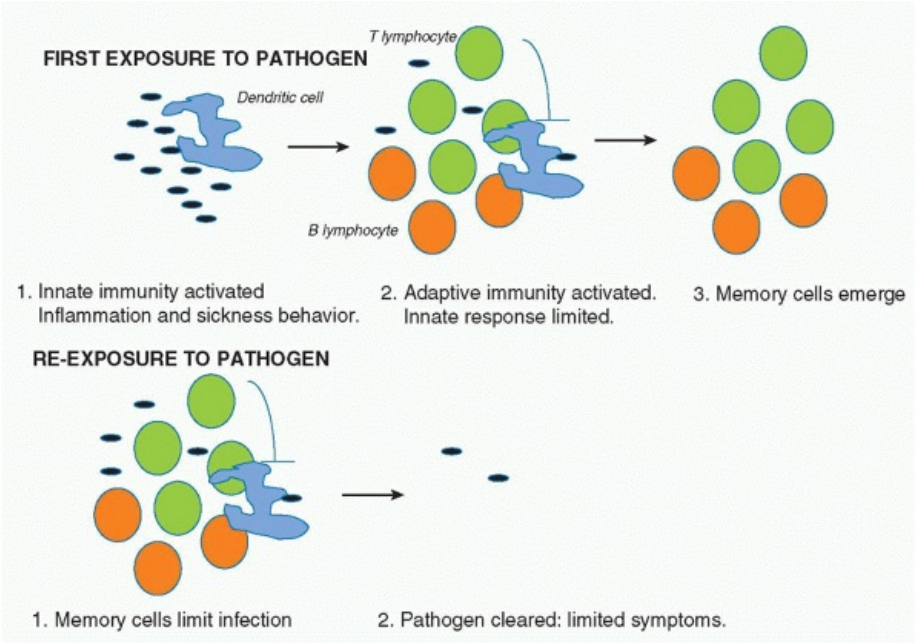

FIG. 15.1. The Relationship between Innate and Adaptive Immunity. A: Upon exposure to a pathogen, the innate immune response is triggered mainly by phagocytes and dendritic cells. Inflammation ensues, which leads to sickness behavior and host defense. The adaptive immune system is then triggered leading to effector T- and B-lymphocytes, some of which become memory cells. Adaptive immunity also has a negative feedback effect on innate immunity, preventing damage. **B:** When an organism is exposed to the same pathogen again, the memory cells can handle the infection, limiting innate immunity such that there are only minor symptoms. The evolution of adaptive immunity therefore provides an advantage to the host: adaptive immunity evolved to clear pathogens and limit innate immunity.

THE FRONTLINE OF INNATE IMMUNITY

The Skin

Innate immunity at its simplest involves barriers that prevent entry of microorganisms in the first place. In mammals, the skin is an obvious barrier that when broken in injury allows pathogens to enter. Many pathogens use insects that can deliver them across the skin into the bloodstream. A good example here is *Plasmodium falciparum*, which causes malaria. It is transmitted by mosquitoes feeding on blood. If the skin is broken, pathogens can enter. The importance of the integrity of the skin barrier can be seen in atopic dermatitis, an inflammatory skin disease. Mutations in the gene encoding fillaggrin, which is important for skin integrity, associate with atopic dermatitis.[5] The altered fillaggrin is less able to maintain the skin barrier, and the dermatitis is likely to be caused by microorganisms penetrating the skin or environmental irritants that provoke allergy.

The Epithelial Barrier

Equally important is the epithelial barrier that lines surfaces inside the body in the respiratory, gastrointestinal, and urogenital tracts. Microbes gain access to these surfaces via processes such as inhalation and digestion. The epithelial cells that make up the epithelium form tight junctions that keep the microorganisms out.[6] Specialized structures called cilia also expel microbes, and damage to cilia, for example caused by cigarette smoke, promotes respiratory tract infections.[7]

DIRECT-ACTING ANTIMICROBIAL FACTORS

The epithelia also produce a range of chemicals that limit microbial growth. Mucus is a key substance that traps microbes and is expelled. The importance of mucus can be seen in cystic fibrosis, where unusually thick mucus is made in lungs that limits expulsion and gives rise to growth of bacteria in the airways.[8] This chronic infection in turn leads to the lung pathology in this disease. In the stomach, acid production by the epithelium leads to low pH that limits growth of microbes. Enzymes that digest microbial proteins are also produced, including pepsin in the gut. A phospholipid-based substance termed pulmonary surfactant is made in the lungs that acts to trap microbes and can also directly lyse bacteria. Similarly, in skin the production of fatty acids in sebum limits bacterial growth. Lysozyme is a prominent enzyme in tears and saliva that breaks down peptidoglycan in the cell walls of gram-positive bacteria. Lysozyme is in fact a prototype of an innate immune protein because it does not affect the host cells, as they lack peptidoglycan. It is made by epithelial cells, but also phagocytes and Paneth cells in the crypts in the small intestine.

Antimicrobial Peptides

Another important example of innate immune proteins produced at barriers is antimicrobial peptides.[9] Epithelial cells are a major source of these peptides, but phagocytes can also make them. They are an ancient form of defense because they are found in most species, including insects and plants. There are three classes: defensins, catelicidins, and histatins. Defensins are 30 to 40 amino acid peptides that usually have three disulphide bonds. Their key property is amphipathicity (they contain both polar and nonpolar properties), and it is this property that is thought to underly their specificity for the membranes of bacteria, fungi, and

certain viruses. Their hydrophobic part inserts into these membranes, which are more positively charged than host cell membranes. A pore is formed, rendering the membrane leaky and leading to lysis.

There are three subfamilies of defensins-: α, β, and θ, which have different specificities in terms of target microorganisms.[10] They are produced as propeptides that require processing by proteases. Neutrophils produce α-defensins and store them in so-called primary granules from which they are secreted upon neutrophil activation. Paneth cells in the gut produce a class of α-defensins called cryptidins, which are processed by trypsin prior to secretion into the gut., β-defensins are made in the urogenital and respiratory tracts, skin, and tongue. Keratinocytes in skin are also a key producer of β-defensins.

Catelicidins lack the disulphide bonds in the α-defensins and are produced by epithelial cells, phagocytes, and keratinocytes. They are also produced as propeptides and in neutrophils are processed by elastases in secondary granules and then secreted in response to microbial stimulation. Histatins are produced in the parotid, sublingual, and submandibular glands in the oral cavity. They are histidine-rich and target fungal membranes.

Phospholipase A2

Phospholipase A2 (PLA2) is another antimicrobial protein found in primary granules of neutrophils and similar to defensins, is secreted during degranulation in response to bacterial activation. It is also secreted by epithelial cells and Paneth cells. PLA2 can directly kill gram-positive bacteria by hydrolysing phospholipids in the microbial membrane.[11] The means by which it distinguishes host membranes from bacterial membranes is because its effect on phospholipids is strongly potentiated by another protein termed bactericidal permeability increasing (BPI), which binds to lipopolysaccharide (LPS) in gram-negative bacterial membranes.[12] As will be discussed subsequently, LPS is one of most potent innate immune activators known; it is sensed by a protein termed toll-like receptor-4 (TLR4). LPS does not occur in host membranes, and so BPI only facilitates the effect of PLA2 on bacterial membranes. BPI disrupts the integrity of the bacterial membrane and presumably allows PLA2 to gain access to phospholipids, where it cleaves fatty acids from the sn-2 position, leading to lysis. Similar to PLA2, BPI is found in primary granules in neutrophils.

Lactoferrin

Lactoferrin is an 80 kDa protein that belongs to the transferrin family and therefore acts to sequester iron. This effect is thought to limit the availability of iron to bacteria, altering their metabolism and thereby compromising their ability to replicate.[13] Lactoferrin is found in the secondary granules of neutrophils and occurs at a high level in breast milk, its cellular origin there being epithelial cells. Lactoferrin can also be processed to generate an antimicrobial peptide called lactoferricin that, similar to other such peptides, can lyse bacterial membranes.

The Microbiota

A final front-line mechanism to keep microbes at bay is commensal bacteria in the healthy intestine. Commensal bacteria live in our intestines and serve several functions, including providing vitamin B_{12} and other nutrients. However, they also maintain epithelial barrier integrity, most likely because they are sensed by innate immune receptors that will be

described in subsequent sections, and these receptors seal the epithelial barrier against infection.[14] However, importantly they also compete with any pathogenic bacteria in the intestines limiting their growth. Similarly, on skin and in the urogenital tract, there are competing microbes.

THE INITIAL RESPONSE ONCE BARRIERS ARE BREACHED

What happens when skin or epithelial barriers are breached? Pathogens have mechanisms to get across the barriers, or if the barrier is damaged by trauma, pathogens will traverse the broken barrier. Two important cascades are triggered in blood that will generate inflammatory factors or act to wall off pathogens and prevent them from spreading. The first cascade to be characterized in detail is the complement cascade, described in detail elsewhere in this volume. Complement generates the membrane attack complex that will lyse bacteria. Complement factors such as C1q also bind to pathogen surfaces and act as opsonins, promoting uptake by neutrophils and phagocytes. The complement cascade also generates chemotactic factors such as C5a, which attracts neutrophils to the site of infection. Coagulation is the other cascade that is triggered, largely by bacteria. This will form clots that prevent bacteria from spreading.

The Neutrophil

A key cell type, however, that is engaged early in infection is the neutrophil, again described in detail elsewhere. Chemotactic peptides present in bacteria, termed fMLP, act as chemoattractants for neutrophils. Both C5a and fMLP also trigger an important process in the neutrophil termed the respiratory burst.[15] This involves an enzyme complex termed the nicotinamide adenine dinucleotide phosphate-oxidase. This is also present in macrophages and is activated either by the G-protein coupled receptors that bind fMLP or C5a, or by the process of phagocytosis. It is a multicomponent enzyme complex that contains three cytosolic subunits: p40phox, p47phox, and p67phox. It also contains a membrane-associated flavocytochrome complex comprising p22phox and p91phox. The subunits form the enzyme complex following activation of the low molecular weight G protein Rac. The nicotinamide adenine dinucleotide phosphate-oxidase generates reactive oxygen species by transferring an electron from its flavin adenine dinucleotide cofactor to molecular oxygen forming the superoxide anion and other oxygen radicals. This all occurs in the so-called phagolysosome, which comprises a fusion between the phagosome that contains the ingested bacteria with the lysosome. The oxidizing environment will be antibacterial, but in addition, will lead to acidification of the phagolysosome due to potassium and hydrogen ions being drawn in to neutralize the charged superoxide ion. The acidification, however, has been shown to lead to dissociation of proteolytic

enzymes such as elastase from the proteoglycan matrix in the phagolysosome.[16] These proteases will also have a direct effect on bacteria.

In addition, superoxide is a substrate for myeloperoxidase, which is stored in primary granules and generates hypochlorous acid and chloramines, as well as other reactive oxygen species that are bactericidal and fungicidal. A final oxygen radical generated in neutrophils is nitric oxide. It is produced by the enzyme nitric oxide synthase, which is induced by cytokines such as interferon-γ. Nitric oxide is also microbicidal.

THE CONCEPT OF PATTERN RECOGNITION: HISTORICAL PERSPECTIVE

Much of the forgoing components of innate immunity had been worked out at least in broad terms by the mid-1990s. However, the issue of the exact recognition mechanism during innate immunity by the host was poorly understood. In 1989, Janeway made the point that the continued focus on adaptive immunity, which was evident at the time, would lead to an asymptote being approached in our understanding of immunity.[17] He speculated that receptors must exist for microbial structures that were known to be proinflammatory, such as LPS from gram-negative bacteria. Receptors such as mannan-binding lectin (MBL), which binds mannose in microbial membranes (and which does not exist in host membranes) and CD14 (which binds LPS), had already been described at that stage, but importantly these proteins did not signal because they possessed no intracellular domains. MBL was shown to be an opsonin, and CD14 had no clear function. Janeway therefore proposed that pattern recognition receptors (PRRs) must exist that recognize microbial "patterns" such as LPS, termed pathogen-associated molecular patterns (PAMPs), and trigger activation of cells such as dendritic cells, which are prodigious producers of cytokines and also present antigen to T cells. At around the same time, Matzinger was proposing that the job of the immune system is to sense "danger."[18] The danger can be in the form of a microbe or in the form of tissue injury. Again, the idea that specific receptors would exist to recognize products of damaged tissue was proposed. In essence, what was being hypothesized was the existence of receptors that would drive the production of cytokines, as several cytokines had been found that could provoke inflammation and also adaptive immunity. LPS was a powerful inducer of such cytokines and yet no receptor had been convincingly described for LPS that would activate transcription factors such as NF-kB, which was known to be required for increased transcription of genes such as that encoding the potent proinflammatory cytokine tumor necrosis factor (TNF).

How did Janeway's insight that PRRs and PAMPs would form the core of innate immunity lead to the discovery of the TLRs, which became the prototypical PRRs? Five important findings were made.

1. The receptor for the proinflammatory cytokine interleukin (IL)-1, which is a key mediator of many aspects of inflammation and which, similar to LPS, could activate NFκB, was shown to have a signalling domain similar to a *Drosphila melanogaster* protein Toll.[19] The Toll protein had been discovered as being important in the generation of dorsal-ventral polarity in the developing *Drosophila* embryo. At first glance, this seemed odd. What was a receptor important for inflammation doing with a signaling domain highly similar to a protein in fruit fly development? Toll drives a transcription factor termed dorsal, however, which is highly similar to NFκB so it seemed as if a similar "machine" was being used in two different contexts: one for inflammation in the case of IL-1 and the other for development in the fruit fly.
2. In 1994, a protein termed N protein was described in tobacco by Whitham and colleagues, which provided resistance to tobacco mosaic virus.[20] This protein also had the domain found in the type I IL-1 receptor and Toll, which was then named the Toll-IL-1 receptor - resistance (TIR) domain. Clearly, the TIR domain was therefore important for innate immunity in very diverse species.

3. This was confirmed when in 1996, Lemaitre and colleagues reported that Toll also has a role in innate immunity in *Drosophila*, being activated in response to fungi and driving the antifungal peptide drosomycin.[21]
4. Mammalian proteins even more similar to Toll than the IL-1 receptor, and termed TLRs, were then reported[22] and one, termed Toll (subsequently renamed TLR4) when overexpressed in dendritic cells, was shown to drive costimulation of T cells, as reported by Medzhitov and colleagues.[23]
5. In 1999, Poltorak and colleagues reported that the lack of responsiveness of a strain of mouse termed C3H/HeJ to LPS was due to a mutation in the gene encoding TLR4, such that a proline in the signaling domain was converted to a histidine.[24] This rendered TLR4 unable to signal, identifying TLR4 as the longsought signaling receptor for LPS. It was therefore clear that TLRs were ideal examples of the PRRs proposed by Janeway. Subsequently,[10] TLRs were described in human, although other species have very many more, notably the sea urchin, which has over 150.[25] TLRs were shown to recognize a diverse range of PAMPs and also in some cases products of damaged tissue, termed danger-associated molecular patterns (DAMPs). Complex signaling pathways were elucidated and links to disease established. The discovery of TLRs also galvanized researchers to seek other PRR types, and there are now several families, notably the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), which sense viral ribonucleic acid (RNA), the nucleotide oligomerization domain (NOD)-like receptors (NLRs), which sense bacteria and DAMPs, and the C-type lectin receptors, which sense fungi.[26] Figure 15.2 illustrates the main families of PRRs. A level of complexity in innate immunity was therefore revealed that has provided great insight into the overall workings of the immune systems of many organisms.

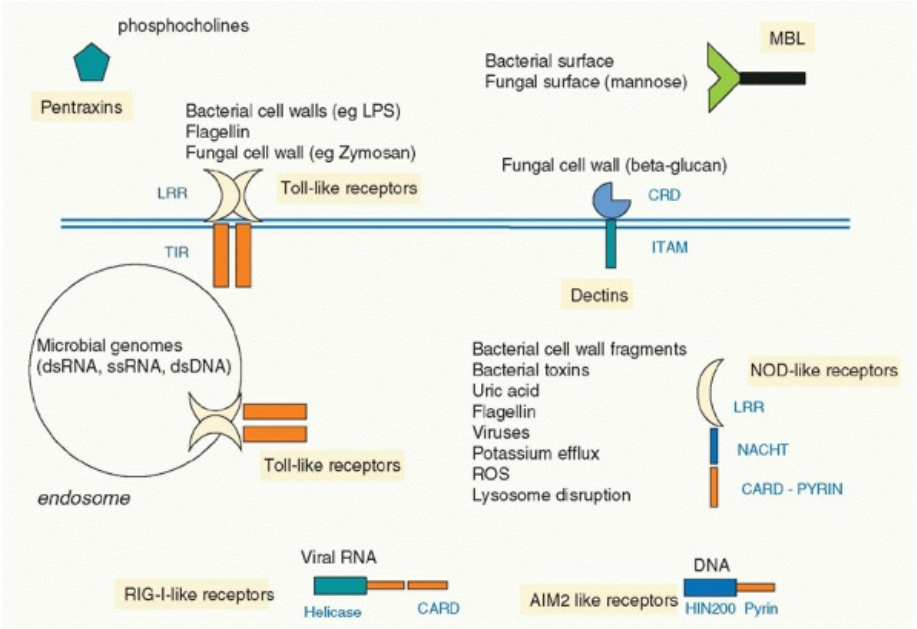

FIG. 15.2. Innate Immune Sensors. Several classes of innate immune sensors have

been found. Soluble factors such as pentraxins and mannoan-binding lectins bind pathogen structures and promote phagocytosis. Toll-like receptors (TLRs) sense bacteria, fungi, and protozoans from the plasma membrane, whereas endosomal TLRs sense microbially derived nucleic acids. C-type lectin receptors sense fungal cell wall components and mycobacteria. In the cytosol nucleotide oligomerization domain-like receptors sense microbial products and also, in the case, of Nlrp3 particles such as uric acid and IAPP. Retinoic acid-inducible gene I-like receptor sense viral ribonucleic acid, whereas AIM-2-like receptors sense microbial deoxyribonucleic acid. All lead to signaling pathways culminating in the induction of immune and inflammatory genes.

Toll-Like Receptors

TLRs are type I transmembrane receptors that have leucinerich repeats (LRRs) and a TIR domain. LRRs are found in many proteins, and that they occur in TLRs is of interest given their capacity to interact with different kinds of ligands. Also of note is that in agnathans, the LRR is used to build a repertoire of variable lymphocyte receptors, which are analogous to the antibody repertoire in human.[27] The LRR domain is therefore of evolutionary interest in that it is used for TLRs but also generation of diversity in the lamprey and the hagfish. The immunoglobulin domain serves this purpose in vertebrates and in fact is fused to the TIR domain in the type I IL-1 receptor. Figure 15.3 illustrates the relationship between these domains.

The 10 TLRs in human have different ligand specificities, and several ligand/TLR structures have been solved.[28] Broadly speaking, TLRs can be split into two families. TLRs 1, 2, 4, 5, and 6 are expressed in the plasma membrane, and their job is to sense bacterial, fungal, and protozoal products. TLRs 3, 7, and 9 are all expressed in endosomal membranes with their LRRs in the lumen and their TIR domains pointing into the cytosol. Their role is to sense viral nucleic acids. There are also differences in relation to signaling,[29] with all of the TLRs with the exception of TLR3 signalling via MyD88, to NF-κB and mitogen-activated protein (MAP) kinases such as p38. TLR2 and especially TLR4 require a bridging adapter termed Mal to recruit MyD88. TLR3 signals via Trif to NF-κB and another transcription factor IRF3. TLR4 can also signal via Trif and does so via the second bridging adapter Tram.

Toll-Like Receptors 2, 1, and 6

TLR2 recognizes multiple ligands. From bacteria, these include lipopeptides, lipoproteins, lipoteichoic acid, and mycobacterial lipoarabinomannan. From yeast, TLR2 recognizes the cell wall structure zymosan. TLR2 also recognizes glycosylphosphoinositol from the parasite *Tryanosoma cruzi*. Alone amongst the TLRs, TLR2 can heterodimerize with other TLRs, and this provides a degree of specificity in ligand recognition.[30] The TLR1/TLR2 dimer binds triacylated lipopeptides whereas TLR2/TLR6 recognizes diacylated lipopepides. In the case of TLR2/6, one acyl chain fits into a hydrophobic pocket in one TLR, whereas the second acyl chain fits into the other TLR.[31] This allows the TLRs to dimerize, an important event for signaling. There is also a coreceptor for TLR2/6 in the form of CD36, which appears to deliver the

lipopeptide to the TLR dimer.[32] TLR2 is expressed on macrophage, dendritic cells, B cells, and also on regulatory T cells.

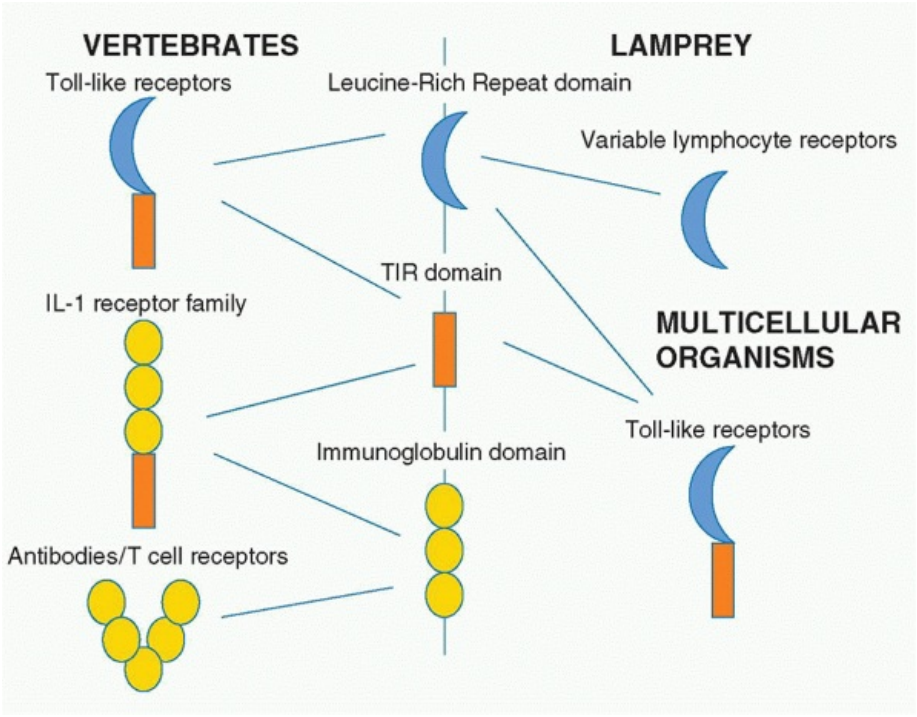

FIG. 15.3. Protein Domains in Innate and Adaptive Immunity. Three protein domains occur in several immune system proteins. The leucine-rich repeat occurs in toll-like receptors in vertebrates and all multicellular organisms combing with the toll-IL-1 receptor-resistance (TIR) domain. The leucine-rich repeat also forms the basis of the repertoire of variable lymphocyte receptors in the hagfish. The immunoglobulin domain only occurs in vertebrates and is found in antibodies and T-cell receptors. However, it also combines with the TIR domain to form interleukin-1 receptor family members.

Toll-Like Receptor 4

TLR4 is the receptor for LPS and is the most studied TLR. It acts as a homodimer but on its own is unable to recognize LPS. Similar to TLR2 and CD36, a coreceptor is needed, termed MD2.[33] LPS is a hexacylated lipid, and five of the acyl chains are buried in MD2, whereas the sixth is on the surface and interacts with TLR4.[34] However, two other proteins are needed to deliver LPS to MD2. LBP occurs in serum and binds LPS monomers, transferring them to CD14.[35] CD14 can occur as a soluble protein or as a glycerophosphatidylinositol anchored protein.[36] The job of CD14 appears to be to concentrate LPS and deliver it to MD2. In B cells, there are two other accessory molecules: RP105 and MD1.[37] These appear to serve an analogous function to CD14 and MD2. Recognition of LPS is therefore highly complex. This is presumably because once TLR4 is activated, there can be lethal consequences in the form of septic shock; therefore, several proteins are needed for LPS recognition. Similar to TLR2, a dimerization event occurs upon ligand binding that allows the intracellular TIR domains to come into juxtaposition and trigger signaling pathways. TLR4 has

been shown to recognize other ligands, notably F protein from respiratory syncytial virus,[38] although the basis for this recognition is not known. Another example is the metal nickel. In contact dermatitis, there is an inflammatory reaction to nickel, and it has been shown that nickel coordinates two key histidines on different TLR4 proteins, causing a cross-linking event that activates TLR4 and drives the inflammatory process in skin.[39] TLR4 is expressed on multiple cell types, notably on macrophages and dendritic cells but also on neutrophils and in the endothelium.

Toll-Like Receptor 3

TLR3 is the receptor for double-stranded RNA, found in viruses or more commonly in the form of the viral RNA analogue polyIC. PolyIC had been known for some time to be a potent inducer of the antiviral type I interferons. Similar to LPS, therefore, the discovery of TLR3 as the receptor for polyIC provided a molecular explanation for how polyIC induces this response. TLR3 is expressed on macrophages and dendritic cells and importantly on CD8+ dendritic cells, which are potent type I interferon producers.

Toll-Like Receptor 5

TLR5 is the receptor for bacterial flagella.[40] Flagellin is a key protein in flagella, which occur in multiple bacteria species. Flagellin is in many ways a prototypical PAMP because it does not occur in humans and can bear few mutations in bacteria. TLR5 is therefore required for host defence against flagellated bacteria such as *Legionella* and *Salmonella*.

Toll-Like Receptor 7

TLR7 recognizes single-stranded RNA from viruses. It is highly expressed in plasmacytoid dendritic cells, where when activated it induces type I interferons. TLR7 was first described as the receptor for a small molecule termed imiquimod, which is used to treat genital warts. This effect is mediated by type I interferons. TLR7's expression on plasmocytoid dendritic cells reveals a key function in antiviral immunity.

Toll-Like Receptor 9

TLR9 is the deoxyribonucleic acid (DNA) sensing TLR that, similar to TLR3 and TLR7, is found in endosomes. The type of DNA recognized is of interest as it binds to hypomethylated CpG-rich DNA, commonly found in bacteria and viruses. TLR9 is also expressed in plasmacytoid dendritic cells as well as in macrophages and in the epithelium. Once activated, again by dimerization, TLR9 induces a strong proinflammatory signal, increasing TNF production as well as type I interferons. A protein termed Unc93b is required to traffic TLR9 from the Golgi to intracellular vesicles. The CpG-rich DNA

from pathogens is taken up by cells in an endosome, which fuses with the TLR9-expressing endosome. In this way, the receptor can interact with its ligand. TLR9 has no known coreceptors, but it has been shown that it requires cleavage by cathepsins for full activation.[41]

The Recognition of Danger-Associated Molecular Patterns by Toll-Like Receptors

TLRs have also been implicated in the recognition of products of damaged tissues.[42] This is an important area as the inflammatory process can occur under conditions where there is apparently no infection. DAMPs include a range of factors released by damaged cells and include high-mobility group protein 1, heat shock proteins such as hsp90, products of damaged connective tissue such as hyaluronic acid fragments, versican and tenascin-C, the acute-phase reactant serum amyloid A, pyrrole-based lipids such as CEP, and coagulation products such as fibrinogen. This area has been somewhat controversial, as in vitro there is a risk of LPS contamination. However, the consensus has emerged that at least some of these are indeed sensed by TLRs although what the mechanism might be is not fully understood. Many of these are sensed by TLR4, notably high-mobility group protein-1, hsps, hyaluronan fragments, and tenascin-C. TLR2 has been shown to sense versican and CEP. Importantly, these factors are only produced by damaged cells, as would occur during tissue injury. There may be coreceptors involved here, which explains the recognition process, because it is difficult to imagine how a single TLR can recognize multiple ligands. A clearer example is the sensing of host nucleic acids in the case of cell damage. Necrotic cells have been shown to release double-stranded RNA, which is sensed by TLR3. TLR7 can sense host RNA, and TLR9 can sense host DNA in the context of immune complexes. This has particular relevance to autoimmune diseases such as systemic lupus erythematosus (SLE).[43]

Toll-Like Receptor Signaling Pathways

The key output from TLR signaling is cytokines, and TLRs filled the missing gap in our understanding of what actually induces cytokines. Because they bind directly to microbial ligands, TLRs bring us close to the initiation of host defense.

The type of cytokine being induced is dependent on the TLR. TLR4 is the most prodigious activator of macrophages known, with many genes induced by TLR4. Other TLRs are less prolific but all share the feature of driving inflammation. Cytokines such as IL-6, TNF, and the chemokine IL-8 are typical responses for plasma membrane receptors. For the endosomal TLRs, type I interferons are the typical readout. What gives rise to signaling specificity? As stated previously, TLRs use different adapter proteins. All have a TIR domain, and upon TLR dimerization, adapter TIR domains are likely to be recruited to the interface formed between two TLRs. This then launches the signaling pathways.

Broadly speaking, TLR signaling pathways can be divided into two: MyD88-dependent and MyD88-independent. All TLRs, with the exception of TLR3, signal via MyD88. IL-1 receptor family members, including the receptor for IL-18, also signal via MyD88.[44] The MyD88 dependent pathway involves the IL-1 receptor-associated kinases (IRAKs). There are four of these: IRAK-1, IRAK-2, IRAK-4, and IRAK-M. They interact with MyD88 via their death domains, because along with the TIR domain, MyD88 has a death domain. The structure of this multicomponent signaling machine, termed the MyDDosome, has been solved.[45,46] Strikingly, it has a stoichiometry of 14. SIx MyD88 subunits assemble and bring four IRAK-4s to the complex. These sit on top of the assembled MyD88 subunits in a helical structure. Four IRAK-2s or IRAK-4s are then recruited. All of the death domain interfaces in this complex have been solved, and it is predicted to have a high degree of cooperativity. The so-called MyDDosome launches signaling pathways activated by TLRs.

Once assembled, IRAK-4 phosphorylates IRAK-1, and this event appears to be key for

signaling. The IRAKs then dissociate from the complex, and IRAK-1 and/or IRAK-2 then interact with the ubiquitin ligase Traf6, a member of the Traf family. Once activated, Traf-6 signals in association with two noncanonical E3 ubiquitin ligases, Ubc13 and Uev1A.[47] This results in polyubiquitination of Traf6. This is a type of ubiquitination termed K63-linked ubiquitination. This covalently modifies Traf-6 causing oligomerization and downstream activation. This activation involves the IkappaB kinase (IKK) complex for the NF-κB pathway. The main form of NF-κB in cells is a p50/p65 dimer. It is, however, kept in check by an inhibitory protein I-κB. Traf6 activates the kinase TAK-1, which in turn activates the IKK complex leading to I-κB degradation (via K48-linked polyubiquitination). This releases the p50/p65 dimer, exposing a nuclear localization sequence and allowing NF-κB to bind its consensus site in target genes. In addition, TAK-1 can activate MAP kinase cascades, notably via MKK3/6, which leads to p38 MAP kinase activation, and MKK7, which leads to activation of Jun N-terminal kinase. Both of these MAP kinases can activate transcription factors, such as ATF3 in the case of p38 and Jun in the case of Jun N-terminal kinase. This in turn will modulate inflammatory gene expression.[48]

MyD88 is also involved in activation of IRF7 by TLR7 and TLR9.[49] This is an important transcription factor for the induction of interferon-alpha, a key antiviral cytokine but also a cytokine implicated in the pathogenesis of SLE.

The importance of the MyD88 pathway can be seen in MyD88-deficient mice, which are unable to mount an effective host defense response to multiple pathogens, including a wide range of bacteria, as well as fungi, protozoans, and certain viruses.[50] In addition, MyD88-deficient mice are resistant to inflammation in a number of disease models including models of SLE and arthritis. However, in the case of humans, the situation is not as clear-cut. This is because of reports of MyD88 deficiency in human.[51] People with this deficiency show higher mortality in childhood but susceptibility to a restricted set of pathogens, the main defect in host defense being against pyogenic infections such as *Streptococci*. This is also the case in subjects with IRAK-4 deficiencies.[52] Once people with these deficiencies reach adulthood, however, no immunodeficiencies are evident. It is likely, therefore, that the MyD88 pathway is somewhat redundant in children and is not required in adults probably

because of the emergence of adaptive immunity. This supports the concept raised at the start of this chapter, that once memory is established, innate immunity is not as important.

The second TIR domain adapter to be found was Mal (also termed TIRAP).[53,54] This protein is required for MyD88-dependent signaling specifically by TLR2 and especially TLR4. It appears to act as a bridging adapter for MyD88 to be recruited to the receptor TIR domains and has a PIP2 binding domain which localizes it to the plasma membrane.[55] It is also a substrate for IRAK-1 and IRAK-4, and upon phosphorylation undergoes ubiquitination and degradation,[56] providing a negative feedback loop in TLR signaling.

The MyD88-independent signalling pathway involves the third adapter, Trif.[57] This adapter is used by TLR3 to signal and activates NF-κB as well as another transcription factor IRF3 via activation of the kinase TBK-1.[58] TLR4 can also activate Trif, but this happens in endosomes. The fourth adapter, Tram, mediates this effect, acting as a bridge for Trif in a

manner similar to Mal acting as a bridge for MyD88.[59] Tram has a myristoylation motif that localizes it to the plasma membrane.[60] However, upon activation of TLR4, Tram undergoes phosphorylation by protein kinase C-epsilon.[61] This leads to dissociation of Tram from the plasma membrane, and it is likely that a TLR/Tram complex localizes to the endosome, where Trif is then recruited, again leading to TBK-1 activation and induction of type I interferons. The TLR4 signal is terminated by a splice variant of Tram termed TAG, which displaces the Trif/Tram complex.[62] It is this dual pathway, Mal/MyD88 and Tram/Trif, occurring at different subcellular localizations, that probably make LPS such a powerful innate immune activator, as a wide range of genes can be induced. Figure 15.4 illustrates the TLR4 signaling pathway.

The final TIR domain adaptor to be found is SARM. This is a large protein with a TIR domain and SAM and ARM domain. SARM is a negative regulator of Trif-dependent signaling, acting as another modulator of TLR4 action.[63] It is of interest that there are several mechanisms to inhibit TLR signaling, including microRNAs, decoy receptors, ubiquitin regulators (eg, A20), and splice variants of MyD88 and IRAK2 that are inhibitory.[64] This tells us that TLRs, and in particular TLR4, must be kept under control otherwise the host can be damaged or even succumb.

Viruses have also been shown to target TLRs. The best examples are two vaccinia virus proteins A46R and A52R.[65] A46R has been shown to block recruitment of TIR domain-containing adapters, particularly Mal and Tram.[66] A52R inhibits activation of the IRAKs.[67] Deletion of A52R leads to attenuation of the virus, confirming its importance for viral evasion.[68]

Conservation of the Toll-Like Receptor System Across Nature

Every multicellular organism has proteins with TIR domains, attesting to their evolutionary importance. A particular case is in plants, which have a number of TLRs.[69] As mentioned previously, N protein from tobacco has a TIR domain and is required for antiviral immunity. Similarly, L protein from flax has a TIR domain and is required for resistance to flax rust. Plants also have IRAK-like kinases activated by these receptors, notably BAK1 and BIK1 in Arabidopsis. The receptor for flagellin in Arabidopsis is sensed by a leucine rich repeat protein termed FLS-2. This receptor has an IRAK-like kinase domain actually attached and also activates BAK-1 and BIK-1. FLS-2 activates MAP kinases such as MEKK1, which probably lead to activation of the AtWRKY family of transcription factors. In addition, ubiquitin ligases termed PUBs ubiquitinate FLS-2, downregulating it.[70] The parallel in human is Triad3a, which ubiquinates TLR4 leading to its degradation.[71] These conserved elements confirm the importance of the TLR pathways across evolutionary time. Table 15.1 illustrates the conservation between mammals, insects, and plants.

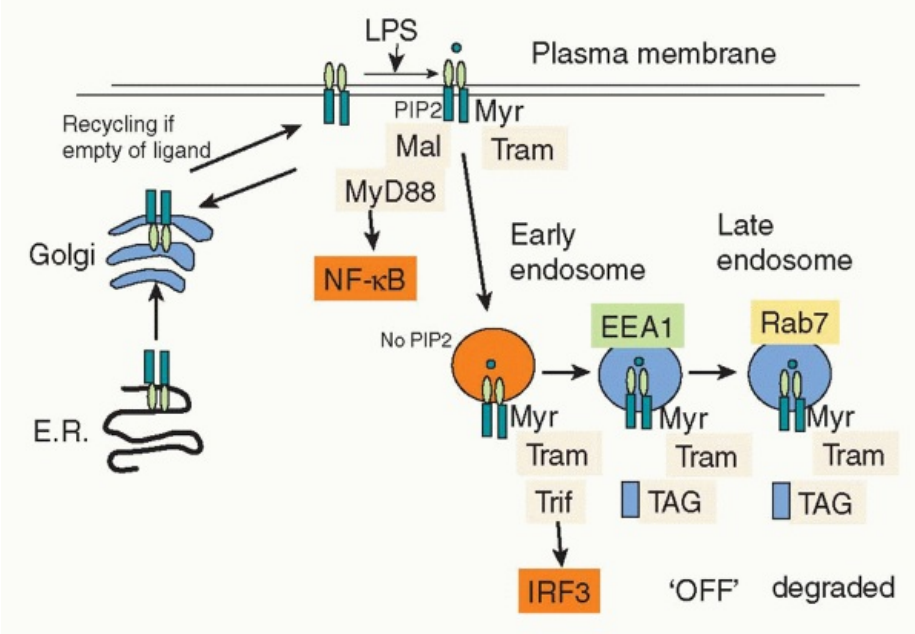

FIG. 15.4. Subcellular Toll-Like Receptor 4 Signaling. The signaling pathway, activated by lipopolysaccharide (LPS) via toll-like receptor-4 (TLR4), is highly complex. In resting cells, TLR4 recycles from the endoplasmic reticulum to the plasma membrane. An LPS/MD2 complex binds TLR4, stabilizing it transiently at the plasma membrane. The activated TLR4 dimer recruits the adapters Mal and MyD88, which then lead to activation of NF-κB, which induces multiple immune and inflammatory genes, such as tumor necrosis factor, interleukin-1beta, and interleukin-6. Mal is retained in the membrane by binding the phospholipid PIP2. Th adapter Tram is also membrane-localized via a myristate in its N-terminus. Tram then undergoes phosphorylation by protein kinase C-epsilon. TLR4 then internalizes in combination with Tram and localizes to an early endosome where it encounters Trif. This adapter activates TBK-1 and promotes IRF3 activation, which regulates the expression of type I interferons and other gene products. The endsome containing TLR4 then matures to a late endosome where TAG is engaged. This protein displaces Trif from Tram, and TLR4 is ultimately degraded.

Therapeutic Targeting of Toll-Like Receptors

TLRs are being targeted clinically, either in the form of new vaccine adjuvants or with inhibitors that would be anti-inflammatory.[72] The best example of an adjuvant approach is imiquimod, a compound found to be antiviral by activating TLR7. This induces type I interferons, and imiquimod is used to treat genital warts. Another example of an adjuvant approach is the use of monophosphoryl lipid A in vaccines for such pathogens as human papilloma virus. This acts via TLR4 but is not as toxic as LPS. In terms of anti-inflammatory

approaches, eritoran, an antagonist toward TLR4, has been tested in septic shock, but with only limited success. As referred to previously, TLR4 has also been implicated in the pathogenesis of contact dermatitis. It is also implicated in rheumatoid arthritis and inflammatory bowel disease. TLR2 has been shown to be important for ischemia/reperfusion injury and may prove interesting as a target to limit this process in transplantation.[73] There is

promise that targeting TLR7 and/or TLR9 might be effective in SLE, as in this disease TLR7 senses host ribonucleoprotein complexes (known to be implicated in SLE) while TLR9 senses DNA/chromatin complexes (again implicated in SLE). Whether inhibiting TLRs will be prove to be to immunosuppressant remains to be seen, although given the redundancy in the TLR system in relation of host defense, this may not pose a problem, particularly if a given TLR has a nonredundant role in inflammatory disease.

TABLE 15.1 Conservation of Innate Immunity across Species

	Human	Fruit Fly	Plant
Receptor (TIR domain)	Toll-like receptors	Toll	Tobacco: N protein Flax: L6
Adapter protein (TIR domain)	MyD88, Mal, Trif, Tram	dMyD88	
Non-RD kinase	IRAK-1	Pelle	Rice: XA21
RD kinase	IRAK-4		Arabidopsis: BIK1
Ankyrin repeat	IkappaB NF-κB	Cactus Relish	Rice: NH

RD, Arginine-Aspartic acid; TIR, toll-IL-1 receptor-resistance.

Notes: Each receptor senses microbial products and activates the adapters and kinases indicated. Human and insects have the NF-κB family transcription factors on these pathways. Plants lack NF-κB but have other transcription factors such as the WRKY family.

NOD-Like Receptors

The second major family of PRRs are the NLRs.[74] Nod-like receptors undergo oligomerization in response to activation. They also have LRRs, similar to TLRs. There are several subfamilies that can be defined by additional domains. The main members are summarized in Table 15.2. Of particular interest are the NLRC family, which have antibacterial NOD1 and NOD2 as members and are defined by the caspase recruitment domain (CARD). The Nlrp subfamily contains a pyrin domain, and the prototypical member is Nlrp3, which is a key component of the inflammasome multiprotein complex that regulates caspase-1 and drives production of the proinflammatory cytokine IL-1beta. The NAIP subfamily contains a Bir domin. All NLRs are intracellular proteins, implying that ligands have to be endocytosed in order to activate them.

Nucleotide Oligomerization Domain 1 and Nucleotide Oligomerization Domain 2

NOD1 and NOD2 sense breakdown products of peptidoglyan, which is a major component of the cell wall of gram-positive and gram-negative bacteria. The finding that NOD2 mutations strongly associate with Crohn disease was an important finding. It supported the hypothesis that aberrant sensing of intestinal microbes is important for the pathogenesis of inflammatory bowel disease.[75] NOD2 detects muramyl dipeptide, whereas NOD1 detects *meso*-diaminopimelic acid. This latter component is more common in gram-negative bacteria. Peptidoglycan is constantly remodeled during bacterial growth, and the component parts are produced constantly, making them ideal PAMPs. How the ligands get into the cytosol is not wholly clear, however. Transport systems such as pannexin, PepT1, and PepT2 have been shown to carry muramyl peptides across the plasma membrane, and there is also evidence of endocytosis.[76] NOD1 and NOD2 signaling leads to NF-κB and MAP kinase activation in a manner similar to TLRs. There are no adapters, however, and they recruit RIP2 kinase to activate these downstream pathways. NOD1 and NOD2 agonists can synergise with TLRs, which is likely to be important in vivo.[77]

Role of Nucleotide Oligomerization Domain 1 in Antimicrobial Responses

The first pathogen shown to be sensed by NOD1 in vivo was *Helicobacter pylori* ; NOD1-deficient mice were demonstrated to be more susceptible.[78] Key outputs from NOD1 in this model are chemokines, antimicrobial peptides, and type I interferons. NOD1 has also been shown to be important in the host defense response to other pathogens,

including *Clostridium difficile*, *Legionella pneumophilia*, and *Listeria monocytogenes*.[79] In all cases, impaired recruitment of neutrophils was a feature of the NOD1-deficient mice, highlighting again the importance of neutrophils in antibacterial host defense. NOD1-deficient mice have also been shown to be impaired in their response to the intracellular parasite *Trypanosome cruzi*.[80] Although the *T. cruzi* ligand is not known, macrophages from the deficient mice have an impaired ability to kill intracellular parasites.

TABLE 15.2 Notable Nucleotide Oligomerization Domain-Like Receptor Family Members

Subfamily	N-terminus	Function
NLRB (NAIP)	BIR	Binds flagellin
NLRC		
NOD1	CARD	Binds ieDAP

NOD2	CARD	Binds MDP
NLRC4	CARD	Binds flagellin/NAIP
NLRP		
NLRP1	Pyrin domain	Binds anthrax lethal toxin
NLRP3	Pyrin domain	Mutiple activators (eg, uric acid, IAPP, cholesterol crystals)

BIR, Baculo Virus Repeat; CARD, caspase recruitment domain; IAPP, islet amyloid polypeptide; ieDAP, Diaminopimelic acid; MDP, Muramyl Dipeptide; NAIP, Neuronal Apoptosis Inhibitory Protein; NLRB, Nod-like receptor with BIR domain; NLRP, Nod-like receptor with Pyrin domain; NOD, nucleotide oligomerization domain.

Role of Nucleotide Oligomerization Domain 2 in the Antimicrobial Response

Similar to NOD1, NOD2 has been shown to be important for host defense against multiple bacteria. One interesting aspect, however, was that NOD2 was only shown to be required for host defense against *L. monocytogenes* when given by the oral route.[81] Intravenous and intraperitoneal infections were handled normally in NOD2-deficient mice. This suggests that other PRRs can compensate for the lack of NOD2 depending on the route of infection. There is also redundancy in relation to NOD1 in the response to *Salmonella typhiurium* infection. NOD1- or NOD2-deficient mice are normal in their response, but the double knockout is impaired, with decreased inflammation and enhanced colonization of the intestine.[82]

Autophagy, Adaptive Immunity, and Nucleotide Oligomerization Domain 1 and Nucleotide Oligomerization Domain 2

Another interesting aspect of NOD1 and NOD2 biology concerns a process termed autophagy. This is a process whereby an organelle called the autophagosome digests material in the cytosol of cells. Autophagosomes are related to lysosomes and were originally shown to be important in development and in starvation where in effect a cell will digest some of its own contents to provide nutrients. Autophagy has also been found to be important in antimicrobial immunity. It is an important part of intracellular innate immunity and may in fact be the means by which cells such as macrophages clear bacteria in a process that was originally thought to involve the lysosome. Autophagosomes can be viewed as a subtype of lysosome. These insights came from the uncovering of proteins required for the formation of the autophagosome, notably ATG16, which is recruited to membranes and allows formation of the autophagosomal double membrane structure. NOD1 and NOD2 activation have been shown to lead to recruitment of ATG16L to the plasma membrane sites where *S. typhimurium* and *Shigella flexneri* intrude.[83] This leads to degradation of bacteria in autophagosomes and importantly to the processing of bacterial antigens for presentation on major histocompatibility complex class II molecules. This is a classic example of the link between

innate and adaptive immunity. Also of interest is the observation of mutations in ATG16L in Crohn disease and the observation that the mutant NOD2 also in Crohn disease is less able to drive ATG16L recruitment.[84] This could mean that impaired antigen presentation to T cells (eg, regulatory T cells that produce IL-10) might be important in Crohn disease pathogenesis.

There is additional evidence that NOD1 and NOD2 promote adaptive immunity. This was first observed when it was realized that muramyl dipeptide was a key component in Freund's adjuvant, a standard adjuvant used in experimental immunology to drive antibody production. This is probably due to NOD2 inducing important T-cell-activating cytokines, but there is also evidence for NOD2 in T cells themselves where it promotes interferon-gamma production.

Inflammasomes

There are 14 members in the Nlrp branch of the NLRs, and those with a known function are components in inflammasomes. The term inflammasome comes from the fact that the regulation of caspase-1 was shown to be due to assembly of a multiprotein complex involving Nlrps and also the scaffold protein Asc.[85] This complex is needed for the activation of caspase-1, the enzyme that processes the pro-forms of IL-1beta and IL-18. Given the importance of these cytokines in inflammation and inflammatory diseases, much attention has focused on inflammasome regulation.

Nlrp3

The Nlrp3 inflammasome has been characterized in detail. Nlrp3 has a carboxy-terminal LRR domain, a central NOD domain, and an amino terminal pyrin domain. The pyrin domain mainly interacts with the key scaffold protein Asc. It is triggered by agents phagocytosed by macrophages, including bacteria and viruses, but also particles such as uric acid, silica, asbestos and cholesterol crystals, and amyloidcontaining proteins such as beta-amyloid and islet amyloid polypeptide (IAPP).[86] Uric acid, cholesterol crystals, betaamyloid, and IAPP all constitute DAMPs. Activation mechanisms are not fully worked out, although potassium efflux is a common event that is required for oligomerization of the Nlrp3/Asc complex. Roles for reactive oxygen species and cathepsins have also been suggested.[87,88] CARD domains in Asc and caspase interact, whereas the pyrin domain in Nlrp3 interacts with the pyrin domain in Asc, allowing for the complex to assemble. This is needed to activate caspase-1. Extracellular adenosine triphosphate is also able to activate the Nlrp3 inflammasome. Also of note is the fact that the system must be primed by other innate receptors, notably the TLRs, which induce Nlrp3 and also pro-IL-1beta expression.

The effect of uric acid on Nlrp3 is of interest because uric acid may be a common danger signal from damaged cells, as well as having a clear role in gout.[89,90] Similarly, the inflammation associated with silica and asbestos is likely to be due Nlrp3 activation.[91] The effect of cholesterol crystals, which form in atherosclerotic plaques, on Nlrp3 implicates the inflammasome in heart disease.[92] Similarly, the effect of beta-amyloid suggests a role in Alzheimer disease.[93] In the case of IAPP, a role in type 2 diabetes is suggested.[94] IL-1 has been implicated in insulin resistance and beta cell loss in type 2 diabetes, but s there is no infection, what drives IL-1beta is not clear. Hyperlipidemia is now known to activate the Nlrp3 inflammasome in adipose tissue.[95] This will

lead to IL-1beta release, which causes insulin resistance. The beta cells in the pancreas respond by secreting insulin, but also IAPP, which modulates insulin action. However, IAPP can form amyloid, and macrophages in the pancreas take up IAPP; the Nlrp3 inflammasome is activated leading to IL-1beta production. These findings support the clinical targeting of IL-1beta in several diseases notably type 2 diabetes and atherosclerosis.[96]

Another important aspect of the Nlrp3 inflammasome concerns genetic mutations. Activating mutations in Nlrp3 lead to autoinflammatory diseases such as Muckle Wells disease and familial Mediteranean fever.[97] Autoinflammatory diseases such as these are prototypical genetic diseases of innate immunity because there are only inflammatory symptoms with no roles for T and B cells and autoimmunity. The diseases are systemic, involving skin inflammation, arthropathy, and amyloidosis. They are treatable with anti-IL-1beta blocking agents, attesting to the potency of this proin-flammatory cytokine.

Nlrp3 in Host Defense

Several bacteria have been shown to activate the Nlrp3 inflammasome. A common feature is pore-forming toxins that cause potassium efflux. *Listeria* produces a toxin called listeriolysin that is required for Nlrp3 activation.[98] Secretion of pneumolysin by *S. pneumoniae* or streptolysin O from *S. pyogenes* both result in activation of the Nlrp3 inflammasome.[99]

Nlrp3 is also activated by viruses. Influenza is a potent Nlrp3 activator, and Nlrp3-deficient mice are less able to survive influenza infection.[100] Viral RNA was shown to prime the inflammasome via TLR7, whereas activation of Nlrp3 was mediated by the influenza-encoded M2 ion channel, which led to potassium efflux.[101] Other viruses such as the DNA viruses vaccinia and varicella zoster, and RNA viruses such as encephalomyocarditis virus and vesicular stomatits virus all activate the Nlrp3 inflammasome.[102] It is therefore likely that the Nlrp3 inflammasome is key for the fever response to all these viruses, as IL-1beta is a key pyrogen during infection.

The Nlrp3 inflammasome can also be activated by fungi, notably *Candida albicans* and *Saccharomyces cerevisiase*.[103] Again, there is interplay with TLRs, as zymosan (a PAMP in the cell wall of yeast) acting via TLR2 primes the Nlrp3 inflammasome for activation. *Aspergillus fumigatus* can also activate the inflammasome.[104]

Finally, parasites can also activate the Nlrp3 inflammasome. *Plasmodium falciparum*, the causative agent of malaria, secretes a crystal called hemazoin, which activates Nlrp3.[105] Malaria severity is less in Nlrp3-deficient mice. Schistosomes have also been shown to activate Nlrp3.

Overall, therefore, fever, a common response to infection, is likely to involve the Nlrp3 inflammasome in many cases.

IPAF

Another inflammasome complex comprises the NLR IPAF, also known as NLRC4. IPAF contains an N-terminal CARD, a central NOD domain, and a C-terminal LRR. It responds to bacterial flagellin, which activates caspase-1 via IPAF.[106] Bacteria such as *Salmonella* are

sensed by IPAF. The interaction between IPAF and caspase-1 again involves CARD domains. More generally, IPAF is activated mainly by gramnegative bacteria that contain bacterial type III or type IV secretion systems. These include *Salmonella*, *Legionella*, *Shigella*, *Pseudomonas*, and *Yersinia*.[107] Several of these strains when deficient in flagellin do not activate caspase-1. Flagellin added extracellularly does not activate the IPAF inflammasome and is likely to enter the cytosol via the type III or type IV secretion systems. IPAF has a coreceptor that is another NLR family member NAIP5. This protein interacts directly with flagellin and is the actual sensor, the signaling occurring via IPAF.[108] Similar to NODs and TLRs, there is also evidence for an interaction between IPAF and TLR5. In a model of immunization with ovalbumin and flagellin, it was shown that IPAF- or TLR5-deficient mice are normal in the humoral response, but a double knockout mouse was deficient in its response.[109] This implies that both IPAF and TLR5 are acting in a redundant manner in this system. More generally, it is no surprise that innate systems would be redundant in terms of host defense, as a lack of redundancy would leave the host open to manipulation by pathogens.

Nlrp1

The Nlrp1 inflammasome was the first to be characterized biochemically, but its activators are not fully understood. It is expressed in many cell types, including granulocytes, monocytes, dendritic cells, B cells, T cells, and neurons. It also interacts with Asc, and there is evidence for both caspase-1 and caspase-5 in the Nlrp1 inflammasome. Anthrax toxin has been shown to be sensed by Nlrp1, and in mice the Nlrp1b isoform has been shown to be a key determinant of susceptibility to infection by *Bacillus anthracis*.[110] A role for Nlrp1 in antiviral immunity is suggested from the observation that Kaposi sarcoma herpes virus encodes a protein called Orf63 that blocks Nlrp1-dependent responses and is required for reactivation and generation of progeny virus.[111] Finally, mutations in Nlrp1 are associated with the skin disease vitiligo.[112]

AIM2

The final inflammasome to be characterized contains AIM2, which also couples to caspase-1 via Asc. AIM2 is a member of the PYHIN family of proteins, which contain a pyrin domain and HIN200 domain, which binds DNA.[113] The pyrin domain in AIM2 allows it to interact with Asc. It is activated directly by DNA and has been shown to be activated by DNA from bacteria such as *Listeria monocytogenes* and *Francisella tularensis*, and viruses such as vaccinia virus and cytomegalovirus.[114] Responses to these pathogens in vivo are all impaired in AIM2-deficient mice.

Retinoic Acid-Inducible Gene I-Like Receptors

RLRs are a family of RNA helicases that contain a DExD/H box as the defining protein domain.[115] They occur in the cytosol of most cell types and sense viral RNA. The key output from RLRs is type I interferons. There are three RLRs: RIG-I, melanoma differentiation associated factor 5 (Mda5), and laboratory of genetics and physiology 2.

Figure 15.5 illustrates RIG-I signaling. RIG-I and Mda5 sense a wide range of viruses and

have shared structural features. They both have two CARD domains, a DEAD box Helicase domain, a repressor domain, and a C-terminal domain. Structural insights have revealed that upon binding RNA, a major conformational change occurs releasing the repressor domain and shifting the two CARDs out of the protein to engage with the downstream signaling molecule MAVS.[116,117,118] Laboratory of genetics and physiology 2 lacks the CARDs and is thought to function as a regulator of RIG-I and Mda5 signaling.

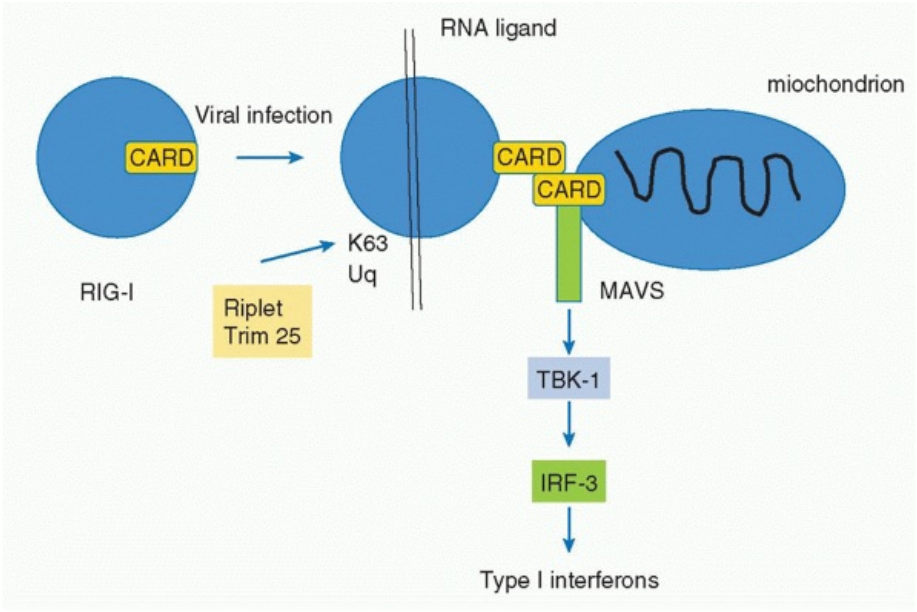

FIG. 15.5. Retinoic Acid-inducible Gene I (RIG-I) Signaling. In response to viruses such as influenza, RIG-I is activated by viral ribonucleic acid. This leads to a dramatic conformational change, such that the caspase recruitment domain (CARD) domains (*orange*) move out from the protein structure and engage with the CARD domain in MAVS, which is in the mitochondrial outer membrane. RIG-I also undergoes K63-linked ubiquitination by Riplet and TRIM25, an event required for activation. MAVS then activates TBK-1, which phosphorylates IRF3, leading to induction of type I interferons and an antiviral response.

Viruses detected by RLRs include Sendai, respiratory syncytial virus, measles, rabies, influenza, Ebola, hepatitis C, myxoma, West Nile virus, dengue, and vaccinia. RIG-I preferentially recognizes RNA sequences that contain 5′triphosphorylated ends, which serves to distinguish them from host RNA sequences.[119] Polyuridine motifs in the RNA are also important for recognition, and such motifs occur in hepatitis C, Ebola, and influenza virus.

Retinoic Acid-Inducible Gene I-Like Receptor Signaling

The key process in the RLR signaling mechanism is the recruitment of the adapter protein MAVS (also known as IPS-1, Visa, and Cardif). MAVS is a membrane-bound CARDcontaining protein that occurs in the outer mitochondrial membrane and also on the membrane of peroxisomes.[120] Activation of RIG-I and Mda5 leads to a translocation to these membranes via the interaction with MAVS. The transcription factors IRF3 and IRF7 are activated by MAVS

via the kinases TBK-1 and IKK-epsilon.[121] These in turn lead to induction of type I interferons and other anti-viral genes, with the type I interferons then activating subsequent antiviral responses via the JAK/STAT signaling pathway. RIG-I can also interact with Asc and activate caspase-1, placing RIG-I as another inflammasome component.[122] Another key protein that interacts with RIG-I is STING. This protein was found to be involved in DNA-dependent signaling but also appears to be involved in RNA-dependent signaling via RIG-I.[123]

An excellent example of immune evasion by viruses is the targeting of MAVS by the hepatitis C protease NS4.[124] This leads to MAVS degradation and impaired RIG-I signaling. NS4 is currently the focus of targeting by a protease inhibitor, which could have utility in the treatment of hepatitis C.[125]

RIG-I signaling is a tightly controlled process as overactivation could kill the host; in fact, this may be the reason for the lethal effects of influenza and other viruses. Ubiquitination is a key process here. K63-linked ubiquitination of RIG-I by the ubiquintin ligase Riplet is required for RIG-I signaling.[126] TRIM25 also causes K63-linked ubiquitination, and this has been shown to enhance the interaction with MAVS.[127] RNF125, on the other hand, causes K48-linked ubiquination of RIG-I, leading to its degradation in the proteosome.[128] The deubiquitinase CYLD removes the K63-linked ubiquitin from RIG-I, inhibiting RIG-I activity.[129] Reversible ubiquitination is therefore a key control mechanism for RIG-I.

Cooperation between Retinoic Acid-Inducible Gene I-Like Receptors and Other Pattern Recognition Receptors in Antiviral Immunity

As is the case with NLRs and TLRs, RLRs and other PRRs also cooperate in host defense, in this case against viruses. A good example is the response to West Nile virus. This virus is transmitted by mosquitoes; upon injection into skin, RIG-I triggers an interferon response from resident Langerhans cells and skin fibroblasts.[130] Some virus will then disseminate to the draining lymph node where it infects resident macrophages and dendritic cells. The TLRs are then engaged, particularly TLR7, and induce cytokines to promote adaptive immunity.[131] Some virus will escape to the spleen, and RIG-I will again be engaged here. The situation for TLR3 is more complex because TLR3-deficient mice have improved survival rates.[132] SARM knockout mice, on the other hand, have increased lethality,[133] consistent with the inhibitory effect of SARM on TLR3 signaling.

Retinoic Acid-Inducible Gene I-Like Receptors and Adaptive Immunity

As with other PRRs, RLRs have also been shown to be key for adaptive immunity via the induction of cytokines, in this case type I interferons. Interferons are key regulators of T cells, promoting their survival and clonal expansion after antigen presentation. Interferons also promote the activity of cytotoxic T cells and promote antibody production in B cells. They also promote major histocompatibility complex class I expression as well as costimulatory molecules on dendritic cells.

PKR, 2′-5′-Oligoadenylate Synthase, and RNaseL

Prior to the RLRs, PKR and 2′-5′-oligoadenylate synthase were known to sense viral RNA. PKR is a serine/threonine protein kinase that contains three double-stranded RNA binding domains in the N terminus and a kinase domain in the C terminus.[134] It is activated by double-stranded RNA and therefore acts as an intracellular PRR. A key substrate for PKR is the translation initiation factor eIF-2alpha.[135] This directly restricts both cellular and viral protein synthesis. PKR can also induce apoptosis in virally infected cells.

Oligoadenylate synthase (OAS) is another doublestranded RNA sensor.[136] It is highly induced by Type I interferons. Oligoadenylate produced by OAS activates RNAseL.[137] This enzyme degrades viral RNA but can also degrade ribosomal RNA, limiting translation of host and viral mRNAs. Of particular note is the observation that RNAseL can also generate ligands for RIG-I from viral RNA.[138] This indicates that the OAS/RNAseL axis can serve to amplify antiviral responses triggered by double-stranded RNA. There is also evidence that RNAseL can also promote Mda5 activation. Based on the nature of RNAseL-generated products, both RIG-I and Mda5 can therefore recognize fragments of RNA that are specific cleavage products from viral RNA.

Cytosolic Sensors of Dexoyribonucleci Acid that Induce Type I Interferons

The process of DNA sensing in the cytosol that leads to type I interferons is not as well understood as RNA sensing. As mentioned previously, TLR9 can sense hypomethylated CpG motifs in DNA, but this occurs in endosomes. Intracellular delivery of mammalian or bacterial DNA can, however, induce type I interferons in a TLR9-independent manner. The pathway was shown to involve IRF3 and TBK-1, as with RLRs. Three mechanisms have been proposed. The first is DNA-dependent activator of IFN-regulatory factor (DAI) that interacts with DNA and enhances type I interferons.[139] The precise role of DAI, however, is not clear because DAI-deficient mice have a normal response to infection with DNA viruses.[140] The second mechanism involves RNA polymerase III. This host enzyme has been shown to convert viral DNA into RNA, which can then be detected by RIG-I.[141] This process has been shown to occur during infection with Epstein-Barr virus, herpes virus. and the bacterium *Legionella pneumoniea*. The third process involves DNA sensing with IFI16.[142] This protein was identified as the product of an interferon-inducible gene and was then shown to bind a 70-base-pair double-stranded DNA sequence from vaccinia. Of particular interest is the structure of IFI16 because, similar to AIM2, it also senses DNA but activates caspase-1. IFI16 has a pyrin domain and two HIN200 domains, placing it in the PYHIN family. AIM2 and IFI16 are therefore part of the AIM2-like receptor family.

IFI16 signals via a protein termed STING, which is found in the endoplasmic reticulum membrane.[143] DAI has also been found to signal via STING. The function of STING is to interact with TBK-1 and promote IRF3 activation. STING is in fact essential for TBK-1 activation. STING-deficient mice have impaired interferon induction in response to bacterial, viral, and mammalian DNA, as well as to several DNA viruses, including herpes simplex virus-1, human cytomegalovirus, vaccinia virus and baculovirus, and *Plasmodium falciparum*.[144,145] STING is also required for the immune response to DNA vaccines, presumably because of the essential role it plays in the induction of interferons.

STING also plays a role in the induction of interferon by *Francisella tularensis* and *Listeria monocytogenes*. Cyclic dinucleotides produced by *Listeria* have been shown to be directly sensed by STING, implying that it is actually a PRR for these dinucleotides.[146]

There is also evidence for viral evasion of DNA-sensing pathways. Cytomegalovirus encodes a protein termed M45 that blocks DAI signaling.[147] Another cytomegalovirus protein is pUL83, which inhibits IFI16.[148] The pox virus protein M13L has been shown to target AIM2.[149]

C-Type Lectin Receptors

The term C-type lectin was first used to describe a family of calcium (hence "C")-dependent carbohydrate-binding (lectin) proteins.[150] The carbohydrate binding region was in a carbohydrate recognition domain and the more general term C-type lectin domain is used to describe this family of receptors. Dectin-1 is the best characterized member. It recognizes β-1,3-linked glucans, which occur in the cell wall of fungi, some bacteria, and plants.[151] It binds a range of fungal pathogens, including *Candida*, *Aspergillu*, *Coccidiodes*, *Pneumocystis*, and also bacterial pathogens such as *Mycobacteria*.[151] Two related proteins are CLEC-2 and DNGR-1.[152,153] CLEC-2 has been shown to bind HIV, whereas DNGR-1 has been shown to bind an unknown ligand sequestered in living cells and exposed upon cell death. All three of these C-type lectin receptors (CLRs) signal via a tyrosine kinase termed Syk.[154] This protein couples to CARD9, which in turn can activate NF-κB and promote inflammatory gene expression. It has also been shown to activate Nlrp3 and promote IL-1beta production,[155] These CLRs can also promote phagocytosis. Similar to other PRRs, there are also synergies. Dectin-1 and TLR2 when both activated strongly promote the production of proinflammatory cytokines.[156]

DC-SIGN is another CLR but instead of signaling via Syk, it activates Raf-1 and promotes MAP kinase activation.[157] It binds to mannose and fucose and has been shown to bind HIV, measles virus, severe acute respiratory syndrome, dengue virus, and mycobacteria. Raf-1 is able to modulate the

NF-κB pathway by regulating phosphorylation of the p65 subunit of NF-κB.

Phagocytic Receptors

The PRRs clearly form a major class of innate immune receptors. What distinguishes them from other innate recognition systems is their ability to change gene expression in target cells, the complexity of the gene expression profiles ultimately giving rise to the specific effector mechanisms required to clear the provoking pathogen. Another group of pathogen sensors, however, are proteins that recognize the pathogen but whose function it is to promote phagocytosis.

Scavenger receptors. Scavenger receptors (SRs) are in the plasma membrane and are defined by their ability to bind modified low-density lipoprotein.[158] Six classes are known but most is known about two: SR-A and macrophage receptor with collagenous structure. Both contain a collagenous region and an SR cysteine-rich domain. Both are trimers. They have a

role in low-density lipoprotein uptake, but also recognize LPS and lipoteichoic acid from bacteria. SR-A-deficient mice are more susceptible to infection by *Listeria monocytogenes*, herpes simplex, and malaria.[159] They are also more susceptible to LPSinduced sepsis, implying a role in LPS clearance. Macrophage receptor with collagenous structure can bind gram-positive and gram-negative bacteria and promote their phagocytosis.[160]

Macrophage mannose receptor. The macrophage mannose receptor (MR) is a protein found in the plasma membrane of macrophages and contains cysteine-rich and fibronectin type 2 domains, followed by eight carbohydrate recognition domains of the C-type lectin family.[161] MR recognizes mannose-rich carbohydrates from bacteria but can also promote phagocytosis of high-mannose glycoproteins from the host. MR has been shown to be required for phagocytosis of multiple bacteria, fungi, and protozoans. There is evidence that MR can complex with TLRs and promote TLR signaling,[162] yet another example of cooperation between PRRs.

Secreted pattern recognition molecules. There are also a range of secreted pattern recognition molecules that have various functions, although a unifying feature is opsonization. They are secreted into the circulation during infection and can reach high concentrations, effectively acting as acute phase proteins. Three classes have been particularly well-studied.

Mannan-binding lectin. MBL is the best characterized of the collectin family, which have a C-type lectin domain.[163] It binds to the terminal mannose and fucose residents on microbial carbohydrates and importantly can recognize the spatial arrangement of these sugars, which differs according to the host forms. It recognizes a wide range of pathogens, including *Staphylococci*, *Streptococci*, *Pseudomonas*, *Klebsiella*, *Salmonella*, *Mycobacteria*, *Candida*, influenza, human immunodeficiency virus, and trypanosomes. As can be seen from this list, MBL can therefore recognize all classes of pathogens. The main function of MBL is to activate the lectin pathway of complement. It is associated with two serine proteases, MASP-1 and MASP-2.[164] These proteases, when activated by ligand-bound MBL, cleave the C2 and C4 complement proteins initiating the complement cascade. MBL can also act as an opsonin, binding to C1qRp. MBL deficiency has been found in humans and leads to increased susceptibility to various infections.[165]

Pentraxins. C-reactive protein (CRP) and serum amyloid P both belong to the pentraxin family.[166] They are acute phase protein in human and mouse, respectively. They are produced mainly by hepatocytes in response to cytokines such as IL-1 and IL-6. They bind to bacterial surfaces and recognize phosphorylcholine. They promote opsonization and can activate the classical complement pathway via C1q. CRP is a classical diagnostic indicator of inflammation. Another pentraxin is PTX3, which plays an important role in host defense against bacteria and fungi.[167] It is expressed on multiple cell types, including dendritic cells, macrophages, and neutrophils. It is strongly induced by TLRs. Similar to CRP and serum amyloid P, it binds directly to bacterial and fungal membranes and promotes phagocytosis.

Peptidoglycan recognition proteins. Peptidoglycan recognition proteins are a family of PRRs that bind peptidoglycan.[168] They are conserved across evolution and were first described in *Drosophila*. They all contain a highly conserved peptidoglycan domain, and some also have transmembrane domains. Most are secreted, however. Four have been described in humans.

Their functions have yet to be fully elucidated, although they can clearly bind peptidoglycan. There is some evidence that they might compete with other peptidoglyan recognition PRRs, such as TLR2, possibly acting to limit inflammation, although this has to be demonstrated.

CONCLUSION

Clearly our knowledge of innate immune processes has greatly improved in the past few years. Multiple mechanisms exist that keep pathogens at bay, and several receptor classes can drive innate defence mechanisms via the production of effector cytokines and other mediators. The innate immune system provides an important link to the adaptive response, mainly via the activation of dendritic cells. Other cell types that participate include mast cells, basophils, and natural killer cells, which are discussed at length elsewhere. Innate immunity therefore provides us with a first line of defense, but via the inflammatory response and the activation of adaptive immunity orchestrates the major mechanisms that lead to restoration of the host after infection.

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REFERENCES

1. Medzhitov R, Janeway CA Jr. Decoding the patterns of self and nonself by the innate immune system. *Science*. 2002;296(5566):298-300.

2. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol*. 2009;27:519-550.

3. Guarda G, Dostert C, Staehli F, et al. T cells dampen innate immune responses through inhibition of NLRP1 and NLRP3 inflammasomes. *Nature*. 2009;460(7252):269-273.

4. O'Neill LAJ. Immunity's early warning system. *Scientific American*. 2005;292:38-45.

5. Irvine AD, McLean WH, Leung DY. Filaggrin mutations associated with skin and allergic diseases. *N Engl J Med.* 2011;365(14):1315-1327.

6. Liu Z, Shi C, Yang J, et al. Molecular regulation of the intestinal epithelial barrier: implication in human diseases. *Front Biosci*. 2011;17: 2903-2909.

7. Foster WM, Langenback EG, Bergofsky EH. Disassociation in the mucociliary function of central and peripheral airways of asymptomatic smokers. *Am Rev Respir Dis.* 1985;132(3):633-639.

8. Fahy JV, Dickey BF. Airway mucus function and dysfunction. *N Engl J Med.* 2010;363(23):2233-2247.

9. Wiesner J, Vilcinskas A. Antimicrobial peptides: the ancient arm of the human immune system. *Virulence*. 2010;1(5):440-464.

10. Levy O. Antimicrobial proteins and peptides of blood: templates for novel antimicrobial agents. *Blood*. 2000;96 8):2664-2672.

11. Laine VJ, Grass DS, Nevalainen TJ. Protection by group II phospholipase A2 against Staphylococcus aureus. *J Immunol* . 1999;162(12): 7402-7408.

12. Elsbach P, Weiss J. Role of the bactericidal/permeability increasing protein in host defence. *Curr Opin Immunol*. 1998;10(1):45-49.

13. Hoek KS, Milne JM, Grieve PA, Dionysius DA, Smith R. Antibacterial activity in bovine lactoferrin-derived peptides. *Antimicrob Agents Chemother.* 1997;41(1):54-59.

14. Wells JM, Rossi O, Meijerink M, van Baarlen P. Epithelial crosstalk at the microbiota-mucosal interface. *Proc Natl Acad Sci U S A.* 2011;108(suppl 1):4607-4614.

15. Bogdan C, Rollinghof M, Diefenbach A. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. *Curr Opin Immunol* . 2000;12(1):64-76.

16. Segal AW. How neutrophils kill microbes. *Annu Rev Immunol* . 2005;23: 197-223.

17. Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology. *Co Spring Harb Symp Quant Biol* . 1989;54(pt 1): 1-13.

18. Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol* . 1994;12:991-1045.

19. Gay NJ, Keith FJ. Drosophila toll and IL-1 receptor. *Nature*. 1991;351 (6325):355-356.

20. Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B. The product of the tobacco mosaic virus resistance gene N: similarity to toll and the interleukin-1 receptor. *Cell* . 1994;78(6):1101-1115. Erratum in: *Cell* . 1995;81(3):466.

21. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spätzle/toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* . 1996;86(6):973-983.

22. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to Drosophila Toll. *Proc Natl Acad Sci U S A.* 1998;95(2):588-593.

23. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature*. 1997;388:394-

397.

24. Poltorak A, Smirnova I, Clisch R, Beutler B. Defective LPS signalling in C3H/HeJ and C57BL/10ScCr mice: mutations in the Tlr4 gene. *Science*. 1998;282(5396):2085-2088.

25. Satake H, Sasaki N. Comparative overview of toll-like receptors in lower animals. *Zoolog Sci*. 2010;27(2):154-161.

26. Medzhitov R. Inflammation 2010: new adventures of an old flame. *Cell*. 2010;140(6):771-776.

27. Herrin BR, Cooper MD. Alternative adaptive immunity in jawless vertebrates. *J Immunol*. 2010;185(3):1367-1374.

28. Kang JY, Lee JO. Structural biology of the toll-like receptor family. *Annu Rev Biochem*. 2011;80:917-941.

29. O'Neill LA, Bowie AG. The family of five: TIR domain containing adapters in TLR signalling. *Nat Rev Immunol* . 2007;7:353-364.

30. Ozinsky A, Underhill DM, Fontenot JD, et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by co-operation between toll-like receptors. *Proc Natl Acad Sci U S A* . 2000;97(25):13766-13771.

31. Jin MS, Kim SE, Heo JY, et al. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell*. 2007;130(6):1071-1082.

32. Hoebe K, Georgel P, Rutschmann S, et al. CD36 is a sensor of diglycerides. *Nature*. 2005;433(7025):523-527.

33. Shimazu R, Akashi S, Ogata H, et al. MD2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor-4. *J Exp Med*. 1999;189:1777-1787.

34. Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature*. 2009;458(7242):1191-1195.

35. Kitchens RL, Thompson PA. Modulatory effects of sCD14 and LBP on LPS-host cell interactions. *J Endotoxin Res*. 2005;11(4):225-229.

36. Haziot A, Ferrero E, Kontgen F, et al. Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. *Immunity*. 1996;4(4):407-414.

37. Miura Y, Shimazu R, Miyake K, et al. RP105 is associated with MD1 and transmits an activation signal in human B cells. *Blood*. 1998;92: 2815-2823.

38. Kurt-Jones EA, Popova L, Kwinn L, et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol* . 2000;1(5):398-401.

39. Schmidt M, Raghavan B, Muller V, et al. Crucial role for human tolllike receptor 4 in the development of contact allergy to nickel. *Nat Immunol* . 2010;11(9):814-819.

40. Hayashi F, Smith KD, Ozinsky A, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*. 2001; 410(6832):1099-1103.

41. Park B, Brinkmann MM, Spooner E, Lee CC, Kim YM, Ploegh HL. Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9. *Nat Immunol* . 2008;9(12):1407-1414.

42. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol* . 2007;81(1):1-5.

43. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature*. 2002; 416(6881):603-607.

44. O'Neill LA. The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. *Immunol Rev* . 2008;226:10-18.

45. Lin SC, Lo YC, Wu H. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. *Nature.* 2010;465(7300):885-890.

46. Gay NJ, Gangloff M, O'Neill LA. What the Myddosome structure tells us about the initiation of innate immunity. *Trends Immunol.* 2011;32(3):104-109.

47. Deng L, Wang C, Spencer E, et al. Activation of the IkappaB kinase complex by Traf6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* . 2000;103(2):351-361.

48. Cohen P. Targeting protein kinases for the development of anti-inflammatory drugs. *Curr Opin Cell Biol.* 2009;21(2):317-324.

49. Honda K, Taniguchi T. IRFs: master regulators of signalling by Tolllike receptors and cytosolic pattern recognition receptors. *Nat Rev Immunol* . 2006;6(9):644-658.

50. Takeuchi O, Akira S. MyD88 as a bottle neck in Toll/IL-1 signaling. *Curr Top Microbiol Immunol.* 2002;270:155-167.

51. von Bernuth H, Picard C, Jin Z, et al. Pyogenic bacterial infections in humans with MyD88 deficiency. *Science*. 2008;321(5889):691-696.

52. Ku CL, von Bernuth H, Picard C, et al. Selective predisposition to bacterial infections in IRAK-4-deficient children: IRAK-4-dependent TLRs are otherwise redundant in protective immunity. *J Exp Med*. 2007;204(10):2407-2422.

53. Fitzgerald KA, Palsson-McDermott EM, Bowie AG, et al. Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature.* 2001;413:78-83.

54. Horng T, Barton GM, Medzhitov R. TIRAP: an adapter molecule in the toll signaling pathway. *Nat Immunol*. 2001;2(9):835-841.

55. Kagan JC, Medzhitov R. Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell*. 2006;125(5):943-955.

56. Dunne A, Carpenter S, Brikos C, et al. IRAK1 and IRAK4 promote phosphorylation, ubiquitination, and degradation of MyD88 adaptorlike (Mal). *J Biol Chem*. 2010;285(24):18276-18282.

57. Yamamoto M, Sato S, Mori K, et al. Cutting edge: a novel toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the toll-like receptor signaling. *J Immunol*. 2002;169(12):6668-6672.

58. Fitzgerald KA, McWhirter SM, Faia KL, et al. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol*. 2003;4(5):491-496.

59. Fitzgerald KA, Rowe DC, Barnes BJ, et al. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J Exp Med.* 2003;198(7):1043-1055.

60. Rowe DC, McGettrick AF, Latz E, et al The myristoylation of TRIF-related adaptor molecule is essential for Toll-like receptor 4 signal transduction. *Proc Natl Acad Sci U S A.* 2006; 103(16):6299-6304.

61. McGettrick A, Brint EK, Palsson-McDermott EM, et al. Trif-related adapter molecule is phosphorylated by protein kinase C epsilon during toll-like receptor 4 signalling. *Proc Natl Acad Sci U S A*. 2006;103(24): 9196-9201.

62. Palsson-McDermott E, Doyle S, McGettrick A, et al. TAG: a GOLD domain-containing splice variant of TRAM that negatively regulates the MyD88-independent pathway in TLR4 signaling. *Nat Immunol*. 2009;10:579-586.

63. Carty M, Goodbody R, Schroder M, Stack J, Moynagh PN, Bowie AG. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent toll-like receptor signaling. *Nat Immunol*. 2006;7(10): 1074-1081.

64. Liew E, Xu D, Brint E, O'Neill LA. Negative regulation of TLR signalling. *Nature Rev Immunol.* 2005;5(6):446-458.

65. Bowie A, Kiss-Toth E, Symons JA, Smoth GL, Dower SK, O'Neill LA. A46R and A52R from vaccinia virus are antagonists of host IL-1 and Toll-like receptor signalling. *Proc Natl Acad Sci U S A*. 2000;97: 10162-10167.

66. Stack J, Haga IR, Schroder M, et al. Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence. *J Exp Med*. 2005;201(6):1007-1018.

67. Maloney G, Schröder M, Bowie AG. Vaccinia virus protein A52R activates p38 mitogen-activated protein kinase and potentiates lipopolysaccharide-induced interleukin-10. *J Biol Chem*. 2005;280(35): 30838-30844.

68. Harte MT, Haga IR, Maloney G, et al. The poxvirus protein A52R targets Toll-like receptor signaling complexes to suppress host defence. *J Exp Med.* 2003;197(3):343-351.

69. Ronald PC, Beutler B. Plant and animal sensors of conserved microbial signatures. *Science*. 2010;333:1061-1064.

70. O'Neill LA. Plant science. Innate immunity in plants goes to the PUB. *Science*. 2011;332(6036):1386-1387.

71. Chuang TH, Ulevitch RJ. Triad3A, an E3 ubiquitin-protein ligase regulating toll-like receptors. *Nat Immunol* . 2004;5(5):495-502.

72. Hennessy EJ, Parker AE, O'Neill LA. Targeting toll-like receptors: emerging therapeutics? *Nat Rev Drug Discov* . 2010;9(4):293-307.

73. Farrar CA, Keogh B, McCormack W, et al. Inhibition of TLR2 promotes graft function in a murine model of renal transplant ischemiareperfusion injury. *FASEB J*. 2012;26(2):799-807.

74. Elinav E, Trowig T, Henao-Mejia J, Flavell RA. Regulation of the antimicrobial response by NLR proteins. *Immunity*. 2011;34:665-679.

75. Cho JH. The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol*. 2008;8:458-466.

76. Vavricka SR, Musch MW, Chang JE, et al. hPepT1 transports muramyl dipeptide, activating NF-kappaB and stimulating IL-8 secretion in human colonic Caco2/bbe cells. *Gastroenterology*. 2004;127:1401-1409.

77. Fritz JH, Girardin SE, Fitting C, et al. Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and NOD1- and NOD2-activating agonists. *Eur J Immunol*. 2005;35:2459-2470.

78. Viala J, Chaput C, Boneca IG, et al. Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. *Nat Immunol*. 2004;5:1166-1174.

79. Hassegawa M, Yamazaki T, Kamada N, et al. Nod1 mediates recognition of Clostridium difficile and induces neutrophil recruitment and protection against the pathogen. *J Immunol*. 2011;186:4872-4880.

80. Silva GK, Gutierrez FR, Guedes PM, et al. Cutting edge: Nod1 - dependent responses account for murine resistance against Trypanosome cruzi infection. *J Immunol*. 2010;184:1148-1152.

81. Kobyashi KS, Chamaillard M, Ogura Y, et al. Nod2-dependent regulation of innate and adaptve immunity in the intestinal tracts. *Science*. 2005;307:731-734.

82. Geddes K, Rubino S, Streutker C, et al. Nod1 and Nod2 regulation of inflammation in the Salmonella colitis model. *Infect Immunity*. 2010;78:5107-5115.

83. Cooney R, Baker J, Brain O, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med*. 2010;16:90-97.

84. Travassos LH, Carneiro LA, Ramjeet M, et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane site of bacterial entry. *Nat Immunol*. 2010;11:55-62.

85. Tschopp J, Martinon F, Burns K. NALPs: a novel protein family involved in inflammation. *Nat Rev Mol Cell Biol* . 2003;4(2):95-104.

86. Masters SL, O'Neill LA. Disease-associated amyloid and misfolded protein aggregates activate the inflammasome. *Trends Mol Med*. 2011; 17(5):276-282.

87. Zhou R, Yazdi AS, Menu P, Tschopp J A role for mitochondria in NLRP3 inflammasome activation. *Nature*. 2011;469:221-225.

88. Hornung V, Bauernfeind F, Halle A, et al. Silica crystals and aluminium salts activate the NALP3 inflammasome through phagosomal destabilisation. *Nat Immunol*. 2008;9;847-856.

89. Shi Y, Evans JE, Rock KL. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature*. 2003;425(6957): 516-521.

90. Martinon F, Pétrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature*. 2006; 440(7081):237-241.

91. Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, Tchopp J. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science*. 2008;320(5876):674-677.

92. Duewell P, Kono H, Rayner KJ, et al. Nlrp3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature*. 2010;464:1357-1361.

93. Halle A, Hornung V, Petzold GC, et al. The NALP3 inflammasome is involved in the innate immune response to amyloid beta. *Nat Immunol* . 2008;9:857-865.

94. Masters SL, Dunne A, Subramanian SL, et al. Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1β in type 2 diabetes. *Nat Immunol* . 2010;11(10):897-904.

95. Wen H, Gris D, Lei Y, et al. Fatty acid-induced Nlrp3-Asc inflammasome activation interferes with insulin signalling. *Nat Immunol* . 2011;12:408-415.

96. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. *Ann Rev Immunol* . 2009;27:519-550.

97. Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nat Genet* . 2001;29(3):301-305.

98. Mariathasan S, Newton K, Monack DM, et al. Differential activation of the inflammasome by caspase-1 adapters Asc and Ipaf. *Nature*. 2004;430:213-218.

99. Harder J, Franchi L, Munoz-Planillo R, Park JH, Reimer T, Nunex G. Activation of the Nlrp3 inflammasome by Streptococcus pyogenes requires streptolysin O and NF-kappaB activation but proceeds independently of TLR signalling and P2X7 receptor. *J Immunol* . 2009;183:5823-5829.

100. Allen IC, Scull MA, Holl EK, et al. The Nlrp3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. *Immunity*. 2009;30:556-565.

101. Ichinohe T, Lee HK, Ogura Y, Flavell R, Iwasaki A. Influenza virus activates inflammasomes via its intracellular M2 ion channel. *Nat Immunol* 2010;11:404-410.

102. Barlan AU, Griffen TM, McGuire KA, Wietoff CM. Adenovirus membrane penetration activates the Nlrp3 inflammasome. *J Virol* . 2011;85:146-155.

103. Hise AG, Tomalka J, Ganesan S, et al. An essential role for the Nlrp3 inflammasome in host defense against the human fungal pathogen Candida albican. *Cell Host Microbe*. 2009;5:487-497.

104. Said-Sadler N, Padilla E, Langsley G, Ojcius DM. Aspergillus fumigatus stimulates the Nlrp3 inflammasome through a pathway requiring ROS production and the Syk tyrosine kinase. *PLoS One*. 2010;5:e10008.

105. Dostert C, Guarda G, Romero JF, et al. Malarial hemozoin is a Nalp3 inflammasome activating danger signal. *PLoS One*. 2009;4:e6510.

106. Sutterwala FS, Mijares LA, Li L, Ogura Y, Kazmierczak BL, Flavell RA. Immune recognition of Pseudomonas aeruginosa mediated by the IPAF/NLRC4 inflammasome. *J Exp Med*. 2007;294:3235-3245.

107. Franchi L, Amer A, Body-Malapel M, et al. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1-beta in Salmonellainfected macrophages. *Nat Immunol*. 2006;7:576-582.

108. Kofoed EM, Vance RE. Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature*. 2011; 477(7366):592-595.

109. Vijay-Kumar M, Carvalho FA, Aitken JD, Fifadara NH, Gewirtz AT. TLR5 or NLRC4 is necessary and sufficient for promotion of humoral immunity by flagellin. *Eur J Immunol* . 2010;40:3582-3534.

110. Boyden ED, Dietrich WF. Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nat Genetics*. 2006;38:240-244.

111. Gregory SM, Davis BK, West JA, et al. Discovery of a viral NLR homolog that inhibits the inflammasome. *Science*. 2011;331:330-334.

112. Jin Y, Mailloux CM, Gowan K, et al. NALP1 in vitiligo-associated multiple autoimmune disease. *N Engl J Med* . 2007;356(12):1216-1225.

113. Schattgen SA, Fitzgerald KA. The PYHIN protein family as mediators of host defenses. *Immunol Rev*. 2011;243(1):109-118.

114. Rathinam VA, Jiang Z, Waggoner SN, et al. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat Immunol*. 2010;11(5):395-402.

115. Loo YM, Gale M Jr. Immune signalling by RIG-I-like receptors. *Immunity*. 2011;34:680-692.

116. O'Neill LA, Bowie AG. The powerstroke and camshaft of the RIG-I antiviral RNA detection machine. *Cell*. 2011;147(2):259-261.

117. Luo D, Ding SC, Vela A, Kohlyway A, Lindenbach BD, Pyle AM. Structural insights into RNA recognition by RIG-I. *Cell*. 2011;147(2):409-422.

118. Kowalinski E, Lunardi T, McCarthy AA, et al. Structural basis for the activation of innate immune pattern-recognition receptor RIG-I by viral RNA. *Cell*. 2011;147(2):423-435.

119. Hornung V, Ellegast J, Kim S, et al. 5′-triphosphate RNA is a ligand for RIG-I. *Science*. 2006;314:994-997.

120. Scott I. The role of mitochondria in the mammalian antiviral defense system. *Mitochondrion*. 2010;10:316-320.

121. Paz S, Sun Q, Nakhaei P, et al. Induction of IRF3 and IRF7 phosphorylation following activation of the RIG-I pathway. *Cell Mol Biol* . 2006;52:17-28.

122. Poeck H, Bscheider M, Gross O, et al. Recognition of RNA virus by RIG-I results in activation of CARD9 and inflammasome signalling for interleukin-1 beta production. *Nat Immunol*. 2010;11:63-69.

123. Ishikawa H, Barber GN. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature*. 2008;455:674-678.

124. Baril M, Racine ME, Penin F, Lamarre D. MAVS dimer is a crucial signaling component of innate immunity and the target of hepatitis C virus NS3/4A protease. *J Virol*. 2009;83(3):1299-1311.

125. Hézode C. Oral combination therapy: future hepatitis C virus treatment? *J Hepatol*. 2011;55(4):933-935.

126. Gao D, Yang YK, Wang RP, et al. REUL is a novel E3 ubiquitin ligase and stimulator of RIG-I. *PLoS One*. 2009;4:e5760.

127. Gack ME, Shin YC, Joo CH, et al. TRIM25 E3-ubiquitin ligase is essential for RIG-I antiviral activity. *Nature*. 2007;446:916-920.

128. Arimoto K, Takahashi H, Hishiki T, Konishi H, Fujita T, Shimotohno K. Negative regulation of RIG-I signalling by the ubiquitin ligase RNF125. *Proc Natl Acad Sci U S A* . 2007;104:7500-7505.

129. Friedman CS, O'Donnell MA, Legarda-Addison D, et al. The tumor suppressor CYLD

is a negative regulator of RIG-I-mediated anti-viral response. *EMBO Rep*. 2008;9:930-936.

130. Frederickson BL, Gale M Jr. West Nile virus evades activation of IRF3 through RIG-I-dependent and -independent pathways without antagonising host defense signalling. *J Virol*. 2006;80:2913-2923.

131. Town T, Bai F, Wang T, et al. TLR7 mitigates lethal West Nile encephalitis via interleukin-23-dependent immune cell infiltration and homing. *Immunity*. 2009;30:242-253.

132. Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA. TLR3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat Med*. 2004;10:1366-1373.

133. Szretter KJ, Samuel MA, Gilfillan S, Fuchs A, Colonna M, Diamond MS. The immune adapter molecule SARM modulates tumor necrosis factor alpha production and microglia activation in the brain stem and restricts West Nile virus pathogenesis. *J Virol*. 2009;83:9329-9338.

134. Seth RB, Sun L, Chen ZJ. Antiviral innate immunity pathways. *Cell Res*. 2006;16:141-147.

135. Williams BR. PKR: sentinel kinase for cellular stress. *Oncogene*. 1999;18:6112-6120.

136. Kumar M, Carmichael GG. Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes. *Microbiol Mol Biol Rev*. 1998;62:6112-6120.

137. Samuel CE. Antiviral actions of interferons. *Clin Microbiol Rev*. 2001; 14:778-809.

138. Malathi K, Saito T, Crochet N, Barton DJ, Gale M Jr, Silverman RH. RNAse L releases small RNA from HCV RNA that refolds into a potent PAMP. *RNA*. 2010;16:2108-2119.

139. Wang Z, Choi MK, Ban T, et al. Regulation of innate immune responses by DAI (DLM-1/ZBP1) and other DNA sensing molecules. *Proc Natl Acad Sci U S A*. 2008;105:5477-5482.

140. Ishii KJ, Kawagoe T, Koyama S, et al. TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. *Nature*. 2008;451:725-729.

141. Ablasser A, Bauernfeind F, Hartmann G, Latz E, Fitzgerald KA, Hornung V. RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. *Nat Immunol*. 2009;10:1065-1072.

142. Unterholzner L, Keating SE, Baran M, et al. IFI16 is an innate immune sensor for intracellular DNA. *Nat Immunol*. 2010;11:997-1004.

143. Keating SE, Baran M, Bowie AG. Cytosolic DNA sensors regulating type I interferon induction. *Trends Immunol*. 2011;32(12):574-581.

144. Ishikawa H, Ma Z, Barber GN. STING regulates intracellular DNAmediated Type I interferon-dependent innate immunity. *Nature*. 2009;461:788-792.

145. Sharma S, DeOliveria RB, Kalantari P, et al. Innate immune recognition of an AT-rich stem-loop DNA motif in the Plasmodium falciparum genome. *Immunity*. 2011;35(2):194-207.

146. Burdette DL, Monroe KM, Sotelo-Troha K, et al. STING is a direct innate immune sensor of cyclic di-GMP. *Nature*. 2011;478(7370): 515-518.

147. Rebsamen M, Heinz LX, Meylan E, et al. DAI/ZBP1 recruits RIP1 and RIP3 thought RIP homotypic interaction motifs to activate NFkappaB. *EMBO Rep*. 2009;10:916-922.

148. Cristea IM, Moorman NJ, Terhune SS, et al. Human cytomegalovirus pUL83 stimulates activity of the viral immediate-early promoter through its interaction with the cellular IFI16 protein. *J Virol*. 2010;84:7803-7814.

149. Johnston JB, Barrett JW, Nazarian SH, et al. A poxvirus-encoded pyrin domain protein interacts with ASC-1 to inhibit host inflammatory and apoptotic responses to infection. *Immunity*. 2005;23:587-598.

150. Osorio F, Reis E, Sousa C. Myeloid C-type lectin receptors in pathogen recognition and host defence. *Immunity*. 2011;651-664.

151. Brown GD. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat Rev Immunol*. 2006;6:33-43.

152. Suzuki-Inoue K, Fuller GL, Garcia A, et al. A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. *Blood*. 2006;107:542-549.

153. Sancho D, Joffre OP, Keller AM, et al. Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature*. 2009;458:899-903.

154. Rogers NC, Slack EC, Edwards AD, et al. Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C-type lectins. *Immunity*. 2005;22:507-517.

155. Gross O, Poeck H, Bscheider M, et al. Syk kinase signalling couples to the Nlrp3

inflammasome for anti-fungal host defence. *Nature*. 2009;459:433-436.

156. Brown GD, Herre J, Williams DL, Willment JA, Marshall AS, Gordon S. Dectin-1 mediates the biological effects of beta-glucans. *J Exp Med*. 2003;197:1119-1124.

157. Geijtenbeek TB, Gringhuis SI. Signaling through C-type lectin receptors: shaping immune responses. *Nat Rev Immunol* . 2009;9:465-479.

158. Krieger M, Herz J. Structure and functions of multiligand lipoprotine receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Ann Rev Biochem*. 1994;63:601-637.

159. Suzuki H, Kurihara Y, Takeya M, et al. A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature*. 1997;386:292-296.

160. Kraal G, van der Laan LJ, Elomaa O, Tryggvason K. The macrophage receptor MARCO. *Microbes Infect.* 2000;2:313-316.

161. Fraser IP, Koziel H, Ezekowitz RA. The serum mannose-binding protein and the macrophage mannose receptor are pattern recognition molecules that link innate and adaptive immunity. *Semin Immunol*. 1998;10:363-372.

162. Linehan SA, Martinez-Pomares L, Gordon S. Macrophage lectins in host defence. *Microbes Infect*. 2000;2:279-286.

163. Clark HW, Reid KB, Sim RB. Collectins and innate immunity in the lung. *Microbes Infect*. 2000;2:273-278.

164. Degn SE, Jensenius JC, Thiel S. Disease-causing mutations in genes of the complement system. *Am J Hum Genet*. 2011;88(6):689-705.

165. Tsutsumi A, Takahashi R, Sumida T. Mannose binding lectin: genetics and autoimmune disease. *Autoimmun Rev*. 2005;4(6):364-372.

166. Garlanda C, Bottazzi B, Salvatori G, et al. Pentraxins in innate immunity and inflammation. *Novartis Found Symp*. 2006;279:80-86.

167. Bottazzi B, Doni A, Garlanda C, Mantovani A. An integrated view of humoral innate immunity: pentraxins as a paradigm. *Annu Rev Immunol*. 2010;28:157-183.

168. Liu C, Xu Z, Gupta D, Dziarski R. Peptidoglycan recognition proteins: a novel family of four human innate immunity pattern recognition molecules. *J Biol Chem*. 2001;276:34686-34694.

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Chapter 16

Dendritic Cells

Kang Liu

Michel C. Nussenzweig

INTRODUCTION

Immunology interfaces with medicine at many points, the most prominent being infectious diseases, cancer, transplantation, allergy, and autoimmunity. Antigen-specific immunity is critical for vaccination and for protective immunity against pathogens and tumors. Moreover, when control over immune responses is lost, the result is autoimmune disease. T cells are an essential element that regulates the balance in immunity by killing infected cells, helping antibody formation, and suppressing autoimmune responses. However, T cells are incapable of recognizing native antigens. Instead, they recognize processed peptides presented by major histocompatibility complex (MHC) molecules. Dendritic cells (DCs) are professional antigen-presenting cells that inform the fight against invasive pathogens while enforcing tolerance to self- and harmless environmental antigens. They capture pathogens and receive signals from pathogens that influence the outcome of immune responses. On the basis of these signals, DCs orchestrate antigen-specific T-cell differentiation toward Th1, Th2, Th17, or Tfh pathways. Alternatively, they can silence self-reactive T cells by inducing deletion, anergy, or active regulation (via regulatory T [T_{reg}] cells). This chapter will focus on the discovery, function, and development of DCs, and the mechanisms by which they link innate immunity to adaptive immunity.

DISCOVERY

DCs were discovered as part of an effort to understand the initiation of immunity. The mouse as an experimental animal was critical because of a system, developed by Mishell and Dutton,[1] in which mouse spleen cell suspensions could be stimulated to generate antibody responses in culture. One of the early observations was that lymphocytes alone were not sufficient to induce antibody-forming cell responses and that an adherent accessory cell was required. The search for the accessory cell led to the discovery of DCs. In this section, we will review the early methods used to identify and study DCs.

Identification and Isolation

DCs were discovered by Steinman and Cohn when they examined spleen adherent cells by phase contrast microscopy.[2] They then employed physical techniques to fractionate spleen cells and purify DCs. These cells were found to adhere to plastic or glass, had low buoyant density, and did not bind to erythrocytes coated with antibody. Sequential steps of density

centrifugation in bovine serum albumin gradients and adherence to glass were originally used to purify DCs.[3]

At the time, macrophages were thought to be the key accessory cell because they composed a major population of adherent cells and also because their role in innate immunity had been long appreciated. Moreover, purified antigen loaded macrophages induced immune responses when reinjected into their hosts.[4] However, macrophages failed to show robust activity in induction of antibody responses in vitro, and they rapidly degraded ingested antigens suggesting that they would be unable to present it to lymphocytes.[5,6,7]

Dendritic Cells Initiate T-Cell Responses

In addition to their morphology, the cell surface composition of the newly discovered DCs proved to be distinct. DCs were found to express high levels of MHC antigens.[8,9] At the time, the precise function of the MHC in antigen presentation was not known, but it was already clear that the MHC was a key genetic determinant of immune responses and that it encoded many of the antigens involved in graft rejection.

The mixed leukocyte reaction (MLR) was considered an in vitro model system to study graft rejection and was used by Steinman and Cohn to examine the function of DCs.[10] They found that DCs were nearly two orders of magnitude more potent than unfractionated spleen cells, B cells, or macrophages in stimulating allogeneic T cells in the MLR.[10,11] On the basis of these experiments, they speculated that DCs were the accessory cells that present antigen to T cells to initiate immune responses.[12] However, their conclusions were not widely accepted by immunologists because unlike traditional immune responses, the MLR did not require priming.[13]

Proof that DCs are antigen-presenting cells came from experiments performed by Nussenzweig and Steinman who cocultured DCs with responding T cells and hapten-modified thymocytes. They showed that DCs induced MHC-restricted cytotoxic T cells specific to the hapten and that macrophages and other purified populations of lymphoid cells were nearly inactive as accessory cells.[14]

Monoclonal Antibodies to Dendritic Cells

Although the morphologic and functional differences between DCs and other leukocytes were striking, few immunology laboratories were equipped to perform the purification procedures required to study DCs until monoclonal antibodies to DCs became available. A series of

mouse DC-restricted monoclonal antibodies were produced by Steinman and others starting in 1980s, including 33D1 (specific for the cell surface receptor DCIR2/Clec4a4),[15] NLDC-145 (specific for the adsorptive endocytosis receptor, DEC205/cluster of differentiation [CD]205),[16] and N418 (specific for the CD11c integrin).[17] These monoclonal antibodies were used to establish the unique functions of DCs within heterogeneous mixtures of leukocytes and to identify DCs in situ. For example, 33D1 was used to selectively deplete DCs from mouse spleen suspensions and shows that this depleted MLR stimulating activity[12] and accessory function in the Mishell-Dutton system.[18]

Dendritic Cell Subsets

Shortman and colleagues first showed that DCs are heterogeneous and suggested the existence of DC subsets.[19,20] These subsets differ in their anatomic distribution, cell surface marker expression, and function. In the mouse, three major groups of DCs exist in the steady state: plasmacytoid DCs (pDCs), conventional DCs (cDCs), and migratory DCs (mDCs). pDCs are important mediators of antiviral immunity through their ability to produce large amounts of type I interferons (IFNs) upon viral infection (see following discussion). cDCs are composed of two major subsets, namely CD8α+ and CD8α-.[21] mDCs are present in nonlymphoid tissues such as the liver, gut, skin, lung, and aorta, and they are composed of two main subsets CD103+ and CD103-.[22] These nonlymphoid tissue DCs are referred to as mDCs because they transit from tissue to lymphoid organs.[21,23]

The two major subsets of mouse spleen cDCs have some overlapping functions. Both can process and present antigens to T cells and also secrete cytokines such as interleukin (IL)-12, which can influence the ultimate polarization of the T-cell response to pathogens. However, the two subsets also have unique functions in vivo and are not redundant.[24,25,26]

Some of the differences in cDC subset function may be accounted for differential expression of toll-like receptors (TLRs) or other mediators of cDC activation. For example, spleen CD8α+ cDCs express TLR3 (recognizing doublestranded ribonucleic acid [RNA]) but lack TLR5 (recognizing flagellin) and TLR7 (recognizing single-stranded RNA), whereas CD8α- cDCs express TLR5 and TLR7 but have low levels of TLR3.[27] Comparative analysis of messenger RNA expression profiles between the two DC subsets revealed that the two are as different from each other as are T- and B-lymphocytes.[28] This is in part reflected in the antigen processing capacity of the two cDCs types. CD8α+ cDCs are specialized for MHCI cross-presentation, and enriched in Tap1, Tap2, calreticulin, calnexin, Sec61, ERp57, ERAAP, as well as cystatin B and C, all of which are involved in MHCI presentation or inhibition of enzymes that process peptides for MHCII presentation. In contrast, CD8α- cDCs, which are biased for MHCII presentation, were enriched in cathepsins C, H, and Z, asparagine endopeptidase, GILT, and H2-Mbeta 1, all of which are implicated in the MHCII antigen-processing pathway.[28] The immunologic consequences of this specialization can be seen after immunization with antigens targeted to one or the other cDC in vivo. Antigens targeted to CD8α+ and CD8α- DCs by chimeric monoclonal antibodies that bind endocytic receptors Dec205/CD205 or DCIR2/Clec4a4 are biased to strong CD8 or CD4 T-cell responses, respectively.[28]

In conclusion, the two major subsets of DCs have distinct microanatomic locations, gene expression profiles, and functions. However the CD8α+ cDC in lymphoid tissue and CD103+ subset in nonlymphoid tissue DCs subsets are far better characterized than the CD8α- cDC or CD103- subset, which are heterogeneous and difficult to distinguish from activated monocytes.

ANATOMIC DISTRIBUTION

Lymphoid Organs

Peripheral lymphoid organs (ie, spleen, lymph nodes, and mucosal associated lymphoid tissues) are the sites where primary immune responses develop, including activation of helper, killer, and antibody-forming cells. Initiation of immune responses is facilitated by the highly organized structure of the peripheral lymphoid organs, with lymphocytes segregated into B- and T-cell areas. In addition to lymphocytes, the T-cell area also contains large interdigitating cells that were initially thought to be macrophages. However, cytologic and functional studies of spleen and lymph nodes in rat and mice revealed that interdigitating cells lack phagosomes and lysosomes, and are poorly phagocytic.[29,30,31] These features distinguished interdigitating cells from macrophages and suggested that they might be related to the cDCs isolated from mouse spleen.

Mouse interdigitating cells were later proven to be cDCs based on immunohistochemistry using DC-specific monoclonal antibodies. In human lymphoid organs, interdigitating cells in the T-cell area express CD83, a member of immunoglobulin superfamily. This marker is not conserved in the mouse where the interdigitating cells in the T-cell area of lymph nodes express CD11c and DEC205/CD205. In contrast, the two cDC subsets in the mouse spleen segregate into distinct microanatomic compartments. CD8α+ cDCs in spleen T-cell areas resemble those in the lymph nodes in that they express DEC-205, whereas those in the bridging channels and red pulp express DCIR2.[28]

In the T-cell area, cDCs form a dense network of cells that are primarily sessile but constantly protrude and retract large membrane folds into their environment.[32] T cells actively migrate through the DC network in search of antigen, and when they find a cDC presenting cognate antigen, they pause for a prolonged interaction.[33] The large DC membrane protrusions are thought to increase the surface area for antigen presentation and facilitate antigen detection by migrating T cells.[34]

Nonlymphoid Organs

In addition to the lymphoid organs, DCs are also found in most nonlymphoid organs and in all epithelial surfaces that contact the environment. In organs such as heart, lung, kidney, the dermal layer of skin, and meninges and choroid

plexus in the brain, they are found in interstitial spaces that are drained by lymphatics.[35,36,37] Like their lymphoid counterparts, interstitial DCs can be distinguished from macrophages by abundant expression of MHCII and low levels of lysosomal hydrolases. In comparison to macrophages, which are also abundant in the interstitial spaces, interstitial DCs are less phagocytic and more sensitive to radiation. Tissue macrophages and DCs can be distinguished by immunohistology; the former typically express F4/80 and lysosomal hydrolase, whereas the latter express MHCII and CD11c. The standard markers for distinguishing DCs and macrophages in interstitial tissues are listed in Table 16.1. Importantly, although these markers help to differentiate macrophage and cDCs in most tissues, they are not sufficient in defining cell lineages unless used in combination with functional and developmental analysis. This is because CD11c and MHCII can also be expressed by lung macrophages and induced on monocytes during inflammation.[38]

TABLE 16.1 Cell Surface Comparison between Dendritic Cells and Macrophage

	DC	Macrophage
MHC II	+	-/lo
FcR (CD16/CD32)	-	+
CD11b	-/lo	+
F4/80	-	+
CD115	-/lo	+

CD, cluster of differentiation; DC, dendritic cell; MHC, major histocompatibility complex.

Afferent Lymphatics

Afferent lymphatics in rats, rabbits, guinea pigs, sheep, and humans contain cells with motile processes that were named veiled cells because of their large membrane protrusions.[34,39,40,41] These cells were found to be poorly phagocytic, express high levels of MHCII, and are potent stimulators of the MLR in vitro. Lymphadenectomy and thoracic duct cannulation in larger animals such as rats and sheep made it possible to collect these cells from afferent lymph. Using this technique, Huang et al. revealed that veiled cells are constantly carrying intestinal epithelial contents via lymphatics to the cDC network in the T-cell area of the mesenteric lymph nodes.[42] Therefore, in the healthy individual, these veiled cells continually carry and present self- and harmless antigen to the adaptive immune system, thereby contributing to the induction of peripheral tolerance (see following discussion); during infection, they carry pathogen-derived antigens and induce specific T-cell activation. Thus veiled cells correspond to mDCs.

Dendritic Cells in the Skin and Other Body Surfaces

Several types of DCs, monocytes, and macrophages exist in the skin and at other body surfaces. Professional antigenpresenting cells in the skin include Langerhans cells (LCs) in the epidermis, and CD103+ and CD103- DCs in the dermis.

DENDRITIC CELL DEVELOPMENT

Origin of Conventional Dendritic Cells in the Lymphoid Organs

DCs, like all other leukocytes, develop from bone-marrow-derived hematopoietic stem cells. Monocytes, macrophages, granulocytes, megakaryocytes, and erythrocytes differentiate from a common myeloid progenitor (CMP) whereas B, T, and natural killer (NK) cells differentiate from a common lymphoid progenitor.[43,44,45] Although early cell transfer and genetic

experiments were interpreted to indicate that DCs originated from lymphoid and myeloid progenitors,[46,47,48,49] subsequent work showed that all DCs are derived from myeloid progenitors.[50]

Relationship between Dendritic Cells and Monocytes

In the steady state, DCs and monocytes can readily be distinguished based on cell surface marker expression. For example, DCs are CD11c+CD115-/loMHCII+ and monocytes are CD11b+CD115+F4/80+ (see Table 16.1). In contrast, under inflammatory conditions or in tissues such as lung and intestine, where they can be exposed to pathogenic and nonpathogenic microbes, monocytes develop many of the characteristic features associated with DCs, and the distinction becomes far more difficult. Moreover, the question of how DCs are related to monocytes and macrophages during development was a vexing problem that was only resolved very recently.

The idea that monocytes might be the direct precursors of DCs in vivo was suggested by elegant experiments with human CD14+ monocytes.[51] Monocytes undergoing reverse transmigration, which simulates their entry into lymphatic vessels from tissues, lose expression of monocyte markers CD14 and CD64 and upregulate human leukocyte antigen-DR and CD54.[51] Phagocytosis further stimulates expression of CD80, CD86, human leukocyte antigen-DR, DC-LAMP, and CD83, all of which are expressed by DCs.[51] Consistent with these cell surface changes, activated monocytes stimulate allogeneic T-cell proliferation in MLRs.

Similarly, in mice, microspheres injected intracutaneously are phagocytosed by CD11b+ F4/80+ monocytes that can then be found in the draining lymph nodes 3 to 4 days after the injection.[52] These cells display high expression of MHCII, MHCI, CD86, and stimulate allogeneic T-cell proliferation; however, unlike DCs, they express low levels of CD11c and no CD8α. Therefore, monocytes in the skin did not appear to become classical cDCs upon migration to lymph nodes; nevertheless, these activated monocytes shared many of the features associated with DCs. Finally, activated monocytes that appear in the spleen during *Listeria monocytogenes* infection expressed many of the cell surface features of cDCs.[53] These cells produce tumor necrosis factor (TNF) and inducible nitric oxide synthase (iNOS) and were named TNF/iNOS-producing DCs (Tip-DCs).[53] Tip-DC deficiency led to lethal *L. monocytogenes* infection; however, this effect was independent of the development of adaptive CD8 T-cell immunity.[53]

The idea that DCs develop from monocytes was tested by several laboratories in direct transfer experiments in

mice without clear success.[54,55,56,57] Indeed, it was always the nonmonocyte fraction of the bone marrow that produced cDCs.[54,56] The only exception to this rule occurred after inflammation induced by Freund's Complete Adjuvant (CFA), when monocytes differentiated into CD11c+CD11b+Mac3+ cells, which again are phenotypically distinct from cDCs.[56]

The inability of monocytes to produce cDCs in steadystate lymphoid organs was further confirmed in a series of elegant genetic studies using reporter mice carrying Rosa26-

$Stop^{flox}EGFP$.[58] In these mice, a lox-flanked Stop transcription signal must be deleted by Cre expression before GFP can be transcribed. Breeding LysM-Cre and Rosa26-$Stop^{flox}EGFP$ mice results in lysozyme promoter-driven Cre expression in monocytes and neutrophils, as well as deletion of the Stop sequence from Rosa26-$Stop^{flox}EGFP$ in these cells. As a result, monocytes and their progeny are irreversibly marked with enhanced green fluorescent protein expression. Classical DCs in the peripheral lymphoid organs remained enhanced green fluorescent protein negative in these mice; therefore, they cannot be derived from monocytes.[58]

In conclusion, experiments in humans and mice show that monocytes can develop some of the features of DCs under conditions of inflammation in vivo, or when cultured with cytokines in vitro, but they are not precursors of cDCs.

Dendritic Cell Progenitors in the Bone Marrow

If monocytes do not produce DCs, the question remains: what is the origin of DCs and where does commitment to this lineage occur? The first clue in solving this problem came from the identification of a common precursor for monocytes, macrophagesm and classical DCs (macrophage-DC progenitors [MDPs][54]). MDP are Lin-CX3CR1+CD11b-CD 115+cKit+CD135+ and account for 0.5% of all bone marrow mononuclear cells in mice.[54,59] When cultured with granulocyte-macrophage-colony stimulating factor (GM-CSF) in vitro or adoptively transferred into mice, these cells produced macrophages and DCs, but not neutrophil granulocytes, B-or T-lymphocytes, or NK cells.[54,59,60] Therefore, MDPs are more restricted than CMPs, from which they are derived.

A DC-restricted progenitor that produces cDCs and pDCs but not monocytes in vitro[61] or in vivo[62] was then identified based on reduced cKit (CD117) and residual Flt3 expression and named common-DC progenitor (CDP; Lin-CD115+Flt3+CD117lo). The CDP is downstream of CMP and MDP because adoptive transfer of either CMP or MDP gives rise to CDP and monocytes.[60] More importantly, these experiments showed that the split between the monocyte and DC lineages occurs in the bone marrow between the MDP and CDP stages of development.[60]

Migratory Pre-Dendritic Cells

cDC precursors must migrate from the bone marrow to the lymphoid organs through the blood. However, MDPs and CDPs are restricted to the bone marrow.[60] The identity of the DC precursor was suggested by the finding that lowdensity CD11c+MHC II-SIRPα^{lo} cells isolated from blood, bone marrow, and periphery had the potential to produce cDCs.[56,63,64] Although this group of cells was heterogeneous, the combination of these markers and persistent expression of Flt3 defined pre-DCs and allowed for their isolation in mice.[60]

Pre-DCs migrate from the bone marrow to the blood and then to peripheral lymphoid organs and nonlymphoid tissues.[60] These cells comprise ˜0.5% of all leukocytes in bone marrow, 0.02% in blood, 0.05% in the spleen, and 0.03% in the lymph nodes. Pre-DCs have a short half-life in the blood of < 1 hour[65]; this, together with the small number of these cells in blood

and tissues, may explain why previous efforts to identify pre-DCs failed and why human pre-DCs have yet to be identified.

Pre-cDCs isolated from the bone marrow, blood, or spleen give rise to both CD8α+ and CD8α- cDCs in lymphoid and nonlymphoid organs.[60] Thus, the pre-DC is a progenitor with significant plasticity. In conclusion, the DC and monocyte lineages split in the bone marrow, where MDPs give rise to both monocytes and CDP; the latter produce pre-DCs, which migrate from bone marrow through the blood to the periphery to give rise to DCs (Fig. 16.1).

Origin of Nonlymphoid Tissue Dendritic Cells

Skin

The skin contains LCs in the epidermis and CD103+ and CD103- DCs in the dermis.

Epidermis. LCs were discovered in 1868 by Paul Langerhans. Although LCs were initially thought to be of neural crest origin,[66,67] this view was changed with the discovery that LCs are closely related to leukocytes,[68,69] express Fc receptors[70] and MHCII antigens,[71] and are competent to present antigen to primed T cells.[72]

Experiments using bone marrow chimeras and parabiotic mice demonstrated that LCs are long-lived cells that divide in situ in the skin.[73] These cells originate from fetal liver-derived progenitors in an Flt3L-independent manner, and they are only replaced by bone marrow-derived hematopoietic cells during inflammation.[73,74] Thus, the origin of the LCs is different from that of cDCs.

Nevertheless, mouse LCs have been valuable as models for studying the differentiation and migration of antigenpresenting cells.[75,76,77,78,79,80,81] LCs in the epithelial layer of the skin can take up particles[82] and contain distinct granules, known as Birbeck granules, which are a type of endocytic organelle enriched in langerin.[83,84] While LCs express Fc receptors and MHCII in situ, the expression of Fc receptors drops and MHCII molecules redistribute from intracellular compartments to the cell surface in cultured LCs that become more highly immunostimulatory.[75,76,85] Cells that resemble LCs are also found in other stratified squamous epithelia such as the vagina, cervix, anus, pharynx, and esophagus.

LCs show many of the phenotypic features of cDCs, including morphology, ability to redistribute large amounts of MHCII from the endocytic system to the cell surface, and capacity to stimulate allogeneic T-cell proliferation in vitro after activation.[75,78,86,87,88] Importantly, however, LCs differ from DCs in that they are resistant to irradiation, they are self-renewing in situ and not of pre-DC origin in the steady

state, and finally, like monocytes and macrophages, their development is dependent on macrophage-colony stimulating factor (M-CSF) and not on Flt3L.[89] LC precursors, which are CX3CR1+CD45+, colonize the dermis during late embryonic development.[90,91] In mice, these precursors complete their differentiation into LCs by postnatal d2, whereupon they undergo a burst of cell division between postnatal d2 and d7, which accounts for the dramatic increase in LCs during this developmental window. The LC precursor in skin appears to

resemble the MDP.[90] Recently, fate-mapping analysis revealed that LCs derive from primitive macrophages during yolk sac stage of embryogenesis, and not from postnatal hematopoietic progenitors.[91] These two lineages can be differentiated by their dependence of Myb gene. The absence of Myb impaired HSC development, but did not affect development of the yolk sac macrophage progenitor that gives rise to LCs, microglia and liver Kupffer cells. This finding places the LC in its own differentiation pathway distinctive from cDC and monocyte.[91]

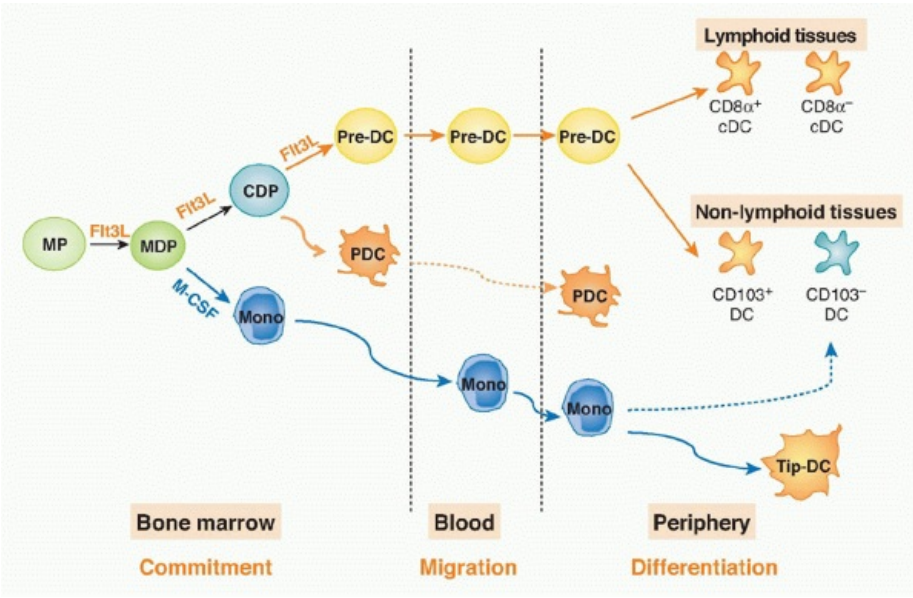

FIG. 16.1. Dendritic Cell and Monocyte Origin and Development.

Dermis. The dermal layer of the skin contains two subsets of DCs: CD103+ and CD103- DCs. CD103+ dermal DCs are langerin+CD11blo, whereas CD103- dermal DCs are langerin-CD11b+.[92,93,94,95] Although both LCs and CD103+ dermal DCs express langerin, CD103+ dermal DCs are more closely related to spleen CD8α+ cDCs than to LCs.[25,37,96] CD103+ dermal DCs, like spleen cDCs, are M-CSF-independent and Flt3-dependent.[37] Unlike LCs that can self-renew in situ, dermal CD103+ DCs are continually replenished from the same bloodborne pre-DCs that give rise to lymphoid organ DCs.[95] In addition, like CD8α+ splenic cDCs, development of CD103+ dermal DCs requires expression of the IFN regulatory factor (IRF)8, Id2, and Batf3 transcription factors (see following discussion). Finally, both dermal CD103+ dermal DCs and CD8α+ cDCs specialize in antigen cross-presentation.[28,96] The CD103- dermal DC compartment appears to be a heterogenous mixture of cells, some of which are Flt3 dependent and of pre-DC origin and others M-CSF-dependent and of monocyte origin.[37]

Skin-draining lymph nodes contain subpopulations of DCs that resemble CD103+ and CD103- dermal DCs.[92,95] Their migration from the skin to the draining lymph nodes is dependent on CCR7 but independent of CD62L. In the steady state, these cells account for 20% of lymph node DCs while the remainder of the DCs are derived from blood pre-DCs.[60]

Inflammation dramatically enhances the migration of dermal DCs to the lymph node.[52,58]

Gut, Kidney, Lung, and Liver

Similar to the skin, CD103+ and CD103- DCs are found in nearly all mouse nonlymphoid tissues.[37,97] However, the origin of these cells was not established until very recently. CD103+ nonlymphoid tissue DCs express Flt3, a marker expressed on most cells in the DC lineage but not on monocytes. Furthermore, mice deficient in Flt3L or its receptor, Flt3, showed significant decreases in CD103+ nonlymphoid tissue DCs but normal numbers of monocytes.[37,98] Finally, adoptive transfer of pre-DCs into naïve recipients, or mice in which CD11c cells had been depleted, resulted in reconstitution of the CD103+ nonlymphoid tissue DC compartment in the liver, lung, kidney, and intestine.[37,57] In contrast, adoptive transfer of 50 times more monocytes failed to show detectable DC progeny in naïve mice.[37,57] Thus, in the steady state, CD103+ DCs in all tissues tested are pre-DC

and not monocyte-derived. Only after depletion of endogenous CD11c+ cells were monocytes able to produce some CD103- DCs.[37,57] The idea that all CD103+ nonlymphoid tissue DCs are derived from pre-DCs and not from monocytes is further supported by genetic experiments that show a common requirement for Batf3, Id2, and IRF8 for lymphoid tissue CD8α+ DCs, and CD103+ nonlymphoid tissue DCs, but not monocytes or macrophages (see following discussion).[24,37,99,100]

In conclusion, in addition to DCs in the lymphoid organs, pre-cDCs also contribute to CD103+ DC development in nonlymphoid tissues. CD103- DCs appear to be more heterogenous; some of these cells are derived from pre-DCs while others may be of monocyte origin.

Plasmacytoid Dendritic Cells

pDCs were described by pathologists as a unique population of cells in the peripheral lymphoid organs that resemble plasma cells and monocytes and were referred to as plasmacytoid monocytes. In 1990s, Svensson et al. described a population of cells in human blood that produces large amounts of type I IFN upon exposure to herpes simplex virus, and they named these cells IFN-producing cells.[101] Both of these populations correspond to the pDCs described by Liu and colleagues.[102,103,104]

pDCs were first purified from human tonsil and blood on the basis of being CD4+CD3-CD11c-. They were then shown to develop DC-like morphology upon culture with IL-3 and anti-CD40. Liu and colleagues discovered that purified pDCs produced 100 to 1,000 times more type I IFN than the other blood cell types following viral activation.[104] Subsequently, pDCs were identified and isolated from lymphoid tissues in mice, and later in rats, monkeys, and pigs. In humans, the pDCs express CD4, MHCII, CD68, CD123, and blood DC antigen 2 (BDCA2), but do not express other lineage marker such as CD3 (T cell), CD14 (monocyte), CD19 (B cell), CD16 and CD56 (NK cell), or CD11c, BDCA1, and BDCA3 (cDC). They are identified as CD4+, CD11c-, and Lin- (CD3, CD14, CD16, CD19, CD56) cells.[105] In mice, pDCs express CD11c (lower levels than cDCs), Gr1, B220, and PDCA1; low levels of MHCII; do not express CD11b or CD19;and they are frequently isolated by virtue of CD11c and B220 expression.[106]

pDCs are closely related to cDCs; they originate from the same progenitor, the CDP. Development of pDCs is also critically dependent on Flt3L. Unlike cDCs, however, pDCs do not express high levels of MHC II in the steady state and therefore, their primary function is not antigen presentation to CD4 T cells. Instead, pDCs focus on sensing infection and producing type I IFN. Depletion of pDC blocks antiviral IFN responses but also results in loss of T_{reg} homeostasis, impaired differentiation of Th cells, and initiation of CD8 T-cell responses (see following discussion).[107,108]

Growth Factors for Dendritic Cells and Monocytes

GM-CSF, M-CSF and Flt3L are essential growth factors for myeloid cell development in vitro and in vivo. During the early stages in myeloid development, shared progenitors of lymphocytes, polymorphonuclear leukocytes, monocytes, and DCs express one or more of the receptors for these growth factors. For example, Flt3 is expressed very early in hematopoiesis on shared progenitors of lymphocytes and all myeloid cells.[109] However, with the exception of pre-DCs, Flt3 expression is lost by nearly all leukocytes by the time they enter circulation.[60] Conversely, M-CSF receptor expression is retained by cells in the monocyte lineage but lost on pre-DCs and DCs.[60]

As might be predicted from their patterns of expression, M-CSF and Flt3L are essential for normal development of the monocyte and DC lineages, respectively. For example, mice deficient in M-CSF or its receptor show deficiencies in monocyte, macrophage, and LC development but have normal spleen and lymph node DCs.[110] In contrast, DC development is impaired in mice deficient in either Flt3L or Flt3, but monocyte development is normal.[59,111] Consistent with these observations, administration or overexpression of Flt3L results in selective expansion of DC populations, including fully differentiated DCs in lymphoid organs.[59,112]

Dendritic Cell Cultures

In the early 1990s, Banchereau and Schuler and their colleagues established in vitro culture systems to produce human monocyte-derived DCs (moDCs), and Inaba and Steinman for the mouse equivalent.[76,113,114] These culture systems are widely used today for basic and clinical studies. Bone marrow cells from mice or monocytes from humans are cultured for 6 days in medium containing GM-CSF to produce cells with dendritic morphology that exhibit modest phagocytic activity, express CD11c and MHCII, and stimulate the MLR. Differentiation to moDCs is further stimulated by addition of lipopolysaccharide, which induces a series of changes that were originally described as maturation. Notably, monocytes do not give rise to DCs in the lymphoid organs in vivo (see previous discussion), and GM-CSF is entirely dispensable for DC development in vivo[115]; thus, the moDCs generated in GM-CSF culture are not entirely representative of the cDCs found in the lymphoid tissues. moDCs may be more closely related to activated monocytes than to steady-state DCs in the lymphoid organs.

Authentic cDCs can be produced in in vitro cultures of bone marrow cells supplemented with Flt3L, which is an essential growth factor for DCs in vivo.[116] Flt3L cultures produce all of the known subsets of DCs, including cells with features of pDCs, CD8α+/CD103+ cDCs, and

CD8α- cDCs.[116]

Transcriptional Regulation of Dendritic Cell Development

A number of different transcription factors are known to regulate DC development, but to date none of these are cDC specific. For example, signal transducer and activator of transcription (STAT)3 is required for cDC development in part because this transcription factor is downstream of Flt3.[117] Binding of Flt3L to its receptor on the surface of DC precursors or cDCs induces activation of the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin signaling

pathway; this leads to activation of STAT3.[118] Activation of STAT3 is also regulated by the transcriptional repressor, Gfi-1, deletion of which leads to reduction of lymphoid DCs and increase of LCs.[119] PU.1, an E-twenty-six family transcription factor, is a third transcription factor that is downstream of Flt3 signaling.[120] Although PU.1 deficiency is lethal, mice reconstituted with PU.1-deficient hematopoietic cells show a number of hematopoietic defects, including strong reduction in CD8α+ and CD8α- cDCs.

The Interferon Regulatory Factors

IRF2, 4, and 8 are prominent transcription factors that regulate DC diversification. IRF2-/- mice exhibit reduced CD8α- cDCs, IRF4-/- mice display reduced CD8α-cDCs and slightly reduced pDCs, and IRF8-/- mice have reduced CD8α+ cDCs, pDCs, and LCs.[100,121,122,123]

NF-kB/Rel

Members of the NF-kB/Rel family also contribute to DC development. CD8α- cDC numbers are dramatically reduced in RelB-/- mice.[124] TRAF6 belongs to TNF receptor-associated factor family and acts upstream of the NF-kB cascade. Consistent with a role for NF-kB in cDC development, TRAF6-/- mice also show a defect in the CD8α- cDC compartment.[125]

Basic Helix-Loop-Helix Transcription Factors

Basic helix-loop-helix factors (E12, E47, HEB, E2-2) interact with helix-loop-helix Id (inhibitors of deoxyribonucleic acid [DNA] binding) proteins to control DC development. Loss of Id2 leads to a severe reduction of CD8α+ cDCs and absence of LCs. Another helix-loop-helix family member, E2-2, plays a crucial role in guiding pDC development and maintaining stability of the pDC lineage. Deletion of E2-2 in developing precursors blocked pDC development, whereas deletion in more mature cells reversed the development, suggesting differentiation plasticity that needs to be constantly reenforced.[126,127] Similar to mice, E2-2 haploinsufficient humans with Pitt-Hopkins syndrome show a pDC defect.[126]

TABLE 16.2 Transcription Factors Determine Dendritic Cell Development and Homeostasis in Vivo

		Classical DC		Tissue DC		
	Plasmacytoid DC	**CD8α+**	**CD8α-**	**CD103+**	**CD103-**	**LC**
PU.1-/-		---	----			
Irf8-/-	----	----	n.a.	----		--
Irf2-/-		n.a.	---			-
Irf4-/-	-	n.a.	---			
RelB-/-		n.a.	-----			n.a.
Gfi1-/-	--	--	--			++
ID2-/-	n.a.	----	n.a.	----		-----
E2-2-/-	----	n.a.	n.a.			
Stat3-/-		----	----			
Stat5-/-	---	---	---			
Ikaros-/-		----	-----			
Xbp1-/-	---	--	--			
Batf3-/-	n.a.	-----	n.a.	-----	n.a.	
Runx3-/-		++	-			-----
RBP-J-/		n.a.	----			
Notch2-/-		-	----	----	n.a.	

-, decrease; +, increase; CD, cluster of differentiation; DC, dendritic cell; LC, Langerhans cell; n.a., not altered.

Batf3

Batf3 is expressed at high levels in DCs and is specifically required for development of CD8α+ cDCs and CD103+ nonlymphoid tissue DCs.[24,128] Batf3-/- mice failed to mount antitumor and antiviral immune responses, demonstrating a key role of CD8α+ cDCs and CD103+ nonlymphoid tissue DCs in priming CD8 T cells.

Notch

Terminal DC differentiation appears to depend on signaling through Notch receptors and their transcriptional effector RBP-J. Inactivation of RBP-J in DCs results in severe reduction and functional impairment of splenic CD8α- cDCs.[129] In addition, Notch2 deletion leads to the loss of CD103+ nonlymphoid tissue DCs in the intestinal lamina propria and to a corresponding decrease of IL-17-producing CD4 T cells in the intestine.[130]

In conclusion, DC differentiation is dependent on a number of interacting transcription factors (Table 16.2). Together, they turn on expression of lineage-specific genes and suppress alternative developmental programs.

Human Dendritic Cell Development

Research on human DCs is difficult because it is largely limited to peripheral blood, whereas in the mouse, most of the work has been done on DCs residing in lymphoid or nonlymphoid tissues. As a result, human DC research has relied on peripheral blood monocytes cultured in the presence of cytokines, which generate moDCs and not cDCs.

Despite these obstacles, three different subsets of DCs have been found in human blood. These subsets are referred to as BDCA1, 2, and 3 based on their expression of cell surface markers.[131] BCDA1+ DCs resemble mouse CD8α- cDCs, BDCA2+CD11c- DCs are equivalent to mouse pDCs, and BDCA3+ DCs are equivalent to mouse CD8α+ cDCs (Fig. 16.2). These interspecies associations were initially based on similarities in gene expression between human and mouse DC subsets,[132] and were recently confirmed in functional experiments.[133,134,135,136,137] Moreover, human DCs can be obtained from cultures of human cord blood supplemented with Flt3L and GM-CSF.[134]

The human equivalents of the bone marrow-derived DC precursors, MDP, CDP, and pre-DC remain to be isolated. However, recent clinical studies revealed genetically defined syndromes associated with DC deficiency, which may shed some light on human DC development. Three different genetic lesions have been associated with the triad of DC deficiency, monocytopenia, and opportunistic infections. GATA2 mutation leads to a loss of DC, monocytes, B cells, and NK cells.[138,139] These patients have normal LCs and macrophages, suggesting that LCs and tissue macrophage have different origin from DCs and monocytes in humans. Mutations in IRF8 are associated with disseminated bacille Calmette-Guérin infection.[140] Two different mutations in IRF8 have been identified. The K108E variant is associated with an autosomal recessive severe immunodeficiency with a complete absence of circulating monocytes and DCs; the T80A variant is an autosomal dominant, milder immunodeficiency and is associated with selective depletion of circulating DCs. Loss of DCs due to IRF8 mutation also leads to myeloproliferation in humans and in mice due to increased serum Flt3L, which induces expansion of myeloid progenitors. In

humans, as in mice, loss of DCs is associated with loss of T_{reg} cells, confirming the existence of a homeostatic feedback loop these two cell types[141] (see following discussion).

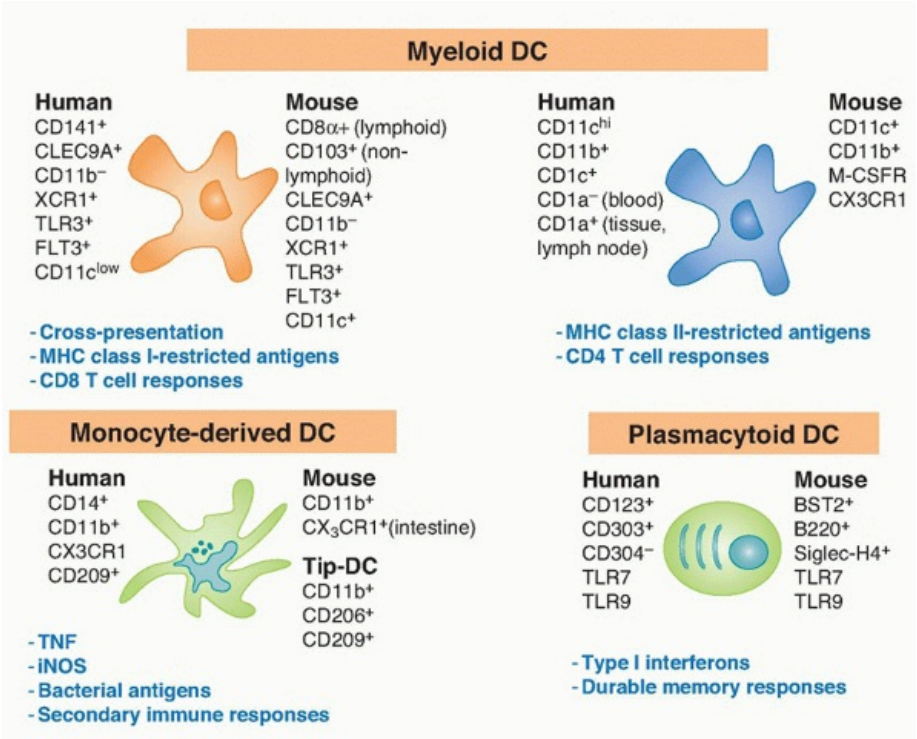

FIG. 16.2. Functionally Correlated Dendritic Cell Subsets in Human and Mouse.

DENDRITIC CELL HOMEOSTASIS

Lymphoid Organs

Based on their rapid labeling kinetics and loss of label in pulse chase experiments with BrdU, it was proposed that DCs have a very short half-life.[136,137] However, these early experiments are difficult to interpret in view of the more recent finding that DCs in lymphoid and nonlymphoid tissues divide in situ.[37,65,142]

The half-life of cDCs in tissues was measured in parabionts and shown to vary from 5 to 7 days in the spleen, lymph nodes, liver, and kidney, and as many as 25 days in the lung.[37,65] cDC homeostasis in all organs is maintained through a dynamic balance of three parameters: continuous input of pre-DCs from the blood, limited cDC division in situ, and cell death. Fitting BrdU incorporation and parabiosis separation data into mathematical equations produced a numerical estimate of the rate of DC precursor input (˜4,300/hour) and DC death (˜9,600/hour) in the mouse spleen.[65]

Regulation of Dendritic Cell Division

cDCs in mouse lymphoid and nonlymphoid tissues divide in situ for 10 to 14 days before being replaced by pre-DCs.[65] In the steady state, their division is regulated by

lymphotoxin-beta and Flt3L. Flt3L impacts nearly all stages of cDC development in lymphoid and nonlymphoid tissues, and is essential for their development.[59,112,143] In contrast, the effects of lymphotoxin-beta appear to be limited to CD4+ spleen DCs.[142] How the levels of circulating Flt3L are regulated is not known, but there appears to be a feedback loop between tissue cDCs and Flt3L that results in upregulation of Flt3L in the serum in response to cDC depletion,[144] loss of T_{reg}s,[60,145,146] or TLR ligand injection.[147] In all instances, increased Flt3L production leads to increased export of DC progenitors from the bone marrow and DC division in the periphery.

Conversely, increasing the number of DCs by Flt3L injection leads to an increase in the number of T_{reg} cells. Thus, the feedback loop between DCs and T_{reg}s is mediated by Flt3L, and that maintains the physiologic numbers of these two cell types in the steady state (Fig. 16.3).[146] Alterations in this mechanism lead to immune imbalance and can alter the course of autoimmune disease in mice.[146,148] For example, increasing the number of DCs in diabetes prone nonobese diabetic mice leads to increased numbers of T_{reg}s and a delay in disease onset. Similarly, Flt3L injection can protect mice from inflammatory bowel disease by increasing the number of cDCs and thereby the number of T_{reg}s. Thus, the Flt3Lmediated homeostatic feedback loop between T_{reg}s and DCs has clinical implication for vaccine design as well as the control of autoimmunity. Finally, this mechanism is entirely consistent with the proposed role of DCs in maintaining tolerance and regulating immunologic responses in vivo.[149]

Dendritic Cell Migration

DC migration from peripheral tissues into lymphoid organs is key to their sentinel and antigen trafficking functions. Upon microbial contact or stimulation by inflammatory cytokines, nonlymphoid tissue resident cDCs traffic through afferent lymphatics to the T-cell areas of the lymph node where they can participate in the initiation of immune responses. Migration is dependent on CCR7, which is upregulated by stimuli such as TLR ligands that activate DCs.

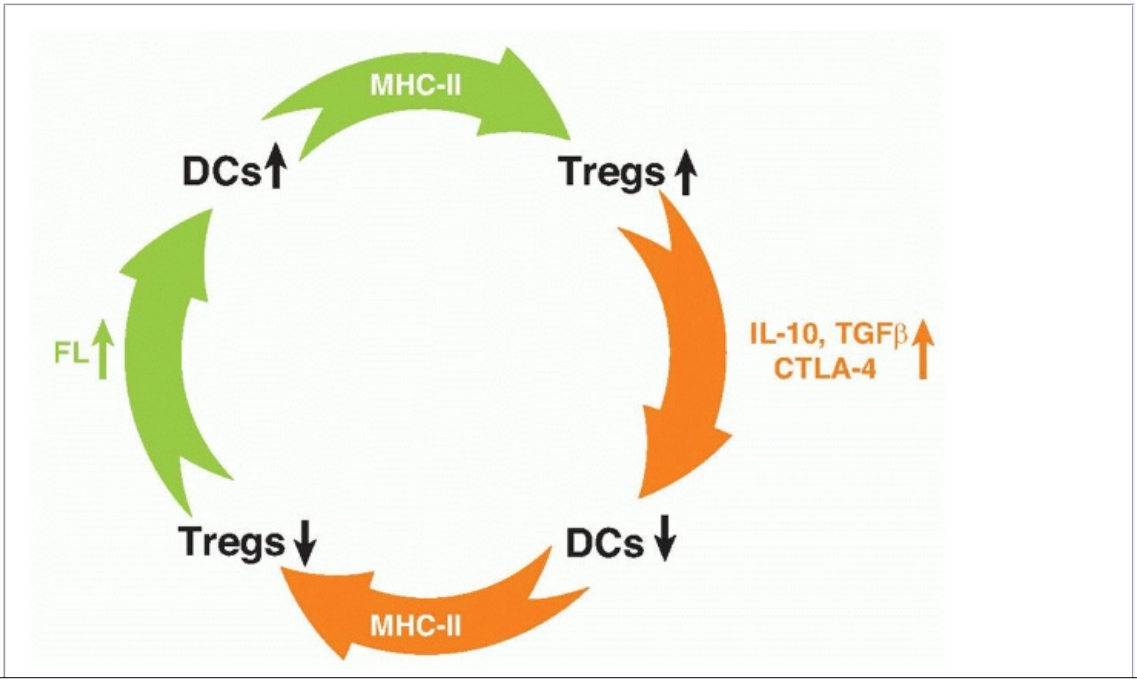

FIG. 16.3. Homeostatic Feedback Loop between Dendritic Cells and Regulatory T Cells.

Even in the absence of invading pathogens, some DCs are always migrating from tissues to lymph nodes. Studying afferent lymph is difficult in mice, but in rats, for example, DCs move along liver sinusoids in hepatic lymphatics to celiac lymph nodes,[150,151] and DCs from intestine migrate to mesenteric lymph nodes.[42,152] These cells are not found in the efferent lymph, indicating that most of the migrating DCs die after their arrival in lymphoid tissues. The DCs that migrate in the steady state might have several functions: to replenish immature populations, to transport self- or environmental antigens, or to be on patrol to identify invaders.

DC migration is a regulated process, controlled at the level of chemokine production and chemokine receptor expression and function.[153] Some immature DCs can express a repertoire of receptors (eg, CCR1, CCR2, CCR5, CCR6, and CXCR4) that bind inflammatory chemokines (eg, CCL5, CCL2, CCL3, CCL4, CCL20, and CXCL12). DC activation due to inflammatory signals is usually associated with downregulation of chemokine inflammatory receptors and the de novo expression of CCR7, the receptor for CCL19 and CCL21. In mice homozygous for an autosomal recessive mutation in CCL21 (paucity of lymph node T cells or plt/plt mice), naive T cells fail to home to secondary lymphoid organs. DCs in these mice also fail to accumulate in the spleen and in the T-cell areas of lymph nodes.[154] Similarly, CCR7-/- mice show defective secondary lymphoid organ architecture, defective homing of DCs and lymphocytes, and defective entry of DCs into lymphatic vessels at peripheral sites both in the steady state and inflammation conditions.[155] CCL19 and CCL21 also increase the maturation and proinflammatory differentiation of cDCs.[156] Therefore, chemokine/chemokine receptor interactions not only orchestrate the DC migration but also influence their immunogenic potential.

There are many other examples in which specific chemokines control the traffic of select populations of DCs (Table 16.3). In skin exposed to ultraviolet light, LCs disappear and are replaced in 2 weeks. The recruitment of LC precursors from blood is dependent on their expression of CCR2.[73] During murine listeriosis, CCR2 is also required for TNF-inducible nitric oxide synthase-producing DCs to migrate into the spleen.[53] Migration of pDCs into inflamed lymph nodes and their redistribution during inflammation depend on CXCR3 and CCR7. CCR6 is used by cDCs to populate epithelial surfaces during inflammation.[157,158,159] In the steady state, CXCL14 is important for LC progenitors to establish themselves in the skin.[160] All these findings indicate that the differential expression of chemokine receptors by DCs (and their subsets) at different stages of their life history determines their location in vivo.

ANTIGEN PRESENTATION

Antigen Capture

Macropinocytosis

DCs in culture continuously form 0.25 to 1.0 um pinocytic vesicles. These vesicles allow DCs to sample a large volume of extracellular fluid and soluble proteins that are present at low concentrations. Expression of aquaporin 3 and 7 on DCs may also contribute to macropinocytosis.[161]

TABLE 16.3. Animal Models Demonstrate Dendritic Cell Migration Depending on Chemokine Receptor

DC migration	
CCR2-/-, CCR5-/-	The recruitment of new Langerhans cells is dependent on their expression of the CCR2 chemokine receptor and on the secretion of CCR2-binding chemokines by inflamed skin.73 Recruitment of TNF/inducible nitric oxide synthase-producing DC subset in spleens of *Listeria monocytogenes*-infected mice is dependent on CCR2 and independent of CCR5.53,246
CCR6-/-	CCR6 mediates DC localization, lymphocyte homeostasis, and immune responses in mucosal tissue. In CCR6-/- mice, DCs expressing CD11c and CD11b are absent from the subepithelial dome region of Peyer patches.247
CCR7-/-, plt/plt (CCL19 and CCL21-/-)	CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs.155 Plt/plt mice lacking CCR7 ligands, CCL19 and CCL21, have defects in lymphocyte homing and DC localization.154 CCR7 also governs skin DC migration under inflammatory and steady-state conditions.248 CCR7 ligands, CCL19 and CCL21, induce a proinflammatory differentiation program in DCs.156
CCR8-/-	CCR7 and CCR8 pathways are used by monocyte-derived DCs during mobilization from skin to lymph nodes.249
CXCR3-/-	Plasmacytoid DCs migrate to inflamed lymph nodes, produce interferon-α, and help lymph node DCs to induce antiviral CTLs.250,251

CTL, cytotoxic T-lymphocyte; DC, dendritic cell; plt, paucity of lymph node T cells; TNF, tumor necrosis factor

Receptor-Mediated Phagocytosis

Phagocytosis is triggered by the attachment of extracellular particles to surface receptors, which mediate particle uptake. Multiple receptors on cDCs enhance recognition and ingestion of particulates including pathogens and dying cells. Importantly, many of the receptors also recognize molecular patterns on pathogens and as a result, activate DCs. In addition, DCs also express Fc receptors that mediate ingestion of opsonized particles and delivery to intracellular compartments that facilitate TAP-1-dependent cross-presentation to CD8 T cells. DCs express both activating and inhibitory forms of Fc receptors.[162] The inhibitory receptors help maintain DCs in an immature tolerogenic state. DCs that lack the FcγRIIB inhibitory receptor are more readily activated because the immunoreceptor-based tyrosine activation motif-associated FcγRIIA receptor is no longer subject to inhibition.[163] These observations have been extended in mice and humans through the identification of monoclonal antibodies that selectively block inhibitory receptors.[164,165]

Other than TLRs, DCs express many different types of pattern recognition receptors including those of the specific intercellular adhesion molecule-3-grabbing nonintegrin (SIGN) family, and multiple C-type lectins such as macrophage mannose receptor (MMR), DEC205, and DCIR2. MMR binds a range of bacteria, yeasts, and viruses through interactions between a mannose-type carbohydrate recognition domain and pathogen-associated high mannose structures. Notably, DCs deficient in any single C-type lectin receptor, such as MMR, DEC205, or DCIR2, develop normal adaptive immune response to dying cells, or pathogens, suggesting that this important class of receptors is redundant.

Dendritic Cell Maturation

DCs exist in two functionally distinct and phenotypically different stages. In the steady state, DCs in most tissues are equipped to capture and present antigens to T cells, but the outcome of antigen presentation by steady-state DCs is tolerance and not immunity. Steady-state DCs express high levels of pattern recognition and activation receptors, allowing them to sense changes in the environment, including pathogens and inflammatory cytokines. These signals induce extensive differentiation to a mature or activated state characterized by increased levels of cell surface MHC and coactivator expression, as well as cytokine secretion. In the activated state, DCs initiate potent and specifically polarized T-cell immune responses. Thus, pathogen sensing by DCs is an important mechanism that links innate pathogen recognition to the adaptive immune response.

Toll-Like Receptor Signaling

Microbes and viruses induce DC activation in part by engaging TLRs. DCs express nearly all known TLRs and also express additional cytoplasmic receptors that recognize pathogen patterns such as melanoma differentiation associated protein 5 (MDA-5), RIG-I, and DDX41, and nucleotide oligomerization domain-like receptors, although expression of specific receptors is restricted to distinct DC subsets.[166,167,168] In many cases, a single microbe will trigger several different TLRs expressed by DCs; therefore, a single TLR may be redundant for inducing immunity to many, but not all, pathogens. Thus, in humans, loss of TLR3 results in susceptibility to herpes simplex virus-1 infection but not to other microbes that also contain the TLR3 ligand.[169] Pathogen recognition by TLR ligation induces rapid DC activation and upregulation of MHC and costimulatory molecules, and also triggers secretion

of inflammatory cytokines such as IL-1α, IL-6, TNFα, IL-18, and IL-12, which are critical in driving T-cell differentiation.[170,171,172]

Cytokines, T, and Natural Killer T Cell Signals

DCs can also sense proinflammatory cytokines such as TNFα and IL-1β, and direct cellular contact with activated T or NKT cells by ligation of CD40, a TNF receptor family molecule that is highly expressed on the surface of DCs. Many of these receptors, including TLRs, IL-1R, and TNF receptors, activate DCs through the NF-κB pathway.

Antigen Processing

DCs process ingested antigens for presentation on both MHC class I and MHC class II. Although DCs are excellent MHCII antigen-presenting cells, they are not unique in this respect. Other cells, including activated B cells and monocytes, can also process and present antigens for presentation by MHCII efficiently. For example, in germinal centers, activated B cells are the key antigen-presenting cell.

Macrophages endocytose antigens and rapidly digest them. In contrast, DCs sequester and preserve the captured antigen for later presentation. Preservation of antigens is critical for immunogenicity and depends on two DC specializations. The first is low lysosomal protease activity. Macrophages contain high levels of lysosomal proteases, including cathepsin S, cathepsin L, cathepsin K, and asparagine endopeptidase. These proteases enable rapid degradation of internalized proteins to single amino acids. Compared to macrophages, DCs express low amounts of proteases, resulting in a limited capacity for lysosomal degradation.[173] This low-level proteolytic capacity appears to be crucial as the peptides loaded on MHC and recognized by T cells consist of peptides between 8 to 17 amino acids. Consistent with this idea, mutations in amino acids in antigens that increase their resistance to lysosomal proteolysis increase immunogenicity by decreasing the rate of proteolysis.[173]

A second feature of DC lysosomes that facilitates antigen presentation is that DC lysosomes are less acidic compared to those in professional phagocytes. In macrophages and neutrophils, the low pH of the phagosome and endosome activates lysosomal proteases delivered to these endocytic vesicles. In DCs, the pH in these compartments is less acidic because the assembly of V-ATPase, an adenosine triphosphate-dependent vacuolar proton pump, appears to be incomplete in the lysosome of steady-state DCs, leading to alkalinization of the endocytic compartment.[174] In addition, DCs show efficient recruitment of Nox2 to endosomes and phagosomes. Nox2 produces reactive oxygen species, which consumes protons and thereby reduces the acidification of the endocytic compartment. NOX2-defective DCs exhibit a higher level acidification of the phagosome and increased proteolysis and decreased antigen presentation.[175] Furthermore, Nox2-deficient patients, who suffer from chronic granulomatous disease, show impaired cross-presentation.[176] Together, the lower levels of proteolytic activity and decreased acidity in endocytic compartment lead to a decrease in the rate of antigen digestion and increased availability of partially processed peptides for loading on MHC. This unique feature of DCs may also help preserve the antigen during the migration of nonlymphoid tissue DCs from the site of antigen capture to the lymph nodes.

Antigens are actively taken up by immature DCs and targeted to MHCII-positive lysosomes. However, these antigens are sequestered in an intact form and are not efficiently utilized for formation of MHCII-peptide complexes. Immature DCs do form stable MHCII dimers, but their presence does not result in immunogenic complexes. DC activation by microbial products or proinflammatory cytokines results in redistribution of MHCII from intracellular compartments to the plasma membrane. In addition, DC activation reduces lysosomal cystatin C, which leads to increased activation of cathepsin S and more efficient processing of Ii.[85]

DENDRITIC CELLS LINK INNATE AND ADAPTIVE IMMUNITY

Plasmacytoid Dendritic Cells

Nucleic acid-sensing pDCs are activated when they sense pathogen or self-derived nucleic acids and are involved in antiviral immunity and autoimmune diseases. Whereas monocytes express TLR4 (lipopolysaccharide sensor) and cDCs express TLR3 (double-stranded RNA sensor), pDCs selectively express TLR7 (single-stranded RNA sensor) and TLR9 (DNA sensor). pDCs endocytose viruses and sense their nucleic acids using TLR7 and TLR9, both of which reside in the endosomal compartment. TLR7 detects single-stranded RNA viruses, such as influenza, respiratory syncytial virus, Sendai, and vesicular stomatitis virus, and synthetic singlestranded RNA analogs such as R848/Imiqimod; TLR9 detects DNA viruses, such as herpes simplex virus-1, herpes simplex virus-2, and murine cytomeglavirus, or cytosinphosphatidyl-guanosin oligodeoxynucleotides. Viruses that enter the cytoplasm of pDCs are detected after autophagy, a conserved cell-autonomous process involving lysosomal degradation of cellular organelles to deliver cytoplasmic RNA to TLR7-containing endosomal compartments. Both TLR7 and TLR9 use MyD88 as the adaptor protein for activation; thus, MyD88-deficient pDCs have defective responses to a wide range of viruses that enter the cytoplasm. Recruitment (binding) of MyD88 to the TLR leads to assembly of a signal-transducing complex that includes IRAK4, TRAF6, Bruton's tyrosine kinase, and IRF7.[177] This signaling complex is critical for IFN production by pDCs. In most cells, production of IFNα depends on binding of IFNβ to interferon-α/β receptor (IFNAR), which induces IRF7 expression. In pDCs, however, constitutive high-level expression of IRF7 facilitates a rapid IFNα response that is independent of IFNAR signaling.[105]

IFN production by pDCs is rapid, starting 4 hours after exposure to TLR ligands and peaking after 24 hours when pDCs become refractory. Type I IFN produced from activated pDCs not only directly inhibits viral replication, but also activates NK, B cells, and cDCs.[178] For example, human immunodeficiency virus (HIV) or herpes simplex virus infection stimulates pDCs to produce type I IFN and CD40L, which in turn activates cDCs and facilitates antigen presentation and development of antiviral immunity. Similarly, pDC nucleic acid sensing and cDC activation occurs in autoimmune diseases. In patients with systemic lupus erythematosus, pDCs are continuously activated by circulating immune complexes composed of self-DNA or RNA leading to high circulating levels of IFNα.[178]

Given the potential pathologic consequences of high-level IFNα production by pDCs, it is not surprising that this response is highly regulated. pDCs express an array of negative regulators to downregulate IFNα production. In humans, two surface molecules, BDCA2 and immunoglobulin-like

transcript 7 (also known as LILRA4), were found to suppress TLR-induced IFN production from pDCs. Both receptors mediate their inhibitory effects through cytoplasmic immunoreceptor-based tyrosine activation motifs, and both BDCA2 and immunoglobulin-like transcript 7 associate with the γ-chain of the high-affinity Fc receptor for immunoglobulin E (FcεRIγ).[179] Other immunoreceptor-based tyrosine activation motif-mediated inhibitors of pDC IFN production include NKp44 and sialic acid binding Ig-like lectin (Siglec)-H, which recruits DAP12.[180]

Interactions with Innate Lymphocytes

DCs interact with innate lymphocytes (eg, NK, NKT, and γδ T cells) in ways that enhance the functions of both cell types. DCs produce IL-12, IL-15, IL-2, and IFNα/β that affect different facets of NK cell function, whereas the innate lymphocytes induce DC activation.[181,182] Injection of activated cDCs leads to the recruitment of NK cells into the draining lymph nodes while exposure of cDCs to TLR stimuli induces IL-2 and IL-12 production, which in turn activate NK cells to produce IFNγ. DCs also present different glycolipids on CD1d molecules to the invariant T-cell receptor on NKT cells. These include endogenous lysosomal glycosphingolipids (eg, iGb3),[183] microbial lipids,[184,185] or synthetic glycolipids (eg, alpha-GalCer)[186,187] to activate NKT cells in vivo.

Type I IFN appears to be one of the essential mediators of DC activation in vivo. For example, antigen targeted to cDCs can elicit strong Th1 immunity in the presence of PolyI:C. PolyI:C binds to TLR3 on the cell surface and intracellular sensor MDA-5. However, PolyI:C does not directly stimulate DC maturation because deficiency of TLR3 or MDA-5 on DCs does not abrogate immunogenicity.[188] Instead, PolyI:C stimulates nonhematopoietic cells to produce large amounts of type I IFN.[188] High levels of systemic type I IFN stimulate functional maturation of cDCs, which consequently induce a robust Th1 response. Thus, microbial stimuli-induced DC maturation in vivo engages a cascade of cellular and molecular mechanisms, which may amplify the environmental signals to facilitate optimal immunity.

Initiating Adaptive T-Cell Immunity

In the steady state, DCs can endocytose a diverse array of antigens through multiple receptors on their cell surface. However, steady-state DCs typically express relatively low levels of surface MHC class I and II products and only low levels of costimulatory molecules (eg, CD80, CD86). Upon receipt of an activation stimulus, DCs undergo extensive differentiation, and they migrate in increased numbers to secondary lymphoid tissues.

DC-T-cell interactions in the lymph nodes have now been studied in the living state with two-photon microscopy.[33,189,190,191,192] Naïve antigen-specific T cells arrest on antigen-presenting DCs, and this stable interaction lasts for at least 18 hours. In the steady state, this stable DC-T-cell interaction leads to tolerance while an activation signal such as TLR ligation allow DCs to initiate immunity. cDC activation results in redistribution of MHCII from intracellular compartments to the plasma membrane; the upregulation of costimulatory molecules such as CD40, CD80, and CD86; and change in profiles of cytokine and chemokines such as TNFα and IL-12. All these changes likely contribute to the initiation of T-cell immunity.

The communication between DCs and T cells is a dialogue in which the DCs also respond to T cells. For example, CD40 and TRANCE/RANK receptor on DCs are ligated by the corresponding TNF family member expressed on activated and memory T cells (ie, CD40L and RANK-L).[193,194,195] This leads to increased DC survival and in the case of CD40 ligation, upregulation of CD80 and CD86, secretion of IL-12, and release of chemokines such as IL-8 and MIP-1α and β.

Controlling the Quality of the T-Cell Response

DCs are involved in critical T-cell fate decisions such as clonal selection, tolerance versus immunity, Th1 versus Th2, and even memory. In the presence of mature DCs producing IL-12 or IFNs (as might occur when DCs are ligated by CD40L or infected with viruses), CD4 T cells differentiate along a Th1 pathway for IFNγ production. The latter in turn activates the antimicrobial activity of macrophages and promotes killer T-cell differentiation. However, in the presence of exogenous IL-4, DCs induce T cells to differentiate into Th2 cells, which secrete IL-4, IL-5, and IL-13. These cytokines help B cells to make antibodies of the IgG1 and IgE isotypes, activate eosinophils, and stimulate fibrosis. A new and striking pathway that was first discovered with human monocyte-derived DCs involves the epithelial-derived cytokine thymic stromal lymphopoietin (TSLP). This cytokine stimulates DCs to induce "inflammatory Th2 cells" that produce TNFα (rather than IL-10) in addition to IL-4, IL-5, and IL-13.[196] Recent studies have shown that TSLP receptor knockout mice exhibit strong Th1 responses, with high levels of IL-12, IFNγ, and IgG2a, but low production of IL-4, IL-5, IL-10, IL-13, and immunoglobulin E.[197] DCs that are activated with either CD40L or TSLP are similar in appearance, being rich in MHCII and CD86 co-stimulatory molecules. However, they differ significantly in cytokine and chemokine production, and the functional consequences for T cells vary.[196]

Cross-Presentation by Conventional Dendritic Cells

CD8 T cells are critical for protective immunity against intracellular pathogens and malignant tumor cells. When autologous T cells are incubated with influenza-infected DCs, strong proliferative and cytotoxic T-lymphocyte (CTL) responses develop within a week. Thus, DCs present endogenous antigens on MHCI to prime CD8+ T cells. Interestingly, DCs can also present exogenous antigen on MHCI, an unconventional presentation path called "cross-presentation." The earliest experiment demonstrating the capacity of DCs in cross-presentation was done in vitro, when trinitrophenyl-modified cells were taken up by DCs. DCs can present trinitrophenyl, an exogenous antigen, and induce antitrinitrophenyl CTLs in vitro in a MHC-restricted manner.[14] Later, DCs were found to take up exogenous influenza

antigen in the form of nonreplicative virus or apoptotic cells, and present them on MHCI to prime CD8 T cells.[198] In the absence of DCs, mice are unable to process several different antigens through the exogenous pathway, indicating that DCs were a major cell type for cross presentation to CD8 T cells in vivo.[199] DCs cross present exogenous antigen taken up as soluble form or via multiple receptors, including FcR, CD91, Lox-1, DEC205/CD205, and DNGR1. CD8+ DCs in mice and their functional equivalent BDCA3+ DCs in humans excel in cross-presenting exogenous antigens.[24,134,200] Exogenous presentation or cross-

presentation is essential for protective immunity against viruses and tumors.

DENDRITIC CELLS CONTROL TOLERANCE

The mechanisms used by DCs to initiate immunity against pathogens pose a major risk for the development of autoimmunity, allergy, and chronic inflammatory disease. For example, infection frequently induces some cell death; therefore, DCs at the site of infection capture pathogens and dying cells, and the two are processed and presented similarly.[201,202] Likewise, at body surfaces, maturing DCs are also capturing environmental antigens to which the body must remain unresponsive. How do maturing DCs focus the immune response on antigens derived from the pathogen and avoid inducing immunity to self- and nonpathogenic environmental antigens?

Thymic Dendritic Cells Contribute to Central Tolerance

Self-reactive thymocytes are deleted by antigen-presenting cells during negative selection. The thymus contains two major populations of antigen-presenting cells that express MHCII, namely, medullary thymic epithelial cells and cDCs. Both cell types are required for efficient negative selection. Medullary thymic epithelial cells express a panoply of selfantigens under the control of the autoimmune regulator "AIRE."[203] Self-antigens for negative selection in the thymus also include antigens expressed by DCs, and antigens that enter the thymus through the bloodstream and are captured by DCs. In the absence of antigen presentation by DCs, negative selection of CD4+ thymocytes is impaired.[204] In addition to negative selection, thymic cDCs also support the development of Foxp3+ T_{reg}s.[205] Thus cDCs contribute to central tolerance in the thymus by more than one mechanism.

Dendritic Cells Mediate Peripheral Tolerance

DCs also mediate tolerance in the periphery. Because central tolerance alone is incomplete, the immune system must continually establish tolerance to harmless or "noninfectious" antigen in the environment. Therefore, the effective control of self-reactive T cells depends on peripheral tolerance. For example, mice and humans deficient in T_{reg}s, which suppress autoreactive T cells in the periphery, succumb to autoimmunity at an early age. DCs constantly carry innocuous antigens from the periphery (eg, from the skin, airways, stomach, intestine, and pancreas) and present them to T cells in lymphoid organs.[206] A critical observation was that bone-marrow-derived cells in the pancreatic lymph nodes present peptides derived from insulin-producing β cells from pancreatic islets, leading to tolerance.[207,208] Although DCs were not implicated directly, a direct role for DCs in maintaining tolerance was established by Hawiger and colleagues by targeting antigens directly to DCs.[209] Furthermore, CD8α+ cDCs are particularly efficient in capturing dying cells[201] and inducing tolerance.[202] Likewise, when DCs capture innocuous proteins from the airway, profound tolerance develops even though the T cells can initially proliferate extensively to the antigen-capturing DCs in the draining lymph nodes.[210]

Deletion and Anergy

Mechanisms for peripheral tolerance can be intrinsic (deletion and anergy) or extrinsic

(through suppressive T_{reg} cells). Interestingly, the former requires expression of B7 family members on the steady state DCs (eg, PD-L1 and CD86), which then ligate PD-1 and CTL antigen-4 on the T cells to be tolerized.

Regulatory T cells

Autoreactive T cells can remain quiescent in the presence of T_{reg} cells. There are two types of T_{reg}s: naturally occurring T_{reg}s derived from thymus (natural T_{reg}s) and T_{reg}s induced from Foxp3-CD4+ T cells in the periphery (induced T_{reg}s).[211]

The groups of Belkaid and Powrie found that a subset of gut DCs expressing CD103, the αEβ7 integrin, are specialized at inducing Foxp3+ T_{reg}s and maintaining oral tolerance.[212] CD103+ DCs from mesenteric lymph nodes or gut-associated lymphoid tissue produce transforming growth factor-β, a cytokine critical for induction of Foxp3+ T_{reg}s.[212] Additionally, CD103+ gut DCs use retinal dehydrogenase to metabolize vitamin A to bioactive retinoic acid, which acts as a cofactor for transforming growth factor-β to induce Foxp3+ T_{reg}s.[212,213] Similarly, skin DCs use vitamin D3 to induce T_{reg}s.[214,215] Therefore, DCs are able to employ environmental signals, vitamin A in the gut, and vitamin D3 in the skin to induce tolerance to harmless foreign antigens. The CD8+ and CD103+ DC subset appears to be specialized at T_{reg} induction. When antigens were targeted to CD8α+ DEC-205+ or CD8α- DCIR2+ DC subsets in vivo, only CD8α+ DEC-205+ DCs were able to induce Foxp3+ T_{reg}s from Foxp3- CD4+ T cells.[216]

DCs also maintain the homeostasis of T_{reg}s. Loss of DCs leads to a loss of T_{reg} cells, and the remaining T_{reg} cells exhibit decreased Foxp3 expression. The DC-dependent loss in T_{reg} cells leads to an increase in the number of T cells producing inflammatory cytokines, such as IFNβ and IL-17.[217] Conversely, increasing the number of DCs leads to increased T_{reg}-cell division and accumulation by a mechanism that requires MHCII expression on DCs.[146] Activation of β-catenin is essential for DC to control T_{reg} and peripheral tolerance. DCs lacking β-catenin show decreased production of immunosuppressive cytokines and a decreased ability to support the differentiation of naïve T cells into T_{reg}s in vitro.[218]

In summary, DCs have the capacity to induce tolerance by several mechanisms.

DENDRITIC CELLS IN CLINICAL IMMUNOLOGY

This section focuses on diseases in which DCs play a pathogenic role or can be targets for therapy.

Dendritic Cells in Transplantation

DCs play a key role in the outcome of organ and hematopoietic transplantation. In organ transplantation, both donor and recipient DCs contribute to graft rejection. Donor DCs in grafted organs migrate to lymphoid organs where they stimulate alloreactive T cells in the recipient to induce organ rejection[219,220]; recipient DCs can also capture antigens from the graft and elicit organ rejection. For example, in hematopoietic transplantation, recipient DCs

initiate T-cell-induced graft-versus-host reactions.[74,221]

Dendritic Cells in Autoimmune Disease

Several human autoimmune diseases appear to involve DCs. Abundant DCs are found in the synovial exudates of rheumatoid arthritis, and TNF-α produced by DCs contributes to the severity of the disease. Similarly, in psoriasis, DCs infiltrate lesional skin, produce TNF-α, and polarize T cells toward Th1/Th17[222,223]; additionally, pDCs in psoriatic lesion become activated to produce type I IFN, which also contributes to the inflammatory response.

In systemic lupus erythematosus, two subsets of DCs contribute to the onset and severity of the disease; pDCs in patients with systemic lupus erythematosus overproduce IFNα, which activates cDCs and interferes with their ability to maintain peripheral tolerance.[178] Finally, DCs have been implicated in mouse models of type I diabetes by carrying self-antigens from the pancreas to draining lymph nodes where diabetogenic T-cell responses are initiated. In addition, DCs cultured from nonobese diabetic mice show an activated phenotype with increased IL-12 and costimulatory molecule expression.[224]

Dendritic Cells in Viral Infections

DCs mediate antiviral immunity by priming T-cell responses. However, a number of viruses have evolved strategies to subvert DCs, and thereby, the immune system.

The interaction between HIV and DCs is a fascinating example of viral immune subversion. DCs carry HIV from peripheral tissues into draining lymph nodes where the virus is transmitted to CD4 T cells. HIV binds to DCs by CCR4, CXCR5, or DC-SIGN, but productive infection is restricted by SAMHD1, a protein encoded by an Aicardi-Goutières syndrome susceptibility gene.[225] Transmission to CD4 T cells is dependent on DC-SIGN, a C-type lectin pathogen-recognition receptor expressed on the surface of DCs that retains the attached virus in an infectious state. In the lymphoid organs, close interaction between DCs and CD4+ T cells facilitate HIV transmission.[226,227]

Interestingly, DC-SIGN also serves as receptor for several other viruses including hepatitis C virus, Ebola virus, cytomegalovirus, dengue virus, and the severe acute respiratory syndrome coronavirus.[228,229,230] Dengue is a mosquitoborne flavivirus that causes a disease that can be associated with hemorrhagic fever. Dengue virus targets DCs directly through DC-SIGN but also enters DCs as a passenger in immune complexes that are taken up by Fc receptors.[231,232] When infection occurs through antibody enhancement mediated by Fc receptor, the infected DCs are involved in induction of the T-cell cytokines that mediate the vascular leak syndrome associated with the infection.

Dendritic Cells in Cancer

Tumors can suppress immunity in part through their effects on DCs. DC differentiation and activation can be suppressed by cancer-derived cytokines, such as IL-6, vascular endothelial growth factor, and IL-10.[233] In contrast to their normal counterparts that activate immune responses, DCs derived from tumors induce Foxp3+ T_{reg}s[234] and IL-13-producing CD4+ T cells,[235] and suppress proliferation of CTLs[236] and NKT cells.

Dendritic Cell-Targeted Vaccines

DC-based vaccines are currently being used in the clinic, and DC-targeted vaccines are being tested.

DC-based immune therapy is currently available for treating prostatic cancer, but it is far from optimized and is not curative. Monocyte-derived DCs are generated ex vivo, loaded with tumor cells or tumor antigens, and reinjected into the patient.[237] Scientific and practical problems exist with this approach, including limited responses possibly due to inefficient migration of monocyte-derived DCs from injection site to the draining lymphoid organs and inefficient antigen presentation.

DC-targeted vaccines are based on the idea that antigens delivered specifically to DCs in conjunction with the appropriate adjuvants will produce strong and lasting immunity.[209] DC-targeted vaccines require that antigens be delivered specifically to endocytic receptors on DCs together with the appropriate stimuli to induce DC activation. For example, antigens have been incorporated into antireceptor monoclonal antibodies, which are then injected into the vaccine recipient.[209,238,239] This paradigm was established using antibodies to DEC205/CD205, which is abundant on DCs and delivers antigens to both MHC class I and II antigen-processing compartments.[240] In mice, antigens targeted to DEC205/CD205 on activated DCs induce strong immunity against tumors and a number of intracellular pathogens, including malaria and *Leishmania*.[238,241,242] Importantly, these responses are broad, often generating immunity against multiple epitopes[243] in mice of several MHC haplotypes and the responses elicit protection from mucosal infections.[244] Other potential DC targets include LOX-1/OLR1, MMR/CD206, DCIR/CLEC4A, DC-SIGN/CD209, DNGR1, langerin (CD207), and CD40.[28,245]

In conclusion, DCs play important roles in a number of different diseases. Moreover, they are excellent targets in designing new approaches to prevention and treatment of these diseases.

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REFERENCES

1. Mishell RI, Dutton RW. Immunization of dissociated spleen cell cultures from normal mice. *J Exp Med*. 1967;126(3):423-442.

2. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med*. 1973;137(5):1142-1162.

3. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. *J Exp Med*. 1974;139(2):380-397.

4. Unanue ER, Cerottini JC. The immunogenicity of antigen bound to the plasma membrane of macrophages. *J Exp Med*. 1970;131(4):711-725.

5. Ehrenreich BA, Cohn ZA. The fate of peptides pinocytosed by macrophages in vitro. *J Exp Med*. 1969;129(1):227-245.

6. Ehrenreich BA, Cohn ZA. The uptake and digestion of iodinated human serum albumin by macrophages in vitro. *J Exp Med*. 1967;126(5):941-958.

7. Steinman RM, Cohn ZA. The interaction of soluble horseradish peroxidase with mouse peritoneal macrophages in vitro. *J Cell Biol* . 1972; 55(1):186-204.

8. Steinman RM, Kaplan G, Witmer MD, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, new surface markers, and maintenance in vitro. *J Exp Med*. 1979;149(1):1-16.

9. Nussenzweig MC, Steinman RM, Unkeless JC, Witmer MD, Gutchinov B, Cohn ZA. Studies of the cell surface of mouse dendritic cells and other leukocytes. *J Exp Med*. 1981;154(1):168-187.

10. Steinman RM, Witmer MD. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc Natl Acad Sci U S A* . 1978;75(10):5132-5136.

11. Nussenzweig MC, Steinman RM. Contribution of dendritic cells to stimulation of the murine syngeneic mixed leukocyte reaction. *J Exp Med*. 1980;151(5):1196-1212.

12. Steinman RM, Gutchinov B, Witmer MD, Nussenzweig MC. Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. *J Exp Med*. 1983;157(2):613-627.

13. Paul WE. Bridging innate and adaptive immunity. *Cell*. 2011;147(6): 1212-1215.

14. Nussenzweig MC, Steinman RM, Gutchinov B, Cohn ZA. Dendritic cells are accessory cells for the development of anti-trinitrophenyl cytotoxic T lymphocytes. *J Exp Med*. 1980;152(4):1070-1084.

15. Nussenzweig MC, Steinman RM, Witmer MD, Gutchinov B. A monoclonal antibody specific for mouse dendritic cells. *Proc Natl Acad Sci U S A* . 1982;79(1):161-165.

16. Kraal G, Breel M, Janse M, Bruin G. Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. *J Exp Med*. 1986;163(4):981-997.

17. Metlay JP, Witmer-Pack MD, Agger R, Crowley MT, Lawless D, Steinman RM. The

distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. *J Exp Med*. 1990;171(5):1753-1771.

18. Inaba K, Steinman RM, Van Voorhis WC, Muramatsu S. Dendritic cells are critical accessory cells for thymus-dependent antibody responses in mouse and in man. *Proc Natl Acad Sci U S A*. 1983;80(19):6041-6045.

19. Ardavin C, Shortman K. Cell surface marker analysis of mouse thymic dendritic cells. *Eur J Immunol* . 1992;22(3):859-862.

20. Vremec D, Zorbas M, Scollay R, et al. The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *J Exp Med*. 1992;176(1):47-58.

21. Shortman K, Liu YJ. Mouse and human dendritic cell subtypes. *Nat Rev Immunol* . 2002;2(3):151-161.

22. Helft J, Ginhoux F, Bogunovic M, Merad M. Origin and functional heterogeneity of non-lymphoid tissue dendritic cells in mice. *Immunol Rev*. 2010;234(1):55-75.

23. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature*. 1998;392(6673):245-252.

24. Hildner K, Edelson BT, Purtha WE, et al. Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science*. 2008;322(5904):1097-1100.

25. Edelson BT, Kc W, Juang R, et al. Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells. *J Exp Med*. 2010;207(4):823-836.

26. Mashayekhi M, Sandau MM, Dunay IR, et al. CD8alpha(+) dendritic cells are the critical source of interleukin-12 that controls acute infection by Toxoplasma gondii tachyzoites. *Immunity*. 2011;35(2):249-259.

27. Edwards AD, Diebold SS, Slack EM, et al. Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines. *Eur J Immunol* . 2003;33(4):827-833.

28. Dudziak D, Kamphorst AO, Heidkamp GF, et al. Differential antigen processing by dendritic cell subsets in vivo. *Science*. 2007;315(5808): 107-111.

29. Veerman AJ. On the interdigitating cells in the thymus-dependent area of the rat spleen: a relation between the mononuclear phagocyte system and T-lymphocytes. *Cell Tissue Res*. 1974;148(2):247-257.

30. Fossum S. The architecture of rat lymph nodes. II. Lymph node compartments. *Scand J Immunol*. 1980;12(5):411-420.

31. Fossum S, Vaaland JL. The architecture of rat lymph nodes. I. Combined light and electron microscopy of lymph node cell types. *Anat Embryol (Berl)*. 1983;167(2):229-246.

32. Lindquist RL, Shakhar G, Dudziak D, et al. Visualizing dendritic cell networks in vivo. *Nat Immunol*. 2004;5(12):1243-1250.

33. Shakhar G, Lindquist RL, Skokos D, et al. Stable T cell-dendritic cell interactions precede the development of both tolerance and immunity in vivo. *Nat Immunol*. 2005;6(7):707-714.

34. Knight SC, Balfour BM, O'Brien J, Buttifant L, Sumerska T, Clarke J. Role of veiled cells in lymphocyte activation. *Eur J Immunol* . 1982;12(12):1057-1060.

35. Hart DN, Fabre JW. Demonstration and characterization of Ia-positive dendritic cells in the interstitial connective tissues of rat heart and other tissues, but not brain. *J Exp Med*. 1981;154(2):347-361.

36. Anandasabapathy N, Victora GD, Meredith M, et al. Flt3L controls the development of radiosensitive dendritic cells in the meninges and choroid plexus of the steady-state mouse brain. *J Exp Med*. 2011;208(8):1695-1705.

37. Ginhoux F, Liu K, Helft J, et al. The origin and development of nonlymphoid tissue CD103+ DCs. *J Exp Med*. 2009;206(13):3115-3130.

38. Landsman L, Varol C, Jung S. Distinct differentiation potential of blood monocyte subsets in the lung. *J Immunol*. 2007;178(4):2000-2007.

39. Kelly RH, Balfour BM, Armstrong JA, Griffiths S. Functional anatomy of lymph nodes. II. Peripheral lymph-borne mononuclear cells. *Anat Rec*. 1978;190(1):5-21.

40. Bujdoso R, Hopkins J, Dutia BM, Young P, McConnell I. Characterization of sheep afferent lymph dendritic cells and their role in antigen carriage. *J Exp Med*. 1989;170(4):1285-1301.

41. Drexhage HA, Mullink H, de Groot J, Clarke J, Balfour BM. A study of cells present in peripheral lymph of pigs with special reference to a type of cell resembling the Langerhans cell. *Cell Tissue Res*. 1979;202(3):407-430.

42. Huang FP, Platt N, Wykes M, et al. A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *J Exp Med*. 2000;191(3):435-444.

43. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell*. 1997;91(5):661-672.

44. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*. 2000;404(6774):193-197.

45. Kondo M, Scherer DC, King AG, Manz MG, Weissman IL. Lymphocyte development from hematopoietic stem cells. *Curr Opin Genet Dev* . 2001;11(5):520-526.

46. Wu L, Li CL, Shortman K. Thymic dendritic cell precursors: relationship to the T lymphocyte lineage and phenotype of the dendritic cell progeny. *J Exp Med*. 1996;184(3):903-911.

47. Wu L, Nichogiannopoulou A, Shortman K, Georgopoulos K. Cellautonomous defects in dendritic cell populations of Ikaros mutant mice point to a developmental relationship with the lymphoid lineage. *Immunity*. 1997;7(4):483-492.

48. Shortman K, Vremec D, Corcoran LM, Georgopoulos K, Lucas K, Wu L. The linkage between T-cell and dendritic cell development in the mouse thymus. *Immunol Rev*. 1998;165:39-46.

49. Guerriero A, Langmuir PB, Spain LM, Scott EW. PU.1 is required for myeloid-derived but not lymphoid-derived dendritic cells. *Blood*. 2000;95(3):879-885.

50. Manz MG, Traver D, Akashi K, et al. Dendritic cell development from common myeloid progenitors. *Ann N Y Acad Sci* . 2001;938:167-173, discussion 173-174.

51. Randolph GJ, Beaulieu S, Lebecque S, Steinman RM, Muller WA. Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. *Science*. 1998;282(5388):480-483.

52. Randolph GJ, Inaba K, Robbiani DF, Steinman RM, Muller WA. Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. *Immunity*. 1999;11(6):753-761.

53. Serbina NV, Salazar-Mather TP, Biron CA, Kuziel WA, Pamer EG. TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity*. 2003;19(1):59-70.

54. Fogg DK, Sibon C, Miled C, et al. A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science*. 2006;311(5757):83-87.

55. Varol C, Landsman L, Fogg DK, et al. Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *J Exp Med*. 2007;204(1):171-180.

56. Naik SH, Metcalf D, van Nieuwenhuijze A, et al. Intrasplenic steadystate dendritic cell precursors that are distinct from monocytes. *Nat Immunol*. 2006;7(6):663-671.

57. Bogunovic M, Ginhoux F, Helft J, et al. Origin of the lamina propria dendritic cell network. *Immunity*. 2009;31(3):513-525.

58. Jakubzick C, Bogunovic M, Bonito AJ, Kuan EL, Merad M, Randolph GJ. Lymph-migrating, tissue-derived dendritic cells are minor constituents within steady-state lymph nodes. *J Exp Med*. 2008;205(12): 2839-2850.

59. Waskow C, Liu K, Darrasse-Jeze G, et al. The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues. *Nat Immunol*. 2008;9(6):676-683.

60. Liu K, Victora GD, Schwickert TA, et al. In vivo analysis of dendritic cell development and homeostasis. *Science*. 2009;324(5925):392-397.

61. Naik SH, Sathe P, Park HY, et al. Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. *Nat Immunol*. 2007;8(11):1217-1226.

62. Onai N, Obata-Onai A, Schmid MA, Ohteki T, Jarrossay D, Manz MG. Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow. *Nat Immunol*. 2007;8(11):1207-1216.

63. del Hoyo GM, Martin P, Vargas HH, Ruiz S, Arias CF, Ardavin C. Characterization of a common precursor population for dendritic cells. *Nature*. 2002;415(6875):1043-1047.

64. Diao J, Winter E, Chen W, Cantin C, Cattral MS. Characterization of distinct conventional and plasmacytoid dendritic cell-committed precursors in murine bone marrow. *J Immunol*. 2004;173(3):1826-1833.

65. Liu K, Waskow C, Liu X, Yao K, Hoh J, Nussenzweig M. Origin of dendritic cells in peripheral lymphoid organs of mice. *Nat Immunol*. 2007;8(6):578-583.

66. Rowden G. The Langerhans cell. *Crit Rev Immunol*. 1981;3(2):95-180.

67. Stingl G, Elbe A, Paer E, Kilgus O, Strohal R, Schreiber S. The role of fetal epithelial tissues in the maturation/differentiation of bone marrow-derived precursors into dendritic epidermal T cells (DETC) of the mouse. *Curr Top Microbiol Immunol*. 1991;173:269-277.

68. Katz SI, Tamaki K, Sachs DH. Epidermal Langerhans cells are derived from cells originating in bone marrow. *Nature*. 1979;282(5736): 324-326.

69. Frelinger JG, Hood L, Hill S, Frelinger JA. Mouse epidermal Ia molecules have a bone marrow origin. *Nature*. 1979;282(5736):321-323.

70. Stingl G, Wolff-Schreiner EC, Pichler WJ, Gschnait F, Knapp W, Wolff K. Epidermal Langerhans cells bear Fc and C3 receptors. *Nature*. 1977;268(5617):245-246.

71. Rowden G, Lewis MG, Sullivan AK. Ia antigen expression on human epidermal Langerhans cells. *Nature*. 1977;268(5617):247-248.

72. Stingl G, Katz SI, Clement L, Green I, Shevach EM. Immunologic functions of Ia-bearing epidermal Langerhans cells. *J Immunol* . 1978;121(5):2005-2013.

73. Merad M, Manz MG, Karsunky H, et al. Langerhans cells renew in the skin throughout life under steady-state conditions. *Nat Immunol* . 2002;3(12):1135-1141.

74. Merad M, Hoffmann P, Ranheim E, et al. Depletion of host Langerhans cells before transplantation of donor alloreactive T cells prevents skin graft-versus-host disease. *Nat Med*. 2004;10(5):510-517.

75. Schuler G, Steinman RM. Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J Exp Med*. 1985;161(3):526-546.

76. Romani N, Lenz A, Glassel H, et al. Cultured human Langerhans cells resemble lymphoid dendritic cells in phenotype and function. *J Invest Dermatol* . 1989;93(5):600-609.

77. Romani N, Schuler G. Structural and functional relationships between epidermal Langerhans cells and dendritic cells. *Res Immunol*. 1989;140(9):895-898, discussion 918-926.

78. Inaba K, Schuler G, Witmer MD, Valinksy J, Atassi B, Steinman RM. Immunologic properties of purified epidermal Langerhans cells. Distinct requirements for stimulation of unprimed and sensitized T lymphocytes. *J Exp Med*. 1986;164(2):605-613.

79. Pure E, Inaba K, Crowley MT, et al. Antigen processing by epidermal Langerhans cells correlates with the level of biosynthesis of major histocompatibility complex class II molecules and expression of invariant chain. *J Exp Med*. 1990;172(5):1459-1469.

80. Larsen CP, Steinman RM, Witmer-Pack M, Hankins DF, Morris PJ, Austyn JM. Migration and maturation of Langerhans cells in skin transplants and explants. *J Exp Med*. 1990;172(5):1483-1493.

81. Macatonia SE, Knight SC, Edwards AJ, Griffiths S, Fryer P. Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein

isothiocyanate. Functional and morphological studies. *J Exp Med*. 1987;166(6):1654-1667.

82. Reis e Sousa C, Stahl PD, Austyn JM. Phagocytosis of antigens by Langerhans cells in vitro. *J Exp Med*. 1993;178(2):509-519.

83. Kissenpfennig A, Ait-Yahia S, Clair-Moninot V, et al. Disruption of the langerin/CD207 gene abolishes Birbeck granules without a marked loss of Langerhans cell function. *Mol Cell Biol*. 2005; 25(1):88-99.

84. Schuler G, Romani N, Linert J, Shevach EM, Stingl G. Subsets of epidermal Langerhans cells as defined by lectin binding profiles. *J Invest Dermatol*. 1983;81(5):397-402.

85. Pierre P, Turley SJ, Gatti E, et al. Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature*. 1997;388(6644): 787-792.

86. Steiner G, Wolff K, Pehamberger H, Stingl G. Epidermal cells as accessory cells in the generation of allo-reactive and hapten-specific cytotoxic T lymphocyte (CTL) responses. *J Immunol*. 1985;134(2): 736-741.

87. Schuler G, Romani N, Steinman RM. A comparison of murine epidermal Langerhans cells with spleen dendritic cells. *J Invest Dermatol*. 1985;85(1)(suppl):99s-106s.

88. Witmer-Pack MD, Olivier W, Valinsky J, Schuler G, Steinman RM. Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells. *J Exp Med*. 1987;166(5):1484-1498.

89. Ginhoux F, Tacke F, Angeli V, et al. Langerhans cells arise from monocytes in vivo. *Nat Immunol*. 2006;7(3):265-273.

90. Chorro L, Sarde A, Li M, et al. Langerhans cell (LC) proliferation mediates neonatal development, homeostasis, and inflammation-associated expansion of the epidermal LC network. *J Exp Med*. 2009;206(13): 3089-3100.

91. Schulz C, Gomez Perdiguero E, Chorro L, et al. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science*. 2012;336(6077):86-90.

92. Bogunovic M, Ginhoux F, Wagers A, et al. Identification of a radio-resistant and cycling dermal dendritic cell population in mice and men. *J Exp Med*. 2006;203(12):2627-2638.

93. Poulin LF, Henri S, de Bovis B, Devilard E, Kissenpfennig A, Malissen B. The dermis contains langerin+ dendritic cells that develop and function independently of epidermal

Langerhans cells. *J Exp Med*. 2007;204(13):3119-3131.

94. Bursch LS, Wang L, Igyarto B, et al. Identification of a novel population of Langerin+ dendritic cells. *J Exp Med*. 2007;204(13):3147-3156.

95. Ginhoux F, Collin MP, Bogunovic M, et al. Blood-derived dermal langerin+ dendritic cells survey the skin in the steady state. *J Exp Med*. 2007;204(13):3133-3146.

96. Bedoui S, Whitney PG, Waithman J, et al. Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. *Nat Immunol*. 2009;10(5):488-495.

97. Sung SS, Fu SM, Rose CE Jr, Gaskin F, Ju ST, Beaty SR. A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. *J Immunol*. 2006;176(4):2161-2172.

98. Varol C, Vallon-Eberhard A, Elinav E, et al. Intestinal lamina propria dendritic cell subsets have different origin and functions. *Immunity*. 2009;31(3):502-512.

99. Hacker C, Kirsch RD, Ju XS, et al. Transcriptional profiling identifies Id2 function in dendritic cell development. *Nat Immunol*. 2003;4(4):380-386.

100. Aliberti J, Schulz O, Pennington DJ, et al. Essential role for ICSBP in the in vivo development of murine CD8alpha + dendritic cells. *Blood*. 2003;101(1):305-310.

101. Svensson H, Johannisson A, Nikkila T, Alm GV, Cederblad B. The cell surface phenotype of human natural interferon-alpha producing cells as determined by flow cytometry. *Scand J Immunol*. 1996;44(2): 164-172.

102. Grouard G, Durand I, Filgueira L, Banchereau J, Liu YJ. Dendritic cells capable of stimulating T cells in germinal centres. *Nature*. 1996;384(6607):364-367.

103. Grouard G, Rissoan MC, Filgueira L, Durand I, Banchereau J, Liu YJ. The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J Exp Med*. 1997;185(6): 1101-1111.

104. Siegal FP, Kadowaki N, Shodell M, et al. The nature of the principal type 1 interferon-producing cells in human blood. *Science*. 1999;284(5421):1835-1837.

105. Liu YJ. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol*. 2005;23: 275-306.

106. Ferrero I, Held W, Wilson A, Tacchini-Cottier F, Radtke F, MacDonald HR. Mouse CD11c(+) B220(+) Gr1(+) plasmacytoid dendritic cells develop independently of the T-cell lineage. *Blood*. 2002;100(8): 2852-2857.

107. Cervantes-Barragan L, Lewis KL, Firner S, et al. Plasmacytoid dendritic cells control T-cell response to chronic viral infection. *Proc Natl Acad Sci U S A* . 2012;109(8):3012-3017.

108. Takagi H, Fukaya T, Eizumi K, et al. Plasmacytoid dendritic cells are crucial for the initiation of inflammation and T cell immunity in vivo. *Immunity*. 2011;35(6):958-971.

109. Karsunky H, Merad M, Cozzio A, Weissman IL, Manz MG. Flt3 ligand regulates dendritic cell development from Flt3+ lymphoid and myeloid-committed progenitors to Flt3+ dendritic cells in vivo. *J Exp Med*. 2003;198(2):305-313.

110. Witmer-Pack MD, Hughes DA, Schuler G, et al. Identification of macrophages and dendritic cells in the osteopetrotic (op/op) mouse. *J Cell Sci* . 1993;104(pt 4):1021-1029.

111. McKenna HJ, Stocking KL, Miller RE, et al. Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood*. 2000;95(11):3489-3497.

112. Maraskovsky E, Brasel K, Teepe M, et al. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J Exp Med*. 1996;184(5):1953-1962.

113. Caux C, Dezutter-Dambuyant C, Schmitt D, Banchereau J. GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. *Nature*. 1992;360(6401):258-261.

114. Inaba K, Inaba M, Romani N, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med*. 1992;176(6):1693-1702.

115. Vremec D, Lieschke GJ, Dunn AR, Robb L, Metcalf D, Shortman K. The influence of granulocyte/macrophage colony-stimulating factor on dendritic cell levels in mouse lymphoid organs. *Eur J Immunol* . 1997;27(1):40-44.

116. Naik SH, Proietto AI, Wilson NS, et al. Cutting edge: generation of splenic CD8+ and CD8- dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *J Immunol* . 2005;174(11): 6592-6597.

117. Laouar Y, Welte T, Fu XY, Flavell RA. STAT3 is required for Flt3Ldependent dendritic cell differentiation. *Immunity*. 2003;19(6): 903-912.

118. Sathaliyawala T, O'Gorman WE, Greter M, et al. Mammalian target of rapamycin controls dendritic cell development downstream of Flt3 ligand signaling. *Immunity*. 2010;33(4):597-606.

119. Rathinam C, Geffers R, Yucel R, et al. The transcriptional repressor Gfi1 controls STAT3-dependent dendritic cell development and function. *Immunity*. 2005;22(6):717-728.

120. Onai N, Obata-Onai A, Tussiwand R, Lanzavecchia A, Manz MG. Activation of the Flt3 signal transduction cascade rescues and enhances type I interferon-producing and dendritic cell development. *J Exp Med*. 2006;203(1):227-238.

121. Tamura T, Tailor P, Yamaoka K, et al. IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity. *J Immunol*. 2005;174(5):2573-2581.

122. Schiavoni G, Mattei F, Borghi P, et al. ICSBP is critically involved in the normal development and trafficking of Langerhans cells and dermal dendritic cells. *Blood*. 2004;103(6):2221-2228.

123. Ichikawa E, Hida S, Omatsu Y, et al. Defective development of splenic and epidermal CD4+ dendritic cells in mice deficient for IFN regulatory factor-2. *Proc Natl Acad Sci U S A*. 2004;101(11):3909-3914.

124. Wu L, D'Amico A, Winkel KD, Suter M, Lo D, Shortman K. RelB is essential for the development of myeloid-related CD8alpha-dendritic cells but not of lymphoid-related CD8alpha+ dendritic cells. *Immunity*. 1998;9(6):839-847.

125. Kobayashi T, Walsh PT, Walsh MC, et al. TRAF6 is a critical factor for dendritic cell maturation and development. *Immunity*. 2003;19(3): 353-363.

126. Cisse B, Caton ML, Lehner M, et al. Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development. *Cell*. 2008;135(1):37-48.

127. Ghosh HS, Cisse B, Bunin A, Lewis KL, Reizis B. Continuous expression of the transcription factor e2-2 maintains the cell fate of mature plasmacytoid dendritic cells. *Immunity*. 2010;33(6):905-916.

128. Edelson BT, Bradstreet TR, Wumesh KC, et al. Batf3-dependent CD11b(low/-) peripheral dendritic cells are GM-CSF-independent and are not required for Th cell priming after subcutaneous immunization. *PLoS One*. 2011;6(10):e25660.

129. Caton ML, Smith-Raska MR, Reizis B. Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. *J Exp Med*. 2007;204(7):1653-1664.

130. Lewis KL, Caton ML, Bogunovic M, et al. Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. *Immunity*. 2011;35(5):780-791.

131. Collin M, Bigley V, Haniffa M, Hambleton S. Human dendritic cell deficiency: the missing ID? *Nat Rev Immunol* . 2011;11(9):575-583.

132. Robbins SH, Walzer T, Dembele D, et al. Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. *Genome Biol*. 2008;9(1):R17.

133. Crozat K, Guiton R, Contreras V, et al. The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells. *J Exp Med*. 2010;207(6): 1283-1292.

134. Poulin LF, Salio M, Griessinger E, et al. Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. *J Exp Med*. 2010;207(6):1261-1271.

135. Lauterbach H, Bathke B, Gilles S, et al. Mouse CD8alpha+ DCs and human BDCA3+ DCs are major producers of IFN-lambda in response to poly IC. *J Exp Med*. 2010;207(12):2703-2717.

136. Kamath AT, Henri S, Battye F, Tough DF, Shortman K. Developmental kinetics and lifespan of dendritic cells in mouse lymphoid organs. *Blood*. 2002;100(5):1734-1741.

137. Kamath AT, Pooley J, O'Keeffe MA, et al. The development, maturation, and turnover rate of mouse spleen dendritic cell populations. *J Immunol* . 2000;165(12):6762-6770.

138. Kazenwadel J, Secker GA, Liu YJ, et al. Loss-of-function germline GATA2 mutations in patients with MDS/AML or MonoMAC syndrome and primary lymphedema reveal a key role for GATA2 in the lymphatic vasculature. *Blood*. 2012;119(5):1283-1291.

139. Hahn CN, Chong CE, Carmichael CL, et al. Heritable GATA2 mutations associated with familial myelodysplastic syndrome and acute myeloid leukemia. *Nat Genet*. 2011;43(10):1012-1017.

140. Hambleton S, Salem S, Bustamante J, et al. IRF8 mutations and human dendritic-cell immunodeficiency. *N Engl J Med* . 2011;365(2):127-138.

141. Bigley V, Haniffa M, Doulatov S, et al. The human syndrome of dendritic cell, monocyte, B and NK lymphoid deficiency. *J Exp Med*. 2011;208(2):227-234.

142. Kabashima K, Banks TA, Ansel KM, Lu TT, Ware CF, Cyster JG. Intrinsic lymphotoxin-beta receptor requirement for homeostasis of lymphoid tissue dendritic cells. *Immunity*. 2005;22(4):439-450.

143. Kingston D, Schmid MA, Onai N, Obata-Onai A, Baumjohann D, Manz MG. The concerted action of GM-CSF and Flt3-ligand on in vivo dendritic cell homeostasis. *Blood*.

2009;114(4):835-843.

144. Birnberg T, Bar-On L, Sapoznikov A, et al. Lack of conventional dendritic cells is compatible with normal development and T cell homeostasis, but causes myeloid proliferative syndrome. *Immunity*. 2008;29(6):986-997.

145. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol*. 2007;8(2):191-197.

146. Darrasse-Jeze G, Deroubaix S, Mouquet H, et al. Feedback control of regulatory T cell homeostasis by dendritic cells in vivo. *J Exp Med*. 2009;206(9):1853-1862.

147. Eidenschenk C, Crozat K, Krebs P, et al. Flt3 permits survival during infection by rendering dendritic cells competent to activate NK cells. *Proc Natl Acad Sci U S A*. 2010;107(21):9759-9764.

148. O'Keeffe M, Brodnicki TC, Fancke B, et al. Fms-like tyrosine kinase 3 ligand administration overcomes a genetically determined dendritic cell deficiency in NOD mice and protects against diabetes development. *Int Immunol*. 2005;17(3):307-314.

149. Steinman RM, Nussenzweig MC. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc Natl Acad Sci U S A*. 2002;99(1):351-358.

150. Matsuno K, Ezaki T, Kudo S, Uehara Y. A life stage of particle-laden rat dendritic cells in vivo: their terminal division, active phagocytosis, and translocation from the liver to the draining lymph. *J Exp Med*. 1996;183(4):1865-1878.

151. Matsuno K, Ezaki T. Dendritic cell dynamics in the liver and hepatic lymph. *Int Rev Cytol*. 2000;197:83-136.

152. Mayrhofer G, Pugh CW, Barclay AN. The distribution, ontogeny and origin in the rat of Ia-positive cells with dendritic morphology and of Ia antigen in epithelia, with special reference to the intestine. *Eur J Immunol*. 1983;13(2):112-122.

153. Randolph GJ, Angeli V, Swartz MA. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat Rev Immunol*. 2005;5(8):617-628.

154. Gunn MD, Kyuwa S, Tam C, et al. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J Exp Med*. 1999;189(3):451-460.

155. Forster R, Schubel A, Breitfeld D, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell*. 1999;99(1):23-33.

156. Marsland BJ, Battig P, Bauer M, et al. CCL19 and CCL21 induce a potent proinflammatory differentiation program in licensed dendritic cells. *Immunity*. 2005;22(4):493-505.

157. Greaves DR, Wang W, Dairaghi DJ, et al. CCR6, a CC chemokine receptor that interacts with macrophage inflammatory protein 3alpha and is highly expressed in human dendritic cells. *J Exp Med*. 1997;186(6): 837-844.

158. Dieu MC, Vanbervliet B, Vicari A, et al. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med*. 1998;188(2):373-386.

159. Iwasaki A, Kelsall BL. Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3alpha, MIP-3beta, and secondary lymphoid organ chemokine. *J Exp Med*. 2000;191(8):1381-1394.

160. Schaerli P, Willimann K, Ebert LM, Walz A, Moser B. Cutaneous CXCL14 targets blood precursors to epidermal niches for Langerhans cell differentiation. *Immunity*. 2005;23(3):331-342.

161. de Baey A, Lanzavecchia A. The role of aquaporins in dendritic cell macropinocytosis. *J Exp Med*. 2000;191(4):743-748.

162. Muta T, Kurosaki T, Misulovin Z, Sanchez M, Nussenzweig MC, Ravetch JV. A 13-amino-acid motif in the cytoplasmic domain of Fc gamma RIIB modulates B-cell receptor signalling. *Nature*. 1994;369(6478):340.

163. Kalergis AM, Ravetch JV. Inducing tumor immunity through the selective engagement of activating Fcgamma receptors on dendritic cells. *J Exp Med*. 2002;195(12):1653-1659.

164. Dhodapkar KM, Kaufman JL, Ehlers M, et al. Selective blockade of inhibitory Fcgamma receptor enables human dendritic cell maturation with IL-12p70 production and immunity to antibody-coated tumor cells. *Proc Natl Acad Sci U S A* . 2005;102(8):2910-2915.

165. Samuelsson A, Towers TL, Ravetch JV. Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor. *Science*. 2001;291(5503):484-486.

166. Gitlin L, Barchet W, Gilfillan S, et al. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proc Natl Acad Sci U S A*. 2006;103(22): 8459-8464.

167. Kato H, Sato S, Yoneyama M, et al. Cell type-specific involvement of RIG-I in antiviral response. *Immunity*. 2005;23(1):19-28.

168. Zhang Z, Yuan B, Bao M, Lu N, Kim T, Liu YJ. The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. *Nat Immunol*. 2011;12(10):959-965.

169. Zhang SY, Jouanguy E, Ugolini S, et al. TLR3 deficiency in patients with herpes simplex encephalitis. *Science*. 2007;317(5844):1522-1527.

170. Kadowaki N, Ho S, Antonenko S, et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med*. 2001;194(6):863-869.

171. Sporri R, Reis e Sousa C. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. *Nat Immunol*. 2005;6(2): 163-170.

172. Kupz A, Guarda G, Gebhardt T, et al. NLRC4 inflammasomes in dendritic cells regulate noncognate effector function by memory CD8 T cells. *Nat Immunol*. 2012;13(2):162-169.

173. Delamarre L, Pack M, Chang H, Mellman I, Trombetta ES. Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science*. 2005;307(5715):1630-1634.

174. Trombetta ES, Ebersold M, Garrett W, Pypaert M, Mellman I. Activation of lysosomal function during dendritic cell maturation. *Science*. 2003;299(5611):1400-1403.

175. Savina A, Jancic C, Hugues S, et al. NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. *Cell*. 2006;126(1):205-218.

176. Mantegazza AR, Savina A, Vermeulen M, et al. NADPH oxidase controls phagosomal pH and antigen cross-presentation in human dendritic cells. *Blood*. 2008;112(12):4712-4722.

177. Ito T, Wang YH, Liu YJ. Plasmacytoid dendritic cell precursors/type I interferon-producing cells sense viral infection by Toll-like receptor (TLR) 7 and TLR9. *Springer Semin Immunopathol*. 2005;26(3):221-229.

178. Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. *Science*. 2001;294(5546):1540-1543.

179. Cao W, Rosen DB, Ito T, et al. Plasmacytoid dendritic cell-specific receptor ILT7-Fc epsilonRI gamma inhibits Toll-like receptor-induced interferon production. *J Exp Med*.

2006;203(6):1399-1405.

180. Sjolin H, Robbins SH, Bessou G, et al. DAP12 signaling regulates plasmacytoid dendritic cell homeostasis and down-modulates their function during viral infection. *J Immunol*. 2006;177(5):2908-2916.

181. Munz C, Steinman RM, Fujii S. Dendritic cell maturation by innate lymphocytes: coordinated stimulation of innate and adaptive immunity. *J Exp Med*. 2005;202(2):203-207.

182. Walzer T, Dalod M, Robbins SH, Zitvogel L, Vivier E. Natural-killer cells and dendritic cells: “l'union fait la force.” *Blood*. 2005;106(7): 2252-2258.

183. Zhou D, Mattner J, Cantu C III, et al. Lysosomal glycosphingolipid recognition by NKT cells. *Science*. 2004;306(5702):1786-1789.

184. Mattner J, Debord KL, Ismail N, et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature*. 2005;434(7032):525-529.

185. Kinjo Y, Wu D, Kim G, et al. Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature*. 2005;434(7032):520-525.

186. Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med*. 2003;198(2):267-279.

187. Hermans IF, Silk JD, Gileadi U, et al. NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. *J Immunol*. 2003;171(10):5140-5147.

188. Longhi MP, Trumpfheller C, Idoyaga J, et al. Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. *J Exp Med*. 2009;206(7):1589-1602.

189. Bousso P, Robey E. Dynamics of CD8+ T cell priming by dendritic cells in intact lymph nodes. *Nat Immunol*. 2003;4(6):579-585.

190. Miller MJ, Safrina O, Parker I, Cahalan MD. Imaging the single cell dynamics of CD4+ T cell activation by dendritic cells in lymph nodes. *J Exp Med*. 2004;200(7):847-856.

191. Mempel TR, Henrickson SE, Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature*. 2004;427(6970):154-159.

192. Hugues S, Fetler L, Bonifaz L, Helft J, Amblard F, Amigorena S. Distinct T cell dynamics in lymph nodes during the induction of tolerance and immunity. *Nat Immunol*. 2004;5(12):1235-1242.

193. van Kooten C, Banchereau J. Functions of CD40 on B cells, dendritic cells and other cells. *Curr Opin Immunol*. 1997;9(3):330-337.

194. Anderson DM, Maraskovsky E, Billingsley WL, et al. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendriticcell function. *Nature*. 1997;390(6656):175-179.

195. Wong BR, Josien R, Lee SY, et al. TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J Exp Med*. 1997;186(12):2075-2080.

196. Soumelis V, Reche PA, Kanzler H, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol*. 2002;3(7):673-680.

197. Al-Shami A, Spolski R, Kelly J, Keane-Myers A, Leonard WJ. A role for TSLP in the development of inflammation in an asthma model. *J Exp Med*. 2005;202(6):829-839.

198. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature*. 1998;392(6671):86-89.

199. Jung S, Unutmaz D, Wong P, et al. In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. *Immunity*. 2002;17(2):211-220.

200. Schreibelt G, Klinkenberg LJ, Cruz LJ, et al. The C-type lectin receptor CLEC9A mediates antigen uptake and (cross-)presentation by human blood BDCA3+ myeloid dendritic cells. *Blood*. 2012;119(10): 2284-2292.

201. Iyoda T, Shimoyama S, Liu K, et al. The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. *J Exp Med*. 2002;195(10):1289-1302.

202. Liu K, Iyoda T, Saternus M, Kimura Y, Inaba K, Steinman RM. Immune tolerance after delivery of dying cells to dendritic cells in situ. *J Exp Med*. 2002;196(8):1091-1097.

203. Anderson MS, Venanzi ES, Klein L, et al. Projection of an immunological self shadow within the thymus by the aire protein. *Science*. 2002;298(5597):1395-1401.

204. Brocker T, Riedinger M, Karjalainen K. Targeted expression of major histocompatibility complex (MHC) class II molecules demonstrates that dendritic cells can induce negative but not positive selection of thymocytes in vivo. *J Exp Med*. 1997;185(3):541-550.

205. Proietto AI, van Dommelen S, Zhou P, et al. Dendritic cells in the thymus contribute to T-regulatory cell induction. *Proc Natl Acad Sci U S A* . 2008;105(50):19869-19874.

206. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. *Annu Rev Immunol*. 2003;21:685-711.

207. Kurts C, Heath WR, Carbone FR, Allison J, Miller JF, Kosaka H. Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J Exp Med*. 1996;184(3):923-930.

208. Kurts C, Kosaka H, Carbone FR, Miller JF, Heath WR. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. *J Exp Med*. 1997;186(2):239-245.

209. Hawiger D, Inaba K, Dorsett Y, et al. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med*. 2001;194(6):769-779.

210. Brimnes MK, Bonifaz L, Steinman RM, Moran TM. Influenza virusinduced dendritic cell maturation is associated with the induction of strong T cell immunity to a coadministered, normally nonimmunogenic protein. *J Exp Med*. 2003;198(1):133-144.

211. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol*. 2005;6(12):1219-1227.

212. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med*. 2007;204(8):1757-1764.

213. Sun CM, Hall JA, Blank RB, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med*. 2007;204(8):1775-1785.

214. van der Aar AM, Sibiryak DS, Bakdash G, et al. Vitamin D3 targets epidermal and dermal dendritic cells for induction of distinct regulatory T cells. *J Allergy Clin Immunol* . 2011;127(6):1532-1540 e7.

215. Sigmundsdottir H, Pan J, Debes GF, et al. DCs metabolize sunlightinduced vitamin D3 to “program” T cell attraction to the epidermal chemokine CCL27. *Nat Immunol*. 2007;8(3):285-293.

216. Yamazaki S, Dudziak D, Heidkamp GF, et al. CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. *J Immunol*. 2008;181(10):6923-

6933.

217. Ohnmacht C, Pullner A, King SB, et al. Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. *J Exp Med*. 2009;206(3):549-559.

218. Manicassamy S, Reizis B, Ravindran R, et al. Activation of beta-catenin in dendritic cells regulates immunity versus tolerance in the intestine. *Science*. 2010;329(5993):849-853.

219. Ochando JC, Homma C, Yang Y, et al. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat Immunol* . 2006;7(6):652-662.

220. Inaba K, Turley S, Yamaide F, et al. Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *J Exp Med*. 1998;188(11):2163-2173.

221. Shlomchik WD, Couzens MS, Tang CB, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science*. 1999;285(5426):412-415.

222. Lowes MA, Chamian F, Abello MV, et al. Increase in TNF-alpha and inducible nitric oxide synthase-expressing dendritic cells in psoriasis and reduction with efalizumab (anti-CD11a). *Proc Natl Acad Sci U S A* . 2005;102(52):19057-19062.

223. Zaba LC, Fuentes-Duculan J, Eungdamrong NJ, et al. Psoriasis is characterized by accumulation of immunostimulatory and Th1/Th17 cell-polarizing myeloid dendritic cells. *J Invest Dermatol*. 2009;129(1):79-88.

224. Poligone B, Weaver DJ Jr, Sen P, Baldwin AS Jr, Tisch R. Elevated NFkappaB activation in nonobese diabetic mouse dendritic cells results in enhanced APC function. *J Immunol*. 2002;168(1):188-196.

225. Laguette N, Sobhian B, Casartelli N, et al. SAMHD1 is the dendritic-and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. *Nature*. 2011;474(7353):654-657.

226. Frankel SS, Wenig BM, Burke AP, et al. Replication of HIV-1 in dendritic cell-derived syncytia at the mucosal surface of the adenoid. *Science*. 1996;272(5258):115-117.

227. Kwon DS, Gregorio G, Bitton N, Hendrickson WA, Littman DR. DC-SIGN-mediated internalization of HIV is required for trans-enhancement of T cell infection. *Immunity*. 2002;16(1):135-144.

228. Alvarez CP, Lasala F, Carrillo J, Muniz O, Corbi AL, Delgado R. C-type lectins DC-

SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in trans. *J Virol*. 2002;76(13):6841-6844.

229. Yang ZY, Huang Y, Ganesh L, et al. pH-dependent entry of severe acute respiratory syndrome coronavirus is mediated by the spike glycoprotein and enhanced by dendritic cell transfer through DCSIGN. *J Virol*. 2004;78(11):5642-5650.

230. Lozach PY, Amara A, Bartosch B, et al. C-type lectins L-SIGN and DCSIGN capture and transmit infectious hepatitis C virus pseudotype particles. *J Biol Chem*. 2004;279(31):32035-32045.

231. Boonnak K, Slike BM, Burgess TH, et al. Role of dendritic cells in antibody-dependent enhancement of dengue virus infection. *J Virol*. 2008;82(8):3939-3951.

232. Tassaneetrithep B, Burgess TH, Granelli-Piperno A, et al. DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J Exp Med*. 2003;197(7):823-829.

233. Kortylewski M, Kujawski M, Wang T, et al. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nat Med*. 2005;11(12):1314-1321.

234. Ghiringhelli F, Puig PE, Roux S, et al. Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation. *J Exp Med*. 2005;202(7):919-929.

235. Aspord C, Pedroza-Gonzalez A, Gallegos M, et al. Breast cancer instructs dendritic cells to prime interleukin 13-secreting CD4+ T cells that facilitate tumor development. *J Exp Med*. 2007;204(5):1037-1047.

236. Diao J, Zhao J, Winter E, Cattral MS. Tumors suppress in situ proliferation of cytotoxic T cells by promoting differentiation of Gr-1(+) conventional dendritic cells through IL-6. *J Immunol*. 2011;186(9):5058-5067.

237. Schuler-Thurner B, Schultz ES, Berger TG, et al. Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocytederived dendritic cells. *J Exp Med*. 2002;195(10):1279-1288.

238. Soares H, Waechter H, Glaichenhaus N, et al. A subset of dendritic cells induces CD4+ T cells to produce IFN-gamma by an IL-12-independent but CD70-dependent mechanism in vivo. *J Exp Med*. 2007;204(5): 1095-1106.

239. Trumpfheller C, Finke JS, Lopez CB, et al. Intensified and protective CD4+ T cell immunity in mice with anti-dendritic cell HIV gag fusion antibody vaccine. *J Exp Med*.

2006;203(3):607-617.

240. Jiang W, Swiggard WJ, Heufler C, et al. The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature*. 1995;375(6527):151-155.

241. Boscardin SB, Hafalla JC, Masilamani RF, et al. Antigen targeting to dendritic cells elicits long-lived T cell help for antibody responses. *J Exp Med*. 2006;203(3):599-606.

242. Trumpfheller C, Longhi MP, Caskey M, et al. Dendritic cell-targeted protein vaccines: a novel approach to induce T cell immunity. *J Intern Med*. 2012;271(2):183-192.

243. Bozzacco L, Yu H, Zebroski HA, et al. Mass spectrometry analysis and quantitation of peptides presented on the MHC II molecules of mouse spleen dendritic cells. *J Proteome Res*. 2011;10(11):5016-5030.

244. Trumpfheller C, Caskey M, Nchinda G, et al. The microbial mimic poly IC induces durable and protective CD4+ T cell immunity together with a dendritic cell targeted vaccine. *Proc Natl Acad Sci U S A* . 2008;105(7):2574-2579.

245. Idoyaga J, Lubkin A, Fiorese C, et al. Comparable T helper 1 (Th1) and CD8 T-cell immunity by targeting HIV gag p24 to CD8 dendritic cells within antibodies to Langerin, DEC205, and Clec9A. *Proc Natl Acad Sci U S A* . 2011;108(6):2384-2389.

246. Zhong MX, Kuziel WA, Pamer EG, Serbina NV. Chemokine receptor 5 is dispensable for innate and adaptive immune responses to Listeria monocytogenes infection. *Infect Immun*. 2004;72(2): 1057-1064.

247. Cook DN, Prosser DM, Forster R, et al. CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. *Immunity*. 2000;12(5):495-503.

248. Ohl L, Mohaupt M, Czeloth N, et al. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity*. 2004;21(2):279-288.

249. Qu C, Edwards EW, Tacke F, et al. Role of CCR8 and other chemokine pathways in the migration of monocyte-derived dendritic cells to lymph nodes. *J Exp Med*. 2004;200(10):1231-1241.

250. Asselin-Paturel C, Brizard G, Chemin K, et al. Type I interferon dependence of plasmacytoid dendritic cell activation and migration. *J Exp Med*. 2005;201(7):1157-1167.

251. Yoneyama H, Matsuno K, Zhang Y, et al. Evidence for recruitment of plasmacytoid dendritic cell precursors to inflamed lymph nodes through high endothelial venules. *Int Immunol*. 2004;16(7):915-928.

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Chapter 17
Natural Killer Cells

Wayne M. Yokoyama

INTRODUCTION

Natural killer (NK) cells were initially described because they spontaneously kill certain tumor targets.[1,2,3,4] However, they are now recognized to play important roles in early innate immune responses, especially to viral infections. They interact with other innate immune components and modulate the subsequent adaptive immune response. These effects are due to NK cell responses to proinflammatory cytokines or susceptible targets, which stimulate NK cells to secrete other cytokines and/or kill targets. In this chapter, we will consider all of these issues in detail by describing how they differ from other lymphocytes, their functions, unique target recognition strategies, tolerance, development, and role in immune responses and human diseases. There will be an emphasis on their target recognition receptors because their discovery made it possible to understand NK cell biology more precisely.

GENERAL DESCRIPTION

Developmental studies have provided strong evidence that NK cells belong to the lymphocyte lineage (discussed in detail in the following). Morphologically, NK cells are typically large lymphocytes containing azurophilic granules.[5] However, the large granular lymphocyte morphology is not invariably associated with NK cells because small, agranular lymphocytes may display natural killing,[6] activated cytotoxic T-lymphocytes (CTLs) can display this morphology,[7] and human large granular lymphocyte leukemias contain NK- and T-cell variants.[8] Among lymphocytes, NK cells more closely resemble T cells than B cells. Thus, it is useful to compare and contrast these two lymphocyte populations as well as consider another enigmatic cell termed the “lymphokine-activated killer” (LAK) cell.

Natural Killer Cells versus T Cells

NK cells are most often confused with T cells because they may have similar morphologies, express several cell surface molecules in common,[9,10] and share functional capabilities. While this confusion was frequent before the molecular description of the T-cell receptor (TCR)/cluster of differentiation (CD)3 complex, their similarities remain a potential source of uncertainty. However, mature NK cells are clearly not T cells by several criteria.[11] Conventional NK cells do not require a thymus for development and are normal in athymic nude mice (though this is not the case for the newly described “thymic” NK cell subset, discussed subsequently). NK cells do not express the TCR on the cell surface, do not

produce mature transcripts for TCR chains, and do not rearrange TCR genes.[12,13] Mice with the *scid* mutation or deficiencies in *Rag1* or *Rag2* lack TCR gene rearrangements and mature T cells but possess NK cells with apparently normal function.[14,15,16,17] Several CD3 components may be found in the cytoplasm of NK cells, particularly immature NK cells, but they are not displayed on the cell surface[18] with the exception of CD3ζ. But CD3ζ is expressed in association with FcγRIII (CD16) and other NK cell activation receptors instead of the TCR/CD3 complex.[19,20] Whereas mice lacking CD3ζ lack most T cells, NK cell number and function are minimally affected.[21] On the other hand, NK cells are completely absent in mice with only partial defects in T-cell subsets, such as in mice lacking components of the IL-15R (see following discussion). NK cells do not require the presence of major histocompatibility complex (MHC) class I (MHC-I) molecules on their targets for lysis in an important functional distinction with CD8+ MHC-I-restricted T cells. Instead, NK cells kill more efficiently when their targets lack MHC-I expression. Thus, NK cells can be clearly distinguished from T cells, even from so-called CD3+ NKT cells that express NK cell markers (see following discussion).

Natural Killer Cells, Lymphokine-Activated Killer Cells, and Interleukin-15

Another area of overlap between NK and T cells concerns cytokine responses. When mouse splenocytes or human peripheral mononuclear cells are exposed to high concentrations of interleukin (IL)-2 (800 to 1000 U/mL), robust lymphocyte proliferation ensues (ie, LAK cells are generated).[22,23,24,25] Although most are CD3- NK cells, TCR/CD3+ T cells are also produced. To distinguish NK cells within this population, they are sometimes called "CD3$^-$ LAK" cells or "IL-2-activated NK cells."

In a related phenomenon, NK cells are activated when mice are injected with polyinosinic-polycytidylic acid (poly-lic) or other agents that trigger through Toll-like receptors (TLRs), often on plasmacytoid dendritic cells (DCs).[26] NK cells can also be activated in vitro with interferon (IFN)α/β, IFNγ, or low concentrations of IL-2 that are insufficient to induce proliferation.[3,4,27]

NK cells activated in these various ways, with or without proliferation, display enhanced killing of typical NKsensitive targets. They also kill a broader panel of targets, including those that are generally resistant to freshly isolated NK cells, such as the murine P815 mastocytoma cells and

freshly explanted tumors. Many agents that enhance killing by NK cells may also activate T cells, such that even T-cell clones may display promiscuous killing of targets that is no longer MHC-restricted.[28] This phenomenon was a frequent source of confusion between NK cells and T cells during the initial characterization of both cell types.

Why cytokine activation leads to enhanced killing is incompletely understood. Interestingly, mouse NK cells constitutively express messenger ribonucleic acid (mRNA) for cytotoxicity components, perforin, and granzymes, but no protein.[29] Cytokine activation enhances expression of mRNA for perforin and granzymes,[30] and translation into expressed proteins that contributes to more lytic capacity,[29,31] but this effect may not explain the capacity of

LAK cells to kill a broader panel of targets. Activated NK cells express additional receptors that may deliver stimulatory signals,[32,33] but their contribution to the LAK phenomenon remains unclear. In mice, cytokine activation of NK cells results in expression of an alternatively spliced form of a receptor termed NKG2D (see subsequent discussion), which may play a role,[34,35] and IL-15 contributes to LAK-like activity of CTLs, although how much this applies to conventional NK cells is not understood.[36] Enhanced killing may also be due to effects on adhesion molecules.[37,38] Nonetheless, it remains unclear how each of these factors contribute to the LAK phenomenon of enhanced and broader killing capacity as compared to resting NK cells.

It seems unlikely that high concentrations of IL-2 can be achieved, even locally, to stimulate NK cells in vivo. Furthermore, NK cells tend to be early responders in immune responses whereas the prime reservoir of large amounts of IL-2 is the activated T cell that produces it somewhat later. Although naïve T cells can make IL-2 soon after stimulation[39,40] and DCs can produce IL-2 to enhance NK lytic activity,[41] whether the resultant IL-2 concentrations are sufficient to generate LAK cells in vivo is unclear.

Interestingly, NK cells are apparently normal in mice with a targeted mutation in the IL-2 gene or the IL-2Rα chain,[42,43] indicating that IL-2 itself is not required for normal NK cell development. Paradoxically, NK cells are deficient in mice with a mutation in either IL-2Rβ or IL-2Rγ.[44,45,46] The discrepancy in NK cell dependence on IL-2 versus IL-2 receptor (IL-2R), as well as the LAK cell phenomenon, may be best understood by comparing the components of the IL-2 and IL-15 receptors.

In brief, the high affinity IL-2R is a heterotrimeric receptor complex comprised of α (p55), β (p75), and γ (p64) chains.[47] Though individual components may bind IL-2 with low affinity, only the intermediate-affinity βγ receptor (K_d ~1 nM) and the high-affinity αβγ receptor (K_d ~10 pM) are capable of signaling. Resting NK cells constitutively express IL-2Rβγ[47,48] and upon activation, may induce IL-2Rα and further upregulate IL-2Rγ chain expression.[47] In contrast, resting T cells generally do not express any functional IL-2 receptors, and most naïve T cells do not respond to high concentrations of IL-2.[49] The IL-2Rγ chain is also termed the common γ subunit (γc) because it is a required component of the multimeric receptor complexes for other cytokines, including IL-15,[50] that is particularly relevant to NK cells. IL-15 does not bind to IL-2Rα but instead utilizes a unique IL-15Rα chain to form a high-affinity complex with IL-2Rβγ.[51,52] The IL-15Rα chain does not directly signal. Its distribution is widespread on numerous cell and tissue lineages including NK cells.

IL-15 has a number of effects on NK cell biology. It is required for NK cell development; mice lacking IL-15 or any component of the trimeric IL-15R complex lack NK cells.[44,45,46,53,54] Not surprisingly, mice deficient in other components of the IL-15R complex and its signaling pathway (IL-2Rβ, Jak3, and STAT5α/β) exhibit similar defects in NK cell development.[44,55,56,57] Depending on its relative concentration, IL-15 has an antiapoptotic or proproliferative effect.[58,59] When NK cells are transferred to NK cell-deficient mice, they undergo “homeostatic” proliferation,[60,61] akin to T-cell homeostatic proliferation.[62] Like

memory CD8+ T-cell homeostasis, NKcell homeostasis is IL-15-dependent,[60,61] to a more or less degree.[63] Finally, LAK cells can be generated with IL-15.[64] These studies strongly suggest that LAK cells are generated because high-dose IL-2 acts through the IL-2Rβγ that is normally expressed with IL-15Rα as components of the constitutively expressed trimeric IL-15R complex on resting NK cells. Thus, the LAK cell phenomenon is related to the role of IL-15 and its receptor in NK cell biology.

Interestingly, IL-15 is expressed at very low levels and is difficult to detect in vivo.[65] The IL-15Rα chain can present IL-15 in *trans* to NK cells that can respond through IL-2/15Rβγ alone.[66] For example, IL-15Rα-deficient NK cells develop in bone marrow (BM) chimeric mice in which IL-15Rα-deficient BM was used to reconstitute IL-15Rα-sufficient animals,[67] indicating that IL-15Rα on another cell can allow IL-15Rα-deficient NK-cell development. In certain inflammatory situations in vivo, DC presentation of IL-15 in *trans* can enhance NK cell responses (also known as priming).[31,68] In DCs expressing both IL-15 and IL-15Ra, the IL-15Ra chain provides a chaperone function to stabilize receptor-cytokine complexes on the cell surface.[69] Taken together, *trans* presentation of IL-15 may be physiologically important to NK cell function.

SELECTIVE NATURAL KILLER CELL SURFACE MARKERS

The constitutive expression on NK cells of IL-15R complex with IL-2Rβ has practical usefulness because anti-IL-2Rβ (CD122) is sometimes used to identify naïve CD3- NK cells or deplete them in mice,[70] but anti-CD122 is less useful during an ongoing immune response and CD122 is expressed on regulatory T cells. Anti-IL-15Rα antibodies have not been widely used. Other markers have proven to be more useful for analysis of NK cells.

In the mouse, the NK1.1 molecule is an especially important marker on NK cells in C57BL strains.[11] NK1.1 is an activation receptor encoded by *Nkrp1c* (*Klrb1c*),[71] a member of the *Nkrp1* gene family (see following discussion). In FACS sorting experiments, the NK1.1+ fraction contained all of the natural killing activity in the spleen.[72] In vivo administration of the anti-NK1.1 mAb PK136[73] completely abrogated natural killing but did not affect adaptive immune responses[74]

(mAb PK136 is available from the American Type Culture Collection [ATCC], Manassas, VA [HB-191] and is an IgG2a isotype [ATCC, and data not shown], not IgG2b as originally described[73]). While mAb PK136 is very efficient at NK-cell depletion and is widely used for this purpose, unfortunately it recognizes an epitope on NK1.1 that is confined to C57BL/6, C57BL/10, and a few other strains.[73] Moreover, in Swiss, NIH and SJL/J mice, mAb PK136 recognizes another NKRP1 family member, NKRP1B.[75,76] However, there are now available NK1.1+ congenic strains, such as BALB.B6- *Cmv1*^{r} (catalogued as C.B6-*Klra8*$^{Cmv1\text{-}r}$ /UwaJ, stock number 002936 at The Jackson Laboratory, Bar Harbor, ME) in which the C57BL/6 allele of NK1.1 has been genetically bred onto the BALB/c background that otherwise lacks the NK1.1 epitope.[77] Similarly, the NK1.1 allele has been introgressed onto the nonobese diabetic (NOD) background.[78]

A subpopulation of T cells expresses NK1.1, described in detail in another chapter 18. These "natural killer T (NKT) cells" express the TCR/CD3 complex and typically are restricted by the nonclassical MHC-I molecule, CD1, which presents glycolipid antigens to NKT cells. NKT cells respond early during the course of an immune response and may potently activate conventional NK cells.[79] Nonetheless, NKT cells can be distinguished from conventional NK cells by expression of the CD3 complex (ie, conventional NK cells are NK1.1+ CD3-).

The NKG2D (Klrk1) activation receptor is expressed on all NK cells in human and all strains of mice examined.[80] In humans, NKG2D is also expressed on all γδTCR+ and CD8+ T cells, whereas in mice, NKG2D is expressed on most NKT and γδTCR+ T cells but not on resting CD8+ T cells.[81,82,83] However, essentially all activated mouse CD8+ T cells express NKG2D. In both humans and mice, CD4+ T cells do not express NKG2D, but it is found on a subset CD4+CD28- T cells in patients with rheumatoid arthritis.[84] Blocking anti-NKG2D mAbs and NKG2D-deficient mice have been described.[80,85,86,87] Regardless, conventional NK cells are NKG2D+ CD3-.

The NKp46 (Ncr1) activation receptor appears to be expressed on all CD3- NK cells in humans and all strains of mice. However, recent reports indicate expression of NKp46 on immune cells in the gut that may be developmentally distinct from conventional NK cells.[88,89,90,91,92] Moreover, depleting anti-NKp46 mAbs have not been described, limiting its usefulness for in vivo functional experiments. Nonetheless, recently developed mice may allow other approaches, such as a mouse where a green fluorescent protein (GFP) cassette was inserted into *Nkp46* and two different transgenic (Tg) mice with a *Nkp46* promoter contruct for expression of Cre or diptheria toxin receptor.[93,94,95]

The mAb DX5 recognizes a molecule that is coexpressed on most NK1.1+ CD3- cells and on small populations of splenocytes in NK1.1- strains, consistent with identification of NK cells in all strains. However, mAb DX5 recognizes the α2 integrin that is widely expressed on other leukocytes, not just NK cells,[96,97] and its expression is regulated.[98] Nevertheless, the DX5 epitope has been used to identify NK cells in mouse strains that do not express NK1.1, but it has been largely supplanted by other nonpolymorphic markers such as NKp46.

The glycolipid determinant asialo-GM_1 is expressed by most if not all murine NK cells and a subpopulation of T cells.[99,100,101] Although the functional significance of this molecule is unknown, polyclonal rabbit anti-asialo-GM_1 (Wako Chemicals USA, Richmond, VA) has been used to effectively deplete NK cells. In more recent studies, the anti-NK1.1 mAb PK136 has become the reagent of choice for NK-cell depletion because of an available defined mAb and its more restricted reactivity with NK cells.[11,72,73,74] However, anti-asialo-GM_1 remains in use for NK-cell depletion when anti-NK1.1 cannot be employed.[73]

In addition to NKG2D and NKp46, human NK cells selectively express CD56. Although it is also found on neural tissues and some tumors, CD56 is generally not expressed by other hematopoietic cells or lymphocytes.[102,103,104] This 140 kDa molecule is derived from alternative splicing of the gene encoding neural cell adhesion molecule (NCAM) involved in nervous system development and cell-cell interactions.[105,106] CD56 may be involved in adhesion between NK cells and their targets,[107] but this function is controversial. Curiously,

mouse CD56 is not expressed on hematopoietic cells,[108] indicating that its role on NK cells is not conserved. Nevertheless, CD56 is particularly useful as a pan-NK-cell marker in humans.

Human NK cells can be functionally divided according to the level of CD56 expressed.[103,109] Most human peripheral blood NK cells are $CD56^{dim}$, a phenotype associated with more cytotoxicity and less cytokine production than a smaller subset of NK cells that express CD56 at higher levels ($CD56^{bright}$). These cells also tend to differentially express receptors involved in target recognition as well as CD16. The $CD56^{bright}$ cells may undergo a maturation process to become $CD56^{dim}$ cells[110] and may be related to a subset of NK cells identified in mice, termed “thymic” NK cells.[111]

Other molecules selectively expressed on NK cells are better discussed below under the general topic of NK cell receptors because they are molecularly defined and their ligands are known.

A MOLECULAR DEFINITION OF NATURAL KILLER CELLS?

A precise molecular definition of NK cells has been elusive. There are no known molecules that are exclusively expressed on NK cells and are responsible for critical functions only displayed by NK cells. The NK cell is therefore still defined by function to the exclusion of other cells, a concept first articulated 25 years ago.[11]

The defining functional feature of NK cells remains their intrinsic ability to perform natural killing (ie, they spontaneously lyse certain tumor cells in a perforin-dependent manner). Unlike other lymphocytes, NK cells do not express surface immunoglobulin or the TCR/CD3 complex, and generally do not require MHC-I expression on targets for lysis. Therefore, a current working definition is that an NK cell is a sIg^-, $TCR/CD3^-$ lymphocyte that can mediate perforin-dependent natural killing against targets that may lack MHC-I expression.

It is noteworthy that T cells were historically defined by an awkward functional definition (thymus-derived, sIg^- lymphocytes responsible for cell mediated immunity).[112]

With the molecular definition of the TCR and coexpressed CD3 molecules, immunologists can now define a T cell as a cell expressing the TCR/CD3 complex.[113] The availability of molecular probes and mAbs directed against this complex provides precise definition even in pathologic tissue sections, without the need for functional analysis (cell mediated immunity, thymus dependence). Similarly, a molecular definition should permit unequivocal identification of NK cells to define their role in normal immune responses and pathologic settings.

Presumably, such a definition will require further knowledge of the molecular basis for NK-cell function, such as the receptors involved in natural killing. On the other hand, one difficulty is that the function that is most attributed to NK cells, natural killing, can be displayed by other cells, such as cytokine-treated T cells. Moreover, NK cells can utilize more than one receptor for target recognition, and individual NK cells can simultaneously express several of these receptors. Thus, there is, as yet, no consensus on the elusive “NK-cell receptor” analogous to the TCR, and the general sentiment in the field is that there is unlikely to be such a receptor.

In the absence of a precise definition, most investigators currently consider the following phenotypes to be surrogate markers of bona fide NK cells. Mouse NK cells are typically NK1.1+ (in appropriate strains), FcγRIII+ (CD16), CD122+, and CD3-. Human NK cells are generally CD56+ and CD3-. In general, mouse and human NK cells also express NKG2D and NKp46, with caveats as elaborated previously.

Note that these markers are generally correlated with cells having natural killing capacity but the markers themselves are not required for natural killing. It should be emphasized that these phenotypes can lead to some confusion due to expression of other molecules on NK cells that are used to help define other immune cells. For example, NK cells express B220 (CD45R) that is often used as a B-cell-specific marker; CD19 is more reliable to distinguish B cells from NK cells.[114] Similarly, NK cells express CD11b, first described as Mac-1 on macrophages.[98] Moreover, NK cells express CD11c, a marker used to define certain DC populations, leading to publications describing a novel type of DC, termed killer DCs.[115,116] However, detailed investigation suggests that these cells are developmentally unrelated to DCs and are actually activated NK cells.[117,118,119] Therefore, markers associated with NK-cell function have been extremely useful in shaping our current concepts of NK cell biology and elaborate their effector functions, but caution may be necessary to avoid confusion with other immune cells.

EFFECTOR FUNCTIONS OF NATURAL KILLER CELLS

Cytotoxicity

A hallmark of NK-cell effector function is target killing, mediated by a process termed granule exocytosis that can be initiated by exposure to susceptible targets or cross-linking of specific activation receptors. Like CTLs, NK cells possess preformed cytoplasmic granules that resemble secretory lysosomes with properties of both secretory granules and lysosomes.[120] Granule formation is affected by Lyst, the molecule defective in humans with Chediak-Higashi syndrome[121,122] in which enlarged lysosomes are observed apparently due to decreased lysosome fission.[123] Normal granules contain perforin and granzymes (granule enzymes). Perforin, a pore-forming protein, is rendered inactive by association with calreticulin and serglycin, and is activated by a cysteine protease.[124] Granzymes are first produced as inactive proenzymes that are activated by N-terminal cleavage by dipeptidyl peptidase I, also known as cathepsin C. However, granzymes are rendered inactive by the acidic pH of the granules. Upon activation by a sensitive target, NK (and T) cells are triggered to rapidly polarize the granules and reposition the microtubule organizing center toward the target in a dynein-dependent manner.[125] The granule membrane ultimately fuses with the plasma membrane, and externalizes, releasing granule contents. Calcium-dependent polymerization of perforin results in “perforation” of the target cell plasma membrane, and granzyme entry by an as yet incompletely understood process. A recent study suggests that perforin induces a plasma membrane repair process that results in endocytosis of perforin and granzymes into enlarged endosomes, called “gigantosomes.”[126] Perforin pores in the gigantosome membrane then allow delivery of granzymes that mediate cleavage of caspases and Bid, ultimately leading to target cell apoptosis.[127]

Recently, many details of the granule exocytosis pathway have come from studies of the heterogeneous human disorder, hemophagocytotic lymphohistiocytosis (HLH).[128,129] In particular, genetic studies of heritable HLH, termed familial HLH (FHL), led to identification of the first described FHL mutation in the perforin gene (*PRF1*), responsible for FHL2. Studies of patients with FHL without *PRF1* mutations led to discovery of other genes whose products (MUNC13-4, syntaxin 11, MUNC18-2) affect granule exocytosis by cytotoxic lymphocytes. Fusion of the cytolytic granule with the plasma membrane requires vesicular RAB27a, a member of the small GTPase superfamily. Defects in RAB27a are associated with the human disorder Griscelli syndrome, type 2. Mice have been described with defects in granule exocytosis components including LYST (*beige*), perforin (*Pfn1-/-*), Unc13d (equivalent to MUNC13-4, also known as *Jinx*), and Rab27a (*ashen*). As highlighted by the names of the mutant mice, many mutations of molecules in the granule exocytosis pathway are associated with skin pigment changes because these molecules also affect melanosomes in melanocytes.[130]

Human T and NK cells also express another pore-forming molecule, granulysin, that is related to a family of saposin-like proteins.[131] Based on crystallographic studies, these molecules appear to be active against bacteria, fungi, and tumor cells by charge association with target membranes and subsequent disruption, leading to target cell lysis.[132] Granulysin is contained in cytolytic granules containing the other cytolytic proteins, such as granzymes.[133] In a perforindependent manner, granulysin causes target apoptosis but is not expressed in mouse cytotoxic lymphocytes.[134]

NK-cell cytotoxcity can be demonstrated in several related ways. Natural killing refers to the process by which NK cells kill certain tumor targets without need for prior host sensitization with the target. Natural killing was first

assessed with a simple in vitro assay for target membrane integrity that is still used today, the standard ^{51}Cr-release assay.[135] The prototypical NK-sensitive tumor target for mouse NK cells is YAC-1 (TIB-160 from ATCC), a thymoma derived from Moloney virus-infected A strain mice, whereas the standard human target is K-562 (CCL-243 from ATCC), an erythroleukemic cell line derived from a human patient with chronic myelogenous leukemia in blast crisis.[136] Maximal killing by enriched, IL-2-activated NK cells usually occurs with effector:target (E:T) ratios of <10:1 whereas unfractionated, freshly isolated peripheral blood or splenocyte preparations usually require E:T ratios of >100:1. Even at high E:T ratios, not all targets are killed, with percentagespecific cytotoxicity typically ranging from ~10% with fresh NK cells to ~80% with activated NK cells. Note that perforin-dependent leakage of ^{51}Cr from the targets is mostly complete within about an hour; 4-hour assays are standard. Longer periods may reflect other apoptotic processes, such as Fas-induced apoptosis.

While ^{51}Cr release is still the gold standard, there are also numerous nonradioactive tests for perforin-dependent killing, including release of intracellular enzymes or use of fluorochromes for target labeling.[137,138,139] The release of granule components, including granzymes, into the supernatant can be determined by conversion of an appropriate substrate, such as granzyme A-mediated cleavage of alpha-N-benzyloxy-carbonyl-L-lysinethiobenzyl ester (also

known as BLT-esterase activity).[140]

A particularly useful new flow cytometric assay exploits the orientation of lysosomal-associated membrane protein-1 (LAMP-1, CD107a) on the luminal side of cytotoxic granules in unactivated NK cells. During granule exocytosis, the granule fuses with the plasma membrane, resulting in externalization of the granule membrane and exposing CD107a on the external surface of the plasma membrane as an indicator of NK-cell activation.[141,142,143] By contrast to other methods, the CD107a assay provides the opportunity for measuring NK-cell responses at the single cell level, isolating triggered NK cells,[144] and possibly simultaneously assessing other NK-cell functions.

Activated NK cells and CTLs also induce perforin-independent target cell killing by expressing Fas ligand (tumor necrosis factor [TNF] superfamily 6) that binds Fas (TNF receptor superfamily 6, TNFRSF6) on the target, triggering apoptosis.[145,146,147,148,149,150] Similarly, other TNF superfamily members, such as TNF-related apoptosis-inducing ligand (TRAIL TNFSF10), can be involved in related processes.[151] However, mice deficient in TNF family members or their receptors may manifest significant alterations in lymphoid organogenesis and splenic architecture, and NK cell number and function,[152,153,154] such that the relative contributions of these pathways to NK-cell function are incompletely understood. Moreover, NK cells from mice deficient in perforin, granzymes, or molecules involved in granule formation or exocytosis demonstrate profound defects in natural killing in vitro.[155,156,157,158] Similar defects have been found with NK cells derived from patients with deficiencies in granule exocytosis.[128] Thus, the available data strongly suggest that granule exocytosis is the predominant mechanism for natural killing.

In addition to natural killing, cytotoxicity by NK cells can be triggered by deliberate cross-linking of activation receptors (discussed in greater detail in the “Activation Receptors” section). Plant lectins can also trigger target killing.[159] In general for all stimuli, cytotoxicity occurs via granule exocytosis and can be measured with the same assays for natural killing.

Cytokine Production

When exposed to NK-sensitive targets or cross-linking of receptors, NK cells also produce cytokines, including IFNγ, TNFα, and granulocyte-macrophage colony stimulating factor (GM-CSF).[160,161,162] They can also be similarly triggered to produce chemokines, such as RANTES, lymphotactin, MIP-1α, and MIP-1β.[163] Moreover, NK cells produce cytokines in response to other cytokines. For example, in response to IL-12, NK cells produce IFNγ.[164] Similarly, NK cells respond to type I IFNs (IFNα/β) produced by DCs stimulated by in vivo administration of poly-I:C and other ligands for TLR and nucleic acid sensors.[165] Cytokine-stimulated responses may obscure detection of specific activation by activation receptors in vivo.[163,166]

While cytokine production can be indirectly measured with RT-PCR for mRNA, it should be noted that resting NK cells typically already express abundant levels of cytokine mRNA even though the proteins are not synthesized,[167] as described previously for granule components

in mouse NK cells.[29] Enzyme-linked immunosorbent assays (ELISA) of tissue culture supernatants are often used, but recent studies have utilized intracellular staining of cytokines, such as IFN, for analysis of individual NK-cell responses that may be more informative, akin to use of the CD107a degranulation assay.[168]

In immune responses, NK-cell production of cytokines should occur relatively early and may thereby influence the subsequent adaptive immune response. Moreover, their responses to cytokines are regulated by complex interacting pathways.[169] A fuller description of NK-cell cytokine responses and production is provided in the following sections on NK cell responses during infections and interactions with DCs.

NATURAL KILLER CELL RECOGNITION OF TARGETS

Molecular dissection of NK-cell recognition of their targets opened new frontiers in NK-cell biology because it not only explained target recognition but it led to identification of receptors that are selectively expressed on NK cells. In addition to providing molecular tools for detailed studies of NK cell function, this analysis yielded several surprises. In contrast to CTL recognition: 1) NK cell receptors are germline encoded and are not strictly "clonotypic" as defined in terms of clonotypic TCRs (unique receptor only expressed by the rare effector clone and its progeny); 2) individual NK cells express both inhibitory and activation receptors for target recognition, and often simultaneously express several different receptors of each type; 3) the receptors are often promiscuous and may have overlapping ligand specificities; and 4) NK-cell receptors specifically bind MHC-I molecules but they are functionally and structurally distinct from other

receptors that bind MHC-I (ie, TCR and CD8). In the following sections, we will discuss NK-cell receptors involved in target recognition by first considering the relationship between target susceptibility to natural killing and expression of MHC-I.

Target Cell Major Histocompatibility Complex-I and Natural Killer Cells: The "Missing-Self" Hypothesis

Whereas initial studies suggested that natural killing was "non-MHC-restricted,"[11] substantial progress in understanding NK-cell recognition began with ascertaining the role of MHC-I molecules in natural killing (Fig. 17.1). Kärre and colleagues discovered that MHC-I-deficient tumors remained susceptible to in vivo rejection, apparently by NK cells.[170] Conversely, target cell expression of MHC-I molecules appeared to have a protective effect against NKcell -mediated lysis in vitro. A number of methods, such as IFNγ treatment, to upregulate MHC-I correlated with target protection but other effects could not be excluded.[171] There was significant variability in capacity of specific MHC-I molecules to protect targets[172,173]; in vitro culture conditions could influence NK-cell specificities,[174] and the specificities of individual human NK cell clones were not easily assignable to specific MHC-I alleles.[175] Thus, the MHC-I effect on natural killing was controversial for some time.

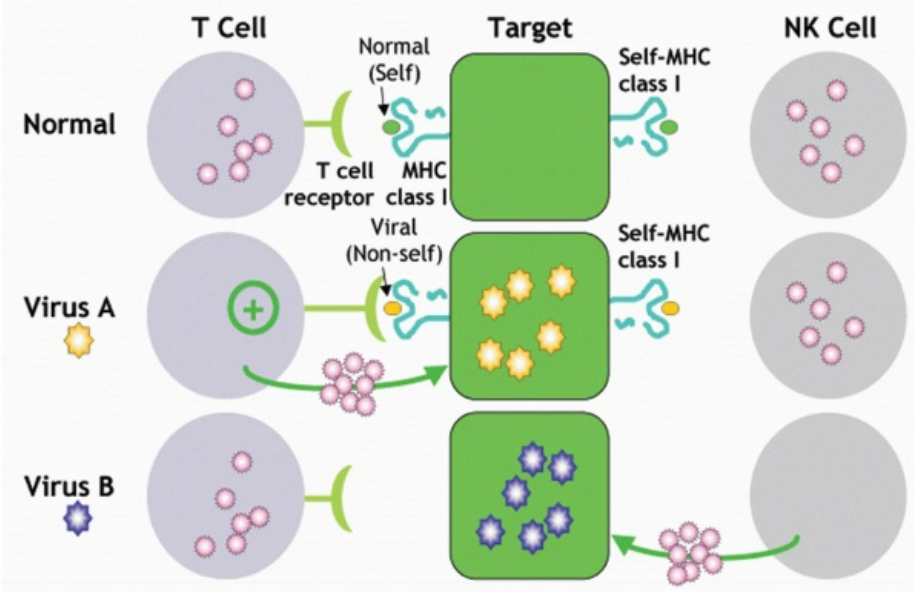

FIG. 17.1. Major Histocompatibility Complex (MHC)-I Expression on Targets is Inversely Related to Natural Killing. Targets expressing MHC-I are more resistant to lysis by natural killer (NK) cells than targets lacking MHC-I expression. This is the exact opposite of the requirements for MHC-I-restricted cyotoxic T-lymphocytes that recognize foreign peptides presented by MHC-I. As depicted, T cells can recognize virus-infected cells, but some viruses may evade T cells by downregulating MHC-I. These infected cells then become more susceptible to NK cells, which generally tend not to discriminate between self- and viral-peptides, though there are some peptide contributions to NK recognition as described in the text.

Several groups, however, observed that MHC-I-expressing parental targets were resistant to natural killing, whereas mutants selected for absence of MHC-I expression became susceptible. The parental (resistant) phenotype could be restored by reconstitution of MHC-I expression by transfected expression of molecules to correct the defect, be it β_2-microglobulin (β2m)[176] or transporter associated with processing (TAP).[177,178] Studies utilizing mice with a targeted mutation in the β2m gene added substantial support to the MHC-I protective effect, as normal expression of MHC-I heavy chains requires β2m.[179,180] β2m-deficient lymphoblasts were susceptible to lysis by normal NK cells. Moreover, β2m-/- BM transplanted into otherwise syngeneic normal hosts was rejected by recipient NK cells.[180,181] These results resembled hybrid resistance whereby NK cells in irradiated F_1 hybrid mice reject parental BM transplants.[182] Hybrid resistance is regulated by parental determinants that are genetically linked to the MHC-I region, H-2D.[183] Thus, in several distinct NK-cell recognition systems, the target cell expression of certain MHC-I molecules correlated with resistance to

natural killing whereas absence of MHC-I was associated with susceptibility to NK cells.

NK cells, therefore, have a different relationship to target cell MHC-I molecules than MHC-I-restricted CTLs (see Fig. 17.1). Strictly speaking, NK cell lysis is “non-MHC-restricted,”[11] at

least as far as MHC restriction is precisely defined for T cells having a requirement for specific self-MHC molecules presenting a given peptide antigen.[184] However, the term "non-MHC-restricted" (and its synonyms) is now somewhat outdated because it implies, when viewed in a broader sense, that MHC plays no role in NK-cell cytotoxicity. Avoidance of these terms will minimize confusion concerning the relationship of target cell MHC-I molecules with NK-cell specificity.

As initially observed and discussed by Kärre in the "missing-self" hypothesis, the relationship between target expression of MHC-I and resistance to natural killing highlights a fundamental distinction between NK and T cells[185] (see Fig. 17.1). Whereas T cells are triggered by detection of "foreign" epitopes, Kärre proposed that NK cells are equipped to detect the absence of "self" epitopes. The [missing-self] hypothesis suggests that NK cells survey tissues for expression of MHC-I molecules that are normally ubiquitously expressed and that somehow prevent NK-cell activity. If MHC-I molecules are downregulated or mutated, NK cells can then lyse the target. The generally opposite requirements of NK and T cells for target cell MHC-I expression may be physiologically important. Several pathogens, including herpes viruses, possess mechanisms that prevent the normal expression of MHC-I molecules on infected cells, providing means to avoid MHC-I-restricted T cells.[186] Moreover, tumorigenesis is frequently associated with alterations in MHC molecules, either mutation in structural genes or decreased expression, again leading to escape from T-cell surveillance.[187,188,189] In either case, however, the MHC-I-deficient cells should become more susceptible to natural killing. The host, therefore, is endowed with two components (T and NK cells) with opposing requirements for self-MHC-I expression. This fail-safe system should eliminate pathologic processes that might otherwise evade immune responses by any alteration of MHC-I expression (either increased to avoid NK cells or decreased to avoid T cells). The missing-self hypothesis thus provided a tentative physiologic explanation for MHC-I-associated resistance, creating a framework for initial attempts to define NK-cell recognition of their targets.

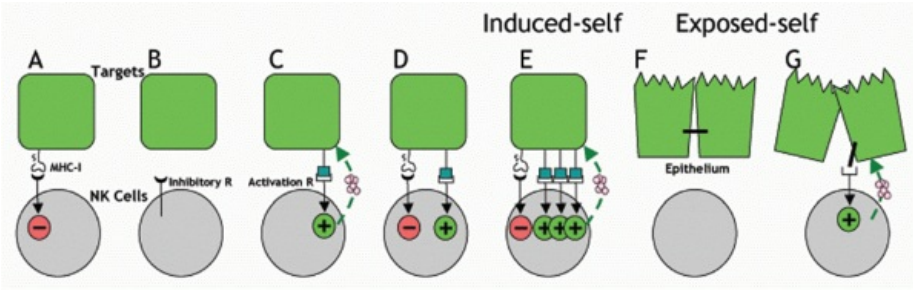

FIG. 17.2. Current Principles of Target Recognition by Natural Killer (NK) Cells. Pictured are several scenarios of interactions between ligands on targets (*top row*) and receptors on NK cells (*bottom row*). Successful activation of NK cells is shown by the *dashed upward line*. **A:** Targets expressing majoe histcompatibility complex (MHC)-I are resistant to lysis by NK cells because of MHC-I-specific inhibitory receptors. **B:** The absence of MHC-I (or lack of receptors specific for target MHC-I, not depicted) does not

automatically result in target killing. **C:** Activation receptor engagement is required to trigger target killing in absence of MHC-I. **D:** In the situation where both inhibitory and activation receptors are engaged, the inhibitory receptor effect often dominates and no killing occurs. **E:** In the induced-self model, induced expression of NKG2D ligands can overcome the inhibitory influence of MHC-I, resulting in NK cell activation. **F:** Normal epithelium masks ligands for NK-cell activation receptors at the tight junctions. **G:** Under pathologic situations, the epithelial architecture may be disrupted, leading to ligand exposure. (Not depicted are inhibitory ligands at the epithelial tight junctions that may inhibit NK cells when they transmigrate through epithelial barriers.) NK cells can also recognize pathogen encoded ligands on infected cells (not shown, but similar to *C* or *E*).

Current Principles of Target Recognition by Natural Killer Cells

The MHC-I-associated resistance to natural killing inspired a panoply of models and their variants to explain not only resistance but also natural killing.[172] The *target interference* or *masking* model predicted that a single NK-cell receptor activates natural killing when it engages its putative target cell ligand.[185] MHC-I molecules mask the putative target cell ligand and block its recognition by the NK-cell receptor. This hypothesis was initially favored because it was the simplest.[190] Moreover, it made the most sense if one considered that NK cells should have only one defined receptor analogous to the TCR. The *effector inhibition* or *inhibitory receptor* model suggested that NK cells are inhibited from natural killing by an NK-cell receptor that binds MHC-I on the target and delivers negative signals overriding a default pathway of activation.[185]

Although the target interference model has not been refuted, it is now known that NK cells express inhibitory receptors that physically bind MHC-I in a manner that may be influenced by MHC-bound peptides[191] (Fig. 17.2).

All known inhibitory receptors contain cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) consisting of V/I/L/SxYxxL/V (single amino acid code where x is any amino acid).[192] Ligand engagement leads to phosphorylation of the ITIM, which leads to inhibition, traditionally thought to be due to recruitment and activation of intracellular phosphatases, although recent evidence suggests more complexity.

MHC-I inhibitory receptors on NK cells have either of two general structures[193]: 1) C-type lectin-like receptors that are disulfide-linked dimers with type II transmembrane topology (extracellular carboxyl termini). These receptors are encoded in the NK gene complex (NKC) and were first described in mice. 2) Immunoglobulin (Ig)-superfamily receptors that have type I transmembrane orientation. These molecules are encoded in a different genetic region, termed the leukocyte receptor complex (LRC), and were first described in humans. Although ongoing studies indicate that both structural types of receptors are expressed on mouse and human NK cells, the lectin-like receptors (Ly49 receptors) are the major MHC-specific inhibitory receptors in mouse whereas the Ig-like receptors (killer Ig-like receptors [KIRs]) predominate in human.

The absence of MHC-I does not always result in killing, indicating that release from inhibition

does not result in activation by default (Fig. 17.2B). Instead, it was suggested that NK cells express two functionally different receptors for target cell ligands.[194,195] In this *two receptor model*, one receptor triggers activation upon ligand binding (Fig. 17.2C) whereas the MHC-I-specific receptor inhibits activation by negative signaling. In many circumstances, the inhibitory receptor effect dominates over the activation receptor (Fig. 17.2D), but the outcome usually reflects the integration of signals from both types of receptors, which can be affected by ligand expression or affinities (not shown).

Many, but not all, NK-cell activation receptors are encoded in the NKC and LRC, having similar structural properties as their inhibitory receptor counterparts except for absence of cytoplasmic ITIMs. The activation receptors typically do not have signaling motifs in their cytoplasmic domains but contain charged transmembrane residues that facilitate association with reciprocally charged residues in the transmembrane domains of signaling chains having immunoreceptor tyrosine-based activation motifs (ITAMs) analogous to ITAMs in TCR and B-cell receptor (BCR) complexes (D/ExxYxxL/Ix$_{6-8}$YxxL/I). NK cells express three ITAM-containing signaling chains: CD3ζ, FcεRIγ, and DAP12 (DNAX associated protein of 12 kDa, also known as killer activating receptor-associated protein, KARAP; Ly83; tyrosine kinase binding protein, Tyrobp). NK cells also express DAP10 (hematopoietic cell signal transducer, Hcst) that lacks ITAMs and instead contains a motif for recruitment of phosphatidylinositol 3-kinase (PI3K) and Grb2. The signaling chains typically provide two major functions: facilitate cell surface expression of the associated activation receptor, and transduce signals.

To date, the ligands for activation receptors fall into several major groups. One group is encoded by the host and is expressed normally. Presumably, NK-cell attack against cells expressing these ligands is limited by inhibitory receptors (Fig. 17.2D). Another group of ligands is characterized by their relatively low expression on normal tissues and induced expression under “stress” conditions (Fig. 17.2E). Other ligands become exposed when tissue architecture is altered (Fig. 17.2F,G). Because the ligands are encoded in the normal host genome, they would be recognized by the NK cell as indicators of pathologic conditions, either as “induced-self” or “exposed-self,” respectively. Another group of ligands is found on infected cells and is encoded by the pathogen (not shown but similar to Fig. 17.2C or E).

Finally, many other NK-cell receptors have been discovered that do not fall neatly into the categories described here. Some appear to have similar inhibitory function as the MHC-specific inhibitory receptors but bind non-MHC ligands, strongly suggesting MHC-independent self-recognition. The function of these and other receptors remains under intense investigation.

NATURAL KILLER CELL RECEPTORS

In the following sections, we will describe the major receptors on NK cells in detail by first discussing the MHCspecific inhibitory receptors that helped elucidate NK recognition paradigms before delving into MHC-independent inhibitory receptors, activation receptors, and other receptors found on NK cells. Given the large number of receptors now identified (Table 17.1), this section will primarily discuss work on the receptors that have been studied most extensively. This summary will illustrate the experimental approaches that led to identification of these receptors, their features, and outline general principles applicable for study of other receptors that will not be discussed in detail due to space constraints. Nonetheless, description of the major activation receptors and their ligands help illustrate and

provide molecular handles on the various functions of NK cells.

Inhibitory Natural Killer-Cell Receptors Specific for Major Histocompatibility Complex-I Molecules

The mouse and human MHC-specific inhibitory receptors are remarkably different in protein structure. Each will be discussed separately.

Mouse Ly49

The Ly49A receptor was the first inhibitory MHC-I-specific NK-cell receptor to be described in molecular terms.[191,196] Ly49A was originally identified as a molecule of unknown function on a T-cell tumor.[197,198] It is a disulfide-linked homodimer (44 kDa subunits) with type II membrane orientation, and C-type lectin superfamily homology.[199,200] Previously termed Ly49, it is now appreciated that Ly49A (Klra1) belongs to a family of highly related molecules.[195,201,202,203] Indeed, genetic analysis revealed that the genes for Ly49A and NK1.1 are linked in the NKC (Fig. 17.3), leading to studies indicating that Ly49A is constitutively expressed on a distinct subpopulation (20%) of NK cells in C57BL/6 mice.[203]

P.403
P.404

TABLE 17.1 The Panoply of Receptors Expressed by Natural Killer Cells[a]

Receptor	H	M	Inhibitory (I) or Activation (A)	Other Names	Ligand
Ly49A		X	I	Ly49, Ly-49, Klra1	$H2D^d$, D^k, Dp, alleles in $H2^r$, and $H2^q$
Ly49C		X	I	Klra3	$H2K^b$, numerous
Ly49E		X	I	Klra5	Urokinase plasminogen activator
Ly49G2		X	I	LGL-1, Klra7	$H2D^d$
Ly49I129		X	I	Klra9	m157

Ly49Q		X	I	Klra17	$H2K^b$
KIR2DL1	X		I	CD158a, p58.1, EB6	HLA-C2 (HLA-Cw2, Cw4, -Cw5, -Cw6)
KIR2DL2/KIR2DL3	X		I	CD158b, p58.2, GL183	HLA-C1 (HLA-Cw1, -Cw3, -Cw7, -Cw8)
KIR2DL4	X		I	CD158d	HLA-G
KIR3DL1	X		I	CD185e1, NKB1, p70, NKAT3	Bw4 (HLA-A and B)
KIR3DL2	X		I	CD158k, p140, NKAT4	HLA-A3, -A11
Lilrb4		X	I	gp49	αvβ3 integrin
CD94/NKG2A	X		I	Kp43	HLA-E
		X	I		Qa-1
LILRB1	X		I	CD85j, ILT2, LIR1	Folded HLA, UL18
LILRB2	X		I	ILT4, LIR2	Folded, free HLA
LAIR-1	X		I		Collagen
Siglec-7	X		I	P75, AIRM1	Carbohydrates
Siglec-10	X		I		Carbohydrates?
Siglec-E		X	I		?
PILRa	X		I		CD99

PILRb	X		I		CD99
FcγRIII	X	X	A	CD16	Fc of IgG
Ly49D		X	A		Chinese hamster MHC-I, $H2D^d$
Ly49H		X	A		m157
$Ly49P^{MA/My}$		X	A		M04 + $H2D^k$
KIR2DS1	X		A	CD158h, p50.1	HLA-Cw7
KIR2DS2	X		A	CD158j, NKAT5, p50.2, clone 49	
KIR2DS3	X		A	NKAT7	
KIR2DS4	X		A	CD158i, NKAT8, clone 39	HLA-Cw4
KIR2DS5	X		A	CD158g, NKAT9	
KIR3DS1	X		A	CD158e2	
CD94/NKG2C	X		A		HLA-E
CD94/NKG2C		X	A		Qa-1
NKG2D		X	A, costimulation	KLRK1	MICA, MICB, ULBP/RAET1
NKG2D	X		A, costimulation	Klrk1	H60, RAE1, MULT1
Nkrp1c		X	A	NK1.1	?
Nkrp1b(d)		X	I		Clrb

Nkrp1f		X	A?		Clrg
NKRP1A	X		I		LLT1
NKp80	X		A		AICL
NKp65	X		A	KLRF2	CLEC2A
2B4	X	X	A,I	SLAMF4	CD48
CD2	X	X	A		CD48
NTBA	X	X	A	SLAMF6, Ly108	NTBA
Ly9	X	X	A	SLAMF3	Ly9
CD84	X	X	A	SLAMF5	CD84
CRACC	X	X	A	SLAMF7	CRACC
NKp46	X	X	A		Hemagglutinin
NKp44	X		A		?
NKp30	X		A		B7-H6
CD69	X	X	A		?
Ly6		X	A		?
Gp42		Rat	A		?
Klrg1		X	I		Caherins
CEACAM1	X		I	CD66a	CEA
CD226	X	X	A	DNAM-1	necl-5 (CD155, PVR), nectin-2 (CD112, PVRL2)
CD96	X		A	Tactile	necl-5

CRTAM	X	A	necl-2
TIGIT	X	I	PVR, PVRL2

AICL, activation-induced C-type lectin; AIRM1, adhesion inhibitory receptor 1; CRTAM, class I-restricted T-cell-associated molecule; LGL, large granular lymphocyte; NK, natural killer; TIGIT, T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain.

a These are the major receptors discovered on NK cells in humans and mice, listed in order of appearance in the text.

Multiple lines of evidence indicate that Ly49A is an inhibitory receptor specific for MHC-I, particularly $H2D^d$: 1) Functional analysis: the Ly49A+ NK-cell subset were equivalent to Ly49A- NK cells in killing several targets, but they could not lyse a large panel of targets that were readily lysed by Ly49A- NK cells. This phenotype was related to MHC-I expression of certain H2 haplotypes on target cells.[191,204,205] Transfected expression of $H\text{-}2D^d$ selectively rendered a susceptible target resistant to natural killing by Ly49A+ NK cells. Moreover, killing through disparate stimuli by Ly49A+ NK cells was also inhibited. 2) Cell binding: Ly49A+ tumor cells bound specifically to immobilized MHC-I molecules[206] and to $H\text{-}2D^d$-transfectants.[207] 3) Antibody blocking: $F(ab')_2$ fragments of mAb directed against either Ly49A or the α1/α2 (but not the α3 domain) of $H\text{-}2D^d$ reversed resistance in killing experiments (permitted lysis) and blocked the cell binding assay.[191,204,206,207] 4) In vivo expression: the apparent level of Ly49A expressed per NK cell was downregulated in MHC congenic and Tg mice expressing $H\text{-}2D^d$.[208,209,210] This was not due to negative selection because the percentage of Ly49A+ NK cells was unchanged. 5) Gene transfer: primary NK cells and T cells expressing a Ly49A transgene and a Ly49A-transfected NK cell line were specifically inhibited by $H\text{-}2D^d$.[211,212] 6) Inhibition by Ly49A is ITIM-dependent, based on gene transfer of mutant Ly49A molecules.[212] 7) $H2D^d$ tetramers bind Ly49A transfectants.[213] 8) Ly49A tetramers bind $H2D^d$ on transfected cells.[214,215] 9) Biophysical studies: recombinant Ly49A binds recombinant $H2D^d$ in surface plasmon resonance (SPR) studies with K_D = ~2.0 μM.[216]

10) Crystallography: the structure of Ly49A complexed with $H2D^d$ was determined.[217] 11) Less extensive studies also indicate that Ly49A recognizes $H\text{-}2D^k$, $H2D^p$, and alleles in $H2^r$ and $H2^q$.[191,208,213,218,219] Therefore, Ly49A is an MHC-I-specific receptor for $H\text{-}2D^d$, $H\text{-}2D^k$, and $H2D^p$, and alleles in $H2^r$ and $H2^q$.

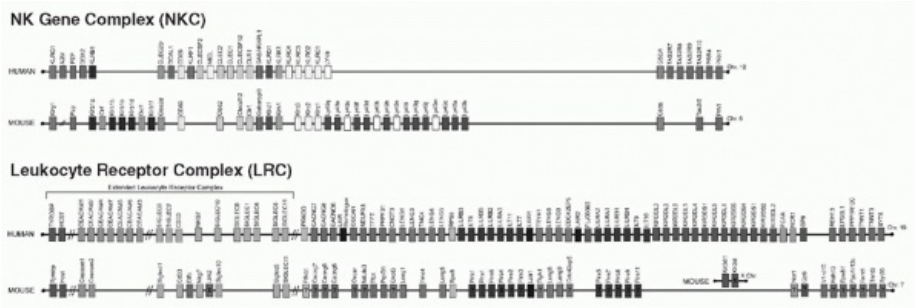

FIG. 17.3. The Genomic Organization of the Natural Killer (NK) Gene Complex and Leukocyte Receptor Complex in Humans and Mice. The figures are not drawn to scale with precise gene locations being modified as new sequence information becomes available (genome.ucsc. edu/ and www.ebi.ac.uk/ipd/kir/). The *grey shading* is coordinated to represent related genes. *Question marks* indicate genes whose precise location is not known. An "*X*" indicates genes not homologous to other aligned genes. *Double slashes* represent large genomic distances. Note that most but not all genes are expressed on NK cells. Many remain orphan genes because the functions of their gene products have not been determined. Modified from Kelley et al.[312]

The nature of the Ly49A interaction with MHC-I, however, is fundamentally different from TCR/MHC-I interactions because the former appears to be relatively independent of the specific peptide bound by $H2D^d$.[220,221] However, bound peptides are required for appropriately folded MHC-I molecules that can be recognized. Despite its structural homology to C-type lectins that are carbohydrate-binding proteins,[222,223] Ly49A does not have the residues for coordinate binding to calcium that is required for lectin binding. Moreover, Ly49A binding to its MHC ligands is not carbohydrate-dependent based on functional, SPR, and crystallographic analyses.[216,224] In the crystallographic structure of Ly49A complexed to $H2D^d$ (2.3Å resolution), the lectin-like structure of Ly49A was confirmed[217] (Fig. 17.4). Two interaction sites were seen between the lectin-like domain of Ly49A and $H2D^d$: site 1 involved the "left" side of the peptide-binding cleft of $H2D^d$ and a wedge-like site 2 involved the undersurface of the peptide-binding cleft. The residues in Ly49A involved in binding either ligand site are overlapping. Mutational analysis revealed that Ly49A binds site 2 where it contacts α1, α2, and α3 of $H2D^d$ and β2m.[214,216,225] This site is near Asn80, an Asn-linked glycosylation site conserved in all MHC-I molecules, leaving open the issue of whether carbohydrates could affect the interaction, such as affinities or kinetic parameters, but this has not been studied in depth. These studies also provide a structural explanation for species-specific β2m requirements as revealed by functional studies.[226] Thus, Ly49A recognizes site 2 in the MHC molecule in terms of *trans* recognition between the NK-cell receptor and target cell MHC-I molecule.

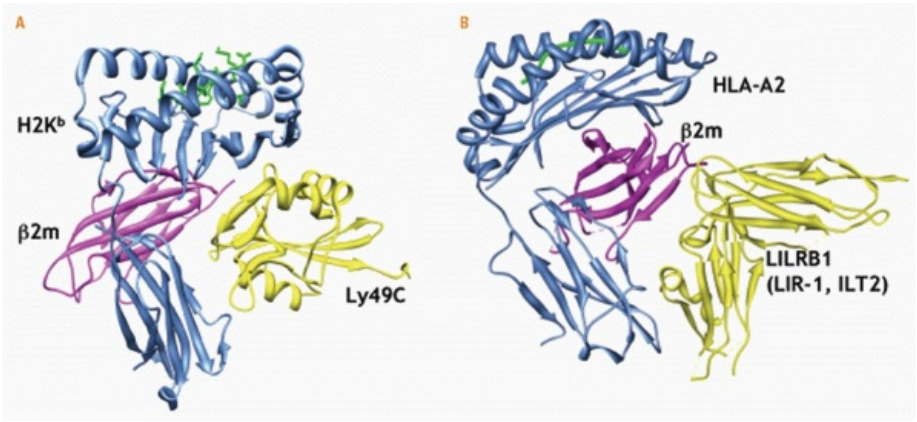

FIG. 17.4. Crystal Structures of Natural Killer (NK)-Cell Receptors in Complex with Their Ligands. A: Mouse Ly49C bound to $H2K_b$ at site 2 (PDB ID = 3C8K).[193,235] Ly49A interaction with site 2 of $H2D^d$ is very similar.[170] Site 1 of Ly49A-$H2D^d$ interaction is approximately located where the $H2K^b$ label is placed. **B:** Human LILRB1 (LIR1, ILT2) bound to human leukocyte antigen-A2 (PDB ID = 1P7Q).[266] Figures were produced using the University of California, San Francisco Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (www.cgl.ucsf.edu/chimera).[1030] The structures are viewed from the side with the NK cell positioned at the top of the figure and the target cell surface at the bottom. The major histocompatibility complex molecules are oriented similarly.

Most but not all other Ly49 receptors are MHCI-specific inhibitory receptors. First noted by Southern blot analysis and cDNA cloning, genome sequence analysis revealed 15 complete Ly49 genes in C57BL/6 mice.[199,200,201,202,227] There is evidence for alternative splicing and alternative transcriptional start sites for the Ly49 genes, though their importance has not been elucidated.[202,228,229] Notably, Ly49C has broad specificity for H2 alleles, as revealed by tetramer staining, and is the only known inhibitory NKcell receptor specific for an $H2^b$ haplotype allele ($H2K^b$) in C57BL/6 mice, notwithstanding unconfirmed reports that Ly49I also binds $H2K^b$.[168,213,230] Ly49C is recognized by two mAbs: 5E6, which also binds Ly49I, and 4LO3311, which has exquisite specificity for Ly49C.[231,232,233] X-ray crystallographic studies have indicated that Ly49C binds $H2K^b$ in a manner similar to Ly49A interaction with $H2D^d$ (see Fig. 17.4).[234,235] Only a site 2 interaction was seen with the contact residues, showing a similar but distinct topology to the Ly49A-$H2D^d$ interaction. Interestingly, however, peptides bound to $H2K^b$ clearly affect functional interactions with Ly49C[236] and affinities as measured by SPR.[234] However, Ly49C does not directly engage the peptide, indicating long-range effects. Finally, both Ly49A and Ly49C

appear to undergo conformational changes upon ligand binding, and a Ly49 dimer can engage two MHC-I molecules.[235,237] Thus, Ly49 receptors bind their MHC ligands in a

structurally related manner.

Recent studies suggest that Ly49 molecules also can bind MHC in a *cis* interaction between receptor and ligand on the NK cell itself.[168,238] For example, an MHC ligand for Ly49A or Ly49C on the same cell prevents binding of MHC tetramer.[168,238] If the cells are briefly exposed to mild acidic conditions, MHC-I expression is lost (due to disruption of the noncovalently linked MHC-I heterotrimer), and Ly49A binding to cognate MHC-tetramers is restored. This *cis* interaction is dependent on site 2 residues in the MHC molecule. These findings may help explain the observation that the presence of self-MHC ligands leads to downregulation of Ly49 expression, as previously noted on primary NK cells in MHC congenic mice.[208,209,210] *Cis* interactions may also explain functional differences in NK cells in MHC-congenic mice or NK cells that do or do not express MHC ligands in Tg mice that are mosaic for MHC expression.[239,240,241] Finally, there are biophysical data supporting a role for the relatively long, flexible stalk region of Ly49 receptors in allowing either *trans* or *cis* interactions.[242] At the moment, the physiologic importance of *cis* interactions is incompletely understood but may be relevant to NK-cell tolerance and education, as discussed below.

Although they have not been studied as extensively as Ly49A and Ly49C, other inhibitory Ly49 receptors and their ligands have been identified[202,230] and characterized with other MHC allele specificities, as detailed in a recent review.[243] Moreover, they are structurally related.[235,244] Individual NK cells may express multiple Ly49 receptors simultaneously,[210,233,245] often (but not always) two or more, suggesting that individual NK cells may be inhibited by more than one MHC-I molecule.

Ontogenetic studies demonstrate that the total repertoire of Ly49 expression does not reach adult levels until sometime after 3 weeks of age, concomitant with attainment of full NK-cell cytolytic activity.[246] Thereafter, the expression of Ly49 receptors is generally thought to be fixed and stable on an individual NK cell. Ly49E is expressed only on fetal NK cells, but NK cells in mice deficient in Ly49E are otherwise normal.[247] In adult mice, developmental studies indicate that Ly49 receptors are first expressed on immature NK cells in the BM, before a phase of constitutive proliferation[98] that appears to be modestly affected by MHC haplotype.[168] There are only modest effects on the final “repertoire” of MHCspecific receptors expressed by splenic NK cells in different MHC environments.[210,248]

The expression of inhibitory Ly49 receptors appears to occur in a stochastic manner. There is evidence for monoallelic expression of Ly49 receptors (expression from one chromosome), initially described as “allelic exclusion,” a term that has fallen out of favor because it has a specific meaning and mechanism for TCRs and BCRs.[249] At least some Ly49 genes possess bidirectional, overlapping promoters directed in opposite orientations.[250] Transcription factors driving transcription in one direction prevent binding of other factors driving transcription in the opposite direction. Directionality and monoallelic expression may also be controlled by DNA methylation.[251] A “probabilistic” model has been proposed to explain these findings that may also explain the stochastic expression of Ly49 genes and their stable expression. However, recent studies of *Ly49* indicate highly variable transcriptional start

sites, suggesting that the probabilistic model may not be correct.[229] Other data indicate that TCF-1 but not LEF-1 in the T-cell factor/lymphoid enhancer family of DNA-binding proteins affects some but not all Ly49 receptor expression.[252,253,254] Thus, the elements controlling Ly49 gene expression are incompletely understood.

Analysis of the Ly49 receptors thus far is largely based on examination of the C57BL/6 alleles, but the Ly49 receptors display extensive polymorphism. The Ly49 family is encoded in the NKC located on mouse chromosome 6 with the syntenic human region being chromosome 12p13.2[195,203,255,256] (see Fig. 17.3). While the NKC also contains genes for other lectin-like receptors, the Ly49 genes are clustered with the exception of *Ly49b*. Corresponding to restriction fragment length polymorphic (RFLP) variants originally detected with the Ly49A cDNA,[203] there is significant allelic polymorphism of the Ly49 cluster between inbred mouse strains with differences in gene number as well as alleles for the *Ly49* genes.[227,257,258,259] In contrast to C57BL/6J mice, genomic sequence analysis shows 8 putative *Ly49* genes in BALB/c mice, 19 in 129 mice (of which at least 9 appear to be pseudogenes) and 22 in NOD mice. Array-based comparative genomic hybridization analysis of 21 mouse strains compared to the reference C57BL/6J strain indicated that these mice could be grouped into five clusters that correspond to or are predictive of restriction fragment length polymorphic patterns on Southern blot analysis.[203,260] There are also multiple alleles for individual *Ly49* family members.[227,249,257,258,261] Thus, there is significant polymorphism of the *Ly49* molecules at both the haplotype (gene numbers) and individual gene (alleles) levels, not unexpected because the Ly49 molecules bind highly polymorphic MHC-I molecules.

The MHC-I specificities have generally been well characterized for only a few Ly49 alleles. Interestingly, mAbs specific for one Ly49 allele may bind another molecule with a different function or specificity in another mouse strain,[262,263,264] similar to what was recognized for mAb reactivity with different MHC alleles.[265] Thus, the polymorphisms also raise practical issues when studying Ly49 molecules in different mouse strains.

Finally, it should be noted that Ly49 receptors may be expressed by other cells; some are selectively expressed on non-NK cells and some may have specificities for non-MHC ligands. NKT and other T-cell subsets may express Ly49 receptors but they have not been thoroughly studied.[266,267] Ly49B and Ly49Q are not expressed on NK cells; rather, they are expressed on myeloid cells.[268,269] Interestingly, Ly49Q recognizes $H2K^b$ and positively regulates TLR signaling.[270,271] On the other hand, Ly49E appears to recognize urokinase plasminogen activator, though physical binding has not been established.[272] Interestingly, Ly49B, Ly49E, and Ly49Q are predicted to be distinct in fine structure from the

known MHC-I-specific Ly49 receptors on NK cells.[235] Thus, while Ly49 receptors are predominantly NK-cell inhibitory receptors for MHC class I, they may have other roles that are less well understood; still other Ly49 receptors have activation function, as discussed subsequently.

Human Killer Immunoglobulin-like Receptors

In contrast to mouse NK cells, human NK cells can be cloned by limiting dilution in the presence of irradiated feeder cells, phytohemagglutinin, and IL-2, leading to establishment of short-term NK-cell clones that have differences in target killing and surface molecules. mAbs were isolated that reacted specifically with these clones; reactivity correlated with the capacity of the clones to kill certain tumors, and the mAbs affected cytotoxicity. This general approach led to the identification of the human NK-cell receptors.

A series of studies[273,274,275] showed that the mAbs GL183 and EB6 identify serologically distinct 55 kDa or 58 kDa molecules, initially termed p58. These molecules had several features: 1) selective expression on overlapping NK cell subsets; 2) expression on NK cell clones correlated with expression of certain human leukocyte antigen (HLA) class I alleles on resistant targets; 3) a target susceptible to a given NK-cell clone bearing p58 molecules reactive with either mAb was made resistant by transfection of cDNAs encoding certain HLA-C molecules; 4) the otherwise resistant, HLA-C-transfected targets could be lysed in the presence of the appropriate anti-p58 mAbs. The mAb effect occurred with $F(ab')_2$ fragments, suggesting that the interaction between p58 and an HLA class I molecule on the target cell inhibits the NK cell. Thus, the p58 molecules displayed features consistent with a role as inhibitory human NK-cell receptors specific for MHC-I, analogous to the mouse Ly49A receptor that was being studied in parallel, as described previously.

Other studies noted that NK-cell specificity was skewed when the NK cells were grown in the presence of cells bearing allo-MHC determinants.[276,277] This specificity correlated with reactivities that mapped to paired residues at position 77 and 80 in the .α1 domain of HLA-C. All known HLA-C molecules could be divided into two groups, one with Asn77-Lys80 (HLA-Cw2, -Cw4, -Cw5, -Cw6) and the other with Ser77-Asn80 (HLA-Cw1, -Cw3, -Cw7, -Cw8). Indeed, transfection analysis showed that p58 specificity for HLA-C molecules was related to expression of the EB6 epitope for the former (specificity 1, now termed HLA-C1), whereas the latter was related to the GL183 epitope (specificity 2, HLAC2) on the NK-cell clones.[276,278,279] Thus, human NK-cell receptors showed promiscuous specificity that was dependent on residues 77 and 80 in HLA-C.

The NKB1 (p70) molecule was serologically similar to p58 molecules with regard to subset expression, and correlation of expression on NK-cell clones to specificity for HLA class I.[280,281] In contrast to p58 molecules, however, NKB1 had a distinct M_r (70 kDa) and specificity for HLA-B. The NKB1+ clones were specifically inhibited by targets expressing transfected HLA-Bw4 molecules, and the anti-NKB1 mAb reversed the inhibition. Analysis of informative HLA-B alleles showed that this specificity was conferred by a region in the α1 domain overlapping the area on HLA-C recognized by p58 molecules.[282] Finally, HLA-A3, -A11-specific receptors have similar properties to p58 and NKB1 except that they appear to be disulfide-linked dimers termed p140,[283] whereas others have found that a monomeric HLA-A3-specific receptor resembles NKB1.[284] Thus, representative alleles of all classical HLA class I loci are capable of inhibiting NK cells through p58/NKB1/p140 receptors although HLA-B and -C alleles dominate human NK-cell specificities, and it is not yet known if there are receptors reactive with each HLA allele.

When the cDNAs for the p58 and NKB1 molecules were cloned, they were surprisingly found to encode type I integral membrane proteins with Ig-like domains[285,286,287] unlike the

lectin-like Ly49 family of type II receptors. The Ig-like receptors are now collectively known as killer Iq-like receptors (KIRs) or CD158.[288,289] The KIR nomenclature is based on whether the receptor has two or three Ig-like external domains (KIR2D or KIR3D, respectively), and possession of a long (L) or short (S) cytoplasmic domain. In general, the L forms are inhibitory because they contain ITIMs, whereas the S forms appear to be activation receptors (see following discussion). Each distinct receptor is also designated by a number. The KIR2DL1 (CD158a, p58.1) molecule bears the original EB6 epitope and is specific for HLA-C (Lys80, specificity 2), whereas KIR2DL2 (CD158b1, p58.2) and KIR2DL3 (CD158b2, p58) have the GL183 epitope and are specific for HLA-C (Asn80, specificity 1). (As detailed in the following, structural analysis supports grouping of HLA-C alleles into two mutually exclusive groups, HLA-C1 and HLA-C2, based on direct interaction of KIR2DL2/3 and KIR2DL1, respectively, with residue 80 of HLA-C, validating original functional groupings but simplifying HLA-C groupings to just residue 80.[276,277]) KIR2DL4 (CD158d, p49) reportedly binds HLA-G[290] but displays both inhibitory and activation functions.[291,292,293] KIR3DL1 (CD158e1, NKB1, NKAT3) is specific for HLA-A and HLA-B molecules with the Bw4 epitope.[282] KIR3DL2 (CD158k, p140, NKAT4) has HLA-A3 and HLA-A11 specificity.[283,284]

There is unequivocal evidence that the KIR2DL and KIR3DL molecules are inhibitory HLA class I-specific receptors. In addition to the data with NK cell clones and mAbs mentioned previously, the following have been described: 1) KIR bind directly to HLA class I: soluble KIR2DL-Fc fusion proteins bind cells expressing the appropriate transfected HLA class I alleles.[294,295] In addition, a soluble KIR2DL molecule containing only the extracellular domain binds specifically to its HLA-C ligand in solution.[296] 2) Gene transfer of KIR: KIR2DL specificity and inhibitory function were transferred when KIR2DL cDNAs were transiently expressed with vaccinia constructs in human NK-cell clones.[294] Similarly, Tg expression of KIR2DL2 in mice conferred inhibition of rejection of BM expressing Tg HLA-Cw3.[297] 3) SPR measurements indicate that the KIRs bind their HLA ligands with K_d = ˜10 μM.[298,299,300,301] Binding is affected by peptide bound by HLA molecule.[300,301] Through histidine-rich domains, the KIRs bind Zn++, which affects KIR multimerization and binding kinetics to HLA ligands.[302,303] 4) Crystallographic studies demonstrate

KIR2DL1 (2.8 Å resolution) and KIR2DL2 (3.0 Å resolution) interactions with their cognate HLA ligands.[299,304] Thus, KIR molecules are clearly MHC-I-specific inhibitory receptors on NK cells.

Interestingly, structural studies indicate that KIR molecules bind HLA class I molecules in a manner analogous to recognition of MHC by TCRs (Fig. 17.5). In particular, both KIR2DL1 and KIR2DL2 use surface loops near their interdomain hinge regions to bind their cognate HLA-C ligands (Cw4 and Cw3, respectively) with a footprint overlying the “right” side of the peptide-binding cleft (when viewed from the “top” in standard depictions of MHC-I molecules).[299,304] The receptors bind both α1 and α2 helices with interactions between KIR2DL1 and Lys80 of HLA-Cw4 and between KIR2DL2 and Asn80 of HLA-Cw3. These interactions with residue 80 of the HLA-C molecules were lost when the reciprocal residues were swapped, accounting for the previously described HLA-C groupings and KIR

specificities in functional studies[276,277] and mutational analysis indicating that residue 80 is more significant for KIR interaction than residue 77.[305,306,307,308] Although neither KIR2DL molecule has extensive contacts with peptides bound to HLA-C, KIR2DL interactions with HLA-C imposes physical constraints on the p8 position of the peptide. This may account for observed peptide preferences in functional studies and antagonism of certain peptides on KIR inhibitory function.[309,310] A recent structure (1.8 Å resolution) of KIR3DL1 complexed to HLA-B*5701 revealed a similar recognition strategy.[301] Thus, KIRs and Ly49s bind their MHC ligands in markedly different ways, despite their analogous functions as MHC-specific inhibitory receptors.

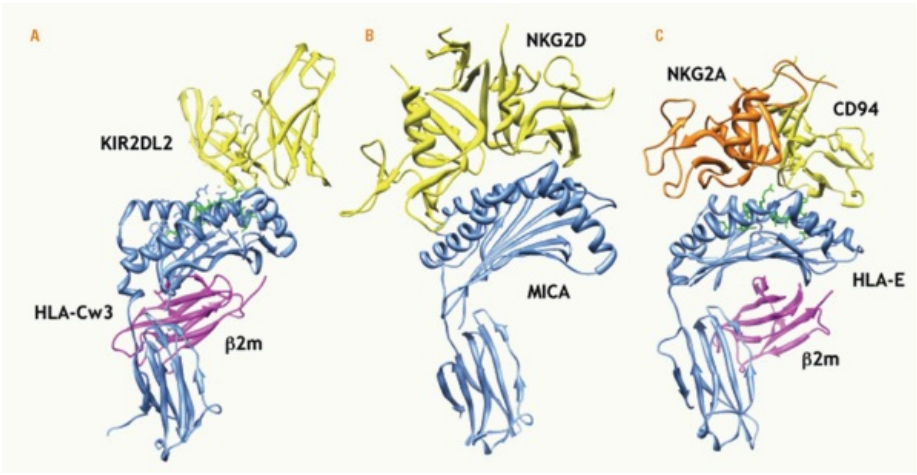

FIG. 17.5. Additional Structures of Natural Killer-Cell Receptors in Complex with Their Ligands. A: Human KIR2DL2 with HLA-Cw3 (PDB ID = 1EFX).236 Two KIR molecules are apparent in the crystal structure with only one molecule (killer Ig-like receptor [KIR] A) contacting the human leukocyte antigen (HLA) molecule. In this view, KIR B molecule is not shown. **B:** Human NKG2D with MICA (PDB ID = 1HYR).[412] **C:** Human CD94/NKG2A with HLA-E (PDB ID=3CDG).[382] The figures were produced and oriented as described in Figure 17.4.

Also unlike the Ly49s, the KIRs are encoded in the LRC on human chromosome 19q13.4 that encodes many other Ig-like receptors (see Fig. 17.3)[311]; the KIR genes are clustered toward the telomeric end of the LRC.[312] Interestingly, the mouse LRC on chromosome 7qA1 does not include genes for KIR-like molecules that instead are encoded on the X chromosome.[313,314] On the other hand, like the Ly49s, the KIRs display remarkable polymorphism with at least 11 genes[315,316,317] (see www.ebi.ac.uk/ipd/kir/index.html for updated database).

The human *KIR* locus also demonstrates considerable haplotype diversity with at least 27 different haplotypes,[315,316,318] recently defined at the sequence level.[317] While there has not been a consensus definition, two major types of haplotypes have been described.[289] Group B contains one or more of the following: *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*,

KIR2DS5, and *KIR3DS1*, whereas group A haplotypes have none of these genes. Reflecting extensive allelic polymorphism of individual genes, a large number of different KIR genotypes have been described, and they are distributed differently in the various ethnic populations.

As with the Ly49s, only a few KIR alleles have been well characterized with respect to HLA class I specificities. Nonetheless, these genetic variants have not only provided clues to new receptors and ligand specificities but also valuable links to the role of NK cells and their receptors in

disease pathogenesis (discussed in the subsequent clinical section), and more broadly, human evolution.

Convergent Evolution of Major Histocompatibility Complex-Specific Natural Killer-Cell Receptors

Despite the controversy surrounding the initial cloning of mouse Ly49s and human KIRs and leukocyte Ig-like receptors (LILRs), additional data have provided new interpretations of the distinctly different receptors used by mouse and human NK cells, respectively, to recognize MHC-I. Detailed genome sequence information is available on the NKC and LRC in mice, humans, and other species. In the mouse, MHC-specific NK-cell receptors with Ig-like domains have not yet been described, although there is conservation of several genes in the broader LRC on mouse chromosome 7[312] (see Fig. 17.3). Activated mouse NK cells do express gp49b (Lilrb4), an Ig-like inhibitory receptor also expressed on other leukocytes, including mast cells.[319,320,321,322] However, it is not expressed by resting NK cells and is not specific for MHC-I. Instead, it binds the integrin αvβ3 and appears more important for responses of other cells, such as neutrophil, eosinophil, and DCs.[323,324,325,326,327,328] Mouse *Kir3dl1* is on the X chromosome (see Fig. 17.3) and expressed in NK and T cells, but its function and ligand remain unknown.[313,314] The Ly49 locus in humans consists only of *LY49L* (*KLRA1*) that is a pseudogene because of a point mutation that gives rise to a splicing abnormality.[329] Thus, current data indicate that mouse NK cells do not express functional KIR orthologues while human NK cells do not express functional Ly49 orthologues.

One reason for this discrepancy may be that the corresponding orthologue is present in the genome but has not been identified. Indeed, genomic sequencing has revealed a multitude of candidate orphan receptors in the genome and specifically the LRC and NKC that have yet to be studied carefully.[311,330] In that regard, identification and functional analyses of NK cell receptors led to the recognition that they are encoded in genomic regions containing gene clusters for related receptors that are expressed on other leukocytes, not just NK cells. Dissection of the expression and function of these receptors is a rich area of research that is beyond the scope of this chapter.

The alternative and currently favored view for the discrepancy is that mice and humans independently evolved analogous receptors to serve the same function. While both human and mouse NK cells express a conserved relatively nonpolymorphic lectin-like receptor, CD94/NKG2, it does not possess many of the features that are shared by mouse Ly49 and human KIRs:

1. both Ly49s and KIRs are constitutively and selectively expressed on naïve, unstimulated

NK cells (with exceptions for rare populations of T cells);

2. both bind MHC-I molecules with intermediate affinity (K^D = 2-10 μM);
3. binding to MHC is promiscuous;
4. MHC-bound peptides have only a modest effect, if at all, on recognition;
5. both use ITIMs to inhibit NK-cell activation;
6. they are expressed in a stochastic fashion on overlapping subsets of NK cells;
7. a single NK cell simultaneously expresses one or more of either type of inhibitory receptor (each may be functional);
8. once they are expressed, their expression appears to be stable;
9. both are germ-line encoded by small families of genes that are clustered in the genome;
10. both display impressive polymorphism, in terms of gene number and alleles for each gene;
11. both are related to molecules that lack ITIMs and instead are activation receptors; and
12. both are involved in NK-cell education by self-MHCI (see following discussion).

Thus, the mouse Ly49 receptors and human KIRs are analogous receptors in an apparently striking example of convergent evolution,[331] whereby each species came up with a different genetic solution to provide extremely important functions for species reproduction and survival.

In other species, *Ly49* and *KIR* genes have been analyzed primarily with respect to sequence and gene number.[332] For example, the *LY49L* gene in baboons appears to be functional but the putative polypeptide lacks an ITIM.[333] In rats, the *Ly49* cluster appears to have markedly expanded with at least 25 genes,[334,335] demonstrating one of the most rapid rates of gene expansion.[336] Dog, cat, and pig appear to have only one *Ly49*, whereas horse represents the only known nonrodent mammal with several *Ly49* genes.[337] The chicken genome has several lectin-like receptor genes that are genetically linked to the MHC.[338,339,340] On the other hand, multiple KIR genes have been described in primates and cattle.[341,342,343,344] Rhesus macaques have a profound plasticity of KIRs with multiple genotypes and haplotypes[345] encoding receptors that bind MHC-I.[346,347] In rat, a KIRlike sequence has been reported,[314] and dogs and cats lack functional KIRs.[348] Interestingly, pigs and marine carnivores each possess a single Ly49 and KIR gene, but it is not clear if these are functional.[348] Finally, several species have no readily identifiable Ly49 or KIR genes[332] (eg, teleost fish instead possess a large number of novel immunetype receptors with sequence homology to mammalian LRC-encoded receptors[349]). Perhaps these species have had alternative convergent evolutionary stratetgies to preserve inhibitory MHC-I-specific receptors.

Additional studies of the Ly49-like and KIR-like molecules as well as potential new orthologues in other species will be of interest to evolutionary biologists for several reasons including prior description of NK-like cells in lower vertebrates and MHC genes that are coevolving.[332,350] Moreover, in primitive chordates, NK-like, missing-self-like recognition

affects histocompatiblity reactions[351] but the molecular determinants of histocompatibility involves molecules unrelated to mammalian Ly49, KIR, or even MHC itself.[352,353,354] Thus, evolutionary studies of NK-cell receptors and their ligands may provide unique insight into missingself and other histocompatiblity reactions.

While we have focused thus far on mouse Ly49 and human KIR as the major MHC-I-specific receptors, there are other well-described NK-cell receptors belonging to

either the lectin-like receptor or Ig-like receptor superfamilies. These receptors also have specificities for MHC-I molecules.

Human and Mouse CD94/NKG2

The analysis of CD94 (Klrd1) and NKG2 (Klrc, excluding NKG2D [Klrk1]) family of molecules was especially challenging and required insightful investigations. Identified by subtractive hybridization, the human NKG2 molecules are type II integral membrane proteins with external C-type lectin domains[355] encoded in the NKC[356] (see Fig. 17.3). Initial attempts to express NKG2 molecules on the cell surface were thwarted. Meanwhile, mAb reactivity suggested that CD94 was variably expressed on human NK cells as a disulfidelinked dimer (70 kDa NR, 43 kDa R),[357] and both activation and inhibition functions for CD94 were described.[358,359,360,361] Surprisingly, cDNA cloning revealed that CD94 has a short seven amino acid cytoplasmic domain, suggesting that it cannot signal on its own.[360] Furthermore, anti-CD94 immunoprecipitates were not detectable from radiolabeled CD94 transfectants, despite easily detectable expression on FACS analysis with the same mAbs.[362,363] These apparent discrepancies were resolved when it became clear that CD94 heterodimerizes with NKG2 molecules[362]; NKG2A is the 43 kDa molecule previously identified as Kp43 with anti-CD94 mAbs.[364,365] While CD94 may be expressed as a homodimer, the NKG2 partner provides the signaling motif, whether activation or inhibition.[364,366] (NKG2B is an alternatively spliced form of NKG2A. The rest of the NKG2 family is discussed in the following.)

The ligand specificity for CD94/NKG2 receptors was also initially thought to be promiscuous as interactions with many classical (class Ia) and nonclassical (class Ib) HLA molecules had been described.[359,362,364,367,368,369,370] However, human CD94/NKG2 receptors directly recognize HLA-E, a MHC-Ib molecule homologous to mouse Qa-1.[371,372,373] HLA-E (and Qa-1) is widely expressed with limited polymorphism.[374,375,376] While HLA-E heavy chain is expressed with β2m and a peptide occupying its peptide-binding cleft, its peptide repertoire is largely derived from the leader sequences of MHC-Ia molecules, as previously noted for mouse Qa-1.[377,378] HLA-E (or Qa-1) expression thus requires normal production of HLA-E (or Qa-1) and synthesis of certain MHC-Ia molecules. Mouse CD94/NKG2 recognizes Qa-1 that shares many features with HLA-E.[379,380] These findings need to be considered in the context of the prevailing view at the time that mouse and human NK cells use structurally different receptors to recognize MHC-I molecules.[381] Clearly, the CD94/NKG2 receptors and their ligands are conserved in humans and mice.

The crystal structure of human CD94/NKG2A bound to HLA-E was resolved to 2.5Å and 4.4Å resolution.[382,383] Remarkably, CD94/NKG2A interfaces with HLA-E in a manner analogous to TCR recognition of peptide-loaded MHC-I,[384] including TCR binding to HLA-E itself.[385] Both innate and adaptive receptors for HLA-E lay across the peptide-binding cleft, though peptide itself plays a relatively minor role in binding CD94/NKG2A.[382,383] Strikingly, this binding is distinct from that of Ly49 receptors to their classical MHC-I ligands (see previous discussion). On the other hand, this binding interface is similar to binding of another NKC-encoded, lectin-like receptor, the NKG2D activation receptor, to its MHC-like ligands (see following discussion). Thus, NKC-encoded, lectin-like receptors surprisingly use different strategies to contact their ligands, even though these ligands have structurally related MHC-I folds.

Despite its conservation between mice and humans, the role of CD94/NKG2 receptors in NK-cell function is still incompletely understood. For example, viruses encode peptides that bind and enhance expression of HLA-E, providing a CD94/NKG2A-dependent mechanism to avoid NK-cell attack.[386,387] By contrast, human NK cells expressing CD94/NKG2C (an activation receptor) expand in response to cytomegalovirus (CMV)-infected targets.[388] When a large number of human NK-cell clones were obtained from two normal individuals, CD94/NKG2 seemed to account for the majority of self-MHC-specific receptors on clones from one individual whereas KIRs dominated the self-specific receptors on clones from the other individual, suggesting that some individuals may depend on CD94/NKG2 for self-tolerance.[389] Qa-1 and HLA-E can present peptides derived from other molecules, including the signal sequence of heat shock protein 60 (Hsp60) that is induced by a number of stimuli,[390,391] a multidrug resistance transporter,[392] or blastocyst MHC expressed in embryonic tissues.[393] This may result in loss or gain of recognition by the inhibitory CD94/NKG2A receptor, suggesting intrinsic mechanisms to perturb inhibition by CD94/NKG2A in certain circumstances.

Yet, CD94 appears to be dispensable in certain strains of mice, such as DBA/2J, that do not appear to have any untoward NK-cell phenotype.[394] This finding has been recapitulated in studies of a CD94 knockout mouse on the 129 strain background.[395] Interestingly, CD94 knockout mice are more susceptible to ectromelia virus,[396] as detailed below.

CD94/NKG2 molecules may be important in T-cell function. CD94/NKG2A is rapidly induced on antigenspecific CD8+ T cells during polyoma virus and other infections,[397,398,399] and CD8+ T cells expressing CD94/NKG2A preferentially proliferate during persistent infection, suggesting that CD94/NKG2 receptors may play a role in memory T-cell responses,[400] and that TCR specificity is correlated with CD94/NKG2A expression by human CTL.[401] Indeed, CD94/NKG2A inhibits antigen-specific cytotoxicity in polyoma virus responses, although this effect is pathogen-dependent. CD94/NKG2A has been studied with other viral infections, including herpes simplex virus,[402] murine CMV,[403] gHV68, and influenza,[404] for example. Although not all studies revealed functional consequences, the recurring theme is the appearance of CD94/NKG2A on previously activated CD8+ T cells. Thus, CD94/NKG2 receptors may regulate T-cell responses.

Other Human Immunoglobulin-like Receptors Specific for Major Histocompatibility Complex-I Molecules

The human LILR family is encoded in the LRC (see Fig. 17.3), just centromeric to the *KIR* genes. There are two general forms of these receptors, subfamily A that appears to be activation receptors, and subfamily B that has the ITIMs

characteristic of inhibitory receptors. The best characterized members, LILRB1 (also known as CD85j, Ig-like transcript 2 [ILT2], or leukocyte IG-like receptor 1 [LILR1]) is broadly expressed whereas LILRB2 (CD85d, ILT4, or LIR2) is not expressed on NK cells but is expressed by myelomonocytic cells, including DCs and monocytes. Both recognize HLA class I molecules[405,406] with LILRB1 exclusively binding folded HLA class I molecules with β2m, whereas LILRB2 can bind both folded and free HLA class I heavy chains.[407] Interestingly, a human CMV protein, UL18, binds LILRB1 with 1000-fold higher affinity than HLA molecules, implicating a role for LILRB1 in host defense.[408] The ligands and functions of other LILRBs (LILRB3 [CD85a, ILT5, LIR3], LILRB4 [CD85k, ILT3, LIR5], LILRB5 [CD85c, LIR8], and LILRB6 [CD85b]) are as yet unknown but they may not be able to bind HLA due to structural constraints.[409]

LILRB1 has four Ig-like domains and binds a conserved region in the α3 domain of most, if not all, classical and nonclassical HLA class I molecules (HLA-A, -B, -C, -E, -F, and -G).[408] Interestingly, the crystal structure of LILRB1 bound to HLA-A2 (3.4Å resolution) reveals that it binds MHC molecules under the peptide-binding domain where it contacts α3 and β2m, more akin to Ly49 engagement of MHC than KIR[409] (see Figs. 17.4 and 17.5). Even though LILRB1 and LILRB2 have differing capacities to bind free HLA molecules, LILRB2 has an HLA class I binding site that overlaps with but is distinct from that for LILRB1.[410] LILRB1 binds UL18 in a manner structurally similar to HLA class I binding.[411]

Interestingly, LILR genes demonstrate allelic polymorphisms,[412] though less so than the adjacent KIR cluster.[413] Nonetheless, polymorphisms in LILRB1 may affect receptor expression.[414] Moreover, polymorphisms in LILR genes are associated with certain autoimmune diseases, such as rheumatoid arthritis.[415]

Major Histocompatibility Complex-Independent Natural Killer-Cell Inhibitory Receptors

As already mentioned, NK cells also express inhibitory receptors for non-MHC ligands, such as mouse gp49b, which binds the αvβ3 integrin,[323] and there is a growing list of other such molecules.[416] Some of these receptors will be discussed in a more appropriate context in the following sections. Most of these receptors contain cytoplasmic ITIMs so their inhibitory function can be predicted even if not directly tested, though some caution is required because the motifs may be involved in other signaling processes.

Human and mouse NK cells express the ITIM-bearing leukocyte-associated Ig-like receptor 1 (LAIR-1, CD305), which is an Ig-like molecule broadly expressed by most leukocytes and encoded in the LRC.[417,418] Initial reports indicating that LAIR-1 binds epithelial cellular

adhesion molecule were irreproducible.[419,420] Instead, LAIR-1 binds multiple forms of collagen,[421] which has been validated in crystallographic and biochemical studies.[422] Interestingly, LAIR-1 mediates inhibition that is independent of Src homology 2 (SH2)-domain-containing phosphatases, and instead recruits C-terminal Src kinase (Csk),[423] suggesting that Csk may be involved in inhibitory signaling. Although the in vivo context for functional interaction awaits further characterization, it is reminiscent of the broader reactivity of the Siglecs.

The CD33-related sialic acid binding Ig-like lectins (CD33rSiglecs) are type I receptors with varying numbers of Ig-like domains expressed on a broad array of cells and encoded in the "extended" LRC[312,424,425] (see Fig. 17.3). Despite having sialic acid recognition in common, the Siglecs appear to show differences in carbohydrate recognition, depending on the specific glycan context.[424] Human NK cells express Siglec-7 (p75, adhesion inhibitory receptor 1 [AIRM1]) and Siglec-10, whereas some mouse NK cells express a related Siglec-E.[425] Siglec-7 has been most extensively studied. As expected, its cytoplasmic ITIM can recruit SHP-1 and inhibit NK-cell functions.[426,427] Moreover, expression of its ligand on targets inhibits NK cells in a Siglec-dependent manner.[428] However, the effects appear to be modulated by *cis* interactions between the Siglec receptor and its carbohydrate ligands on the NK cell itself,[424,428,429] reminiscent of *cis* interactions between Ly49 and MHC ligands, as previously discussed.

The Ig-like receptors, termed paired Ig-like receptor (PILR)α and β, are not encoded in the LRC, rather on human chromosome 7 (mouse chromosome 5).[430,431] Mouse PILRα is an ITIM-containing inhibitory receptor, whereas PILRβ is an activation receptor that couples to DAP12.[430,432] Both receptors are expressed on NK (and other immune) cells and recognize CD99. Interestingly, sialylated O-linked glycans on CD99 are involved in recognition by PILRs.[433]

Thus, NK cells (and other leukocytes) express multiple inhibitory receptors that are capable of MHC-independent recognition. How they participate in NK-cell responses and contribute to MHC-dependent effects are beginning to be elucidated, and some appear to play a specific role in the context of activation receptors, as discussed in the following.

Natural Killer-Cell Activation Receptors

NK cells clearly kill MHC-I-deficient targets more efficiently than MHC-I-sufficient targets. However, this enhanced killing does not occur simply because a nonspecific default pathway is unleashed when MHC-I is absent. Instead, it is clear that susceptible targets express ligands for NK-cell activation receptors. In general, these receptors and their ligands were defined following description of the inhibitory receptors.

Approaches to Identification of Activation Receptors

Initial progress in elucidating NK-cell activation receptors was difficult. The approaches that yielded the molecular definition of the TCR, such as subtractive hybridization, mutagenesis of T-cell tumors, and anticlonotypic mAbs,[434,435,436] were of limited success.[437,438] Unlike the working paradigm of MHC restriction that guided the molecular identification of the TCR,

the principles guiding NK-cell activation by targets were unclear. Breakthroughs in identifying NK-cell activation receptors thus required other approaches.

Some activation receptors were recognized because they were first identified on other cells, such as FcγRIII. Other

activation receptors were identified by genetic means, such as cDNA clones for molecules resembling the inhibitory receptors but lacking cytoplasmic ITIMs. Others were identified by a genetic positional cloning approach. Specific stimulation of NK cells through mAbs proved useful for the initial identification of candidate activation receptors and to validate the activation function of receptors identified by other means.

NK cells can be stimulated to mediate antibody-dependent cellular cytotoxicity (ADCC) through the FcγRIII (CD16) receptor that binds the Fc portion of the IgG coating a target. In a related way, anti-FcγRIII can also trigger through CD16 in a process termed “redirected lysis” or “reverse ADCC” because the antibody binds in the opposite orientation to ADCC. A few mAbs against other NK-cell surface molecules also can activate in the redirected lysis assay, highlighting a relatively unique functional property of the recognized molecules (and mAbs), because activation does not occur when most NK-cell surface molecules are cross-linked. First, popularized for analysis of anti-TCR antibodies,[439] redirected lysis occurs when IgG reacts specifically with the NK-cell receptor, and its Fc portion binds a target cell Fc receptor (FcγR) that apparently provides bridging and cross-linking effects.[439] Target lysis does not occur if FcγR binding on the target is prevented with FcγR-deficient targets, $F(ab')_2$ fragments of the anti-NK-cell receptor antibody, or anti-target cell FcγR Ab blockade. In the latter case, Fc regions must be removed to prevent inadvertent triggering of conventional ADCC via CD16 on the NK cell. Thus, the redirected lysis assay is a helpful experimental tool.

Gene transfer studies have been helpful adjuncts to study NK-cell receptors. NK cells are difficult to transfect, and there are few useful tumors with the notable exception of RNK-16, a rat NK tumor line.[440] Viral vectors, such as vaccinia virus, have been useful for gene transfer with the caveat that functional experiments have to be performed within a small time frame before untoward effects occur.[441] Recent use of lentiviral vectors also show promise for gene transfer into primary NK cells.[442]

Recent studies have exploited reporter cell assay systems similar to those used to identify TCR ligands.[443] ITAMmediated signaling leads to inducible, nuclear factor of activated T cells (NFAT)-dependent expression of a reporter molecule, such as β-galactosidase or GFP. Even an inhibitory receptor can be used to activate the reporter cell by fusion of its extracellular domain to a suitable transmembrane and cytoplasmic domain containing ITAMs. Such reporter cells can then be used to detect ligands.[444,445,446]

In the following sections, we will describe NK-cell activation receptors with an emphasis on those with known ligands.

Activation Receptors Related to Major Histocompatibility Complex-Specific Inhibitory Receptors

Despite initial characterization as inhibitory receptors for MHC-I, the Ly49 family contains

other members (ie, Ly49D and Ly49H in C57BL/6 mice) without cytoplasmic ITIMs. Instead, they are activation receptors containing charged transmembrane residues for association with DAP12 that facilitates expression and provides signal transduction capacity.[447,448,449,450] Although Ly49H will be considered below in the context of viral infection, Ly49D has no known role in viral defense. A positional cloning approach indicated that Ly49D is the product of the *Chok* locus, which controls NKcell specificity for killing of a xenogeneic target, Chinese hamster ovary cells,[159,451] due to recognition of a Chinese hamster MHC-I molecule.[452] Interestingly, when Ly49D was transfected into RNK-16 cells, Ly49D can recognize $H2D^d$,[440] but $H2D^d$ tetramers do not bind Ly49D for unclear reasons, although potentially reflecting lower avidity.[213,262] Several other Ly49 receptors have been identified in non-C57BL/6 mouse strains that have properties of activation receptors (charged transmembrane residues, no ITIMs) but they have been less well characterized,[257,258] except for Ly49P in MA/My mice, which is involved in controlling viral infection that is related to MHC-I recognition[453] (see subsequent details). Thus, some members of the Ly49 family are activation receptors with apparent specificity for MHC-I, potentially with less avidity.

Molecular cloning of the KIR family also led to the identification of two domain receptors (also known as p50) or three domain Ig-like receptors with short cytoplasmic domains lacking the ITIM.[454,455] These molecules are now known as the KIR2DS or KIR3DS, respectively, with numbers for specific molecules (ie, KIR2DS1 [CD158h, p50.1], KIR2DS2 [CD185j, NKAT5, p50.2, clone 49], KIR2DS3 [NKAT7], KIR2DS4 [CD158i, NKAT8, clone 39], KIR2DS5 [CD158g, NKAT9], and KIR3DS1 [CD158e2]). Expression of these molecules may be difficult to determine because mAbs for KIR2DL molecules cross-react with KIR2DS molecules.[456] KIR2DS molecules can associate with DAP12 and activate NK cells in the redirected lysis assay.[454,457] KIR2DS and KIR3DS molecules can recognize HLA class I molecules with specificities similar to corresponding inhibitory KIRs, apparently with lower avidity.[458,459,460,461] However, KIR2DS4 and HLA-C alleles and KIR3DS1 and HLA-B alleles influence their respective interactions.[462,463] Thus, further analysis is needed to support the hypothesis that activating forms of KIRs bind HLA alleles less well than the inhibitory receptors as a potential explanation for dominance of inhibition over activation (see Fig. 17.2D).

It remains possible that the activating forms of the Ly49s and KIRs have other ligands and perhaps their MHC specificities are instead somehow related to their physiologically relevant ligand. Indeed, this has been demonstrated for Ly49H, an activation receptor that recognizes a virusencoded ligand with an MHC-I-like fold.[444,446,464] Moreover, KIR2DS4 may recognize a non-MHC ligand.[465] Thus, further analysis is required for understanding the role of Ly49 and KIR activation receptors in MHC-I recognition and with respect to their inhibitory counterparts.

In addition to NKG2A, the NKG2 family also contains NKG2C, NKG2E, and NKG2F, which are products of different genes.[355,466] (NKG2B is an alternatively spliced isoform of NKG2A, whereas NKG2H is an alternatively spliced isoform of NKG2E.) NKG2C and NKG2E lack cytoplasmic ITIMs and contain charged transmembrane residues for association with

DAP12.[467] While the role of NKG2F is unknown

because it lacks an external domain and remains inside the cell, associated with DAP12 but not with CD94,[468] NKG2C and NKG2E form functional heterodimers with CD94.[364] Like CD94/NKG2A receptors, these heterodimers recognize HLA-E or Qa-1, but unlike CD94/NKG2A receptors, they activate NK cells.[469,470] Interestingly, the inhibitory form binds with higher affinity to HLA-E than the activating form.[382,383,471,472] There also appears to be some peptide preference between the different functional forms[382,383,473] that may be relevant in certain physiologic situations. Thus, the CD94/NKG2 receptors may discriminate between subtle differences in their MHC-Ib ligands.

FcγRIII (CD16)

Frequently overlooked but perhaps the first molecularly defined activation receptor on NK cells is FcγRIII (CD16), through which NK cells mediate ADCC against IgG-coated targets.[474,475] Unlike other Fcγ receptor-bearing effector cells, NK cells are generally thought to express only one of the known Fcγ receptors that binds IgG with low affinity,[476] although others suggest that human NK cells may express FcγRII isoforms.[477] There are two human FcγRIII isoforms with identical extracellular domains.[476] Human NK cells express only FcγRIIIA., which is a transmembrane molecule, whereas FcγRIIIB has a glycosylphosphatidyl-inositol (GPI) linkage and is expressed by neutrophils. In mice, only the transmembrane isoform (FcγRIII) is present[478] and displays 95% sequence conservation with muFcγRII. The recently described FcγRIV is a newly recognized orthologue of human CD16A but is not expressed on mouse NK cells.[479,480] There are species differences in CD16 binding to mouse IgG isotypes; mouse IgG3 mAbs bind human CD16 the most efficiently (3 > 2a > 2b >> 1), whereas they bind mouse CD16 with the lowest affinity (2b > 2a > 1 >> 3).[476] In the laboratory, a rabbit antimouse Ig polyclonal Ab, whose Fc portion binds strongly to both human and mouse CD16, could be added to facilitate Fc receptor binding.

The transmembrane FcγRIII molecules are physically associated with FcεRIγ and less commonly with CD3ζ.[481] FcγRIII can also associate with γζ heterodimers. The associated chains are required for optimal cell surface expression of FcγRIII and for signal transduction. After cross-linking, FcγRIII activates biochemical events that are reminiscent of T-cell activation, leading to granule exocytosis and cytokine production.[161,482,483,484,485] In vivo, ADCC may be useful in host defense against pathogens or infected cells if Abs are bound to their surface, triggering not only killing but also cytokine production and other NK-cell responses. Although NK cells are generally thought to participate early in a primary immune response (see subsequent discussion), the delay required for isotype switching to IgG production suggests that CD16 cross-linking on NK cells plays a role in secondary immune responses in vivo.

Regardless, ADCC is remarkably similar to natural killing and was important for the initial establishment of the concept of NK-cell activation receptors.[161] Yet, CD16 is not required for NK-cell target recognition because human CD16-CD3- lymphocytes can still mediate natural killing.[486] Moreover, CD3ζ is phosphorylated upon CD16 ligation but not when NK cells are

exposed to NK-sensitive targets. Deficiency of γ chain abrogated ADCC but not natural killing.[474] Thus, CD16 is not involved in natural killing.

CD16-related artifacts must be considered when studying NK cells. Flow cytometry experiments may be flawed if CD16 binding is not taken into account. To eliminate this possibility, $F(ab')_2$ fragments should be used. Alternatively, blockade of FcγRIII binding may be sufficient with protein A or G (that bind Fc region on Ig) or anti-FcγRIII mAbs, such as unlabeled mAb 2.4G2 (ATCC HB 197) that reacts with both mouse FcγRII and FcγRIII.[478] Similarly, as discussed previously, antibody blockade experiments should be done with caution if the antibody specifically reacts with the target because ADCC may be stimulated.

NKG2D

NKG2D (KLRK1) was first cloned from human NK cells as a cDNA related to NKG2A and C.[355] However, NKG2D is distinct from other NKG2 molecules for several reasons. There is only limited sequence homology between NKG2D and other NKG2 molecules (28% amino acid identity for the lectin-like domain) whereas other NKG2 molecules are closely related to each other (70% identity). Rather than heterodimerizing with CD94, NKG2D is expressed as a disulfide-linked homodimer on all NK cells in humans and mice. In humans, NKG2D is also expressed on all γδTCR+ and CD8+ T cells, whereas in mice, NKG2D is expressed on most NKT and γδTCR+ T cells but not on resting CD8+ T cells.[81,82,83] However, essentially all activated mouse CD8+ T cells express NKG2D. In both humans and mice, CD4+ T cells do not express NKG2D, but it is found on a subset of CD4+ T cells in patients with rheumatoid arthritis.[84] Finally, NKG2D has functional properties and ligand specificities that distinguish it from the other NKG2 molecules, indicating that NKG2D should not be considered as a member of the NKG2 family.

NKG2D does not have any known cytoplasmic motif and was first shown in humans to preferentially associate with a signaling chain termed DAP10, encoded by a gene localized 130 bp away from the gene for DAP12.[487] DAP10 does not have any ITAMs; instead, it contains a YxxM motif for recruitment of PI3K.[487] This motif is similar to that found in CD28, and functional studies indicate that NKG2D can act as a costimulatory molecule on T cells.[488,489,490] Moreover, DAP10 has a site for recruitment of Grb2.[491] Thus, NKG2D may provide qualitatively different signals, resulting in different cytokine production, for example,[490] than activation receptors associated with ITAM-signaling chains.

Other studies have suggested that NKG2D functions as a primary activation (triggers alone) rather than costimulatory receptor (does not stimulate unless it synergizes with another receptor) on NK cells.[80,492] Such studies need to be reconsidered in light of several factors. 1) Many studies of NKG2D function use targets that are poorly killed by NK cells. When these targets are transfected with NKG2D ligands, killing is enhanced in an NKG2D-dependent manner. However, such studies do not distinguish whether NKG2D functions as a primary activation receptor or as a costimulatory receptor (analogous to CD28 requirement for

full activation of T cells) as the same experimental outcome is anticipated in either case. 2) When cross-linking is done with immobilized mAbs alone and CD16 coengagement on NK

cells is avoided, NKG2D functions as a costimulatory receptor on mouse IL-2-activated NK cells.[80] 3) In mice but not humans, there are two alternatively spliced isoforms of NKG2D.[34,493] A long form (NKG2D-L) contains a 13 amino acid extension at the amino terminus (cytoplasmic domain) as compared to the short form (NKG2D-S). Resting NK cells predominantly express NKG2D-L that preferentially associates with DAP10. However, activation of NK cells with cytokines causes a transient increase in NKG2D-S that associates with ITAM-containing DAP12 as well as DAP10. However, others have found that NKG2D-L can associate with DAP12, albeit to a lesser degree, and that both isoforms are present in resting NK cells.[494] Regardless, in the absence of DAP10, mouse NKG2D can associate with DAP12,[35] allowing it to signal akin to a primary activation receptor. Thus, mouse NKG2D is an unusual example of a receptor with the same extracellular domain but with potentially different functional outcomes (primary activation versus costimulation), depending on its associated partner chain.

There is remarkable plasticity for NKG2D in that it can bind many apparently disparate ligands that are only superficially related to each other by sequence alignment, and human NKG2D can bind mouse ligands and vice versa. Based on soluble MICA binding, the first human NKG2D ligands were found to be MICA and MICB (MHC-I chain-related, A and B) encoded on chromosome 6p21.3, centromeric to HLA-B locus.[81] Subsequently binding studies of UL16 from human CMV (HCMV) identified the ULBP (UL16 binding protein) family that in turn binds NKG2D.[495] The UL16-binding proteins (ULBPs) are encoded by genes in the retinoic acid expressed transcript (*RAET1*) gene family (official HUGO nomenclature) encoded on chromosome 6q24.2-q25.3 and discovered by genomic mining. Whereas *RAET1F*, *RAET1J*, *RAET1K*, and *RAET1M* are pseudogenes, other *RAET1* genes give rise to ULBP1 (RAET1I), ULBP2 (RAET1H), ULBP3 (RAET1N), ULBP4 (RAET1E), ULBP5 (RAET1G), and ULBP6 (RAET1L), some of which have been extensively studied as ligands for NKG2D.[495,496,497,498] MICA, MICB, ULBP4, and ULBP5 are considered to be transmembrane proteins whereas the other RAET1 proteins are GPI-linked.[499]

Mouse NKG2D ligands were discovered with soluble NKG2D that was used for expression cloning.[82,83] Two types of ligands were originally defined: the minor histocompatiblity antigen H60 and members of the retinoic acid early inducible gene-1 (RAE-1) family. It is now clear that the mouse NKG2D ligands are the H-60 family, consisting of H60a, b, and c, the RAE-1 family, consisting of RAE-1α, β, γ, δ, and ε (also known as Raet1a-e), and murine ULBP-like transcript (MULT1).[82,83,500,501,502] There are strain-specific differences in ligand expression. For example, BALB/c mice express H60a, RAE-1α, β, and γ, whereas C57BL/6 mice do not express these molecules and express RAE-1δ and ε.[503] In addition, there are allelic forms of NKG2D ligands (eg, 80 different alleles of MICA and 33 alleles of MICB [hla.alleles.org/classo.html]), b ut these have not been fully characterized in humans (or mice).

Where studied, all NKG2D ligands have distant amino acid similarity to MHC-I, although they share only about ˜25% amino acid identity with each other. Within a family, however, they may be much more closely related (up to 90% identity). None of the NKG2D ligands associates with β2m or binds peptides.[504,505,506,507] Many contain only the α1/α2 platforms and

many are GPI-linked to the plasma membrane. The ligands display binding to NKG2D in two ways based on affinity.[501,502,504,508,509] For example, RAE1α, β, γ, and δ, and H60b and c demonstrate low-affinity interactions with mouse NKG2D, similar to MICA to human NKG2D (K_D = ~300 to 8700 nM). In contrast, RAE1ε (also described as RAE1B6),[509] H60a and MULT1 show a much higher affinity interaction (K_D = 6 to 30 nM). Thus, closely related molecules within a family may display wide disparity in affinities whereas distantly related molecules can share high affinity with NKG2D.

Structural studies at 2.6 to 3.5 Å resolution indicate that the NKG2D ligands have MHC-I-like folds, though the "peptide-binding cleft" is closed.[504,505,510] NKG2D binds its ligands more analogous to TCR docking on MHC and unlike Ly49 recognition (see Figs. 17.4 and 17.5), despite the relationship of NKG2D and Ly49 receptors as NKCencoded, lectin-like homodimers that bind MHC-related molecules.[504,510,511] Indeed, NKG2D interactions with its ligands are much more aligned with CD94/NKG2A recognition of HLA-E, as described previously. Moreover, NKG2D uses largely nonoverlapping patches to engage a similar orthogonal footprint on its disparate ligands. Interestingly, the putative immunoevasion molecule, UL16, binds MICB in essentially the same manner as NKG2D is predicted to bind, even though UL16 has a different three-stranded β-sheet structure.[512] Additional structures of NKG2D complexed with other ligands should yield additional insight as to how NKG2D can bind a panoply of distantly related ligands with varying affinities.

NKG2D function has been described in the "inducedself" model (see Fig. 17.2E) because expression of its ligands can be inducible and can override inhibitory influences of MHC-I.[513,514,515] In many cases, low basal transcriptional levels are markedly upregulated in pathologic conditions. For example, MICA and MICB expression is markedly enhanced on epithelial tissues in inflammatory bowel disease.[516] Whereas this notion was initially thought to be related to heat shock elements in MICA promoter, recent detailed studies indicate that NKG2D ligand transcription in some cells is not affected by heat shock or hypoxia in vitro.[517] Instead, transcription of NKG2D ligands can be induced by several other stimuli. For example, DNA damage from ionizing radiation or chemotherapy agents results in upregulation of human and mouse NKG2D ligand transcripts and concomitant surface protein expression.[517] This process was dependent on activation of the ataxia telangiectasia, mutated (ATM), and ATM- and Rad3 (ATR)-related pathways. Interestingly, several micro-ribonucleic acids (miRNAs) can regulate MICA and MICB expression, and they are decreased following heat shock, allowing upregulation of MICA and MICB.[518] On the other hand, Dicer knockdown also results in a DNA damage response that results

in enhanced MICA and MICB expression.[519] Regardless, NKG2D ligand expression due to genotoxic responses may be relevant to chemotherapy effects in cancer, such as multiple myeloma.[520]

Other external stimuli, such as phorbol ester, retinoic acid, cytokines, and TLR stimulation, can induce NKG2D ligand expression on mouse and human cells.[500,521,522] The phorbol ester effect is regulated by the transcription factor JunB[523] and retinoic acid stimulation was

the impetus for original identification of the RAE-1 family.[524] Cytokines can also regulate NKG2D ligands. For example, IFNγ can downregulate transcripts for H60a expression on tumor cells and alter NK-cell susceptibility.[525] For MICA, the IFNγ effect is mediated through miRNAs.[526] Interestingly, TLRinduced primarily MICA and not MICB, through pathways dependent on ATM, ATR, and miRNAs.[522] Viral infection can also induce NKG2D ligands,[527] perhaps not surprisingly because viruses target NKG2D and its ligands (described in the following). For example, murine CMV (MCMV) infection induced RAE-1 mRNA and surface expression within 18 hours. This effect was independent of the DNA damage response but required PI3K activation.[527] However, PI3K activation alone was insufficient to induce ligand expression, suggesting that additional signals are required for NKG2D ligand expression by viral infections and perhaps other stimuli.

For other NKG2D ligands, transcripts are constitutively expressed in a wide variety of tissues[495,501]; posttranslational modifications alter surface protein expression levels. For example, mRNA for MULT1 is widely expressed in normal tissues[501] and MULT1 surface expression is regulated by ubiquitination.[528] MULT1 has a long cytoplasmic domain, unlike most other NKG2D ligands, which are GPI-linked. Under normal circumstances, MULT1 is ubiquitinated on its cytoplasmic Lys residues and is targeted for degradation. However, cellular stress, such as heat shock or ultraviolet irradiation, reduced its ubiquitination and allowed surface expression. In particular, MULT1 expression is regulated by the E3 ubiquitin ligases, membrane-associated RING-CH (MARCH) 4 and membrane-associated MARCH 9.[529] A human virus, Kaposi sarcoma-associated herpesvirus (KSHV), exploits this pathway to avoid NK-cell attack (detailed in the following).

Despite the nearly universal acceptance of the inducedself model for NKG2D function, it is not clear if this hypothesis is applicable to all of its ligands.[530,531] It remains possible that constitutive expression or affinity of some NKG2D ligands may be too low to permit NKG2D activation to override MHC-I-dependent and - independent inhibitory receptors. Continued analysis at the protein level is anticipated, as well as study of NKG2D ligands in specific tissues, exemplied by analysis of H60c in the skin where it is selectively expressed and can also costimulate dendritic epidermal T cells.[532]

On the other hand, chronic exposure to membranebound or soluble NKG2D ligands results in downregulation of NKG2D expression and lower functional responsiveness.[85,533,534,535] Patients with tumors expressing MIC frequently contain soluble MIC in their peripheral blood, presumably as a result of proteolytic cleavage of membrane-expressed ligands,[533,536] though the enzyme required is unclear, and the process apparently requires palmitoylation of MICA.[537] Mice Tg for NKG2D ligands show impotent NKG2D responses,[534,538] a topic better discussed subsequently in the context of NK cell tolerance and education. On the other hand, acute upregulation of an NKG2D ligand alone can rapidly induce immune responses[539] (albeit effects not examined for NK cells). Taken together, these findings may explain why tumors frequently express NKG2D ligands that would otherwise enhance their susceptibility to NK-cell attack, supporting a role for NKG2D in tumor surveillance and also the general concept that NKG2D-mediated activation is balanced between acute, induced-

self, and chronic expression of its ligands.

Additional studies support a role for NKG2D in tumor surveillance. For example, mice are more sensitive to developing methylcholanthrene-induced fibrosacromas when NKG2D is neutralized by chronic anti-NKG2D mAb administration[86] or targeted deletion.[540] Moreover, mice lacking γδT cells are more susceptible to carcinogenesis apparently due to an NKG2D-dependent effect.[500] IFNγ is a known host mediator that shapes the tumor phenotypes in a broader process known as "immunoediting,"[541,542] and IFNγ also mediates downregulation of H60 expression on tumors.[525] Thus, NKG2D and its ligands are important in the host response (or lack thereof) to tumors.

A role for NKG2D in antiviral responses is indicated by studies that show that viruses use different strategies to interfere with ligand recognition by NKG2D. This was first noted when the HCMV protein UL16 was found to bind ULBPs.[495] In infected cells, UL16 retains some (MICB, ULBP1, and ULBP2) but not all NKG2D ligands in the endoplasmic reticulum and cis-Golgi, preventing their expression on the cell surface and protecting from NK cell lysis.[543,544,545] HCMV also encodes UL142, which retains certain alleles of MICA in the cis-Golgi.[546] In mice, MCMV encodes four molecules that downregulate expression of all NKG2D ligands. gp40 from the *m152* open reading frame (ORF) downregulates all five RAE-1 ligands but has no effect on H60 or MULT1.[503,547] H60 is downregulated by the product of the *m155* ORF at a post-Golgi level, perhaps by targeting H60 for proteasomal degradation[548,549] and MULT1 expression is affected by *m145*.[550] The herpes virus Fc receptor (fcr-1), the product of the *m138* ORF, targets RAE-1ε, H60, and MULT1.[551,552] Interestingly, KSHV encodes an E3 ligase, termed K5, that can downregulate MICA and MICB expression.[553] This effect was due to ubiquitinated internalization of MICA, but not degradation, and protected cells from NK-cell cytotoxicity, suggesting that KSHV targets a normal pathway regulating NKG2D ligand expression. HCMV and other herpes viruses (KSHV, Epstein-Barr virus) encode miRNAs that affect MICA and/or MICB expression.[554,555] Non-herpes viruses also target the NKG2D pathway; adenovirus E3/19K sequesters MICA and MICB in the endoplastic reticulum,[556] whereas the orthopoxviruses, cowpox, and monkeypox viruses encode a secreted, high-affinity NKG2D ligand that antagonizes NKG2D recognition and activation.[557] Thus, the multitude of viral strategies that affect NKG2D recognition

strongly suggests that NKG2D plays an important role in the host response to viral infections.

Surprisingly, an NKG2D knockout mouse displayed enhanced resistance to MCMV infections.[87] This mouse also displayed modest changes in NK-cell development with faster NK-cell maturation that were not demonstrated in another NKG2D knockout,[540] even though both were generated directly in C57BL/6-derived embryonic stem cells. A subsequent retargeting of the *Klrk1* locus recapitulated the enhanced resistance to MCMV,[558] which for the moment is at odds with viral neutralization of NKG2D.

Finally, NKG2D may play a role in autoimmune disorders. In autoimmune type I diabetes mellitus (TIDM), MICA polymorphisms are reportedly associated with increased risk,[559] but

MICA is closely linked with *HLA*, which clearly imparts significant risk so additional analysis of large patient cohorts is needed to segregate the *MICA* effect from that of *HLA* alleles in TIDM or any other HLA-associated autoimmune disease.[560] Nonetheless, NKG2D ligands are expressed in the prediabetic NOD mouse pancreas and anti-NKG2D blockade prevents TIDM.[561] In human patients with inflammatory syndromes, such as rheumatoid arthritis, Wegener granulomatosis, and unstable angina, there is an unusual population of CD4+ CD28- NKG2D+ T cells that is expanded.[562,563,564] NKG2D can provide a costimulatory signal for T cells, at least for CD4+ CD28- T cells in rheumatoid arthritis and rheumatoid arthritis synovium expresses NKG2D ligands.[84,489] IL-15 induces NKG2D expression on intestinal epithelial T-lymphocytes that then display a LAK cell-like promiscuous killing capacity against enterocytes which upregulate NKG2D ligands from gliadin exposure, suggesting that NKG2D may play a pathogenic role in celiac sprue.[36,565,566] On the other hand, others have postulated that CD4+ NKG2D+ T cells may be normally immunosuppressive as they are inversely correlated with disease activity in patients with systemic lupus erythematosus.[567] Thus, NKG2D may play a pathogenic role, albeit somewhat unclear, in several autoimmune disorders in humans and mice.

Natural Killer Gene Complex-encoded Lectin-like Receptors Recognize Natural Killer Gene Complex-encoded Lectin-like Ligands

First identified by functional studies in the rat, Nkrp1 (Klrb1) molecules belong to a family of lectin-like, disulfidelinked homodimers with type II orientation encoded in the mouse and rat NKC[71,255,330,568,569,570,571] (see Fig. 17.3). Expression is relatively selective for NK cells, although Nkrp1 molecules are also expressed by NKT cells in rats and mice.[572,573] There is only a single gene (*NKRP1A*, CD161) in humans that is expressed on a subpopulation of NK cells.[574] The continuing allure of the Nkrp1 family stems from observations that NK1.1 (the most widely known specific serologic marker of NK cells) is encoded by *Nkpr1c* (*Klrb1c*) in C57BL/6 mice,[71] Nkrp1 receptors are conserved, and they have interesting genetics with respect to their ligands.[575]

Initial functional studies indicated that rodent Nkrp1 molecules can activate NK cells through redirected lysis,[32,571,576,577,578] which can be prevented by inhibitory Ly49 receptor engagement.[140] Mouse Nkrp1c (NK1.1) is functionally associated with FcεRIγ, although its deficiency curiously does not affect NK1.1 expression.[579] An Nkrp1a loss mutant of rat RNK-16 cells failed to kill certain targets; transfection restored killing capacity, suggesting that rat Nkrp1a is an activation receptor specific for target determinants.[580] Thus, Nkrp1 molecules were among the first described NKcell-specific activation receptors though inhibitory forms were later described.

Inasmuch as Nkrp1 molecules are homologous to C-type lectins, the ligands for Nkrp1 molecules were initially presumed to be carbohydrates but Nkpr1 molecules lack residues for coordinate binding of calcium that is required for authentic C-type lectin recognition of carbohydrates.[222] While the ligands for Nkrp1c have not yet been identified, Nkrp1f recognizes C-type lectin related g (Clrg, also known as Clec2i, Dcl1).[445,581,582,583] Nkrp1f

is presumed to be an activation receptor because it has a charged transmembrane residue and no cytoplasmic signaling motifs. The inhibitory receptor, Nkrp1d, is expressed on all NK cells in C57BL/6 mice and is specific for Clrb (Clec2d; osteoclast inhibitory lectin, Ocil). Thus, the Nkrp1 family is an MHC-independent system that is presumably involved in self-tolerance and is the first example of a lectin-like receptor recognizing a lectin-like ligand unlike MHC-like ligands for other NKC-encoded receptors.

Genome sequence analysis indicates that *Nkrp1d* in C57BL/6 mice is represented by *Nkrp1b* in other strains.[75,76,569,584] The Nkrp1b molecule from SJL and SW mice was discovered to be reactive with the anti-NK1.1 mAb, highlighting the close similarities of these molecules and serologic cross-reactivity. Whereas the older literature suggested that there are also strain differences in transcript expression with markedly lower expression in BALB/c NK cells,[585] more recent data demonstrate abundant expression of Nkrp1 transcripts.[584] Moreover, the BALB/c allele of Nkpr1b also reacts with Clrb, indicating conserved specificity and function.

The Clr molecules belong to an NKC-encoded family with type II orientation and C-type lectin homology.[581] They are most closely related to the CD69 molecule encoded by an adjacent gene. In C57BL/6 mice, seven Clr genes have been identified at the genomic level. RT-PCR analysis indicates that *Clrb* is broadly expressed, whereas *Clrg* and *Clrf* genes are present in restricted and nonoverlapping tissues, including NK cells, and *Clra* and *Clrc* transcripts have not yet been identified. *Clre*, a probable pseudogene, has numerous stop codons in its expected open reading frame. Genomic analysis indicates the existence of a rat *Clr* family,[570] whereas in humans, three genes, *LLT1*,[586] *AICL*,[587] and *DCAL-1*[588] are localized next to CD69 and are related to the Clr family of genes.

Human NKRP1A binds LLT1, a functional homolog of mouse Clr.[589,590] Moreover, activation-induced C-type lectin (AICL CLEC2B) is the ligand for the NK-cell receptor activation receptor, NKp80 (KLRF1), which is also encoded in the NKC but is absent in rodents.[591,592,593] Finally, CLEC2A (keratinocyte-associated C-type lectin, KACL) is primarily expressed in the skin and is recognized by another NK-cell activation receptor, termed NKp65 (KLRF2).[594] All of these

receptors and their ligands are lectin-like molecules encoded in the human NKC.

The genetics of the *Nkrp1* and *Clr* loci is especially interesting from several viewpoints. 1) These loci are co-mingled in the NKC, from rodents to humans[445,570,575,584,589,590] (see Fig. 17.3). 2) In mice, there is limited allelic polymorphism, with conservation of gene order and content, despite genetic proximity to the highly polymorphic *Ly49* cluster.[445,584,595] For example, compare the conservation of the Nkrp1 gene cluster with polymorphism of the Ly49 cluster in C57BL/6 and 129 strain mice.[227,257,260,445,595] 3) At the individual gene level, *Nkpr1* alleles appear to be much less divergent than the corresponding *Ly49* alleles in the same inbred mouse strains.[257,445,584,595] 4) The *Nkpr1-Clr* interval of the NKC appears to be genetically protected with suppression of recombination.[596,597] Genetic linkage of Nkrp1 and its ligands thus resembles the tight genetic linkage of receptor and ligand genes and recombinational suppression of the self-incompatibility loci in plants to prevent self-

fertilization and related mating loci in other species.[598,599] Furthermore, the coevolution of such linked genes for receptors and ligands also represents an interesting issue in evolutionary biology because reciprocal mutations in both receptor and ligand genes are simultaneously needed to generate new specificities.[598] Nevertheless, the genetic pressure to conserve the *Nkrp1-Clr* gene order and coding sequences may therefore reflect a critical role for Nkrp1 and Clr molecules in innate immune cell interactions and functions.[575]

While the in vivo role of Nkrp1 and Clr molecules is incompletely understood, the best evidence for their functional importance comes from studies in rats. Rat CMV encodes RCTL (rat CMV C type lectin-like), which closely resembles rat Clrb and regulates its expression on infected cells.[600] RCTL interacts with the Nkrp1b inhibitory receptor and inhibits NK cell killing of infected cells. An RCTL loss mutant rat CMV is attenuated in vivo in an NK-cell-dependent manner. These studies, indicating that viruses encode decoy ligands for Nkrp1 receptors, support the importance of Nkpr1-Clr interactions in immune responses.

2B4, CD2, and Signaling Lymphocytic Activation Molecule Family of Receptors

The 2B4 (CD244, SLAMF4) molecule was originally identified on mouse NK cells with a mAb that perturbed mouse NK-cell function.[601] 2B4 is a type I integral membrane protein that belongs to the family of Ig-like molecules, including signaling lymphocytic activation molecule (SLAM) (SLAMF4, CD150), NK, T, and B cell antigen (NTBA, also known as SLAMF6 or Ly108), Ly9 (SLAMF3, CD229), CD84 (SLAMF5), and CD2-like receptor activating cytotoxic cells (CRACCs; SLAMF7, CD319).[602] Although these receptors may be broadly expressed, 2B4, NTBA, Ly9, CD84, and CRACC are expressed on NK cells. NTBA, Ly9, CD84, and CRACC are involved in homophilic interactions whereas 2B4 recognizes CD48, a GPI-linked molecule expressed on hematopoietic cells.[603,604] CD48 itself is also recognized by CD2, albeit at a ninefold lower affinity than by 2B4 (K_d = ~16 μM). Anti-CD2 mAbs can stimulate NK cells in the redirected lysis assay[605] and granule exocytosis,[606] but CD2-NK cells can still mediate natural killing.[9] The cytoplasmic domains of SLAM, 2B4, NTBA, Ly9, CD84, and CRACC contain a motif with sequence similarly to the ITIM, termed the immunoreceptor tyrosine-based switch motif (ITSM) consisting of TxYxxV/I consensus sequence.[607] The ITSMs allow interactions with a signaling adapter, SLAMassociated protein (SAP, also known as SH2D1A). The importance of SAP and its associated receptors is highlighted by the X-linked lymphoproliferative (XLP) syndrome, a human immunodeficiency involving abnormal proliferation of T and B cells during Epstein-Barr virus infections due to mutations in SAP.[608] SAP is related to Ewing sarcoma-associated transcript (EAT2, also known as SH2D1B1) and EAT2-related transducer (ERT, also known as SH2D1B2), which is present in rodents and only expressed in NK cells.[604] SAP and the SAP-related molecules contain a single SH2 domain for interaction with the ITSMs of presumably all SLAM family members except CRACC.[609,610,611,612,613] Instead, CRACC recruits EAT2, which functionally substitutes for SAP.[612] Subsequently, the SAP-related proteins can recruit a wide variety of downstream signaling molecules including Fyn, a Src-family tyrosine kinase, and tyrosine phosphatases, inositol phosphatases, and adaptor molecules.[614]

The complexities of this receptor-ligand signaling pathway are evident from the previous description. Receptors have overlapping ligand specificities; they recruit different adaptors that in turn can recruit downstream signaling molecules, even those with opposing functions. In this regard, functional studies of SLAM family molecules on NK cells have often been ambiguous, often depending on the experimental approach.[602] For example, 2B4 cross-linking with mAb can both stimulate as well as inhibit mouse NK cells,[601] whereas a 2B4-deficient mouse primarily displays effects consistent with 2B4 being primarily an inhibitory receptor.[615] However, the inhibitory effect of 2B4 is more apparent in mice than humans.[604] Some of these outcomes may depend on spatial distribution, 2B4 isoforms, or differential recruitment of SAP or its related molecules.[602] Further analysis should enlighten NK-cell function and signaling because 2B4 and related receptors demonstrate MHCindependent regulation of NK cells, and mediate inhibition and activation in a functionally distinct manner from the MHC-specific inhibitory receptors and ITAM-signaling chain associated receptors, respectively.

Natural Cytotoxicity Receptors

A series of mAbs that redirected lysis of human NK cell clones lacking KIRs led to the identification of the "natural cytotoxicity receptors" (NCRs), NKp46 (NCR1, CD335), NKp44 (NCR2, CD336), and NKp30 (NCR3, CD337).[616,617,618,619,620] These molecules are selectively expressed on NK cells, though NKp44 is expressed only upon activation. Whereas NKp46 is encoded in the LRC (see Fig. 17.3), NKp44 and NKp30 are encoded in the class III region of the MHC on human chromosome 6.[621,622] cDNA cloning revealed that they are type I integral proteins with one (NKp30, NKp44) or two (NKp46) Ig-like extracellular domains.

They contain charged transmembrane residues for association with ITAM-signaling chains (ie, ζγ heterodimers (NKp46, NKp30) or DAP12 (NKp44). In the mouse, only the gene for NKp46 is present in the syntenic region of chromosome 7 with the genes for other NCRs being absent (NKp44) or a pseudogene (NKp30).[623] The NCRs appear to play a role in cytotoxicity against tumors of varying origins because anti-NCR antibodies block target killing.[624] A mouse NKp46-Ig fusion protein binds RMA-S targets and deficiency of NKp46 leads to impaired in vivo clearance of RMA-S cells.[93] Interestingly, human NCRs apparently recognize mouse tumors and vice versa, suggesting conservation of these receptor-ligand pairs across species but identity of their target ligands were elusive,[624] until recently for NKp30 and NKp46.

In functional and structural studies, NKp30 clearly recognizes B7-H6, a member of the CD28 family that also includes CTLA-4 and PD-1.[625,626] B7-H6 is not expressed normally, is found on tumor cells, and sensitizes targets to NKp30-dependent cytotoxicity by NK cells. In a 2.0 Å structure of NKp30 complexed to B7-H6, NKp30 engages its ligand in an antibody-like manner distinct from the way other receptors bind B7 family molecules. Other potential NKp30 ligands include pp65 of HCMV, though it is a tegument protein and not expressed on the plasma membrane of infected cells.[627] HLA-B-associated transcript 3 (BAT3) was also proposed, but it is a nuclear protein that is released with DNA damage or endoplasmic reticulum stress.[628] Finally, NKp30 reportedly binds poxviral hemagglutinin independent of

B7-H6 and BAT3.[629] Nonetheless, at least one ligand is now well characterized for NKp30.

Human NKp46 appears to recognize influenza hemagglutinin on infected cells.[629,630] The interaction is dependent on sialic acid residues on oligosaccharides on NKp46 itself,[630] but this specificity is difficult to explain due to the ubiquitous expression of sialylated saccharides. NKp46 and NKp30 may recognize heparan sulfate proteoglycans on their cellular targets, though this is also controversial.[631,632] Nevertheless, NKp46-knockin (GFP)/knockout mice are susceptible to influenza, consistent with a role for NKp46 in defense against influenza,[93] albeit possibly in an indirect manner.[633] These studies may provide important clues to understanding of a receptor (NKp46) conserved in humans and mice.

NKp46 Tg mice have been proposed to be useful for NKcell analysis.[95] These mice are Tg for a construct consisting of 400 bp from the human NKp46 promoter driving expression of enhanced GFP and the human diptheria toxin receptor. However, NKp46+ cells in the gut may be derived from a non-NK-cell lineage.

Human NK-22 cells were identified in the gut as being CD3- CD56+ NKp44+ cells that produce IL-22 but not IL-17 upon stimulation with IL-23.[88] In the mouse, a similar functional population producing IL-22 was identified among NKp46+ cells and to a lesser degree, among NK1.1+ cells.[89,90,91,634] Immature human NK cells in secondary lymphoid tissue and the uterus may produce IL-22.[635,636] However, other reports indicate that NK-22 cells are more closely related to lymphoid tissue inducer (LTi) cells.[634,637,638,639] Thus, there may be heterogeneity among IL-22-producing NKp46+ cells.

Other Activation Receptors

Several other NK-cell-expressed molecules can activate cytolysis. For example, mAbs against mouse CD69 and Ly-6 and rat gp42 can trigger killing.[32,33] CD69 is encoded in the NKC and is structurally related to other NKC-encoded receptors. Interestingly, CD69 expression is upregulated upon stimulation,[322] and its expression is not confined to NK cells.[640] CD69 is functionally active on a large variety of hematopoietic cells when cross-linked by anti-CD69 mAbs. Its ligand is unknown, but it regulates the function of the sphingosine 1-phosphate receptor-1 through a membrane interaction.[641] Ly-6 belongs to a large family of small (15 to 18 kDa) GPI-anchored molecules that can activate lymphocytes when cross-linked.[642,643] Rat gp42 is a GPI-anchored protein with two Ig-like domains that was originally identified on IL-2-activated NK cells and the rat RNK-16 NK cell line.[33] Anti-gp42 can activate RNK-16 but not IL-2-activated NK cells. However, CD69, Ly-6, and gp42 are not expressed on freshly isolated NK cells and are expressed only after activation through other pathways; they are therefore not involved in triggering natural killing by freshly isolated NK cells. Although their physiological role is unknown, their activation potential and enhanced killing by IL-2-activated NK cells suggest that these molecules may contribute to this phenotype.

Receptors Involved in Recognition of Epithelial Tissues

NK cells possess receptors that are specific for ligands expressed at cell-cell junctions in epithelial tissues. Interestingly, ligands, such as cadherins, carcinoembryonic antigen (CEA)-

related adhesion molecules (CEACAMs), nectins, and nectin-like proteins (necls), are involved in forming adherens junctions and are engaged in homotypic or heterotypic interactions, and thus, are not normally exposed.[644] KLRG1 (also known as mast cell-associated function antigen, MAFA) is a lectin-like receptor with a single cytoplasmic ITIM.[645,646,647] Unlike human *KLRG1*, mouse *Klrg1* resides relatively distant and centromeric from the rest of the NKC, consistent with gene duplication and chromosomal inversion events. First discovered on rat mast cells, it has a broader distribution in human and mouse, including NK and T cells but not mast cells. Expression of Klrg1 on NK cells is downregulated in MHC-I-deficient mice, unlike the Ly49s that are modestly upregulated, but no MHC binding has been observed for Klrg1.[648] Instead, mouse Klrg1 binds three of the seven "classical" cadherins, E-, N-, and R-cadherin, leading to inhibition of NK lysis.[649,650] Biophysical studies show the K_D = 120 mM for mouse KLRG1-mouse E-caherin.[651] The structure of KLRG1 at 1.8 Å resolution revealed a typical lectin-like domain for KLRG1, which binds E-cadherin at a site distinct from where the integrin $\alpha_E\beta_7$ (CD103) binds E-cadherin. A KLRG1-deficient mouse demonstrates that it is dispensable for NK- and T-cell differentiation and antiviral responses, but it has not been

exhaustively studied, especially for epithelial responses.[652] Human CEACAM1 (CD66a) contains a cytoplasmic ITIM and can directly bind CEA, thereby potentially regulating NK-cell activities in human MHC-I-deficient patients and during pregnancy.[653,654,655] Mouse NK cells also express CEACAM1.[656] Inasmuch as CEACAM1 binds homophilic and heterophilic ligands expressed on epithelial and other cells,[657] NK cells thus express several inhibitory receptors for ligands normally expressed on epithelial cells.

NK cells also express cell-cell adhesion Ig-like receptors for molecules located at the adherens junction (ie, nectins and necls).[658,659] Specifically, they express CD226 (DNAM-1, DNAX accessory molecule-1) that recognizes necl-5 (CD155, poliovirus receptor, PVR) and nectin-2 (CD112, PVRL2).[660] NK cells also express CD96 (Tactile, T-cell activation, increased late expression) that binds necl-5 but not nectin-2, and class I-restricted T-cell-associated molecule (CRTAM), a receptor that recognizes necl-2.[661,662,663] DNAM-1 associates with lymphocyte function-associated antigen (LFA-1) and regulates its capacity to bind its ligand, ICAM-1.[660,664] In addition, DNAM-1 can recruit actin-binding proteins to form an adhesive complex with other cells. DNAM-1-deficient mice have defective NK and T cells, and fail to control tumors, both transferred and de novo carcinogeninduced.[665,666] Although these receptors can activate Src and other downstream pathways,[659] they are thought to function on NK cells as adhesion receptors that enhance cytotoxicity, rather than as primary activation receptors. Conversely, NK cells also express T-cell immunoglobulin and ITIM domain (TIGIT), which recognizes PVR and PVRL2 but not PVRL3, and inhibits NK-cell killing.[667,668]

These studies suggest that these receptors may affect different NK-cell functions as related to epithelial tissues.[644,649] For example, the inhibitory receptors may regulate NK-cell transmigration across an epithelial barrier or prevent NKcell attack against normal

tissues.[649,669] While the activation receptors could also affect NK-cell transmigration,[649,669,670] they also could be poised to attack cells that have disordered cell-cell junctions, such as tumors that typically lose cadherin expression and expose nectins and necls, potentially making them more susceptible to NK attack, consistent with an "exposed-self" model for NK-cell activation (see Fig. 17.2F, G). On the other hand, tumors may become resistant to NK cells by altering expression of these molecules. Current evidence provides some support to these complex scenarios that will require further investigation.[644]

Accessory Molecules

The role of accessory molecules in NK-cell activation has been difficult to address without knowledge of the "NK-cell (activation) receptor." Nevertheless, NK-cell cytotoxicity is critically dependent on classical accessory and adhesion molecules, such as LFA-1.[671,672] Insect cells expressing ICAM-1, a ligand for LFA-1, can be killed by human NK cells through an LFA-1-dependent process, suggesting that LFA-1 alone is sufficient to trigger killing.[673] LFA-1 cross-linking does not lead to granule polarization, suggesting that it may be insufficient for triggering the killing process by itself.[143] This issue is further complicated by the capacity of receptors like DNAM-1 to modulate LFA-1 association and function on NK cells.[660] Moreover, NK cells express multiple receptors capable of binding target ligands, with each receptor potentially affecting different downstream signaling pathways. When pairs of these receptors (eg, CD16, NKp46, NKG2D, 2B4, DNAM-1, or CD2) are cross-linked, synergistic NK-cell activation in terms of cytokine production and target killing can be seen for some but not all pairs.[674] However, this approach has not been used for the panoply of NK-cell receptors. Thus, how "accessory" molecules or other receptors contribute to each activation receptor function on NK cells remains to be systematically determined.

Ligands Recognized by Both Activation and Inhibitory Receptors

Evident from the previous discussion is the general concept that NK cells usually express both activation and inhibitory receptors simultaneously. Moreover, some of these receptors with opposing functions bind essentially identical ligands. For example, both activation and inhibitory receptors in the Ly49 and KIR families reportedly bind MHC-I molecules, and CD94/NKG2A and CD94/NKG2C bind HLA-E/Qa1. In general, where studied, the inhibitory receptors tend to bind ligands with higher affinities than their corresponding activation receptor counterpart, perhaps accounting for the common observation that inhibition tends to dominate over activation. However, the overall relevance of paired activation and inhibitory receptors to NK-cell immune responses remains to be elaborated.

SIGNAL TRANSDUCTION IN NATURAL KILLER CELLS

NK cells receive two basic types of external stimuli from cytokines and chemokines, and from target cell recognition. In general, their cytokine and chemokine responses are related to those found in other cells responding to the same pathways. However, as can be appreciated from the previous description of individual target recognition receptors, the pathways leading to target killing are complicated because some receptors are coupled to ITAM-containing signaling chains (CD3ζ, FcεRIγ, or DAP12). Indeed, some receptors use more than one of

these signaling chains, which are generally thought to be equivalent but not dissected in detail. Other receptors, such as NKG2D (through DAP10), have Tyr motifs for recruitment of PI3K and Grb2. Still others (2B4 and related receptors) have ITSMs. Moreover, integrin signaling also affects NK-cell activation by targets, and it was reported that NKG2D signaling through DAP10 can affect IL15 responses and vice versa,[675] which highlights the possible cross-talk of signaling pathways more broadly in immune cells.[676] Finally, because individual NK cells can express (and use) multiple different receptors simultaneously, and some but not all work synergistically,[674] the outcomes of these activation pathways may reflect profound complexity, even without consideration of inhibitory receptor signaling events.[677]

The study of NK-cell signal transduction also comes with some caveats. Most studies have been performed on bulk populations of NK cells, NK cell clones, or in vitro adapted cell lines that may not be representative of pathways triggered in individual NK cells which may express only some but not all signaling molecules detected in the entire NKcell population. When NK cells are triggered by target cells, there may be unknown stimulation through other receptors because unknown target ligands may inadvertently trigger or modulate functions of other NK-cell receptors. Finally, there may be species (human versus mouse) differences in signaling. Despite these complexities, themes have emerged in understanding of NK-cell signal transduction.

For the signal transduction pathways stimulated by NK-cell activation receptors coupled to ITAM-containing signaling chains, downstream events resemble those found in TCR and BCR signaling.[678] Activation receptor crosslinking leads to ITAM phosphorylation by Src family tyrosine kinases, recruitment and activation of Syk family tyrosine kinases, and subsequent downstream activation events. Inasmuch as TCR and BCR signaling is covered in detail elsewhere, we will highlight here notable differences with TCR and BCR signaling. 1) Individual NK cells may simultaneously express multiple activation receptors, each of which may associate with different ITAM-signaling chains that could be phosphorylated by different Src family tyrosine kinases and/or lead to different downstream signaling events. 2) NK cells express multiple Src kinases, including Lck, Fyn, Src, Yes, Lyn, and Fgr,[679] with redundancies in their contributions to ITAM phosphorylation. For example, Fyn is activated following Ly49D (DAP12) cross-linking but NK cells deficient in Fyn, Lck, or both still kill in a Ly49D-dependent manner.[680] 3) NK cells express both Syk family tyrosine kinases, ZAP-70, and Syk itself, whereas T and B cells express either, respectively, but not both. Deficiency of either ZAP-70, Syk, or both has only minimal effects on NK cell killing,[681] suggesting other pathways for transmitting NK activation signals. 4) NK cells express many adapter molecules found in T and B cells, but their contributions to NK signal transduction are less well defined. For example, SLP-76 and linker for activation of T cells are dispensable for NK-cell signaling, although they are both required for T-cell signaling.[682,683] 5) Different isoforms of signal transduction molecules are responsible for NK-cell activation. For example, mouse NK cells utilize phospholipase C-γ2 (PLCγ2) as a critical signaling mediator[684] more like B cells than T cells.[685] 6) In contrast to T and B cells, NK-cell signaling components are generally not required for normal NK-cell development. For example, NK cells are not deficient in number or maturation state in PLCγ2-deficient mice, unlike B cells, which require PLCγ2 for

development.[684,685] Taken together, these findings highlight differences between NK-cell and T- and B-cell signaling.

Illustrating some of the aforementioned issues are studies on the Vav family of guanine nucleotide exchange factors, Vav1, Vav2, and Vav3, which are all expressed in mouse NK cells.[686] Vav2 and Vav3 are required for FcRγ and DAP12 signaling, whereas Vav1 is required for DAP10 signal transduction. Additionally, NK-cell number and differentiation are not apparently affected by Vav deficiencies even when they are required for NK-cell activation. In contrast, deficiencies in Vav family members lead to T- and B-cell development defects.[687] These observations also indicate the redundancies in NK-cell receptor signaling.

On the other hand, Vav1 appears to be central to human NK-cell activation by targets[688,689] even though the role of other Vav isoforms in human NK cells is less clear. Vav1 is also involved in macromolecular complexes with c-Cbl that are formed during NK-cell activation.[690] Interestingly, c-Cbl provides an inhibitory influence on NK-cell activation.[689] During NKG2D and 2B4 synergistic signaling, strong Vav1 signals override the c-Cbl effect to allow NK-cell activation, again placing Vav1 as a central player in human NK-cell activation.

Recruitment and activation of PI3K appears to be critical to NK-cell activation for target killing.[691,692] How PI3K is activated depends on the receptor and its proximal signal transduction events.[677] In the case of NKG2D, the YxxM motif in DAP10 is phosphorylated and recruits PI3K directly,[693] which appears to be sufficient to activate NK-cell killing in the absence of DAP12 or Syk family tyrosine kinases.[694] The pathway may be more complex in human NK cells where evidence suggests that DAP10 recruits a Grb2-Vav1 intermediate for activation.[491] For other activation receptors, Syk activation is upstream of PI3K[695] and may directly recruit PI3K.[696] PI3K in turn activates a mitogen activated protein kinase (MEK) for stimulation of extracellular signal-regulated kinase (ERK).[691] Ultimately, actin polymerization occurs, granules become polarized toward the target, and exocytosis occurs, resulting in target apoptosis, as discussed previously. Interestingly, in human NK cells, engagement of LFA-1 by ICAM-1 on insect cells was sufficient to induce granule polarization but not degranulation,[143,674] suggesting additional complexity regarding the signals required for polarization versus degranulation.

High-resolution microscopy has enabled visualization of events following NK-cell activation. Like CTLs, NK cells form discrete protein clusters at the site of contact with their targets, termed the immunological synapse,[697,698] though obviously the TCR is not involved. Following target contact, the NK immunological synapse (NKIS) is formed at the NK-target interface in discrete stages whereby receptor signaling, filamentous (F)-actin rearrangement, and polarization of granules occur.[699,700] CD2, F-actin, LFA-1, and Mac-1 accumulate in the peripheral supramolecular activation complex (pSMAC), whereas granules (perforin) and the activation receptor accumulate centrally (cSMAC).[701] Other peripheral supramolecular activation complex and centrally supramolecular activation complex components and patterns are found depending on whether or not the contacts lead to cytolysis.[698,702] With this basic outline of microscopic events, molecules required for NKIS formation and granule polarization are being dissected.[125,692,703,704,705]

Mechanism of Inhibition by Natural Killer-Cell Receptors

The now widely accepted view is that the NK-cell inhibitory receptor mechanism consists of ligand binding and receptor cross-linking followed by ITIM phosphorylation, and preferential recruitment of SHP-1, and that this mechanism

operates with both structural types of inhibitory receptors. A typical human KIR molecule has two ITIMs separated by ~24 residues. In contrast, Ly49A has only one ITIM per chain, but Ly49 molecules are normally expressed as homodimers. CD94 has a minimal cytoplasmic tail, but NKG2A has two ITIMs. Each ITIM can be phosphorylated upon receptor cross-linking (though difficult to visualize) or tyrosine phosphatase inhibition,[212,706,707,708,709,710] presumably by a Src family tyrosine kinase as in B cells,[711] although redundant expression and function of these kinases have precluded identification of a single kinase required for ITIM phosphorylation in NK cells. ITIM phosphorylation results in recruitment and activation of the intracellular tyrosine phosphatase, SH2-containing protein tyrosine phosphatase (SHP)-1 (also known as hematopoietic cell phosphatase (HCP), protein tyrosine phosphatase 1C (PTPIC), and protein-tyrosine phosphatase, nonreceptor-type, 6(PTPN6)).[212,706,707,708,712] Two ITIMs are required for sequential binding of the two SH2 domains in SHP-1.[710,713] The NK-cell MHC-specific inhibitory receptors preferentially recruit SHP-1 rather than SH2-containing inositol polyphosphate 5-phosphatase (SHIP), though they may occasionally bind SHP-2.[706,714,715] Inhibitory receptor engagement results in early KIR and SHP-1 recruitment to areas surrounded by LFA-1.[716] Moreover, early events in NK-cell activation are blocked by inhibitory receptors, such as formation of the NKIS.[716,717,718] On the other hand, KIR phosphorylation occurs within clusters at the NKIS, which may serve to focus inhibition on downstream targets,[719] such as Vav1, which is recruited into the mature synapse during NKG2D/DAP10 signaling.[704] Indeed, the downstream target of the ITIM-recruited tyrosine phosphatase appears to be Vav1 in human NK cells activated by targets.[688] Thus, the general consensus has long been that the inhibitory receptors recruit tyrosine phosphatases that dephosphorylate molecules in the activation receptor cascade, such as Vav1.

On the other hand, related ITIM-containing inhibitory receptors bind other effector molecules, such as Csk by LAIR-1 and LILRB1,[423,720] suppressor of cytokine signaling 3 (SOCS3) by Siglecs,[721] and SHIP by KLRG1[722] and FcγRIIB.[723] Even a KIR ITIM may recruit other molecules such as β-arrestin[724] and PI3K.[725] Morever, a SHIP-deficient mouse demonstrates abnormalities in NK-cell function and Ly49 receptor expression,[726] even though Ly49 receptors recruit SHP-1. The functional significance of these signaling effector molecules in NK-cell inhibition and the specificity for ITIM recruitment of selective molecules are poorly understood and is potentially related to the complexity of inhibitory receptor signaling.

Emerging data suggest that inhibitory receptor signaling may not be as simple as phosphatase recruitment to dephosphorylate proximal molecules in the activation pathway that then prevents all downstream signaling events. For example, inhibitory receptors

efficiently blocked granule polarization but inefficiently prevented degranulation.[727] During ongoing NK-cell activation, KIR2DL2 engagement by photoactivated HLA-C ligand disrupted the NKIS but had little effect on ongoing calcium flux.[728] Thus, some but not all of the signaling events downstream of NK-cell activation receptors may be effectively regulated by the inhibitory receptors, directing future studies to selected aspects of NK-cell signal transduction.

Inhibitory signaling also induces other events. Normal human NK-cell activation by susceptible targets led to Tyr phosphorylation of Vav1 and its association with c-Cbl, which was also Tyr phosphorylated.[690] In turn, c-Cbl was associated with a signaling complex consisting of an adaptor protein (Crk, either CrkII or CrkL, depending on the cell), a scaffold protein (p130CAS,[130] kDa Crk-associated substrate), and C3G (Crk SH3 domain-binding guanine-nucleotide exchange factor). These c-Cbl complexes have the potential to signal a wide variety of cellular pathways including Rap1 GTPase activation, actin reorganization, cell adhesion, and mitogen-activated protein kinase activation,[729] which appear to be critical for NK killing. By contrast, ligand engagement of either KIR or CD94/NKG2A blocked the formation of Cbl-Crk-p130CAS-C3G complexes.[690] Moreover, ligand engagement of either KIR or CD94/NKG2A induced the association of the tyrosine kinase c-Abl with Crk, and Crk phosphorylation that was shown to be required for active dissociation of Crk from Cbl and for inhibition. Thus, these studies showed that inhibitory signaling ironically involved Tyr phosphorylation and disruption of macromolecular signaling complexes, which in turn, suggest that NK-cell activation (and inhibition) may be subject to more qualitative controls than previously recognized.

Another possible explanation for inhibitory signaling is related to the inhibitory influence that c-Cbl has on NK-cell activation[689] and other immune cells, such as T and B cells.[730] Although Cbl family molecules exhibit E3 ubiquitin ligase activity, and may exert their negative effects on lymphocyte signaling by enhancing degradation of signaling components,[731] there are as yet little data supporting this mechanistic role for Cbl in NK-cell activation.[689,690] Nonetheless, current information suggests additional aspects of NK-cell inhibitory receptor signaling are yet to be discovered.

Finally, Ly49 receptors on NK cells can acquire their cognate MHC-I ligands from surrounding cells, resulting in display of both molecules on the NK cells.[732,733] Broadly speaking, this effect is probably related to trogocytosis, a poorly understood phenomenon of intercellular transfer of surface molecules.[734] How much this contributes to *cis* effects on inhibitory receptor function and its physiological relevance require further evaluation.

NATURAL KILLER-CELL TOLERANCE

NK cells display potent effector functions that must be controlled to prevent inadvertent damage to normal tissues (ie, NK cells must demonstrate tolerance to self). Although the missing-self hypothesis provides a rationale and starting point for dissection of NK-cell tolerance, it has a number of caveats that need to be considered before delving into current understanding of this aspect of NK-cell biology.

Influence of Host Major Histocompatibility Complex-I Environment

The missing-self hypothesis implies that there should be overt NK-cell autoreactivity in MHC-I-deficient hosts, but this was not observed in humans or mice.[179,180,181,735,736,737,738] Instead,

NK cells from MHC-I-deficient mice demonstrate poor killing of MHC-I-deficient targets or rejection of MHC-I-deficient BM, even though they appear normal in number, tissue distribution, and expression of activation receptors. Another very difficult issue is related to hybrid resistance whereby host NK cells in an F_1 hybrid animal can reject BM grafts from either inbred parent.[739,740] It was difficult to understand how NK cells discriminate between cells expressing the full complement of self-MHC alleles versus those otherwise normal cells expressing only some self-MHC molecules. NK-cell-mediated rejection of MHC-I-sufficient BM grafts also depends on which host MHC-I allele is present.[741,742] Thus, NK cells are regulated by host MHC environment, not just by the target cell in effector responses.

The MHC-specific inhibitory receptors were likely to be involved in self-tolerance because they explain the influence of MHC expression on NK-cell effector functions against target cells. However, how these receptors are related to self-tolerance in vivo was a challenging issue. Several hypotheses were proposed. An individual NK cell can simultaneously express multiple inhibitory receptors but some may not recognize self-MHC. The “at least one receptor” model suggests tolerance is achieved as long as each NK cell expresses at least one receptor with self-MHC specificity.[389,743] There are changes in the repertoire of MHC-I-specific inhibitory receptors, depending on the MHC haplotype, but these differences are modest, regardless of whether the receptor is self-specific or not.[210,248,389,743,744] As determined by antibody reactivity, the expression level of an NK receptor on an individual NK cell decreases when the host expresses the MHC ligand for that receptor.[208,209,745] Functional analyses of NK cells with “downregulated” receptor expression suggest that such NK cells are more sensitive to small changes in MHC ligand expression on the target, as explained by the “receptor calibration” model.[744,746] However, these studies involved in vitro conditions that could alter their intrinsic functional capacities. Thus, several hypotheses based on the MHC-specific inhibitory receptors were proposed to account for NK-cell self-tolerance.

Also unexplained was the observation that NK cells were found in wild-type mice expressing none of the known self-MHC-specific inhibitory receptors. These cells were functionally “hyporesponsive,” showing defective antiactivation receptor cross-linking and killing of NK-sensitive targets,[747] but it was possible that other, yet to be defined receptors were playing a role. Another issue was the appropriate pairing of polymorphic inhibitory receptors with their highly polymorphic cognate MHC ligands.[260,317] The genes for the receptors and their ligands are located on different chromosomes (ie, the receptor and ligand genes segregate independently). Thus, there must be mechanisms to provide NK cells with the appropriate inhibitory receptors with specificity for self-MHC because the appropriate pairs are not inherited together, unlike the closely linked *Nkpr1-Clr* gene pairs.[575]

The Licensing Hypothesis

Studies with target cell-free stimulation and single cell assays of NK-cell responsiveness

revealed insight into NKcell tolerance.[168,747,748] Freshly explanted, resting murine NK cells from MHC-I-deficient mice were functionally defective in triggering by immobilized antiactivation receptor antibodies.[168] Conversely, in wild-type mice, functional competence correlated with expression of a Ly49 inhibitory receptor for self-MHC-I.[168] CD94/NKG2 receptors appeared not to be relevant,[168] as confirmed by studies of a CD94-deficient mouse.[395] Particularly informative were studies with a single chain trimer MHC-I molecule, consisting of antigenic peptide-linker-β2m-linker-$H2K^b$ as a single polypeptide that binds only Ly49C.[168] In Tg mice expressing only this MHC-I molecule, only Ly49C+ NK cells were functionally competent. Although crystallographic studies revealed two potential sites on MHC-I that could be recognized by Ly49 receptors, studies of Tg mice bearing mutant MHC molecules indicate that the Ly49 receptors recognize the same site on their MHC ligands (site 2) for both education and effector inhibition.[749] Thus, NK cells use their MHCspecific receptors interacting with self-MHC-I to become competent to be triggered through their activation receptors by (Fig. 17.6), an education process termed “licensing.”

Despite initial confusion over the term licensing,[750] most investigators now concur that licensing or education by self-MHC-specific receptors results in fuctionally competent NK cells.[751] Moreover, human NK cells are also subjected to a similar process involving KIR and self-HLA ligands[752,753] that may also explain clinical studies relating KIR and HLA alleles and disease and even human immunodeficiency virus (HIV) control.[754,755] Such KIR-HLA relationships frequently involve pairs with high affinities and resolution of chronic infections[756] that are difficult to explain when only considering effector inhibitory function of the KIRs. Thus, licensing may be clinically relevant and may be applicable to other clinical uses of NK cells (see following discussion) because it is a second function of the NK-cell receptors for MHC.

Licensing leads to appropriate pairing of inhibitory receptors with self-MHC and strongly suggests that there are two types of self-tolerant NK cells (see Fig. 17.6).[750,757] Regardless of the MHC-I environment, licensed NK cells are tolerant because they have inhibitory receptors for self-MHC, the same receptors involved in licensing. Unlicensed NK cells are also tolerant because they are not functionally competent and have no need for inhibition by self-MHC under steady-state conditions. In hosts heterozygous for MHC alleles, each MHC allele could potentially license different NK-cell populations. This aspect of licensing is relevant to hybrid resistance because an (A × B)F_1 hybrid animal should have NK cells that are separately licensed on different MHC alleles.[750] F_1 hybrid NK cells that were licensed by MHC alleles from parent A should be inhibited by A alleles but not B alleles, and thus reject BM from parent B. The converse should also be true. In the F_1 animal itself, NK cells are licensed by either parental allele so all NK cells should be inhibited by normal tissues that codominantly express both MHC alleles. Licensing potentially explains how NK cells distinguish cells expressing the full complement of MHC-I from those expressing only some alleles.

Although initial studies on licensing were focused on only a few receptors and MHC alleles to demonstrate convincing effects, subsequent studies have demonstrated that

the licensing status of an individual NK cell is dependent on the Ly49 receptor, MHC allele, and the number of expressed self-MHC-specific Ly49 receptors.[219,758] For example, different MHC alleles appear to be more potent in licensing Ly49A+ NK cells, in a hierarchical manner.[219] The same hierarchy of MHC alleles was seen with Ly49A downregulation and inhibition of Ly49A+ NK cells in effector responses. An MHC allele was more potent at inhibition as compared to licensing, providing a margin of safety against NK-cell autoreactivity.[219,759] Interestingly, MHC heterozygosity was comparable to homozygosity with capacity of an MHC allele to license.[219] Studies of mice bearing one, two, or more MHC alleles also show similar effects.[758] These results have prompted a refinement of the licensing hypothesis (ie, the "tuning" or "rheostat" model) whereby the strength of inhibitory receptor engagement by self-MHC determines the degree to which an NK cell is capable of being activated.[750,760]

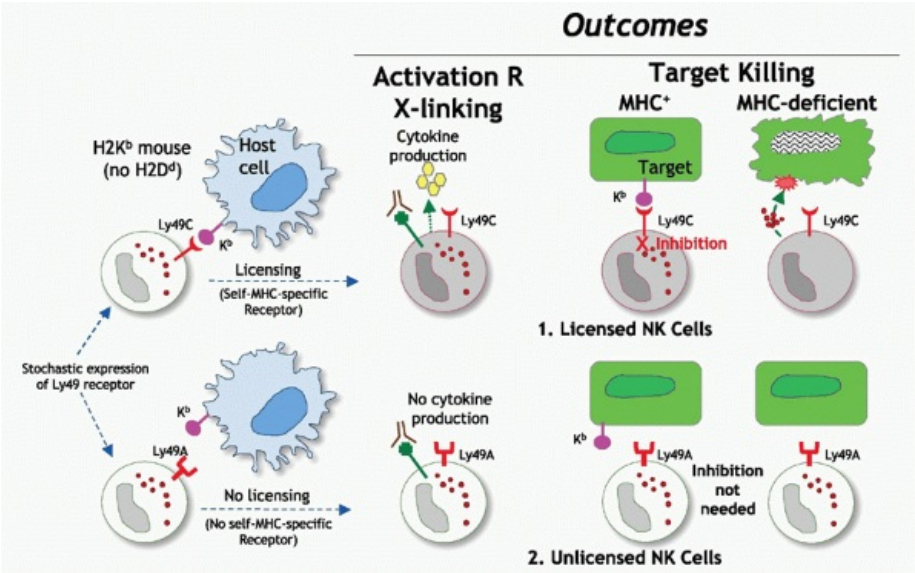

FIG. 17.6. Licensing of Natural Killer (NK) Cells by Host Major Histocompatibility Complex (MHC)-I. Depicted is the situation in a mouse expressing only $H2K^b$ that is a ligand for Ly49C but not Ly49A. Cells expressing Ly49C therefore have a self-specific receptor, whereas those expressing Ly49A do not. Ly49C engagement by self-MHC-I results in a licensed NK cell that has functional competence to be triggered through its activation receptors. These cells can be inhibited by self-MHC through the same receptor that conferred licensing. Ly49A+ NK cells remain self-tolerant because they are unlicensed. For clarity, only one MHC allele and cells expressing one Ly49 receptor are shown. Activation receptors are also not shown for the target killing depiction. Normally, the host has multiple MHC alleles, each of which could license different NK cells, and individual NK cells express multiple Ly49s simultaneously. Each NK cell is likely to be licensed separately, potentially by different MHC alleles, and through different Ly49s. Modified from Elliott and Yokoyama.[750]

Where and with whom licensing occurs is currently unclear. While original studies suggested that licensing occurs during development in the BM,[168] adoptive transfer of otherwise unlicensed NK cells from an MHC-deficient host into an MHC-sufficient environment results in licensed donor NK cells.[761,762] The converse was also observed (ie, loss of function when wild-type NK cells were transferred into an MHC-deficient environment). These studies add to the discussion of whether an NK cell interacts with self-MHC in *trans* (on another cell) on in *cis* (on the same NK cell).[763] What is difficult to exclude is the possibility that NK cells may take up MHC-I from other cells via their MHC-specific receptors,[732,733] but the transfer studies indicate that the NK cell itself does not need to synthesize MHC-I. Thus, licensing may be a dynamic process in which NK cells constantly test the same site on their MHC-I ligands to simultaneously maintain functional competency and effector inhibition.

Although licensing is a positive outcome (ie, the acquisition of functional competence), it requires the ITIM of the self-specific Ly49 receptor to deliver signals that ultimately result in a licensed NK cell[168] (see Fig. 17.6). The nature of such signals is not yet known, and at least two qualitatively different models are being considered. The MHC-specific receptor could confer licensing in a positive way by delivery of signals that directly induce a differentiation process and

license NK cells, akin to a stimulatory receptor and also known as the “arming” hypothesis[764] In the second model, also known as the “disarming” hypothesis,[764] the MHCspecific receptors decrease the effect of a putative, as yet uncharacterized, self-specific activation receptor. If unimpeded by self-MHC-I, NK cells become hyporesponsive due to overstimulation through the activation receptor. This model is somewhat akin to T-cell anergy and places the self-MHC-specific receptor in a familiar light as an inhibitory receptor. Both models are similar to those previously proposed,[765] but engagement of the MHC-specific receptor with self-MHC is now known to be a required key step[168] and is common to both models. Also, the models are not mutually exclusive and either could be affected by coreceptors or adhesion molecules. Regardless, licensing may be explained by either the arming or disarming hypothesis.[750]

For the arming hypothesis, it is difficult to reconcile the involvement of the ITIM in the self-MHC specific receptor in licensing,[168] though recent analysis shows that inhibitory receptor signaling is more complex than previously recognized.[689,690] Recent studies also suggest that there are few differences in genes expressed by licensed verus unlicensed NK cells.[766] Instead, in licensed NK cells, the activation receptors appeared to be localized in nanodomains in the plasma membrane, whereas in unlicensed cells, the activation receptors were apparently confined to the actin meshwork.[766] Future studies will be needed to validate this correlation and how the self-MHC-specific receptors mediate these changes.

For the disarming model, studies on mice constitutively expressing activation receptor ligands by transgensis or retroviral transduction of hematopoietic stem cells have been informative.[534,538,767,768] In support of the disarming hypothesis, continuous engagement of NKG2D or Ly49H resulted in hypofunctional NK cells, akin to anergy. Indeed, m157 Tg mice could not resist MCMV infections normally, and the Ly49H+ NK-cell subset was

selectively hyporesponsive. However, the anergic phenotype was unaffected by expression of a self-MHC-specific receptor.[767] Although it remains possible that receptor-ligand interactions that normally govern NK-cell self-tolerance were not well represented in these Tg mice, the current data nonetheless do not support the disarming hypothesis as an explanation for licensing and suggest that "anergy" may be a distinct tolerance mechanism for NK cells, perhaps analogous to separable tolerance mechanisms for T and B cells.[769]

In addition to bone marrow rejection, licensed NK cells may provide primary protection in viral infections and may enhance CD8+ T-cell responses.[770,771,772] On the other hand, unlicensed NK cells may gain functional competence by exposure to cytokines.[168] During MCMV infection, unlicensed NK cells (ie, cells without self-MHC-specific receptors) appear to control viral replication in both MHC-deficient and -sufficient mice.[773,774,775] Thus, the role of self-tolerant NK cells in immune responses requires additional study.

NATURAL KILLER-CELL DEVELOPMENT

Early evidence indicated that the complete phenotypic and functional maturation of NK cells occurs in the BM and requires an intact microenvironment because BM ablation or congenital BM defects lead to abnormal NK cells.[776,777,778,779,780] Indeed, certain aspects of NK-cell development are regulated by direct interactions between developing NK cells and stromal elements, such as interactions between membrane lymphotoxin-α (LTα)-expressing NK-cell precursors (NKPs) and LTα-responsive stromal cells that are necessary for normal development.[154,781] In vitro, NK cells can be generated from early hematopoietic cells in a cytokine cocktail consisting of stem cell factor (c-kit ligand), IL-7, flt-3 ligand, and IL-15.[782,783,784,785,786,787,788,789] Direct contact with stromal cells appears to be required for acquisition of Ly49 receptors by developing NK cells. Moreover, the Tyro3 family of receptors (Tyro3, Mer, Axl) on NK cells and their ligands (Gas6, protein S) on stromal cells are apparently necessary for expression of NKcell receptors and functional differentiation in vitro and in vivo.[790,791] Thus, NK-cell development requires certain cytokines and direct stromal cell contact.

Although the general topic of NK-cell development is currently an active area of investigation, and is thus subject to future modifications, several major themes have emerged that will be summarized here. The earliest step involves the commitment of hematopoietic stem cells in the BM to the common lymphoid progenitor that can give rise to NK, T, and B cells, but not to myeloid cell lineages.[792,793] Mice deficient in various transcription factors, including Ikaros and PU.1, display severe defects in the development of all lymphoid cells including NK cells, while myeloid and erythroid lineages are less affected.[794,795,796,797,798] Thus, NK-cell development appears to share a common pathway with other lymphocytes, providing evidence that NK cells belong to the lymphocyte lineage.

Commitment to the NK-cell lineage from the common lymphoid progenitor (CLP) appears to involve an intermediary cell with T- and NK-cell potential (T/NK progenitor), perhaps reflecting the close resemblance of T- and NK-cell effector functions. Consistent with this bipotentiality, CD3ε and FcεRIγTg mice exhibit selective defects in both NK- and T-cell development.[799,800] Moreover, deficiencies in several genes, including transcription factors

and IL-15,[54,801] affect development of NK-cell and certain T-cell subpopulations, particularly NKT cells and memory T cells. T/NK progenitor populations, including immature thymocytes, can give rise to T and/or NK cells but not to other lineages, depending on culture conditions, such as Notch signaling.[802,803,804,805,806,807,808] Interestingly, the transcription factor Bcl11b is required to maintain T-cell fate by apparently repressing NK-cell fate. Specifically, Bcl11b is required for T-cell specification,[809,810,811,812] and its deletion, in committed T-cell precursors or even mature T cells, results in loss of T-cell phenotype and instead acquisition of NK cell-like phenotype,[809] highlighting the developmental pathways shared by these cell lineages.

NK cells also have a developmental relationship to Lymphoid Tissue inducer (LTi) cells. In addition to phenotypic similarities as previously discussed for NK-22 cells, LTi and NK cells also share a relatively unique feature in expressing surface LTα, which influences development of lymphoid tissues and NK cells, respectively.[154,781] Moreover, deficiencies of either Ikaros or Id2 result in defects in LTi and NK cells, though other cells are also affected.[813,814] Interestingly,

mice lacking the thymocyte selection-associated high mobility group box protein DNA-binding protein lack both LTi and CD122+ NK cells.[815] The NK-cell defect is NK cell-intrinsic, and Id2 overexpression could not compensate for thymocyte selection-associated high mobility group box protein (TOX)-deficiency.

Until recently, the acquisition of IL-2/15Rβ subunit (CD122) was considered to mark the earliest identifiable committed NKP.[805,816] However, an earlier progenitor population (pre-NKP) has been identified that has not yet expressed CD122.[817] Upon adoptive transfer, these lineagenegative (lin-) cKit- Flk2- CD27+ CD244+ IL-7Rα+ CD122- cells from the BM could give rise to NK cells in the BM, blood, and spleen but no other cell lineages. Thus, IL-15 is not required for commitment to the NK cell lineage, even though it is required for subsequent NK-cell developmental stages as illustrated by the persistent expression of CD122.[44,53,54,55,56,57]

CD122 expression is regulated by the combined action of the T-box transcription factors, T-bet (Tbx21) and Eomesodermin (Eomes) that have redundant function in NK-cell development.[801,818] Specifically, *Tbx21-/- Eomes*+/- mice have marked decreases in CD122 expression and NK cells, whereas *Tbx21-/- Eomes*+/+ mice manifest no defect in CD122 expression and more modest defects in NK-cell number. Moreover, Eomes targets the promoter of *Cd122*. IL-15 appears to regulate the basic leucine zipper (bZip) transcription factor E4BP4 (E4 binding protein 4,[819] also known as nuclear factor IL-3[820]) in NK-cell development. *E4bp4*-/- mice display profound NK-cell deficiency.[821,822,823] Gene transduction of *E4bp4*-/- hematopoietic stem cells with *E4bp4* rescued NK-cell development in vitro even in the absence of IL-15, suggesting that E4BP4 acts downstream of IL-15 signaling. *E4bp4*-/- mice show no alterations in other hematopoietic cells except for a defect in CD8a+ cell development. [824] This latter finding may be relevant to phenotypic overlap between NK cells and DCs.[115,116,117,118,119] Regardless, Eomes affects NK-cell development by promoting CD122 expression, whereas E4BP4 affects commitment to the

NK-cell lineage by apparently acting downstream of IL-15.

Committed NKPs next differentiate into mature NK cells in a series of putative developmental intermediate stages that occur in the BM.[98,816] Originally defined by correlating phenotypic markers, including NK-cell receptors, integrins, and other molecules with apparently distinct developmental stages,[825,826] these stages have been confirmed by studies following serial acquisition of markers during NK-cell differentiation in vitro.[789] Regardless, these stages of committed NKPs are undergoing constant refinement as new rate-limiting steps are being uncovered, such as in studies of mice lacking specific transcription factors or as new markers and tools are developed.[827] Nonetheless, a few highlights should be noted.

NK-cell receptors involved in target recognition are expressed at immature stages, with NK1.1 and CD94/NKG2 being among the first, followed by NKp46, and NKG2D then the Ly49s.[827] Following Ly49 acquisition, developing NK cells undergo spontaneous proliferation at an immature stage.[98] Thereafter, as NK cells acquire high-level expression of Mac-1 (αMβ1) and CD43, proliferation markedly decreases unless challenged by pathogens such as viruses that can stimulate mature NK-cell proliferation.[98,166] NK cells then gain functional activities associated with splenic NK cells, indicating that Mac-1 expression correlates with a late maturation stage. A selective NK-cell deficiency in a Tg mouse is manifested by a failure to become mature, functional Mac-1^{hi} cells,[828,829] consistent with this differentiation step. Finally, while CD27 is expressed from the very earliest stages of NK-cell development,[817] its diminished expression within the Mac-1+ subset is associated with a more responsive capacity.[830] Thus, Mac-1 and CD27 expression are now commonly used to ascertain NKcell maturation in the mouse.

In addition to the transcription factors mentioned previously, several other transcription factors, including Ets-1, Id2, IRF-1, MEF, and GATA-3, have been implicated in NK-cell development and function.[813,831,832,833,834,835,836] The need for Id2 in NK-cell development can be overcome by deletion of the gene for the E protein, E2A.[814] IRF-1-deficient mice lack NK cells, and this defect can be overcome by the addition of IL-15, suggesting that stromal cells produce IL-15 in an IRF-1-dependent manner.[813] Expression of Bcl2 restored the CD8+ T-cell deficiency but not the NK-cell deficiency in IRF-1-deficient mice.[837] IRF-2-deficient mice manifest a late defect in NK-cell maturation with normal constitutive proliferation but increased apoptosis thereafter.[838,839] In contrast to the other transcription factors, GATA-3 does not affect NK cell number; rather, it controls their IFNγ production and their homing to the liver.[836] Similarly, CCAAT/enhancer binding protein (C/EBP) γ-deficient NK cells display defective effector functions.[840] The relationships of the transcription factors to each other are just beginning to be elaborated in terms of regulating NK-cell development and function.

As for mouse NK cells, human NK-cell developmental stages have been identified by correlating markers on in vivo subsets.[841,842,843,844] In general, these stages have been recapitulated by in vitro development of functional human NK cells from hemapoietic stem cells from umbilical cord blood or differentiated embryonic stem cells.[845,846] As in the

mouse, a combination of cytokines and stromal cells is necessary for in vitro differentiation.

These studies suggest that human NK cells differentiate in secondary lymphoid tissues as revealed by studies of $CD56^{bright}$ and $CD56^{dim}$ NK cells.[110,847] $CD56^{bright}$ NK cells preferentially express CD94/NKG2 receptors (versus KIRs), are CD16-, and are better cytokine producers and less efficient killers than the $CD56^{dim}$ subset that tend to be KIR+ and CD16+.[109] A CD34+ CD45RA+ hematopoietic precursor cell (HPC) was identified that expressed the integrin $\alpha_4\beta_7$ at high levels.[847] This population was found at low levels (< 1% of HPCs) in the BM but was markedly enriched in lymph node (LN; > 95% of HPCs), and could be differentiated into $CD56^{bright}$ NK cells in vitro. On the other hand, in nonreactive LNs, NK cells tend to be $CD56^{bright}$ whereas in reactive LNs, they tend to be $CD56^{dim}$, the predominant population in peripheral blood.[110] $CD56^{bright}$ cells acquire the $CD56^{dim}$ phenotype when stimulated in vitro with proinflammatory

cytokines, such as IL-12, or with IL-15 in *trans* in a humanized mouse model.[848] Taken together, these data suggest that human NK cells differentiate in secondary lymphoid tissues in response to inflammation by first becoming $CD56^{bright}$ then completing maturation to the $CD56^{dim}$ phenotype.

Although the maturation of mouse NK cells in secondary lymphoid tissues has not been studied carefully, there is an abundance of NK cells with an immature phenotype in organs, such as the liver.[98] On the other hand, recent studies also provide evidence for thymic maturation of a subpopulation of mouse NK cells, termed “thymic” NK cells[111] These cells are characteristically CD127+ $CD69^{high}$ $Ly49^{low}$ $CD11b^{low}$ in contrast to conventional resting splenic NK cells that are CD127- CD69- $Ly49^{hi}$ $CD11b^{hi}$. Thymic NK cells were enriched in LNs and are absent in GATA-3-deficient and in athymic nude mice, indicating that these cells require GATA-3 and an intact thymus for their development. Indeed, thymic NK cells can develop in vivo and in vitro from double negative (CD4- CD8-) 1 (DNI) subsets of immature thymocytes.[789] Interestingly, mouse thymic NK cells bear some phenotypic markers that tend to be found on human $CD56^{bright}$ NK cells.[111] The contribution of secondary lymphoid tissues to NK-cell development, the role of thymic NK cells in immune responses, and the relationship of these mouse and human NK-cell subsets remain to be clarified.

ROLE OF NATURAL KILLER CELLS IN IMMUNE RESPONSES

Mature NK cells in the periphery are involved in rapid innate defense. However, they constitute only a small population of cells (about 2.5% of splenic leukocytes in C57BL/6 mice). How can this small population quickly respond with enough of a critical mass to effect significant innate defense? One mechanism involves the expression of multiple activation receptors by individual NK cells.[449] By contrast to clonally distributed TCRs endowing the individual T cell with the ability to respond only to one antigen, an individual NK cell appears capable of responding to multiple activation receptor ligands. Furthermore, the naïve T-cell population contains only rare cells with a TCR for the relevant antigen, whereas large percentages of the NK-cell population express any given activation receptor in an

overlapping fashion. This multiple activation receptor expression on sizeable subpopulations would allow a substantial number of NK cells to quickly respond to a given specific insult. Another mechanism is related to their constitutive expression of cytokine receptors that permit many NK cells to be stimulated by proinflammatory cytokines produced early in the course of an immune response. Finally, NK cells appear poised to respond rapidly. They constitutively express mRNA for effector molecules, such as granzymes and cytokines, but no protein due to translational control, and can rapidly produce these molecules upon stimulation.[29] Thus, large numbers of NK cells can rapidly respond to a particular stimulus through their activation or cytokine receptors.

Where do NK cells respond? In the mouse, mature conventional NK cells are found primarily in the spleen, blood, and liver. Interestingly, NK cells are localized to the red pulp of the spleen.[849,850] In the liver, they are in the sinusoidal regions rather than the parenchyma, and few NK cells are present in other solid organs. In viral infections, NK cells infiltrate the liver parenchyma in the vicinity of infected foci.[849,851] Several chemokines, including MIP1α, have been implicated in NK-cell localization to liver parenchyma during immune responses,[852] for example, but the NK cell "chemokine code" needs to be clarified.[853] Surprisingly, there are relatively few NK cells in naïve lymph nodes,[849,854] and the thymic NK-cell phenotype is overrepresented among the few resident NK cells,[111] although NK cells can be recruited to draining LNs.[855,856] However, human NK cells are relatively abundant in LNs where they appear to mature, as discussed previously. Study of human NK-cell responses in solid tissues has been limited for obvious reasons. Nonetheless, NK cells seem best suited for surveying the blood for transformed or infected cells and pathogens during acute immune responses but can be recruited to pathologic sites as needed.

As early innate immune responders, NK cells have the capacity to shape the adaptive immune response. For example, early NK-cell control of viral infection essentially limits antigen load and presentation by DCs thereby limiting CD4+ T-cell responses.[857] By contrast, early control also reduces activation of plasmacytoid DCs, thereby lessening the detrimental effects of type I IFNs, preserves the conventional DC compartment, and accelerates CD8+ T-cell responses.[858] Early IFNγ production by NK cells can prime and polarize T-helper responses.[855,859,860,861] In leishmania infections, later production of IL-10 by NK cells has an inhibitory effect on T cells.[862] In addition, NK-cell lysis of targets also enhances T-cell responses.[863] Thus, NK cells have the capacity to enhance, suppress, and polarize adaptive immune responses.

Natural Killer Cells and Tumor Surveillance

An abundant early literature supported a role for NK cells in resisting tumor growth and metastasis.[3] Most prior work utilized experimental protocols involving adoptive transfer of tumor cells into mice where NK cells may eliminate > 90% of tumor cells within the first 24 hours.[864] Several long-term assays of in vivo tumor clearance are available, such as survival or lesion size, but T-cell responses need to be excluded even in syngeneic hosts due to the possibility of tumor-specific peptides. Clearance of intravenously administered radiolabeled tumor cells can be measured with radioactivity of the lung as an index of tumor burden.

Because this lung clearance assay can be performed as early as 4 hours after tumor inoculation, it is relatively confined to innate NK-cell responses in the unimmunized host.[15] In these assays, NK-deficient mice are unable to clear adoptively transferred tumors.[828]

On the other hand, few studies are available on control of primary tumor formation by NK cells (ie, tumor surveillance).[865] Probably the most systematic studies in a single experimental model involve fibrosarcoma development after subcutaneous methylcholanthrene challenge.[866,867,868] In brief, NK cells collaborate with NKT cells in preventing

fibrosarcomas through mechanisms involving perforin, TRAIL, and IFNγ. There is also evidence for involvement of NKG2D[86] and its ligands with respect to the immunoediting of tumors by NK cells.[525,542]

In humans, epidemiological data indicates a strong correlation between high cytotoxic activity of peripheral blood lymphocytes and reduced cancer risk.[869] In addition, ongoing studies of BM transplantation for leukemia demonstrate a relationship between KIR, HLA alleles, and reduced risk of relapse from leukemia in some circumstances.[870,871] Thus, current data support the initial concept that NK cells are involved in tumor immunosurveillance.

Natural Killer Cells in Host Defense Against Pathogens

While NK cells reportedly respond to a wide variety of microorganisms including viruses, bacteria, parasites, and fungi,[872,873] the role of NK cells in infections is probably best illustrated by human patients with selective NK-cell deficiencies.[874,875] Although the molecular basis for most human NK-cell deficiencies is unknown, several points can be gleaned. 1) NK-cell deficiency is associated with a propensity for severe or recurrent virus infections, particularly herpes viruses. 2) Difficulty with tumors is not a common feature, except for virus-related lesions. 3) The disorder is rare, perhaps because patients succumb to overwhelming infection before the syndrome is recognized. 4) Defective NK cells can be found in other genetic and acquired immunodeficiency disorders that affect other immune components. For example, NK cell activity is significantly diminished in AIDS.[876,877] NK-cell infection with herpesvirus 6 induces de novo expression of CD4 rendering susceptibility to HIV-1 infection,[878] perhaps accounting, in part, for increased susceptibility of patients with acquired immunodeficiency syndrome to opportunistic infections, such as severe CMV.[879] Immature NK-cell number and function in the developing fetus may be clinically relevant to the classic “TORCH” syndrome, birth defects associated with maternal toxoplasma, rubella, CMV, and herpes virus infections.[880] Thus, NK cells appear to be especially important in controlling infections, especially from herpes viruses.

In mouse models, detailed evaluation of NK-cell responses against *Listeria* and viral infections have been especially revealing.[881,882] In vivo antibody depletion of NK cells results in marked viral replication in internal organs (spleen, liver), and lethality with MCMV, vaccinia virus, or mouse hepatitis virus.[883,884,885] A similar phenotype was observed in Tg mice lacking NK cells.[886,887] Interestingly, if depleting antibody was given to wild-type mice

later in the infection, there was no untoward effect.[884] Thus, NK cells are significant in early, innate immunity to infections.

Natural Killer-Cell Cytokine Responses and Production during Infection

During infection, NK cells can respond to several different cytokines resulting in production of other cytokines. In listeriosis, the classic model for T-cell-dependent resistance, *scid* mice achieve acute control of infection despite absence of T cells,[888] due to early NK-cell production of IFNγ.[889] However, NK cells do not appear to respond directly to *Listeria*. Rather, macrophages produce IL-12 that then stimulate NK-cell secretion of IFNγ and infection control.[164,890] Furthermore, TNFα can synergize with IL-12 to induce NK-cell production of IFNγ, whereas IL-10 is antagonistic.[164] The increased susceptibility of mice lacking IL-12 receptor, IFNγ, or the IFNγ receptor signaling pathway[891,892,893,894] are consistent with macrophage production of IL-12 that stimulates NK cells to secrete IFNγ in listeriosis.

While a similar IL-12-IFNγ pathway is also operational in MCMV infections,[886,895,896] IL-18 also contributes somewhat to NK cell control of MCMV.[897] However, IL-12 appears to be more critical than IL-18 because uniform lethality was observed in IL-12p35-/-mice challenged with MCMV while all IL-18-/- mice survive.[897] On the other hand, neutralization of IL-18 is a common feature of orthopox-viruses.[898] For example, ectromelia virus (mousepox) contains an ORF for an IL-18 binding protein (IL18BP) that effectively neutralizes the effects of IL-18 on NK cells.[899] In addition to augmented IFNγ production, IL-18 enhances perforin-dependent cytotoxicity.[900,901,902]

Importantly, not all viral infections are controlled by NK cells. For example, NK-cell depletion has little effect on lymphocytic choriomeningitis virus (LCMV) infections.[883,896,903] Yet, during infections, even with LCMV, cytotoxicity of NK cells is enhanced and proliferation ensues. These events constitute systemic effects directly or indirectly mediated by cytokines, such as IL-12, IL-18, and IFNα/β.[904] However, IFNγ production is not seen in LCMV infections.[895,905] This apparent paradox is due to an inhibitory effect of IFNα/β on IL-12-dependent IFNγ production.[905] Inhibition by IFNα/β is mediated through the STAT1 signaling pathway. In the absence of STAT1, IL-12 responsiveness is restored and IFNα/β induces IFNγ production. These studies indicate that the NK-cell cytokine response to infection varies with the pathogen even though many responses may appear to be similar.

A challenging issue is the role of IL-15 in NK cell responses in vivo[906] because IL-15 and IL-15Rα-deficient mice lack NK cells.[53,54] Nevertheless, NK cells can be stimulated during infection by IL-15 and control viral replication during in vitro cultures.[906,907,908,909,910] Moreover, IL-15 can provide protection to herpes simplex viral infections.[910] IFNα/β can stimulate IL-15 production that can drive NK-cell proliferation.[911] Interestingly, however, NK-cell proliferation can occur in an IFNα/β- or IL-15-independent manner.[912,913]

NK-cell responses to cytokines are also regulated by other innate lymphocytes. For example,

in TCRδ-/- mice, *Listeria* infection is enhanced compared to TCRβ-/- mice and is associated with diminished production of IFNγ by NK cells, suggesting that γδT cells regulate NK-cell responses.[914] Similarly, NKT cells can regulate NK cells because administration of α-galactosylceramide, a potent ligand for the TCR on NKT cells, results in nearly concomitant activation of NK cells[79] that may be exploited as a potential cancer immunotherapy. [915] The mechanism behind the NK and NKT cell cross-talk involves IFNγ, apparently produced initially by an activated macrophage.[916] Inasmuch as NKT cells can

recognize glycolipid antigens in mycobacteria,[917,918] these studies indicate a potential physiologically important mechanism and therapeutic intervention for NK-cell activation in innate control of these organisms.[919]

Natural Killer-Cell Activation Receptors in Infection

There are several observations from studies of viral evasion indicating the importance of NK-cell activation receptors in infection. Because viruses have evolved numerous strategies to downregulate MHC-I molecules on infected cells to avoid MHC-I-restricted cytotoxic T-lymphocytes,[186] virally infected cells should have enhanced susceptibility to NK-cell lysis. However, viruses also encode proteins that evade NK cells.[920] In many cases, viral interference of NK cells is related to enhanced function of inhibitory MHCI-specific NK-cell receptors. For example, murine and rat CMV contain ORFs *m144* and *r144*, respectively, that encode molecules with sequence and structural homology to MHC-I and enhance in vivo virulence presumably by interacting with as yet unidentified NK-cell inhibitory receptors.[921,922,923,924,925] MCMV also encodes m157 that can bind the inhibitory receptor Ly49I in 129 strain mice (see following discussion), as well as other MHC-I-like molecules of unknown function.[444,446,464,926] HCMV encodes an MHC-I-like molecule (UL18) that binds LIR1 (ILT2), an Ig-like inhibitory receptor on NK cells,[405,411,927] though its functional significance is somewhat unclear.[928] HCMV also encodes a peptide that binds and enhances expression of HLA-E that in turn binds CD94/NKG2A, a lectin-like NK-cell inhibitory receptor.[386,929,930,931] Another example is the selective downregulation of MHC-I by HIV-1.[932,933] In this case, the virus downregulates HLA-A and B but not HLA-C or E; the former HLA molecules tend to be restricting elements for MHC-I-restricted CTLs, whereas the latter are selectively recognized by human KIRs and CD94/NKG2A. Therefore, viruses have evolved mechanisms to selectively engage inhibitory receptors that presumably prevent the action of NK-cell activation receptors.

Viruses can also directly block triggering of NK-cell activation receptors. For example, both HCMV and MCMV encode multiple proteins that block NKG2D ligand expression by intracellular retention with functional consequences in vitro and in vivo,[920] as previously detailed. HIV-1 Nef also blocks NKG2D ligand expression.[934] Cowpox and monkeypox viruses use a different strategy; they encode a highaffinity, soluble antagonist that binds NKG2D and blocks its ligand recognition.[557] HCMV, Epstein-Barr virus, and KSHV use yet another strategy by encoding miRNAs that target MICB mRNA.[554,555] More generally, KSHV also avoids NK-cell activation through K5 that downregulates expression of ICAM-1 and B7-

2, ligands for NK cell coreceptors involved in target-induced stimulation.[935] Thus, viruses use multiple strategies to specifically thwart NK-cell responses through their activation receptors.

The viral evasion strategies implicate NK-cell activation receptors that specifically recognize infected cells; several have been identified. Human NKp46 binds hemagglutinin of influenza virus and hemagglutinin-neuraminidase of parainfluenza virus, suggesting it may be involved in resistance to these viruses.[630] However, this interaction is dependent on sialic acid residues that are widely expressed, and the in vivo significance of these findings is difficult to assess in humans. Nevertheless, susceptibility of NKp46-deficient mice to influenza infections corroborates these findings.[93] The autosomal dominant *Cmv1* gene in the NKC is responsible for resistance of certain mouse strains to MCMV[596,597,936,937]; MCMV-resistant C57BL/6 mice are susceptible when depleted of NK cells.[938] Extensive genetic and immunological evidence established that *Ly49h* is responsible for genetic resistance to MCMV.[449,938,939,940,941,942] A DAP12-signaling mutant mouse could not resist MCMV,[943] consistent with in vitro signaling studies showing that Ly49H signals through DAP12.[448,939] Thus, Ly49H is an NK-cell activation receptor responsible for genetic resistance to MCMV.

The ligand for Ly49H is encoded by the *m157* ORF in MCMV.[444,446] m157 and 11 other putative MCMV molecules have predicted MHC-I folds, now validated by crystallography.[464,926] Interestingly, in mice lacking adaptive immunity, m157 mutant MCMV clones emerge during MCMV infection, indicating selection pressure from Ly49H+ NK cells result in escape mutant viruses.[944] The reasons for maintenance of *m157* in the MCMV genome are still under investigation, but several observations suggest *m157* may be advantageous to the virus under certain circumstances. 1) m157 binds to the Ly49I inhibitory receptor in 129 mice.[446] 2) *m157*-deletion viruses cause a modest decrease in viral titers in mice lacking both Ly49I and Ly49H, suggesting yet another immune evasion role.[945] 3) A unique aspect of herpes virus biology is the capacity to become latent. If the virus kills the host during the acute viral replicative phase during which Ly49H mediates its control, then there will be no latent phase. 4) Host-virus coevolution often results in attenuated viruses,[946] and other MCMV proteins have positive effects on host responses.[947] Indeed, as one aspect of this coevolution, natural variants of m157 show differential binding activities to a broader range of inhibitory receptors in different mouse strains, including inhibitory Ly49C from B6 mice.[948,949] Thus, studies of m157 reflect the coevolution of the host and virus.

Interestingly, other Ly49 activation receptors also recognize MCMV-encoded proteins. In MA/My mice, the Ly49P activation receptor is responsible for resistance in the context of $H2^k$.[453,950,951,952] This receptor-ligand interaction is more complex than Ly49H-m157 because Ly49P appears to recognize the cell surface combination of $H2D^k$ in association with m04/gp34 encoded by MCMV. However, there appears to be another molecule required for Ly49P recognition as m04 expression on $H2D^k$-expressing cells was insufficient for recognition; infection with Δ*m04* MCMV was required.[453] Recent studies demonstrate that

other Ly49 activation receptors from different mouse strains (BALB/c, NOD/LtJ, PWK/Pas) can also recognize MCMV-infected cells in an m04-H2 allele-dependent manner, providing in vivo control.[953] Taken together, these remarkable studies indicate NK cells recognize MCMV in an "MHC-restricted" manner that is fundamentally different than MHC-restriction as defined for T-cell biology.

Loci for resistance to other pathogens, such as ectromelia virus and herpes simplex virus, have also been genetically mapped to the NKC.[954,955] These loci are termed *Rmp1* and *Rhs1*, respectively, and C57BL/6 mice are resistant. *Rmp1* was initially mapped using DBA/2 mice that lack a functional *Cd94* gene.[394,954] Recent studies of a CD94-deficient mouse show ectromelia virus susceptibility, suggesting that *Rmp1* is encoded by *Cd94*.[396] CD94/NKG2E appears to recognize ectromelia virus-infected cells via Qa-1 and synergizing with NKG2D. Thus, NKC-encoded NK-cell activation receptors mediate NK-cell-dependent resistance to viruses.

In addition to target killing, NK-cell activation receptors also trigger cytokine production in vitro. For example, Ly49H+ NK cells can be selectively activated to produce IFNγ and chemokines within 6 to 8 hours after coculture in vitro with MCMV-infected macrophages or m157 transfectants,[444,956] and m157 itself is expressed soon after infection.[957] However, during early MCMV infection in vivo, IFNγ production was not confined to the Ly49H+ NK-cell subset in C57BL/6 mice, indicating early relative "nonspecific" stimulation of NK cells.[166] Moreover, infection stimulates NK-cell proliferation[958,959] but early (days 1 to 2 postinfection) in vivo NK-cell proliferation was nonselective with respect to Ly49H expression. This proliferation resembled the cytokine-driven "bystander proliferation" observed in T cells in response to viral infections or stimulation with IFNα/β,[960] suggesting that the initial phase of viral-induced NK-cell proliferation represents a nonspecific response to proinflammatory cytokines (IL-12, IFNα/β) and proliferative cytokines such as IL-15.[912] The nonselective proliferation phase was followed by a period of preferential proliferation of Ly49H+ NK cells peaking at days 4 to 6 of MCMV infection.[166] The selective phase of NK-cell proliferation reflected the augmentation of pro-proliferative cytokine stimulation by Ly49H signaling mediated via DAP12.[59] Other activation receptors also trigger specific proliferation in MCMV infection, such as Ly49L+ NK cells in BALB/c mice.[953] However, in infection with vaccinia virus, which lacks a ligand for Ly49H, the initial phase of nonspecific NK-cell proliferation was found but the later specific proliferation of Ly49H+ NK cells was absent.[166] Thus, initial virus-specific NK-cell responses may be masked by generic cytokine responses and only later become detectable.

In humans, how NK cells participate in viral infections has been challenging to understand. However, an unprecedented opportunity to study human NK-cell responses during an epidemic of *Puumala hantavirus* revealed rapid and sustained expansion of NK cells.[961] Most cells expressed CD94/NKG2C and a self-HLA-specific KIR, consistent with expansion of licensed NK cells. Interestingly, expansion of CD94/NKG2C+ NK cells occurs in HCMV+ individuals but not with Epstein-Barr virus or herpes simplex virus infections,[962] though this could not explain the finding in the hantavirus-infected individuals. Regardless, children

infected with HCMV show a similar expansion of CD94/NKG2C+ NK cells; in one case of a T-cell-deficient infant, the expanded NK-cell population was associated with resolution of infection, suggesting that human NK cells can control HCMV.[963,964] Moreover, expansion of CD94/NKG2C+ NK cells also occurs in the setting of acute Chikungunya virus infection.[965] In vitro studies revealed that human NK cells can respond to HCMV-infected fibroblasts by producing cytokines and degranulating. These responses were enhanced by IL-12 and type I IFNs, and there was evidence for stimulation through NKp46 and DNAM-1. However, there was no apparent involvement of the CD94/NKG2C activation receptor,[966] despite expansion of CD94/NKG2C+ NK cells with prolonged culture.[388] Thus, the factors driving human NK-cell expansion and control of viral infection are not completely understood.

MEMORY-LIKE NATURAL KILLER RESPONSES

Although innate immunity is typified by the absence of memory responses, several recent studies suggest that NK cells have memory-like responses, more typical of adaptive immunity, apparently blurring the distinction between innate and adaptive immune responses.[751] In classic contact hypersensitivity (CHS) assays in vivo, NK cells appear to mediate hapten-specific responses in a T- and B-cell-independent manner.[967,968] Adoptive transfer of Ly49C/I+ liver NK cells from sensitized C57BL/6 mice transferred CHS. In addition, hapten-specificity was long lived, as NKdependent responses lasted at least 4 weeks. Subsequent studies indicated that exposure to virus and viral particles, specifically influenza virus, vesicular stomatitis virus, HIV-1, and MCMV, can also induce virus-specific memory-like NK cells.[969,970] Upon adoptive transfer, NK cells can then protect against lethal challenge with the sensitizing virus. While MCMV protection apparently occurred with sensitized splenic NK cells, protection from other viral challenges required liver NK cells expressing CXCR6. Thus, emerging studies suggest that NK cells can display memory-like responses.

The role and basis for NK-cell memory-like responses will require detailed investigation. It is not yet clear if this aspect of NK-cell function is relevant to “vaccination” and how much it contributes to immune protection when immune T and B cells are present. It is unclear how NK cells can demonstrate “antigen” specificity in the absence of the somatic rearrangement mechanism underlying T- and B-cell antigen receptor gene recombination because NK memory-like responses were seen in *Rag2*-/- mice.[967] Moreover, cytokine stimulation alone, without “antigen” stimulation, can induce NK cells to have more robust responses later.[971] Finally, there appear to be significant differences in the inflammatory characteristics and histological features of CHS related to NK-cell responses,[972] suggesting that there may be aspects to be considered that differ from conventional concepts of T-cell-mediated CHS.[968] Thus, although mechanistic details should be forthcoming, this topic has generated intense interest as indicated by review articles which now outnumber the primary literature by about three to one, because the concept of NK-cell memory breaks traditional views on innate and adaptive immunity, and potentially opens the door for examination of related processes in other innate immune components.

NATURAL KILLER CELL AND DENDRITIC CELL INTERACTIONS

Given that both NK cells and DCs, when separately studied, are critical early responders in host immune defense, it was not surprising that these cells could communicate in a bidirectional manner.[973] Since the initial description of this “cross-talk,” innumerable studies have been published indicating that NK cells can respond to signals derived from the various DC subsets and vice versa, in a variety of different immune responses in mice and humans.

Data support both cell contact-independent and -dependent pathways. The contact-independent pathways are perhaps better understood. For example, a variety of stimuli, such as certain TLR ligands,[26,974,975] can activate DCs to produce cytokines. Indeed, activated DCs can produce cytokines, including IFNα/β, IL-12, IL-15, IL-18, and apparently IL-2,[26,975,976,977,978,979] that can stimulate NK-cell production of other cytokines, such as IFNγ. Moreover, these cytokines enhance NK-cell cytotoxicity by increasing protein expression of perforin and granzymes[29] as well as perforin-independent cytotoxic pathways. The cytokines can also enhance responses mediated by ITAM-signaling chain-associated receptors.[59] The in vivo relevance of TLR-induced, DC-produced cytokines is evident in studies showing that TLR9-dependent activation of plasmacytoid DCs in MCMV infections is critical for appropriate Ly49H-dependent NK-cell control of infection.[974]

Direct NK-DC cell-cell contact can also enhance NKcell activation.[973] Several molecules have been implicated in this process, including cytokine-induced DC expression of ligands for human or mouse NKG2D.[980,981] Human DCs can also activate resting NK cells via the NKp30 receptor.[982] Other studies indicate that mouse NK cells can be activated by DCs in a TREM-2-dependent manner through the DAP12 signaling pathway.[983] DCs can also present IL-15 in *trans* that promotes NK-cell development and primes NK cells to respond to bacterial and viral pathogens.[31,984] This process may also be relevant to human NK-cell differentiation.[848]

On the other hand, NK-DC interactions can induce DC maturation.[985] For example, MHC-I-deficient targets can activate NK cells that then induce DC maturation.[986] NK-DC interactions also enhance TH1 polarization apparently through NK-cell production of IFNγ .[855] During MCMV infections, recognition of virus-infected cells by NK cells is critical for maintenance of DC subsets in the spleen.[987] While NK-DC cell contact is also subject to inhibitory effects and even killing of DCs by NK cells,[988,989] the capacity of NK-DC interactions to induce T-cell responses[855,981,986,990] has led to interest in exploiting these interactions for therapeutic vaccines.[991]

A hallmark of DC maturation is migration to a draining LN. Interestingly, however, deliberate introduction of DCs into the lymphatics leads to robust recruitment of activated NK cells.[855] Real-time imaging indicates that NK cells contact DCs in the superficial regions of the LNs where they are less motile and their interactions with DCs are more extensive than T-cell motility and contacts with DCs.[992] *Leishmania* infection led to NK-cell secretion of IFNγ and migration to the paracortex where CD4 T-cell activation occurred, indicating dynamic interactions in the LN not previously appreciated. In humans, DCs in LNs may be involved in differentiation of $CD56^{bright}$ NK cells into $CD56^{dim}$ NK cells.[110]

NATURAL KILLER CELLS AND MATERNAL-FETAL INTERACTIONS

One enigmatic area of continuing interest concerns NK cells in maternal-fetal interactions.[993,994,995] Several observations are related: 1) Initially described as granulated metrial gland cells, maternal uterine NK (uNK) cells accumulate in the uterus, near the fetal trophoblast layer, in all mammalian species examined thus far. 2) At the time of implantation, uNK cells are the most abundant leukocyte present. 3) In humans, uNK cells closely resemble $CD56^{bright}$ subpopulation, but there are phenotypic differences, including expression of activation markers, and different repertoire of MHC-specific receptors. 4) In some mice lacking NK cells, reproductive defects have been described.[996,997] 5) Trophoblasts can stimulate mouse uNK cells.[998,999] 6) There are significant differences in the anatomy of the maternalfetal interface that may limit extrapolation of experimental results from one species to another,[994] although recent studies suggest that rodent uNK cells may interact with trophoblast tissues in a manner similar to human uNK cells.[1000,1001] Thus, uNK cells may provide important clues to understanding maternal-fetal interactions.

Interestingly, counterintuitive insight has come from epidemiological data regarding KIR and HLA genotype associations with reproductive failure.[1002,1003] The role for HLA-G in these disorders is unclear, though early studies suggested that human trophoblasts express the nonclassical MHC-I molecule, HLA-G,[1004] but not other MHC-I or -II molecules; HLA-G may protect the fetus from uNK cell attack.[1005] However, HLA-G is relatively nonpolymorphic, so this may not be able to explain alloantigenicity of the fetus. By contrast, recent studies, using newly available staining reagents, indicate that trophoblast cells predominantly express HLAC.[1003] Furthermore, protection from reproductive failure is associated with maternal KIR haplotypes encoding the KIR2DS1 activation receptor that is specific for HLA-C2, whereas unopposed inhibitory receptors are associated with increased risk. In addition, protection increased when the fetus carried HLA-C2. The implication of this work is that uNK cells sense fetal tissues via their activation and inhibitory receptors to guide the dynamic process of placental remodeling,[1006] and suggest a mechanism for selection of KIR and HLA genotypes in different populations.

Indeed, uNK cells appear to regulate the placental vasculature. Mice lacking NK cells show defects in placental blood vascular remodeling that appears to be partially IFNγ-dependent.[1007] In addition, human uNK cells produce angiogenic factors, particularly early in pregnancy.[1008] Recent studies in mice and rats recapitulate some of the findings observed in humans,[1000,1001,1003] providing new experimental approaches to better understand the role of uNK cells in maternal-fetal tolerance.

NATURAL KILLER CELLS IN HUMAN DISEASE

Throughout this chapter, we have used human disorders involving NK cells to help illustrate their biology. Whereas NK-cell dysfunction has been associated with a large number of other diseases, it is not yet clear if they are clearly involved in a pathogenic manner. However, KIR and HLA genotypes have been linked with an increasing number of human ailments,[754]

providing strong evidence implicating a pathogenetic role for NK cells in these diseases. For example, hepatitis C virus can cause a chronic, persistent infection, although some patients resolve the infection. Interestingly, patients who are homozygous for a KIR gene (KIR2DL3) are more likely to clear hepatitis C virus if they are also homozygous for the HLA-C alleles recognized by KIR2DL3.[756] In papillomavirus infections that can go on to produce cervical carcinomas, resistance to developing neoplasia is similarly associated with genotypes encoding certain KIR-HLA receptor-ligand pairs.[1009] In HIV-1 infections, certain KIR and HLA alleles are associated with reduced risk of infection and slower progression.[1010,1011,1012,1013] These associations are even more compelling given a recent report indicating that the KIR genotypes are associated with amino acid polymorphisms in HIV-1 itself, suggesting that NK cells through their KIRs selected specific HIV-1 genotypes.[1014] These sequences enhance binding of inhibitory KIRs to HIV-1-infected CD4+ T cells and reduce the antiviral activity of NK cells, providing a putative mechanism to explain the association of specific KIR and HLA genotypes with HIV-1 progression. Thus, while the means by which other KIR-HLA combinations modify disease progression is a topic under current investigation, the emerging data nonetheless demonstrate the importance of NK cells and KIR specificities in human disorders and treatments.

Studies also suggest that KIR specificities may be clinically useful to guide treatment. For example, in BM transplantation for leukemia, donor NK cells may help provide as in anti-leukemia effect against residual malignant cells in the recipient if there is a mismatch between the donor and recipient HLA alleles.[870] While this observation was not uniformly observed in other transplant centers,[871,1015,1016] improvements in determing KIR polymorphisms have guided donor selection strategies, leading to superior outcomes in BM transplant therapy for leukemia and neuroblastoma.[1017,1018] Thus, KIR genotyping is clinically useful.

Dysfunctional NK cells are found in patients with FHL who typically demonstrate HLH, as described previously. NK-cell dysfunction is also found in systemic onset juvenile idiopathic arthritis and macrophage activation syndrome associated with that disease.[1019] However, it is not yet clear if NK dysfunction is a cause or effect of systemic illness.

Early studies used LAK cells for adoptive immunotherapy of cancers refractory to conventional therapy.[1020] Several cases of complete remission were reported, but the treatment required intravenous administration of high doses of IL-2 that has significant toxicity. Improved understanding of NK-cell biology and their receptors and specificities has renewed interest in using NK cells for adoptive immunotherapy.[1021,1022] In addition, clinical use of IL-15 may allow for more specific NK-cell activation while sparing the toxicity seen with IL-2.[1023] However, these approaches are still in their infancy and will require controlled studies.

Finally, NK-cell proliferative disorders are associated with both chronic viral infections (Epstein-Barr virus) and autoimmune phenomenon.[8,1024,1025] NK-cell malignancies are particularly aggressive and can appear as lethal midline granuloma due to the propensity of NK lymphomas to present in the sinus and nasopharyngeal passages where they are especially destructive.[1026] NK cells are also associated with hydroa vacciniforme and

hypersensitivity to mosquito bites.[1027]

CONCLUSION

Detailed studies have moved NK cells from the fringes of immunology into the mainstream. Along the way, there have been innumerable challenges, some from "mistaken notions" based on existing paradigms for other immune cells, because NK cells have novel mechanisms of participating in immune defense and tolerance.[1028] Nonetheless, as immunologists and physicians gain more appreciation for the molecular basis for NK-cell biology, the future holds promise for insight into a number of enigmatic and unusual human diseases as well as common disorders, such as hepatitis C virus and HIV infections, and exploitation of NK cells for immunotherapy.

Finally, the elucidation of KIR genotypes and their role in infection and pregnancy support a broader view. The net outcome of selection for certain KIR and HLA genotypes may reflect a short-term survival advantage in terms of infection and a longer-term survival advantage as determined by reproductive influences. Similar processes must also be ongoing in other mammalian species and support the basis for convergent evolution of the NK-cell inhibitory receptors for MHC-I. Thus, "NK cells are centrally involved in both immunity and reproduction."[1029]

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REFERENCES

1. Kiessling R, Klein E, Wigzell H. Natural killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells: specificity and distribution according to genotype. *Eur J Immunol* . 1975;5:112-117.

2. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity. *Int J Cancer* . 1975;16:216.

3. Herberman R. *NK Cells and Other Natural Effector Cells* . New York, NY: Academic Press; 1982.

4. Trinchieri G. Biology of natural killer cells. *Adv Immunol* . 1989;47:187-376.

5. Timonen T, Ortaldo JR, Herberman RB. Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. *J Exp Med* . 1981;153:569-582.

6. Inverardi L, Witson JC, Fuad SA, et al. CD3 negative "small agranular lymphocytes"

are natural killer cells. *J Immunol*. 1991;146:4048-4052.

7. Shortman K, Wilson A, Scollay R, et al. Development of large granular lymphocytes with anomalous, non-specific cytotoxicity in clones derived from Ly 2+ T cells. *Proc Natl Acad Sci U S A*. 1983;80:2728.

8. Lamy T, Loughran TP Jr. Clinical features of large granular lymphocyte leukemia. *Semin Hematol*. 2003;40:185-195.

9. Lanier LL, Phillips JH. A map of the cell surface antigens expressed on resting and activated human natural killer cells. In: Reiherz EL, ed. *Leukocyte Typing II*. New York, NY: Springer-Verlag; 1986:165-180.

10. Barclay AN, Birkeland ML, Brown MH, et al. *The Leucocyte Antigen Factsbook*. London, United Kingdom: Academic Press, 1993.

11. Lanier LL, Phillips JH, Hackett J Jr, et al. Natural killer cells: definition of a cell type rather than a function. *J Immunol*. 1986;137:2735-2739.

12. Ritz J, Campen TJ, Schmidt RE, et al. Analysis of T-cell receptor gene rearrangement and expression in human natural killer clones. *Science*. 1985;228:1540.

13. Lanier LL, Cwirla S, Federspiel N, et al. Human natural killer cells isolated from peripheral blood do not rearrange T cell receptor b chain genes. *J Exp Med*. 1986;163:209.

14. Dorshkind K, Pollack SB, Bosma MJ, et al. Natural killer (NK) cells are present in mice with severe combined immunodeficiency (scid). *J Immunol*. 1985;134:3798-3801.

15. Hackett J Jr, Bosma GC, Bosma MJ, et al. Transplantable progenitors of natural killer cells are distinct from those of T and B lymphocytes. *Proc Natl Acad Sci U S A*. 1986;83:3427-3431.

16. Mombaerts P, Iacomini J, Johnson RS, et al. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*. 1992;68:869-877.

17. Shinkai Y, Rathbun G, Lam KP, et al. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell*. 1992;68:855-867.

18. Phillips JH, Hori T, Nagler A, et al. Ontogeny of human natural killer (NK) cells: fetal NK cells mediate cytolytic function and express cytoplasmic CD3 epsilon,delta proteins. *J Exp Med*. 1992;175:1055-1066.

19. Anderson P, Caligiuri M, Ritz J, et al. CD3-negative natural killer cells express zeta TCR as part of a novel molecular complex. *Nature*. 1989;341:159-162.

20. Lanier LL, Cwirla S, Yu G, et al. Membrane anchoring of a human IgG Fc receptor (CD16) determined by a single amino acid. *Science*. 1989;246:1611-1613.

21. Liu CP, Ueda R, She J, et al. Abnormal T cell development in CD3-zeta-/- mutant mice and identification of a novel T cell population in the intestine. *EMBO J*. 1993;12:4863-4875.

22. Grimm EA, Mazumder A, Zhang HZ, et al. Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. *J Exp Med*. 1982;155:1823-1841.

23. Trinchieri G, Matsumoto-Kobayashi M, Clark SC, et al. Response of resting human peripheral blood natural killer cells to interleukin 2. *J Exp Med*. 1984;160:1147-1169.

24. Phillips JH, Lanier LL. Dissection of the lymphokine-activated killer phenomenon. Relative contribution of peripheral blood natural killer cells and T lymphocytes to cytolysis. *J Exp Med*. 1986;164:814-825.

25. Ortaldo JR, Mason A, Overton R. Lymphokine-activated killer cells. Analysis of progenitors and effectors. *J Exp Med*. 1986;164:1193-1205.

26. Gerosa F, Gobbi A, Zorzi P, et al. The reciprocal interaction of NK cells with plasmacytoid or myeloid dendritic cells profoundly affects innate resistance functions. *J Immunol*. 2005;174:727-734.

27. Reynolds CW, Wiltrout RH. *Functions of the Natural Immune System*. New York, NY: Plenum Press, 1989.

28. Brooks CG, Holscher M, Urdal D. Natural killer activity in cloned cytotoxic T lymphocytes: regulation by interleukin 2, interferon, and specific antigen. *J Immunol*. 1985;135:1145-1152.

29. Fehniger TA, Cai SF, Cao X, et al. Acquisition of murine NK cell cytotoxicity requires the translation of a pre-existing pool of granzyme B and perforin mRNAs. *Immunity*. 2007;26:798-8113.

30. Salcedo TW, Azzoni L, Wolf SF, et al. Modulation of perforin and granzyme messenger RNA expression in human natural killer cells. *J Immunol*. 1993;151:2511-2520.

31. Lucas M, Schachterle W, Oberle K, et al. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. *Immunity*. 2007;26: 503-517.

32. Karlhofer FM, Yokoyama WM. Stimulation of murine natural killer (NK) cells by a monoclonal antibody specific for the NK1.1 antigen. IL-2-activated NK cells possess additional specific stimulation pathways. *J Immunol*. 1991;146:3662-3673.

33. Seaman WE, Niemi EC, Stark MR, et al. Molecular cloning of gp42, a cell-surface molecule that is selectively induced on rat natural killer cells by interleukin 2: glycolipid membrane anchoring and capacity for transmembrane signaling. *J Exp Med*. 1991;173:251-260.

34. Diefenbach A, Tomasello E, Lucas M, et al. Selective associations with signaling molecules determine stimulatory versus costimulatory activity of NKG2D. *Nat Immunol*. 2002;3:1142-1149.

35. Gilfillan S, Ho EL, Cella M, et al. NKG2D recruits two distinct adapters to trigger natural killer cell activation and costimulation. *Nat Immunol*. 2002;3:1150-1155.

36. Meresse B, Chen Z, Ciszewski C, et al. Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity*. 2004;21:357-366.

37. Umehara H, Takashima A, Minami Y, et al. Signal transduction via phosphorylated adhesion molecule, LFA-1 beta (CD18), is increased by culture of natural killer cells with IL-2 in the generation of lymphokine-activated killer cells. *Int Immunol*. 1993;5:19-27.

38. Nishio M, Spielman J, Lee RK, et al. CD80 (B7.1) and CD54 (intracellular adhesion molecule-1) induce target cell susceptibility to promiscuous cytotoxic T cell lysis. *J Immunol*. 1996;157:4347-4353.

39. Sojka DK, Bruniquel D, Schwartz RH, et al. IL-2 secretion by CD4+ T cells in vivo is rapid, transient, and influenced by TCR-specific competition. *J Immunol*. 2004;172:6136-6143.

40. D'Souza WN, Lefrancois L. Frontline: An in-depth evaluation of the production of IL-2 by antigen-specific CD8 T cells in vivo. *Eur J Immunol* . 2004;34:2977-2985.

41. Granucci F, Feau S, Angeli V, et al. Early IL-2 production by mouse dendritic cells is the result of microbial-induced priming. *J Immunol*. 2003;170:5075-5081.

42. Schorle H, Holtschke T, Hunig T, et al. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature*. 1991;352:621-624.

43. Willerford DM, Chen J, Ferry JA, et al. Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. *Immunity*. 1995;3:521-530.

44. Suzuki H, Duncan GS, Takimoto H, et al. Abnormal development of intestinal

intraepithelial lymphocytes and peripheral natural killer cells in mice lacking the IL-2 receptor beta chain. *J Exp Med*. 1997;185: 499-505.

45. DiSanto JP, Muller W, Guy-Grand D, et al. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor gamma chain. *Proc Natl Acad Sci U S A* . 1995;92:377-381.

46. Cao X, Shores EW, Hu-Li J, et al. Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. *Immunity*. 1995;2:223-238.

47. Taniguchi T, Minami Y. Minireview: the IL-2/IL-2 receptor system: a current overview. *Cell*. 1993;73:5-8.

48. Nakarai T, Robertson MJ, Streuli M, et al. Interleukin 2 receptor g chain expression on resting and activated lymphoid cells. *J Exp Med*. 1994;180:241-251.

49. Perussia B. Lymphokine-activated killer cells, natural killer cells and cytokines. *Curr Opin Immunol*. 1991;3:49-55.

50. Sugamura K, Asao H, Kondo M, et al. The interleukin-2 receptor gamma chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID. *Ann Rev Immunol*. 1996;14:179-205.

51. Giri JG, Kumaki S, Ahdieh M, et al. Identification and cloning of a novel IL-15 binding protein that is structurally related to the alpha chain of the IL-2 receptor. *EMBO J*. 1995;14:3654-663.

52. Tagaya Y, Bamford RN, DeFilippis AP, et al. IL-15: a pleiotropic cytokine with diverse receptor/signaling pathways whose expression is controlled at multiple levels. *Immunity*. 1996;4:329-336.

53. Lodolce JP, Boone DL, Chai S, et al. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity*. 1998;9:669-676.

54. Kennedy MK, Glaccum M, Brown SN, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J Exp Med*. 2000;191:771-780.

55. Park SY, Saijo K, Takahashi T, et al. Developmental defects of lymphoid cells in Jak3 kinase-deficient mice. *Immunity*. 1995;3:771-782.

56. Imada K, Bloom ET, Nakajima H, et al. Stat5b is essential for natural killer cell-mediated proliferation and cytolytic activity. *J Exp Med*. 1998;188:2067-2074.

57. Moriggl R, Topham DJ, Teglund S, et al. Stat5 is required for IL-2-induced cell cycle

progression of peripheral T cells. *Immunity*. 1999;10:249-259.

58. Cooper MA, Bush JE, Fehniger TA, et al. In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. *Blood*. 2002;100:3633-3638.

59. French AR, Sjolin H, Kim S, et al. DAP12 signaling directly augments proproliferative cytokine stimulation of NK cells during viral infections. *J Immunol*. 2006;177:4981-4990.

60. Prlic M, Blazar BR, Farrar MA, et al. In vivo survival and homeostatic proliferation of natural killer cells. *J Exp Med*. 2003;197:967-976.

61. Ranson T, Vosshenrich CA, Corcuff E, et al. IL-15 is an essential mediator of peripheral NK-cell homeostasis. *Blood*. 2003;101:4887-4893.

62. Jameson SC. Maintaining the norm: T-cell homeostasis. *Nat Rev Immunol*. 2002;2:547-556.

63. Jamieson AM, Isnard P, Dorfman JR, et al. Turnover and proliferation of NK cells in steady state and lymphopenic conditions. *J Immunol*. 2004;172:864-870.

64. Carson WE, Giri JG, Lindemann MJ, et al. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J Exp Med*. 1994;180:1395-1403.

65. Burkett PR, Koka R, Chien M, et al. Coordinate expression and trans presentation of interleukin (IL)-15Ralpha and IL-15 supports natural killer cell and memory CD8+ T cell homeostasis. *J Exp Med*. 2004;200:825-834.

66. Dubois S, Mariner J, Waldmann TA, et al. IL-15Ralpha recycles and presents IL-15 In trans to neighboring cells. *Immunity*. 2002;17:537-547.

67. Koka R, Burkett PR, Chien M, et al. Interleukin (IL)-15R[alpha]-deficient natural killer cells survive in normal but not IL-15R[alpha]-deficient mice. *J Exp Med*. 2003;197:977-984.

68. Koka R, Burkett P, Chien M, et al. Cutting edge: murine dendritic cells require IL-15R alpha to prime NK cells. *J Immunol*. 2004;173: 3594-3598.

69. Mortier E, Woo T, Advincula R, et al. IL-15Ralpha chaperones IL-15 to stable dendritic cell membrane complexes that activate NK cells via trans presentation. *J Exp Med*. 2008;205:1213-1225.

70. Tanaka T, Kitamura F, Nagasaka Y, et al. Selective long-term elimination of natural killer cells in vivo by an anti-interleukin 2 receptor beta chain monoclonal antibody in mice. *J Exp Med*. 1993;178:1103-1107.

71. Ryan JC, Turck J, Niemi EC, et al. Molecular cloning of the NK1.1 antigen, a member of the NKR-P1 family of natural killer cell activation molecules. *J Immunol*. 1992;149:1631-1635.

72. Hackett J Jr, Tutt M, Lipscomb M, et al. Origin and differentiation of natural killer cells. II. Functional and morphologic studies of purified NK-1.1+ cells. *J Immunol*. 1986;136:3124-3131.

73. Koo GC, Peppard JR. Establishment of monoclonal anti-Nk-1.1 antibody. *Hybridoma*. 1984;3:301-303.

74. Seaman WE, Sleisenger M, Eriksson E, et al. Depletion of natural killer cells in mice by monoclonal antibody to NK-1.1. Reduction in host defense against malignancy without loss of cellular or humoral immunity. *J Immunol*. 1987;138:4539-4544.

75. Carlyle JR, Martin A, Mehra A, et al. Mouse NKR-P1B, a novel NK1.1 antigen with inhibitory function. *J Immunol*. 1999;162:5917-5923.

76. Kung SK, Su RC, Shannon J, et al. The NKR-P1B gene product is an inhibitory receptor on SJL/J NK cells. *J Immunol*. 1999;162:5876-5887.

77. Scalzo AA, Lyons PA, Fitzgerald NA, et al. The BALB.B6-Cmv1r mouse: a strain congenic for Cmv1 and the NK gene complex. *Immunogenetics*. 1995;41:148-151.

78. Carnaud C, Gombert J, Donnars O, et al. Protection against diabetes and improved NK/NKT cell performance in NOD.NK1.1 mice congenic at the NK complex. *J Immunol*. 2001;166:2404-2411.

79. Carnaud C, Lee D, Donnars O, et al. Cutting edge: cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J Immunol*. 1999;163:4647-4650.

80. Ho EL, Carayannopoulos LN, Poursine-Laurent J, et al. Costimulation of multiple NK cell activation receptors by NKG2D. *J Immunol*. 2002;169:3667-3675.

81. Bauer S, Groh V, Wu J, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science*. 1999;285: 727-729.

82. Cerwenka A, Bakker ABH, McClanahan T, et al. Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity*. 2000;12:721-727.

83. Diefenbach A, Jamieson AM, Liu SD, et al. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat Immunol*.

2000;1:119-126.

84. Groh V, Bruhl A, El-Gabalawy H, et al. Stimulation of T cell autoreactivity by anomalous expression of NKG2D and its MIC ligands in rheumatoid arthritis. *Proc Natl Acad Sci U S A*. 2003;100:9452-9457.

85. Ogasawara K, Hamerman JA, Hsin H, et al. Impairment of NK cell function by NKG2D modulation in NOD mice. *Immunity*. 2003;18: 41-51.

86. Smyth MJ, Swann J, Cretney E, et al. NKG2D function protects the host from tumor initiation. *J Exp Med*. 2005;202:583-588.

87. Zafirova B, Mandaric S, Antulov R, et al. Altered NK cell development and enhanced NK cell-mediated resistance to mouse cytomegalovirus in NKG2D-deficient mice. *Immunity*. 2009;31(2):270-282.

88. Cella M, Fuchs A, Vermi W, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature*. 2009;457:722-725.

89. Satoh-Takayama N, Vosshenrich CA, Lesjean-Pottier S, et al. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity*. 2008;29:958-970.

90. Sanos SL, Bui VL, Mortha A, et al. RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nat Immunol*. 2009;10:83-91.

91. Luci C, Reynders A, Ivanov, II, et al. Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin. *Nat Immunol*. 2009;10:75-82.

92. Satoh-Takayama N, Dumoutier L, Lesjean-Pottier S, et al. The natural cytotoxicity receptor NKp46 is dispensable for IL-22-mediated innate intestinal immune defense against Citrobacter rodentium. *J Immunol*. 2009;183:6579-6587.

93. Gazit R, Gruda R, Elboim M, et al. Lethal influenza infection in the absence of the natural killer cell receptor gene Ncr1. *Nat Immunol*. 2006;7:517-523.

94. Eckelhart E, Warsch W, Zebedin E, et al. A novel Ncr1-Cre mouse reveals the essential role of STAT5 for NK-cell survival and development. *Blood*. 2011;117:1565-1573.

95. Walzer T, Blery M, Chaix J, et al. Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. *Proc Natl Acad Sci U S A* . 2007;104:3384-3389.

96. Arase H, Saito T, Phillips JH, et al. Cutting edge: the mouse NK cell-associated antigen recognized by DX5 monoclonal antibody is CD49b (alpha 2 integrin, very late antigen-2). *J Immunol*. 2001;167: 1141-1144.

97. Edelson BT, Li Z, Pappan LK, et al. Mast cell-mediated inflammatory responses require the alpha 2 beta 1 integrin. *Blood*. 2004;103: 2214-2220.

98. Kim S, Iizuka K, Kang HS, et al. In vivo developmental stages in murine natural killer cell maturation. *Nat Immunol*. 2002;3:523-528.

99. Kasai M, Iwamori M, Nagai Y, et al. A glycolipid on the surface of mouse natural killer cells. *Eur J Immunol* . 1980;10:175-180.

100. Young WW Jr, Hakomori SI, Durdik JM, et al. Identification of ganglio-N-tetraosylceramide as a new cell surface marker for murine natural killer (NK) cells. *J Immunol*. 1980;124:199-201.

101. Suttles J, Schwarting GA, Stout RD. Flow cytometric analysis reveals the presence of asialo GM1 on the surface membrane of alloimmune cytotoxic T lymphocytes. *J Immunol*. 1986;136:1586-1591.

102. Hercend T, Griffin JD, Bensussan A, et al. Generation of monoclonal antibodies to a human natural killer clone: characterization of two natural killer associated antigens, NKH1A and NKH2, expressed on subsets of large granular lymphocytes. *J Clin Invest*. 1985;75:932.

103. Lanier LL, Le AM, Civin CI, et al. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J Immunol*. 1986;136:4480.

104. Lanier LL, Testi R, Bindl J, et al. Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. *J Exp Med*. 1989;169:2233.

105. Cunningham BA, Hemperly JJ, Murray BA, et al. Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science*. 1987;236:799.

106. Rutishauser U, Acheson A, Hall AK, et al. The neural cell adhesion molecule (NCAM) as a regulator of cell-cell interactions. *Science*. 1988;240:53.

107. Nitta T, Yagita H, Sato K, et al. Involvement of CD56 (NKH1/Leu-19 antigen) as an adhesion molecule in natural killer-target cell interactions. *J Exp Med*. 1989;170:1757.

108. Barclay AN, Brown MH, Law SKA, et al. *The Leucocyte Antigen Factsbook*. San Diego, CA: Academic Press, 1997.

109. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol*. 2001;22:633-640.

110. Romagnani C, Juelke K, Falco M, et al. CD56brightCD16- killer Iglike receptor- NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation. *J Immunol*. 2007;178:4947-4955.

111. Vosshenrich CA, Garcia-Ojeda ME, Samson-Villeger SI, et al. A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. *Nat Immunol*. 2006;7: 1217-1224.

112. Paul WE. *Fundamental Immunology*. New York, NY: Raven Press, 1984.

113. Paul WE. *Fundamental Immunology*. 3rd ed. New York, NY: Raven Press, 1993.

114. Rolink A, ten Boekel E, Melchers F, et al. A subpopulation of B220+ cells in murine bone marrow does not express CD19 and contains natural killer cell progenitors. *J Exp Med*. 1996;183:187-194.

115. Chan CW, Crafton E, Fan HN, et al. Interferon-producing killer dendritic cells provide a link between innate and adaptive immunity. *Nat Med*. 2006;12:207-213.

116. Taieb J, Chaput N, Menard C, et al. A novel dendritic cell subset involved in tumor immunosurveillance. *Nat Med*. 2006;12:214-219.

117. Vosshenrich CA, Lesjean-Pottier S, Hasan M, et al. CD11cloB220+ interferon-producing killer dendritic cells are activated natural killer cells. *J Exp Med*. 2007;204:2569-2578.

118. Caminschi I, Ahmet F, Heger K, et al. Putative IKDCs are functionally and developmentally similar to natural killer cells, but not to dendritic cells. *J Exp Med*. 2007;204:2579-2590.

119. Blasius AL, Barchet W, Cella M, et al. Development and function of murine B220+CD11c+NK1.1+ cells identify them as a subset of NK cells. *J Exp Med*. 2007;204:2561-2568.

120. Clark R, Griffiths GM. Lytic granules, secretory lysosomes and disease. *Curr Opin Immunol*. 2003;15:516-521.

121. Perou CM, Moore KJ, Nagle DL, et al. Identification of the murine beige gene by YAC complementation and positional cloning. *Nat Gen*. 1996;13:303-308.

122. Barbosa MD, Nguyen QA, Tchernev VT, et al. Identification of the homologous beige

and Chediak-Higashi syndrome genes. *Nature*. 1996;382:262-265.

123. Durchfort N, Verhoef S, Vaughn MB, et al. The enlarged lysosomes in beige(j) cells result from decreased lysosome fission and not increased lysosome fusion. *Traffic*. 2011;13(1):108-119.

124. Lieberman J. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat Rev Immunol* . 2003;3:361-370.

125. Mentlik AN, Sanborn KB, Holzbaur EL, et al. Rapid lytic granule convergence to the MTOC in natural killer cells is dependent on dynein but not cytolytic commitment. *Mol Biol Cell*. 2010;21:2241-2256.

126. Thiery J, Keefe D, Boulant S, et al. Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. *Nat Immunol*. 2011;12:770-777.

127. Afonina IS, Cullen SP, Martin SJ. Cytotoxic and non-cytotoxic roles of the CTL/NK protease granzyme B. *Immunol Rev*. 2010;235:105-116.

128. de Saint Basile G, Menasche G, Fischer A. Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. *Nat Rev Immunol* . 2010;10:568-579.

129. Wood SM, Ljunggren HG, Bryceson YT. Insights into NK cell biology from human genetics and disease associations. *Cell Mol Life Sci* . 2011;68:3479-3493.

130. Bahadoran P, Aberdam E, Mantoux F, et al. Rab27a: a key to melanosome transport in human melanocytes. *J Cell Biol* . 2001;152:843-850.

131. Clayberger C, Krensky AM. Granulysin. *Curr Opin Immunol* . 2003;15: 560-565.

132. Anderson DH, Sawaya MR, Cascio D, et al. Granulysin crystal structure and a structure-derived lytic mechanism. *J Mol Biol* . 2003;325:355-365.

133. Schmidt H, Gelhaus C, Nebendahl M, et al. Effector granules in human T lymphocytes: proteomic evidence for two distinct species of cytotoxic effector vesicles. *J Proteome Res*. 2011;10:1603-1620.

134. Saini RV, Wilson C, Finn MW, et al. Granulysin delivered by cytotoxic cells damages endoplasmic reticulum and activates caspase-7 in target cells. *J Immunol* . 2011;186:3497-3504.

135. Brunner KT, Mauel J, Cerottini JC, et al. Quantitative assay of the lytic action of immune lymphoid cells on 51Cr-labelled allogeneic target cells in vitro; inhibition by

isoantibody and by drugs. *Immunol*. 1968;14:181.

136. Lozzio CB, Lozzio BB. Human chronic myelogenous leukemia cellline with positive Philadelphia chromosome. *Blood*. 1975;45:321-334.

137. Korzeniewski C, Callewaert DM. An enzyme-release assay for natural cytotoxicity. *J Immunol Methods*. 1983;64:313-320.

138. Wierda WG, Mehr DS, Kim YB. Comparison of fluorochrome-labeled and 51Cr-labeled targets for natural killer cytotoxicity assay. *J Immunol Methods*. 1989;122:15-24.

139. Radosevic K, Garritsen HS, Van Graft M, et al. A simple and sensitive flow cytometric assay for the determination of the cytotoxic activity of human natural killer cells. *J Immunol Methods*. 1990;135:81-89.

140. Kim S, Yokoyama WM. NK cell granule exocytosis and cytokine production inhibited by Ly-49A engagement. *Cell Immunol*. 1998;183:106-112.

141. Betts MR, Brenchley JM, Price DA, et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J Immunol Methods*. 2003;281:65-78.

142. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. *J Immunol Methods*. 2004;294:15-22.

143. Bryceson YT, March ME, Barber DF, et al. Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. *J Exp Med*. 2005;202:1001-1012.

144. Penack O, Gentilini C, Fischer L, et al. CD56dimCD16neg cells are responsible for natural cytotoxicity against tumor targets. *Leukemia*. 2005;19:835-840.

145. Henkart PA. Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *Immunity*. 1994;1:343-346.

146. Montel AH, Bochan MR, Hobbs JA, et al. Fas involvement in cytotoxicity mediated by human NK cells. *Cell Immunol*. 1995;166:236-246.

147. Arase H, Arase N, Saito T. Fas-mediated cytotoxicity by freshly isolated natural killer cells. *J Exp Med*. 1995;181:1235-1238.

148. Lee RK, Spielman J, Zhao DY, et al. Perforin, Fas ligand, and tumor necrosis factor are the major cytotoxic molecules used by lymphokine-activated killer cells. *J Immunol*. 1996;157:1919-1925.

149. Oshimi Y, Oda S, Honda Y, et al. Involvement of Fas ligand and Fas-mediated pathway in the cytotoxicity of human natural killer cells. *J Immunol* . 1996;157:2909-2915.

150. Screpanti V, Wallin RP, Ljunggren HG, et al. A central role for death receptor-mediated apoptosis in the rejection of tumors by NK cells. *J Immunol* . 2001;167:2068-2073.

151. Takeda K, Hayakawa Y, Smyth MJ, et al. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nat Med*. 2001;7:94-100.

152. Gommerman JL, Browning JL. Lymphotoxin/light, lymphoid microenvironments and autoimmune disease. *Nat Rev Immunol* . 2003;3: 642-655.

153. Ware CF. Network communications: lymphotoxins, LIGHT, and TNF. *Annu Rev Immunol*. 2005;23:787-819.

154. Iizuka K, Chaplin DD, Wang Y, et al. Requirement for membrane lymphotoxin in natural killer cell development. *Proc Natl Acad Sci U S A* . 1999;96:6336-6340.

155. Kagi D, Ledermann B, Burki K, et al. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature*. 1994;369:31-37.

156. Revell PA, Grossman WJ, Thomas DA, et al. Granzyme B and the downstream granzymes C and/or F are important for cytotoxic lymphocyte functions. *J Immunol* . 2005;174:2124-2131.

157. Pham CT, Ley TJ. Dipeptidyl peptidase I is required for the processing and activation of granzymes A and B in vivo. *Proc Natl Acad Sci U S A* . 1999;96:8627-8632.

158. Crozat K, Hoebe K, Ugolini S, et al. Jinx, an MCMV susceptibility phenotype caused by disruption of Unc13d: a mouse model of type 3 familial hemophagocytic lymphohistiocytosis. *J Exp Med*. 2007;204:853-863.

159. Idris AH, Smith HRC, Mason LH, et al. The natural killer cell complex genetic locus, Chok, encodes Ly49D, a target recognition receptor that activates natural killing. *Proc Natl Acad Sci U S A*. 1999;96:6330-6335.

160. Degliantoni G, Murphy M, Kobayashi M, et al. Natural killer (NK) cell-derived hematopoietic colony-inhibiting activity and NK cytotoxic factor. Relationship with tumor necrosis factor and synergism with immune interferon. *J Exp Med*. 1985;162:1512-1530.

161. Anegon I, Cuturi MC, Trinchieri G, et al. Interaction of Fc receptor (CD16) ligands induces transcription of interleukin 2 receptor (CD25) and lymphokine genes and expression of their products in human natural killer cells. *J Exp Med*. 1988;167:452-472.

162. Cuturi MC, Anegon I, Sherman F, et al. Production of hematopoietic colony stimulating factors by human natural killer cells. *J Exp Med*. 1989;169:569.

163. Dorner BG, Scheffold A, Rolph MS, et al. MIP-1alpha, MIP-1beta, RANTES, and ATAC/lymphotactin function together with IFN-gamma as type 1 cytokines. *Proc Natl Acad Sci U S A*. 2002;99:6181-6186.

164. Tripp CS, Wolf SF, Unanue ER. Interleukin 12 and tumor necrosis factor alpha are costimulators of interferon gamma production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. *Proc Natl Acad Sci U S A* . 1993;90:3725-3729.

165. McCartney S, Vermi W, Gilfillan S, et al. Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells. *J Exp Med*. 2009;206:2967-2976.

166. Dokun AO, Kim S, Smith HR, et al. Specific and nonspecific NK cell activation during virus infection. *Nat Immunol* . 2001;2:951-956.

167. Stetson DB, Mohrs M, Reinhardt RL, et al. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J Exp Med*. 2003;198:1069-1076.

168. Kim S, Poursine-Laurent J, Truscott SM, et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature*. 2005;436:709-713.

169. Biron CA. Interferons alpha and beta as immune regulators—a new look. *Immunity*. 2001;14:661-664.

170. Kärre K, Ljunggren H-G, Piontek G, et al. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature*. 1986;319:675-678.

171. Piontek GE, Taniguchi K, Ljuggren HG, et al. YAC-1 MHC class I variants reveal an association between decreased NK sensitivity and increased H-2 expression after interferon treatment or in vivo passage. *J Immunol* . 1985;135:4281-4288.

172. Ljunggren HG, Karre K. In search of the “missing self”: MHC molecules and NK cell recognition. *Immunol Today*. 1990;11:237-244.

173. Storkus WJ, Salter RD, Alexander J, et al. Class I-induced resistance to natural killing: identification of nonpermissive residues in HLA-A2. *Proc Natl Acad Sci U S A* . 1991;88:5989-5992.

174. Colonna M, Brooks EG, Falco M, et al. Generation of allospecific natural killer cells by stimulation across a polymorphism of HLA-C. *Science*. 1993;260:1121-1124.

175. Litwin V, Gumperz J, Parham P, et al. Specificity of HLA class I antigen recognition by human NK clones: evidence for clonal heterogeneity, protection by self and non-self alleles, and influence of the target cell type. *J Exp Med*. 1993;178:1321-1336.

176. Quillet A, Presse F, Marchiol-Fournigault C, et al. Increased resistance to non-MHC-restricted cytotoxicity related to HLA A,B expression. Direct demonstration using beta 2-microglobulin-transfected Daudi cells. *J Immunol*. 1988;141:17-20.

177. Franksson L, George E, Powis S, et al. Tumorigenicity conferred to lymphoma mutant by major histocompatibility complex-encoded transporter gene. *J Exp Med*. 1993;177:201-205.

178. Salcedo M, Momburg F, Hammerling GJ, et al. Resistance to natural killer cell lysis conferred by TAP1/2 genes in human antigen-processing mutant cells. *J Immunol*. 1994;152:1702-1708.

179. Liao NS, Bix M, Zijlstra M, et al. MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. *Science*. 1991;253:199-202.

180. Höglund P, Ohlén C, Carbone E, et al. Recognition of beta 2-microglobulin- negative (beta 2m-) T-cell blasts by natural killer cells from normal but not from beta 2m- mice: nonresponsiveness controlled by beta 2m- bone marrow in chimeric mice. *Proc Natl Acad Sci U S A*. 1991;88:10332-10336.

181. Bix M, Liao NS, Zijlstra M, et al. Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice. *Nature*. 1991;349: 329-331.

182. Yu YYL, Kumar V, Bennett M. Murine natural killer cells and marrow graft rejection. *Ann Rev Immunol*. 1992;10:189-213.

183. Rembecki RM, Kumar V, David CS, et al. Bone marrow cell transplants involving intra-H-2 recombinant inbred mouse strains. Evidence that hemopoietic histocompatibility-1 (Hh-1) genes are distinct from H-2D or H-2L. *J Immunol*. 1988;141:2253-22560.

184. Jorgensen JL, Reay PA, Ehrici EW, et al. Molecular components of T-cell recognition. *Ann Rev Immunol*. 1992;10:835-873.

185. Kärre K. Role of target histocompatibility antigens in regulation of natural killer activity: a reevaluation and a hypothesis. In: Herberman RB, Callewaert D, eds. *Mechanisms of Cytotoxicity by NK Cells* . Orlando, FL: Academic Press; 1985:81-103.

186. Hansen TH, Bouvier M. MHC class I antigen presentation: learning from viral

evasion strategies. *Nat Rev Immunol* . 2009;9:503-513.

187. Holtkamp B, Cramer M, Rajewsky K. Somatic variation of H-2Kk expression and structure in a T-cell lymphoma: instability, stabilization, high production and structural mutation. *EMBO J*. 1983;2: 1943-1951.

188. Gattoni-Celli S, Kirsch K, Timpane R, et al. Beta 2-microglobulin gene is mutated in a human colon cancer cell line (HCT) deficient in the expression of HLA class I antigens on the cell surface. *Cancer Res* . 1992;52:1201-1204.

189. Korkolopoulou P, Kaklamanis L, Pezzella F, et al. Loss of antigen-presenting molecules (MHC class I and TAP-1) in lung cancer. *Br J Cancer* . 1996;73:148-153.

190. Storkus WJ, Dawson JR. Target structures involved in natural killing (NK): characteristics, distribution, and candidate molecules. *Crit Rev Immunol* . 1991;10:393-416.

191. Karlhofer FM, Ribaudo RK, Yokoyama WM. MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. *Nature*. 1992;358:66-70.

192. Long EO. Regulation of immune responses through inhibitory receptors. *Annu Rev Immunol* . 1999;17:875-904.

193. Yokoyama WM. Natural killer cells. Right-side-up and up-side-down NK-cell receptors. *Curr Biol* . 1995;5:982-985.

194. Yokoyama WM. Recognition structures on natural killer cells. *Curr Opin Immunol* . 1993;5:67-73.

195. Yokoyama WM, Seaman WE. The Ly-49 and NKR-P1 gene families encoding lectin-like receptors on natural killer cells: the NK gene complex. *Ann Rev Immunol* . 1993;11:613-635.

196. Yokoyama WM, Daniels BF, Seaman WE, et al. A family of murine NK cell receptors specific for target cell MHC class I molecules. *Sem Immunol* . 1995;7:89.101.

197. Chan PY, Takei F. Expression of a T cell receptor-like molecule on normal and malignant murine T cells detected by rat monoclonal antibodies to nonclonotypic determinants. *J Immunol* . 1986;136:1346.

198. Nagasawa R, Gross J, Kanagawa O, et al. Identification of a novel T cell surface disulfide-bonded dimer distinct from the alpha/beta antigen receptor. *J Immunol* . 1987;138:815-824.

199. Chan PY, Takei F. Molecular cloning and characterization of a novel murine T cell

surface antigen, YE1/48. *J Immunol*. 1989;142:1727.

200. Yokoyama WM, Jacobs LB, Kanagawa O, et al. A murine T lymphocyte antigen belongs to a supergene family of type II integral membrane proteins. *J Immunol*. 1989;143:1379-1386.

201. Wong S, Freeman JD, Kelleher C, et al. Ly-49 multigene family. New members of a superfamily of type II membrane proteins with lectinlike domains. *J Immunol*. 1991;147:1417-1423.

202. Smith HRC, Karlhofer FM, Yokoyama WM. Ly-49 multigene family expressed by IL-2-activated NK cells. *J Immunol*. 1994;153: 1068-1079.

203. Yokoyama WM, Kehn PJ, Cohen DI, et al. Chromosomal location of the Ly-49 (A1, YE1/48) multigene family. Genetic association with the NK 1.1 antigen. *J Immunol*. 1990;145:2353-2358.

204. Karlhofer FM, Ribaudo RK, Yokoyama WM. The interaction of Ly-49 with H-2Dd globally inactivates natural killer cell cytolytic activity. *Trans Assoc Am Physicians*. 1992;105:72-85.

205. Correa I, Corral L, Raulet DH. Multiple natural killer cell-activating signals are inhibited by major histocompatibility complex class I expression in target cells. *Eur J Immunol*. 1994;24:1323-1331.

206. Kane KP. Ly-49 mediates EL4 lymphoma adhesion to isolated class I major histocompatibility complex molecules. *J Exp Med*. 1994;179: 1011-1015.

207. Daniels BF, Karlhofer FM, Seaman WE, et al. A natural killer cell receptor specific for a major histocompatibility complex class I molecule. *J Exp Med*. 1994;180:687-692.

208. Karlhofer FM, Hunziker R, Reichlin A, et al. Host MHC class I molecules modulate in vivo expression of a NK cell receptor. *J Immunol*. 1994;153:2407-2416.

209. Olsson MY, Karre K, Sentman CL. Altered phenotype and function of natural killer cells expressing the major histocompatibility complex receptor Ly-49 in mice transgenic for its ligand. *Proc Natl Acad Sci U S A* . 1995;92:1649-1653.

210. Held W, Dorfman JR, Wu MF, et al. Major histocompatibility complex class I dependent skewing of the natural killer cell LY49 receptor repertoire. *Eur J Immunol* . 1996;26:2286-2292.

211. Held W, Cado D, Raulet DH. Transgenic expression of the Ly49A natural killer cell receptor confers class I major histocompatibility complex (MHC)-specific inhibition and prevents bone marrow allograft rejection. *J Exp Med*. 1996;184:2037-2041.

212. Nakamura MC, Niemi EC, Fisher MJ, et al. Mouse Ly-49A interrupts early signaling events in natural killer cell cytotoxicity and functionally associates with the Shp-1 tyrosine phosphatase. *J Exp Med*. 1997;185:673-684.

213. Hanke T, Takizawa H, McMahon CW, et al. Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors. *Immunity*. 1999;11:67-77.

214. Matsumoto N, Mitsuki M, Tajima K, et al. The functional binding site for the C-type lectin-like natural killer cell receptor Ly49A spans three domains of its major histocompatibility complex class I ligand. *J Exp Med*. 2001;193:147-158.

215. Matsumoto N, Yokoyama WM, Kojima S, et al. The NK cell MHC class I receptor Ly49A detects mutations on H-2D(d) inside and outside of the peptide binding groove. *J Immunol*. 2001;166:4422-4428.

216. Wang J, Whitman MC, Natarajan K, et al. Binding of the natural killer cell inhibitory receptor Ly49A to its major histocompatibility complex class I ligand. Crucial contacts include both H-2Dd and beta 2-microglobulin. *J Biol Chem*. 2002;277:1433-1442.

217. Tormo J, Natarajan K, Margulies DH, et al. Crystal structure of a lectin-like natural killer cell receptor bound to its MHC class I ligand. *Nature*. 1999;402:623-631.

218. Olsson-Alheim MY, Sundback J, Karre K, et al. The MHC class I molecule H-2Dp inhibits murine NK cells via the inhibitory receptor Ly49A. *J Immunol*. 1999;162:7010-7014.

219. Jonsson AH, Yang L, Kim S, et al. Effects of MHC class I alleles on licensing of Ly49A+ NK cells. *J Immunol*. 2010;184:3424-3432.

220. Correa I, Raulet DH. Binding of diverse peptides to MHC class I molecules inhibits target cell lysis by activated natural killer cells. *Immunity*. 1995;2:61-71.

221. Orihuela M, Margulies DH, Yokoyama WM. The natural killer cell receptor Ly-49A recognizes a peptide-induced conformational determinant on its major histocompatibility complex class I ligand. *Proc Natl Acad Sci U S A* . 1996;93:11792-11797.

222. Weis WI, Drickamer K. Structural basis of lectin-carbohydrate recognition. *Ann Rev Biochem*. 1996;65:441-473.

223. Natarajan K, Dimasi N, Wang J, et al. Structure and function of natural killer cell receptors: multiple molecular solutions to self, nonself discrimination. *Annu Rev Immunol*. 2002;20:853-885.

224. Matsumoto N, Ribaudo RK, Abastado J-P, et al. The lectin-like NK cell receptor Ly-

49A recognizes a carbohydrate-independent epitope on its MHC class I ligand. *Immunity*. 1998;8:245-254.

225. Mitsuki M, Matsumoto N, Yamamoto K. A species-specific determinant on beta2-microglobulin required for Ly49A recognition of its MHC class I ligand. *Int Immunol*. 2004;16:197-204.

226. Michaelsson J, Achour A, Rolle A, et al. MHC Class I Recognition by NK Receptors in the Ly49 Family Is Strongly Influenced by the beta(2)-Microglobulin Subunit. *J Immunol*. 2001;166:7327-7334.

227. Wilhelm BT, Gagnier L, Mager DL. Sequence analysis of the ly49 cluster in C57BL/6 mice: a rapidly evolving multigene family in the immune system. *Genomics*. 2002;80:646-661.

228. Silver ET, Elliott JF, Kane KP. Alternatively spliced LY-49D and H transcripts are found in IL-2-activated NK cells. *Immunol Today*. 1996;44:14-17.

229. Gays F, Koh AS, Mickiewicz KM, et al. Comprehensive analysis of transcript start sites in ly49 genes reveals an unexpected relationship with gene function and a lack of upstream promoters. *PLoS One*. 2011;6:e18475.

230. Brennan J, Mager D, Jefferies W, et al. Expression of different members of the Ly-49 gene family defines distinct natural killer cell subsets and cell adhesion properties. *J Exp Med*. 1994;180: 2287-2295.

231. Lemieux S, Ouellet-Talbot F, Lusignan Y, et al. Identification of murine natural killer cell subsets with monoclonal antibodies derived from 129 anti-C57BL/6 immune spleen cells. *Cell Immunol*. 1991;134:191-204.

232. Stoneman ER, Bennett M, An J, et al. Cloning and characterization of 5E6(Ly-49C), a receptor molecule expressed on a subset of murine natural killer cells. *J Exp Med*. 1995;182:305-313.

233. Brennan J, Lemieux S, Freeman JD, et al. Heterogeneity among Ly-49C natural killer (NK) cells - characterization of highly related receptors with differing functions and expression patterns. *J Exp Med*. 1996;184:2085-2090.

234. Dam J, Guan R, Natarajan K, et al. Variable MHC class I engagement by Ly49 natural killer cell receptors demonstrated by the crystal structure of Ly49C bound to H-2K(b). *Nat Immunol*. 2003;4:1213-1222.

235. Deng L, Cho S, Malchiodi EL, et al. Molecular architecture of the major histocompatibility complex class I-binding site of Ly49 natural killer cell receptors. *J Biol Chem*. 2008;283:16840-16849.

236. Franksson L, Sundback J, Achour A, et al. Peptide dependency and selectivity of the NK cell inhibitory receptor Ly-49C. *Eur J Immunol* . 1999;29:2748-2758.

237. Dam J, Baber J, Grishaev A, et al. Variable dimerization of the Ly49A natural killer cell receptor results in differential engagement of its MHC class I ligand. *J Mol Biol* . 2006;362:102-113.

238. Doucey MA, Scarpellino L, Zimmer J, et al. Cis association of Ly49A with MHC class I restricts natural killer cell inhibition. *Nat Immunol* . 2004;5:328-336.

239. Johansson MH, Bieberich C, Jay G, et al. Natural killer cell tolerance in mice with mosaic expression of major histocompatibility complex class I transgene. *J Exp Med*. 1997;186:35-64.

240. Andersson M, Freland S, Johansson MH, et al. MHC class I mosaic mice reveal insights into control of Ly49C inhibitory receptor expression in NK cells. *J Immunol* . 1998;161:6475-6479.

241. Chalifour A, Scarpellino L, Back J, et al. A Role for cis interaction between the inhibitory Ly49A receptor and MHC class I for natural killer cell education. *Immunity*. 2009;30:337-347.

242. Back J, Malchiodi EL, Cho S, et al. Distinct conformations of Ly49 natural killer cell receptors mediate MHC class I recognition in trans and cis. *Immunity*. 2009;31:598-608.

243. Orr MT, Lanier LL. Inhibitory Ly49 receptors on mouse natural killer cells. *Curr Top Microbiol Immunol*. 2011;350:67-87.

244. Dimasi N, Sawicki MW, Reineck LA, et al. Crystal structure of the Ly49I natural killer cell receptor reveals variability in dimerization mode within the Ly49 family. *J Mol Biol* . 2002;320:573-585.

245. Kubota A, Kubota S, Lohwasser S, et al. Diversity of NK cell receptor repertoire in adult and neonatal mice. *J Immunol* . 1999;163:212-216.

246. Dorfman JR, Raulet DH. Acquisition of Ly49 receptor expression by developing natural killer cells. *J Exp Med*. 1998;187:609-618.

247. Aust JG, Gays F, Hussain F, et al. Mice lacking Ly49E show normal NK cell development and provide evidence for probabilistic expression of Ly49E in NK cells and T cells. *J Immunol* . 2011;186:2013-2023.

248. Salcedo M, Diehl AD, Olsson-Alheim MY, et al. Altered expression of Ly49 inhibitory receptors on natural killer cells from MHC class I-deficient mice. *J Immunol* .

1997;158:3174-3180.

249. Held W, Roland J, Raulet DH. Allelic exclusion of Ly49-family genes encoding class I MHC-specific receptors on NK cells. *Nature*. 1995;376:355-358.

250. Saleh A, Davies GE, Pascal V, et al. Identification of probabilistic transcriptional switches in the Ly49 gene cluster: a eukaryotic mechanism for selective gene activation. *Immunity*. 2004;21:55-66.

251. Rouhi A, Gagnier L, Takei F, et al. Evidence for epigenetic maintenance of Ly49a monoallelic gene expression. *J Immunol*. 2006;176: 2991-2999.

252. Kunz B, Held W. Positive and negative roles of the trans-acting T cell factor-1 for the acquisition of distinct Ly-49 MHC class I receptors by NK cells. *J Immunol*. 2001;166:6181-6187.

253. Ioannidis V, Kunz B, Tanamachi DM, et al. Initiation and limitation of Ly-49A NK cell receptor acquisition by T cell factor-1. *J Immunol*. 2003;171:769-775.

254. Held W, Clevers H, Grosschedl R. Redundant functions of TCF-1 and LEF-1 during T and NK cell development, but unique role of TCF-1 for Ly49 NK cell receptor acquisition. *Eur J Immunol* . 2003;33:1393-1398.

255. Yokoyama WM, Ryan JC, Hunter JJ, et al. cDNA cloning of mouse NKR-P1 and genetic linkage with Ly-49. Identification of a natural killer cell gene complex on mouse chromosome 6. *J Immunol*. 1991;147: 3229-3236.

256. Yokoyama WM, Plougastel BF. Immune functions encoded by the natural killer gene complex. *Nat Rev Immunol* . 2003;3:304-316.

257. Makrigiannis AP, Patel D, Goulet ML, et al. Direct sequence comparison of two divergent class I MHC natural killer cell receptor haplotypes. *Genes Immun*. 2005;6:71-83.

258. Anderson SK, Dewar K, Goulet ML, et al. Complete elucidation of a minimal class I MHC natural killer cell receptor haplotype. *Genes Immun*. 2005;6:481-492.

259. Belanger S, Tai LH, Anderson SK, et al. Ly49 cluster sequence analysis in a mouse model of diabetes: an expanded repertoire of activating receptors in the NOD genome. *Genes Immun*. 2008;9:509-521.

260. Higuchi DA, Cahan P, Gao J, et al. Structural variation of the mouse natural killer gene complex. *Genes Immun*. 2010;11:637-648.

261. Sundback J, Karre K, Sentman CL. Cloning of minimally divergent allelic forms of

the natural killer (NK) receptor LY-49C, differentially controlled by host genes in the MHC and NK gene complexes. *J Immunol*. 1996;157:3936-3942.

262. Mehta IK, Smith HRC, Wang J, et al. A "chimeric" C57L-derived Ly49 inhibitory receptor resembling the Ly49D activation receptor. *Cell Immunol*. 2001;209:29-41.

263. Makrigiannis AP, Pau AT, Saleh A, et al. Class i mhc-binding characteristics of the 129/j ly49 repertoire. *J Immunol*. 2001;166:5034-5043.

264. Mehta IK, Wang J, Roland J, et al. Ly49A allelic variation and MHC class I specificity. *Immunogenetics*. 2001;53:572-583.

265. Hansen TH, Carreno BM, Sachs DH. The major histocompatibility complex. In: Paul WE, ed. *Fundamental Immunology*. 3rd ed. New York, NY: Raven Press; 1993:577-628.

266. Maeda M, Lohwasser S, Yamamura T, et al. Regulation of NKT cells by Ly49: analysis of primary NKT cells and generation of NKT cell line. *J Immunol*. 2001;167:4180-4186.

267. Coles MC, McMahon CW, Takizawa H, et al. Memory CD8 T lymphocytes express inhibitory MHC-specific Ly49 receptors. *Eur J Immunol* . 2000;30:236-244.

268. Toyama-Sorimachi N, Tsujimura Y, Maruya M, et al. Ly49Q, a member of the Ly49 family that is selectively expressed on myeloid lineage cells and involved in regulation of cytoskeletal architecture. *Proc Natl Acad Sci U S A* . 2004;101:1016-1021.

269. Gays F, Aust JG, Reid DM, et al. Ly49B Is expressed on multiple subpopulations of myeloid cells. *J Immunol*. 2006;177:5840-5851.

270. Tai LH, Goulet ML, Belanger S, et al. Positive regulation of plasmacytoid dendritic cell function via Ly49Q recognition of class I MHC. *J Exp Med*. 2008;205:3187-3199.

271. Yoshizaki M, Tazawa A, Kasumi E, et al. Spatiotemporal regulation of intracellular trafficking of Toll-like receptor 9 by an inhibitory receptor, Ly49Q. *Blood*. 2009;114:1518-1527.

272. Van Den Broeck T, Stevenaert F, Taveirne S, et al. Ly49E-dependent inhibition of natural killer cells by urokinase plasminogen activator. *Blood*. 2008;112:5046-5051.

273. Moretta A, Tambussi G, Bottino C, et al. A novel surface antigen expressed by a subset of human CD3- CD16+ natural killer cells. Role in cell activation and regulation of cytolytic function. *J Exp Med*. 1990;171:695.

274. Moretta A, Bottino C, Pende D, et al. Identification of four subsets of human CD3-CD16+ natural killer (NK) cells by the expression of clonally distributed functional surface

molecules: correlation between subset assignment of NK clones and ability to mediate specific alloantigen recognition. *J Exp Med*. 1990;172:1589-1598.

275. Moretta A, Biassoni R, Bottino C, et al. Major histocompatibility complex class I-specific receptors on human natural killer and T lymphocytes. *Immunol Rev*. 1997;155:105-117.

276. Colonna M, Borsellino G, Falco M, et al. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc Natl Acad Sci U S A* . 1993;90:12000-12004.

277. Colonna M. Specificity and function of immunoglobulin superfamily NK cell inhibitory and stimulatory receptors. *Immunol Rev*. 1997;155:127-133.

278. Ciccone E, Pende D, Viale O, et al. Involvement of HLA class I alleles in natural killer (NK) cell-specific functions: expression of HLA-Cw3 confers selective protection from lysis by alloreactive NK clones displaying a defined specificity (specificity 2). *J Exp Med*. 1992;176:963.

279. Moretta A, Vitale M, Bottino C, et al. P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. *J Exp Med*. 1993;178:597-604.

280. Litwin V, Gumperz J, Parham P, et al. NKB1: a natural killer cell receptor involved in the recognition of polymorphic HLA-B molecules. *J Exp Med*. 1994;180:537-543.

281. Lanier LL, Corliss B, Phillips JH. Arousal and inhibition of human NK cells. *Immunol Rev*. 1997;155:145-154.

282. Gumperz JE, Litwin V, Phillips JH, et al. The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. *J Exp Med*. 1995;181: 1133-1144.

283. Pende D, Biassoni R, Cantoni C, et al. The natural killer cell receptor specific for HLA-A allotypes: a novel member of the p58/p70 family of inhibitory receptors that is characterized by three immunoglobulinlike domains and is expressed as a 140-kD disulphide-linked dimer. *J Exp Med*. 1996;184:505-518.

284. Dohring C, Scheidegger D, Samaridis J, et al. A human killer inhibitory receptor specific for HLA-A. *J Immunol* . 1996;156:3098-3101.

285. Wagtmann N, Biassoni R, Cantoni C, et al. Molecular clones of the p58 NK cell receptor reveal immunoglobulin-related molecules with diversity in both the extra- and intracellular domains. *Immunity*. 1995;2:439-449.

286. Colonna M, Samaridis J. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science*. 1995;268:405-408.

287. D'Andrea A, Chang C, Franz-Bacon K, et al. Molecular cloning of NKB1. A natural killer cell receptor for HLA-B allotypes. *J Immunol* .1995;155:2306-2310.

288. Long EO, Colonna M, Lanier LL. Inhibitory MHC class I receptors on NK and T cells: a standard nomenclature. *Immunol Today*. 1996;17:100.

289. Marsh SG, Parham P, Dupont B, et al. Killer-cell immunoglobulinlike receptor (KIR) nomenclature report, 2002. *Tissue Antigens*. 2003; 62:79-86.

290. Rajagopalan S, Long EO. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J Exp Med*. 1999;189:1093-1100.

291. Cantoni C, Verdiani S, Falco M, et al. P49, a putative HLA class I-specific inhibitory NK receptor belonging to the immunoglobulin superfamily. *Eur J Immunol* . 1998;28:1980-1990.

292. Faure M, Long EO. KIR2DL4 (CD158d), an NK cell-activating receptor with inhibitory potential. *J Immunol*. 2002;168:6208-6214.

293. Miah SM, Hughes TL, Campbell KS. KIR2DL4 differentially signals downstream functions in human NK cells through distinct structural modules. *J Immunol*. 2008;180:2922-2932.

294. Wagtmann N, Rajagopalan S, Winter CC, et al. Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. *Immunity*. 1995;3:801-809.

295. Dohring C, Colonna M. Human natural killer cell inhibitory receptors bind to HLA class I molecules. *Eur J Immunol* . 1996;26:365-369.

296. Fan QOR, Garboczi DN, Winter CC, et al. Direct binding of a soluble natural killer cell inhibitory receptor to a soluble human leukocyte antigen-Cw4 class I major histocompatibility complex molecule. *Proc Natl Acad Sci U S A* . 1996;93:7178-7183.

297. Cambiaggi A, Verthuy C, Naquet P, et al. Natural killer cell acceptance of H-2 mismatch bone marrow grafts in transgenic mice expressing HLA-Cw3 specific killer cell inhibitory receptor. *Proc Natl Acad Sci U S A* . 1997;94:8088-8092.

298. Vales-Gomez M, Reyburn HT, Mandelboim M, et al. Kinetics of interaction of HLA-C ligands with natural killer cell inhibitory receptors. *Immunity*. 1998;9:337-344.

299. Boyington JC, Motyka SA, Schuck P, et al. Crystal structure of an NK cell immunoglobulin-like receptor in complex with its class I MHC ligand. *Nature*. 2000;405:537-543.

300. Maenaka K, Juji T, Nakayama T, et al. Killer cell immunoglobulin receptors and T cell receptors bind peptide-major histocompatibility complex class I with distinct thermodynamic and kinetic properties. *J Biol Chem*. 1999;274:28329-28334.

301. Vivian JP, Duncan RC, Berry R, et al. Killer cell immunoglobulin-like receptor 3DL1-mediated recognition of human leukocyte antigen B. *Nature*. 2011;479:401-405.

302. Rajagopalan S, Long EO. Zinc bound to the killer cell-inhibitory receptor modulates the negative signal in human NK cells. *J Immunol*. 1998;161:1299-1305.

303. Vales-Gomez M, Erskine RA, Deacon MP, et al. The role of zinc in the binding of killer cell Ig-like receptors to class I MHC proteins. *Proc Natl Acad Sci U S A* . 2001;98:1734-1739.

304. Fan QR, Long EO, Wiley DC. Crystal structure of the human natural killer cell inhibitory receptor KIR2DL1-HLA-Cw4 complex. *Nat Immunol*. 2001;2:452-460.

305. Cella M, Longo A, Ferrara GB, et al. NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80. *J Exp Med*. 1994;180:1235-1242.

306. Luque I, Solana R, Galiani MD, et al. Threonine 80 on HLA-B27 confers protection against lysis by a group of natural killer clones. *Eur J Immunol* . 1996;26:1974-1977.

307. Mandelboim O, Reyburn HT, Valesgomez M, et al. Protection from lysis by natural killer cells of group 1 and 2 specificity is mediated by residue 80 in human histocompatibility leukocyte antigen C alleles and also occurs with empty major histocompatibility complex molecules. *J Exp Med*. 1996;184:913-922.

308. Mandelboim O, Reyburn HT, Sheu EG, et al. The binding site of NK receptors on HLA-C molecules. *Immunity*. 1997;6:341-350.

309. Rajagopalan S, Long EO. The direct binding of a p58 killer cell inhibitory receptor to human histocompatibility leukocyte antigen (HLA)-Cw4 exhibits peptide selectivity. *J Exp Med*. 1997;185:1523-1528.

310. Fadda L, Borhis G, Ahmed P, et al. Peptide antagonism as a mechanism for NK cell activation. *Proc Natl Acad Sci U S A* . 2010;107:10160-10165.

311. Barrow AD, Trowsdale J. The extended human leukocyte receptor complex: diverse

ways of modulating immune responses. *Immunol Rev*. 2008;224:98-123.

312. Kelley J, Walter L, Trowsdale J. Comparative genomics of natural killer cell receptor gene clusters. *PLoS Genet*. 2005;1:129-139.

313. Welch AY, Kasahara M, Spain LM. Identification of the mouse killer immunoglobulin-like receptor-like (Kirl) gene family mapping to chromosome X. *Immunogenetics*. 2003;54:782-790.

314. Hoelsbrekken SE, Nylenna O, Saether PC, et al. Cutting edge: molecular cloning of a killer cell Ig-like receptor in the mouse and rat. *J Immunol*. 2003;170:2259-2263.

315. Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol*. 2002;20:217-251.

316. Bashirova AA, Martin MP, McVicar DW, et al. The killer immunoglobulin-like receptor gene cluster: tuning the genome for defense. *Annu Rev Genomics Hum Genet*. 2006;7:277-300.

317. Pyo CW, Guethlein LA, Vu Q, et al. Different patterns of evolution in the centromeric and telomeric regions of group A and B haplotypes of the human killer cell Ig-like receptor locus. *PLoS One*. 2010;5:e15115.

318. Hsu KC, Chida S, Geraghty DE, et al. The killer cell immunoglobulinlike receptor (KIR) genomic region: gene-order, haplotypes and allelic polymorphism. *Immunol Rev*. 2002;190:40-52.

319. Wang LL, Mehta IK, LeBlanc PA, et al. Mouse natural killer cells express gp49B1, a structural homologue of human killer inhibitory receptors. *J Immunol*. 1997;158:13-17.

320. Rojo S, Burshtyn DN, Long EO, et al. Type I transmembrane receptor with inhibitory function in mouse mast cells and NK cells. *J Immunol*. 1997;158:9-12.

321. Katz HR, Vivier E, Castells MC, et al. Mouse mast cell gp49B1 contains two immunoreceptor tyrosine-based inhibition motifs and suppresses mast cell activation when coligated with the high-affinity Fc receptor for IgE. *Proc Natl Acad Sci U S A*. 1996;93:10809-10814.

322. Wang LL, Chu DT, Dokun AO, et al. Inducible expression of the gp49B inhibitory receptor on NK cells. *J Immunol*. 2000;164:5215-5220.

323. Castells MC, Klickstein LB, Hassani K, et al. gp49B1-alpha(v) beta3 interaction inhibits antigen-induced mast cell activation. *Nat Immunol*. 2001;2:436-442.

324. Norris HH, Peterson ME, Stebbins CC, et al. Inhibitory receptor gp49B regulates

eosinophil infiltration during allergic inflammation. *J Leukoc Biol* . 2007;82:1531-1541.

325. Kasai S, Inui M, Nakamura K, et al. A novel regulatory role of gp49B on dendritic cells in T-cell priming. *Eur J Immunol* . 2008;38:2426-2437.

326. Breslow RG, Rao JJ, Xing W, et al. Inhibition of Th2 adaptive immune responses and pulmonary inflammation by leukocyte Ig-like receptor B4 on dendritic cells. *J Immunol*. 2010;184:1003-1013.

327. Zhou JS, Friend DS, Feldweg AM, et al. Prevention of lipopolysaccharide-induced microangiopathy by gp49B1: evidence for an important role for gp49B1 expression on neutrophils. *J Exp Med*. 2003;198: 1243-1251.

328. Zhou JS, Friend DS, Lee DM, et al. gp49B1 deficiency is associated with increases in cytokine and chemokine production and severity of proliferative synovitis induced by anti-type II collagen mAb. *Eur J Immunol* . 2005;35:1530-1538.

329. Westgaard IH, Berg SF, Orstavik S, et al. Identification of a human member of the Ly-49 multigene family. *Eur J Immunol* . 1998;28:1839-1846.

330. Flornes LM, Nylenna O, Saether PC, et al. The complete inventory of receptors encoded by the rat natural killer cell gene complex. *Immunogenetics*. 2010;62:521-530.

331. Barten R, Torkar M, Haude A, et al. Divergent and convergent evolution of NK-cell receptors. *Trends Immunol*. 2001;22:52-57.

332. Yoder JA, Litman GW. The phylogenetic origins of natural killer receptors and recognition: relationships, possibilities, and realities. *Immunogenetics*. 2011;63:123-141.

333. Mager DL, McQueen KL, Wee V, et al. Evolution of natural killer cell receptors: coexistence of functional Ly49 and KIR genes in baboons. *Curr Biol* . 2001;11:626-630.

334. Wilhelm BT, Mager DL. Rapid expansion of the Ly49 gene cluster in rat. *Genomics*. 2004;84:218-221.

335. Nylenna O, Naper C, Vaage JT, et al. The genes and gene organization of the Ly49 region of the rat natural killer cell gene complex. *Eur J Immunol* . 2005;35:261-272.

336. Hao L, Nei M. Genomic organization and evolutionary analysis of Ly49 genes encoding the rodent natural killer cell receptors: rapid evolution by repeated gene duplication. *Immunogenetics*. 2004;56:343-354.

337. Gagnier L, Wilhelm BT, Mager DL. Ly49 genes in non-rodent mammals. *Immunogenetics*. 2003;55:109-115.

338. Kaufman J, Milne S, Gobel TW, et al. The chicken B locus is a minimal essential major histocompatibility complex. *Nature*. 1999;401:923-925.

339. Rogers SL, Gobel TW, Viertlboeck BC, et al. Characterization of the chicken C-type lectin-like receptors B-NK and B-lec suggests that the NK complex and the MHC share a common ancestral region. *J Immunol* . 2005;174:3475-3483.

340. Hao L, Klein J, Nei M. Heterogeneous but conserved natural killer receptor gene complexes in four major orders of mammals. *Proc Natl Acad Sci U S A* . 2006;103:3192-3197.

341. Khakoo SI, Rajalingam R, Shum BP, et al. Rapid evolution of NK cell receptor systems demonstrated by comparison of chimpanzees and humans. *Immunity*. 2000;12:687-698.

342. Hershberger KL, Shyam R, Miura A, et al. Diversity of the killer cell Ig-like receptors of rhesus monkeys. *J Immunol* . 2001;166:4380-4390.

343. Rajalingam R, Hong M, Adams EJ, et al. Short KIR haplotypes in pygmy chimpanzee (Bonobo) resemble the conserved framework of diverse human KIR haplotypes. *J Exp Med*. 2001;193:135-146.

344. Storset AK, Slettedal IO, Williams JL, et al. Natural killer cell receptors in cattle: a bovine killer cell immunoglobulin-like receptor multigene family contains members with divergent signaling motifs. *Eur J Immunol* . 2003;33:980-990.

345. Blokhuis JH, van der Wiel MK, Doxiadis GG, et al. The extreme plasticity of killer cell Ig-like receptor (KIR) haplotypes differentiates rhesus macaques from humans. *Eur J Immunol* . 2011;41:2719-2728.

346. Older Aguilar AM, Guethlein LA, Hermes M, et al. Rhesus macaque KIR bind human MHC class I with broad specificity and recognize HLA-C more effectively than HLA-A and HLA-B. *Immunogenetics*. 2011;63:577-585.

347. Rosner C, Kruse PH, Hermes M, et al. Rhesus macaque inhibitory and activating KIR3D interact with Mamu-A-encoded ligands. *J Immunol* . 2011;186:2156-2163.

348. Hammond JA, Guethlein LA, Abi-Rached L, et al. Evolution and survival of marine carnivores did not require a diversity of killer cell Ig-like receptors or Ly49 NK cell receptors. *J Immunol* . 2009;182:3618-3627.

349. Yoder JA, Mueller MG, Wei S, et al. Immune-type receptor genes in zebrafish share genetic and functional properties with genes encoded by the mammalian leukocyte receptor cluster. *Proc Natl Acad Sci U S A* . 2001;98:6771-6776.

350. Hao L, Nei M. Rapid expansion of killer cell immunoglobulin-like receptor genes in primates and their coevolution with MHC Class I genes. *Gene*. 2005;347:149-159.

351. De Tomaso AW. Sea squirts and immune tolerance. *Dis Model Mech*. 2009;2:440-445.

352. De Tomaso AW, Nyholm SV, Palmeri KJ, et al. Isolation and characterization of a protochordate histocompatibility locus. *Nature*. 2005;438:454-459.

353. Nyholm SV, Passegue E, Ludington WB, et al. fester, a candidate allorecognition receptor from a primitive chordate. *Immunity*. 2006;25:163-173.

354. McKitrick TR, Muscat CC, Pierce JD, et al. Allorecognition in a basal chordate consists of independent activating and inhibitory pathways. *Immunity*. 2011;34:616-626.

355. Houchins JP, Yabe T, McSherry C, et al. DNA sequence analysis of NKG2, a family of related cDNA clones encoding type II integral membrane proteins on human natural killer cells. *J Exp Med*. 1991;173: 1017-1020.

356. Ho EL, Heusel JW, Brown MG, et al. Murine Nkg2d and Cd94 are clustered within the natural killer complex and are expressed independently in natural killer cells. *Proc Natl Acad Sci U S A*. 1998;95:6320-6325.

357. Aramburu J, Balboa MA, Izquierdo M, et al. A novel functional cell surface dimer (Kp43) expressed by natural killer cells and gamma/delta TCR+ T lymphocytes. II. Modulation of natural killer cytotoxicity by anti-Kp43 monoclonal antibody. *J Immunol*. 1991;147:714-721.

358. Rubio G, Aramburu J, Ontanon J, et al. A novel functional cell surface dimer (kp43) serves as accessory molecule for the activation of a subset of human gamma delta T cells. *J Immunol*. 1993;151:1312-1321.

359. Moretta A, Vitale M, Sivori S, et al. Human natural killer cell receptors for HLA-class I molecules. Evidence that the Kp43 (CD94) molecule functions as receptor for HLA-B alleles. *J Exp Med*. 1994;180:545-555.

360. Chang C, Rodriguez A, Carretero M, et al. Molecular characterization of human CD94: a type II membrane glycoprotein related to the C-type lectin superfamily. *Eur J Immunol*. 1995;25:2433-2437.

361. Perez-Villar JJ, Melero I, Rodriguez A, et al. Functional ambivalence of the Kp43 (CD94) NK cell-associated surface antigen. *J Immunol*. 1995;154:5779-5788.

362. Phillips JH, Chang CW, Mattson J, et al. CD94 and a novel associated protein (94ap) form a NK cell receptor involved in the recognition of HLA-A, HLA-B, and HLA-C

allotypes. *Immunity*. 1996;5:163-172.

363. Perez-Villar JJ, Carretero M, Navarro F, et al. Biochemical and serologic evidence for the existence of functionally distinct forms of the CD94 NK cell receptor. *J Immunol*. 1996;157:5367-5374.

364. Lazetic S, Chang C, Houchins JP, et al. Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. *J Immunol*. 1996;157:4741-4745.

365. Carretero M, Cantoni C, Bellon T, et al. The CD94 and NKG2-A C-type lectins covalently assemble to form a natural killer cell inhibitory receptor for HLA class I molecules. *Eur J Immunol* . 1997;27:563-567.

366. Houchins JP, Lanier LL, Niemi EC, et al. Natural killer cell cytolytic activity is inhibited by NKG2-A and activated by NKG2-C. *J Immunol*. 1997;158:3603-3609.

367. Pazmany L, Mandelboim O, Valesgomez M, et al. Protection from natural killer cell-mediated lysis by HLA-G expression on target cells. *Science*. 1996;274:792-795.

368. Sivori S, Vitale M, Bottino C, et al. CD94 functions as a natural killer cell inhibitory receptor for different HLA class I alleles: identification of the inhibitory form of CD94 by the use of novel monoclonal antibodies. *Eur J Immunol* . 1996;26:2487-2492.

369. Pende D, Sivori S, Accame L, et al. HLA-G recognition by human natural killer cells. Involvement of CD94 both as inhibitory and as activating receptor complex. *Eur J Immunol*. 1997;27:1875-1880.

370. Perez-Villar JJ, Melero I, Navarro F, et al. The CD94/NKG2-A inhibitory receptor complex is involved in natural killer cell-mediated recognition of cells expressing HLA-G1. *J Immunol*. 1997;158:5736-5743.

371. Lee N, Llano M, Carretero M, et al. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc Natl Acad Sci U S A* . 1998;95:5199-5204.

372. Braud VM, Allen DSJ, O'Callaghan CA, et al. HLA-E binds to naturalkiller-cell receptors CD94/NKG2A, B and C. *Nature*. 1998;391:795-799.

373. Borrego F, Ulbrecht M, Weiss EH, et al. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J Exp Med*. 1998;187:813-818.

374. Wei XH, Orr HT. Differential expression of HLA-E, HLA-F, and HLA-G transcripts in human tissue. *Human Immunol*. 1990;29:131-142.

375. Koller BH, Geraghty DE, Shimizu Y, et al. HLA-E. A novel HLA class I gene expressed in resting T lymphocytes. *J Immunol*. 1988;141:897-904.

376. Ulbrecht M, Honka T, Person S, et al. The HLA-E gene encodes two differentially regulated transcripts and a cell surface protein. *J Immunol*. 1992;149:2945-2953.

377. Aldrich CJ, DeCloux A, Woods AS, et al. Identification of a Tapdependent leader peptide recognized by alloreactive T cells specific for a class Ib antigen. *Cell*. 1994;79:649-658.

378. Soloski MJ, DeCloux A, Aldrich CJ, et al. Structural and functional characteristics of the class IB molecule, Qa-1. *Immunol Rev*. 1995;147:67-89.

379. Vance RE, Kraft JR, Altman JD, et al. Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical major histocompatibility complex (MHC) class I molecule Qa-1(b). *J Exp Med*. 1998;188:1841-1848.

380. Salcedo M, Bousso P, Ljunggren HG, et al. The Qa-1b molecule binds to a large subpopulation of murine NK cells. *Eur J Immunol* . 1998;28:4356-4361.

381. Gumperz JE, Parham P. The enigma of the natural killer cell. *Nature*. 1995;378:245-248.

382. Petrie EJ, Clements CS, Lin J, et al. CD94-NKG2A recognition of human leukocyte antigen (HLA)-E bound to an HLA class I leader sequence. *J Exp Med*. 2008;205:725-735.

383. Kaiser BK, Pizarro JC, Kerns J, et al. Structural basis for NKG2A/CD94 recognition of HLA-E. *Proc Natl Acad Sci U S A* . 2008;105:6696-6701.

384. Garcia KC, Adams JJ, Feng D, et al. The molecular basis of TCR germline bias for MHC is surprisingly simple. *Nat Immunol*. 2009;10: 143-147.

385. Hoare HL, Sullivan LC, Pietra G, et al. Structural basis for a major histocompatibility complex class Ib-restricted T cell response. *Nat Immunol*. 2006;7:256-264.

386. Tomasec P, Braud VM, Rickards C, et al. Surface expression of HLAE, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. *Science*. 2000;287:1031.

387. Nattermann J, Nischalke HD, Hofmeister V, et al. The HLA-A2 restricted T cell epitope HCV core 35-44 stabilizes HLA-E expression and inhibits cytolysis mediated by natural killer cells. *Am J Pathol*. 2005;166:443-453.

388. Guma M, Budt M, Saez A, et al. Expansion of CD94/NKG2C+ NK cells in response to human cytomegalovirus-infected fibroblasts. *Blood*. 2006;107:3624-3631.

389. Valiante NM, Uhrberg M, Shilling HG, et al. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity*. 1997;7:739-751.

390. Lo WF, Woods AS, DeCloux A, et al. Molecular mimicry mediated by MHC class Ib molecules after infection with gram-negative pathogens. *Nat Med*. 2000;6:215-218.

391. Michaelsson J, Teixeira de Matos C, Achour A, et al. A signal peptide derived from hsp60 binds HLA-E and interferes with CD94/NKG2A recognition. *J Exp Med*. 2002;196:1403-1414.

392. Wooden SL, Kalb SR, Cotter RJ, et al. Cutting edge: HLA-E binds a peptide derived from the ATP-binding cassette transporter multidrug resistance-associated protein 7 and inhibits NK cell-mediated lysis. *J Immunol*. 2005;175:1383-1387.

393. Tajima A, Tanaka T, Ebata T, et al. Blastocyst MHC, a putative murine homologue of HLA-G, protects TAP-deficient tumor cells from natural killer cell-mediated rejection in vivo. *J Immunol*. 2003;171:1715-1721.

394. Vance RE, Jamieson AM, Cado D, et al. Implications of CD94 deficiency and monoallelic NKG2A expression for natural killer cell development and repertoire formation. *Proc Natl Acad Sci U S A*. 2002;99:868-873.

395. Orr MT, Wu J, Fang M, et al. Development and function of CD94-deficient natural killer cells. *PLoS One*. 2010;5:e15184.

396. Fang M, Orr MT, Spee P, et al. CD94 is essential for NK cell-mediated resistance to a lethal viral disease. *Immunity*. 2011;34:579-589.

397. Moser JM, Gibbs J, Jensen PE, et al. CD94-NKG2A receptors regulate antiviral CD8+ T cell responses. *Nat Immunol*. 2002;3:189-195.

398. Miller JD, Peters M, Oran AE, et al. CD94/NKG2 expression does not inhibit cytotoxic function of lymphocytic choriomeningitis virus-specific CD8(+) T cells. *J Immunol*. 2002;169:693-701.

399. McMahon CW, Zajac AJ, Jamieson AM, et al. Viral and bacterial infections induce expression of multiple NK cell receptors in responding CD8(+) T cells. *J Immunol*. 2002;169:1444-1452.

400. Byers AM, Andrews NP, Lukacher AE. CD94/NKG2A expression is associated with proliferative potential of CD8 T cells during persistent polyoma virus infection. *J Immunol*.

2006;176:6121-6129.

401. Jabri B, Selby JM, Negulescu H, et al. TCR specificity dictates CD94/NKG2A expression by human CTL. *Immunity*. 2002;17:487-499.

402. Suvas S, Azkur AK, Rouse BT. Qa-1b and CD94-NKG2a interaction regulate cytolytic activity of herpes simplex virus-specific memory CD8+ T cells in the latently infected trigeminal ganglia. *J Immunol*. 2006;176:1703-1711.

403. Cavanaugh VJ, Raulet DH, Campbell AE. Upregulation of CD94/NKG2A receptors and Qa-1b ligand during murine cytomegalovirus infection of salivary glands. *J Gen Virol*. 2007;88:1440-1445.

404. Zhou J, Matsuoka M, Cantor H, et al. Cutting edge: engagement of NKG2A on CD8+ effector T cells limits immunopathology in influenza pneumonia. *J Immunol*. 2008;180:25-29.

405. Cosman D, Fanger N, Borges L, et al. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. *Immunity*. 1997;7:273-282.

406. Colonna M, Navarro F, Bellon T, et al. A common inhibitory receptor for MHC class I molecules on human lymphoid and myelomonocytic cells. *J Exp Med*. 1997;186:1809-1818.

407. Jones DC, Kosmoliaptsis V, Apps R, et al. HLA class I allelic sequence and conformation regulate leukocyte Ig-like receptor binding. *J Immunol*. 2011;186:2990-2997.

408. Chapman TL, Heikeman AP, Bjorkman PJ. The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18. *Immunity*. 1999;11:603-613.

409. Willcox BE, Thomas LM, Bjorkman PJ. Crystal structure of HLA-A2 bound to LIR-1, a host and viral major histocompatibility complex receptor. *Nat Immunol*. 2003;4:913-919.

410. Shiroishi M, Kuroki K, Rasubala L, et al. Structural basis for recognition of the nonclassical MHC molecule HLA-G by the leukocyte Ig-like receptor B2 (LILRB2/LIR2/ILT4/CD85d). *Proc Natl Acad Sci U S A*. 2006;103:16412-16417.

411. Yang Z, Bjorkman PJ. Structure of UL18, a peptide-binding viral MHC mimic, bound to a host inhibitory receptor. *Proc Natl Acad Sci U S A*. 2008;105:10095-10100.

412. Hirayasu K, Ohashi J, Tanaka H, et al. Evidence for natural selection on leukocyte immunoglobulin-like receptors for HLA class I in northeast Asians. *Am J Hum Genet*. 2008;82:1075-1083.

413. Wilson MJ, Torkar M, Haude A, et al. Plasticity in the organization and sequences of human KIR/ILT gene families. *Proc Natl Acad Sci U S A* . 2000;97:4778-4783.

414. Davidson CL, Li NL, Burshtyn DN. LILRB1 polymorphism and surface phenotypes of natural killer cells. *Hum Immunol*. 2010;71:942-949.

415. Kuroki K, Tsuchiya N, Shiroishi M, et al. Extensive polymorphisms of LILRB1 (ILT2, LIR1) and their association with HLA-DRB1 shared epitope negative rheumatoid arthritis. *Hum Mol Genet*. 2005;14:2469-2480.

416. Kumar V, McNerney ME. A new self: MHC-class-I-independent natural-killer-cell self-tolerance. *Nat Rev Immunol* . 2005;5:363-374.

417. Meyaard L, Adema GJ, Chang C, et al. LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. *Immunity*. 1997;7:283-290.

418. Lebbink RJ, de Ruiter T, Verbrugge A, et al. The mouse homologue of the leukocyte-associated Ig-like receptor-1 is an inhibitory receptor that recruits Src homology region 2-containing protein tyrosine phosphatase (SHP)-2, but not SHP-1. *J Immunol* . 2004;172:5535-5543.

419. Meyaard L, van der Vuurst de Vries AR, de Ruiter T, et al. The epithelial cellular adhesion molecule (Ep-CAM) is a ligand for the leukocyte-associated immunoglobulin-like receptor (LAIR). *J Exp Med*. 2001;194:107-112.

420. Meyaard L, van der Vuurst de Vries AR, de Ruiter T, et al. Retraction. *J Exp Med*. 2003;198:1129.

421. Lebbink RJ, de Ruiter T, Adelmeijer J, et al. Collagens are functional, high affinity ligands for the inhibitory immune receptor LAIR-1. *J Exp Med*. 2006;203:1419-1425.

422. Brondijk TH, de Ruiter T, Ballering J, et al. Crystal structure and collagen-binding site of immune inhibitory receptor LAIR-1: unexpected implications for collagen binding by platelet receptor GPVI. *Blood*. 2010;115:1364-1373.

423. Verbrugge A, Rijkers ES, de Ruiter T, et al. Leukocyte-associated Iglike receptor-1 has SH2 domain-containing phosphatase-independent function and recruits C-terminal Src kinase. *Eur J Immunol* . 2006;36: 190-198.

424. Varki A, Angata T. Siglecs—the major subfamily of I-type lectins. *Glycobiol*. 2006;16:1R-27R.

425. Cao H, Crocker PR. Evolution of CD33-related siglecs: regulating host immune functions and escaping pathogen exploitation? *Immunol*. 2011;132:18-26.

426. Falco M, Biassoni R, Bottino C, et al. Identification and molecular cloning of p75/AIRM1, a novel member of the sialoadhesin family that functions as an inhibitory receptor in human natural killer cells. *J Exp Med*. 1999;190:793-802.

427. Avril T, Floyd H, Lopez F, et al. The membrane-proximal immunoreceptor tyrosine-based inhibitory motif is critical for the inhibitory signaling mediated by Siglecs-7 and -9, CD33-related Siglecs expressed on human monocytes and NK cells. *J Immunol*. 2004;173:6841-6849.

428. Nicoll G, Avril T, Lock K, et al. Ganglioside GD3 expression on target cells can modulate NK cell cytotoxicity via siglec-7-dependent and -independent mechanisms. *Eur J Immunol*. 2003;33:1642-1648.

429. Avril T, North SJ, Haslam SM, et al. Probing the cis interactions of the inhibitory receptor Siglec-7 with {alpha}2,8-disialylated ligands on natural killer cells and other leukocytes using glycan-specific antibodies and by analysis of {alpha}2,8-sialyltransferase gene expression. *J Leukoc Biol*. 2006;80(4):787-796.

430. Mousseau DD, Banville D, L'Abbe D, et al. PILRalpha, a novel immunoreceptor tyrosine-based inhibitory motif-bearing protein, recruits SHP-1 upon tyrosine phosphorylation and is paired with the truncated counterpart PILRbeta. *J Biol Chem*. 2000;275:4467-4474.

431. Wilson MD, Cheung J, Martindale DW, et al. Comparative analysis of the paired immunoglobulin-like receptor (PILR) locus in six mammalian genomes: duplication, conversion, and the birth of new genes. *Physiol Genomics*. 2006;27:201-218.

432. Shiratori I, Ogasawara K, Saito T, et al. Activation of natural killer cells and dendritic cells upon recognition of a novel CD99-like ligand by paired immunoglobulin-like type 2 receptor. *J Exp Med*. 2004;199: 525-533.

433. Wang J, Shiratori I, Satoh T, et al. An essential role of sialylated O-linked sugar chains in the recognition of mouse CD99 by paired Ig-like type 2 receptor (PILR). *J Immunol*. 2008;180:1686-1693.

434. Hedrick SM, Cohen DI, Nielsen EA, et al. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature*. 1984; 308:149-153.

435. Weiss A, Stobo JD. Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. *J Exp Med*. 1984;160:1284-1299.

436. Samelson LE, Germain RN, Schwartz RH. Monoclonal antibodies against the antigen receptor on a cloned T-cell hybrid. *Proc Natl Acad Sci U S A* . 1983;80:6972-6976.

437. Houchins JP, Yabe T, McSherry C, et al. Isolation and characterization of NK cell or NK/T cell-specific cDNA clones. *J Mol Cell Immunol* . 1990;4:295-304.

438. Seaman WE, Eriksson E, Dobrow R, et al. Inositol trisphosphate is generated by a rat natural killer cell tumor in response to target cells or to crosslinked monoclonal antibody OX-34: possible signaling role for the OX-34 determinant during activation by target cells. *Proc Natl Acad Sci U S A* . 1987;84:4239-4243.

439. Leo O, Sachs DH, Samelson LE, et al. Identification of monoclonal antibodies specific for the T cell receptor complex by Fc receptor-mediated CTL lysis. *J Immunol* . 1986;137:3874-3880.

440. Nakamura MC, Linnemeyer PA, Niemi EC, et al. Mouse Ly-49D recognizes H-2Dd and activates natural killer cell cytotoxicity. *J Exp Med*. 1999;189:493-500.

441. Rajagopalan S, Long EO. Use of vaccinia virus for functional gene transfer in natural killer cells. *Methods Mol Biol* . 2000;121:265-272.

442. Tran J, Kung SK. Lentiviral vectors mediate stable and efficient gene delivery into primary murine natural killer cells. *Mol Ther* . 2007;15: 1331-1339.

443. Sanderson S, Shastri N. LacZ inducible, antigen/MHC-specific T cell hybrids. *Int Immunol* . 1994;6:369-376.

444. Smith HR, Heusel JW, Mehta IK, et al. Recognition of a virus-encoded ligand by a natural killer cell activation receptor. *Proc Natl Acad Sci U S A* . 2002;99:8826-8831.

445. Iizuka K, Naidenko OV, Plougastel BF, et al. Genetically linked C-type lectin-related ligands for the NKRP1 family of natural killer cell receptors. *Nat Immunol* . 2003;4:801-807.

446. Arase H, Mocarski ES, Campbell AE, et al. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science*. 2002;296:1323-1326.

447. Mason LH, Anderson SK, Yokoyama WM, et al. The Ly-49D receptor activates murine natural killer cells. *J Exp Med*. 1996;184: 2119-2128.

448. Smith KM, Wu J, Bakker AB, et al. Cutting edge: Ly-49D and Ly-49H associate with mouse DAP12 and form activating receptors. *J Immunol* . 1998;161:7-10.

449. Smith HR, Chuang HH, Wang LL, et al. Nonstochastic coexpression of activation receptors on murine natural killer cells. *J Exp Med*. 2000;191:1341-1354.

450. Bakker AB, Hoek RM, Cerwenka A, et al. DAP12-deficient mice fail to develop

autoimmunity due to impaired antigen priming. *Immunity*. 2000;13:345-353.

451. Nakamura MC, Naper C, Niemi EC, et al. Natural killing of xenogeneic cells mediated by the mouse Ly-49D receptor. *J Immunol* . 1999;163:4694-4700.

452. Furukawa H, Iizuka K, Poursine-Laurent J, et al. A ligand for the murine NK activation receptor Ly-49D: activation of tolerized NK cells from beta(2)-microglobulin-deficient mice. *J Immunol* . 2002;169:126-136.

453. Kielczewska A, Pyzik M, Sun T, et al. Ly49P recognition of cytomegalovirus-infected cells expressing H2-Dk and CMV-encoded m04 correlates with the NK cell antiviral response. *J Exp Med*. 2009;206:515-523.

454. Moretta A, Sivori S, Vitale M, et al. Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. *J Exp Med*. 1995;182:875-884.

455. Biassoni R, Cantoni C, Falco M, et al. The human leukocyte antigen (HLA)-C-specific “activatory” or “inhibitory” natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. *J Exp Med*. 1996;183:645-650.

456. Sivori S, Carlomagno S, Falco M, et al. Natural killer cells expressing the KIR2DS1-activating receptor efficiently kill T-cell blasts and dendritic cells: implications in haploidentical HSCT. *Blood*. 2011;117: 4284-4292.

457. Lanier LL, Cortiss BC, Wu J, et al. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature*. 1998;391:703-707.

458. Biassoni R, Pessino A, Malaspina A, et al. Role of amino acid position 70 in the binding affinity of p50.1 and p58.1 receptors for HLA-Cw4 molecules. *Eur J Immunol* . 1997;27:3095-3099.

459. Stewart CA, Laugier-Anfossi F, Vely F, et al. Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors. *Proc Natl Acad Sci U S A* . 2005;102:13224-13229.

460. Chewning JH, Gudme CN, Hsu KC, et al. KIR2DS1-positive NK cells mediate alloresponse against the C2 HLA-KIR ligand group in vitro. *J Immunol* . 2007;179:854-868.

461. Vales-Gomez M, Reyburn HT, Erskine RA, et al. Differential binding to HLA-C of p50-activating and p58-inhibitory natural killer cell receptors. *Proc Natl Acad Sci U S A* . 1998;95:14326-14331.

462. Graef T, Moesta AK, Norman PJ, et al. KIR2DS4 is a product of gene conversion with KIR3DL2 that introduced specificity for HLA-A*11 while diminishing avidity for HLA-C. *J Exp Med*. 2009;206:2557-2572.

463. O'Connor GM, Yamada E, Rampersaud A, et al. Analysis of binding of KIR3DS1*014 to HLA suggests distinct evolutionary history of KIR3DS1. *J Immunol*. 2011;187:2162-2171.

464. Adams EJ, Juo ZS, Venook RT, et al. Structural elucidation of the m157 mouse cytomegalovirus ligand for Ly49 natural killer cell receptors. *Proc Natl Acad Sci U S A*. 2007;104:10128-10133.

465. Katz G, Gazit R, Arnon TI, et al. MHC class I-independent recognition of NK-activating receptor KIR2DS4. *J Immunol*. 2004;173:1819-1825.

466. Plougastel B, Trowsdale J. Cloning of NKG2-F, a new member of the NKG2 family of human natural killer cell receptor genes. *Eur J Immunol*. 1997;27:2835-2839.

467. Wu J, Cherwinski H, Spies T, et al. DAP10 and DAP12 form distinct, but functionally cooperative, receptor complexes in natural killer cells. *J Exp Med*. 2000;192:1059-1068.

468. Kim DK, Kabat J, Borrego F, et al. Human NKG2F is expressed and can associate with DAP12. *Mol Immunol*. 2004;41:53-62.

469. Llano M, Lee N, Navarro F, et al. HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/NKG2 receptors: preferential response to an HLA-G-derived nonamer. *Eur J Immunol*. 1998;28:2854-2863.

470. Vance RE, Jamieson AM, Raulet DH. Recognition of the class Ib molecule Qa-1(b) by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells. *J Exp Med*. 1999;190:1801-1812.

471. Vales-Gomez M, Reyburn HT, Erskine RA, et al. Kinetics and peptide dependency of the binding of the inhibitory NK receptor CD94/NKG2-A and the activating receptor CD94/NKG2-C to HLA-E. *EMBO J*. 1999;18:4250-4260.

472. Kaiser BK, Barahmand-Pour F, Paulsene W, et al. Interactions between NKG2x immunoreceptors and HLA-E ligands display overlapping affinities and thermodynamics. *J Immunol*. 2005;174:2878-2884.

473. Kraft JR, Vance RE, Pohl J, et al. Analysis of Qa-1(b) peptide binding specificity and the capacity of CD94/NKG2A to discriminate between Qa-1-peptide complexes. *J Exp Med*. 2000;192:613-624.

474. Takai T, Li M, Sylvestre D, et al. FcR gamma chain deletion results in pleiotrophic

effector cell defects. *Cell*. 1994;76:519-529.

475. Hazenbos WLW, Gessner JE, Hofhuis FMA, et al. Impaired IgGdependent anaphylaxis and arthus reaction in Fc-gamma-RIII (CD16) deficient mice. *Immunity*. 1996;5:181-188.

476. Ravetch JV, Kinet JP. Fc receptors. *Ann Rev Immunol*. 1991;9:457-491.

477. Metes D, Ernst LK, Chambers WH, et al. Expression of functional CD32 molecules on human NK cells is determined by an allelic polymorphism of the FcgammaRIIC gene. *Blood*. 1998;91:2369-2380.

478. Perussia B, Tutt MM, Qiu WQ, et al. Murine natural killer cells express functional Fc gamma receptor II encoded by the Fc gamma R alpha gene. *J Exp Med*. 1989;170:73-86.

479. Nimmerjahn F, Ravetch JV. Fcgamma receptors: old friends and new family members. *Immunity*. 2006;24:19-28.

480. Nimmerjahn F, Lux A, Albert H, et al. FcgammaRIV deletion reveals its central role for IgG2a and IgG2b activity in vivo. *Proc Natl Acad Sci U S A*. 2010;107:19396-19401.

481. Letourneur O, Kennedy IC, Brini AT, et al. Characterization of the family of dimers associated with Fc receptors (Fc epsilon RI and Fc gamma RIII). *J Immunol*. 1991;147:2652-2656.

482. Cassatella MA, Anegon I, Cuturi MC, et al. Fc gamma R(CD16) interaction with ligand induces Ca2+ mobilization and phosphoinositide turnover in human natural killer cells. Role of Ca2+ in Fc gamma R(CD16)-induced transcription and expression of lymphokine genes. *J Exp Med*. 1989;169:549-567.

483. Azzoni L, Kamoun M, Salcedo TW, et al. Stimulation of Fc gamma RIIIA results in phospholipase C-gamma 1 tyrosine phosphorylation and p56lck activation. *J Exp Med*. 1992;176:1745-1750.

484. Kanakaraj P, Duckworth B, Azzoni L, et al. Phosphatidylinositol-3 kinase activation induced upon Fc gamma RIIIA-ligand interaction. *J Exp Med*. 1994;179:551-558.

485. Aramburu J, Azzoni L, Rao A, et al. Activation and expression of the nuclear factors of activated T cells, NFATp and NFATc, in human natural killer cells: regulation upon CD16 ligand binding. *J Exp Med*. 1995;182:801-810.

486. Lanier LL, Ruitenberg JJ, Phillips JH. Functional and biochemical analysis of CD16 antigen on natural killer cells and granulocytes. *J Immunol*. 1988;136:3478-3485.

487. Wu J, Song Y, Bakker AB, et al. An activating immunoreceptor complex formed by

NKG2D and DAP10. *Science*. 1999;285:730-732.

488. Groh V, Rhinehart R, Randolph-Habecker J, et al. Costimulation of CD8ab T cells by NKG2D via engagement by MIC induced on virusinfected cells. *Nat Immunol*. 2001;2:255-260.

489. Fasth AE, Bjorkstrom NK, Anthoni M, et al. Activating NK-cell receptors co-stimulate CD4(+)CD28(-) T cells in patients with rheumatoid arthritis. *Eur J Immunol* . 2010;40:378-387.

490. Barber A, Sentman CL. NKG2D receptor regulates human effector T-cell cytokine production. *Blood*. 2011;117:6571-6581.

491. Upshaw JL, Arneson LN, Schoon RA, et al. NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. *Nat Immunol*. 2006;7:524-532.

492. Pende D, Cantoni C, Rivera P, et al. Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of nonepithelial origin. *Eur J Immunol* . 2001;31:1076-1086.

493. Rosen DB, Araki M, Hamerman JA, et al. A Structural basis for the association of DAP12 with mouse, but not human, NKG2D. *J Immunol*. 2004;173:2470-2478.

494. Rabinovich B, Li J, Wolfson M, et al. NKG2D splice variants: a reexamination of adaptor molecule associations. *Immunogenetics*. 2006;58:81-88.

495. Cosman D, Mullberg J, Sutherland CL, et al. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity*. 2001;14:123-133.

496. Kubin M, Cassiano L, Chalupny J, et al. ULBP1, 2, 3: novel MHC class I-related molecules that bind to human cytomegalovirus glycoprotein UL16, activate NK cells. *Eur J Immunol*. 2001;31:1428-1437.

497. Bacon L, Eagle RA, Meyer M, et al. Two human ULBP/RAET1 molecules with transmembrane regions are ligands for NKG2D. *J Immunol*. 2004;173:1078-1084.

498. Radosavljevic M, Cuillerier B, Wilson MJ, et al. A cluster of ten novel MHC class I related genes on human chromosome 6q24.2-q25.3. *Genomics*. 2002;79:114-123.

499. Eagle RA, Trowsdale J. Promiscuity and the single receptor: NKG2D. *Nat Rev Immunol*. 2007;7:737-744.

500. Girardi M, Oppenheim DE, Steele CR, et al. Regulation of cutaneous malignancy by

gd T Cells. *Science*. 2001;294:605-609.

501. Carayannopoulos L, Naidenko O, Fremont D, et al. Cutting edge: Murine UL16-binding protein-like transcript 1: a newly described transcript encoding a high-affinity ligand for murine NKG2D. *J Immunol* . 2002;169:4079-4083.

502. Takada A, Yoshida S, Kajikawa M, et al. Two novel NKG2D ligands of the mouse H60 family with differential expression patterns and binding affinities to NKG2D. *J Immunol* . 2008;180:1678-1685.

503. Lodoen M, Ogasawara K, Hamerman JA, et al. NKG2D-mediated natural killer cell protection against cytomegalovirus is impaired by viral gp40 modulation of retinoic acid early inducible 1 gene molecules. *J Exp Med* . 2003;197:1245-1253.

504. Li P, Morris DL, Willcox BE, et al. Complex structure of the activating immunoreceptor NKG2D and its MHC class I-like ligand MICA. *Nat Immunol* . 2001;2:443-451.

505. Radaev S, Rostro B, Brooks AG, et al. Conformational plasticity revealed by the cocrystal structure of NKG2D and its class I MHC-like ligand ULBP3. *Immunity*. 2001;15:1039-1049.

506. McFarland BJ, Kortemme T, Yu SF, et al. Symmetry recognizing asymmetry: analysis of the interactions between the C-type lectin-like immunoreceptor NKG2D and MHC class I-like ligands. *Structure (Camb)*. 2003;11:411-422.

507. Li P, Willie ST, Bauer S, et al. Crystal structure of the MHC class I homolog MIC-A, a gammadelta T cell ligand. *Immunity*. 1999;10:577-584.

508. O'Callaghan CA, Cerwenka A, Willcox BE, et al. Molecular competition for NKG2D: H60 and RAE1 compete unequally for NKG2D with dominance of H60. *Immunity*. 2001;15:201-211.

509. Carayannopoulos LN, Naidenko OV, Kinder J, et al. Ligands for murine NKG2D display heterogeneous binding behavior. *Eur J Immunol* . 2002;32:597-605.

510. Li P, McDermott G, Strong RK. Crystal structures of RAE-1beta and its complex with the activating immunoreceptor NKG2D. *Immunity*. 2002;16:77-86.

511. Wolan DW, Teyton L, Rudolph MG, et al. Crystal structure of the murine NK cell-activating receptor NKG2D at 1.95 A. *Nat Immunol* . 2001;2:248-254.

512. Muller S, Zocher G, Steinle A, et al. Structure of the HCMV UL16-MICB complex elucidates select binding of a viral immunoevasin to diverse NKG2D ligands. *PLoS Pathog*. 2010;6:e1000723.

513. Cerwenka A, Baron JL, Lanier LL. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc Natl Acad Sci U S A* . 2001;98:11521-11526.

514. Diefenbach A, Jensen ER, Jamieson AM, et al. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature*. 2001;413:165-171.

515. Regunathan J, Chen Y, Wang D, et al. NKG2D receptor-mediated NK cell function is regulated by inhibitory Ly49 receptors. *Blood*. 2005;105:233-240.

516. Groh V, Bahram S, Bauer S, et al. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. *Proc Natl Acad Sci U S A*. 1996;93:12445-12450.

517. Gasser S, Orsulic S, Brown EJ, et al. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature*. 2005;436:1186-1190.

518. Stern-Ginossar N, Gur C, Biton M, et al. Human microRNAs regulate stress-induced immune responses mediated by the receptor NKG2D. *Nat Immunol* . 2008;9:1065-1073.

519. Tang KF, Ren H, Cao J, et al. Decreased Dicer expression elicits DNA damage and up-regulation of MICA and MICB. *J Cell Biol* . 2008;182:233-239.

520. Soriani A, Zingoni A, Cerboni C, et al. ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype. *Blood*. 2009;113:3503-3511.

521. Hamerman JA, Ogasawara K, Lanier LL. Cutting edge: toll-like receptor signaling in macrophages induces ligands for the NKG2D receptor. *J Immunol* . 2004;172:2001-2005.

522. Eissmann P, Evans JH, Mehrabi M, et al. Multiple mechanisms downstream of TLR-4 stimulation allow expression of NKG2D ligands to facilitate macrophage/NK cell crosstalk. *J Immunol* . 2010;184:6901-6909.

523. Nausch N, Florin L, Hartenstein B, et al. Cutting edge: the AP-1 subunit JunB determines NK cell-mediated target cell killing by regulation of the NKG2D-ligand RAE-1epsilon. *J Immunol* . 2006;176:7-11.

524. Zou Z, Nomura M, Takihara Y, et al. Isolation and characterization of retinoic acid-inducible cDNA clones in F9 cells: a novel cDNA family encodes cell surface proteins sharing partial homology with MHC class I molecules. *J Biochem (Tokyo)*. 1996;119:319-328.

525. Bui JD, Carayannopoulos LN, Lanier LL, et al. IFN-dependent down-regulation of the NKG2D ligand H60 on tumors. *J Immunol*. 2006;176:905-913.

526. Yadav D, Ngolab J, Lim RS, et al. Cutting edge: down-regulation of MHC class I-related chain A on tumor cells by IFN-gamma-induced microRNA. *J Immunol*. 2009;182:39-43.

527. Tokuyama M, Lorin C, Delebecque F, et al. Expression of the RAE-1 family of stimulatory NK-cell ligands requires activation of the PI3K pathway during viral infection and transformation. *PLoS Pathog*. 2011;7:e1002265.

528. Nice TJ, Coscoy L, Raulet DH. Posttranslational regulation of the NKG2D ligand Mult1 in response to cell stress. *J Exp Med*. 2009;206:287-298.

529. Nice TJ, Deng W, Coscoy L, et al. Stress-regulated targeting of the NKG2D ligand Mult1 by a membrane-associated RING-CH family E3 ligase. *J Immunol*. 2010;185:5369-5376.

530. Eagle RA, Jafferji I, Barrow AD. Beyond stressed self: evidence for NKG2D ligand expression on healthy cells. *Curr Immunol Rev*. 2009;5:22-34.

531. Champsaur M, Lanier LL. Effect of NKG2D ligand expression on host immune responses. *Immunol Rev*. 2010;235:267-285.

532. Whang MI, Guerra N, Raulet DH. Costimulation of dendritic epidermal gammadelta T cells by a new NKG2D ligand expressed specifically in the skin. *J Immunol*. 2009;182:4557-4564.

533. Groh V, Wu J, Yee C, et al. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature*. 2002;419:734-738.

534. Wiemann K, Mittrucker HW, Feger U, et al. Systemic NKG2D downregulation impairs NK and CD8 T cell responses in vivo. *J Immunol*. 2005;175:720-729.

535. Ogasawara K, Benjamin J, Takaki R, et al. Function of NKG2D in natural killer cell-mediated rejection of mouse bone marrow grafts. *Nat Immunol*. 2005;6:938-945.

536. Salih HR, Rammensee HG, Steinle A. Cutting edge: down-regulation of MICA on human tumors by proteolytic shedding. *J Immunol*. 2002;169:4098-4102.

537. Aguera-Gonzalez S, Gross CC, Fernandez-Messina L, et al. Palmitoylation of MICA, a ligand for NKG2D, mediates its recruitment to membrane microdomains and promotes its shedding. *Eur J Immunol*. 2011;41(12):3667-3676.

538. Oppenheim DE, Roberts SJ, Clarke SL, et al. Sustained localized expression of

ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance. *Nat Immunol*. 2005;6:928-937.

539. Strid J, Roberts SJ, Filler RB, et al. Acute upregulation of an NKG2D ligand promotes rapid reorganization of a local immune compartment with pleiotropic effects on carcinogenesis. *Nat Immunol*. 2008;9:146-154.

540. Guerra N, Tan YX, Joncker NT, et al. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity*. 2008;28:571-580.

541. Dunn GP, Bruce AT, Sheehan KC, et al. A critical function for type I interferons in cancer immunoediting. *Nat Immunol*. 2005;6:722-729.

542. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. *Annu Rev Immunol*. 2004;22:329-360.

543. Welte SA, Sinzger C, Lutz SZ, et al. Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein. *Eur J Immunol*. 2003;33:194-203.

544. Wu J, Chalupny NJ, Manley TJ, et al. Intracellular retention of the MHC class I-related chain B ligand of NKG2D by the human cytomegalovirus UL16 glycoprotein. *J Immunol*. 2003;170:4196-4200.

545. Dunn C, Chalupny NJ, Sutherland CL, et al. Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity. *J Exp Med*. 2003;197:1427-1439.

546. Ashiru O, Bennett NJ, Boyle LH, et al. NKG2D ligand MICA is retained in the cis-Golgi apparatus by human cytomegalovirus protein UL142. *J Virol*. 2009;83:12345-12354.

547. Krmpotic A, Busch DH, Bubic I, et al. MCMV glycoprotein gp40 confers virus resistance to CD8+ T cells and NK cells in vivo. *Nat Immunol*. 2002;3:529-535.

548. Lodoen MB, Abenes G, Umamoto S, et al. The cytomegalovirus m155 gene product subverts natural killer cell antiviral protection by disruption of H60-NKG2D interactions. *J Exp Med*. 2004;200:1075-1081.

549. Hasan M, Krmpotic A, Ruzsics Z, et al. Selective down-regulation of the NKG2D ligand H60 by mouse cytomegalovirus m155 glycoprotein. *J Virol*. 2005;79:2920-2930.

550. Krmpotic A, Hasan M, Loewendorf A, et al. NK cell activation through the NKG2D ligand MULT-1 is selectively prevented by the glycoprotein encoded by mouse cytomegalovirus gene m145. *J Exp Med*. 2005;201:211-220.

551. Lenac T, Budt M, Arapovic J, et al. The herpesviral Fc receptor fcr-1 down-regulates the NKG2D ligands MULT-1 and H60. *J Exp Med*. 2006;203:1843-1850.

552. Arapovic J, Lenac Rovis T, Reddy AB, et al. Promiscuity of MCMV immunoevasin of NKG2D: m138/fcr-1 down-modulates RAE-1epsilon in addition to MULT-1 and H60. *Mol Immunol*. 2009;47:114-122.

553. Thomas M, Boname JM, Field S, et al. Down-regulation of NKG2D and NKp80 ligands by Kaposi's sarcoma-associated herpesvirus K5 protects against NK cell cytotoxicity. *Proc Natl Acad Sci U S A* . 2008;105: 1656-1661.

554. Stern-Ginossar N, Elefant N, Zimmermann A, et al. Host immune system gene targeting by a viral miRNA. *Science*. 2007;317:376-381.

555. Nachmani D, Stern-Ginossar N, Sarid R, et al. Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. *Cell Host Microbe*. 2009;5:376-385.

556. McSharry BP, Burgert HG, Owen DP, et al. Adenovirus E3/19K promotes evasion of NK cell recognition by intracellular sequestration of the NKG2D ligands major histocompatibility complex class I chainrelated proteins A and B. *J Virol*. 2008;82:4585-4594.

557. Campbell JA, Trossman DS, Yokoyama WM, et al. Zoonotic orthopoxviruses encode a high-affinity antagonist of NKG2D. *J Exp Med*. 2007;204:1311-1317.

558. Zafirova B, Wensveen FM, Gulin M, et al. Regulation of immune cell function and differentiation by the NKG2D receptor. *Cell Mol Life Sci*. 2011;68:3519-3529.

559. Gambelunghe G, Brozzetti A, Ghaderi M, et al. MICA gene polymorphism in the pathogenesis of type 1 diabetes. *Ann N Y Acad Sci* . 2007;1110:92-98.

560. Choy MK, Phipps ME. MICA polymorphism: biology and importance in immunity and disease. *Trends Mol Med*. 2010;16:97-106.

561. Ogasawara K, Hamerman JA, Ehrlich LR, et al. NKG2D blockade prevents autoimmune diabetes in NOD mice. *Immunity*. 2004;20:757-767.

562. Moosig F, Csernok E, Wang G, et al. Costimulatory molecules in Wegener's granulomatosis (WG): lack of expression of CD28 and preferential up-regulation of its ligands B7-1 (CD80) and B7-2 (CD86) on T cells. *Clin Exp Immunol*. 1998;114:113-118.

563. Namekawa T, Snyder MR, Yen JH, et al. Killer cell activating receptors function as costimulatory molecules on CD4+CD28null T cells clonally expanded in rheumatoid

arthritis. *J Immunol*. 2000;165:1138-1145.

564. Liuzzo G, Goronzy JJ, Yang H, et al. Monoclonal T-cell proliferation and plaque instability in acute coronary syndromes. *Circulation*. 2000;101:2883-2888.

565. Roberts AI, Lee L, Schwarz E, et al. Cutting edge: NKG2D receptors induced by IL-15 costimulate CD28-negative effector CTL in the tissue microenvironment. *J Immunol*. 2001;167:5527-5530.

566. Hue S, Mention JJ, Monteiro RC, et al. A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. *Immunity*. 2004;21:367-377.

567. Dai Z, Turtle CJ, Booth GC, et al. Normally occurring NKG2D+CD4+ T cells are immunosuppressive and inversely correlated with disease activity in juvenile-onset lupus. *J Exp Med*. 2009;206:793-805.

568. Giorda R, Trucco M. Mouse NKR-P1. A family of genes selectively coexpressed in adherent lymphokine-activated killer cells. *J Immunol*. 1991;147:1701-1708.

569. Plougastel B, Matsumoto K, Dubbelde C, et al. Analysis of a 1-Mb BAC contig overlapping the mouse Nkrp1 cluster of genes: cloning of three new Nkrp1 members, Nkrp1d, Nkrp1e, and Nkrp1f. *Immunogenetics*. 2001;53:592-598.

570. Kveberg L, Back CJ, Dai KZ, et al. The novel inhibitory NKR-P1C receptor and Ly49s3 identify two complementary, functionally distinct NK cell subsets in rats. *J Immunol*. 2006;176:4133-4140.

571. Chambers WH, Vujanovic NL, DeLeo AB, et al. Monoclonal antibody to a triggering structure expressed on rat natural killer cells and adherent lymphokine-activated killer cells. *J Exp Med*. 1989;169: 1373-1389.

572. Bendelac A. Mouse NK1+ T cells. *Curr Opin Immunol*. 1995;7:367-374.

573. Knudsen E, Seierstad T, Vaage JT, et al. Cloning, functional activities and in vivo tissue distribution of rat NKR-P1+ TCR alpha beta + cells. *Int Immunol*. 1997;9:1043-1051.

574. Lanier LL, Chang C, Phillips JH. Human NKR-P1A. A disulfide-linked homodimer of the C-type lectin superfamily expressed by a subset of NK and T lymphocytes. *J Immunol*. 1994;153:2417-2428.

575. Plougastel BF, Yokoyama WM. Extending missing-self? Functional interactions between lectin-like NKrp1 receptors on NK cells with lectin-like ligands. *Curr Top Microbiol Immunol*. 2006;298:77-89.

576. Ryan JC, Niemi EC, Goldfien RD, et al. NKR-P1, an activating molecule on rat natural killer cells, stimulates phosphoinositide turnover and a rise in intracellular calcium. *J Immunol*. 1991;147:3244-3250.

577. Ljutic B, Carlyle JR, Filipp D, et al. Functional requirements for signaling through the stimulatory and inhibitory mouse NKR-P1 (CD161) NK cell receptors. *J Immunol*. 2005;174:4789-4796.

578. Arase H, Arase N, Saito T. Interferon gamma production by natural killer (NK) cells and NK1.1+ T cells upon NKR-P1 cross-linking. *J Exp Med*. 1996;183:2391-2396.

579. Arase N, Arase H, Park SY, et al. Association with FcR-g is essential for activation signal through NKR-P1 (CD161) in natural killer (NK) cells and NK1.1+ T cells. *J Exp Med*. 1997;186:1957-1963.

580. Ryan JC, Niemi EC, Nakamura MC, et al. NKR-P1A is a target-specific receptor that activates natural killer cell cytotoxicity. *J Exp Med*. 1995; 181:1911-1915.

581. Plougastel B, Dubbelde C, Yokoyama WM. Cloning of Clr, a new family of lectin-like genes localized between mouse Nkrp1a and Cd69 genes. *Immunogenetics*. 2001;53:209-214.

582. Carlyle JR, Jamieson AM, Gasser S, et al. Missing self-recognition of Ocil/Clr-b by inhibitory NKR-P1 natural killer cell receptors. *Proc Natl Acad Sci U S A* . 2004;101:3527-3532.

583. Zhou H, Kartsogiannis V, Hu YS, et al. A novel osteoblast-derived C-type lectin that inhibits osteoclast formation. *J Biol Chem*. 2001; 276:14916-14923.

584. Carlyle JR, Mesci A, Ljutic B, et al. Molecular and genetic basis for strain-dependent NK1.1 alloreactivity of mouse NK cells. *J Immunol*. 2006;176:7511-7524.

585. Giorda R, Weisberg EP, Ip TK, et al. Genomic structure and strainspecific expression of the natural killer cell receptor NKR-P1. *J Immunol*. 1992;149:1957-1963.

586. Boles KS, Barten R, Kumaresan PR, et al. Cloning of a new lectin-like receptor expressed on human NK cells. *Immunogenetics*. 1999;50:1-7.

587. Hamann J, Montgomery KT, Lau S, et al. AICL: a new activation-induced antigen encoded by the human NK gene complex. *Immunogenetics*. 1997;45:295-300.

588. Ryan EJ, Marshall AJ, Magaletti D, et al. Dendritic cell-associated lectin-1: a novel dendritic cell-associated, C-type lectin-like molecule enhances T cell secretion of IL-4. *J Immunol*. 2002;169:5638-5648.

589. Rosen DB, Bettadapura J, Alsharifi M, et al. Cutting edge: lectin-like transcript-1 is a ligand for the inhibitory human NKR-P1A receptor. *J Immunol*. 2005;175:7796-7799.

590. Aldemir H, Prod'homme V, Dumaurier MJ, et al. Cutting edge: lectin-like transcript 1 is a ligand for the CD161 receptor. *J Immunol*. 2005;175:7791-7795.

591. Roda-Navarro P, Arce I, Renedo M, et al. Human KLRF1, a novel member of the killer cell lectin-like receptor gene family: molecular characterization, genomic structure, physical mapping to the NK gene complex and expression analysis. *Eur J Immunol*. 2000;30:568-576.

592. Vitale M, Falco M, Castriconi R, et al. Identification of NKp80, a novel triggering molecule expressed by human NK cells. *Eur J Immunol*. 2001;31:233-242.

593. Welte S, Kuttruff S, Waldhauer I, et al. Mutual activation of natural killer cells and monocytes mediated by NKp80-AICL interaction. *Nat Immunol*. 2006;7:1334-1342.

594. Spreu J, Kuttruff S, Stejfova V, et al. Interaction of C-type lectin-like receptors NKp65 and KACL facilitates dedicated immune recognition of human keratinocytes. *Proc Natl Acad Sci U S A*. 2010;107:5100-5105.

595. Chen P, Belanger S, Aguilar OA, et al. Analysis of the mouse 129-strain Nkrp1-Clr gene cluster reveals conservation of genomic organization and functional receptor-ligand interactions despite significant allelic polymorphism. *Immunogenetics*. 2011;63:627-640.

596. Depatie C, Muise E, Lepage P, et al. High-resolution linkage map in the proximity of the host resistance locus CMV1. *Genomics*. 1997;39: 154-163.

597. Forbes CA, Brown MG, Cho R, et al. The Cmv1 host resistance locus is closely linked to the Ly49 multigene family within the natural killer cell gene complex on mouse chromosome 6. *Genomics*. 1997;41:406-413.

598. Nasrallah JB. Recognition and rejection of self in plant reproduction. *Science*. 2002;296:305-308.

599. Ferris PJ, Armbrust EV, Goodenough UW. Genetic structure of the mating-type locus of Chlamydomonas reinhardtii. *Genetics*. 2002;160:181-200.

600. Voigt S, Mesci A, Ettinger J, et al. Cytomegalovirus evasion of innate immunity by subversion of the NKR-P1B:Clr-b missing-self axis. *Immunity*. 2007;26:617-627.

601. Garni-Wagner BA, Purohit A, Mathew PA, et al. A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells. *J Immunol*. 1993;151:60-70.

602. Veillette A. SLAM-family receptors: immune regulators with or without SAP-family adaptors. *Cold Spring Harb Perspect Biol* . 2010;2:a002469.

603. Brown MH, Boles K, Anton van der Merwe P, et al. 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. *J Exp Med*. 1998;188:2083-2090.

604. Veillette A. Immune regulation by SLAM family receptors and SAPrelated adaptors. *Nat Rev Immunol* . 2006;6:56-66.

605. Siliciano RF, Pratt JC, Schmidt RE, et al. Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein. *Nature*. 1985;317:428-430.

606. Umehara H, Huang JY, Kono T, et al. Involvement of protein tyrosine kinase p72syk and phosphatidylinositol 3-kinase in CD2-mediated granular exocytosis in the natural killer cell line, NK3.3. *J Immunol* . 1997;159:1200-1207.

607. Shlapatska LM, Mikhalap SV, Berdova AG, et al. CD150 association with either the SH2-containing inositol phosphatase or the SH2-containing protein tyrosine phosphatase is regulated by the adaptor protein SH2D1A. *J Immunol* . 2001;166:5480-5487.

608. Engel P, Eck MJ, Terhorst C. The SAP and SLAM families in immune responses and X-linked lymphoproliferative disease. *Nat Rev Immunol* . 2003;3:813-821.

609. Tangye SG, Lazetic S, Woollatt E, et al. Cutting edge: human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP. *J Immunol* . 1999;162:6981-6985.

610. Bottino C, Falco M, Parolini S, et al. NTB-A, a novel SH2D1Aassociated surface molecule contributing to the inability of natural killer cells to kill Epstein-Barr virus-infected B cells in X-linked lymphoproliferative disease. *J Exp Med*. 2001;194:235-246.

611. Bouchon A, Cella M, Grierson HL, et al. Cutting edge: activation of NK cell-mediated cytotoxicity by a SAP-independent receptor of the CD2 family. *J Immunol* . 2001;167:5517-5521.

612. Tassi I, Colonna M. The cytotoxicity receptor CRACC (CS-1) recruits EAT-2 and activates the PI3K and phospholipase Cgamma signaling pathways in human NK cells. *J Immunol*. 2005;175:7996-8002.

613. Cruz-Munoz ME, Dong Z, Shi X, et al. Influence of CRACC, a SLAM family receptor coupled to the adaptor EAT-2, on natural killer cell function. *Nat Immunol* . 2009;10:297-305.

614. Schwartzberg PL, Mueller KL, Qi H, et al. SLAM receptors and SAP influence lymphocyte interactions, development and function. *Nat Rev Immunol* . 2009;9:39-46.

615. Lee KM, McNerney ME, Stepp SE, et al. 2B4 acts as a non-major histocompatibility complex binding inhibitory receptor on mouse natural killer cells. *J Exp Med*. 2004;199:1245-1254.

616. Sivori S, Vitale M, Morelli L, et al. p46, a novel natural killer cellspecific surface molecule that mediates cell activation. *J Exp Med*. 1997;186:1129-1136.

617. Pessino A, Sivori S, Bottino C, et al. Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. *J Exp Med*. 1998;188:953-960.

618. Pende D, Parolini S, Pessino A, et al. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *J Exp Med*. 1999;190:1505-1516.

619. Cantoni C, Bottino C, Vitale M, et al. NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily. *J Exp Med*. 1999;189:787-796.

620. Vitale M, Bottino C, Sivori S, et al. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *J Exp Med*. 1998;187:2065-2072.

621. Neville MJ, Campbell RD. A new member of the Ig superfamily and a V-ATPase G subunit are among the predicted products of novel genes close to the TNF locus in the human MHC. *J Immunol* . 1999;162:4745-4754.

622. Allcock RJ, Barrow AD, Forbes S, et al. The human TREM gene cluster at 6p21.1 encodes both activating and inhibitory single IgV domain receptors and includes NKp44. *Eur J Immunol* . 2003;33:567-577.

623. Hollyoake M, Campbell RD, Aguado B. NKp30 (NCR3) is a pseudogene in 12 inbred and wild mouse strains, but an expressed gene in Mus caroli. *Mol Biol Evol* . 2005;22:1661-1672.

624. Moretta L, Bottino C, Pende D, et al. Surface NK receptors and their ligands on tumor cells. *Semin Immunol*. 2006;18:151-158.

625. Brandt CS, Baratin M, Yi EC, et al. The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. *J Exp Med*. 2009;206:1495-1503.

626. Li Y, Wang Q, Mariuzza RA. Structure of the human activating natural cytotoxicity receptor NKp30 bound to its tumor cell ligand B7-H6. *J Exp Med*. 2011;208:703-714.

627. Arnon TI, Achdout H, Levi O, et al. Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus. *Nat Immunol*. 2005;6:515-523.

628. Pogge von Strandmann E, Simhadri VR, von Tresckow B, et al. Human leukocyte antigen-B-associated transcript 3 is released from tumor cells and engages the NKp30 receptor on natural killer cells. *Immunity*. 2007;27:965-974.

629. Jarahian M, Fiedler M, Cohnen A, et al. Modulation of NKp30- and NKp46-mediated natural killer cell responses by poxviral hemagglutinin. *PLoS Pathog*. 2011;7:e1002195.

630. Mandelboim O, Lieberman N, Lev M, et al. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature*. 2001;409:1055-1060.

631. Bloushtain N, Qimron U, Bar-Ilan A, et al. Membrane-associated heparan sulfate proteoglycans are involved in the recognition of cellular targets by NKp30 and NKp46. *J Immunol*. 2004;173:2392-2401.

632. Warren HS, Jones AL, Freeman C, et al. Evidence that the cellular ligand for the human NK cell activation receptor NKp30 is not a heparan sulfate glycosaminoglycan. *J Immunol*. 2005;175:207-212.

633. Spies T, Groh V. Natural cytotoxicity receptors: influenza virus in the spotlight. *Nat Immunol*. 2006;7:443-444.

634. Satoh-Takayama N, Lesjean-Pottier S, Vieira P, et al. IL-7 and IL-15 independently program the differentiation of intestinal CD3-NKp46+ cell subsets from Id2-dependent precursors. *J Exp Med*. 2010;207:273-280.

635. Hughes T, Becknell B, McClory S, et al. Stage 3 immature human natural killer cells found in secondary lymphoid tissue constitutively and selectively express the TH 17 cytokine interleukin-22. *Blood*. 2009;113:4008-4010.

636. Male V, Hughes T, McClory S, et al. Immature NK cells, capable of producing IL-22, are present in human uterine mucosa. *J Immunol*. 2010;185:3913-3918.

637. Cupedo T, Crellin NK, Papazian N, et al. Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells. *Nat Immunol*. 2009;10:66-74.

638. Crellin NK, Trifari S, Kaplan CD, et al. Human NKp44+IL-22+ cells and LTi-like cells constitute a stable RORC+ lineage distinct from conventional natural killer cells. *J Exp Med*. 2010;207:281-290.

639. Vivier E, Spits H, Cupedo T. Interleukin-22-producing innate immune cells: new players in mucosal immunity and tissue repair? *Nat Rev Immunol* . 2009;9:229-234.

640. Sancho D, Gomez M, Sanchez-Madrid F. CD69 is an immunoregulatory molecule induced following activation. *Trends Immunol*. 2005;26:136-140.

641. Bankovich AJ, Shiow LR, Cyster JG. CD69 suppresses sphingosine 1-phosophate receptor-1 (S1P1) function through interaction with membrane helix 4. *J Biol Chem*. 2010;285:22328-22337.

642. Gumley TP, McKenzie IF, Sandrin MS. Tissue expression, structure and function of the murine Ly-6 family of molecules. *Immunol Cell Biol* . 1995;73:277-296.

643. Bamezai A. Mouse Ly-6 proteins and their extended family: markers of cell differentiation and regulators of cell signaling. *Arch Immunol Ther Exp (Warsz)* . 2004;52:255-266.

644. Colonna M. Cytolytic responses: cadherins put out the fire. *J Exp Med*. 2006;203:261-264.

645. Guthmann MD, Tal M, Pecht I. A secretion inhibitory signal transduction molecule on mast cells is another C-type lectin. *Proc Natl Acad Sci U S A* . 1995;92:9397-9401.

646. Butcher S, Arney KL, Cook GP. MAFA-L, an ITIM-containing receptor encoded by the human NK cell gene complex and expressed by basophils and NK cells. *Eur J Immunol* . 1998;28:3755-3762.

647. Blaser C, Kaufmann M, Pircher H. Cutting edge: virus-activated CD8 T cells and lymphokine-activated NK cells express the mast cell function-associated antigen, an inhibitory C-type lectin. *J Immunol* . 1998;161:6451-6454.

648. Corral L, Hanke T, Vance RE, et al. NK cell expression of the killer cell lectin-like receptor G1 (KLRG1), the mouse homolog of MAFA, is modulated by MHC class I molecules. *Eur J Immunol* . 2000;30:920-930.

649. Ito M, Maruyama T, Saito N, et al. Killer cell lectin-like receptor G1 binds three members of the classical cadherin family to inhibit NK cell cytotoxicity. *J Exp Med*. 2006;203:289-295.

650. Grundemann C, Bauer M, Schweier O, et al. Cutting edge: identification of E-cadherin as a ligand for the murine killer cell lectin-like receptor G1. *J Immunol* . 2006;176:1311-1315.

651. Li Y, Hofmann M, Wang Q, et al. Structure of natural killer cell receptor KLRG1

bound to E-cadherin reveals basis for MHC-independent missing self recognition. *Immunity*. 2009;31:35-46.

652. Grundemann C, Schwartzkopff S, Koschella M, et al. The NK receptor KLRG1 is dispensable for virus-induced NK and CD8+ T-cell differentiation and function in vivo. *Eur J Immunol* . 2010;40:1303-1314.

653. Stern N, Markel G, Arnon TI, et al. Carcinoembryonic antigen (CEA) inhibits NK killing via interaction with CEA-related cell adhesion molecule 1. *J Immunol* . 2005;174:6692-6701.

654. Markel G, Mussaffi H, Ling KL, et al. The mechanisms controlling NK cell autoreactivity in TAP2-deficient patients. *Blood*. 2004;103:1770-1778.

655. Markel G, Wolf D, Hanna J, et al. Pivotal role of CEACAM1 protein in the inhibition of activated decidual lymphocyte functions. *J Clin Invest*. 2002;110:943-953.

656. Thirion G, Feliu AA, Coutelier JP. CD66a (CEACAM1) expression by mouse natural killer cells. *Immunology*. 2008;125:535-540.

657. Gray-Owen SD, Blumberg RS. CEACAM1: contact-dependent control of immunity. *Nat Rev Immunol* . 2006;6:433-446.

658. Fuchs A, Colonna M. The role of NK cell recognition of nectin and nectin-like proteins in tumor immunosurveillance. *Semin Cancer Biol* . 2006;16:359-366.

659. Takai Y, Miyoshi J, Ikeda W, et al. Nectins and nectin-like molecules: roles in contact inhibition of cell movement and proliferation. *Nat Rev Mol Cell Biol* . 2008;9:603-615.

660. Shibuya K, Lanier LL, Phillips JH, et al. Physical and functional association of LFA-1 with DNAM-1 adhesion molecule. *Immunity*. 1999;11:615-623.

661. Fuchs A, Cella M, Giurisato E, et al. Cutting edge: CD96 (tactile) promotes NK cell-target cell adhesion by interacting with the poliovirus receptor (CD155). *J Immunol* . 2004;172:3994-3998.

662. Tahara-Hanaoka S, Shibuya K, Onoda Y, et al. Functional characterization of DNAM-1 (CD226) interaction with its ligands PVR (CD155) and nectin-2 (PRR-2/CD112). *Int Immunol* . 2004;16:533-538.

663. Boles KS, Barchet W, Diacovo T, et al. The tumor suppressor TSLC1/NECL-2 triggers NK-cell and CD8+ T-cell responses through the cell-surface receptor CRTAM. *Blood*. 2005;106:779-786.

664. Shibuya A, Campbell D, Hannum C, et al. DNAM-1, a novel adhesion molecule

involved in the cytolytic function of T lymphocytes. *Immunity*. 1996;4:573-581.

665. Gilfillan S, Chan CJ, Cella M, et al. DNAM-1 promotes activation of cytotoxic lymphocytes by nonprofessional antigen-presenting cells and tumors. *J Exp Med*. 2008;205:2965-2973.

666. Iguchi-Manaka A, Kai H, Yamashita Y, et al. Accelerated tumor growth in mice deficient in DNAM-1 receptor. *J Exp Med*. 2008;205: 2959-2964.

667. Yu X, Harden K, Gonzalez LC, et al. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat Immunol*. 2009;10:48-57.

668. Stanietsky N, Simic H, Arapovic J, et al. The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. *Proc Natl Acad Sci U S A* . 2009;106:17858-17863.

669. Inngjerdingen M, Rolstad B, Ryan JC. Activating and inhibitory Ly49 receptors modulate NK cell chemotaxis to CXC chemokine ligand (CXCL) 10 and CXCL12. *J Immunol*. 2003;171:2889-2895.

670. Reymond N, Imbert AM, Devilard E, et al. DNAM-1 and PVR regulate monocyte migration through endothelial junctions. *J Exp Med*. 2004;199:1331-1341.

671. Melero I, Balboa MA, Alonso JL, et al. Signaling through the LFA-1 leucocyte integrin actively regulates intercellular adhesion and tumor necrosis factor-alpha production in natural killer cells. *Eur J Immunol* . 1993;23:1859-1865.

672. Helander TS, Carpen O, Turunen O, et al. ICAM-2 redistributed by ezrin as a target for killer cells. *Nature*. 1996;382:265-268.

673. Barber DF, Faure M, Long EO. LFA-1 contributes an early signal for NK cell cytotoxicity. *J Immunol*. 2004;173:3653-3659.

674. Bryceson YT, March ME, Ljunggren HG, et al. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood*. 2006;107:159-166.

675. Horng T, Bezbradica JS, Medzhitov R. NKG2D signaling is coupled to the interleukin 15 receptor signaling pathway. *Nat Immunol*. 2007;8:1345-1352.

676. Ivashkiv LB. Cross-regulation of signaling by ITAM-associated receptors. *Nat Immunol*. 2009;10:340-347.

677. Watzl C, Long EO. Signal transduction during activation and inhibition of natural

killer cells. *Curr Protoc Immunol* . 2010;Chapter 11:Unit 11 9B.

678. MacFarlane AWT, Campbell KS. Signal transduction in natural killer cells. *Curr Top Microbiol Immunol*. 2006;298:23-57.

679. Salcedo TW, Kurosaki T, Kanakaraj P, et al. Physical and functional association of p56lck with Fc gamma RIIIA (CD16) in natural killer cells. *J Exp Med*. 1993;177:1475-1480.

680. Mason LH, Willette-Brown J, Taylor LS, et al. Regulation of Ly49D/DAP12 signal transduction by Src-family kinases and CD45. *J Immunol*. 2006;176:6615-6623.

681. Colucci F, Schweighoffer E, Tomasello E, et al. Natural cytotoxicity uncoupled from the Syk and ZAP-70 intracellular kinases. *Nat Immunol*. 2002;3:288-294.

682. Peterson EJ, Clements JL, Ballas ZK, et al. NK cytokine secretion and cytotoxicity occur independently of the SLP- 76 adaptor protein. *Eur J Immunol* . 1999;29:2223-2232.

683. Zhang W, Sommers CL, Burshtyn DN, et al. Essential role of LAT in T cell development. *Immunity*. 1999;10:323-332.

684. Tassi I, Presti R, Kim S, et al. Phospholipase C-gamma 2 is a critical signaling mediator for murine NK cell activating receptors. *J Immunol*. 2005;175:749-754.

685. Wang D, Feng J, Wen R, et al. Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. *Immunity*. 2000;13:25-35.

686. Cella M, Fujikawa K, Tassi I, et al. Differential requirements for Vav proteins in DAP10- and ITAM-mediated NK cell cytotoxicity. *J Exp Med*. 2004;200:817-823.

687. Fujikawa K, Miletic AV, Alt FW, et al. Vav1/2/3-null mice define an essential role for Vav family proteins in lymphocyte development and activation but a differential requirement in MAPK signaling in T and B cells. *J Exp Med*. 2003;198:1595-1608.

688. Stebbins CC, Watzl C, Billadeau DD, et al. Vav1 dephosphorylation by the tyrosine phosphatase SHP-1 as a mechanism for inhibition of cellular cytotoxicity. *Mol Cell Biol* . 2003;23:6291-6299.

689. Kim HS, Das A, Gross CC, et al. Synergistic signals for natural cytotoxicity are required to overcome inhibition by c-Cbl ubiquitin ligase. *Immunity*. 2010;32:175-186.

690. Peterson ME, Long EO. Inhibitory receptor signaling via tyrosine phosphorylation of the adapter CRK. *Immunity*. 2008;29:578-588.

691. Jiang K, Zhong B, Gilvary DL, et al. Pivotal role of phosphoinositide-3 kinase in

regulation of cytotoxicity in natural killer cells. *Nat Immunol*. 2000;1:419-425.

692. Segovis CM, Schoon RA, Dick CJ, et al. PI3K links NKG2D signaling to a CrkL pathway involved in natural killer cell adhesion, polarity, and granule secretion. *J Immunol*. 2009;182:6933-6942.

693. Wu J, Song Y, Bakker AB, et al. An activating immunoreceptor complex formed by NKG2D and DAP10. *Science*. 1999;285:730-732.

694. Zompi S, Hamerman JA, Ogasawara K, et al. NKG2D triggers cytotoxicity in mouse NK cells lacking DAP12 or Syk family kinases. *Nat Immunol*. 2003;4:565-572.

695. Jiang K, Zhong B, Gilvary DL, et al. Syk regulation of phosphoinositide 3-kinase-dependent NK cell function. *J Immunol*. 2002;168:3155-3164.

696. Moon KD, Post CB, Durden DL, et al. Molecular basis for a direct interaction between the Syk protein-tyrosine kinase and phosphoinositide 3-kinase. *J Biol Chem*. 2005;280:1543-1551.

697. Bromley SK, Burack WR, Johnson KG, et al. The immunological synapse. *Annu Rev Immunol*. 2001;19:375-396.

698. Davis DM, Dustin ML. What is the importance of the immunological synapse? *Trends Immunol*. 2004;25:323-327.

699. Orange JS. Formation and function of the lytic NK-cell immunological synapse. *Nat Rev Immunol*. 2008;8:713-725.

700. Bryceson YT, Long EO. Line of attack: NK cell specificity and integration of signals. *Curr Opin Immunol*. 2008;20:344-352.

701. Orange JS, Harris KE, Andzelm MM, et al. The mature activating natural killer cell immunologic synapse is formed in distinct stages. *Proc Natl Acad Sci U S A*. 2003;100:14151-14156.

702. Vyas YM, Mehta KM, Morgan M, et al. Spatial organization of signal transduction molecules in the NK cell immune synapses during MHC class I-regulated noncytolytic and cytolytic interactions. *J Immunol*. 2001;167:4358-4367.

703. Krzewski K, Chen X, Orange JS, et al. Formation of a WIP-, WASp-, actin-, and myosin IIA-containing multiprotein complex in activated NK cells and its alteration by KIR inhibitory signaling. *J Cell Biol*. 2006;173:121-132.

704. Graham DB, Cella M, Giurisato E, et al. Vav1 controls DAP10-mediated natural

cytotoxicity by regulating actin and microtubule dynamics. *J Immunol*. 2006;177:2349-2355.

705. Sinai P, Nguyen C, Schatzle JD, et al. Transience in polarization of cytolytic effectors is required for efficient killing and controlled by Cdc42. *Proc Natl Acad Sci U S A*. 2010;107:11912-11917.

706. Burshtyn DN, Scharenberg AM, Wagtmann N, et al. Recruitment of tyrosine phosphatase HCP by the killer cell inhibitor receptor. *Immunity*. 1996;4:77-85.

707. Campbell KS, Dessing M, Lopez-Botet M, et al. Tyrosine phosphorylation of a human killer inhibitory receptor recruits protein tyrosine phosphatase 1C. *J Exp Med*. 1996;184:93-100.

708. Fry AM, Lanier LL, Weiss A. Phosphotyrosines in the killer cell inhibitory receptor motif of NKB1 are required for negative signaling and for association with protein tyrosine phosphatase 1C. *J Exp Med*. 1996;184:295-300.

709. Faure M, Barber DF, Takahashi SM, et al. Spontaneous clustering and tyrosine phosphorylation of NK cell inhibitory receptor induced by ligand binding. *J Immunol*. 2003;170:6107-6114.

710. Kabat J, Borrego F, Brooks A, et al. Role that each NKG2A immunoreceptor tyrosine-based inhibitory motif plays in mediating the human CD94/NKG2A inhibitory signal. *J Immunol*. 2002;169: 1948-1958.

711. Smith KGC, Tarlinton DM, Doody GM, et al. Inhibition of the B cell by CD22: a requirement for Lyn. *J Exp Med*. 1998;187:807-811.

712. Olcese L, Lang P, Vely F, et al. Human and mouse killer-cell inhibitory receptors recruit PTP1C and PTP1D protein tyrosine phosphatases. *J Immunol*. 1996;156:4531-4534.

713. Wang LL, Blasioli J, Plas DR, et al. Specificity of the SH2 domains of SHP-1 in the interaction with the immunoreceptor tyrosine-based inhibitory motif-bearing receptor gp49B. *J Immunol*. 1999;162:1318-1323.

714. Gupta N, Scharenberg AM, Burshtyn DN, et al. Negative signaling pathways of the killer cell inhibitory receptor and Fc gamma RIIb1 require distinct phosphatases. *J Exp Med*. 1997;186:473-478.

715. Yusa S, Campbell KS. Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2) can play a direct role in the inhibitory function of killer cell Ig-like receptors in human NK cells. *J Immunol*. 2003;170:4539-4547.

716. Vyas YM, Maniar H, Lyddane CE, et al. Ligand binding to inhibitory killer cell Ig-like receptors induce colocalization with Src homology domain 2-containing protein tyrosine phosphatase 1 and interruption of ongoing activation signals. *J Immunol*. 2004;173:1571-1578.

717. Standeven LJ, Carlin LM, Borszcz P, et al. The actin cytoskeleton controls the efficiency of killer Ig-like receptor accumulation at inhibitory NK cell immune synapses. *J Immunol*. 2004;173:5617-5625.

718. Masilamani M, Nguyen C, Kabat J, et al. CD94/NKG2A inhibits NK cell activation by disrupting the actin network at the immunological synapse. *J Immunol*. 2006;177:3590-3596.

719. Treanor B, Lanigan PM, Kumar S, et al. Microclusters of inhibitory killer immunoglobulin-like receptor signaling at natural killer cell immunological synapses. *J Cell Biol*. 2006;174:153-161.

720. Sayos J, Martinez-Barriocanal A, Kitzig F, et al. Recruitment of C-terminal Src kinase by the leukocyte inhibitory receptor CD85j. *Biochem Biophys Res Commun*. 2004;324:640-647.

721. Orr SJ, Morgan NM, Elliott J, et al. CD33 responses are blocked by SOCS3 through accelerated proteasomal-mediated turnover. *Blood*. 2007;109:1061-1068.

722. Xu R, Abramson J, Fridkin M, et al. SH2 domain-containing inositol polyphosphate 5′-phosphatase is the main mediator of the inhibitory action of the mast cell function-associated antigen. *J Immunol*. 2001;167:6394-6402.

723. Ono M, Bolland S, Tempst P, et al. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc(gamma)RIIB. *Nature*. 1996;383:263-266.

724. Yu MC, Su LL, Zou L, et al. An essential function for beta-arrestin 2 in the inhibitory signaling of natural killer cells. *Nat Immunol*. 2008;9(8):898-907.

725. Marti F, Xu CW, Selvakumar A, et al. LCK-phosphorylated human killer cell-inhibitory receptors recruit and activate phosphatidylinositol 3-kinase. *Proc Natl Acad Sci U S A*. 1998;95:11810-11815.

726. Wang JW, Howson JM, Ghansah T, et al. Influence of SHIP on the NK repertoire and allogeneic bone marrow transplantation. *Science*. 2002;295:2094-2097.

727. Das A, Long EO. Lytic granule polarization, rather than degranulation, is the preferred target of inhibitory receptors in NK cells. *J Immunol*. 2010;185:4698-4704.

728. Abeyweera TP, Merino E, Huse M. Inhibitory signaling blocks activating receptor clustering and induces cytoskeletal retraction in natural killer cells. *J Cell Biol* . 2011;192:675-690.

729. Feller SM. Crk family adaptors-signalling complex formation and biological roles. *Oncogene*. 2001;20:6348-6371.

730. Huang F, Gu H. Negative regulation of lymphocyte development and function by the Cbl family of proteins. *Immunol Rev*. 2008;224:229-238.

731. Schmitz ML. Activation of T cells: releasing the brakes by proteolytic elimination of Cbl-b. *Sci Signal* . 2009;2:pe38.

732. Sjostrom A, Eriksson M, Cerboni C, et al. Acquisition of external major histocompatibility complex class I molecules by natural killer cells expressing inhibitory ly49 receptors. *J Exp Med*. 2001;194:1519-1530.

733. Zimmer J, Ioannidis V, Held W. H-2D ligand expression by Ly49A+ natural killer (NK) cells precludes ligand uptake from environmental cells: implications for NK cell function. *J Exp Med*. 2001;194: 1531-1539.

734. Davis DM. Intercellular transfer of cell-surface proteins is common and can affect many stages of an immune response. *Nat Rev Immunol* . 2007;7:238-243.

735. Furukawa H, Yabe T, Watanabe K, et al. Tolerance of NK and LAK activity for HLA class I-deficient targets in a TAP1-deficient patient (bare lymphocyte syndrome type I). *Hum Immunol*. 1999;60:32-40.

736. Vitale M, Zimmer J, Castriconi R, et al. Analysis of natural killer cells in TAP2-deficient patients: expression of functional triggering receptors and evidence for the existence of inhibitory receptor(s) that prevent lysis of normal autologous cells. *Blood*. 2002;99: 1723-1729.

737. Ljunggren HG, Van Kaer L, Ploegh HL, et al. Altered natural killer cell repertoire in Tap-1 mutant mice. *Proc Natl Acad Sci U S A* . 1994;91:6520-6524.

738. Dorfman JR, Zerrahn J, Coles MC, et al. The basis for self-tolerance of natural killer cells in beta2-microglobulin- and TAP-1- mice. *J Immunol* . 1997;159:5219-5225.

739. Cudkowicz G, Stimpfling JH. Hybrid resistance to parental marrow grafts: association with the K Region of H-2. *Science*. 1964;144: 1339-1340.

740. Bennett M. Biology and genetics of hybrid resistance. *Adv Immunol* . 1987;41:333-445.

741. Ohlen C, Kling G, Hoglund P, et al. Prevention of allogeneic bone marrow graft rejection by H-2 transgene in donor mice. *Science*. 1989;246:666-668.

742. Bennett M, Yu YY, Stoneman E, et al. Hybrid resistance: “negative” and “positive” signaling of murine natural killer cells. *Sem Immunol*. 1995;7:121-127.

743. Raulet DH, Vance RE, McMahon CW. Regulation of the natural killer cell receptor repertoire. *Annu Rev Immunol*. 2001;19:291-330.

744. Sentman CL, Olsson MY, Karre K. Missing self recognition by natural killer cells in MHC class I transgenic mice. A “receptor calibration” model for how effector cells adapt to self. *Sem Immunol*. 1995;7: 109-119.

745. Dorfman JR, Raulet DH. Major histocompatibility complex genes determine natural killer cell tolerance. *Eur J Immunol* . 1996;26:151-155.

746. Olsson-Alheim MY, Salcedo M, Ljunggren HG, et al. NK cell receptor calibration: effects of MHC class I induction on killing by Ly49Ahigh and Ly49Alow NK cells. *J Immunol*. 1997;159:3189-3194.

747. Fernandez NC, Treiner E, Vance RE, et al. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood*. 2005;105:4416-4423.

748. Jonsson AH, Yokoyama WM. Assessing licensing of NK cells. *Methods Mol Biol*. 2010;612:39-49.

749. Choi T, Ferris ST, Matsumoto N, et al. Ly49-dependent NK cell licensing and effector inhibition involve the same interaction site on MHC ligands. *J Immunol*. 2011;186:3911-3917.

750. Elliott JM, Yokoyama WM. Unifying concepts of MHC-dependent natural killer cell education. *Trends Immunol*. 2011;32:364-372.

751. Vivier E, Raulet DH, Moretta A, et al. Innate or adaptive immunity? The example of natural killer cells. *Science*. 2011;331:44-49.

752. Anfossi N, Andre P, Guia S, et al. Human NK cell education by inhibitory receptors for MHC class I. *Immunity*. 2006;25:331-342.

753. Kim S, Sunwoo JB, Yang L, et al. HLA alleles determine differences in human natural killer cell responsiveness and potency. *Proc Natl Acad Sci U S A* . 2008;105:3053-3058.

754. Kulkarni S, Martin MP, Carrington M. The Yin and Yang of HLA and KIR in human disease. *Semin Immunol*. 2008;20:343-352.

755. Altfeld M, Fadda L, Frleta D, et al. DCs and NK cells: critical effectors in the immune response to HIV-1. *Nat Rev Immunol* . 2011;11:176-186.

756. Khakoo SI, Thio CL, Martin MP, et al. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science*. 2004;305:872-874.

757. Jonsson AH, Yokoyama WM. Natural killer cell tolerance licensing and other mechanisms. *Adv Immunol* . 2009;101:27-79.

758. Johansson S, Johansson M, Rosmaraki E, et al. Natural killer cell education in mice with single or multiple major histocompatibility complex class I molecules. *J Exp Med*. 2005;201:1145-1155.

759. Brodin P, Lakshmikanth T, Mehr R, et al. Natural killer cell tolerance persists despite significant reduction of self MHC class I on normal target cells in mice. *PLoS One*. 2010;5:e13174.

760. Hoglund P, Brodin P. Current perspectives of natural killer cell education by MHC class I molecules. *Nat Rev Immunol* . 2010;10:724-734.

761. Elliott JM, Wahle JA, Yokoyama WM. MHC class I-deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I-sufficient environment. *J Exp Med*. 2010;207:2073-2079.

762. Joncker NT, Shifrin N, Delebecque F, et al. Mature natural killer cells reset their responsiveness when exposed to an altered MHC environment. *J Exp Med*. 2010;207:2065-2072.

763. Held W, Mariuzza RA. Cis interactions of immunoreceptors with MHC and non-MHC ligands. *Nat Rev Immunol* . 2008;8:269-278.

764. Raulet DH, Vance RE. Self-tolerance of natural killer cells. *Nat Rev Immunol* . 2006;6:520-531.

765. Wu MF, Raulet DH. Class I-deficient hemopoietic cells and nonhemopoietic cells dominantly induce unresponsiveness of natural killer cells to class I-deficient bone marrow cell grafts. *J Immunol* . 1997;158:1628-1633.

766. Guia S, Jaeger BN, Piatek S, et al. Confinement of activating receptors at the plasma membrane controls natural killer cell tolerance. *Sci Signal* . 2011;4:ra21.

767. Tripathy SK, Keyel PA, Yang L, et al. Continuous engagement of a self-specific activation receptor induces NK cell tolerance. *J Exp Med*. 2008;205:1829-1841.

768. Sun JC, Lanier LL. Tolerance of NK cells encountering their viral ligand during development. *J Exp Med*. 2008;205:1819-1828.

769. Mathis D, Benoist C. Levees of immunological tolerance. *Nat Immunol*. 2010;11:3-6.

770. Xie X, Stadnisky MD, Brown MG. MHC class I Dk locus and Ly49G2+ NK cells confer H-2k resistance to murine cytomegalovirus. *J Immunol*. 2009;182:7163-7171.

771. Xie X, Stadnisky MD, Coats ER, et al. MHC class I D(k) expression in hematopoietic and nonhematopoietic cells confers natural killer cell resistance to murine cytomegalovirus. *Proc Natl Acad Sci U S A*. 2010;107:8754-8759.

772. Stadnisky MD, Xie X, Coats ER, et al. Self MHC class I-licensed NK cells enhance adaptive CD8 T-cell viral immunity. *Blood*. 2011;117:5133-5141.

773. Tay CH, Welsh RM, Brutkiewicz RR. NK cell response to viral infections in beta 2-microglobulin-deficient mice. *J Immunol*. 1995;154:780-789.

774. Sun JC, Lanier LL. Cutting edge: viral infection breaks NK cell tolerance to “missing self.” *J Immunol*. 2008;181:7453-7457.

775. Orr MT, Murphy WJ, Lanier LL. “Unlicensed” natural killer cells dominate the response to cytomegalovirus infection. *Nat Immunol*. 2010;11:321-327.

776. Seaman WE, Blackman MA, Gindhart TD, et al. beta-Estradiol reduces natural killer cells in mice. *J Immunol*. 1978;121:2193-2198.

777. Seaman WE, Gindhart TD, Greenspan JS, et al. Natural killer cells, bone, and the bone marrow: studies in estrogen-treated mice and in congenitally osteopetrotic (mi/mi) mice. *J Immunol*. 1979;122:2541-2547.

778. Kumar V, Ben-Ezra J, Bennett M, et al. Natural killer cells in mice treated with 89strontium: normal target-binding cell numbers but inability to kill even after interferon administration. *J Immunol*. 1979;123:1832-1838.

779. Puzanov IJ, Bennett M, Kumar V. IL-15 can substitute for the marrow microenvironment in the differentiation of natural killer cells. *J Immunol*. 1996;157:4282-4285.

780. Ito A, Kataoka TR, Kim DK, et al. Inhibitory effect on natural killer activity of microphthalmia transcription factor encoded by the mutant mi allele of mice. *Blood*. 2001;97:2075-2083.

781. Wu Q, Sun Y, Wang J, et al. Signal via lymphotoxin-beta R on bone marrow stromal

cells is required for an early checkpoint of NK cell development. *J Immunol*. 2001;166:1684-1689.

782. Miller JS, Alley KA, McGlave P. Differentiation of natural killer (NK) cells from human primitive marrow progenitors in a stroma-based long-term culture system: identification of a CD34+7+ NK progenitor. *Blood*. 1994;83:2594-2601.

783. Shibuya A, Nagayoshi K, Nakamura K, et al. Lymphokine requirement for the generation of natural killer cells from CD34+ hematopoietic progenitor cells. *Blood*. 1995;85:3538-3546.

784. Mrozek E, Anderson P, Caligiuri MA. Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells. *Blood*. 1996;87:2632-2640.

785. Williams NS, Moore TA, Schatzle JD, et al. Generation of lytic natural killer 1.1+, Ly-49-cells from multipotential murine bone marrow progenitors in a stroma-free culture: definition of cytokine requirements and developmental intermediates. *J Exp Med*. 1997;186:1609-1614.

786. Yu H, Fehniger TA, Fuchshuber P, et al. Flt3 ligand promotes the generation of a distinct CD34(+) human natural killer cell progenitor that responds to interleukin-15. *Blood*. 1998;92:3647-3657.

787. Williams NS, Klem J, Puzanov IJ, et al. Differentiation of NK1.1+, Ly49+ NK cells from flt3+ multipotent marrow progenitor cells. *J Immunol*. 1999;163:2648-2656.

788. Roth C, Carlyle JR, Takizawa H, et al. Clonal acquisition of inhibitory Ly49 receptors on developing NK cells is successively restricted and regulated by stromal class I MHC. *Immunity*. 2000;13:143-153.

789. Vargas CL, Poursine-Laurent J, Yang L, et al. Development of thymic NK cells from double-negative 1 thymocyte precursors. *Blood*. 2011;118:3570-3578.

790. Caraux A, Lu Q, Fernandez N, et al. Natural killer cell differentiation driven by Tyro3 receptor tyrosine kinases. *Nat Immunol*. 2006;7:747-754.

791. Park IK, Giovenzana C, Hughes TL, et al. The Axl/Gas6 pathway is required for optimal cytokine signaling during human natural killer cell development. *Blood*. 2009;113:2470-2477.

792. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell*. 1997;91: 661-672.

793. Galy A, Travis M, Cen D, et al. Human T, B, natural killer, and dendritic cells arise

from a common bone marrow progenitor cell subset. *Immunity*. 1995;3:459-473.

794. Georgopoulos K, Bigby M, Wang JH, et al. The Ikaros gene is required for the development of all lymphoid lineages. *Cell*. 1994;79:143-156.

795. Wang JH, Nichogiannopoulou A, Wu L, et al. Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. *Immunity*. 1996;5:537-549.

796. Scott EW, Fisher RC, Olson MC, et al. PU.1 functions in a cell-autonomous manner to control the differentiation of multipotential lymphoid-myeloid progenitors. *Immunity*. 1997;6:437-447.

797. DeKoter RP, Singh H. Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science*. 2000;288:1439-1441.

798. Colucci F, Samson SI, DeKoter RP, et al. Differential requirement for the transcription factor PU.1 in the generation of natural killer cells versus B and T cells. *Blood*. 2001;97:2625-2632.

799. Wang B, Biron C, She J, et al. A block in both early T lymphocyte and natural killer cell development in transgenic mice with high-copy numbers of the human CD3E gene. *Proc Natl Acad Sci U S A* . 1994;91: 9402-9406.

800. Flamand V, Shores EW, Tran T, et al. Delayed maturation of CD4-CD8-Fc gamma RII/III+ T and natural killer cell precursors in Fc epsilon RI gamma transgenic mice. *J Exp Med*. 1996;184:1725-1735.

801. Townsend MJ, Weinmann AS, Matsuda J, et al. T-bet regulates the terminal maturation and homeostasis of NK and Va14i NKT cells. *Immunity*. 2004;20:477-494.

802. Rodewald HR, Moingeon P, Lucich JL, et al. A population of early fetal thymocytes expressing Fc gamma RII/III contains precursors of T lymphocytes and natural killer cells. *Cell*. 1992;69:139-150.

803. Carlyle JR, Michie AM, Furlonger C, et al. Identification of a novel developmental stage marking lineage commitment of progenitor thymocytes. *J Exp Med*. 1997;186:173-182.

804. Carlyle JR, Zuniga-Pflucker JC. Requirement for the thymus in alphabeta T lymphocyte lineage commitment. *Immunity*. 1998;9:187-197.

805. Ikawa T, Kawamoto H, Fujimoto S, et al. Commitment of common T/Natural killer (NK) progenitors to unipotent T and NK progenitors in the murine fetal thymus revealed by a single progenitor assay. *J Exp Med*. 1999;190:1617-1626.

806. Douagi I, Colucci F, Di Santo JP, et al. Identification of the earliest prethymic bipotent T/NK progenitor in murine fetal liver. *Blood*. 2002;99:463-471.

807. Porritt HE, Rumfelt LL, Tabrizifard S, et al. Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. *Immunity*. 2004;20:735-745.

808. Zuniga-Pflucker JC. T-cell development made simple. *Nat Rev Immunol* . 2004;4:67-72.

809. Li P, Burke S, Wang J, et al. Reprogramming of T cells to natural killer-like cells upon Bcl11b deletion. *Science*. 2010;329:85-89.

810. Ikawa T, Hirose S, Masuda K, et al. An essential developmental checkpoint for production of the T cell lineage. *Science*. 2010;329:93-96.

811. Li L, Leid M, Rothenberg EV. An early T cell lineage commitment checkpoint dependent on the transcription factor Bcl11b. *Science*. 2010;329:89-93.

812. Di Santo JP. Immunology. A guardian of T cell fate. *Science*. 2010;329:44-45.

813. Yokota Y, Mansouri A, Mori S, et al. Development of peripheral lymphoid organs and natural killer cells depends on the helixloop-helix inhibitor Id2. *Nature*. 1999;397:702-706.

814. Boos MD, Yokota Y, Eberl G, et al. Mature natural killer cell and lymphoid tissue-inducing cell development requires Id2-mediated suppression of E protein activity. *J Exp Med*. 2007;204:1119-1130.

815. Aliahmad P, de la Torre B, Kaye J. Shared dependence on the DNAbinding factor TOX for the development of lymphoid tissue-inducer cell and NK cell lineages. *Nat Immunol*. 2010;11:945-952.

816. Rosmaraki EE, Douagi I, Roth C, et al. Identification of committed NK cell progenitors in adult murine bone marrow. *Eur J Immunol* . 2001;31:1900-1909.

817. Fathman JW, Bhattacharya D, Inlay MA, et al. Identification of the earliest natural killer cell-committed progenitor in murine bone marrow. *Blood*. 2011;118:5439-5447.

818. Intlekofer AM, Takemoto N, Wherry EJ, et al. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat Immunol* . 2005;6:1236-1244.

819. Cowell IG, Skinner A, Hurst HC. Transcriptional repression by a novel member of the bZIP family of transcription factors. *Mol Cell Biol* . 1992;12:3070-3077.

820. Zhang W, Zhang J, Kornuc M, et al. Molecular cloning and characterization of NF-IL3A, a transcriptional activator of the human interleukin-3 promoter. *Mol Cell Biol*. 1995;15:6055-6063.

821. Gascoyne DM, Long E, Veiga-Fernandes H, et al. The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development. *Nat Immunol*. 2009;10:1118-1124.

822. Kamizono S, Duncan GS, Seidel MG, et al. Nfil3/E4bp4 is required for the development and maturation of NK cells in vivo. *J Exp Med*. 2009;206:2977-2986.

823. Kashiwada M, Levy DM, McKeag L, et al. IL-4-induced transcription factor NFIL3/E4BP4 controls IgE class switching. *Proc Natl Acad Sci U S A*. 2010;107:821-826.

824. Kashiwada M, Pham NL, Pewe LL, et al. NFIL3/E4BP4 is a key transcription factor for CD8alpha dendritic cell development. *Blood*. 2011;117:6193-6197.

825. Yokoyama WM, Kim S, French AR. The dynamic life of natural killer cells. *Annu Rev Immunol*. 2004;22:405-429.

826. Di Santo JP. Natural killer cell developmental pathways: a question of balance. *Annu Rev Immunol*. 2006;24:257-286.

827. Narni-Mancinelli E, Chaix J, Fenis A, et al. Fate mapping analysis of lymphoid cells expressing the NKp46 cell surface receptor. *Proc Natl Acad Sci U S A*. 2011;108:18324-18329.

828. Kim S, Iizuka K, Aguila HL, et al. In vivo natural killer cell activities revealed by natural killer cell-deficient mice. *Proc Natl Acad Sci U S A*. 2000;97:2731-2736.

829. Kim S, Song YJ, Higuchi DA, et al. Arrested natural killer cell development associated with transgene insertion into the Atf2 locus. *Blood*. 2006;107:1024-1030.

830. Hayakawa Y, Smyth MJ. CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. *J Immunol*. 2006;176:1517-1524.

831. Barton K, Muthusamy N, Fischer C, et al. The Ets-1 transcription factor is required for the development of natural killer cells in mice. *Immunity*. 1998;9:555-563.

832. Duncan GS, Mittrucker HW, Kagi D, et al. The transcription factor interferon regulatory factor-1 is essential for natural killer cell function in vivo. *J Exp Med*. 1996;184:2043-2048.

833. Ogasawara K, Hida S, Azimi N, et al. Requirement for IRF-1 in the microenvironment supporting development of natural killer cells. *Nature*. 1998;391:700-703.

834. Ohteki T, Yoshida H, Matsuyama T, et al. The transcription factor interferon regulatory factor 1 (IRF-1) is important during the maturation of $NK1.1^+$ T cell receptor-ab^+ ($NK1^+$ T) cells, natural killer cells, and intestinal intraepithelial T cells. *J Exp Med*. 1998;187: 967-972.

835. Lacorazza HD, Miyazaki Y, Di Cristofano A, et al. The ETS protein MEF plays a critical role in perforin gene expression and the development of natural killer and NK-T cells. *Immunity*. 2002;17: 437-449.

836. Samson SI, Richard O, Tavian M, et al. GATA-3 promotes maturation, IFN-gamma production, and liver-specific homing of NK cells. *Immunity*. 2003;19:701-711.

837. Ohteki T, Maki C, Koyasu S. Overexpression of Bcl-2 differentially restores development of thymus-derived CD4-8+ T cells and intestinal intraepithelial T cells in IFN-regulatory factor-1-deficient mice. *J Immunol*. 2001;166:6509-6513.

838. Lohoff M, Duncan GS, Ferrick D, et al. Deficiency in the transcription factor interferon regulatory factor (IRF)-2 leads to severely compromised development of natural killer and T helper type 1 cells. *J Exp Med*. 2000;192:325-336.

839. Taki S, Nakajima S, Ichikawa E, et al. IFN regulatory factor-2 deficiency revealed a novel checkpoint critical for the generation of peripheral NK cells. *J Immunol*. 2005;174:6005-6012.

840. Kaisho T, Tsutsui H, Tanaka T, et al. Impairment of natural killer cytotoxic activity and interferon gamma production in CCAAT/enhancer binding protein gamma-deficient mice. *J Exp Med*. 1999;190:1573-1582.

841. Freud AG, Yokohama A, Becknell B, et al. Evidence for discrete stages of human natural killer cell differentiation in vivo. *J Exp Med*. 2006;203:1033-1043.

842. Freud AG, Caligiuri MA. Human natural killer cell development. *Immunol Rev*. 2006;214:56-72.

843. Huntington ND, Vosshenrich CA, Di Santo JP. Developmental pathways that generate natural-killer-cell diversity in mice and humans. *Nat Rev Immunol*. 2007;7:703-714.

844. Caligiuri MA. Human natural killer cells. *Blood*. 2008;112:461-469.

845. Woll PS, Martin CH, Miller JS, et al. Human embryonic stem cellderived NK cells acquire functional receptors and cytolytic activity. *J Immunol*. 2005;175:5095-5103.

846. Woll PS, Grzywacz B, Tian X, et al. Human embryonic stem cells differentiate into a homogeneous population of natural killer cells with potent in vivo antitumor activity. *Blood*. 2009;113:6094-6101.

847. Freud AG, Becknell B, Roychowdhury S, et al. A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells. *Immunity*. 2005;22:295-304.

848. Huntington ND, Legrand N, Alves NL, et al. IL-15 trans-presentation promotes human NK cell development and differentiation in vivo. *J Exp Med*. 2009;206:25-34.

849. Dokun AO, Chu DT, Yang L, et al. Analysis of in situ NK cell responses during viral infection. *J Immunol*. 2001;167:5286-5293.

850. Andrews DM, Farrell HE, Densley EH, et al. NK1.1+ cells and murine cytomegalovirus infection: what happens in situ? *J Immunol*. 2001;166:1796-1802.

851. Salazar-Mather TP, Lewis CA, Biron CA. Type I interferons regulate inflammatory cell trafficking and macrophage inflammatory protein 1alpha delivery to the liver. *J Clin Invest*. 2002;110:321-330.

852. Salazar-Mather TP, Orange JS, Biron CA. Early murine cytomegalovirus (MCMV) infection induces liver natural killer (NK) cell inflammation and protection through macrophage inflammatory protein 1-alpha (MIP-1-alpha)-dependent pathways. *J Exp Med*. 1998;187:1-14.

853. Walzer T, Vivier E. G-protein-coupled receptors in control of natural killer cell migration. *Trends Immunol*. 2011;32:486-492.

854. Fehniger TA, Cooper MA, Nuovo GJ, et al. CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. *Blood*. 2003;101:3052-3057.

855. Martin-Fontecha A, Thomsen LL, Brett S, et al. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat Immunol*. 2004;5:1260-1265.

856. Fang M, Lanier LL, Sigal LJ. A role for NKG2D in NK cell-mediated resistance to poxvirus disease. *PLoS Pathog*. 2008;4:e30.

857. Andrews DM, Estcourt MJ, Andoniou CE, et al. Innate immunity defines the capacity of antiviral T cells to limit persistent infection. *J Exp Med*. 2010;207:1333-1343.

858. Robbins SH, Bessou G, Cornillon A, et al. Natural killer cells promote early CD8 T cell responses against cytomegalovirus. *PLoS Pathog*. 2007;3:e123.

859. Scharton TM, Scott P. Natural killer cells are a source of interferon gamma that drives differentiation of CD4+ T cell subsets and induces early resistance to Leishmania major in mice. *J Exp Med*. 1993;178: 567-577.

860. Laouar Y, Sutterwala FS, Gorelik L, et al. Transforming growth factorbeta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma. *Nat Immunol*. 2005;6:600-607.

861. Morandi B, Bougras G, Muller WA, et al. NK cells of human secondary lymphoid tissues enhance T cell polarization via IFN-gamma secretion. *Eur J Immunol* . 2006;36:2394-2400.

862. Maroof A, Beattie L, Zubairi S, et al. Posttranscriptional regulation of Il10 gene expression allows natural killer cells to express immunoregulatory function. *Immunity*. 2008;29:295-305.

863. Krebs P, Barnes MJ, Lampe K, et al. NK-cell-mediated killing of target cells triggers robust antigen-specific T-cell-mediated and humoral responses. *Blood*. 2009;113:6593-6602.

864. Herberman RB, Gorelik E. Role of the natural immune system in control of primary tumors and metastasis. In: Reynolds CW, Wiltrout RH, eds. *Functions of the Natural Immune System*. New York, NY: Plenum Press; 1989:3-37.

865. Smyth MJ, Dunn GP, Schreiber RD. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. *Adv Immunol*. 2006;90:1-50

866. Smyth MJ, Crowe NY, Godfrey DI. NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. *Int Immunol*. 2001;13:459-463.

867. Smyth MJ, Cretney E, Takeda K, et al. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gammadependent natural killer cell protection from tumor metastasis. *J Exp Med*. 2001;193:661-670.

868. Takeda K, Smyth MJ, Cretney E, et al. Critical role for tumor necrosis factor-related apoptosis-inducing ligand in immune surveillance against tumor development. *J Exp Med*. 2002;195:161-169.

869. Imai K, Matsuyama S, Miyake S, et al. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet*. 2000;356:1795-1799.

870. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell

alloreactivity in mismatched hematopoietic transplants. *Science*. 2002;295:2097-2100.

871. Hsu KC, Keever-Taylor CA, Wilton A, et al. Improved outcome in HLA-identical sibling hematopoietic stem-cell transplantation for acute myelogenous leukemia predicted by KIR and HLA genotypes. *Blood*. 2005;105:4878-4884.

872. Zucchini N, Crozat K, Baranek T, et al. Natural killer cells in immunodefense against infective agents. *Expert Rev Anti Infect Ther* . 2008;6:867-885.

873. Bouzani M, Ok M, McCormick A, et al. Human NK cells display important antifungal activity against Aspergillus fumigatus, which is directly mediated by IFN-gamma release. *J Immunol*. 2011;187:1369-1376.

874. Biron CA, Byron KS, Sullivan JL. Severe herpesvirus infections in an adolescent without natural killer cells. *New Eng J Med* . 1989;320: 1731-1735.

875. Orange JS. Human natural killer cell deficiencies. *Curr Opin Allergy Clin Immunol* . 2006;6:399-409.

876. Rook AH, Masur H, Lane HC, et al. Interleukin-2 enhances the depressed natural killer and cytomegalovirus-specific cytotoxic activities of lymphocytes from patients with the acquired immune deficiency syndrome. *J Clin Invest*. 1983;72:398-403.

877. Bonavida B, Katz J, Gottlieb M. Mechanism of defective NK cell activity in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. I. Defective trigger on NK cells for NKCF production by target cells, and partial restoration by IL 2. *J Immunol*. 1986;137:1157-1163.

878. Lusso P, Malnati MS, Garzino-Demo A, et al. Infection of natural killer cells by human herpesvirus 6. *Nature*. 1993;362:458-462.

879. Fauci AS, Mavilio D, Kottilil S. NK cells in HIV infection: paradigm for protection or targets for ambush. *Nat Rev Immunol* . 2005;5: 835-843.

880. Epps RE, Pittelkow MR, Su WP. TORCH syndrome. *Sem Dermatol*. 1995;14:179-186.

881. Unanue ER. Inter-relationship among macrophages, natural killer cells and neutrophils in early stages of Listeria resistance. *Curr Opin Immunol* . 1997;9:35-43.

882. Biron CA. Expansion, maintenance, and memory in NK and T cells during viral infections: responding to pressures for defense and regulation. *PLoS Pathog*. 2010;6:e1000816.

883. Bukowski JF, Woda BA, Habu S, et al. Natural killer cell depletion enhances virus

synthesis and virus-induced hepatitis in vivo. *J Immunol*. 1983;131:1531-1538.

884. Bukowski JF, Woda BA, Welsh RM. Pathogenesis of murine cytomegalovirus infection in natural killer cell-depleted mice. *J Virol*. 1984;52:119-128.

885. Welsh RM, Dundon PL, Eynon EE, et al. Demonstration of the antiviral role of natural killer cells in vivo with a natural killer cell-specific monoclonal antibody (NK 1.1). *Nat Immun Cell Growth Regul*. 1990;9: 112-120.

886. Orange JS, Wang B, Terhorst C, et al. Requirement for natural killer cell-produced interferon gamma in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. *J Exp Med*. 1995;182:1045-1056.

887. Loh J, Chu DT, O'Guin AK, et al. Natural killer cells utilize both perforin and gamma interferon to regulate murine cytomegalovirus infection in the spleen and liver. *J Virol*. 2005;79:661-667.

888. Bancroft GJ, Schreiber RD, Unanue ER. Natural immunity: a T-cellindependent pathway of macrophage activation, defined in the scid mouse. *Immunol Rev*. 1991;124:5-24.

889. Dunn PL, North RJ. Early gamma interferon production by natural killer cells is important in defense against murine listeriosis. *Infect Immun*. 1991;59:2892-2900.

890. Tripp CS, Gately MK, Hakimi J, et al. Neutralization of IL-12 decreases resistance to Listeria in SCID and C.B-17 mice. Reversal by IFN-gamma. *J Immunol*. 1994;152:1883-1887.

891. Brombacher F, Dorfmuller A, Magram J, et al. IL-12 is dispensable for innate and adaptive immunity against low doses of Listeria monocytogenes. *Int Immunol*. 1999;11:325-332.

892. Harty JT, Bevan MJ. Specific immunity to Listeria monocytogenes in the absence of IFN gamma. *Immunity*. 1995;3:109-117.

893. Huang S, Hendriks W, Althage A, et al. Immune response in mice that lack the interferon-gamma receptor. *Science*. 1993;259:1742-1745.

894. Meraz MA, White JM, Sheehan KC, et al. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell*. 1996;84:431-442.

895. Orange JS, Biron CA. An absolute and restricted requirement for IL-12 in natural killer cell IFN-gamma production and antiviral defense. Studies of natural killer and T cell

responses in contrasting viral infections. *J Immunol*. 1996;156:1138-1142.

896. Biron CA, Nguyen KB, Pien GC, et al. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol*. 1999;17:189-220.

897. Pien GC, Satoskar AR, Takeda K, et al. Cutting edge: selective IL-18 requirements for induction of compartmental IFNgamma responses during viral infection. *J Immunol*. 2000;165: 4787-4791.

898. Smith VP, Bryant NA, Alcami A. Ectromelia, vaccinia and cowpox viruses encode secreted interleukin-18-binding proteins. *J Gen Virol*. 2000;81(pt 5):1223-1230.

899. Born TL, Morrison LA, Esteban DJ, et al. A poxvirus protein that binds to and inactivates IL-18, and inhibits NK cell response. *J Immunol*. 2000;164:3246-3254.

900. Akira S. The role of IL-18 in innate immunity. *Curr Opin Immunol*. 2000;12:59-63.

901. Hyodo Y, Matsui K, Hayashi N, et al. IL-18 up-regulates perforin-mediated NK activity without increasing perforin messenger RNA expression by binding to constitutively expressed IL-18 receptor. *J Immunol*. 1999;162:1662-1668.

902. Takeda K, Tsutsui H, Yoshimoto T, et al. Defective NK cell activity and Th1 response in IL-18-deficient mice. *Immunity*. 1998;8:383-390.

903. Bukowski JF, Warner JF, Dennert G, et al. Adoptive transfer studies demonstrating the antiviral effect of natural killer cells in vivo. *J Exp Med*. 1985;161:40-52.

904. Guidotti LG, Chisari FV. Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu Rev Immunol*. 2001;19:65-91.

905. Nguyen KB, Cousens LP, Doughty LA, et al. Interferon a/b-mediated inhibition and promotion of interferon-g: STAT1 resolves a paradox. *Nat Immunol*. 2000;1:70-76.

906. Waldmann TA, Tagaya Y. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Ann Rev Immunol*. 1999;17:19-49.

907. Gosselin J, Tomolu A, Gallo RC, et al. Interleukin-15 as an activator of natural killer cell-mediated antiviral response. *Blood*. 1999;94:4210-4219.

908. Fawaz LM, Sharif-Askari E, Menezes J. Up-regulation of NK cytotoxic activity via IL-15 induction by different viruses: a comparative study. *J Immunol*. 1999;163:4473-4480.

909. Ahmad A, Sharif-Askari E, Fawaz L, et al. Innate immune response of the human host to exposure with herpes simplex virus type 1: in vitro control of the virus infection by

enhanced natural killer activity via interleukin-15 induction. *J Virol*. 2000;74:7196-7203.

910. Tsunobuchi H, Nishimura H, Goshima F, et al. A protective role of interleukin-15 in a mouse model for systemic infection with herpes simplex virus. *Virol*. 2000;275:57-66.

911. Nguyen KB, Salazar-Mather TP, Dalod MY, et al. Coordinated and distinct roles for IFN-alphabeta, IL-12, and IL-15 regulation of NK cell responses to viral infection. *J Immunol*. 2002;169:4279-4287.

912. Geurs TL, Zhao YM, Hill EB, et al. Ly49H engagement compensates for the absence of type I interferon signaling in stimulating NK cell proliferation during murine cytomegalovirus infection. *J Immunol*. 2009;183:5830-5836.

913. Sun JC, Ma A, Lanier LL. Cutting edge: IL-15-independent NK cell response to mouse cytomegalovirus infection. *J Immunol*. 2009;183: 2911-2914.

914. Ladel CH, Blum C, Kaufmann SH. Control of natural killer cellmediated innate resistance against the intracellular pathogen Listeria monocytogenes by gamma/delta T lymphocytes. *Infect Immun*. 1996;64:1744-1749.

915. Smyth MJ, Wallace ME, Nutt SL, et al. Sequential activation of NKT cells and NK cells provides effective innate immunotherapy of cancer. *J Exp Med*. 2005;201:1973-1985.

916. Wesley JD, Robbins SH, Sidobre S, et al. Cutting edge: IFN-gamma signaling to macrophages is required for optimal Valpha14i NK T/NK cell cross-talk. *J Immunol*. 2005;174:3864-3868.

917. Moody DB, Reinhold BB, Guy MR, et al. Structural requirements for glycolipid antigen recognition by CD1b- restricted T cells. *Science*. 1997;278:283-286.

918. Moody DB, Ulrichs T, Muhlecker W, et al. CD1c-mediated T-cell recognition of isoprenoid glycolipids in Mycobacterium tuberculosis infection. *Nature*. 2000;404:884-888.

919. Sada-Ovalle I, Skold M, Tian T, et al. Alpha-galactosylceramide as a therapeutic agent for pulmonary Mycobacterium tuberculosis infection. *Am J Respir Crit Care Med*. 2010;182:841-847.

920. Lisnic VJ, Krmpotic A, Jonjic S. Modulation of natural killer cell activity by viruses. *Curr Opin Microbiol*. 2010;13:530-539.

921. Farrell HE, Vally H, Lynch DM, et al. Inhibition of natural killer cells by a cytomegalovirus MHC class I homologue in vivo. *Nature*. 1997;386:510-514.

922. Kloover JS, Grauls GE, Blok MJ, et al. A rat cytomegalovirus strain with a disruption of the r144 MHC class I-like gene is attenuated in the acute phase of infection in neonatal rats. *Arch Virol*. 2002;147:813-824.

923. Cretney E, Degli-Esposti MA, Densley EH, et al. m144, a murine cytomegalovirus (MCMV)-encoded major histocompatibility complex class I homologue, confers tumor resistance to natural killer cell-mediated rejection. *J Exp Med*. 1999;190:435-444.

924. Kubota A, Kubota S, Farrell HE, et al. Inhibition of NK cells by murine CMV-encoded class I MHC homologue m144. *Cell Immunol*. 1999;191:145-151.

925. Natarajan K, Hicks A, Mans J, et al. Crystal structure of the murine cytomegalovirus MHC-I homolog m144. *J Mol Biol*. 2006;358:157-171.

926. Mans J, Natarajan K, Balbo A, et al. Cellular expression and crystal structure of the murine cytomegalovirus major histocompatibility complex class I-like glycoprotein, m153. *J Biol Chem*. 2007;282:35247-35258.

927. Reyburn HT, Mandelboim O, Vales-Gomez M, et al. The class I MHC homologue of human cytomegalovirus inhibits attack by natural killer cells. *Nature*. 1997;386:514-517.

928. Wagner CS, Ljunggren HG, Achour A. Immune modulation by the human cytomegalovirus-encoded molecule UL18, a mystery yet to be solved. *J Immunol*. 2008;180:19-24.

929. Ulbrecht M, Martinozzi S, Grzeschik M, et al. Cutting edge: the human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis. *J Immunol*. 2000;164:5019-5022.

930. Cerboni C, Mousavi-Jazi M, Wakiguchi H, et al. Synergistic effect of IFN-gamma and human cytomegalovirus protein UL40 in the HLAE-dependent protection from NK cell-mediated cytotoxicity. *Eur J Immunol*. 2001;31:2926-2935.

931. Wang EC, McSharry B, Retiere C, et al. UL40-mediated NK evasion during productive infection with human cytomegalovirus. *Proc Natl Acad Sci U S A*. 2002;99:7570-7575.

932. Cohen GB, Gandhi RT, Davis DM, et al. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity*. 1999;10:661-671.

933. Bonaparte MI, Barker E. Killing of human immunodeficiency virusinfected primary T-cell blasts by autologous natural killer cells is dependent on the ability of the virus to alter the expression of major histocompatibility complex class I molecules. *Blood*. 2004;104:2087-2094.

934. Cerboni C, Neri F, Casartelli N, et al. Human immunodeficiency virus 1 Nef protein downmodulates the ligands of the activating receptor NKG2D and inhibits natural killer cell-mediated cytotoxicity. *J Gen Virol*. 2007;88:242-250.

935. Ishido S, Choi JK, Lee BS, et al. Inhibition of natural killer cell-mediated cytotoxicity by Kaposi's sarcoma-associated herpesvirus K5 protein. *Immunity*. 2000;13:365-374.

936. Scalzo AA, Fitzgerald NA, Simmons A, et al. Cmv-1, a genetic locus that controls murine cytomegalovirus replication in the spleen. *J Exp Med*. 1990;171:1469-1483.

937. Scalzo AA, Lyons PA, Fitzgerald NA, et al. Genetic mapping of Cmv1 in the region of mouse chromosome 6 encoding the NK gene complexassociated loci Ly49 and musNKR-P1. *Genomics*. 1995;27:435-441.

938. Scalzo AA, Fitzgerald NA, Wallace CR, et al. The effect of the Cmv-1 resistance gene, which is linked to the natural killer cell gene complex, is mediated by natural killer cells. *J Immunol*. 1992;149:581-589.

939. Brown MG, Dokun AO, Heusel JW, et al. Vital involvement of a natural killer cell activation receptor in resistance to viral infection. *Science*. 2001;292:934-937.

940. Lee SH, Girard S, Macina D, et al. Susceptibility to mouse cytomegalovirus is associated with deletion of an activating natural killer cell receptor of the C-type lectin superfamily. *Nat Genet*. 2001;28:42-425.

941. Daniels KA, Devora G, Lai WC, et al. Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49h. *J Exp Med*. 2001;194:29-44.

942. Lee SH, Zafer A, de Repentigny Y, et al. Transgenic expression of the activating natural killer receptor Ly49H confers resistance to cytomegalovirus in genetically susceptible mice. *J Exp Med*. 2003;197: 515-526.

943. Sjolin H, Tomasello E, Mousavi-Jazi M, et al. Pivotal role of KARAP/DAP12 adaptor molecule in the natural killer cell-mediated resistance to murine cytomegalovirus infection. *J Exp Med*. 2002;195:825-834.

944. French AR, Pingel JT, Wagner M, et al. Escape of mutant doublestranded DNA virus from innate immune control. *Immunity*. 2004;20:747-756.

945. Bubic I, Wagner M, Krmpotic A, et al. Gain of virulence caused by loss of a gene in murine cytomegalovirus. *J Virol*. 2004;78:7536-7544.

946. Virgin HWt. Pathogenesis of viral infection. In: Knipe DM, Howley PM, Griffin DE,

eds. *Fields Virology*. 5th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2007.

947. Holtappels R, Gillert-Marien D, Thomas D, et al. Cytomegalovirus encodes a positive regulator of antigen presentation. *J Virol*. 2006;80:7613-7624.

948. Voigt V, Forbes CA, Tonkin JN, et al. Murine cytomegalovirus m157 mutation and variation leads to immune evasion of natural killer cells. *Proc Natl Acad Sci U S A*. 2003;100:13483-13488.

949. Corbett AJ, Coudert JD, Forbes CA, et al. Functional consequences of natural sequence variation of murine cytomegalovirus m157 for Ly49 receptor specificity and NK cell activation. *J Immunol*. 2011;186: 1713-1722.

950. Desrosiers MP, Kielczewska A, Loredo-Osti JC, et al. Epistasis between mouse Klra and major histocompatibility complex class I loci is associated with a new mechanism of natural killer cell-mediated innate resistance to cytomegalovirus infection. *Nat Genet*. 2005;37:593-599.

951. Dighe A, Rodriguez M, Sabastian P, et al. Requisite H2k role in NK cell-mediated resistance in acute murine cytomegalovirus-infected MA/My mice. *J Immunol*. 2005;175:6820-6828.

952. Xie X, Dighe A, Clark P, et al. Deficient major histocompatibility complex-linked innate murine cytomegalovirus immunity in MA/My.L-H2b mice and viral downregulation of H-2k class I proteins. *J Virol*. 2007;81:229-236.

953. Pyzik M, Charbonneau B, Gendron-Pontbriand EM, et al. Distinct MHC class I-dependent NK cell-activating receptors control cytomegalovirus infection in different mouse strains. *J Exp Med*. 2011;208:1105-1117.

954. Delano ML, Brownstein DG. Innate resistance to lethal mousepox is genetically linked to the NK gene complex on chromosome 6 and correlates with early restriction of virus replication by cells with an NK phenotype. *J Virol*. 1995;69:5875-5877.

955. Pereira RA, Scalzo A, Simmons A. Cutting edge: a NK complex-linked locus governs acute versus latent herpes simplex virus infection of neurons. *J Immunol*. 2001;166:5869-5873.

956. Dorner BG, Smith HRC, French AR, et al. Coordinate expression of cytokines and chemokines by natural killer cells during murine cytomegalovirus infection. *J Immunol*. 2004;172:3119-3131.

957. Tripathy SK, Smith HRC, Holroyd EA, et al. Expression of m157, a murine cytomegalovirus-encoded putative major histocompatibility class I (MHC-I)-like protein, is independent of viral regulation of host MHC-I. *J Virol*. 2006;80:545-550.

958. Biron CA, Sonnenfeld G, Welsh RM. Interferon induces natural killer cell blastogenesis in vivo. *J Leukoc Biol*. 1984;35:31-37.

959. Orange JS, Biron CA. Characterization of early IL-12, IFN-alphabeta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection. *J Immunol*. 1996;156:4746-4756.

960. Tough DF, Borrow P, Sprent J. Induction of bystander T cell proliferation by viruses and type I interferon in vivo. *Science*. 1996;272:1947-1950.

961. Bjorkstrom NK, Lindgren T, Stoltz M, et al. Rapid expansion and long-term persistence of elevated NK cell numbers in humans infected with hantavirus. *J Exp Med*. 2011;208:13-21.

962. Guma M, Angulo A, Vilches C, et al. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood*. 2004;104: 3664-3671.

963. Monsivais-Urenda A, Noyola-Cherpitel D, Hernandez-Salinas A, et al. Influence of human cytomegalovirus infection on the NK cell receptor repertoire in children. *Eur J Immunol*. 2010;40:1418-1427.

964. Kuijpers TW, Baars PA, Dantin C, et al. Human NK cells can control CMV infection in the absence of T cells. *Blood*. 2008;112:914-915.

965. Petitdemange C, Becquart P, Wauquier N, et al. Unconventional repertoire profile is imprinted during acute chikungunya infection for natural killer cells polarization toward cytotoxicity. *PLoS Pathog*. 2011;7:e1002268.

966. Magri G, Muntasell A, Romo N, et al. NKp46 and DNAM-1 NKcell receptors drive the response to human cytomegalovirus-infected myeloid dendritic cells overcoming viral immune evasion strategies. *Blood*. 2011;117:848-856.

967. O'Leary JGO, Goodarzi M, Drayton DL, et al. T and B cell-independent adaptive immunity mediated by natural killer cells. *Nat Immunol*. 2006;7:507-516.

968. Yokoyama WM. Contact hypersensitivity: not just T cells! *Nat Immunol*. 2006;7:437-439.

969. Paust S, Gill HS, Wang BZ, et al. Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. *Nat Immunol*. 2010;11:1127-1135.

970. Sun JC, Beilke JN, Lanier LL. Adaptive immune features of natural killer cells. *Nature*. 2009;457:557-561.

971. Cooper MA, Elliott JM, Keyel PA, et al. Cytokine-induced memorylike natural killer cells. *Proc Natl Acad Sci U S A* . 2009;106:1915-1919.

972. Rouzaire P, Luci C, Blasco E, et al. Natural killer cells and T cells induce different types of skin reactions during recall responses to haptens. *Eur J Immunol* . 2011;42(1):80-88.

973. Fernandez NC, Lozier A, Flament C, et al. Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. *Nat Med*. 1999;5:405-411.

974. Krug A, French AR, Barchet W, et al. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity*. 2004;21:107-119.

975. Zanoni I, Foti M, Ricciardi-Castagnoli P, et al. TLR-dependent activation stimuli associated with Th1 responses confer NK cell stimulatory capacity to mouse dendritic cells. *J Immunol* . 2005;175:286-292.

976. Nishioka Y, Nishimura N, Suzuki Y, et al. Human monocyte-derived and CD83(+) blood dendritic cells enhance NK cell-mediated cytotoxicity. *Eur J Immunol* . 2001;31:2633-2641.

977. Ferlazzo G, Pack M, Thomas D, et al. Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. *Proc Natl Acad Sci U S A*. 2004;101: 16606-16611.

978. Foti M, Granucci F, Pelizzola M, et al. Dendritic cells in pathogen recognition and induction of immune responses: a functional genomics approach. *J Leukoc Biol* . 2006;79:913-916.

979. Stober D, Schirmbeck R, Reimann J. IL-12/IL-18-dependent IFNgamma release by murine dendritic cells. *J Immunol* . 2001;167: 957-965.

980. Jinushi M, Takehara T, Kanto T, et al. Critical role of MHC class I-related chain A and B expression on IFN-alpha-stimulated dendritic cells in NK cell activation: impairment in chronic hepatitis C virus infection. *J Immunol* . 2003;170:1249-1256.

981. Adam C, King S, Allgeier T, et al. DC-NK cell cross talk as a novel CD4+ T-cell-independent pathway for antitumor CTL induction. *Blood*. 2005;106:338-344.

982. Ferlazzo G, Tsang ML, Moretta L, et al. Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. *J Exp Med*. 2002;195:343-351.

983. Terme M, Tomasello E, Maruyama K, et al. IL-4 confers NK stimulatory capacity to murine dendritic cells: a signaling pathway involving KARAP/DAP12-triggering receptor expressed on myeloid cell 2 molecules. *J Immunol* . 2004;172:5957-5966.

984. Castillo EF, Stonier SW, Frasca L, et al. Dendritic cells support the in vivo development and maintenance of NK cells via IL-15 trans-presentation. *J Immunol* . 2009;183:4948-4956.

985. Munz C, Steinman RM, Fujii S. Dendritic cell maturation by innate lymphocytes: coordinated stimulation of innate and adaptive immunity. *J Exp Med*. 2005;202:203-207.

986. Mocikat R, Braumuller H, Gumy A, et al. Natural killer cells activated by MHC class I(low) targets prime dendritic cells to induce protective CD8 T cell responses. *Immunity*. 2003;19:561-569.

987. Andrews DM, Scalzo AA, Yokoyama WM, et al. Functional interactions between dendritic cells and NK cells during viral infection. *Nat Immunol* . 2003;4:175-181.

988. Piccioli D, Sbrana S, Melandri E, et al. Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. *J Exp Med*. 2002;195:335-341.

989. Ferlazzo G, Morandi B, D'Agostino A, et al. The interaction between NK cells and dendritic cells in bacterial infections results in rapid induction of NK cell activation and in the lysis of uninfected dendritic cells. *Eur J Immunol* . 2003;33:306-313.

990. Mailliard RB, Son YI, Redlinger R, et al. Dendritic cells mediate NK cell help for Th1 and CTL responses: two-signal requirement for the induction of NK cell helper function. *J Immunol*. 2003;171: 2366-2373.

991. Ferlazzo G. Natural killer and dendritic cell liaison: recent insights and open questions. *Immunol Lett*. 2005;101:12-17.

992. Bajenoff M, Breart B, Huang AY, et al. Natural killer cell behavior in lymph nodes revealed by static and real-time imaging. *J Exp Med*. 2006;203:619-631.

993. Croy BA. Granulated metrial gland cells: hypotheses concerning possible functions during murine gestation. *J Reprod Immunol* . 1994;27:85-94.

994. Moffett A, Loke C. Immunology of placentation in eutherian mammals. *Nat Rev Immunol*. 2006;6:584-594.

995. Manaster I, Mandelboim O. The unique properties of uterine NK cells. *Am J Reprod Immunol*. 2010;63:434-444.

996. Guimond MJ, Luross JA, Wang BP, et al. Absence of natural killer cells during murine pregnancy is associated with reproductive compromise in TGE26 mice. *Biol Reprod*. 1997;56:169-179.

997. Guimond MJ, Wang B, Croy BA. Engraftment of bone marrow from severe combined immunodeficient (SCID) mice reverses the reproductive deficits in natural killer cell-deficient tg epsilon 26 mice. *J Exp Med*. 1998;187:217-223.

998. Erlebacher A, Lukens AK, Glimcher LH. Intrinsic susceptibility of mouse trophoblasts to natural killer cell-mediated attack in vivo. *Proc Natl Acad Sci U S A* . 2002;99:16940-16945.

999. Carayannopoulos LN, Barks JL, Yokoyama WM, et al. Murine trophoblast cells induce NK cell interferon-gamma production through KLRK1. *Biol Reprod*. 2010;83:404-414.

1000. Madeja Z, Yadi H, Apps R, et al. Paternal MHC expression on mouse trophoblast affects uterine vascularization and fetal growth. *Proc Natl Acad Sci U S A* . 2011;108:4012-4017.

1001. Chakraborty D, Rumi MA, Konno T, et al. Natural killer cells direct hemochorial placentation by regulating hypoxia-inducible factor dependent trophoblast lineage decisions. *Proc Natl Acad Sci U S A* . 2011;108:16295-16300.

1002. Hiby SE, Walker JJ, O'Shaughnessy K M, et al. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J Exp Med*. 2004;200:957-965.

1003. Hiby SE, Apps R, Sharkey AM, et al. Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. *J Clin Invest*. 2010;120:4102-4110.

1004. Schmidt CM, Orr HT. Maternal/fetal interactions: the role of the MHC class I molecule HLA-G. *Crit Rev Immunol* . 1993;13:207-224.

1005. Rouas-Freiss N, Goncalves RM, Menier C, et al. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. *Proc Natl Acad Sci U S A*. 1997;94:11520-11525.

1006. Parham P, Guethlein LA. Pregnancy immunogenetics: NK cell education in the womb? *J Clin Invest*. 2010;120:3801-3804.

1007. Ashkar AA, Di Santo JP, Croy BA. Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. *J Exp Med*. 2000;192:259-270.

1008. Lash GE, Schiessl B, Kirkley M, et al. Expression of angiogenic growth factors by uterine natural killer cells during early pregnancy. *J Leukoc Biol*. 2006;80:572-580.

1009. Carrington M, Wang S, Martin MP, et al. Hierarchy of resistance to cervical neoplasia mediated by combinations of killer immunoglobulin-like receptor and human leukocyte antigen loci. *J Exp Med*. 2005;201: 1069-1075.

1010. Martin MP, Gao X, Lee JH, et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet*. 2002;31: 429-434.

1011. Martin MP, Qi Y, Gao X, et al. Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. *Nat Genet*. 2007;39:733-740.

1012. Boulet S, Sharafi S, Simic N, et al. Increased proportion of KIR3DS1 homozygotes in HIV-exposed uninfected individuals. *AIDS*. 2008;22: 595-599.

1013. Bashirova AA, Thomas R, Carrington M. HLA/KIR restraint of HIV: surviving the fittest. *Annu Rev Immunol*. 2011;29:295-317.

1014. Alter G, Heckerman D, Schneidewind A, et al. HIV-1 adaptation to NK-cell-mediated immune pressure. *Nature*. 2011;476:96-100.

1015. Davies SM, Ruggieri L, DeFor T, et al. Evaluation of KIR ligand incompatibility in mismatched unrelated donor hematopoietic transplants. Killer immunoglobulin-like receptor. *Blood*. 2002;100:3825-3827.

1016. Parham P, McQueen KL. Alloreactive killer cells: hindrance and help for haematopoietic transplants. *Nat Rev Immunol*. 2003;3:108-122.

1017. Cooley S, Weisdorf DJ, Guethlein LA, et al. Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. *Blood*. 2010;116:2411-2419.

1018. Venstrom JM, Zheng J, Noor N, et al. KIR and HLA genotypes are associated with disease progression and survival following autologous hematopoietic stem cell transplantation for high-risk neuroblastoma. *Clin Cancer Res*. 2009;15:7330-7334.

1019. Grom AA, Villanueva J, Lee S, et al. Natural killer cell dysfunction in patients with systemic-onset juvenile rheumatoid arthritis and macrophage activation syndrome. *J Pediatr*. 2003;142:292-296.

1020. Rosenberg SA, Lotze MT, Muul LM, et al. Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *N Engl J Med*. 1985;313:1485-1492.

1021. Bachanova V, Burns LJ, McKenna DH, et al. Allogeneic natural killer cells for refractory lymphoma. *Cancer Immunol Immunother* . 2010;59:1739-1744.

1022. Lundqvist A, Berg M, Smith A, et al. Bortezomib treatment to potentiate the anti-tumor immunity of ex-vivo expanded adoptively infused autologous natural killer cells. *J Cancer*. 2011;2:383-385.

1023. Waldmann TA, Lugli E, Roederer M, et al. Safety (toxicity), pharmacokinetics, immunogenicity, and impact on elements of the normal immune system of recombinant human IL-15 in rhesus macaques. *Blood*. 2011;117:4787-4795.

1024. Nava VE, Jaffe ES. The pathology of NK-cell lymphomas and leukemias. *Adv Anat Pathol*. 2005;12:27-34.

1025. Dearden CE, Johnson R, Pettengell R, et al. Guidelines for the management of mature T-cell and NK-cell neoplasms (excluding cutaneous T-cell lymphoma). *Br J Haematol*. 2011;153:451-485.

1026. Chim CS, Ma SY, Au WY, et al. Primary nasal natural killer cell lymphoma: long-term treatment outcome and relationship with the International Prognostic Index. *Blood*. 2004;103:216-221.

1027. Zhang Y, Nagata H, Ikeuchi T, et al. Common cytological and cytogenetic features of Epstein-Barr virus (EBV)-positive natural killer (NK) cells and cell lines derived from patients with nasal T/NK-cell lymphomas, chronic active EBV infection and hydroa vacciniforme-like eruptions. *Br J Haematol* . 2003;121:805-814.

1028. Yokoyama WM. Mistaken notions about natural killer cells. *Nat Immunol* . 2008;9:481-485.

1029. Parham P. MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol* . 2005;5:201-214.

1030. Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera— a visualization system for exploratory research and analysis. *J Comput Chem*. 2004;25:1605-1612.

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Chapter 18

CD1d-Restricted Natural Killer T Cells

Albert Bendelac

INTRODUCTION AND DEFINITION

Natural killer T (NKT) cells are cluster of differentiation (CD)1d-restricted T cells that use semi-invariant αβ T-cell receptors (TCRs) and reside as long-lived effector cells in lymphoid tissues and in the microvasculature of organs such as the lung and the liver. They promptly release a broad range of cytokines and chemokines in response to microbial and self-lipid antigens presented by CD1d glycoproteins, exerting a protective or pathogenic role in infection, inflammation, allergy, and cancer. The NKT lineage is best defined by expression of *Zbtb16*, which encodes promyelocytic leukemia zinc factor (PLZF), a master transcription factor that directs acquisition of innate-like effector properties during thymic development.

The NKT lineage is a member of the larger family of so-called innate-like lymphocytes, which includes B1 B cells, marginal zone B cells, intraepithelial γδ T-cell sublineages, the CD8αα TCRαβ population of intestinal lymphocytes, and the MR1-restricted mucosal associated invariant T cells.[1] Like NKT cells, these lineages express semi-invariant TCRs or B-cell receptors encoding specificity for conserved microbial and self-ligands. Their “canonical” antigen receptors are sufficient to instruct lineage differentiation during development in the thymus or bone marrow, in a process matching antigen specificity with specialized effector functions and homing to dedicated tissue environments. These stereotypical properties, which are reminiscent of truly innate lymphoid cells, such as natural killer (NK) cells, represent a distinct host defense strategy, perhaps corresponding to an early phase of evolution of adaptive T- and B-cell immunity.

CANONICAL NATURAL KILLER T T-CELL RECEPTORS

In mice, the vast majority of CD1d-restricted NKT cells express the semi-invariant Vα14-Jα18 TCRα chain paired with β chains made of Vβ8, Vβ7, or Vβ2 joined to variable DβJβ segments, whereas the homologous Vα24-Jα18 chain associated with Vβ11 is used in humans[2] (Table 18.1). The “canonical” sequence of the TCR alpha chain is entirely encoded in the genome, although alterations due to nucleotide trimming and N additions can be tolerated if they preserve the antigenic specificity of the TCR. These canonical rearrangements arise randomly and at very low frequency in both fetal and adult life, but massive thymic expansion post-TCR expression ensures a high frequency of NKT cells among recent thymic emigrants.[3]

Other semi-invariant TCRs have been identified among mouse CD1d-restricted NKT cells, including Vα10-Jα50/Vβ8,[4] Vα3.2-Jα9/Vβ8, and Vα8/Vβ8,[5] but their combined frequency is modest compared to the dominant Vα14-Jα18 TCR. Nevertheless, these cells appear to adopt a similar NKT effector phenotype, likely because they follow the same thymic developmental pathway.

ANTIGENIC LIGANDS OF NATURAL KILLER T CELLS

Cluster of Differentiation 1d

CD1d is one of five members of the mammalian family of lipid-presenting, β2-microglobulin-associated, major histocompatibility complex (MHC)-like CD1 molecules, and the only one conserved in mouse.[6,7] The mouse CD1 locus contains *cd1d1* encoding a functional surface glycoprotein and a duplicated gene *cd1d2*, which is generally poorly expressed and, in the C57 background, is inactivated by a frameshift mutation preventing surface expression.[8] CD1d is found constitutively on the cell surface of most antigen-presenting cells, including dendritic cells (DCs), macrophages, and B cells, with particularly high levels on marginal zone B cells.[9] Of relevance to NKT-cell development, CD1d is also prominently but transiently displayed on cortical thymocytes. It is also expressed by endothelial cells and hepatocytes.

CD1d is assembled in the endoplasmic reticulum by association with β2-microglobulin before reaching the cell surface and undergoing extensive rounds of internalization and recycling between the late endosome/lysosome and the plasma membrane[10,11,12] (Fig. 18.1). The rapid rate of internalization depends upon a tyrosine motif encoded in its cytoplasmic tail, which binds adaptor protein (AP)-2 and AP-3 in mouse, and AP-2 in human. This intense recycling accounts for the steady state accumulation of CD1d in the late endosome/lysosome for mouse or late endosome for human. While a diversity of exogenous and endogenous lipids can load CD1d in various compartments,[13,14,15] the late endosome/lysosome environment is optimized for lipid antigen acquisition due to the presence of efficient glycolipid processing enzymes and lipid transfer proteins such as saposins,[16,17,18] Gm2 activator,[16] Niemann-Pick type C2 protein,[19] and, in humans, CD1e.[20] In this acidic compartment, short or polyunsaturated lipids loaded at the cell surface are quickly removed and replaced by long and saturated lipids that bind more stably to CD1d but require lysosomal transfer proteins for loading.[21] Unlike MHC class II, CD1d expression and its presentation of lipid antigens at the cell surface are

independent of toll-like receptor (TLR) signaling and DC maturation.[22] Thus, immature DCs can readily present purified lipid antigens to NKT cells and become activated upon CD40 engagement by NKT cells.

TABLE 18.1 Semi-invariant T-Cell Receptors of Cluster of Differentiation 1d-Restricted Natural Killer T Cells

Human										
	TRAV10	GTG	GTG	AGC	G					
	TRAJ18			C	GAC	AGA	GGC	TCA	ACC	
Canonical hVα24		GTG	GTG	AGC	GAC	AGA	GGC	TCA	ACC	Variable hVβ11
		V	V	S	D	R	G	S	T	
Mouse										
	TRAV11	GTG	GTG	GGC	G					

	TRAJ18			TA	GAT	AGA	GGT	TCA	GCC	
Canonical mVα14		GTG	GTG	GGC	GAT	AGA	GGT	TCA	GCC	Variable mVβ8, mVβ7, mVβ2,
		V	V	G	D	R	G	S	A	

Lipid Ligands of Natural Killer T cells

bial α-glycosylated Lipid Ligands

The first universal NKT ligand emerged from studies of marine sponge extracts that prolonged survival of mice bearing B16 melanoma.[23] The active principle of the sponge *Agelas mauritianus* was an alpha-branched galactosylceramide, which after slight modification led to the synthesis of an extremely potent variant named KRN7000, commonly referred to as αGalCer[24,25] (Fig. 18.2). Over 95% of mouse and human NKT cells recognize αGalCer, irrespective of their variable CDR3 β sequence.[26,27] Furthermore, the mouse CD1d-αGalCer tetramers stain the NKT cells of both human and nonhuman primates,[28,29] attesting to the high degree of conservation of this recognition system.

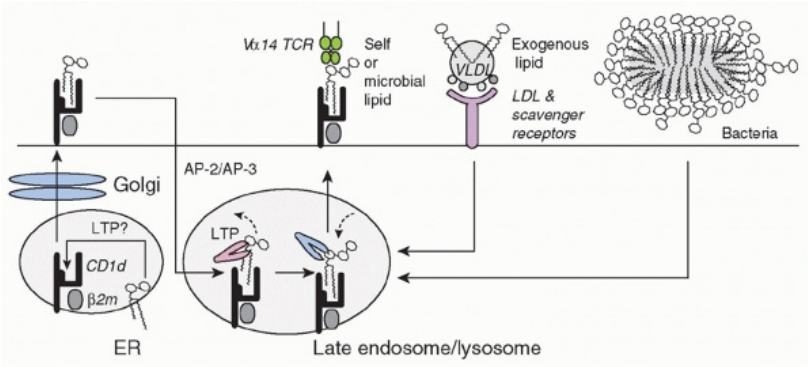

FIG. 18.1. Presentation of Lipid Antigens by Cluster of Differentiation (CD)1d. CD1d acquires lipid antigens after biosynthesis in the endoplastic reticulum and Golgi prior to reaching the cell surface. Multiple rounds of AP-2/AP-3 clathrinmediated internalization to the late endosome/lysosome and recycling to the plasma membrane allow lipid exchange. Lysosomal degradation pathways provide lipid antigens for exchange in a process assisted by lysosomal lipid transfer proteins such as saposins, Gm2 activator, NPC-2, and CD1e. Exogenous lipids bound to lipoproteins enter the cell through scavenger receptors and low-density lipoprotein receptor. Microbial organisms access the lysosome after phagocytosis.

The marine sponge lipid turned out to be closely related to microbial lipids found in some gram-negative lipopolysaccharide (LPS)-negative bacteria. Notably, *Sphingomonas*, a member of the class of α-proteobacteria and a ubiquitous bacterium found in terrestrial and marine environments (including as a bacterial symbiont of sponges), uses alphabranched glycuronylceramides as a substitute for LPS in the outer membrane of its cell wall.[30] It can

infect dendritic cells and macrophages to activate NKT cells.[31,32] Other structurally related α-glycosylated lipids, such as α-galactosyldiacylglycerol, found in *Borrelia burgdorferi*, the agent of Lyme disease,[33] and α-glucosyldiacylglycerol, found in *Streptococcus pneumoniae*,[34] also activate mouse and human NKT cells. Such α-branched glycolipids have not been reported in vertebrates, suggesting that they represent a pathogen signature that the canonical NKT TCR may have evolved to recognize.

Endogenous β-glycosylated Lipid Ligands

Vα14 NKT cells are spontaneously autoreactive to CD1dexpressing cells.[35] For example, NKT hybridomas or clones

can be activated to secrete modest amounts of cytokines when cultured in the presence of fresh thymocytes, DCs, and various tumor cell lines.[8,35,36,37,38] This autoreactivity and the agonist ligands involved appear to be central not only for NKT-cell thymic development,[36] but also for their response to various microbial infections, especially when the pathogens themselves do not express NKT ligands.[31,39] The identification of self-ligands is therefore of major relevance to key aspects of the biology of NKT cells.

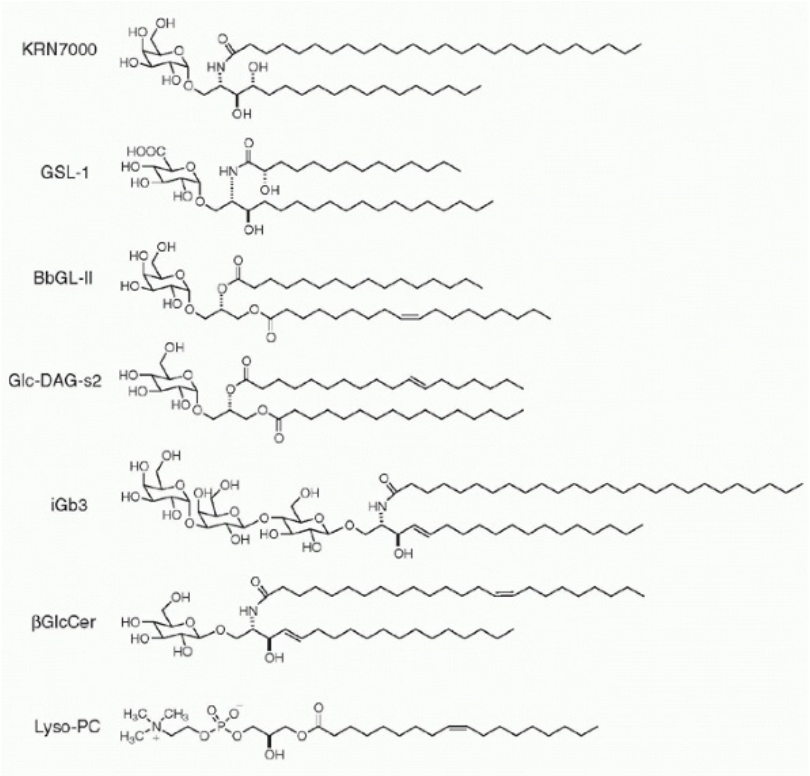

FIG. 18.2. Self- and Foreign-Lipid Antigens Recognized by Natural Killer T Cells. From top to bottom, KRN7000, or reference α-GalCer, synthetic version of the original marine sponge antigen; *Sphingomonas* GSL-1; *Borrelia burgdorferi* BbGL-II, α-galactosyldiacylglycerol; *Streptococcus pneumoniae*, α-glucosyldiacylglycerol; isoglobotrihexosylceramide iGb3; β-glucosylceramide; and lyso-phophatidylcholine.

In the mouse system, recognition of self-ligands by Vα14 NKT cells is largely dependent upon CD1d endosomal trafficking,[40,41] suggesting the recognition of endosomal/lysosomal lipid antigens. This may not be the case for their human counterpart, Vα24 NKT cells.[42] The

search for a lysosomally-loaded self-ligand recognized by NKT cells led to the identification of a trihexosylceramide, iGb3, which is synthesized in the Golgi as an intermediate in the biosynthetic pathway of iGb4 and is also generated in the lysosome after degradation of iGb4 by β-hexosaminidase AB.[43] iGb3 is further degraded into LacCer by α-galactosidase A (Fig. 18.3). Mice lacking β-hexosaminidase B lacked 95% of their NKT cells, a defect associated with the impaired ability of their thymocytes to stimulate autoreactive NKT hybridomas. Conversely, unlike αGalCer, iGb3 is recognized with a Vβ7 > Vβ8 > Vβ2 hierarchy of affinities that mirrors the relative selection of NKT thymic precursors.[44] Furthermore, DCs lacking α-galactosidase A exhibited greater spontaneous stimulation of NKT cells,[45] consistent with the demonstrated accumulation of iGb3.[46,47,48]

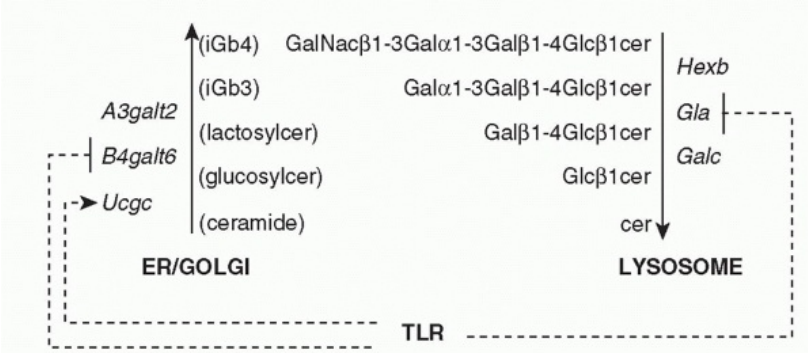

FIG. 18.3. Toll-Like Receptor (TLR)-Mediated Regulation of the Synthesis and Degradation of Natural Killer T Cell Glycosphingolipid Antigens. TLR signaling downregulates *B4galt6* encoding lactosylceramide synthase and *Gla* encoding α-galactosidase A, but upregulates Ucgc encoding β-glucosylceramide synthase. These changes result in the net accumulation of β-glucosylceramide and iGb3.

Among several genes encoding Galα1-3Gal transferases, *a3galt2* is considered more specialized for iGb3 synthesis. As *a3galt2* null mutant mice did not show NKT cell defects[49] and the human homologous gene appeared dysfunctional,[50] iGb3 may not be the sole endogenous glycosylceramide recognized by mouse and human NKT cells. Other reports have suggested that β-galactosylsylceramide[51] and β-glucosylceramide[52] might also function as natural ligands of NKT cells, but these claims have not yet been widely replicated. While the respective importance of all these candidate ligands remains to be further studied in physiological and pathological conditions, it is possible that NKT cells might recognize multiple endogenous β-glycosylceramides as weak agonists.

Importantly, several enzymes involved in the synthesis and degradation of β-glycosylceramides appear to be coordinately regulated in conditions associated with NKT-cell activation[45,52,53] (see Fig. 18.3). For example, TLR signaling was shown to specifically downregulate α-galactosidase A in the degradation pathway,[45] leading to increased iGb3.[46] In the biosynthetic pathway, TLR signaling upregulated β-glucosylceramide synthase while downregulating lactosylceramide synthase, thus increasing β-glucosylceramide.[52] Collectively, these findings suggest that microbial organisms lacking NKT ligands can nevertheless activate NKT cells through TLR-mediated accumulation of endogenous glycosphingolipid ligands.

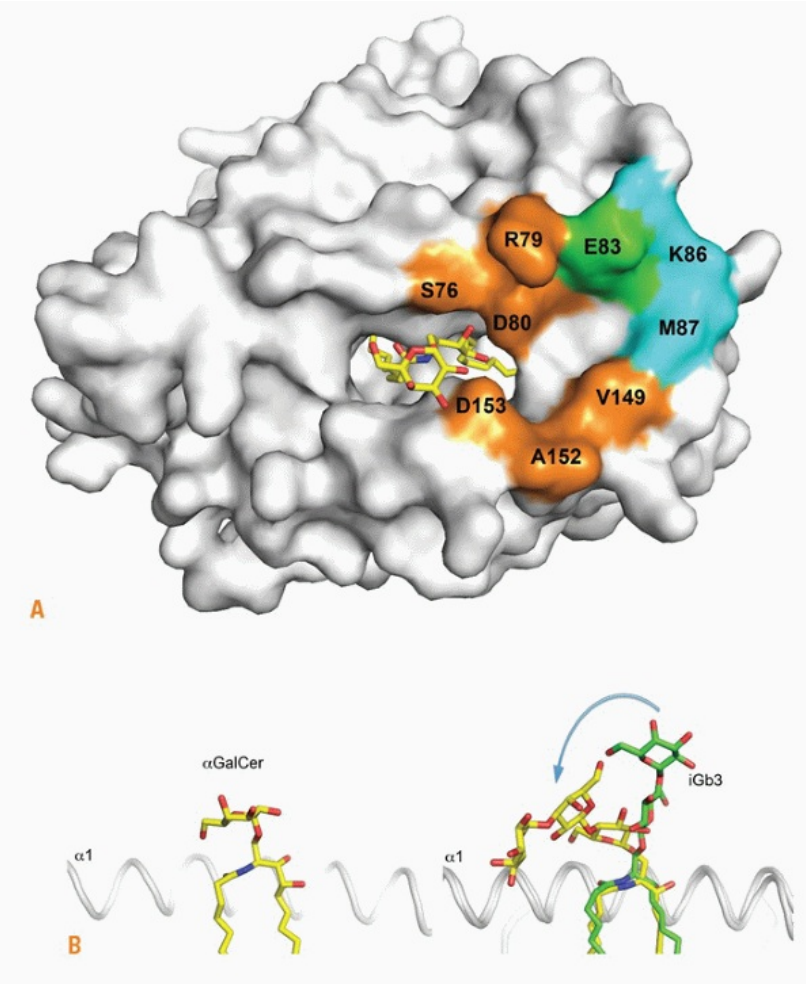

FIG. 18.4. Natural Killer T (NKT) T-Cell Receptor (TCR) Recognition of αGalCer and iGb3. A: Conserved footprint of the mouse NKT TCR on the surface of cluster of differentiation (CD)1d-αGalCer and CD1d-iGb3 (the lipid shown is αGalCer): *orange*, CDR3α; *cyan*, CDR2β; *green*, CDR3α and CDR2β. **B:** Binding mode of αGalCer and iGb3 to CD1d and TCR. *Left*, αGalCer (same structure before and after TCR binding); *right*, iGb3 before (*green sticks*) and after (*yellow sticks*) TCR binding. Note that the distal sugar is not represented before TCR binding. Courtesy of Dirk Zajonc (La Jolla Institute of Allergy and Immunology, San Diego, CA).

Lysophosphatidylcholine was also reported to stimulate a fraction of human but not mouse NKT cells,[54,55] suggesting that upregulation of this ligand in multiple myeloma and other inflammatory conditions might also contribute to NKT-cell activation.

Structural Basis of Lipid Recognition by Natural Killer T cells

The lipid-binding pocket of CD1d is particularly well adapted to bind self- and microbial glycosphingolipids, with the acyl chain in the A' hydrophobic pocket and the sphingosine chain in the F' hydrophobic channel.[56,57,58] The hydroxyl groups of the sphingosine emerge from the groove to establish hydrogen bonds with the α1 helix Arg79 and Asp80, while the galactose is stabilized though hydrogen bonds between its 2- and 3-hydroxyl groups and Asp153 of the α2 helix. Thus, the protruding sugar is solidly anchored in a position parallel to the plane of the α-helices, explaining the exquisite stimulatory properties of several carbohydrate hydroxyl groups. In contrast, the trisaccharide chain of iGb3 protrudes in orthogonal orientation to the plane of the α-helices, with the proximal glucose forming

hydrogen bonds with the α2 helix, in particular Asp153 and Thr156, whereas the position of the ceramide backbone is similar to αGalCer (Fig. 18.4).

How could such highly dissimilar structures be recognized by the same TCR? In the ternary structure of the human TCR-CD1d-αGalCer complex, the TCR docked parallel to and at the extreme end (F' pocket) of the CD1d binding groove, enabling a lock-and-key type of interaction with the solidly anchored, rigid αGalCer antigen. The docking was parallel to the long axis of the binding groove, contrasting with the diagonal footprints observed for MHC-restricted TCRs.[59] The CDR3α loop encoded by the conserved germline Jα18 segment provided a majority of contacts, straddling the antigen binding cleft and engaging electrostatic interactions with α-GalCer, the α1 and the α2 helices of CD1d. CDR1α interacted solely with αGalCer, and CDR2β formed a stretch of interactions with the α1 helix. Consistent with the great diversity of CDR3β usage, this loop contact was limited to a single van der Waals contact with Gln150 of the α2 helix.

The ternary structure of the TCR-CD1d-iGb3 complex was recently elucidated. Unsurprisingly, given the conservation of Jα18 and CD1d in both structures, the overall footprint of the TCR on the CD1d-glycolipid surface was unchanged compared with CD1d-αGalCer. Strikingly, however, the TCR appeared to simply “squash” the trisaccharide headgroup of iGb3 over the α2 helix of CD1d, with the proximal β-linked glucose molded into an orientation similar to the α-linked galactose of αGal-Cer (see Fig. 18.4). Each of the three sugars made stabilizing polar and van der Waals interactions with CD1d residues. The proximal and second sugars also contacted the TCR CDR2α loop, which is not involved in CD1d-αGalCer recognition. The importance of the distal sugar is reflected by the lack of detectable stimulation by lactosylceramide where the third sugar is absent. These conformational changes imply a highly dynamic interaction process during the association phase. The energy penalty incurred for binding self-ligands could be overcome in specific conditions that strengthen the immune synapse. For example, this may occur during TLR-induced inflammation, which upregulates lymphocyte function-associated antigen (LFA-1) and intercellular adhesion molecule (ICAM-1) integrin interactions, or during thymic development, which involves homophilic engagement of signaling lymphocytic activation molecule (SLAM) family receptors.

Additional crystallographic studies further illustrated the difference between self- and foreign-antigen recognition. The α-glycosyldiacylglycerol antigens of *Borrelia burgdorferi*[33,60,61] and *Streptococcus pneumoniae*[34] adopted configurations relatively similar to αGalCer, although some induced fit was observed for both CD1d and the glycolipid upon TCR binding. In the case of *Streptococcus pneumoniae*, an unusual sn2 alkyl chain, vaccenic acid (with an unsaturation at C7), was important for favorable positioning of the glucose. After insertion of a stretch of hydrophobic aminoacids in the CDR3β loop in order to enhance binding to CD1d, other self-lipids such as β-GalCer, Galα1-4GlcCer (lactosylceramide), and phosphatidylinositol could be recognized.[62] Although somewhat contrived, this system revealed a similar bending of the β-linkage between the proximal sugar and the ceramide backbone as seen for iGb3, raising the possibility that several β-linked self glycolipids might serve as autoantigens.

DEVELOPMENT AND HOMEOSTASIS OF NATURAL KILLER T CELLS

Development

The major determinant of NKT cell development is the semi-invariant TCR, which, upon binding to CD1d ligands expressed by cortical thymocytes, provides the signals required for induction of the lineage-specific transcription factor PLZF. Therefore, insights into the peculiar nature and context of TCR engagement and its downstream signaling have

considerably advanced our understanding of the molecular mechanisms of NKT-cell development.

T-Cell Receptor Expression and Positive Selection

The use of CD1d-αGalCer tetramers specific for the canonical mVα14-Jα18/hVα24-Jα18 TCRs has revealed a sequence of selection, expansion, and differentiation events preceding the terminally differentiated NK1.1+ stage.[3,63,64] As illustrated in Figure 18.5, NKT cells originate from mainstream thymocyte precursors that transit through the pre-TCRα+TCR+ stage to reach the CD4+CD8+ double positive (DP) stage, where stochastic Vα-Jα rearrangements lead to expression of canonical Vα14-Jα18/Vβ8, Vβ7 or Vβ2 TCRs. These rearrangements involve distal gene segments that require prolonged cell survival mediated by Bcl-xL, whose expression depends on HEB-induced RORγt.[65,66,67] All Vβ families can pair with the Vα14-Jα18 chain, but only the biased set of Vβs confers specificity for the endogenous ligands. Although Vβ8 predominates among mature NKT cells, the most enriched Vβ family relative to preselection frequency is Vβ7, followed by Vβ8 and Vβ2.[44] This is consistent with the observation that endogenous ligands displayed on the surface of thymocytes activate the Vα14-Jα18 TCRs with a Vβ7 > Vβ8 > Vβ2 hierarchy of affinity, and that in mice expressing very low levels of CD1d, NKT cells showed increased representation of Vβ7.[44,68,69,70,71] Notably, this hierarchy of affinity parallels that of iGb3 but is different from αGalCer, which favors Vβ8. Thus, the naturally selected Vβ repertoire seems to precisely reflect the affinities of the preselected repertoire for endogenous ligands, suggesting that negative selection plays little role in shaping the Vβ repertoire. However, this does not imply that NKT cells are impervious to negative selection because transgenic overexpression of CD1d decreased the relative frequency of the high affinity Vβ7+ cells and exposure to αGalCer-induced deletion of NKT thymocytes.[69]

Developmental Stages

The earliest signaled NKT precursors are rare CD4+CD8+ DP cells that express the semi-invariant TCR. These cells become CD4+ single positive (SP) cells expressing high levels of CD24, a marker of immature cortical thymocytes, as well as CD69, a marker of positive selection.[64] This is the socalled stage 0 of NKT development. As $CD24^{high}$CD4+ cells mature into the next $CD24^{low}$CD4+ stage 1, they undergo several rounds of cell division.[3] Cell cycle may be initiated as early as stage 0, as suggested from the analysis of cyclin D2-or c-Myc-deficient mice.[72,73] The lineage expansion following positive selection ensures the high frequency that is critical for innate immunity. During this phase, a fraction of CD4+

cells downregulates CD4 to become CD4-CD8- double negative (DN) T cells,[64] acquiring a more restricted Th1 cytokine and chemokine profile and an enhanced ability to reject tumors.[74,75,76] Dividing cells first activate their interleukin (IL)-4 locus (stage 1) then interferon (IFN)-γ (independently of both stat6 and stat4) as they upregulate CD44 and downregulate CD62L (stage 2).[3,63,77] Thus, unlike other αβT cells, postselection NKT lineage cells undergo a sequence of events that is reminiscent of the antigen-driven activation, expansion, and effector differentiation of mature T cells.[78]

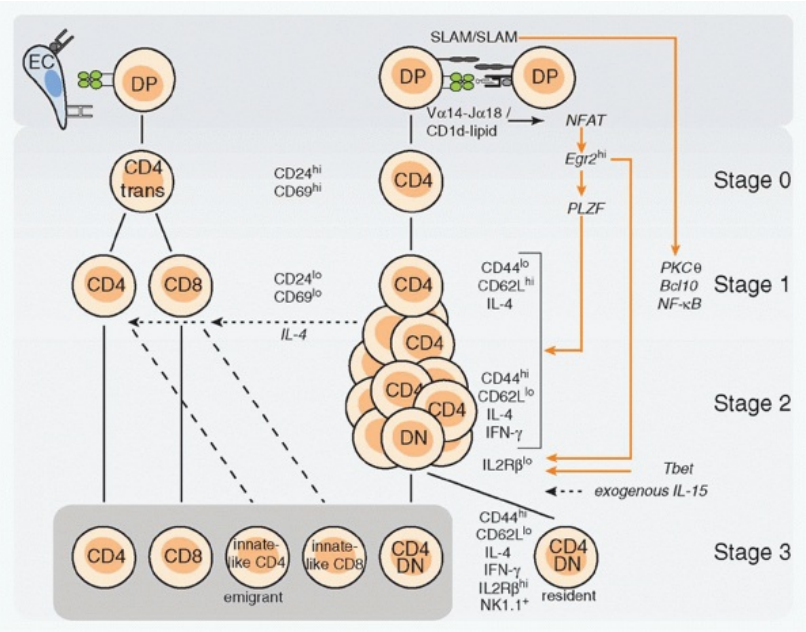

FIG. 18.5. Development of Natural Killer T (NKT) Cells. Thymic stages of NKT-cell development are aligned with corresponding stages of cluster of differentiation (CD)4 and CD8 T-cell development. Stage 0 NKT cells correspond to the transitional CD4 cells. Note the cross-talk between NKT cells and CD4 and CD8 T cells, which is mediated by interleukin-4, and results in the differentiation of “innate-like” CD4 and CD8 T cells in Balb/C mice. DP, CD4+CD8+ double positive; DN, CD4-CD8- double negative; EC, epithelial cell.

NKT lineage cells emigrate from the thymus after upregulation of the S1P1 receptor,[79] as dividing effector-type cells. They represent up to 5% of recent thymic emigrants in the mouse spleen.[3,80] Within a couple of days after emigration, they express NK lineage receptors, including the activating NK1.1 and NKG2D receptors and the inhibitory CD94/NKG2A, Ly49A, C/I, and G2 receptors. Acquisition of this terminal differentiation program is associated with cessation of division and with upregulation of IL2Rβ, which is necessary for IL-15 signaling. Intriguingly, a fraction of NKT thymocytes downregulate the S1P1R and remain as permanent residents in the thymic medulla, where they undergo the same terminal maturation program.[79] These thymic residents appear to be absent in humans.[81]

Recent thymic NKT emigrants and their immediate precursors, stage 2 NKT thymocytes, express neuropilin 1 (Nrp-1), a transmembrane receptor for vascular endothelium growth factor and semaphorin family members.[82] Nrp-1 represents a convenient marker of recent thymic NKT emigrants, as it remains expressed for a few days and is downregulated after terminal maturation to stage 3.

Homotypic Thymocyte-Thymocyte Interactions

The thymic cell types involved in presenting NKT ligands have been thoroughly investigated. Unlike MHC class I or class II, CD1d is prominently expressed on cortical thymocytes, which is consistent with the ability of cortical thymocytes to stimulate NKT hybridomas.[36] Bone marrow chimera experiments demonstrated that NKT-cell development required CD1d expression by cortical thymocytes but not radioresistant stromal cells.[83,84,85] This is radically different from conventional T-cell development that is driven by MHC expression on thymic epithelial cells. Transgenic experiments using promoters for Lck, MHC class I, or MHC

class II to redirect CD1d to various cell compartments in CD1d-deficient hosts suggested that expression on cortical thymocytes was necessary and sufficient for lineage development.[70,86,87,88] This conclusion was recently confirmed after

conditional deletion of CD1d using Cd4-Cre.[89] Mixed chimera experiments using CD1d knockout pLck-CD1d transgenic TCR Cα knockout bone marrow (where thymocyte development is arrested at the DP stage) as the sole source of CD1d expression demonstrated that expression of CD1d solely on cortical thymocytes was sufficient for the full differentiation of NKT cells.[87] Intriguingly, however, the transition to the NK1.1 positive stage was partially impaired after emigration to peripheral tissues, although it was preserved for the cells remaining as residents in the thymus. Terminal differentiation was fully restored if CD1d was re-expressed on MHC class II expressing cells or if the cells were transferred into wild-type recipients.[80,87]

Homophilic Signaling Lymphocytic Activation Molecule-f Family Interactions

A major pathway that is selectively recruited by the thymocyte-thymocyte interactions involves homophilic binding of SLAM family receptors, mainly Slamf1 (SLAM) and Slamf6 (Ly108), which are expressed by cortical thymocytes, but not by thymic epithelial cells.[90] These partially redundant receptors signal through the adaptor SLAM-associated protein (SAP) and the kinase Fyn, explaining earlier reports of developmental arrest in SAP- or Fyn-deficient NKT precursors.[91,92,93,94,95] SAP- and Fyn-deficient NKT thymocytes were blocked at stage 0 and could not be rescued by expression of Bcl-2 or Bcl-xL. They showed lower induction of CD69,[90] suggesting defective signaling by the TCR, perhaps due to a role of Slamf-SAP-Fyn signaling in stabilizing the immune synapse.[96] Individual ablation of Slamf1 or Slamf6 resulted in a modest twofold defect in the expansion of the NKT lineage between stages 1 and 2. However, in "pseudo-double mutant" mixed chimeras where Slamf1/CD1d double deficient NKT precursors were forced to see their ligands on Slamf6-deficient thymocytes, a > 10-fold reduction of NKT cells was observed, demonstrating a requirement of Slamf receptors at the time of TCR engagement by CD1d ligands.[90] Notably, the Slamf locus exhibits considerable polymorphism, which may underlie some of the reported variations in NKT-cell frequencies in different mouse strains and in humans. Genetic studies have provided support for this hypothesis by identifying the lack of expression of Slamf1 by cortical thymocytes in the nonobese diabetic (NOD) strain, which is spontaneously NKT deficient.[97]

Signaling in the Natural Killer T-Cell Microenvironment

Engagement of the semi-invariant TCR activates the same Ras/MAP kinase and calcineurin pathways as reported for MHC-restricted TCRs during positive selection.[98,99] Notably, NKT thymocytes show elevated and sustained expression of the early growth response (Egr) factors 1 and 2 compared with the weak and transient expression observed in MHCrestricted thymocytes.[100] Egr1 is thought to be downstream of the Ras/MAP kinase pathway, whereas Egr2 is mainly induced by the calcineurin/nuclear factor of activated T cells (NFAT) pathway. Egr1 and Egr2 mediate the survival of MHC-restricted T-cell precursors after positive selection through induction of Bcl2 and Bcl-xL.[101,102,103] In the case of NKT cells, however, sustained Egr elevation has specific lineage-determining consequences. Egr2 directly binds to the promoters of NKT lineage-specific genes such as *Zbtb16*, encoding PLZF, and *Il2rb*, encoding the β chain of the IL-15 receptor, and it is required for their induction. This suggests a direct connection between the peculiar signaling emanating from the TCR synapse and these NKT lineage checkpoints.[100] The sustained elevated Egr levels

likely result from the TCR recognition of agonist ligands[36,43] or from the Slamf-SAP-mediated stabilization of the immune synapse.[96] As some *Zbtb16* messenger ribonucleic acid induction was detected in SAP-deficient NKT precursors, SAP may not be absolutely required for PLZF induction.[104]

TCR and Slamf-SAP-Fyn signaling both involve the canonical NF-κB pathway through PKCθ and Bcl-10.[105,106] Mice lacking these downstream signaling components showed partial defects in NKT-cell development,[107,108] as further detailed in the following.

Thus, NKT-cell development is tightly dependent on the signaling elicited through the TCR and Slamf-SAP-Fyn pathways, the specific contributions of which remain to be dissected. Notably, redirecting the expression of MHC class II proteins on cortical thymocytes through ectopic expression of the transcription factor CIITA led to the differentiation of "innate-like" CD4+ thymocytes in a SAP-dependent manner. These cells resembled stage 2 NKT cells and expressed PLZF, reinforcing the notion that homotypic thymocyte-thymocyte interactions, Slamf-SAP-Fyn signaling, and PLZF define a dedicated thymic pathway for the production of innate-like effector T cells.[109,110,111,112] In that context, the reciprocal expression patterns of CD1 and Slamf receptors by thymocytes and of MHC proteins by epithelial cells may well serve the primary purpose of creating different niches for different thymic lineages.[90]

Cluster of Differentiation 4 and Cluster of Differentiation 8 Coreceptor Expression

NKT cells originate from the same pool of DP precursors as MHC-restricted T cells and emerge from thymic selection as CD4 SP cells expressing the CD4 lineage factor ThPOK/c-Krox in a Gata-3-dependent manner.[113,114] A fraction goes on to downregulate CD4 and acquire the DN phenotype, but they still stably express ThPOK, which is essential to downregulate CD8. In humans, some NKT cells can express CD8αα homodimers. ThPOK-deficient NKT cells did not express CD4, and some of them also failed to repress CD8, but otherwise they appeared to develop normally.[113] Their functional properties, however, have not been fully assessed. CD8α-deficient mice showed a modest but significant bias toward the selection of high-affinity Vβ7 TCRs compared with littermate controls.[83] A similar bias was observed after anti-CD8 antibody treatment, suggesting a minor role of CD8 as a coreceptor for CD1d. Transgenic expression of CD8α resulted in the disappearance of NKT thymocytes, suggesting a role in negative selection,[83] a conclusion subsequently challenged when the transgenic model was found to have impaired Vα-Jα rearrangements.[114]

Thus, while NKT-cell development does not appear to rely on CD4 or CD8 coreceptors, the induction of ThPOK and CD4 in this lineage may simply reflect the path of high

affinity TCRs whose signaling is coreceptor independent.[115] However, the subsequent CD4 downregulation in up to 50% to 70% of NKT cells, despite persistent ThPOK expression, remains to be explained.

Cluster of Differentiation 28-B7 Interactions

A modest decrease in the thymic expansion of NKT cells was reported in mice lacking CD28 or B7,[116,117] but the nature of the B7-expressing cell type (epithelial cells or DCs) has not been determined.

Transcriptional Control of Natural Killer T-Cell Development: The Central Role of Promyelocytic Leukemia Zinc Factor

The transcription factor PLZF encoded by *Zbtb16* was identified as the signature master

transcription factor of the NKTcell lineage.[104,118] PLZF is a member of the BTB-ZF family of transcription factors, which also includes Bcl6 and ThPOK. It is composed of a bric-a-brac, tramtrack, and broad (BTB) homodimerizing domain and nine Kruppel-type Cys2His2 zinc fingers. The BTB domain binds a corepressor complex made of histone deacetylases, N-Cor, and SMRT, whereas deoxyribonucleic acid binding is mediated by the zinc fingers. PLZF is induced in NKT precursors just after TCR signaling, with high amounts detected on nearly half of stage 0 and all of stage 1 and 2 cells, and lower levels found in stage 3 cells.

Mice lacking PLZF through either the *luxoid* mutation, which induces a frameshift leading to a truncated BTB protein without zinc fingers, or deletion of exon 2 encoding the BTB domain, exhibited a block in NKT-cell development,[104,118] as well as defects in spermatogonial cell maintenance and osteoblast differentiation.[119,120] PLZF-deficient NKT precursors identified by CD1d-αGalCer tetramers were blocked at stage 1, unable to acquire effector characteristics. Instead, they maintained a naïve phenotype and function, recirculating between blood, lymph node, and spleen, rather than homing to liver and lung, and producing IL-2 instead of IL-4 and IFNγ upon stimulation. Intriguingly, however, they incorporated BrdU at the same high rate as wild type, but without showing expansion, suggesting aborted division or increased cell death.

Ectopic expression of PLZF under the CD4 or Lck promoter induced a typical stage 2 NKT program in CD4 T cells, with downregulation of CD62L, upregulation of CD44 and LFA-1, dual secretion of IL-4 and IFNγ, and homing to liver and lung.[104,121,122,123] Notably, the full effector conversion depended on expression of PLZF at high levels comparable to stage 1 and 2 NKT thymocytes, which was only achieved in the CD4 promoter transgenic model.[124] In these mice, nearly 100% of CD4 cells acquired the effector phenotype at the CD4 SP stage. This effector conversion was also observed in MHC class II-restricted TCR/RAG knockout transgenic models. Thus, while the induction of PLZF requires the signaling environment of NKT cells, the NKT effector program can be transferred by PLZF alone. The molecular basis of the extensive gene reprogramming induced by PLZF, in particular the direct target genes and the biochemical mechanisms of gene activation or repression, are currently being studied.

Other Transcription Factors

The induction of Tbet represents an essential step at the transition between stage 2 and stage 3,[125,126,127] coinciding with the relative decrease in PLZF expression. Both Tbet and Egr2 contribute to *Il2rb* induction,[100] allowing responsiveness to IL-15. While the pathways leading to Tbet induction independently of Stat1 or Stat4 remain unclear, a role for Ets1 or MEF has been suggested.[127,128]

Mice lacking Gata-3 exhibited severe but complex NKTcell defects[129] consisting of a considerable reduction of most of their thymic and peripheral NKT cells, with the strange exception of their stage 3 thymocytes which accumulated normally. Gata-3 is required for ThPOK induction, explaining the loss of CD4 by residual NKT cells.[113]

Mice lacking TGFβRII had severe NKT-cell developmental defects, particularly in a competitive chimera setting.[130] Mutant NKT cells had defective expression of the IL-7 receptor and increased apoptosis at stage 1, but a normal rate of cell division at stage 2. Tbet and IL2Rβ were expressed by a larger than normal fraction of stage 2 cells. These effects were mediated by different branches of TGFβ signaling, including Tif-1γ, Smad4, and the Smad4/Tif-1-independent pathway.

Complex and somewhat conflicting results have been reported regarding the role of different NF-κB factors. Mice lacking IKKβ or expressing a degradation resistant form of I-κBα

generally exhibited severe cell-intrinsic defects at stage 1 and 2, due in part to apoptosis. These effects appeared to be mediated by the partly redundant functions of NF-κB1, RelA, and c-Rel.[131,132,133,134] The upstream activator of NF-κB may include signaling through TCR, SLAM/SAP/Fyn, or CD28/B7. In contrast, ablation of RelB in the alternate pathway impaired early NKT cells development nonspecifically by disrupting the thymic stroma.

While early reports suggested some cell-intrinsic role for lymphotoxin signaling in NKT-cell development,[135] more recent analysis indicated that the major attrition in lymphotoxin-deficient animals occurred during the transition from the thymus to the periphery, possibly related to a defect in NKT thymocyte emigration in the absence of interaction between LTβR on thymic stromal cells and lymphotoxin on bone marrow-derived cells.[136]

cMyc is induced as early as stage 0, when NKT cells enter S phase. Its conditional ablation resulted in developmental arrest at stage 0,[72] similar to cyclin D2-deficient mice (unpublished data), suggesting that entry into cell cycle occurred at stage 0, one stage earlier than previously thought.[64]

Micro-Ribonucleic Acids

Conditional ablation of *Dicer* in thymocytes led to a massive loss of NKT thymocytes but the micro-ribonucleic acids involved have eluded identification.[137,138,139,140]

Other Promyelocytic Leukemia Zinc Factor-Positive Lineages

γδT cells expressing the semi-invariant Vγ1Vδ6 receptor were previously shown to express the NK1.1 marker, home to the liver, and produce IL-4, like Vα14-Jα18 NKT cells.[141]

These cells were not only dependent on SAP for their differentiation, but also expressed PLZF.[142,143,144] In OP-9 supported thymocyte cultures, PLZF could be induced after crosslinking with Vγ-specific TCR antibodies. Likewise, TCR αβ thymocytes upregulated *Zbtb16* messenger ribonucleic acid after injection of anti-TCRβ antibodies in vivo.[100] These findings support the notion that agonist signaling induces PLZF to generate innate-like αβ and γδ lineages. MR-1-specific Mucosal-associated invariant T cells were also reported to express PLZF[104] and home to liver and gut.[145] Together with experiments showing that CD4 T cells selected by MHC class II expressed on thymocytes also acquired PLZF in a SAP-dependent manner,[109,110,111,112] these observations reveal an authentic cellular and molecular pathway dedicated to the generation of innate-like lymphocytes.

Deficiencies or mutations affecting certain genes associated with TCR signaling produced a phenotype characterized by a cell-intrinsic expansion of the PLZF-expressing thymic lineages, including Vγ1Vδ6 T cells and Vα14-Jα18 NKT cells. This expansion was associated with a bystander conversion of MHC-restricted thymocytes, mainly CD8 T cells, into innate-like CD62L^{lo} CD44^{hi} CD122^{hi} eomesodermin^{hi} effectors, caused by IL-4 secreted by the PLZF-expressing cells. This complex phenotype was observed in mice lacking the Tec kinases Itk or Rlk,[146,147,148] or bearing the Y145F mutation in the scaffold protein SLP76, which impairs binding of these kinases after TCR signaling.[143] Mice lacking the inhibitor of E proteins Id3, which can be induced downstream of the TCR-activated MAP kinases, showed a strikingly similar phenotype,[143,149,150] as did mice after conditional ablation of CREB binding protein,[151] a general transcriptional coactivator, and KLF2, a transcription factor that controls CD62L and S1P1 receptor upregulation after positive selection.[148,152]

The convergent impact of these different genes suggests the existence of a negative regulatory pathway that physiologically contains the expansion and function of innatelike T

cells. Notably, wild-type BALB/c mice spontaneously exhibited a similar, albeit milder, phenotype as mutant C57BL/6 mice, including a relative expansion of PLZFexpressing NKT thymocytes and a bystander induction of CD44, CD122, eomesodermin, and the rapid production of cytokines by CD8 SP thymocytes.[148] This phenotype was dependent on *klf13*.[153] Thus, there is a natural crosstalk between innate-like and conventional T cells during thymic development, whose variations may have a profound impact on the immune system.

Homeostasis

NKT cells are produced at a similar rate from fetal to adult life, and they persist indefinitely after adult thymectomy. The mechanisms ensuring their long life in peripheral tissues are detailed in the following.

Interleukin-15

Like NK cells and CD8 memory cells, mature NKT cells critically depend on IL-15 for their terminal maturation as well as their survival and homeostatic renewal.[154,155,156] Cell-type-specific ablation of IL-15Rα, which is essential to present IL-15 at the surface of IL-15-producing cells, revealed that the sole source of IL-15 for development was a nonhemopoietic radiation-insensitive stromal cell type, most likely a medullary epithelial cell.[157] In contrast, both radiation-resistant and hemopoietic cells served as partly redundant sources of IL-15 for peripheral NKT cells.

CXCL16

CXCR6 signaling, in response to CXCL16 produced by endothelial cells, is critical for the survival of liver NKT cells in basal conditions, but not for their specific accumulation or their crawling behavior in the liver sinusoids.[158]

Cluster of Differentiation 1d Ligands

In mice lacking CD1d in peripheral tissues, the full terminal maturation of NKT cells after thymic export is partially altered but the overall frequency, tissue distribution, survival, and functional properties of NKT cells are not significantly impaired.[80,87] Thus, peripheral NKT cells do not require contact with self-antigens for homeostasis.

Microbiota

NKT cells did not exhibit major disturbances in their development or function in germ-free mice.[78,159,160] However, mice lacking NKT cells were reported to harbor changes in the composition of their microbiota.[160]

TISSUE DISTRIBUTION AND RECIRCULATION

In C57BL/6 mice, NKT cells represent ˜0.2% of the T-cell population in blood and peripheral lymph nodes; ˜2.5% of T cells in spleen, mesenteric, and pancreatic lymph nodes; 5% in the lung; and up to 40% in the liver. Furthermore, parabiotic experiments have shown that, unlike most lymphocytes, NKT cells do not recirculate and are lifelong residents of lymphoid tissues, lung, and liver.[161]

Tissue and Intravascular Residents

This tissue residency is particularly remarkable in the liver and lung where NKT cells are mainly found within the capillary microvasculature.[158,161] Intravital imaging using confocal microscopy of the liver of $CXCR6^{GFP}$ mice, where a majority of brightly fluorescent liver

lymphocytes are Vα14 NKT cells, demonstrated their presence inside the sinusoid capillaries, adhering to the luminal side of endothelial cells and crawling, without directional bias, at a speed of ˜10μm/min (Fig. 18.6). While other effector T cells and monocytes can adopt a similar crawling behavior transiently, before they extravasate to enter inflamed tissues, NKT cells are constitutively and permanently crawling in steady-state conditions.

The expression of high amount of CXCR6 by NKT cells matches the production of the corresponding chemokine ligand CXCL16 by endothelial cells lining the sinusoids. CXCR6 appeared to be important for the survival of liver NKT cells[158] as well as NK cells,[162] but it was not required for liver accumulation. Furthermore, the adhesion and crawling processes were unaltered by pertussis toxin treatment, suggesting independence from $G\alpha_i$ protein signaling.[163]

Thus, the constitutive activation of LFA-1 was neither a consequence of TCR signaling, as NKT cells normally accumulated in the liver of mice lacking CD1d outside of the thymus,[80,87] nor of chemokine-induced $G\alpha_i$ protein signaling. Notably, transgenic expression of PLZF in MHC class II-restricted CD4 T cells was sufficient to induce their liver retention, indicating that the adhesion and crawling behavior are an integral component of the PLZF-induced functional program.[161]

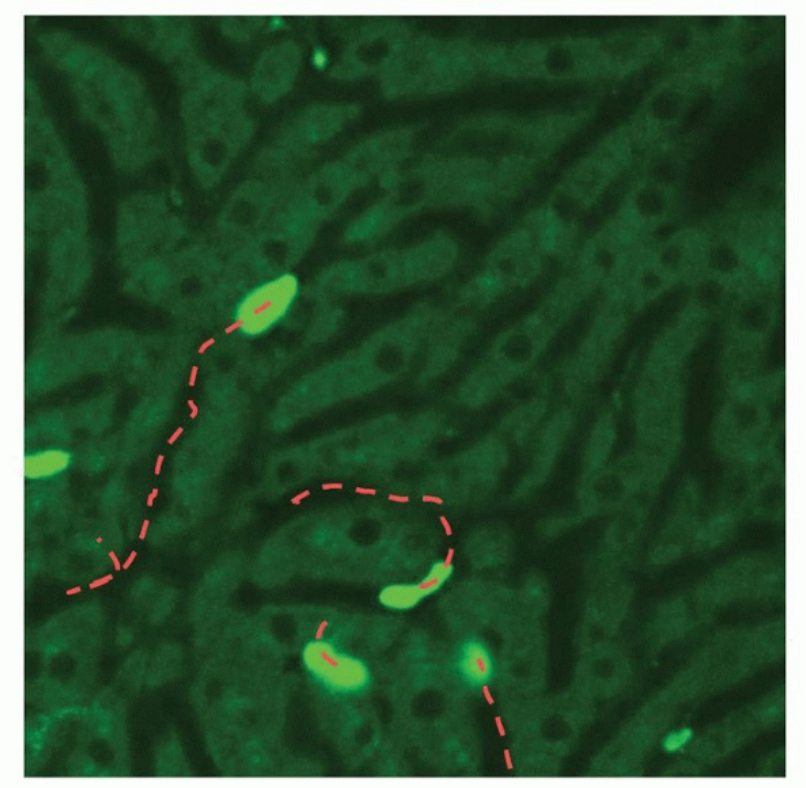

FIG. 18.6. Intravascular Patrolling by Liver Natural Killer T (NKT) Cells. Fluorescent NKT cells in CXCR6-GFP "knock-in" mice are crawling on the luminal side of sinusoid endothelial cells. *Dashed lines* show their path over a period of 10 mn in a live imaging study. (Courtesy of Frederic Geissmann, King's College, London, UK, and Dan Littman, New York University, New York, NY, USA)

Organ-specific Natural Killer T-Cell Sublineages

DN NKT cells expressing Rorγt and producing IL-17 and IL-22 but not IL-4 or IFN-γ selectively resided in skin and draining axillary and inguinal lymph nodes.[164,165] While they shared the usual CD44^{hi}CD62L^{lo} effector phenotype of other NKT cells, they did not express the stage 3 markers Tbet, IL2Rβ, CD69, or NK receptors and instead displayed a CD103^{hi} CCR6+ CD121a+ phenotype. The identification of a minor cell population in the thymus with a similar phenotype suggested that they constituted a separate developmental sublineage.[166] Intriguingly, their expression of Nrp-1, a marker of recent thymic emigrants and their rapid disappearance after adult thymectomy, suggested that, unlike most NKT cells, they represented a short-lived population.[82]

A subset of NKT cells expressing the IL-25 receptor and biased toward production of IL-4, IL-3, and IL-9 was found in the thymus, spleen, and lung, but was poorly represented in the liver and bone marrow.[167] Whether these cells constitute a tissue-specific sublineage that differentiates in the thymus or in the periphery remains to be determined.

NATURAL KILLER T-CELL FUNCTIONS

Functional Properties

DC activation and the explosive release of cytokines and chemokines by NKT cells have well-documented functional consequences in a variety of pathological or therapeutic conditions involving lymphoid tissues and body organs. Important factors biasing the Th1, Th2, or Th17 outcome of NKT-cell activation in vivo have recently emerged. They include the structure of the lipid antigen, the antigen-presenting cell type, the tissue, and the coexposure to TLR ligands. These will be discussed in two separate contexts: the administration of synthetic NKT ligands, which function as potent vaccine adjuvants, and the activation of NKT cells in the context of microbial infection.

Vaccine Adjuvant Properties

Dendritic Cell-based Network of Activation

Several studies have characterized a cascade of activation events following the exogenous administration of NKT ligands such as αGalCer (Fig. 18.7). The central feature was a cross-activation between NKT cells and DCs initiated upon the presentation of αGalCer by resting DCs to NKT cells. Activated NKT cells upregulated CD40L, Th1 and Th2 cytokines, and chemokines; CD40 crosslinking induced DCs to upregulate CD40, B7.1, and B7.2 and IL-12p40, which in turn enhanced NKT-cell activation and cytokine production.[168,169] DCs upregulated MHC class I- and class II-mediated antigen presentation, particularly cross-priming, and secreted CCL17,[170] a chemoattractant for naïve CCR4+ CD8 T cells. IL-12 released by DCs promoted prolonged IFN-γ production by NK cells and activated their cytolytic properties.[171,172] Thus, αGalCer promotes robust CD4 and CD8 T-cell-mediated adaptive immune responses against coadministered nonreplicating protein antigens.[173,174,175] Furthermore, although TLR signaling is not involved in the innate response to αGalCer, the combination of TLR and NKT ligands was synergistic.[176]

Th1 and Th2 Variants

Different synthetic agonists showed biased Th1 versus Th2 outcomes mainly due to their differential ability to induce IL-12 from DCs and to transactivate NK cells. Variants of the original αGalCer (KRN7000) with short or unsaturated lipid chains induced similar Th2 but decreased Th1 cytokines.[177,178,179] While early reports pointed to the lower TCR affinity of a variant with truncated phytosphingosine chain (psC9:0, called “OCH”),[180] several other

Th2 variants, including acylC8:0 or acylC20:2, demonstrated identical interactions based on affinity measurements and crystal structures.[56,181,182] All these Th2 variants had increased solubility in water and could be rapidly loaded onto CD1d molecules at the cell surface. In contrast, αGalCer was almost exclusively loaded in the lysosome due to a requirement for lipid transfer proteins. Whereas αGalCer remained stably associated with CD1d, the short or unsaturated variants were rapidly ejected upon recycling to the acidic endosome.[21] Interestingly, αGalCer was found associated with surface CD1d molecules on lipid rafts, whereas the short or unsaturated variants were loaded to CD1d molecules outside of these rafts, perhaps as a consequence of their different loading compartments.[182] However,

this is unlikely to explain the Th2 bias, as the variants induced the same balance of cytokines as αGalCer when tested in vitro with purified antigen-presenting cells. A more likely explanation for the Th2 bias was suggested by in vivo studies with mice carrying a floxed CD1d allele. Indeed, while αGalCer presentation was mostly limited to lysosomally active DCs and macrophages, which produced IL-12, the Th2 variants were broadly presented by all CD1d-expressing cell types, most of which did not produce IL-12.[89] Thus, structural differences in lipids resulted in the involvement of different antigen-presenting cell types, altering the cellular network and the outcome of NKT-cell activation (Fig. 18.8).

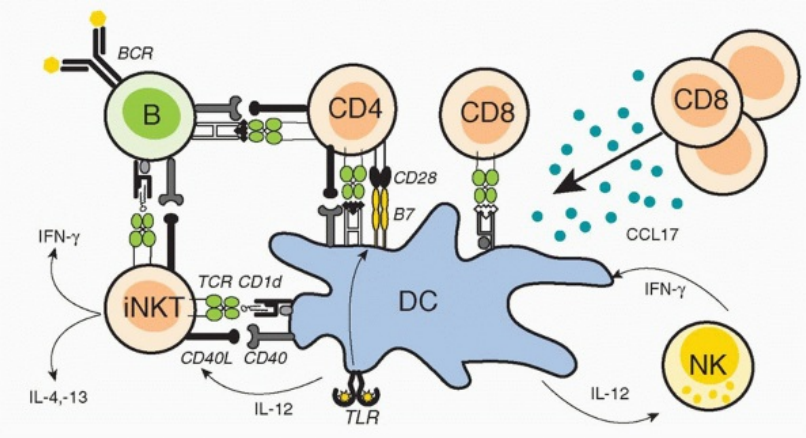

FIG. 18.7. Cellular and Molecular Network Activated by the Natural Killer T (NKT) Ligand αGalCer. Cross-activation between NKT cells and dendritic cells (DCs) lead to cluster of differentiation (CD)40L-CD40 interactions, activating the DC to present peptide antigens to CD4 and also to CD8 cells (cross-priming). NKT cells promptly release Th1 and Th2 cytokines. Naïve CD8 T cells are chemoattracted through CCL17 secreted by DCs. NK cells are "transactivated" by DCs and interleukin-12 to release interferon-γ in a prolonged manner. B cells seeing antigens can receive cognate help from NKT cells and/or from CD4 cells.

Anergy

A limitation to the adjuvant properties of NKT ligands is the rapid NKT TCR downregulation and apoptosis, followed by a long-lasting depletion and a state of anergy.[183,184,185] Anergy could be partially avoided by CD28 costimulation or PD1 blockade,[186,187] or by the administration of αGalCer-pulsed DCs, or αGalCer conjugated to nanoparticles.[188,189,190] The mechanism of NKT-cell anergy remains obscure.

Natural Killer T-Cell Help to B Cells

NKT-cell activation by agonist ligands promoted antibody production, affinity maturation, and switching against associated antigens. Help could be indirect, through CD40L-CD40 mediated activation of the DCs presenting peptide antigen to CD4 T cells, which in turn provided MHC II-restricted help to B cells recognizing a linked antigen. Alternatively, direct NKT-B cell cognate interactions have been demonstrated in vitro and in vivo.[191,192,193,194,195,196,197] The relevance of this direct cognate help was illustrated by infection with *Sphingomonas*, which carries abundant glycosphingolipid ligands in its cell wall.[198] The production of immunoglobulin (Ig)G antibodies against the bacterial membrane appended enzyme pyruvate dehydrogenase complex-E2 depended on CD1d expression by B cells, as evidenced in mixed bone marrow chimeras where CD1d expression was limited to one Ig allotype-marked, B-cell compartment. NKT cells engaged in cognate and prolonged interactions with B cells, after acquiring a stereotypical Bcl-6 dependent follicular helper program with induction of CXCR5, PD1, and IL-21, and migration to the germinal center.[196,197] Intriguingly, immunization with αGalCer-nitrophenol (NP) induced faster but less sustained germinal center formation with fewer long-lived plasma cells and inferior memory compared with keyhole limpet hemocyanin-NP or ovalbumin-loaded (OVA)-NP,[196,197] suggesting that NKT cell help to B cells was intrinsically inferior to conventional T-cell help. However, other variables might have contributed to these differences, including the monovalency of NP in the case of αGalCer, differences in bioavailability, cellular targeting, half-life, and processing of the different immunogen preparations. It is also possible that the rapid induction of NKT-cell anergy after exposure to αGalCer might limit the interactions required for effective B-cell help.

Splenic marginal zone B cells constitutively express approximately eight times more CD1d on their surface than other B-cell subsets.[9] They responded to antigen in an accelerated manner with increased sensitivity conferred by the complement receptor CD21, which lowers the B-cell receptor signaling threshold to complement-coated antigen.[199] These characteristics have long suggested a special function in CD1d-mediated B-cell responses. In support of such

NKT-MZB interactions in vivo, depletion of marginal zone B cells impaired the IgM anti-NP antibody response after systemic administration of αGalCer-NP.[200]

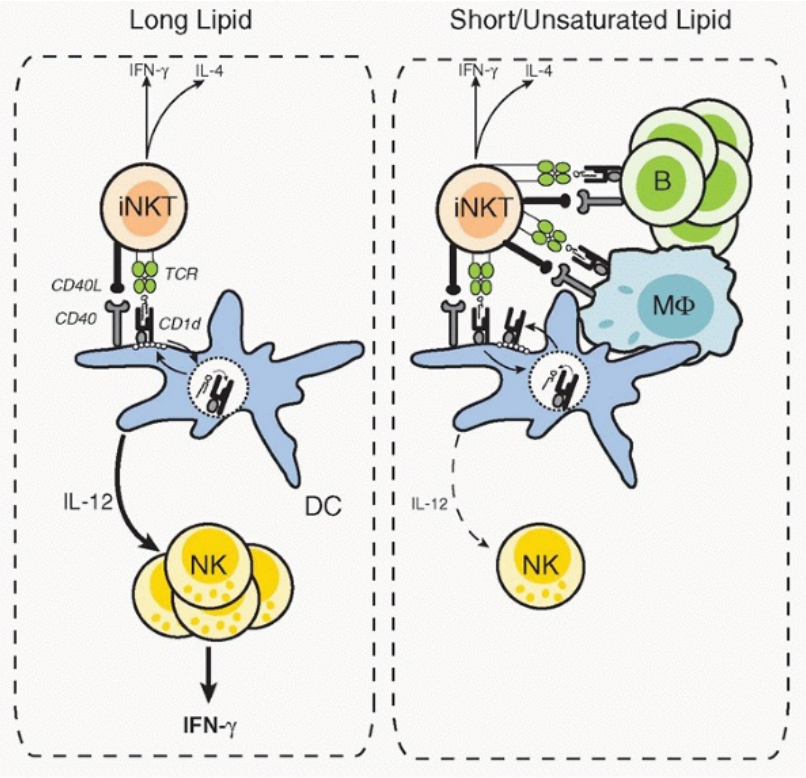

FIG. 18.8. Th1 and Th2 Agonist Ligands of Natural Killer T (NKT) Cells. *Left*, long and saturated αGal-Cer variants (including KRN7000) load cluster of differentiation (CD)1d in the lysosomal compartment due to their requirement for lipid transfer proteins. The main antigen-presenting cell is a dendritic cell (DC), which produces lots of interleukin (IL)-12 and transactivates NK cells. *Right*, short or polyunsaturated lipids load CD1d directly at the cell surface, but are rapidly ejected upon recycling to the lysosome due to displacement by longer, hydrophobic peptides at acidic pH. These short or polyunsaturated lipids are promiscuously presented by all CD1d expressing cells, limiting DC interactions, interleukin-12 production, and natural killer transactivation. CD1d complexed with long and saturated variants sit in lipid rafts, whereas CD1d complexed with short or polyunsaturated variants are excluded from lipid rafts.

Natural Killer T-Cell Help to Macrophages and Myeloid Suppressor Cells

Subcapsular CD169+ macrophages efficiently captured and presented αGalCer to NKT cells in lymph nodes after footpad injection of 200 nm silica beads coated with the lipid antigen.[201] This selective capture further emphasizes the importance of the formulation of αGalCer in targeting different antigenpresenting cell types. Notably, agonist-mediated NKT-cell interaction with myeloid suppressor cells could reverse their suppressive properties. Furthermore, natural interactions between NKT cells and myeloid suppressor cells have been suggested in transplanted cancers and influenza infection.[202,203,204]

Microbial Infections

The recognition of either microbial α-linked glycolipid antigens or self-β-linked antigens induced by TLR signaling ensures the recruitment of NKT cells in most microbial infections (Fig. 18.9). Which of these modes of activation predominates for individual microorganisms is somewhat controversial, as TLR signaling induces IL-12, which is a potent amplifier of IFN-γ secretion, and NKT cells upregulate their IL-12 receptor upon TCR signaling.

Direct Microbial Lipid Recognition

Glycosphingolipids closely related to αGalCer were found in the cell wall of *Sphingomonas*,[30,205] a prominent gram-negative LPS-negative member of α-proteobacteria, an abundant class of bacteria. These glycosphingolipids included the dominant α-branched glucuronyl and galacturonyl ceramides (GSL-1) and the less abundant di- (GSL-2), tri- (GSL-3), and tetraglycosylated (GSL-4) species. Although they form structures that are reminiscent of LPS, their synthesis pathway and their role in the microbial cell wall are not well understood. GSL-1 activates large proportions of mouse and human NKT cells,[31,32,206,207] but it is unclear at present whether the more complex GSL-2, -3, and -4 can be recognized by NKT cells or even whether they can be efficiently processed into GSL-1 by host antigen-presenting cells. During infection, *Sphingomonas* was phagocytosed by macrophages and DCs, and elicited an activation cascade similar to exogenous αGal-Cer. NKT-deficient mice had 15- to 1000-fold more residual bacteria than their wild-type littermates in the first few days after infection.[31,32] High doses of *Sphingomonas* induced a lethal toxic shock in wild-type but not NKT-deficient littermates. These observations have led to the hypothesis that NKT cells and their canonical TCR specificity might have evolved to meet the challenges of these gram-negative LPSnegative bacteria. While *Sphingomonas* can cause severe infection, particularly in immunocompromised hosts, other more deadly members of the class of α-proteobacteria may have provided stronger evolutionary pressures on the NKTcell system. Particularly interesting is the case of *Ehrlichia*, a tick-borne pathogen and member of the *Rickettsiales*, which is of widespread significance for mammals, including wild and domesticated ruminants, dogs, and humans in some regions of the world such as Africa and east Asia. *Ehrlichia muris*

activated NKT cells independently of MyD88, and its clearance was profoundly impaired in CD1d- or Jα18-deficient animals.[31] However, the cell wall composition of *Ehrlichia*, a gram-negative, LPS-negative obligate intracellular bacterium, has not been elucidated.

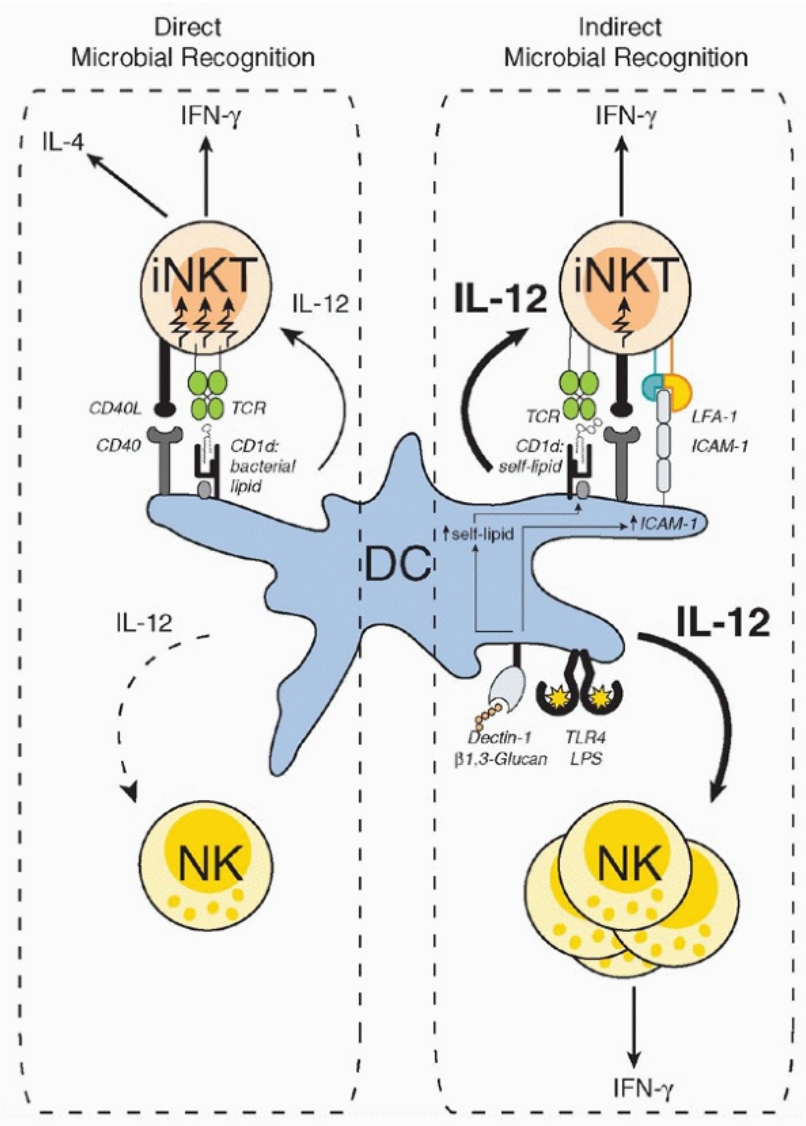

FIG. 18.9. Dual Recognition of Self- and Microbial Ligands in Microbial Infections. *Left*, microbial organisms expressing agonist ligands can elicit direct activation of natural killer T (NKT) cells. *Right*, microbial organisms lacking NKT ligands activate NKT cells through the enhanced recognition of self NKT ligands. These self ligands are upregulated by toll-like receptor- or dectin-1-mediated signals, and their recognition is enhanced by upregulation of intercellular adhesion molecule at the immune synapse. Interleukin-12 secreted by dendritic cells amplifies interferon-γ produced by NKT cells and by natural killer cells.

Other bacteria such as *Streptococcus*[34,208] and *Borrelia*[33] expressed α-linked diacyglycerol lipids that bound CD1d and could directly engage the mouse and human NKT TCR, resulting in accelerated microbial clearance. *Borrelia* was normally cleared by NKT-deficient mice, except at later time points in the joints.[163,209] Intravital microscopy of the liver response to an intravenous inoculation of *Borrelia burgdorferi* showed rapid microbial uptake by stationary Kupffer cells followed by the attraction and arrest of NKT cells in the sinusoids. Stable, CD1d-mediated contacts between Kupffer cells and NKT cells depended on the secretion of CXCL9 by Kupffer cells and the pertussis toxin-sensitive response of CXCR3-expressing NKT cells.

Self-Lipid Recognition

Many bacteria that do not harbor NKT ligands nevertheless induce massive amounts of IFN-γ in a NKT- and CD1d-dependent manner.[31,39] This secretion of IFN-γ is considerably amplified through TLR-induced IL-12 released by DCs and macrophages and the transactivation of NK cells (see Fig. 18.9). TLR signaling regulates several glycosphingolipid enzymes, leading to the accumulation of stimulatory glycosphingolipids (see Fig. 18.3), and activation of LFA-1/ICAM-1 interactions further facilitate NKT-cell recognition of these low-affinity ligands. However, contrary to an early report,[210] NKT cells do not usually constitute the predominant cell-type producing IFN-γ in response to IL-12

in vivo,[39,211] which explains why they generally do not appear to play an essential role in many bacterial infections. Nevertheless, bacterial clearance and neutrophils were reportedly decreased in the lungs of CD1d-deficient mice after infection with *Pseudomonas aeruginosa*.[212] This may not be the case at other sites of infection.[213] Different conclusions were also reported regarding the importance of NKT cells versus NK cells in lethal LPS-induced toxic shock.[214,215]

Fungal and Parasitic Infections

The production of IgG antibodies to the malaria circumsporozoite antigen, a key component of protective immune responses in humans, was suggested to depend on NKTcell recognition of malarial glycosylphosphatidylinositol antigens in a mouse model.[216] However, additional experiments failed to detect a CD1d-dependent component to this antibody response and glycosylphosphatidylinositols have not been identified as NKT-cell antigens in other reports.[217,218] In the context of helminth infection, DCs pulsed with *Schistosoma mansoni* eggs activated NKT cells to secrete Th1 and Th2 cytokines in vitro in a β-hexosaminidase B-dependent but MyD88-independent manner, suggesting recognition of the self-ligand iGb3 in the absence of TLR signaling.[219]

Intratracheal infection by the fungal pathogen *Aspergillus fumigatus* indirectly stimulated NKT-cell production of IFN-γ through activation of the dectin-1/MyD88 pathway by the major cell wall polysaccharides, β-1,3 glucans. Clearance of the pathogen was impaired in NKT-deficient mice.

Viral Infections

Viruses can activate TLR signaling through the adaptors MyD88 and TRIF, which in turn activate autoreactive NKT cells. Relatively modest defects in viral clearance have been reported in CD1d-deficient mice infected with encephalomyocarditis virus[220] or coxsackie B3,[221] but these defects were not observed in Jα18-deficient mice, ruling out a specific role of Vα14 NKT cells. Influenza virus clearance was normal or modestly impaired in mice lacking NKT cells.[222,223] Infections with lymphocytic choriomeningitis virus, mouse cytomegalovirus, vaccinia virus, and coronavirus were unaffected. In humans, a profibrotic role of Vα24 NKT cells was suggested in hepatitis C[224] and non-Vα24 CD1d-restricted T cells were found in the liver.[225] Although a specific role of Vα14 NKT cells in herpes simplex virus infection is controversial,[226,227] studies have suggested that viral invasion may be associated with countermeasures against CD1d or NKT cells. For example, herpes simplex virus-1 drastically and specifically impaired CD1d recycling from lysosome to plasma membrane, an essential pathway for glycolipid antigen presentation to NKT cells.[228] Kaposi sarcoma-associated herpes virus encodes two modulators of immune recognition MIR1 and MIR2 that downregulated CD1d along with other immunologically relevant molecules such as

MHC class I, CD86, and ICAM-1 through ubiquitination of lysine residues in their cytoplasmic tail.[229] The lethal outcome of infections with Epstein-Barr virus in patients with X-linked lymphoproliferative immunodeficiency syndrome due to SAP mutations was hypothesized to result from the absence of NKT cells.[93] Which of these effects or associations reflect specific viral evasion/immune defense strategies and the nature of the putative NKT ligands involved in these infectious conditions remain to be determined.

Autoimmunity, Inflammation, and Allergy

Asthma

Studies in the OVA/Alum model of asthma in mouse and a report that humans with asthma harbored high percentages of Vα24 NKT cells in their bronchoalveolar lavage suggested a broad role of NKT cells in various forms of airway allergic inflammation. However, these studies have not been widely confirmed.[230]

Other studies have focused on the presence of airborne NKT ligands in natural environments, for example in house dust or in air samples where *Sphingomonas* is one of the predominant microbial species identified.[231,232] Airway exposure to these NKT ligands resulted in the rapid CCL17-induced recruitment of resident intravascular NKT cells into the lung with formation of eosinophilic granulomas. Notably, a single airway exposure to protein antigen associated with NKT ligands led to massive recall allergic airway inflammation upon airborne challenge with protein alone. Likewise, coadministration of house dust extracts containing unidentified NKT ligands promoted allergic sensitization to airborne OVA in an NKT-dependent manner.

Thus, emerging studies indicate that natural exposure to airborne NKT ligands in the environment may constitute a previously unappreciated cause of allergic sensitization.

Diabetes and Autoimmune Diseases

Many reports have suggested a role of NKT cells, usually of regulatory nature, in autoimmune diseases such as type I diabetes, arthritis, and lupus, and in atherosclerosis and inflammatory bowel disease.[233] These reports mostly relied on comparisons between wild-type versus Jα18- or CD1ddeficient mice but did not offer precise mechanistic insights into the putative function of NKT cells and their mode of recruitment. Some of these studies did not use littermates as wild-type controls and have not been widely reproduced. Furthermore, the recent discovery that NKT thymocytes impact the function of MHC-restricted T cells in a bystander manner, through the secretion of IL-4,[148] suggests that alternative, indirect mechanisms might contribute to these observations.

Primary Biliary Cirrhosis

Primary biliary cirrhosis (PBC) is an enigmatic disease characterized by the presence of antimitochondrial antibodies, liver lymphocytic infiltrates, and the chronic destruction of the biliary epithelium leading to cirrhosis.[234] The autoantibodies recognize an epitope of the mitochondrial pyruvate dehydrogenase complex-E2 enzyme that is particularly well conserved in *Novosphingobium aromaticivorans*, a strain of *Sphingomonas*. Further, patients with PBC, including those

lacking antimitochondrial antibodies, were specifically seropositive against *Sphingomonas*, which was detected by polymerase chain reaction in stool samples of 25% of diseased or healthy individuals, suggesting that PBC may be induced by aberrant host reactivity to this bacterium.[235] Patients with PBC also showed an enrichment of Vα24 NKT cells in liver biopsies and a depletion in blood.[236] In light of the recent finding that *Sphingomonas* cell

wall glycolipids specifically activate NKT cells, these studies suggested that NKT cells might play a key role in the pathogenesis of PBC by promoting aberrant responses to *Sphingomonas*. An experimental model of infection induced a chronic liver disease with PBClike granulomas and antimicrobial autoantibodies, which could be transferred by lymphocytes. These provocative findings support the hypothesis that PBC might be the consequence of cryptic episodes of bacterial infections, perhaps triggered by commensals containing NKT ligands, leading to NKT cell-assisted breakdown of tolerance to shared microchondrial/bacterial antigens.

Cancer

A role of Jα18 and non-Jα18 CD1d-restricted NKT cells in cancer surveillance has been inferred from reports that CD1d- and Jα18-deficient mice seemed to differ from wildtype mice in their susceptibility to a variety of spontaneous and transplanted cancers.[237] Vα14 NKT cells were suggested to exert protection against spontaneous sarcomas. These included fibrosarcomas in mice injected intramuscularly with methylcholantrene,[238] osteosarcomas and hemopoietic tumors in p53+/- mice,[239] and carcinomas in transgenic adenocarcinoma of the mouse prostate mice.[240] Some transplanted cancers seemed aggravated in their progression by the presence of non-Vα14 NKT cells, apparently because of their secretion of IL-13 and interaction with myeloid suppressor cells.[202,203] These cancer studies have commonly concluded that endogenous ligands might be induced and presented by the tumor themselves or by antigen-presenting cells. The crosstalk between myeloid-derived cells and NKT cells resulted in suppression, or on the contrary, to exacerbation of immune responses against cancer. In most cases, however, the nature of these putative ligands has escaped precise identification. One exception lies with the proposed role of lysophosphatidylcholine as a tumor antigen in myeloma patients.[54]

A serious challenge to the interpretation of these studies is their near exclusive reliance on comparisons between wild-type and NKT-deficient mice and the lack of direct evidence of NKT cell involvement in the antitumor response. Moreover, some studies did not use proper littermate controls, or could not be independently reproduced. For example, the longstanding but isolated report that methylcholantrene-induced sarcomas were naturally controlled by Vα14 NKT cells[238] could not be confirmed in recent double-blind studies comparing large groups of CD1d- or Jα18-deficient mice and their littermate controls.[241] In addition, some of the reported results may be explained by an indirect rather than a direct function of NKT cells, for example due to their cross-talk with conventional T cells.[148]

CONCLUSION

Recent Advances

In the past 4 years, pivotal discoveries have advanced our understanding of the biology of NKT cells, particularly regarding their dual recognition of self- and foreign-lipid antigens; their role in infectious and allergic diseases; their specialized effector functions within microenvironments; their expression of a lineage-specific master transcription factor, PLZF; and their cross-talk with MHC-restricted T cells.

Remaining Challenges

The nature and the hierarchy of self- and foreign antigens recognized by NKT cells and their proposed role in a variety of diseases remain a work in progress. Are there other, perhaps more broadly relevant NKT ligands and what controls their expression?

Which class of microorganisms might explain the evolution of this elaborate lineage? Might NKT cells protect against diseases that have recently disappeared, at least in Western societies? In that regard, typhus-like disease after infection by tick-borne *Ehrlichia* may be

comparable to malarial disease in its devastating impact in endemic regions.

What are the signaling events underlying NKT-cell differentiation, particularly the signals inducing PLZF, the molecular mechanisms of PLZF function, and the acquisition of NK receptors? Are there master transcription factors, similar to PLZF, that control other innate-like lineages such as CD8αα TCRαβ IELs and B1 B cells?

Finally, will NKT agonists demonstrate efficiency and safety as vaccine adjuvants to combat infections and cancer in humans?

Other Cluster of Differentiation 1-restricted T Cells?

Although there is strong evidence that the majority of CD1drestricted αβ T cells in mice belong to the NKT lineage,[5] little is known about the human populations of αβT cells restricted by CD1d or other CD1 isotypes such as CD1a, b, and c.

Convergent studies reviewed in this chapter suggested that thymic selection by ligands predominantly expressed on cortical thymocytes or other hemopoietic cells was a key determinant of innate-like lineage decision. As CD1a, b, c, and d are all predominantly expressed on cortical thymocytes, they might mainly select PLZF-expressing T cells. This hypothesis remains to be directly tested in fresh human T-cell populations. Notably, humanized mice expressing a CD1b-restricted human TCR together with human CD1b under its own regulatory elements developed effector-like T cells that expressed PLZF.[242] Other insights have come from studies of a small subpopulation of human αGalCer-specific T cells that did not express Vα24, but used the remaining conserved elements of the NKT TCR, Jα18 and Vβ11. These cells expressed intermediate amounts of PLZF and kept a naïve $CD62L^{hi}$ $CD45RO^{hi}$ phenotype.[124] This natural example suggests that PLZF expression may be a reliable marker of T cells selected by CD1 molecules, but that different levels of expression may be imparted depending on as yet unknown factors such as perhaps, the intensity of TCR

P.447

signaling. Other studies based on the large scale cloning of fresh T cells have suggested that 3% to 10% of the cord blood T-cell population may be CD1-restricted and naïvelike, with a larger proportion of memory-type cells appearing in adults.[243] These important studies could not, however, determine whether the CD1-restricted T cells were selected by CD1 molecules or were merely cross-reactive cells primarily selected by MHC.

Most fresh CD1-restricted T cells identified so far in mice and humans have shown a CD4+ or CD4-CD8β- DN phenotype, with occasional expression of CD8α but rarely CD8β.[124,243] This coreceptor expression pattern parallels the one found in mouse and human CD1d-restricted NKT cells where it is associated with expression of ThPOK/cKrox, suggesting that most CD1-restricted T cells might also express this transcription factor.

These observations provide a background for studies of fresh, unimmunized CD1-restricted T cells in humans, which are now possible through the use of tetramers. Together with studies of “humanized” mice expressing human CD1-restricted TCRs and CD1 molecules, a more definitive characterization of the different populations of CD1-restricted T cells should emerge. Will they demonstrate the heavy contribution of germline TCR segments and the basal autoreactivity that is a hallmark of NKT cells? Will they display adaptive or innate-like features? Forthcoming answers to these fundamental questions will not only be crucial for clinical applications, but will also shed light on the evolutionary logics of lipid antigen recognition.

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REFERENCES

1. Bendelac A, Bonneville M, Kearney JF. Autoreactivity by design: innate B and T lymphocytes. *Nat Rev Immunol* . 2001;1:177-186.

2. Lantz O, Bendelac A. An invariant T cell receptor a chain is used by a unique subset of MHC class I-specific $CD4^+$ and $CD4^-$ 8^- T cells in mice and humans. *J Exp Med*. 1994;180:1097-1106.

3. Benlagha K, Kyin T, Beavis A, Teyton L, Bendelac A. A thymic precursor to the NKT cell lineage. *Science*. 2002;296:553-555.

4. Uldrich AP, Patel O, Cameron G, et al. A semi-invariant Valpha10+ T cell antigen receptor defines a population of natural killer T cells with distinct glycolipid antigen-recognition properties. *Nat Immunol* . 2011;12:616-623.

5. Park SH, Weiss A, Benlagha K, Kyin T, Teyton L, Bendelac A. The mouse CD1d-restricted repertoire is dominated by a few autoreactive t cell receptor families. *J Exp Med*. 2001;193:893-904.

6. Calabi F, Jarvis JM, Martin LH, Milstein C. Two classes of CD1 genes. *Eur J Immunol* . 1989;19:285-292.

7. Miller MM, Wang C, Parisini E, et al. Characterization of two avian MHC-like genes reveals an ancient origin of the CD1 family. *Proc Natl Acad Sci U S A* . 2005;102:8674-8679.

8. Park S-H, Roark JH, Bendelac A. Tissue specific recognition of mouse CD1 molecules. *J Immunol* . 1998;160:3128-3134.

9. Roark JH, Park S-H, Jayawardena J, Kavita U, Shannon M, Bendelac A. CD1.1 expression by mouse antigen presenting cells and marginal zone B cells. *J Immunol* . 1998;160:3121-3127.

10. Jayawardena-Wolf J, Benlagha K, Chiu YH, Mehr R, Bendelac A. CD1d endosomal trafficking is independently regulated by an intrinsic CD1d-encoded tyrosine motif and by the invariant chain. *Immunity*. 2001;15:897-908.

11. Kang SJ, Cresswell P. Calnexin, calreticulin, and ERp57 cooperate in disulfide bond formation in human CD1d heavy chain. *J Biol Chem*. 2002;277:44838-44844.

12. Zhu Y, Zhang W, Veerapen N, Besra G, Cresswell P. Calreticulin controls the rate of assembly of CD1d molecules in the endoplasmic reticulum. *J Biol Chem*. 2010;285:38283-38292.

13. Cox D, Fox L, Tian R, et al. Determination of cellular lipids bound to human CD1d molecules. *PloS One*. 2009;4:e5325.

14. Muindi K, Cernadas M, Watts GF, et al. Activation state and intracellular trafficking contribute to the repertoire of endogenous glycosphingolipids presented by CD1d [corrected]. *Proc Natl Acad Sci U S A* . 2010;107:3052-3057.

15. Yuan W, Kang SJ, Evans JE, Cresswell P. Natural lipid ligands associated with human CD1d targeted to different subcellular compartments. *J Immunol* . 2009;182:4784-4791.

16. Zhou D, Cantu C lll, Sagiv Y, et al. Editing of CD1d-bound lipid antigens by endosomal lipid transfer proteins. *Science*. 2004;303:523-527.

17. Winau F, Schwierzeck V, Hurwitz R, et al. Saposin C is required for lipid presentation by human CD1b. *Nat Immunol* . 2004;5:169-174.

18. Kang SJ, Cresswell P. Saposins facilitate CD1d-restricted presentation of an exogenous lipid antigen to T cells. *Nat Immunol* . 2004;5:175-181.

19. Schrantz N, Sagiv Y, Liu Y, Savage PB, Bendelac A, Teyton L. The Niemann-Pick type C2 protein loads isoglobotrihexosylceramide onto CD1d molecules and contributes to the thymic selection of NKT cells. *J Exp Med* . 2007;204:841-852.

20. de la Salle H, Mariotti S, Angenieux C, et al. Assistance of microbial glycolipid antigen processing by CD1e. *Science*. 2005;310:1321-1324.

21. Bai L, Sagiv Y, Liu Y, et al. Lysosomal recycling terminates CD1dmediated presentation of short and polyunsaturated variants of the NKT cell lipid antigen alphaGalCer. *Proc Natl Acad Sci U S A* . 2009;106:10254-10259.

22. Moody DB. TLR gateways to CD1 function. *Nat Immunol* . 2006;7:811-817.

23. Kobayashi E, Motoki K, Uchida T, Fukushima H, Koezuka Y. KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol Res*. 1995;7:529-534.

24. Morita M, Motoki K, Akimoto K, et al. Structure-activity relationship of alpha-galactosylceramides against B16-bearing mice. *J Med Chem*. 1995;38:2176-2187.

25. Kawano T, Cui J, Koezuka Y, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science*. 1997;278:1626-1629.

26. Benlagha K, Weiss A, Beavis A, Teyton L, Bendelac A. In vivo identification of glycolipid antigen specific T cells using fluorescent CD1d tetramers. *J Exp Med*. 2000;191:1895-1903.

27. Matsuda JL, Naidenko OV, Gapin L, et al. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers [see comments]. *J Exp Med*. 2000;192:741-754.

28. Brossay L, Chioda M, Burdin N, et al. CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J Exp Med*. 1998;188:1521-1528.

29. Liu Y, Goff RD, Zhou D, et al. A modified alpha-galactosyl ceramide for staining and

stimulating natural killer T cells. *J Immunol Methods*. 2006;312:34-39.

30. Kawahara K, Moll H, Knirel YA, Seydel U, Zahringer U. Structural analysis of two glycosphingolipids from the lipopolysaccharide-lacking bacterium Sphingomonas capsulata. *Eur J Biochem*. 2000;267:1837-1846.

31. Mattner J, Debord KL, Ismail N, et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature*. 2005;434:525-529.

32. Kinjo Y, Wu D, Kim G, et al. Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature*. 2005;434:520-525.

33. Kinjo Y, Tupin E, Wu D, et al. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nat Immunol*. 2006;7:978-986.

34. Kinjo Y, Illarionov P, Vela JL, et al. Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. *Nat Immunol*. 2011;12:966-974.

35. Bendelac A, Lantz O, Quimby ME, Yewdell JW, Bennink JR, Brutkiewicz RR. CD1 recognition by mouse NK1+ T lymphocytes. *Science*. 1995;268: 863-865.

36. Bendelac A. Positive selection of mouse NK1+ T cells by CD1-expressing cortical thymocytes. *J Exp Med*. 1995;182:2091-2096.

37. Behar SM, Podrebarac TA, Roy CJ, Wang CR, Brenner MB. Diverse TCRs recognize murine CD1. *J Immunol*. 1999;162:161-167.

38. Couedel C, Peyrat MA, Brossay L, et al. Diverse CD1d-restricted reactivity patterns of human T cells bearing "invariant" AV24BV11 TCR. *Eur J Immunol* . 1998;28:4391-4397.

39. Brigl M, Bry L, Kent SC, Gumperz JE, Brenner MB. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat Immunol*. 2003;4:1230-1237.

40. Chiu YH, Jayawardena J, Weiss A, et al. Distinct subsets of CD1d-restricted T cells recognize self-antigens loaded in different cellular compartments [in process citation]. *J Exp Med*. 1999;189:103-110.

41. Chiu YH, Park SH, Benlagha K, et al. Multiple defects in antigen presentation and T cell development by mice expressing cytoplasmic tailtruncated CD1d. *Nat Immunol*. 2002;3:55-60.

42. Chen X, Wang X, Keaton JM, et al. Distinct endosomal trafficking requirements for presentation of autoantigens and exogenous lipids by human CD1d molecules. *J Immunol*. 2007;178:6181-6190.

43. Zhou D, Mattner J, Cantu C III, et al. Lysosomal glycosphingolipid recognition by NKT cells. *Science*. 2004;306:1786-1789.

44. Wei DG, Curran SA, Savage PB, Teyton L, Bendelac A. Mechanisms imposing the Vbeta bias of Valpha14 natural killer T cells and consequences for microbial glycolipid recognition. *J Exp Med*. 2006;203: 1197-1207.

45. Darmoise A, Teneberg S, Bouzonville L, et al. Lysosomal alpha-galactosidase controls the generation of self lipid antigens for natural killer T cells. *Immunity*. 2010;33:216-228.

46. Li Y, Thapa P, Hawke D, et al. Immunologic glycosphingolipidomics and NKT cell development in mouse thymus. *J Proteome Res*. 2009;8: 2740-2751.

47. Li Y, Teneberg S, Thapa P, Bendelac A, Levery SB, Zhou D. Sensitive detection of isoglobo and globo series tetraglycosylceramides in human thymus by ion trap mass spectrometry. *Glycobiol*. 2008;18:158-165.

48. Li Y, Zhou D, Xia C, Wang PG, Levery SB. Sensitive quantitation of isoglobotriaosylceramide in the presence of isobaric components using electrospray ionization-ion trap mass spectrometry. *Glycobiol*. 2008;18:166-176.

49. Porubsky S, Speak AO, Luckow B, Cerundolo V, Platt FM, Grone HJ. Normal development and function of invariant natural killer T cells in mice with isoglobotrihexosylceramide (iGb3) deficiency. *Proc Natl Acad Sci U S A* . 2007;104:5977-5982.

50. Christiansen D, Milland J, Mouhtouris E, et al. Humans lack iGb3 due to the absence of functional iGb3-synthase: implications for NKT cell development and transplantation. *PLoS Biol*. 2008;6:e172.

51. Parekh VV, Singh AK, Wilson MT, et al. Quantitative and qualitative differences in the in vivo response of NKT cells to distinct alpha- and beta-anomeric glycolipids. *J Immunol*. 2004;173:3693-3706.

52. Brennan PJ, Tatituri RV, Brigl M, et al. Invariant natural killer T cells recognize lipid self antigen induced by microbial danger signals. *Nat Immunol*. 2011;12:1202-1211.

53. De Libero G, Moran AP, Gober HJ, et al. Bacterial infections promote T cell recognition of self-glycolipids. *Immunity*. 2005;22:763-772.

54. Chang DH, Deng H, Matthews P, et al. Inflammation-associated lysophospholipids as ligands for CD1d-restricted T cells in human cancer. *Blood*. 2008;112:1308-1316.

55. Fox LM, Cox DG, Lockridge JL, et al. Recognition of lyso-phospholipids by human natural killer T lymphocytes. *PLoS Biol*. 2009;7:e1000228.

56. Zajonc DM, Cantu C III, Mattner J, et al. Structure and function of a potent agonist for the semi-invariant natural killer T cell receptor. *Nat Immunol*. 2005;6:810-818.

57. Wu D, Zajonc DM, Fujio M, et al. Design of natural killer T cell activators: structure and function of a microbial glycosphingolipid bound to mouse CD1d. *Proc Natl Acad Sci U S A*. 2006;103:3972-3977.

58. Zajonc DM, Maricic I, Wu D, et al. Structural basis for CD1d presentation of a sulfatide derived from myelin and its implications for autoimmunity. *J Exp Med*. 2005;202:1517-1526.

59. Borg NA, Wun KS, Kjer-Nielsen L, et al. CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor. *Nature*. 2007;448:44-49.

60. Wang J, Li Y, Kinjo Y, et al. Lipid binding orientation within CD1d affects recognition of Borrelia burgorferi antigens by NKT cells. *Proc Natl Acad Sci U S A* . 2010;107:1535-1540.

61. Li Y, Girardi E, Wang J, et al. The Valpha14 invariant natural killer T cell TCR forces microbial glycolipids and CD1d into a conserved binding mode. *J Exp Med*. 2010;207:2383-2393.

62. Pellicci DG, Clarke AJ, Patel O, et al. Recognition of beta-linked self glycolipids mediated by natural killer T cell antigen receptors. *Nat Immunol* . 2011;12:827-833.

63. Pellicci DG, Hammond KJ, Uldrich AP, Baxter AG, Smyth MJ, Godfrey DI. A natural killer T (NKT) cell developmental pathway involving a thymus-dependent NK1.1(-)CD4(+) CD1d-dependent precursor stage. *J Exp Med*. 2002;195:835-844.

64. Benlagha K, Wei DG, Veiga J, Teyton L, Bendelac A. Characterization of the early stages in thymic NKT cell development. *J Exp Med*. 2005;202:485-492.

65. Bezbradica JS, Hill T, Stanic AK, Van Kaer L, Joyce S. Commitment toward the natural T (iNKT) cell lineage occurs at the CD4+8+ stage of thymic ontogeny. *Proc Natl Acad Sci U S A*. 2005;102:5114-5119.

66. Egawa T, Eberl G, Taniuchi I, et al. Genetic evidence supporting selection of the Valpha14i NKT cell lineage from double-positive thymocyte precursors. *Immunity*. 2005;22:705-716.

67. D'Cruz LM, Knell J, Fujimoto JK, Goldrath AW. An essential role for the transcription factor HEB in thymocyte survival, Tcra rearrangement and the development of natural killer T cells. *Nat Immunol* . 2010; 11:240-249.

68. Schumann J, Voyle RB, Wei BY, MacDonald HR. Cutting edge: influence of the TCR V beta domain on the avidity of CD1d:alphagalactosylceramide binding by invariant V alpha 14 NKT cells. *J Immunol* . 2003;170:5815-5819.

69. Chun T, Page MJ, Gapin L, et al. CD1d-expressing dendritic cells but not thymic epithelial cells can mediate negative selection of NKT cells. *J Exp Med*. 2003;197:907-918.

70. Xu H, Chun T, Colmone A, Nguyen H, Wang CR. Expression of CD1d under the control of a MHC class Ia promoter skews the development of NKT cells, but not CD8+ T cells. *J Immunol* . 2003;171:4105-4112.

71. Schumann J, Mycko MP, Dellabona P, Casorati G, MacDonald HR. Cutting edge: influence of the TCR Vbeta domain on the selection of semi-invariant NKT cells by endogenous ligands. *J Immunol* . 2006;176:2064-2068.

72. Dose M, Sleckman BP, Han J, Bredemeyer AL, Bendelac A, Gounari F. Intrathymic proliferation wave essential for Valpha14+ natural killer T cell development depends on c-

Myc. *Proc Natl Acad Sci U S A* . 2009;106:8641-8646.

73. Mycko MP, Ferrero I, Wilson A, et al. Selective requirement for c-Myc at an early stage of V(alpha)14i NKT cell development. *J Immunol* . 2009;182:4641-4648.

74. Crowe NY, Coquet JM, Berzins SP, et al. Differential antitumor immunity mediated by NKT cell subsets in vivo. *J Exp Med*. 2005;202:1279-1288.

75. Lee PT, Benlagha K, Teyton L, Bendelac A. Distinct functional lineages of human valpha24 natural killer T cells. *J Exp Med*. 2002;195:637-641.

76. Gumperz JE, Miyake S, Yamamura T, Brenner MB. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med*. 2002;195:625-636.

77. Stetson DB, Mohrs M, Reinhardt RL, et al. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J Exp Med*. 2003;198:1069-1076.

78. Park SH, Benlagha K, Lee D, Balish E, Bendelac A. Unaltered phenotype, tissue distribution and function of Valpha14(+) NKT cells in germ-free mice. *Eur J Immunol* . 2000;30:620-625.

79. Allende ML, Zhou D, Kalkofen DN, et al. S1P1 receptor expression regulates emergence of NKT cells in peripheral tissues. *FASEB J*. 2008;22:307-315.

80. McNab FW, Berzins SP, Pellicci DG, et al. The influence of CD1d in postselection NKT cell maturation and homeostasis. *J Immunol* . 2005;175:3762-3768.

81. Berzins SP, Cochrane AD, Pellicci DG, Smyth MJ, Godfrey DI. Limited correlation between human thymus and blood NKT cell content revealed by an ontogeny study of paired tissue samples. *Eur J Immunol* . 2005;35:1399-1407.

82. Milpied P, Massot B, Renand A, et al. IL-17-producing invariant NKT cells in lymphoid organs are recent thymic emigrants identified by neuropilin-1 expression. *Blood*. 2011;118:2993-3002.

83. Bendelac A, Killeen N, Littman D, Schwartz RH. A subset of CD4+ thymocytes selected by MHC class I molecules. *Science*. 1994;263:1774-1778.

84. Ohteki T, MacDonald HR. Major histocompatibility complex class I related molecules control the development of CD4+8- and CD4-8-subsets of natural killer 1.1+ T cell receptor-a/b+ cells in the liver of mice. *J Exp Med*. 1994;180:699-704.

85. Coles MC, Raulet DH. NK1.1+ T cells in the liver arise in the thymus and are selected by interactions with class I molecules on CD4+CD8+ cells. *J Immunol* . 2000;164:2412-2418.

86. Forestier C, Park SH, Wei D, Benlagha K, Teyton L, Bendelac A. T cell development in mice expressing CD1d directed by a classical MHC class II promoter. *J Immunol* . 2003;171:4096-4104.

87. Wei DG, Lee H, Park SH, et al. Expansion and long-range differentiation of the NKT cell lineage in mice expressing CD1d exclusively on cortical thymocytes. *J Exp Med*. 2005;202:239-248.

88. Zimmer MI, Colmone A, Felio K, Xu H, Ma A, Wang CR. A cell-type specific CD1d expression program modulates invariant NKT cell development and function. *J Immunol*. 2006;176:1421-1430.

89. Bai L, Constantinides MG, Thomas SY, et al. Distinct antigen-presenting cells expalin the cytokine bias of -a-galactosylceramide variants in vivo. *J Immunol*. 2012188(7):3053-3061.

90. Griewank K, Borowski C, Rietdijk S, et al. Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. *Immunity*. 2007;27:751-762.

91. Gadue P, Stein PL. NK T cell precursors exhibit differential cytokine regulation and require Itk for efficient maturation. *J Immunol*. 2002;169:2397-2406.

92. Eberl G, Lowin-Kropf B, MacDonald HR. Cutting edge: NKT cell development is selectively impaired in Fyn- deficient mice. *J Immunol*. 1999;163:4091-4094.

93. Pasquier B, Yin L, Fondaneche MC, et al. Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. *J Exp Med*. 2005;201:695-701.

94. Nichols KE, Hom J, Gong SY, et al. Regulation of NKT cell development by SAP, the protein defective in XLP. *Nat Med*. 2005;11:340-345.

95. Chung B, Aoukaty A, Dutz J, Terhorst C, Tan R. Signaling lymphocytic activation molecule-associated protein controls NKT cell functions. *J Immunol*. 2005;174:3153-3157.

96. Cannons JL, Qi H, Lu KT, et al. Optimal germinal center responses require a multistage T cell:B cell adhesion process involving integrins, SLAM-associated protein, and CD84. *Immunity*. 2010;32:253-265.

97. Jordan MA, Fletcher JM, Jose R, et al. Role of SLAM in NKT cell development revealed by transgenic complementation in NOD mice. *J Immunol*. 2011;186:3953-3965.

98. Hu T, Gimferrer I, Simmons A, Wiest D, Alberola-Ila J. The Ras/MAPK pathway is required for generation of iNKT cells. *PloS One*. 2011;6:e19890.

99. Lazarevic V, Zullo AJ, Schweitzer MN, et al. The gene encoding early growth response 2, a target of the transcription factor NFAT, is required for the development and maturation of natural killer T cells. *Nat Immunol*. 2009;10:306-313.

100. Seiler MP, Mathew R, Liszewski MK, et al. Elevated and sustained expression of the transcription factors Egr1 and Egr2 controls NKT lineage differentiation in response to TCR signaling. *Nat Immunol*. 2012;13(3):264-271.

101. Shao H, Kono DH, Chen LY, Rubin EM, Kaye J. Induction of the early growth response (Egr) family of transcription factors during thymic selection. *J Exp Med*. 1997;185:731-744.

102. Carter JH, Lefebvre JM, Wiest DL, Tourtellotte WG. Redundant role for early growth response transcriptional regulators in thymocyte differentiation and survival. *J Immunol*. 2007;178:6796-6805.

103. Lawson VJ, Weston K, Maurice D. Early growth response 2 regulates the survival of thymocytes during positive selection. *Eur J Immunol* . 2010;40:232-241.

104. Savage AK, Constantinides MG, Han J, et al. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity*. 2008;29:391-403.

105. Veillette A, Dong Z, Perez-Quintero LA, Zhong MC, Cruz-Munoz ME. Importance and mechanism of 'switch' function of SAP family adapters. *Immunol Rev*. 2009;232:229-239.

106. Cannons JL, Yu LJ, Hill B, et al. SAP regulates T(H)2 differentiation and PKC-theta-mediated activation of NF-kappaB1. *Immunity*. 2004;21:693-706.

107. Schmidt-Supprian M, Tian J, Grant EP, et al. Differential dependence of CD4+CD25+ regulatory and natural killer-like T cells on signals leading to NF-kappaB activation. *Proc Natl Acad Sci U S A* . 2004;101:4566-4571.

108. Stanic AK, Bezbradica JS, Park JJ, Van Kaer L, Boothby MR, Joyce S. Cutting edge: the ontogeny and function of Va14Ja18 natural T lymphocytes require signal processing by protein kinase C theta and NFkappa B. *J Immunol*. 2004;172:4667-4671.

109. Li W, Sofi MH, Rietdijk S, Wang N, Terhorst C, Chang CH. The SLAM-associated protein signaling pathway is required for development of CD4+ T cells selected by homotypic thymocyte interaction. *Immunity*. 2007;27:763-774.

110. Lee YJ, Jeon YK, Kang BH, et al. Generation of PLZF+ CD4+ T cells via MHC class II-dependent thymocyte-thymocyte interaction is a physiological process in humans. *J Exp Med*. 2010;207:237-246.

111. Choi EY, Jung KC, Park HJ, et al. Thymocyte-thymocyte interaction for efficient positive selection and maturation of CD4 T cells. *Immunity*. 2005;23:387-396.

112. Li W, Kim MG, Gourley TS, McCarthy BP, Sant'Angelo DB, Chang CH. An alternate pathway for CD4 T cell development: thymocyteexpressed MHC class II selects a distinct T cell population. *Immunity*. 2005;23:375-386.

113. Wang L, Carr T, Xiong Y, et al. The sequential activity of Gata3 and Thpok is required for the differentiation of CD1d-restricted CD4+ NKT cells. *Eur J Immunol* . 2010;40:2385-2390.

114. Engel I, Hammond K, Sullivan BA, et al. Co-receptor choice by V alpha14i NKT cells is driven by Th-POK expression rather than avoidance of CD8-mediated negative selection. *J Exp Med*. 2010;207:1015-1029.

115. Sarafova SD, Erman B, Yu Q, et al. Modulation of coreceptor transcription during positive selection dictates lineage fate independently of TCR/coreceptor specificity. *Immunity*. 2005;23:75-87.

116. Williams JA, Lumsden JM, Yu X, et al. Regulation of thymic NKT cell development by the B7-CD28 costimulatory pathway. *J Immunol*. 2008;181:907-917.

117. Zheng X, Zhang H, Yin L, Wang CR, Liu Y, Zheng P. Modulation of NKT cell development by B7-CD28 interaction: an expanding horizon for costimulation. *PloS One*. 2008;3:e2703.

118. Kovalovsky D, Uche OU, Eladad S, et al. The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nat Immunol*. 2008;9:1055-1064.

119. Barna M, Hawe N, Niswander L, Pandolfi PP. Plzf regulates limb and axial skeletal patterning. *Nat Genet*. 2000;25:166-172.

120. Buaas FW, Kirsh AL, Sharma M, et al. Plzf is required in adult male germ cells for stem cell self-renewal. *Nat Genet*. 2004;36:647-652.

121. Savage AK, Constantinides MG, Bendelac A. Promyelocytic leukemia zinc finger turns on the effector T cell program without requirement for agonist TCR signaling. *J Immunol*. 2011;186:5801-5806.

122. Kovalovsky D, Alonzo ES, Uche OU, Eidson M, Nichols KE, Sant'Angelo DB. PLZF induces the spontaneous acquisition of memory/effector functions in T cells independently of NKT cell-related signals. *J Immunol*. 2010;184:6746-6755.

123. Raberger J, Schebesta A, Sakaguchi S, et al. The transcriptional regulator PLZF induces the development of CD44 high memory phenotype T cells. *Proc Natl Acad Sci U S A*. 2008;105:17919-17924.

124. Constantinides MG, Picard D, Savage AK, Bendelac A. A naive-like population of human CD1d-restricted T cells expressing intermediate levels of promyelocytic leukemia zinc finger. *J Immunol*. 2011;187:309-315.

125. Intlekofer AM, Takemoto N, Wherry EJ, et al. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat Immunol*. 2005;6:1236-1244.

126. Matsuda JL, Zhang Q, Ndonye R, Richardson SK, Howell AR, Gapin L. T-bet concomitantly controls migration, survival, and effector functions during the development of Valpha14i NKT cells. *Blood*. 2006;107:2797-2805.

127. Townsend MJ, Weinmann AS, Matsuda JL, et al. T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. *Immunity*. 2004;20:477-494.

128. Lacorazza HD, Miyazaki Y, Di Cristofano A, et al. The ETS protein MEF plays a critical role in perforin gene expression and the development of natural killer and NK-T cells. *Immunity*. 2002;17:437-449.

129. Kim PJ, Pai SY, Brigl M, Besra GS, Gumperz J, Ho IC. GATA-3 regulates the development and function of invariant NKT cells. *J Immunol*. 2006;177:6650-6659.

130. Doisne JM, Bartholin L, Yan KP, et al. iNKT cell development is orchestrated by different branches of TGF-beta signaling. *J Exp Med*. 2009;206:1365-1378.

131. Stanic AK, Bezbradica JS, Park JJ, et al. NF-kappa B controls cell fate specification, survival, and molecular differentiation of immunoregulatory natural T lymphocytes. *J Immunol*. 2004;172:2265-2273.

132. Stankovic S, Gugasyan R, Kyparissoudis K, et al. Distinct roles in NKT cell maturation and function for the different transcription factors in the classical NF-kappaB pathway. *Immunol Cell Biol*. 2011;89: 294-303.

133. Sivakumar V, Hammond KJ, Howells N, Pfeffer K, Weih F. Differential requirement for Rel/nuclear factor kappa B family members in natural killer T cell development. *J Exp Med*. 2003;197:1613-1621.

134. Vallabhapurapu S, Powolny-Budnicka I, Riemann M, et al. Rel/NFkappaB family member RelA regulates NK1.1- to NK1.1+ transition as well as IL-15-induced expansion of NKT cells. *Eur J Immunol*. 2008;38:3508-3519.

135. Iizuka K, Chaplin DD, Wang Y, et al. Requirement for membrane lymphotoxin in natural killer cell development. *Proc Natl Acad Sci U S A*. 1999;96:6336-6340.

136. Franki AS, Van Beneden K, Dewint P, et al. A unique lymphotoxin {alpha}beta-dependent pathway regulates thymic emigration of V{alpha}14 invariant natural killer T cells. *Proc Natl Acad Sci U S A*. 2006;103:9160-9165.

137. Fedeli M, Napolitano A, Wong MP, et al. Dicer-dependent microRNA pathway controls invariant NKT cell development. *J Immunol*. 2009;183:2506-2512.

138. Zhou L, Seo KH, He HZ, et al. Tie2cre-induced inactivation of the miRNA-processing enzyme Dicer disrupts invariant NKT cell development. *Proc Natl Acad Sci U S A*. 2009;106:10266-10271.

139. Seo KH, Zhou L, Meng D, Xu J, Dong Z, Mi QS. Loss of microRNAs in thymus perturbs invariant NKT cell development and function. *Cell Mol Immunol*. 2010;7:447-453.

140. Bezman NA, Chakraborty T, Bender T, Lanier LL. miR-150 regulates the development of NK and iNKT cells. *J Exp Med*. 2011;208:2717-2731.

141. Gerber DJ, Azuara V, Levraud JP, Huang SY, Lembezat MP, Pereira P. IL-4-producing gamma delta T cells that express a very restricted TCR repertoire are preferentially localized in liver and spleen. *J Immunol*. 1999;163:3076-3082.

142. Kreslavsky T, Savage AK, Hobbs R, et al. TCR-inducible PLZF transcription factor required for innate phenotype of a subset of gammadelta T cells with restricted TCR diversity. *Proc Natl Acad Sci U S A*. 2009;106:12453-12458.

143. Alonzo ES, Gottschalk RA, Das J, et al. Development of promyelocytic zinc finger and ThPOK-expressing innate gamma delta T cells is controlled by strength of TCR signaling and Id3. *J Immunol*. 2010;184:1268-1279.

144. Felices M, Yin CC, Kosaka Y, Kang J, Berg LJ. Tec kinase Itk in gammadeltaT cells is pivotal for controlling IgE production in vivo. *Proc Natl Acad Sci U S A* . 2009;106:8308-8313.

145. Dusseaux M, Martin E, Serriari N, et al. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood*. 2011;117:1250-1259.

146. Atherly LO, Lucas JA, Felices M, Yin CC, Reiner SL, Berg LJ. The tec family tyrosine kinases itk and rlk regulate the development of conventional CD8(+) T cells. *Immunity*. 2006;25:79-91.

147. Broussard C, Fleischecker C, Horai R, et al. Altered development of CD8(+) T cell lineages in mice deficient for the tec kinases itk and rlk. *Immunity*. 2006;25:93-104.

148. Weinreich MA, Odumade OA, Jameson SC, Hogquist KA. T cells expressing the transcription factor PLZF regulate the development of memory-like CD8+ T cells. *Nat Immunol*. 2010;11:709-716.

149. Verykokakis M, Boos MD, Bendelac A, Adams EJ, Pereira P, Kee BL. Inhibitor of DNA binding 3 limits development of murine slam-associated adaptor protein-dependent “innate” gammadelta T cells. *PloS One*. 2010;5:e9303.

150. Verykokakis M, Boos MD, Bendelac A, Kee BL. SAP protein-dependent natural killer T-like cells regulate the development of CD8(+) T cells with innate lymphocyte characteristics. *Immunity*. 2010;33:203-215.

151. Fukuyama T, Kasper LH, Boussouar F, Jeevan T, van Deursen J, Brindle PK. Histone acetyltransferase CBP is vital to demarcate conventional and innate CD8+ T-cell development. *Mol Cell Biol* . 2009;29: 3894-3904.

152. Weinreich MA, Takada K, Skon C, Reiner SL, Jameson SC, Hogquist KA. KLF2 transcription-factor deficiency in T cells results in unrestrained cytokine production and upregulation of bystander chemokine receptors. *Immunity*. 2009;31:122-130.

153. Lai D, Zhu J, Wang T, et al. KLF13 sustains thymic memory-like CD8(+) T cells in BALB/c mice by regulating IL-4-generating invariant natural killer T cells. *J Exp Med*. 2011;208:1093-1103.

154. Ohteki T, Ho S, Suzuki H, Mak TW, Ohashi PS. Role for IL-15/IL-15 receptor beta-chain in natural killer 1.1+ T cell receptor-alpha beta+ cell development. *J Immunol* . 1997;159:5931-5935.

155. Kennedy MK, Glaccum M, Brown SN, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice [see comments]. *J Exp Med*. 2000;191:771-780.

156. Matsuda JL, Gapin L, Sidobre S, et al. Homeostasis of V alpha 14i NKT cells. *Nat*

Immunol. 2002;3:966-974.

157. Castillo EF, Acero LF, Stonier SW, Zhou D, Schluns KS. Thymic and peripheral microenvironments differentially mediate development and maturation of iNKT cells by IL-15 transpresentation. *Blood*. 2010;116:2494-2503.

158. Geissmann F, Cameron TO, Sidobre S, et al. Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. *PLoS Biol*. 2005;3:e113.

159. Wei B, Wingender G, Fujiwara D, et al. Commensal microbiota and CD8+ T cells shape the formation of invariant NKT cells. *J Immunol*. 2010;184:1218-1226.

160. Nieuwenhuis EE, Matsumoto T, Lindenbergh D, et al. Cd1d-dependent regulation of bacterial colonization in the intestine of mice. *J Clin Invest*. 2009;119:1241-1250.

161. Thomas SY, Scanlon ST, Griewank KG, et al. PLZF induces an intravascular surveillance program mediated by long-lived LFA-1-ICAM-1 interactions. *J Exp Med*. 2011;208:1179-1188.

162. Paust S, Gill HS, Wang BZ, et al. Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. *Nat Immunol*. 2010;11:1127-1135.

163. Lee WY, Moriarty TJ, Wong CH, et al. An intravascular immune response to Borrelia burgdorferi involves Kupffer cells and iNKT cells. *Nat Immunol*. 2010;11:295-302.

164. Doisne JM, Becourt C, Amniai L, et al. Skin and peripheral lymph node invariant NKT cells are mainly retinoic acid receptor-related orphan receptor (gamma)t+ and respond preferentially under inflammatory conditions. *J Immunol*. 2009;183:2142-2149.

165. Doisne JM, Soulard V, Becourt C, et al. Cutting edge: crucial role of IL-1 and IL-23 in the innate IL-17 response of peripheral lymph node NK1.1- invariant NKT cells to bacteria. *J Immunol*. 2011;186:662-666.

166. Coquet JM, Chakravarti S, Kyparissoudis K, et al. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. *Proc Natl Acad Sci U S A* . 2008;105:11287-11292.

167. Terashima A, Watarai H, Inoue S, et al. A novel subset of mouse NKT cells bearing the IL-17 receptor B responds to IL-25 and contributes to airway hyperreactivity. *J Exp Med*. 2008;205:27272733.

168. Tomura M, Yu WG, Ahn HJ, et al. A novel function of Valpha14+CD4+NKT cells: stimulation of IL-12 production by antigen-presenting cells in the Innate Immune System. *J Immunol*. 1999;163:93-101.

169. Kitamura H, Iwakabe K, Yahata T, et al. The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)- 12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J Exp Med*. 1999;189:11211128.

170. Semmling V, Lukacs-Kornek V, Thaiss CA, et al. Alternative crosspriming through CCL17-CCR4-mediated attraction of CTLs toward NKT cell-licensed DCs. *Nat Immunol*. 2010;11:313-320.

171. Carnaud C, Lee D, Donnars O, et al. Cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J Immunol (Cutting Edge)* . 1999;163:4647-4650.

172. Eberl G, MacDonald HR. Selective induction of NK cell proliferation and cytotoxicity by activated NKT cells. *Eur J Immunol* . 2000;30: 985-992.

173. Gonzalez-Aseguinolaza G, Van Kaer L, Bergmann CC, et al. Natural killer T cell ligand alpha-galactosylceramide enhances protective immunity induced by malaria vaccines. *J Exp Med*. 2002;195:617-624.

174. Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med*. 2003;198:267279.

175. Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med*. 2004;199:1607-1618.

176. Silk JD, Hermans IF, Gileadi U, et al. Utilizing the adjuvant properties of CD1d-dependent NK T cells in T cell-mediated immunotherapy. *J Clin Invest*. 2004;114:1800-1811.

177. Miyamoto K, Miyake S, Yamamura T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature*. 2001;413:531-534.

178. Goff RD, Gao Y, Mattner J, et al. Effects of lipid chain lengths in alphagalactosylceramides on cytokine release by natural killer T cells. *J Am Chem Soc*. 2004;126:13602-13603.

179. Yu KO, Im JS, Molano A, et al. Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of alpha-galactosylceramides. *Proc Natl Acad Sci U S A* . 2005;102:3383-3388.

180. Oki S, Chiba A, Yamamura T, Miyake S. The clinical implication and molecular mechanism of preferential IL-4 production by modified glycolipid-stimulated NKT cells. *J Clin Invest*. 2004;113:1631-1640.

181. Wun KS, Cameron G, Patel O, et al. A molecular basis for the exquisite CD1d-restricted antigen specificity and functional responses of natural killer T cells. *Immunity*. 2011;34:327-339.

182. Im JS, Arora P, Bricard G, et al. Kinetics and cellular site of glycolipid loading control the outcome of natural killer T cell activation. *Immunity*. 2009;30:888-898.

183. Eberl G, MacDonald HR. Rapid death and regeneration of NKT cells in anti-CD3epsilon- or IL-12- treated mice: a major role for bone marrow in NKT cell homeostasis. *Immunity*. 1998;9:345-353.

184. Uldrich AP, Crowe NY, Kyparissoudis K, et al. NKT cell stimulation with glycolipid antigen in vivo: costimulation-dependent expansion, Bim-dependent contraction, and hyporesponsiveness to further antigenic challenge. *J Immunol*. 2005;175:3092-3101.

185. Parekh VV, Wilson MT, Olivares-Villagomez D, et al. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest*. 2005;115:2572-2583.

186. Wang J, Cheng L, Wondimu Z, Swain M, Santamaria P, Yang Y. Cutting edge: CD28 engagement releases antigen-activated invariant NKT cells from the inhibitory effects of PD-1. *J Immunol*. 2009;182: 6644-6647.

187. Parekh VV, Lalani S, Kim S, et al. PD-1/PD-L blockade prevents anergy induction and enhances the anti-tumor activities of glycolipidactivated invariant NKT cells. *J Immunol*. 2009;182:2816-2826.

188. Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFNgamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol*. 2002;3:867-874.

189. Chang DH, Osman K, Connolly J, et al. Sustained expansion of NKT cells and antigen-specific T cells after injection of alpha-galactosylceramide loaded mature dendritic cells in cancer patients. *J Exp Med*. 2005;201:1503-1517.

190. Thapa P, Zhang G, Xia C, et al. Nanoparticle formulated alpha-galactosylceramide activates NKT cells without inducing anergy. *Vaccine*. 2009;27:3484-3488.

191. Tonti E, Galli G, Malzone C, Abrignani S, Casorati G, Dellabona P. NKT-cell help to B lymphocytes can occur independently of cognate interaction. *Blood*. 2009;113:370-376.

192. Lang GA, Exley MA, Lang ML. The CD1d-binding glycolipid alphagalactosylceramide enhances humoral immunity to T-dependent and T-independent antigen in a CD1d-dependent manner. *Immunol*. 2006;119:116-125.

193. Galli G, Nuti S, Tavarini S, et al. CD1d-restricted help to B cells by human invariant natural killer T lymphocytes. *J Exp Med*. 2003;197:1051-1057.

194. Leadbetter EA, Brigl M, Illarionov P, et al. NK T cells provide lipid antigen-specific cognate help for B cells. *Proc Natl Acad Sci U S A* . 2008;105:8339-8344.

195. Barral P, Eckl-Dorna J, Harwood NE, et al. B cell receptor-mediated uptake of CD1d-restricted antigen augments antibody responses by recruiting invariant NKT cell help in vivo. *Proc Natl Acad Sci U S A* . 2008;105:8345-8350.

196. Chang PP, Barral P, Fitch J, et al. Identification of Bcl-6-dependent follicular helper NKT cells that provide cognate help for B cell responses. *Nat Immunol*. 2012;13:35-43.

197. King IL, Fortier A, Tighe M, et al. Invariant natural killer T cells direct B cell

responses to cognate lipid antigen in an IL-21-dependent manner. *Nat Immunol*. 2012;13:44-50.

198. Mattner J, Savage PB, Leung P, et al. Liver autoimmunity triggered by microbial activation of natural killer T cells. *Cell Host Microbe*. 2008;3:304-315.

199. Oliver AM, Martin F, Gartland GL, Carter RH, Kearney JF. Marginal zone B cells exhibit unique activation, proliferative and immunoglobulin secretory responses. *Eur J Immunol*. 1997;27:2366-2374.

200. Muppidi JR, Arnon TI, Bronevetsky Y, et al. Cannabinoid receptor 2 positions and retains marginal zone B cells within the splenic marginal zone. *J Exp Med*. 2011;208:1941-1948.

201. Barral P, Polzella P, Bruckbauer A, et al. CD169(+) macrophages present lipid antigens to mediate early activation of iNKT cells in lymph nodes. *Nat Immunol*. 2010;11:303-312.

202. Terabe M, Matsui S, Noben-Trauth N, et al. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat Immunol*. 2000;1:515-520.

203. Terabe M, Swann J, Ambrosino E, et al. A nonclassical non-Valpha14Jalpha18 CD1d-restricted (type II) NKT cell is sufficient for down-regulation of tumor immunosurveillance. *J Exp Med*. 2005;202:1627-1633.

204. De Santo C, Salio M, Masri SH, et al. Invariant NKT cells reduce the immunosuppressive activity of influenza A virus-induced myeloidderived suppressor cells in mice and humans. *J Clin Invest*. 2008; 118(12):4036-4048.

205. Kawasaki S, Moriguchi R, Sekiya K, et al. The cell envelope structure of the lipopolysaccharide-lacking gram-negative bacterium Sphingomonas paucimobilis. *J Bacteriol*. 1994;176:284-290.

206. Sriram V, Du W, Gervay-Hague J, Brutkiewicz RR. Cell wall glycosphingolipids of Sphingomonas paucimobilis are CD1d-specific ligands for NKT cells. *Eur J Immunol*. 2005;35:1692-1701.

207. Wu D, Xing GW, Poles MA, et al. Bacterial glycolipids and analogs as antigens for CD1d-restricted NKT cells. *Proc Natl Acad Sci U S A*. 2005;102:1351-1356.

208. Brigl M, Tatituri RV, Watts GF, et al. Innate and cytokine-driven signals, rather than microbial antigens, dominate in natural killer T cell activation during microbial infection. *J Exp Med*. 2011;208:1163-1177.

209. Tupin E, Benhnia MR, Kinjo Y, et al. NKT cells prevent chronic joint inflammation after infection with Borrelia burgdorferi. *Proc Natl Acad Sci U S A*. 2008;105:19863-19868.

210. Cui J, Shin T, Kawano T, et al. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science*. 1997;278:1623-1626.

211. Park SH, Kyin T, Bendelac A, Carnaud C. The contribution of NKT cells, NK cells, and other gamma-chain-dependent non-T non-B cells to IL-12-mediated rejection of tumors. *J Immunol*. 2003;170:1197-1201.

212. Nieuwenhuis EE, Matsumoto T, Exley M, et al. CD1d-dependent macrophage-mediated clearance of Pseudomonas aeruginosa from lung. *Nat Med*. 2002;8:588-593.

213. Muhlen KA, Schumann J, Wittke F, et al. NK cells, but not NKT cells, are involved in Pseudomonas aeruginosa exotoxin A-induced hepatotoxicity in mice. *J Immunol*. 2004;172:3034-3041.

214. Dieli F, Sireci G, Russo D, et al. Resistance of natural killer T cell-deficient mice to systemic Shwartzman reaction. *J Exp Med*. 2000;192:1645-1652.

215. Emoto M, Miyamoto M, Yoshizawa I, et al. Critical role of NK cells rather than V alpha 14(+)NKT cells in lipopolysaccharide-induced lethal shock in mice. *J Immunol*. 2002;169:1426-1432.

216. Schofield L, McConville MJ, Hansen D, et al. CD1d-restricted immunoglobulin G formation to GPI-anchored antigens mediated by NKT cells. *Science*. 1999;283:225-229.

217. Molano A, Park SH, Chiu YH, Nosseir S, Bendelac A, Tsuji M. Cutting edge: the IgG response to the circumsporozoite protein is MHC class Ildependent and CD1d-independent: exploring the role of GPIs in NK T cell activation and antimalarial responses. *J Immunol*. 2000;164:5005-5009.

218. Romero JF, Eberl G, MacDonald H, Corradin G. CD1d-restricted NK T cells are dispensable for specific antibody responses and protective immunity against liver stage malaria infection in mice. *Parasite Immunol*. 2001;23:267-269.

219. Mallevaey T, Zanetta JP, Faveeuw C, et al. Activation of invariant NKT cells by the helminth parasite Schistosoma mansoni. *J Immunol*. 2006;176:2476-2485.

220. Ilyinskii PO, Wang R, Balk SP, Exley MA. CD1d mediates T-celldependent resistance to secondary infection with encephalomyocarditis virus (EMCV) in vitro and immune response to EMCV infection in vivo. *J Virol*. 2006;80:7146-7158.

221. Huber S, Sartini D, Exley M. Role of CD1d in coxsackievirus B3-induced myocarditis. *J Immunol*. 2003;170:3147-3153.

222. Paget C, Ivanov S, Fontaine J, et al. Potential role of invariant NKT cells in the control of pulmonary inflammation and CD8+ T cell response during acute influenza A virus H3N2 pneumonia. *J Immunol*. 2011;186:5590-5602.

223. De Santo C, Salio M, Masri SH, et al. Invariant NKT cells reduce the immunosuppressive activity of influenza A virus-induced myeloid-derived suppressor cells in mice and humans. *J Clin Invest*. 2008;118:4036-4048.

224. de Lalla C, Galli G, Aldrighetti L, et al. Production of profibrotic cytokines by invariant NKT cells characterizes cirrhosis progression in chronic viral hepatitis. *J Immunol*.

2004;173:1417-1425.

225. Durante-Mangoni E, Wang R, Shaulov A, et al. Hepatic CD1d expression in hepatitis C virus infection and recognition by resident proinflammatory CD1d-reactive T cells. *J Immunol*. 2004;173:2159-2166.

226. Grubor-Bauk B, Simmons A, Mayrhofer G, Speck PG. Impaired clearance of herpes simplex virus type 1 from mice lacking CD1d or NKT cells expressing the semivariant V alpha 14-J alpha 281 TCR. *J Immunol*. 2003;170:1430-1434.

227. Cornish AL, Keating R, Kyparissoudis K, Smyth MJ, Carbone FR, Godfrey DI. NKT cells are not critical for HSV-1 disease resolution. *Immunol Cell Biol*. 2006;84:13-19.

228. Yuan W, Dasgupta A, Cresswell P. Herpes simplex virus evades natural killer T cell recognition by suppressing CD1d recycling. *Nat Immunol*. 2006;7:835-842.

229. Sanchez DJ, Gumperz JE, Ganem D. Regulation of CD1d expression and function by a herpesvirus infection. *J Clin Invest*. 2005;115: 1369-1378.

230. Thomas SY, Chyung YH, Luster AD. Natural killer T cells are not the predominant T cell in asthma and likely modulate, not cause, asthma. *J Allergy Clin Immunol*. 2010;125:980-984.

231. Wingender G, Rogers P, Batzer G, et al. Invariant NKT cells are required for airway inflammation induced by environmental antigens. *J Exp Med*. 2011;208:1151-1162.

232. Scanlon ST, Thomas SY, Ferreira CM, et al. Airborne lipid antigens mobilize resident intravascular NKT cells to induce allergic airway inflammation. *J Exp Med*. 2011;208:2113-2124.

233. Wu L, Van Kaer L. Natural killer T cells and autoimmune disease. *Curr Mol Med*. 2009;9:4-14.

234. Kaplan MM, Gershwin ME. Primary biliary cirrhosis. *New Engl J Med*. 2005;353:1261-1273.

235. Selmi C, Balkwill DL, Invernizzi P, et al. Patients with primary biliary cirrhosis react against a ubiquitous xenobiotic-metabolizing bacterium. *Hepatol*. 2003;38:1250-1257.

236. Kita H, Naidenko OV, Kronenberg M, et al. Quantitation and phenotypic analysis of natural killer T cells in primary biliary cirrhosis using a human CD1d tetramer. *Gastroenterol*. 2002;123:1031-1043.

237. Berzofsky JA, Terabe M. The contrasting roles of NKT cells in tumor immunity. *Curr Mol Med*. 2009;9:667-672.

238. Smyth MJ, Thia KY, Street SE, et al. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med*. 2000;191:661-668.

239. Swann JB, Uldrich AP, van Dommelen S, et al. Type I natural killer T cells suppress tumors caused by p53 loss in mice. *Blood*. 2009;113:6382-6385.

240. Bellone M, Ceccon M, Grioni M, et al. iNKT cells control mouse spontaneous carcinoma independently of tumor-specific cytotoxic T cells. *PloS One*. 2010;5:e8646.

241. Kammertoens T, Qin Z, Briesemeister D, Bendelac A, Blankenstein T. Methylcholanthrene-induced carcinogenesis is promoted by B-cells and IL-4 but there is no evidence for a role of T / NKT-cells and their effector molecules (Fas-ligand, TNF-alpha, Perforin). *Int J Cancer* . 2011. [Epub ahead of print.]

242. Li S, Choi HJ, Felio K, Wang CR. Autoreactive CD1b-restricted T cells: a new innate-like T-cell population that contributes to immunity against infection. *Blood*. 2011;118:3870-3878.

243. de Lalla C, Lepore M, Piccolo FM, et al. High-frequency and adaptivelike dynamics of human CD1 self-reactive T cells. *Eur J Immunol* . 2011;41:602-610.

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Chapter 19

Macrophages and Phagocytosis

Siamon Gordon

INTRODUCTION

Macrophages (Mø) represent a family of mononuclear leukocytes that are widely distributed throughout the body within and outside of lymphohemopoietic organs. They vary considerably in lifespan and phenotype, depending on their origin and local microenvironment. Mature Mø are highly phagocytic, relatively long-lived cells that are adaptable in their biosynthetic responses to antigens and microbial stimuli. The functions of Mø within tissues are homeostatic, regulating the local and systemic milieu through diverse plasma membrane receptors and varied secretory products. They react to, and themselves generate, signals that influence growth, differentiation, and death of other cells, recognizing and engulfing senescent and abnormal cells. These activities contribute substantially to recognition and defense functions against invading microorganisms, foreign particulates, and other immunogens. Innate immune functions of Mø complement their contributions to acquired humoral and cellular immunity, in which they regulate activation of T-and B-lymphocytes; this is achieved in part through their specialized derivatives, dendritic cells (DCs) of myeloid origin. Mø, with or without DCs, process and present antigen; produce chemokines and cytokines such as interleukin (IL)-1, IL-6, IL-12, IL-18, IL-23, tumor necrosis factor (TNF)-α, IL-10, and TGFβ; and phagocytose apoptotic and necrotic cells. Acting directly or under the influence of other immune cells, Mø capture extra- and intracellular pathogens, eliminate invaders, and deliver them to appropriate subcompartments of lymphoid organs. As key regulators of the specific as well as the natural immune response, Mø boost as well as limit induction and effector mechanisms of the specific immune response by positive and negative feedback.

The properties and roles of DCs, especially in antigen presentation, are described in detail elsewhere in this volume. Here, we focus on other members of the Mø lineage, consider their interrelationship, and outline specialized properties that underlie their roles in the execution and regulation of immune responses. A number of texts and presentations deal with the history and broad aspects of Mø immunobiology.[1,2,3,4,5,6,7,8]

SOME LANDMARKS IN THE STUDY OF MACROPHAGES

Our understanding of Mø developed in parallel with the growth of immunology as an experimental science. Metchnikoff, a comparative developmental zoologist, is widely credited for his recognition of phagocytosis and leukocyte recruitment as a fundamental host defense mechanisms of primitive, as well as highly developed multicellular organisms.[3,4,7] The Nobel awards of 2011 to Bruce Beutler,[9] Jules Hoffman,[10] and Ralph Steinman[11] reflect the paradigm shift of immune recognition from lymphocytes to innate antigen-presenting cells (APCs). Metchnikoff already clearly stated the link between capture of infectious microorganisms by the spleen and subsequent appearance of reactive substances (antibodies) in the blood, although mistakenly ascribing their production to the phagocytes themselves. The importance of systemic clearance of particles by Mø, especially Kupffer cells in liver and other endothelial cells, was enshrined in the term reticuloendothelial system. Although it was rejected by influential investigators in the field in favor of the term mononuclear phagocyte system, the appreciation that sinuslining Mø in liver and elsewhere

share common properties with selected endothelial cells is worth preserving. 3 Earlier studies by Florey and his students, including Gowans, established that circulating monocytes give rise to tissue Mø. Van Furth and his colleagues investigated the life history of Mø by kinetic labeling methods; subsequently, the development of membrane antigen markers facilitated a more precise definition of specialized Mø subpopulations in tissues such as brain. The appearance and potential importance of Mø during development also became evident as a result of sensitive immunocytochemical methods. Morphologic and functional studies by Humphrey and many others drew attention to striking diversity among Mø-like cells in secondary lymphoid organs, especially within the marginal zone of the spleen, where complex particulates and polysaccharides are captured from the circulation.

The era of modern cell biology impinged on Mø studies following the studies of Cohn,[12] Hirsch, and their colleagues. Their work touched on many aspects of cell structure and function, including phagocytosis (the zipper mechanism of Silverstein), fluid- and receptor-mediated endocytosis, secretion, and antimicrobial resistance. Isolation and in vitro culture systems became available for cells from mice and humans, especially after the identification of specific growth and differentiation factors such as colony-stimulating factor-1 (CSF-1). It is perhaps fitting that the earliest known natural knockout (ko) affecting Mø, a natural mutation in the op gene in the osteopetrotic mouse, should involve CSF-1.[13] Cell lines retaining some but not all features of

mature Mø have been useful for many biochemical and cellular studies. Macrophages and dendritic cells can be derived from embryonic stem cells and induced pluripotent cells by growth in appropriate culture conditions and transfection of selected transcription factors.

The role of Mø as antigen-processing cells able to initiate adaptive immune responses had false trails (“immunogenic ribonucleic acid [RNA]” was thought to be involved at one time) and encompassed early genetic strategies (Mø of mice selected for high antisheep erythrocyte antibody responses by Biozzi and colleagues displayed enhanced degradative properties; adherent cells from defined guinea pig strains were shown to play an important role in major histocompatibility complex [MHC] Ia-restricted antiinsulin responses). For many years, the APC functions of adherent cells were highly controversial as promoted by Unanue, who concentrated on intracellular processing by Mø, and Steinman, who discovered the specialized role of “DCs” in antigen presentation to naive T-lymphocytes. The importance of Mø as effector cells in immunity to intracellular pathogens such as *Mycobacterium tuberculosis* was recognized early by Lurie and Dannenberg. Mackaness used *Listeria monocytogenes* and bacille Calmette-Guérin (BCG) infection in experimental models and developed the concept of Mø activation as an antigen-dependent but immunologically nonspecific enhancement of antimicrobial resistance. The subsequent delineation of T-lymphocyte subsets and characterization of interferon (IFN)-*γ* as the major cytokine involved in macrophage activation, including MHC II induction, merged with increasing knowledge of the role of reactive oxygen and, later, nitrogen metabolites as cytotoxic agents. The role of virus-infected Mø as MHC I-restricted targets for antigen-specific CD8+ killer cells was part of the initial characterization of this phenomenon by Zinkernagel and Doherty. D'Arcy Hart was an early investigator of the intracellular interactions between Mø and invaders of the vacuolar system, especially mycobacteria, which survive within Mø by inhibiting acidification and phagosomelysosome fusion, thus evading host resistance mechanisms. Mouse breeding studies by several groups defined a common genetic locus involved in resistance to BCG, *Leishmania*, and *Salmonella* organisms. The host phenotype was shown to depend on expression in Mø and, many years later, the gene (termed N-ramp for natural resistance-associated membrane protein) was identified by positional cloning by Skamene, Gros, and their colleagues. Positional cloning by Beutler and associates led to the identification of the gene responsible for lipopolysaccharide (LPS) resistance in particular mouse strains. Together with studies by Hoffmann and his colleagues on the toll pathway in *Drosophila*, this work resulted in an explosion of interest in the identification of mammalian tolllike receptors (TLRs) and their role in innate immunity to infection. At the same time, it became apparent that some malignant tumors contain macrophage populations that may favor their growth.

This brief survey concludes with the identification of Mø as key target cells for infection, dissemination, and persistence of human immunodeficiency virus (HIV), tropic for Mø by virtue of their expression of cluster of differentiation (CD)4, chemokine coreceptors, and DCSIGN, a C-type lectin also expressed by DCs. Although Mø had been implicated by earlier workers such as Mims as important in antiviral resistance generally, their role in this regard was neglected before the emergence of HIV as a major pathogen.

Many molecules have been identified as important in Mø functions in immunity and serve as valuable markers to study their properties in mice and humans. These include Fc and complement receptors, which are important in opsonic phagocytosis, killing, and immunoregulation; scavenger receptors originally implicated in foam cell formation and atherogenesis by Brown and Goldstein; nonopsonic lectin receptors, such as the mannose receptor (MR) and *β*-glucan receptor (dectin-1) and secretory products such as lysozyme, neutral proteinases, TNFα, chemokines, and many other cytokines. A range of membrane antigens expressed by human and rodent mononuclear phagocytes has been characterized and reagents made available for further study of Mø in normal and diseased states. Recently, the role of deoxyribonucleic acid (DNA)-binding transcription factors including members of the NF-*κ*B and ETS (Pu-1) families has received increased attention in the study of differential gene expression by Mø. Gene inactivation has confirmed the important role of many of these molecules within the intact host, and use has been made of cell-specific or conditional ko to uncover the role of Mø in immunologic processes. Naturally occurring inborn errors in humans such as the leukocyte adhesion deficiency syndrome and chronic granulomatous disease have contributed to the analysis of important leukocyte functions, including those of Mø, in host resistance to infection. Mutations in a monocyte-expressed gene (nucleotide oligomerization domain [NOD]-2), involved in cytosolic sensing of microbial products and NF-*κ*B activation, have been implicated in a subset of individuals with an enhanced susceptibility to Crohn disease. The validity of murine ko models for human genetic deficiencies has been confirmed for key molecules involved in Mø activation, such as IFNγ and IL-12. *N*-ethyl *N*-nitrosourea mutagenesis has begun to reveal new macrophage innate immune functions, as has increasing application of system biology tools for microarray, proteomic, epigenetic, and microRNA analysis.

PROPERTIES OF MACROPHAGES AND THEIR RELATION TO IMMUNE FUNCTIONS

Introduction

Mø participate in the production, mobilization, activation, and regulation of all immune effector cells. They interact reciprocally with other cells while their own properties are modified to perform specialized immunologic functions. As a result of cell surface and auto- and paracrine interactions, Mø display marked heterogeneity in phenotype,[14,15] a source of interest and considerable confusion to the investigator. Increasing knowledge of cellular and molecular properties of Mø bears strongly on our understanding of their role in the immune response. These will be reviewed

briefly, with emphasis on functional significance, and attention will be drawn to unresolved and controversial issues.

Growth and Differentiation: Life History and Turnover

In contrast to T- and B-lymphocytes, monocytes from blood give rise to terminally differentiated Mø that cannot recirculate or reinitiate DNA replication except in a limited way. Unlike other myeloid granulocytic cells, Mø can be long lived and retain the ability to synthesize RNA and protein to a marked extent, even when in a relatively quiescent state as “resident” cells. These are distributed throughout the tissues of the body and constitute a possible alarm-response system, but they also mediate homeostatic and poorly understood trophic functions. Following inflammatory and immune stimuli, many more monocytes can be recruited to local sites and give rise to “elicited” or “immunologically activated” Mø with

altered surface, secretory, and cytotoxic properties. The origins of Mø from precursors are well known: from yolk sac (and possibly earlier paraaortic progenitors), migrating to fetal liver, then spleen and bone marrow, before and after birth.[16] Yolk sac precursor cells may contribute to the establishment of selected tissue macrophages such as Langerhans cells in the adult.[17] In the fetus, mature Mø proliferate actively during tissue remodeling in developing organs. In the normal adult, tissue Mø do not self-renew extensively except in specialized microenvironments such as epidermis,[18] nervous system, or lung; after TH2-type parasitic infection, there can be considerable further replication at local sites of inflammation.[19] Growth and differentiation are tightly regulated by specific growth factors and their receptors (eg, IL-3, CSF-1, granulocyte-macrophage [GM]-CSF/IL-34, IL-4, IL-13) and inhibitors (eg, IFNα/β, transforming growth factor [TGF]-β, leukemia inhibitory factor), which vary considerably in their potency and selectivity. These processes are modulated by interactions with adjacent stromal and other cells (eg, through c-kit/ligand and Flt-3/ligand interactions). The growth-response of the target cell to an extrinsic stimulus decreases progressively and markedly (from 10^8 or more to 10^0) during differentiation from stem cell to committed precursor to monoblast, monocyte, and Mø, yet even the most terminally differentiated Mø such as microglial cells can be "reactivated" to a limited extent by local stimuli. Elicited/activated Mø respond more vigorously than resident Mø to growth stimuli in vivo and in vitro, but the molecular basis for their enhanced proliferation is unknown.

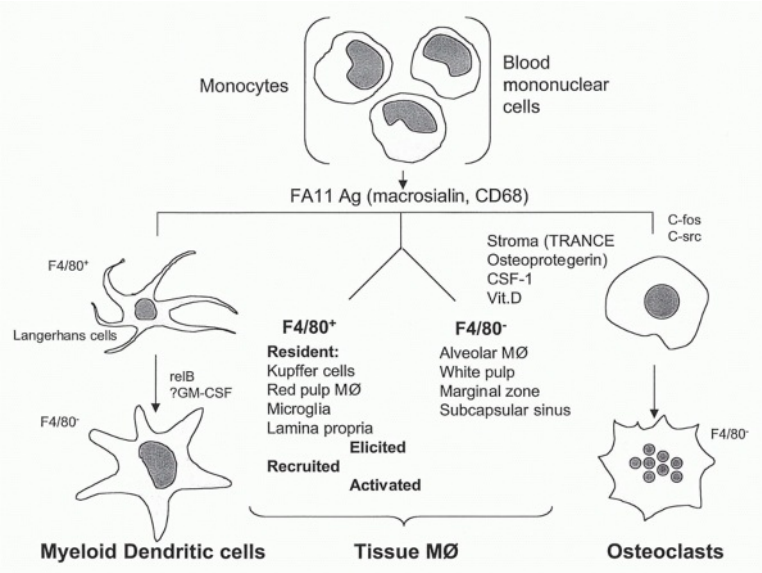

FIG. 19.1. Differentiation of mononuclear phagocytes based on antigen markers FA-11 (macrosialin, murine CD68) and F4/80. See text for detailed discussion.

Although this general picture of blood monocyte-totissue Mø differentiation has been accepted for some time as a result of parabiosis, adoptive transfer, and irradiation-reconstitution experiments, recent studies in mouse and man have demonstrated monocyte heterogeneity and distinct properties,[20,21,22,23] with a subpopulation remaining within the vasculature, to perform a patrolling function. Our understanding of DCs and osteoclast differentiation is still compatible with a relatively simple model (Fig. 19.1) in which major Mø populations in mouse tissues can be characterized by selected antigen markers such as F4/80 (Emrl,

a member of a family of EGF-TM7 molecules) and macrosialin (CD68), a pan-Mø endosomal glycoprotein related to the lysosome-associated membrane protein (LAMP) family. The DCs of myeloid origin (see elsewhere in this volume) share many properties with

monocyte/macrophages,[24] but are specialized to capture, process, and present antigens to naïve lymphocytes. Circulating precursors of DCs and and macrophages are normally present in the mononuclear fraction of blood in small numbers[25]; studies in the mouse may not reflect the origin and differentiation of precursor cells in humans.[26] Monocytes that have crossed the endothelium may be induced to "reverse migrate" into the circulation by selected stimuli in tissues.[27] Finally, the mouse spleen has been shown to serve as a reservoir of monocyte/Mø for recruitment to sites of inflammation.[28]

Circulating mononuclear precursors for osteoclasts are less defined and differentiate into mononucleate cells, recruited in response to sphingosine-1-phosphate to bone, for example,[29] where they fuse to form multinucleate bone-resorbing osteoclasts.[30] Local stromal cells, growth factors such as CSF-1, steroids (vitamin D metabolites), and hormones (eg, calcitonin, for which osteoclasts express receptors) all contribute to local maturation. Osteoprotegerin, a naturally occurring secreted protein with homology to members of the TNF-receptor family, interacts with TRANCE, a TNF-related protein, to regulate osteoclast differentiation and activation in vitro and in vivo.

Use of antigen markers such as CD34 on progenitors, CD14 and CD16 on monocytes, and chemokine receptors and multichannel fluorescein-activated cell sorter analysis have made it possible to isolate leukocyte subpopulations and study their progeny and differential responses in different mouse tissues and models of disease.[31] The mononuclear fraction of blood may contain precursors of other tissue cells, including mesenchymal stem cells able to synthesize matrix proteins such as collagen, and some endothelial cells. Perhaps the mysterious follicular dendritic cells (FDCs) with mixed hemopoietic and mesenchymal properties fall in this category.

The large-scale production of immature and mature DClike cells from bulk monocytes in cytokine-supplemented culture systems (IL-4, GM-CSF, TNFα) has revolutionized the study of these specialized APCs. Individually, the same cytokines give rise to Mø-like cells, and early during in vitro differentiation, the cellular phenotype is reversible. Later, when mature DCs with high MHC II, APC function, and other characteristic markers are formed, differentiation is irreversible. This process is independent of cell division, although earlier progenitors in bone marrow and GM-CSF-mobilized blood mononuclear cells can be stimulated to multiply, as well as differentiate, in vitro. These examples of terminal differentiation observed with DCs and osteoclasts may extend to other specialized, more obvious Mø-like cells. Mature Mø can be derived by growth and differentiation in steroid-supplemented media in Dexter-type long-term bone marrow cultures that contain stromal fibroblasts and hemopoietic elements. These Mø express adhesion molecules responsible for divalent cation-dependent cluster formation with erythroblasts (EbR). This receptor, possibly related to V-CAM, cannot be induced on terminally differentiated peritoneal Mø if these are placed in the same culture system. This contrasts sharply with the ready adaptation of many tissue Mø to conventional cell culture conditions, when the cells often adopt a common, standard phenotype. Irreversible stages of Mø differentiation may therefore occur in specialized microenvironments in vitro or in vivo.

Little is known about determinants of Mø longevity and turnover. Growth factors such as CSF-1 enhance Mø survival and prevent induction of an apoptotic program. The expression of Fas-L and Fas on Mø has been less studied than on lymphocytes; they and other members of the TNF and its receptor family may play a major role in determining Mø survival, especially in induced populations, where cell turnover is markedly enhanced. Tissue Mø vary greatly in their lifespan, from days to months. Apart from inflammatory and microbial stimuli, local and systemic environmental factors such as salt loading and hormones, including estrogen, are known to influence Mø turnover.

Tissue Distribution and Phenotypic Heterogeneity of Resident Macrophages in Lymphoid and Nonlymphoid Organs

The use of the F4/80 plasma membrane antigen made it possible to detect mature Mø in

developing and adult murine tissues and define their anatomic relationship to other cells in endothelium, epithelium, and connective tissue, as well as the nervous system.[32,33] Subsequently, other membrane antigens,[34] macrosialin, sialoadhesin, and others were identified as useful markers for Mø in situ (Table 19.1). Mø subpopulations in different tissues display considerable heterogeneity in expressing these and selected receptor antigens (eg, complement receptor [CR]3 and class A scavenger receptor [SR-A]), drawing attention to unknown mechanisms of homing, emigration, and local adaptation to particular microenvironments. From the viewpoint of immune responses, a few aspects deserve comment.

Fetal Liver and Bone Marrow

Mature Mø form an integral part of the hemopoietic microenvironment and play a key role in the production, differentiation, and destruction of all hemopoietic cells. The fetal liver is a major site of definitive erythropoiesis from midgestation.[16] The bone marrow becomes active in the production of hemopoietic cells from shortly before birth, and Mø are a prominent component of the hemopoietic stroma throughout adult life. Mature "stromal" Mø in fetal liver and adult bone marrow express nonphagocytic adhesion molecules such as sialoadhesin (Sn), an immunoglobulin (Ig)-superfamily sialic acid-binding lectin (Table 19.1), and the EbR referred to previously, which is also involved in adhesion of developing myeloid and possibly lymphoid cells (Fig. 19.2). VLA-4 has been implicated as a ligand for EbR. Ligands for Sn include CD43 on developing granulocytes and on lymphocyte subpopulations. Sn clusters at sites of contact between stromal Mø and myeloid but not erythroid cells. Chemokines are able to induce polarized expression of adhesion molecules such as intercellular adhesion molecules and CD43 in leukocytes, but the significance

of altered ligand distribution for interactions between Mø and bound hemopoietic cells is unknown. Adhesion of immature cells to stromal Mø may play a role in regulating their intermediate stages of development before release into the bloodstream, whereas fibroblasts in the stroma associate with earlier progenitors, as well as with Mø. Discarded nuclei of mammalian erythroid cells are rapidly engulfed by stromal Mø, but the receptors involved in their binding and phagocytosis are unknown. Mø also phagocytose apoptotic hemopoietic cells generated in bone marrow, including large numbers of myeloid and B cells. We know little about the plasma membrane molecules and cytokine signals operating within this complex milieu, but it is clear that stromal Mø constitute a neglected constituent within the hemopoietic microenvironment.

TABLE 19.1 Selected Differentiation Antigens Used to Study Murine Macrophage Heterogeneity

Ab	Ag	Structure	Ligands	Cellular Expression	Function	Comment
F4/80	F4/80 (EMR1)	EGF-TM7	?	Mature Mø, absent T areas	Peripheral tolerance	Useful marker development, CNS
FA-11	Macrosialin (CD68)	Mucin-LAMP	OX-LDL	Pan-Mø, DC	Late endosomal	Glycoforms regulated by inflammation and phagocytosis
5C6	CR3	β2-integrin	iC3b, ICAM	Monocytes,	Phagocytosis,	Important in

	(CD11b, CD18)			microglia, PMN, NK cells	adhesion	inflammatory recruitment, PMN apoptosis
2F8	SR-A (I, II)	Collagenous, type II glycoprotein	Polyanions, LTA, LPS, bacterial proteins		Adhesion, endocytosis	Protects host against LPS-induced shock
		Isoforms differ, cysteine-rich domain	Modified proteins β-amyloid apolipoprotein A, E			
				Mø, sinusoidal endothelium	Phagocytosis of apoptotic cells and bacteria	Promotes atherosclerosis
SER-4	Sn (Siglec-1)	Ig superfamily	Sialyl glycoconjugates (eg, CD43)	Subsets tissue Mø	Lectin	Strongly expressed
3D6						Marginal zone metallophils in spleen and subcapsular sinus of lymph nodes

CNS, central nervous system; DC, dendritic cell; ICAM, intercellular adhesion molecule; Ig, immunoglobulin; LAMP, lysosome-associated membrane protein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; Mø, macrophages; NK, natural killer; OX-LDL, oxidised low density lipoprotein; PMN, polymorphonuclear neutrophil; Sn, sialoadhesin; SR-A, type A scavenger receptor.

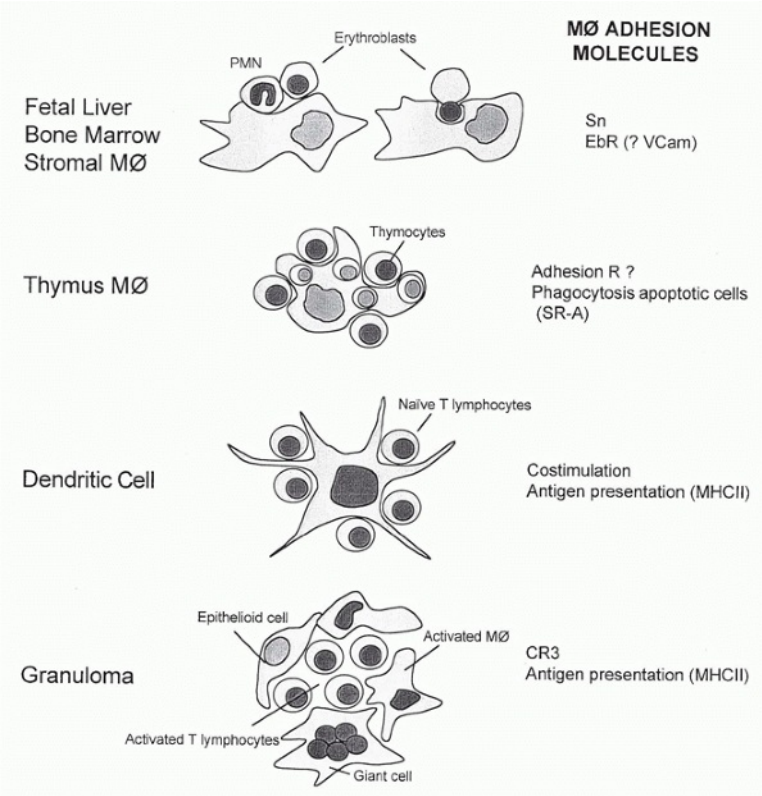

FIG. 19.2. Associations of tissue macrophages with other hemopoietic cells to illustrate variations on a common theme. See text for details.

Thymus

Apart from their remarkable capacity to remove apoptotic thymocytes, the possible role of Mø in positive and negative selection of thymocytes has been almost totally overlooked; more attention has been given to local DCs and their specialized properties. Mature Mø with unusual features are also present in cortex and medulla. Clusters of viable thymocytes and Mø can be isolated from the thymus of young animals by collagenase digestion and adherence to a substratum (see Fig. 19.2). The nonphagocytic adhesion receptors responsible for cluster formation are more highly expressed by thymic than other Mø, but their nature is unknown (N. Platt, unpublished observations). These Mø also express MHC class II antigens and other receptors such as the SR-A (see subsequent discussion), which contributes to phagocytosis of apoptotic thymocytes in vitro, but is redundant in vivo; other markers, such as the F4/80 antigen, are poorly expressed in situ but can be readily detected after cell isolation. A striking difference between thymic and several other tissue Mø subpopulations is their independence of CSF-1; the CSF-1-deficient *op/op* mouse lacks osteoclasts and some Mø populations, including monocytes, peritoneal cells, and Kupffer cells, but contains normal numbers of thymic Mø, as well as DCs and selected Mø in other sites. A second ligand for the CSF-1 receptor Fms, IL-34, may account for CSF-1 independence.[35] Factors involved in constitutive recruitment of thymic Mø are unknown; following death of thymocytes induced by ionizing radiation or glucocorticoids, intensely phagocytic Mø appear in large numbers; it is not known what proportion arises locally and by recruitment.

Spleen

From the viewpoint of the Mø, the spleen is perhaps the most complex organ in the body.[36,37] It contributes to hemopoiesis, which persists postnatally in some species or can be induced by increased demand, can serve as a reservoir as noted previously, and

contributes to the turnover of all blood elements at the end of their natural lifespan. The spleen filters a substantial proportion of total cardiac output, captures particulate and other antigenic materials from the bloodstream, and plays an important role in natural and acquired humoral and cellular immunity. The organ is rich in subpopulations of Mø that differ in microanatomic localization, phenotype, life history, and functions (Fig. 19.3). Mø are central to antigen capture, degradation, transport, and presentation to T- and B-lymphocytes, and contribute substantially to antimicrobial resistance. Recent work has unveiled an unexpected role in facilitating activation of other lymphocyte subsets, such as invariant natural killer T cells[37]; CD 169+ macrophages also activate CD8 T cells in response to dead cell-associated antigens in lymph nodes and by transferring antigen to DCs in the spleen. Because other hemopoietic and secondary lymphoid organs can replace many of these functions after maturation of the immune system, the unique properties of the spleen have been mainly recognized in the immature host and in immune responses to complex polysaccharides. Splenectomy in the adult renders the host susceptible to infection by pathogenic bacteria such as pneumococci that contain saccharide-rich capsular antigens; the marginal zone of the spleen in particular may play an essential role in this aspect of host resistance.

The properties of Mø in the unstimulated mature mouse spleen are different according to their localization in red or white pulp and the marginal zone. Mø are intimately associated with the specialized vasculature. Species differences in splenic anatomy and phenotype are well recognized, although Mø display broadly common features in humans and rodents. Subpopulations of Mø, DCs, and cells with mixed phenotypes have been characterized by in situ analysis by antigen markers, liposome or diphtheria toxin-depletion studies, various immunization and infection protocols, and cytokine and receptor gene ko models in the mouse. The results raise questions about the dynamics and molecular basis of cell production, recruitment, differentiation, emigration, and death within each distinct splenic compartment. Cell isolation methods are still primitive in correlating in vitro properties with those of Mø subpopulations in vivo and remain an important challenge. Detailed aspects of splenic architecture, DC origin and function, and T- and B-lymphocyte induction and differentiation are described elsewhere in this volume. Here, some features of Mø in the normal and immunoreactive organ are highlighted.

Marginal Zone Macrophages

The marginal zone of spleen consists of a complex mixture of resident cells (reticular and other fibroblasts, endothelium), Mø, DCs, and lymphoid cells, including subpopulations of B-lymphocytes. It constitutes an important interface with the circulation that delivers cells, particulates, or soluble molecules directly into the marginal sinus or via the red pulp. Resident Mø are present as specialized metallophilic cells in the inner marginal zone, and other Mø are found in the outer zone; the latter may be more phagocytic. Sn is strongly expressed by the marginal metallophils, compared with only weak expression in red pulp and virtual absence in the white pulp. Sn+ cells appear in this zone 2 to 4 weeks postnatally in the mouse as the white pulp forms. Liposomes containing clodronate, a cytotoxic drug, can be delivered systemically and deplete Sn+ cells and other Mø; regeneration of different Mø subpopulations in spleen occurs at different times, and this procedure has been used to correlate their reappearance with distinct immunologic functions. Marginal

zone Mø lack F4/80 but may express an undefined ligand for F4/80 on circulating activated DCs, which mediates peripheral tolerance to anterior chamber or gut-derived antigens.[38] Marginal zone Mø express phagocytic receptors, such as SR-A, which is more widely present on tissue Mø, as well as MARCO, a distinct collagenous scavenger receptor, which is almost exclusively present on these Mø in the normal mouse. The structures and possible role of these pattern recognition receptors in uptake of microbes are discussed subsequently. In vivo studies have shown that an Mø lectin, the MR, may be involved in transfer of mannosylated ligands to the site of an immune response in the white pulp.[39] The MR contains a highly conserved cysteine-rich domain, not involved in mannosyl recognition, that reacts strongly with ligands on a subset of marginal metallophilic Mø, sulfated glycoforms of Sn, and CD45,

among others; this has been demonstrated with a chimeric probe of the cysteine-rich domain of the MR and human Fc (CR-Fc) and by immunochemical analysis of tissue sections and affinity chromatography of spleen ligands. After immunization, this probe additionally labels undefined cells in the FDC network of germinal centers, as well as tingible body Mø. It is possible that marginal zone Mø can be induced to migrate into white pulp as described after LPS injection; alternatively, they may shed complexes of soluble MR-glycoprotein ligand for transfer to other CR-Fc+ cells, which may be resident or newly recruited mononuclear cells. Finally, the marginal metallophilic Mø population depends on CSF-1 for its appearance and on members of the TNF receptor family, as shown with *op/op* and experimentally produced ko mice.

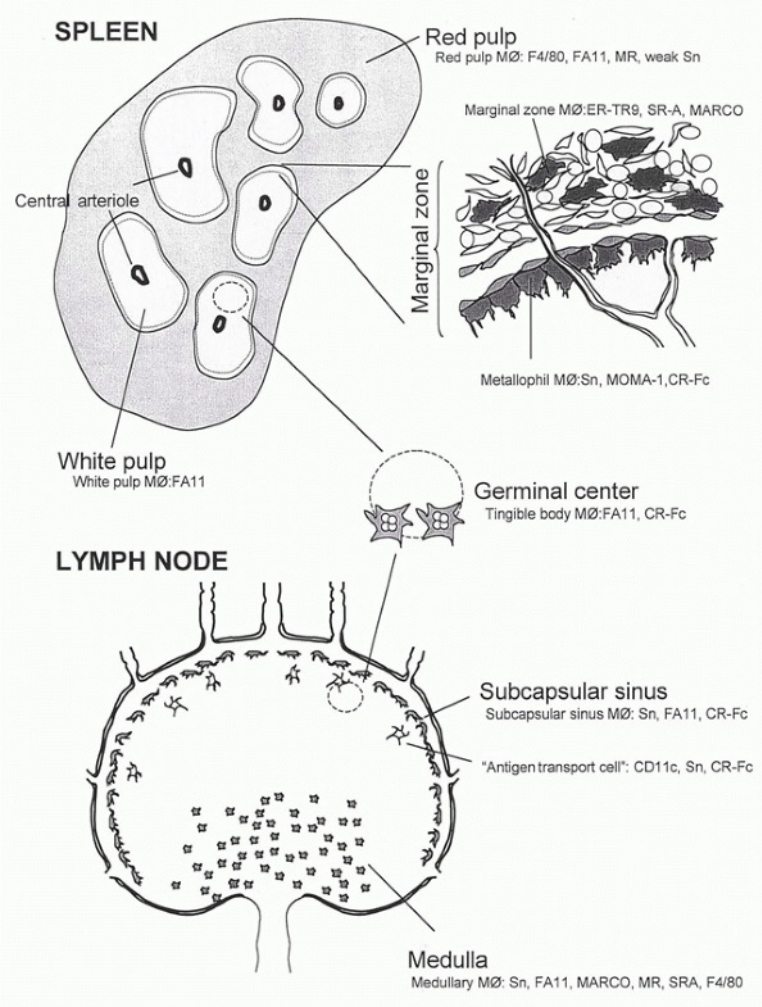

FIG. 19.3. Microheterogeneity of macrophages in spleen, resting, and antigen-stimulated lymph nodes. See text for markers and details.

White Pulp Macrophages

The F4/80 antigen is strikingly absent on murine white pulp Mø, which do express FA-11 (macrosialin), the murine homolog of CD68. Actively phagocytic Mø express this intracellular glycoprotein in abundance compared with DCs. After uptake of a foreign particle (eg, sheep erythrocytes or an infectious agent, such as BCG or *Plasmodium yoellii*), white pulp Mø become more prominent, although it is not known whether there is migration of cells into the white pulp or transfer of phagocytosed material and reactivation of previous resident Mø. Tingible body Mø appear to be involved in uptake and digestion of apoptotic B-lymphocytes.

Red Pulp Macrophages

These express F4/80 antigen and MR strongly and in the mouse include stromal-type Mø involved in hemopoiesis. Extensive phagocytosis of senescent erythrocytes results in accumulation of bile pigments and ferritin, and play an important role in iron turnover[40] and tolerance.[41] The role of various phagocytic receptors in clearance of host cells and pathogens by red pulp Mø requires further study.

There is no evidence that Mø, other than interdigitating DCs, associate directly with CD4+ T-lymphocytes in the normal spleen. Following infection by BCG, for example, or by other microorganisms such as *Salmonella*, there is massive recruitment and local production of Mø, many of which associate with T-lymphocytes. Newly formed granulomata often appear first in the marginal zone (focal accumulations of activated Mø and activated T cells). As infections spread into the white and red pulp, the granulomata become confluent and less localized, obscuring and/or disrupting the underlying architecture of the spleen. The possible role of activated Mø in T-cell apoptosis and clearance in spleen has not been defined.

Lymph Nodes

F4/80 antigen is relatively poorly expressed in lymph node (see Fig. 19.3), but many macrosialin (CD68)+ cells are present. The subcapsular sinus is analogous to the marginal zone and contains strongly Sn+ cells; this is the site where afferent lymph enters, containing antigen and migrating DCs derived from skin and mucosal surfaces. The medulla contains Sn+, CD68+ Mø, which also express high levels of SR-A. As in the spleen marginal zone, subcapsular sinus Mø are strongly labeled by the CR-Fc probe. Following primary or secondary immunization, the staining pattern moves deeper into the cortex and eventually becomes concentrated in germinal centers. The kinetics of this process strongly suggests a transport process by Mø-related cells resembling antigen transport cells described previously. CR-Fc+ cells can be isolated by digestion of lymph nodes and form clusters with CR-Fc- lymphocytes. Adoptive transfer has shown that fluorescein-activated cell sorter-isolated CR-Fc+ cells resemble DCs in their ability to home to T-cell areas and to present antigen to naive T and B cells. Overall, there is considerable heterogeneity in the population of migratory APCs involved in antigen capture, transport, and delivery to T and B cells, and it may turn out that specialized tissue Mø as well as myeloid-type DCs can migrate in response to immunologic stimuli, especially TLR ligands.[42]

Peyer Patch

Although less studied, the Mø in Peyer patch resemble the CD68+, F4/80- cells described in spleen and white pulp and in other T-cell-rich areas. They are well placed to interact with gut-derived antigens and pathogens taken up via specialized epithelial M cells in the dome, and deliver antigens to afferent lymphatics, as myeloid DCs. These cells are distinct from abundant F4/80+ cells in the lamina propria found all the way down the gastrointestinal tract and may play a role in the induction of mucosal immunity. Recent studies have described heterogeneous populations of resident and recruited macrophages and DC in the mouse intestine.[43] The role of the microbiome[44] has received a great deal of attention in regard to innate cell phenotype and epithelial integrity in the gut.

Nonlymphoid Organs

Regional F4/80+ and CD68+ Mø are well described in liver (Kupffer cells), dermis, neuroendocrine and reproductive organs, and serosal cavities, where they are able to react to systemic and local stimuli. In the lung, alveolar Mø are strongly CD68+ but only weakly F4/80+ and are distinct from interstitial Mø and intraepithelial DCs. In the lamina propria of the intestine, Mø display a downregulated phenotype, ascribed to TGFβ of local origin.[45] In addition, resident Mø are found throughout connective tissue and within the interstitium of organs, including heart, kidney, and pancreas. These cells vary greatly depending on their local microenvironment; for example, in the central nervous system, microglia within the

neuropil differ strikingly from Mø in the meninges or choroid plexus.[46] Perivascular Mø in the brain can be distinguished from resident microglia by their expression of endocytic receptors (eg, the SR-A and MR, and of MHC I and II antigens). Microglia are highly ramified, terminally differentiated cells of monocytic origin; many Mø markers are downregulated. Their phenotype is influenced by the blood-brain barrier, normally absent in circumventricular organs, and disrupted by inflammatory stimuli. Microglia can be reactivated by local LPS and neurocytotoxins; they are then difficult to distinguish from newly recruited monocytes, which acquire microglial features once they enter the parenchyma of the brain. Resting microglia are unusual among many tissue Mø in that they constitutively express high levels of CR3 and respond to CR3 ligands, such as mAb, by induced DNA synthesis and apoptosis. In other sites, such as lung and liver, CR3 expression is a feature of recent myeloid recruitment, including monocytes. Resident Kupffer cells lack constitutive CR3 but express a novel CR implicated in clearance function.

Resident tissue macrophages in human tissues express CD68 antigen, but their phenotypic diversity and microheterogeneity in different organs remain poorly defined. Access to skin biopsies, bronchoalveolar lavage, and placenta, for example, provides material for further analysis.

Enhanced Recruitment of Monocytes by Inflammatory and Immune Stimuli: Activation in Vivo

In response to local tissue and vascular changes, partly induced by resident Mø during (re)activation by inflammatory, infectious, and immunologic stimuli, monocytes are recruited from marrow pools and blood in increased numbers; they diapedese and differentiate into Mø with altered effector functions as they enter the tissues.[47] These Mø are classified as "elicited" when cells are generated in the absence of IFNγ and as "immunologically activated" after exposure to IFNγ. Enhanced recruitment can also involve that of other myeloid or lymphoid cells; selectivity of the cellular response depends on the nature of the evoking stimulus (immunogenic or not), the chemokines produced,

and the receptors expressed by different leukocytes. Mø and other cells produce a range of different chemokines and express multiple seven-transmembrane, G protein-coupled chemokine receptors. The chemokines can also act in the marrow compartment, especially if anchored to matrix and glycosaminoglycans, may display other growth regulatory functions, and can control egress. Locally bound or soluble chemokines induce the surface expression and activity of adhesion molecules on circulating white cells, as well as directing their migration through and beyond endothelium. Feedback mechanisms from periphery to central stores and within the marrow stroma may depend on cytokines and growth factors such as macrophage inflammatory protein-1α and GM-CSF, which inhibit or enhance monocyte production, respectively. The adhesion molecules involved in recruitment of monocytes, originally defined by studies in humans with inborn errors and by use of inhibitory antibodies in experimental animal models,[48] overlap with those of polymorphonuclear neutrophils and lymphocytes and include L-selectin, β2-integrins, especially CR3, CD31, an Ig-superfamily molecule, and CD99; additional monocyte adhesion molecules for activated endothelium include CD44, vascular cell adhesion molecules, β_1-integrins, and newly described receptors such as EMR2 and CD97, members of the EGF-TM7 family.[33] The mechanisms of constitutive entry of monocytes into developing and adult tissues, in the absence of an inflammatory stimulus, are unknown.

By contrast with the uncertain precursors of resident Mø and DC populations, distinct monocyte subsets have been implicated in the enhanced mobilization and turnover in response to inflammatory, infectious, and metabolic stimuli, as noted previously (Fig. 19.4). Differential expression of the fractalkine receptor, CCR2, and other chemokine receptors, together with antigen markers (Gr-1 in mouse and CD14 in human), have made it possible to define monocyte heterogeneity. Although such subsets seem to be conserved across several species, their properties may reflect stages of cell activation along a continuous spectrum

rather than true differentiation.

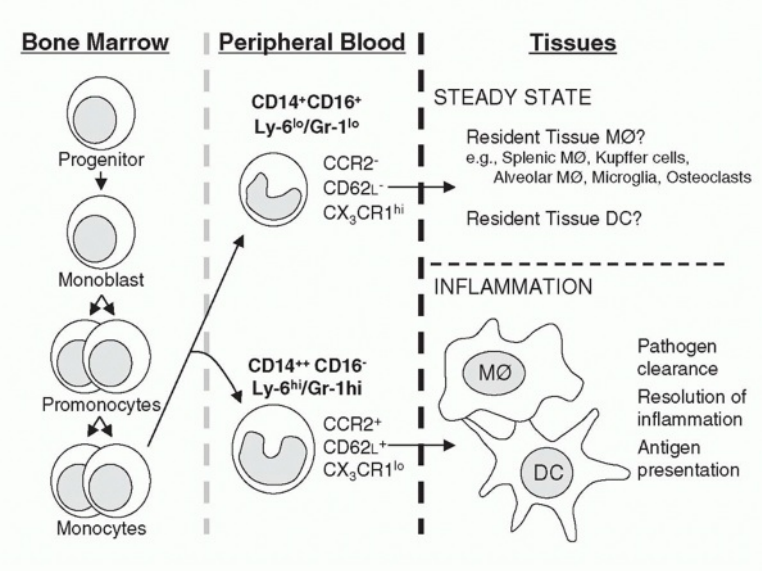

FIG. 19.4. Distinct monocyte subsets give rise to inflammatory and resident macrophages and dendritic cells. The origin of different resident subpopulations is not clear. See text for further details.

The migration and differentiation of newly recruited monocytes once they have left the circulation are poorly understood. They are able to enter all tissues, undergoing alterations in membrane molecules and secretory potential under the influence of cytokines and surface interactions with endothelial cells, leukocytes, and other local cells. Phenotypic changes mentioned in the following section have been characterized by a range of in vitro and in vivo studies. Well-studied examples include murine peritoneal Mø—resident, elicited by thioglycollate broth or biogel polyacrylamide beads, and immunologically activated by BCG infection. The latter provides a useful model of granuloma formation in solid organs but does not fully mimic the human counterpart associated with *M. tuberculosis* infection. Granuloma Mø vary in their turnover and immune effector functions and display considerable heterogeneity; lesions contain recently recruited monocytes, mature, epithelioid Mø (described as secretory cells), and Langhans giant cells. Interactions with T-lymphocytes, other myeloid cells, DCs, fibroblasts, and microorganisms yield a dynamic assembly of cells as the granuloma evolves, heals, and resolves (see Fig. 19.2). Apoptosis and necrosis of Mø and other cells contribute to the balance of continued recruitment and local proliferation. The emigration of Mø rather than DCs from sites of inflammation is less evident, although it has become clear that elicited Mø within the peritoneal cavity, for example, migrate actively to draining lymph nodes.[49]

Gene ko models have confirmed the role of molecules previously implicated in recruitment, activation, and granuloma formation. These include the adhesion molecules listed previously, their ligands, such as intercellular adhesion molecule-1, and key cytokines such as IFN*γ*, IL-12, IL-23, and TNF*α*, as well as their receptors. Antimicrobial resistance and Mø cytotoxicity resulting from production of reactive oxygen and nitrogen metabolites are now accessible to study

in knockouts of the phagocyte oxidase and inducible nitric oxide synthase. kos of membrane molecules of immunologic interest expressed by Mø and other cells include MHC class II and I, CD4, and CD40L, other accessory molecules such as B7-1 and B7-2, and the Mø-restricted intracellular molecule N-ramp.

The study of inborn errors in humans[50,51] and of disease models in genetically modified

mice has brought insight into essential, nonredundant contributions of molecules that regulate Mø activation in vivo, including immunopathology syndromes such as septic shock and autoimmunity. Examples include myeloid antigens such as TREM-1/2,[52] associated with DAP-12, receptor-ligand pairs such as CD200/CD200 receptor,[53] and suppressors of cytokine signaling proteins.[54,55] TNFα is essential for host resistance to infection[56] and also contributes to immunopathology. Highly effective anti-TNF*α* therapy for chronic inflammatory diseases such as rheumatoid arthritis can result in reactivation of latent tuberculosis.

The potential for innate,Th1- and Th2-type regulation of Mø demonstrated in vitro, and discussed subsequently, can result in highly complex, often coexistent, heterogeneity of Mø phenotype in situ (see Fig. 19.4). Although almost all granuloma Mø express lysozyme,[57] only subpopulations express cytokines such as IL-1*β*, IL-6, and TNF*α*. Pro- and anti-inflammatory cytokines, IL-12, IL-18, IL-10, and TGF*β*, produced by Mø themselves and other cells, modulate the phenotype of Mø in vivo.

Intravital imaging has provided insights into the dynamics of interactions of myeloid and lymphoid cells in granuloma formation.[58] Studies of human granulomatous, chronic inflammation provide opportunities to dissect genetic and acquired influences (eg, in NOD-2 deficiency in Blau syndrome[59] and Crohn disease[60]). The mouse resistance protein Irgm1 (LRG-47)[61] has also been implicated in pathogen defense.[62]

Apart from the local interactions outlined, Mø regulate systemic host reactions to immune and infectious stimuli by producing circulating cytokines such as IL-6 and arachidonate- and other lipid-derived metabolites, including resolvins, that contribute to the resolution of acute inflammation.[49] These mediators also act on neural and endocrine centers, crossing the blood-brain barrier, or are generated locally by reactive microglia and Mø. MicroRNAs have been identified that play a role in resolution.[63] Glucocorticosteroids are powerful immunomodulators[64] and form part of a network that regulates monocyte recruitment and Mø functions through circulating mediators such as migration inhibition factor. Mø contain potent enzymes involved in steroid biosynthesis and catabolism.[65]

Although the immunologic relevance of Mø-induced responses may seem evident, many aspects remain unclear. For example, what is the role and phenotype of monocyte/macrophages in the immune reconstitution inflammatory syndrome, associated with dual acquired immunodeficiency syndrome/tuberculosis infection, recipitated by antiretroviral treatment[66]? Do Mø actively suppress or destroy activated T-lymphocytes, thus contributing to regulation of immune responses and peripheral tolerance, or are Mø only passive removers of dying cells? Do Mø contribute to recruitment, differentiation, and death of DCs at sites of inflammation before their migration to secondary lymphoid organs? Do adjuvant-stimulated Mø interact with B-lymphocytes, directing their migration into germinal centers? Are interactions of activated Mø with antibody and complement, through different Fc and complement receptors, implicated in fine-tuning humoral responses? Are activated Mø themselves cytocidal for infected host cells, and to what extent do they in turn interact with and provide targets for attack by natural killer cells and cytotoxic T-lymphocytes? Study of a range of experimental models and disease processes in vivo should yield new insights, as well as extend and confirm mechanisms already defined in vitro.

Phagocytic Recognition and Intracellular Infection

The initiation and localization of an immune response depends on recognition by Mø and other cells of particulate agents or soluble proteins that are foreign or modified-self.[67] Phagocytic and endocytic recognition by Mø and DCs depends, in turn, on opsonic (mainly antibody, complement) and nonopsonic pattern recognition receptors that interact with a range of related ligands (Fig. 19.5). Innate and acquired responses are thus interlinked. Different FcR are involved in uptake and destruction of targets as well as in negative

regulation of effector functions.[68] CRs[69] are also heterogeneous; CR3 interacts with C3-derived ligands formed by activation of the classical, alternate, or lectin pathways and mediate phagocytosis, cell migration, and cell activation. Other ligands include intercellular adhesion molecules. CR3 functions are modulated by fibronectin, via integrins, other adhesion molecules, and inflammatory stimuli. FcR ligation and cross-linking activates tyrosine kinases such as syk that are essential for phagocytosis; CR3 signaling is less defined and may not trigger a respiratory burst or arachidonate release, unlike FcR, thus favoring pathogen entry. Antibodymediated uptake targets an organism or soluble antigen to a different, degradative compartment[70] (Fig. 19.6A, 19.6B) and usually results in its neutralization and destruction, although enhancement of infection can also occur in Mø.[71] Flavivirus infection in the presence of specific antibody can result in the dengue hemorrhagic shock syndrome. Immune complexes, with or without complement, localize antigens to FDCs and other FcR+ CR+ cells. Mø themselves are able to produce all components of the complement cascade in significant amounts at local sites, which may be less accessible to circulating proteins made by hepatocytes.

Nonopsonic receptors reacting directly with ligands on microorganisms[72] include CR3, lectins, especially the MR and *β*-glucan receptor, the scavenger receptors SR-A and MARCO, and the family of TLRs. MRs are present on Mø, DCs, and sinusoidal endothelium.[39] They mediate phagocytosis and endocytosis, including macropinocytosis, and structurally resemble another multilectin, Dec 205, present on DCs as well as tissue Mø and epithelial cells in thymus; carbohydrate recognition by the latter has not been demonstrated. The MR has eight C-type lectin domains, homologous to the mannose-binding protein, a circulating

hepatocyte-derived, acute-phase reactant. Mannose-binding protein, also known as mannan-binding lectin, contains a single lectin domain per polypeptide, which oligomerizes like other collectins to achieve multivalent interactions and activate complement via associated serine proteases. MR expression on Mø is selectively down- and upregulated by IFNγ and IL-4/13, respectively. A possible role of the cysteine-rich domain in transport of immunogenic glycopeptides within secondary lymphoid organs has been proposed.[73]

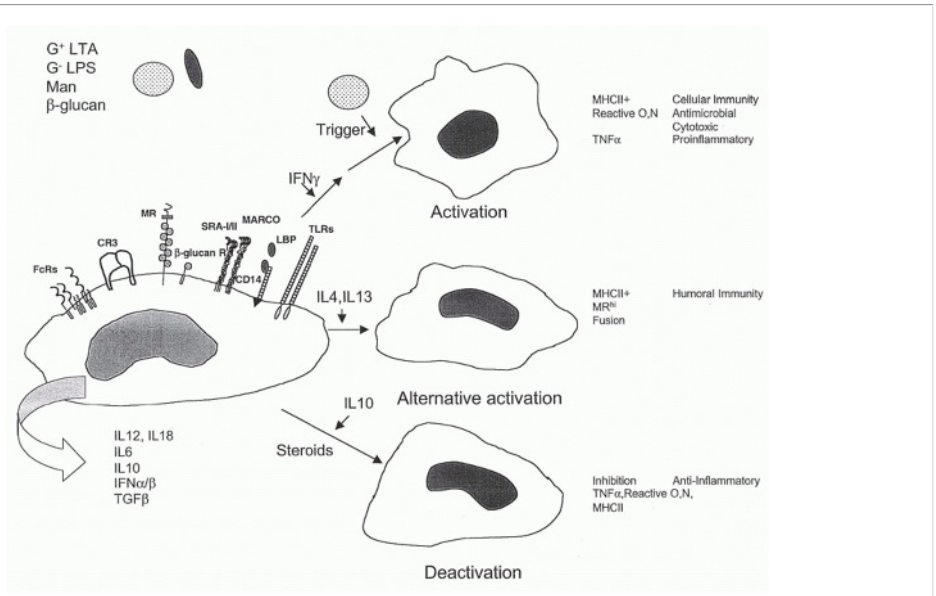

FIG. 19.5. Macrophage activation and the role of microbial stimuli and cytokines. See text for details.

The *β*-glucan receptor, previously reported as dectin-1, is related to C-type lectins and is

responsible for phagocytic recognition of unopsonized zymosan and for Mø activation (Figs. 19.7 and 19.8).[74] It contains an immunoreceptor tyrosine-based activation motif-like motif in its cytoplasmic domain that is essential for phagocytosis and induced secretory responses (eg, TNF*α*). It cooperates with TLR2 and synergizes with other TLRs in cell activation, utilizing syk and CARD9,[75] and can promote TH17 differentiation.[76] It is essential for resistance to a range of fungal particles in vivo, as shown by studies with ko mice and in human primary innate immunodeficiency.[77] Dectin-2, a distinct mannan-binding lectin, has also been implicated in TH17 differentiation.[45,78]

SR-A mediates endocytosis of modified proteins (eg, acetylated lipoproteins) and selected polyanions, such as apolipoprotein A1 and E,[79] LPS, and lipoteichoic acid.[72] In addition, it can serve as an adhesion molecule and contributes to phagocytic clearance of apoptotic thymocytes and gram-negative as well as gram-positive bacteria. MARCO, a related collagenous receptor,[72] mediates cell adhesion and phagocytosis of bacteria but is independently regulated, as discussed subsequently. MARCO[80] and Mincle,[81] a lectin-like receptor, contribute to macrophage responses to trehalose dimycolate, a virulence factor of pathogenic *M. tuberculosis*. An initial report of a phosphatidylserine receptor implicated in the recognition of novel lipid ligands expressed on the surface of apoptotic cells was not confirmed. CD36 (thrombospondin receptor), vitronectin receptors, CD91, and CD44 have all been implicated in the uptake of senescent polymorphonuclear neutrophils by Mø.[82] Other opsonins for apoptotic cell clearance include milk fat globule protein (lactadherin). A role for Mø SR-A in immune induction has not been demonstrated, but studies in SR-A ko mice have revealed an important inhibitory role in limiting TNFα production by immunologically activated Mø. Wild-type, BCGprimed mice produce granulomata rich in SR-A+ Mø; SR-A ko mice restrict growth of this organism and form normal granulomata containing activated, MHC II+ Mø; on additional challenge with LPS, the ko mice die more readily than wild-type animals. TNF*α* levels in the circulation rise markedly because of unopposed triggering via CD14, a receptor for the LPS-binding protein, and contribute to septic shock, because blocking anti-TNF mAb protects these mice.

The family of TLRs consists of homo- or heterodimeric transmembrane molecules related to the IL-1 receptor, which are involved in innate immunity to microbial constituents and activation of Mø responses, and are discussed elsewhere in this volume.[83] Downstream signaling depends on association with other soluble and membrane molecules, as well as with intracellular proteins. MyD88, for example,

has been implicated in many but not all TLR-induced signaling resulting in transcription factor regulation, cell activation, or apoptosis.

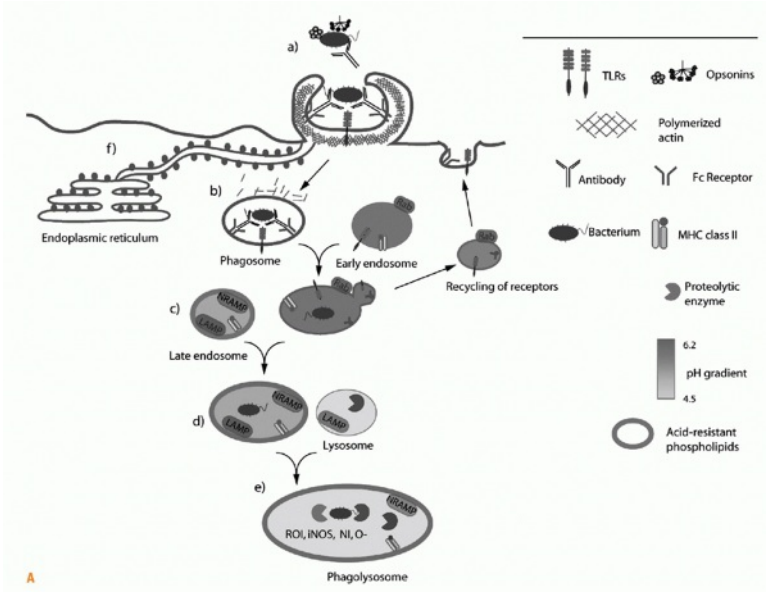

FIG. 19.6. Phagocytic Pathway in Macrophages. A: Heterophagy. Fc receptor-mediated phagocytosis. **a:** Microbes are coated with a variety of opsonins, including complement, pentraxin 3, and antibodies. A number of receptors are involved in initial recognition of microbes and induction of proinflammatory signaling (eg, the toll-like receptors [TLRs], and especially TLRs 2, 4, and 5), but these receptors are not phagocytic. Receptors involved in phagocytosis include the complement receptors, Fc receptors, and others. **b:** Fc receptor ligation initiates a signaling cascade that results in actin polymerization and extension of the plasma membrane. Phagocytosis mediated by this method occurs via the "zipper" mechanism (ie, sequential binding between the Fc receptors and their ligands along the length of the microbe). **c:** Fusion with the early endosome results in a slight drop in pH that results in the uncoupling of receptors with their ligands. Receptor recycling is facilitated by the Rab proteins, which also confer the ability to undergo subsequent fusion. The developing phagosome now contains major histocompatibility complex (MHC) class II and TLRs, including those that signal from within the developing endosome (eg, TLR9). **d:** Fusion with the late endosome results in the addition of the lysosome-associated membrane proteins, the accumulation of acid-resistant phospholipids, and a subsequent drop in pH. **e:** On fusion with lysosomes, the low pH results in the activation of a number of proteolytic enzymes. These are necessary for both direct antimicrobial activity and the creation of peptides for presentation via MHC class II. The phagolysosome is a highly oxidative environment, exposing the pathogen to destructive reactive oxygen and reactive nitrogen intermediates. **f:** Under certain circumstances, the phagosome may contain markers specific for the endoplasmic reticulum (ER). The ER may contribute directly to the creation of the phagocytic membrane in some circumstances (eg, phagocytosis of latex beads), or ER-derived vacuoles may contribute MHC class I and other molecules to the endosomes or the developing phagolysosome. *(continued)*

Naturally occurring microbial ligands for these nonopsonic receptors are still poorly defined; individual receptors mediate microbial binding and uptake of microorganisms, although each contributes only part of total binding (see Fig. 19.6A).[72,84] Particle uptake involves multiple membrane receptors, the cytoskeleton, bulk membrane flow, and remodeling, including formation of a phagocytic synapse and signaling pathways[85,86] (see Fig. 19.8[87]). Phagosome formation and maturation resemble endocytic uptake, initiating Mø vesicle trafficking and recirculation, fusion with lysosomes, acidification, ion fluxes, and digestion. Table 19.2 lists immunologic and other markers

used to identify intracellular compartments.[88] Guanosine triphosphate-binding proteins and complex signaling cascades play an important role in these dynamic events.[62] A key issue that needs to be resolved is how cell and receptor functions are modulated so that microbial phagocytosis or invasion induces inflammatory responses, unlike the uptake of apoptotic cells. The MHC II biosynthesis and subcellular localization and proteolytic processing of peptide antigens in vacuolar and cytosolic compartments of APCs are discussed elsewhere in this volume. Cytokines, especially IL-4/13, IL-10, and IFNγ, influence endocytosis via MR-dependent and -independent pathways and selectively alter vesicle dynamics.

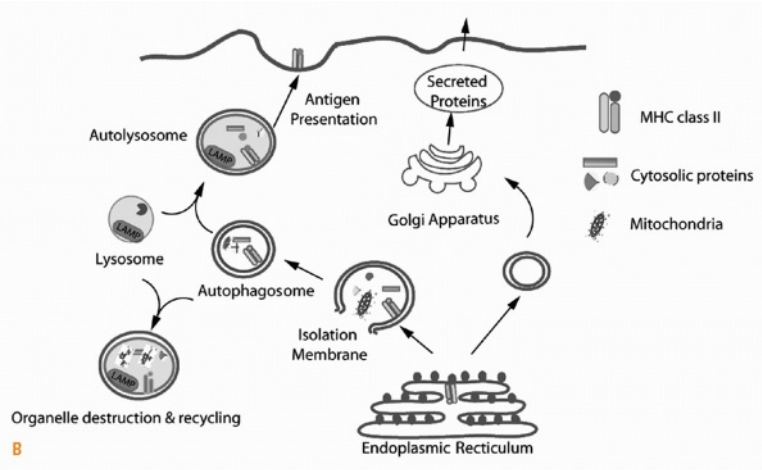

FIG. 19.6. *(continued)* **B:** The autophagy pathway. Autophagy is a homeostatic process that can be further enhanced in macrophages in the presence of interferons or by starvation. Cytosolic proteins and organelles are found in ER-derived cytoplasmic vesicles. These vesicles fuse with lysosomes, and the proteins and organelles are degraded and recycled. Conventional wisdom states that endogenous and cytoplasmic proteins are presented by MHC class I molecules, whereas exogenous peptides are presented by MHC class II molecules; however, it has become clear that peptide presentation is altered considerably on induction of autophagy. The presentation of peptides from intracellular and lysosomal source proteins is increased on MHC-II.

Pathogens vary in using Mø plasma membrane molecules for entry and evasion of host defenses, and modify the composition of the resultant phagosome membrane (Fig. 19.9A, 19.9B).[89] Mycobacteria, for example, employ a range of mechanisms to evade killing by Mø, including delayed maturation of phagosomes and inhibition of fusion with lysosomes and acidification (Fig. 19.9A, 19.9B and 19.10).[90,91] *Listeria monocytogenes* escapes into the cytosol by disruption of the phagosome membrane,[92,93] whereas *Leishmania* multiplies in phagolysosomes.[94] Humoral (antibody, complement) and cellular (IFNγ) mechanisms overcome parasitization of Mø by diversion to lysosomes or induce killing via oxygen/nitrogen (O/N)- dependent and other mechanisms.

Entry of microbial constituents such as muramyldipeptide from vacuolar compartments to the cytosol can result in sensing by NOD-like receptors, inflammasome assembly, activation of caspase-1, and processing and release of IL-1 β.[95,96,97] Nucleic acid recognition results in cytoplasmic and mitochondrial-associated protein signaling responsible for type 1 interferon gene expression (see Fig. 19.9A, 19.9B).[98] A novel ligand, c-di-AMP, secreted by cytosolic *Listeria monocytogenes*, activates a host type I IFN response[92] and may serve as a sensing mechanism for many intracellular pathogens (see Fig. 19.10).[99] STING is a direct innate immune sensor of cyclic di-GMP, another cytosolic metabolite of intracellular microbial infection.[100] It has recently become clear that induction of autophagy and apoptosis by

intracellular pathogens, including *Mycobacterium tuberculosis*, provides important host-protective responses[101] (see Figs. 19.6B and 19.9A, B). For a review on autophagy in immunity and inflammation, see Levine et al.[102,102a]

Although the "canonical" entry pathway described here and illustrated in Figure 19.6A is used and modified by many pathogens, recent evidence has shown that organisms such as *Legionella pneumophila*[102,103] (see Fig. 19.9C, D) and *Brucella abortus*[104] induce vacuoles with novel membrane components or colonize compartments derived from the Golgi apparatus and the endoplasmic reticulum. The relative contributions of plasma membrane and endoplasmic reticulum (see Fig. 19.6A) to vacuole formation varies considerably, depending on the nature of the phagocytic cargo or invading pathogen.[105] Opsonins such as antibody are able to divert the cargo to lysosomes. IFNγ can induce

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guanosine triphosphate-binding proteins that associate with vacuoles inhabited by a range of intracellular pathogens (eg, *Toxoplasma gondii*), thus marking them for destruction within the macrophage.[62,106]

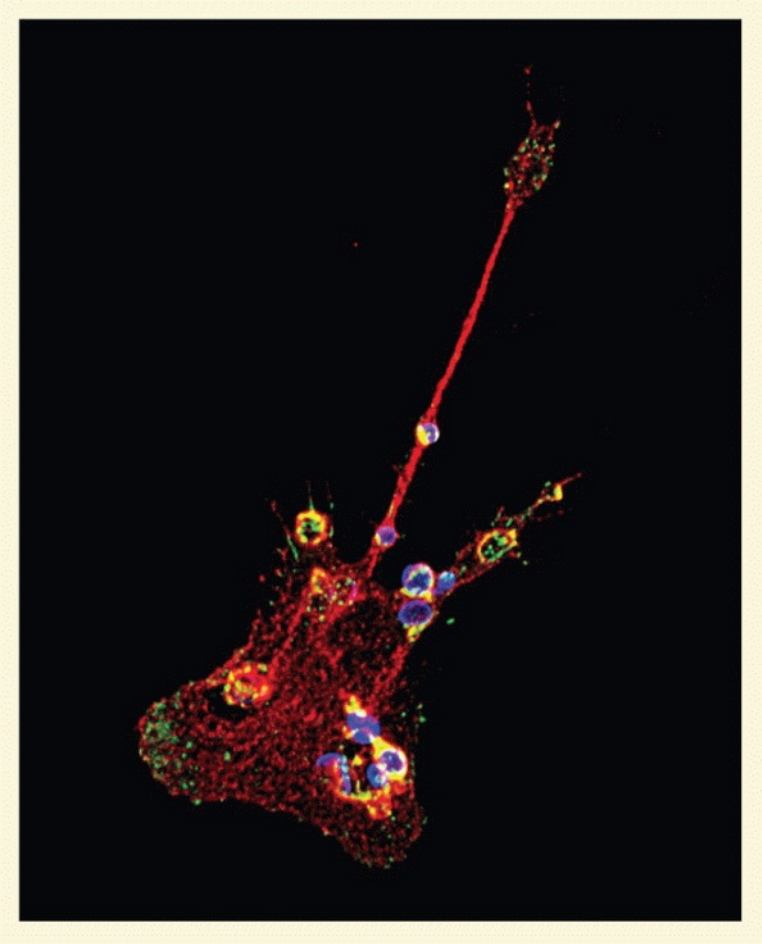

FIG. 19.7. Macrophages Rock! Macrophage engaging zymosan particles. Macrophages are adherent but motile. They use focal adhesions to anchor themselves to the extracellular matrix. Membrane ruffles and lamellipodia form at the leading edge of the cell to enable them to crawl around, and they extend pseudopodia to explore their environment and capture microbes and dead cells for phagocytosis. Consequently, their morphology is highly variable and constantly changing. © Helen Goodridge and David

Underhill

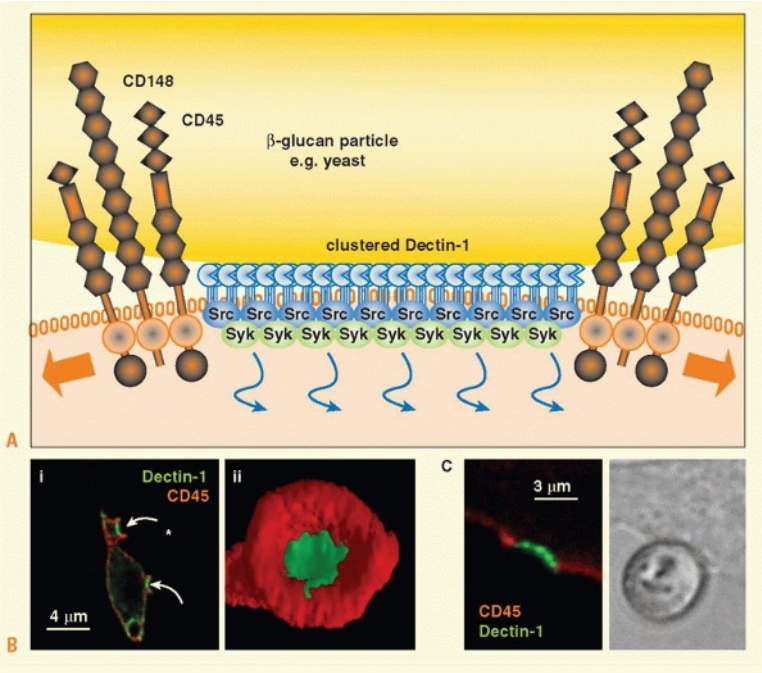

FIG. 19.8. The Dectin-1 Phagocytic Synapse. A: To permit sectin-1 signaling, cluster of differentiation (CD)45 and CD148 tyrosine phosphatases (which negatively regulate signal transduction downstream of dectin-1) are excluded from the contact site upon detection of beta-glucan particles by dectin-1 at the macrophage surface.The phagocytic synapse is similar to the immunological synapse that forms between an antigen presenting cell and a T cell. **B:** Confocal imaging of a macrophage encountering zymosan particles (**i**) shows two phagocytic synapses (arrows), and a three-dimensional isosurface model (**ii**) of one of them (*asterisk*). **C:** A phagocytic synapse that formed upon contact of a swollen *Aspergillus fumigatus* conidium with a macrophage. Images from Goodridge et al.,[87] with permission from Nature Publishing Group.

TABLE 19.2 Membrane or Content Markers Used to Identify Phagosomes as Resembling a Given Endocytic Compartment or the Endoplasmic Reticulum

Compartment	Markers
Early endosome	Membrane markers: TfR, Rab5, annexins, I, II, and III Proteases: immature cathepsin D
Late endosome	Membrane markers: M6PR, Rab7, LAMP1, LAMP2, CD63, CD68 Hydrolases: acid phosphatase, aryl sulfatase, trimetaphosphatase Proteases: cathepsin B, D, H, dipeptidyl peptidase I and II Phospholipid: LBPA
Lysosome	Membrane markers: LAMP1, LAMP2, CD63 Hydrolases: acid phosphatase, aryl sulfatase, trimetaphosphatase

	Proteases: cathepsin B, D, H, dipeptidyl peptidase I and II
ER	Membrane markers: calnexin, calreticulin Enzyme: glucose-6-phosphatase

CD, cluster of differentiation; ER, endoplasmic reticulum; LAMP, lysosome-associated membrane protein; LBPA, lysobisphosphatidic acid; M6PR, mannose-6-phosphate receptor; TfR, transferrin receptor. From de Chastellier C. Electron microscopy. In: Cossart P, Boquet P, Normark, et al., eds. *Cellular Microbiology* , 2nd ed. Washington, DC: ASM Press, 2005:451, with permission.

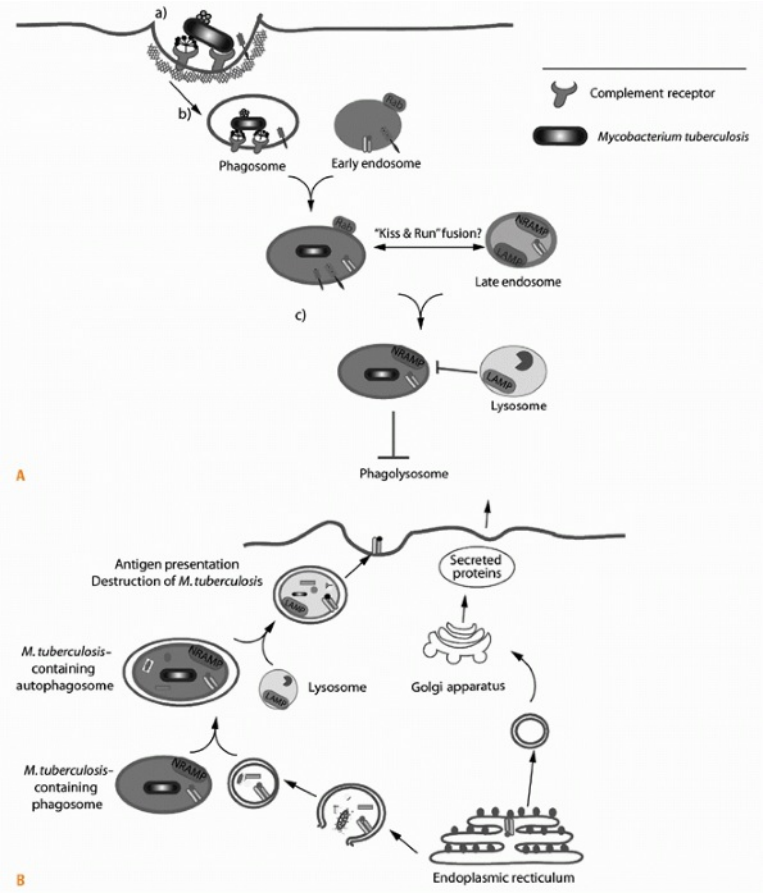

FIG. 19.9. Interactions of Selected Intracellular Pathogens with the Phagocytic Pathway. A: *Mycobacterium tuberculosis* evades destruction by subverting normal phagolysosome maturation. **a:** Phagocytosis of *M. tuberculosis* occurs via the complement pathway (although it may not require direct binding of complement to the bacterium) and is characterized by (**b**) "sinking" phagocytosis (ie, very little filopodia formation or actin polymerization). **c:** The *M. tuberculosis*-containing vacuoles contain markers of the early and late endosomes such as Rab5 and Nrampl but are devoid of most lysosomal markers, including the lysosome-associated membrane proteins, and do not undergo normal acidification. It has been proposed that the colocalization of some but not the normal allotment of endosomal markers can be explained by the concept of "kiss and run" fusion. This implies that the early and late endosomes may have transient contact with the *M. tuberculosis*-containing vacuoles, and there may be a selective transfer of markers rather than a complete fusion. The high pH of the *M. tuberculosis*-containing vacuole (pH 6.2) does not allow optimum loading of major histocompatibility complex (MHC) class II molecules, and thus they remain loaded with nonmycobacterial

peptides. Elevated pH also inhibits production of inducible nitric oxide synthase, which is required for killing. In the presence of interferon (IFN)-γ, normal acidification may be restored, resulting in destruction of the pathogen. **B:** *M. tuberculosis*-containing phagosomes are targeted to the autophagy pathway on treatment with IFNγ. IFNγ treatment can both restore the normal process of acidification and alter the expression of a number of endoplasmic reticulum proteins, the result of which is the targeting of *M. tuberculosis*-containing phagosomes to the autophagosomes. The fusion between the *M. tuberculosis*-containing phagosomes and lysosomes results in an autolysosome with low pH that destroys *M. tuberculosis* and results in *M. tuberculosis* peptides being presented via MHC class II. *(continued)*

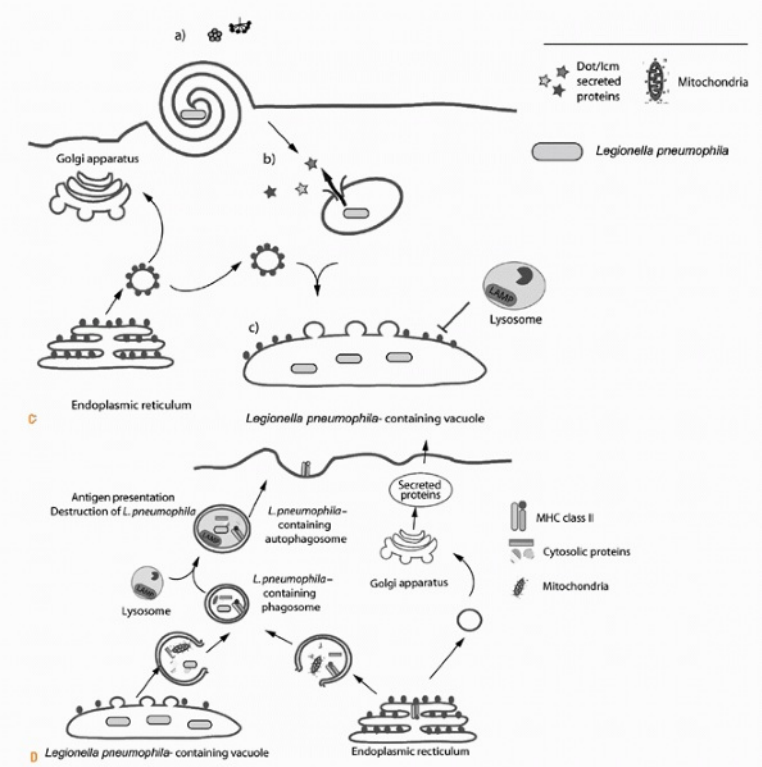

FIG. 19.9. *(continued)* **C:** *Legionella pneumophila* survives intracellularly by subverting phagosome maturation at an early stage. **a:** The recognition and uptake of *L. pneumophila* are not well characterized, but phagocysis occurs via a "spiral" mechanism. **b:** Once inside the cell, *L. pneumophila* uses a specialized secretion system (ie, Dot/Icm secretion system) to secrete proteins directly into the cytosol. These proteins alter the morphology of the vacuole in a number of ways, for example, by actively recruiting vesicles in transit from the endoplastic reticulum to the Golgi apparatus and inhibiting the fusion of lysosomes. **c:** The *L. pneumophila*-containing vacuole thus has many similarities to the Golgi apparatus and endoplastic reticulum, and is rich in peptides, the primary carbon source for *L. pneumophila.* **D:** Macrophages enhance autophagy in response to *L. pneumophila*. A consequence of residing in a vacuole that so closely resembles the endoplastic reticulum is that it is subject to autophagy. *L. pneumophila*-containing phagosomes fuse with lysosomes, resulting in destruction of the pathogen and antigen presentation. Factors secreted from *L. pneumophila* cause macrophages to increase the number of autophagosomes, although autophagosomes containing *L. pneumophila* mature more slowly, and thus it is believed that the bacteria encode factors to delay normal progression.

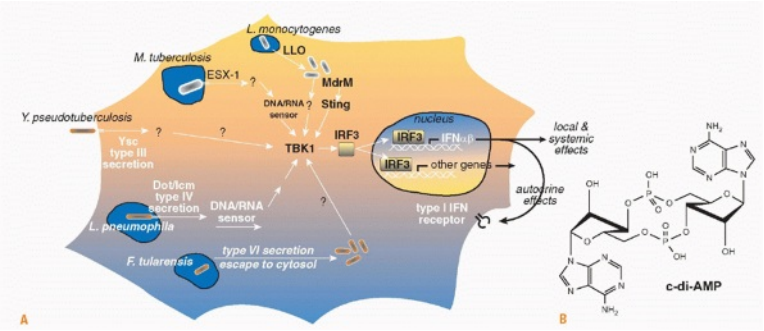

FIG. 19.10. Host Pathways Leading to the Expression of Interferon (IF) Beta and Coregulated Genes. A: Virulent, but not avirulent, intracellular pathogens activate a cytosolic surveillance pathway of innate immunity. Induction of IFN beta by all depicted bacterial species is MyD88 and TRIF-independent, indicating that toll-like receptors are not required. Sting and TBK1 are necessary for IRF3 activation.Wild-type *Listeria monocytogenes* enters the cytosol and activates the expression of IFN beta, while LLO-minus mutants that fail to enter the cytosol do not. Mutants in the multidrug efflux pump, MdrM, enter the cytosol, but induce threefold less IFN beta expression. The *L. monocytogenes* ligand is c-di-AMP **(B)**, while the receptor remains unknown. Mycobacterium tuberculosis resides in a phagosome that resembles early endosomes. Mutants in the ESX-1 auxiliary secretion system and phthiocerol dimycocerosate synthesis fail to induce IFN beta expression. Both TBK-1 and IRF3 are required. The infectious spore from *Histoplasma capsulatum* induces the expression of IFN beta, whereas the parasitic yeast form does not. *Legionella pneumophila* resides in a modified vacuolar compartment that has features of the endoplasmic reticulum. The Dot/Icm-type IV secretion system is necessary for intracellular growth and, independently, for induction of IFN beta expression. Both MDA5 and MAVS are required, suggesting that the ligand may be ribonucleic acid. *Francisella tularensis* needs to enter the cytosol to induce expression of IFN beta. The putative type VI secretion system is required for entry into the cytosol and induction of IFN beta. In each case, nucleic acids may be the ligands specifically recognized by cytosolic receptors, leading to the activation of IFN beta and co-regulated genes. Courtesy of D.A. Portnoy and R.E. Vance. For further information see Vance et al.[91] and Woodward et al.[92]

Clearance of proteinase-inhibitor complexes (eg, by CD91) and of haptoglobin-hemoglobin complexes by the Mø-receptor CD163, and protection by hemeoxygenase-1, are essential homeostatic functions of tissue Mø, limiting potentially injurious extracellular molecules.[107] Other molecules released by injured infected cells can serve as alarmins[108] and danger signals (eg, heat shock proteins[109] and S100 family members,[110] inducers of inflammatory and immune responses).

Major unsolved questions remain concerning phagocytosis, intracellular infection, and immune responses. How do sterile particulate antigens[111] and microbial agents induce T=cell responses, and what are the relative contributions to this process of Mø and DCs, abundant and sparser professional phagocytes, respectively? What is the role of Scavenger and RAGE[72,110] receptors mediating entry of diverse ligands in subsequent adaptive immunity? Does TLR engagement within vacuoles determine the kinetics of phagosome maturation as well as induce local intracellular responses? What determines the balance between total antigen degradation and loading of MHC molecules? What interactions take place between intracellular pathogens and host Mø, especially in regard to hypoxia[112] and nutritional requirements of the host and microbe? What is the role of pathogen-derived secretory products in the vacuolar milieu, in recruitment of organelles such as endoplasmic reticulum and mitochondria, and in effects on host cell biosynthesis? What are the

intracellular killing mechanisms, and how can organisms survive, or become latent, within Mø? To what extent do extracellular pathogens interact with macrophages and DCs[113]? Finally, what receptor-mediated signals induce the secretion of Mø molecules such as IL-12, IL-23, and IL-10 that direct and regulate the resultant specific immune response?

Gene Expression and Secretion

Knowledge of Mø gene expression and protein synthesis is growing rapidly from the application of gene array and proteomic technologies. After surface and endocytic stimulation, the mature Mø is able to secrete a very large range of high- and low-molecular weight products. These include enzymes involved in antimicrobial resistance (lysozyme), neutral proteinases and arachidonate metabolites that

contribute to inflammation and tissue repair, cytokines such as IL-1 and TNFα that modulate the activities of other leukocytes and endothelium, and reactive oxygen and nitrogen intermediates implicated in host defense. Proinflammatory cytokines account for part of the effects of immune adjuvants in promoting, broadening, and sustaining humoral responses. The ability to release these products depends on the prior history of the Mø, whether resident, recruited, or activated (primed), its encounters with microbial wall products, including LPS acting via TLRs, or with apoptotic cells, and exposure to cytokines and other immunomodulatory molecules in its immediate environment. Ligation of specific receptors induces various signaling pathways[114] and is able to alter gene expression in the Mø selectively. NF-κB,[115] Pu-1 and other transcription factors,[116,117,118,119] and IFN regulatory factor families[119,120] contribute to Mø-restricted or activation-dependent changes in gene expression, as does epigenetic regulation.[121] Product expression depends further on translational regulation, posttranslational modification such as proteolytic processing, intracellularly or at the cell surface, and coexpression of inhibitors such as IL-10. Messenger RNA turnover varies greatly for different products due to the presence or absence of specific 3′ instability sequences. Many Mø products are labile and act close to the cell surface; overproduction results in tissue catabolism and systemic effects associated with widespread infection or chronic inflammation, often as a result of an immunologically driven disease process.

TABLE 19.3. Modulation of Macrophage Phenotype

Category	Stimulus	Selected Marker Changes	Function
Innate activation	Microbial products (eg, LPS, other TLR ligands)	Costimulatory molecule expression, MARCO upregulation	Phagocytosis, adaptive immunity
Classic activation	Interferon γ	MHC II upregulation, proinflammatory cytokine secretion, inducible NO synthase	Cell-mediated immunity (eg, intracellular pathogens)
Alternative activation	IL-4/IL-13	Upregulation of MHC II, arginase, mannose receptor, Ym1, FIZZ1 (resistin-like), production of selected chemokines, macrophage fusion	Parasitic and allergic immunity, repair

Innate and acquired deactivation	Apoptotic cells, IL-10, glucocorticoids, TGF*β*, PGE2	Various surface and secretory markers, (eg, anti-TNF*α* actions)	Anti-inflammatory and altered immunity

IL, interleukin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; NO, nitric oxide; PGE2, prostaglandin E2; TGF*β*, transforming growth factor-*β*; TLR, toll-like receptor; TNF*α*, tumor necrosis factor-*α*.

Whereas most bioactivities have been defined in vitro, there is evidence that expression of Mø secretory activities may be quite different in situ; lysozyme production is characteristic of all Mø in culture but is downregulated on most resident cells in vivo, and its expression by granuloma Mø, for example, depends on induction by immune or phagocytic stimuli. 5′ promoter sequences of human lysozyme and CD68 transgenes have been used to target tissue- and Mø activation-specific expression of reporter molecules in vivo. The promoters of these and other Mø-restricted molecules may, in due course, make it possible to direct Mø biosynthetic activities precisely, to boost or inhibit immune responses.

Modulation of Macrophage Activation in Vitro

Our understanding of Mø activation derives from studies of induction of MHC II and costimulatory antigens; of effector functions such as proteinase, TNF*α*, reactive oxygen intermediate and reactive nitrogen intermediate release; of expression of membrane receptors such as MRs; and of resistance to infectious agents, for example, *Mycobacteria*, *Listeria*, *Candida*, and HIV. Generalizations can be made, but it must be remembered that organisms vary considerably in their ability to evade or survive Mø restriction mechanisms, and they interact with Mø in individual ways. Various inhibitory cell surface molecules (eg, CD200, SIRPα,[122] and TAM receptors[123]) are known to regulate Mø activation through interactions with other activating plasma membrane receptors. Receptors including FcR*γ* use paired immunoreceptor tyrosine-based activation motif and immunoreceptor tyrosine-based inhibitory motif intracellular signaling motifs.

Figure 19.4 and Table 19.3 illustrate various pathways and markers of Mø activation that result from microbial, cellular, and cytokine interactions. Knowledge is based mainly on in vitro experiments and in vivo challenge of selected animal models. Innate activation depends on direct stimulation by microbial products, independent of cytokines, although often enhanced by concomitant stimulation (eg, by IFN*γ*). Newly discovered markers of innate activation of mouse peritoneal macrophages include upregulation of MARCO, SR-A, via a TLR pathway, and of CD200, a more widely expressed IgSF membrane glycoprotein able to inhibit TLR, NOD-like receptors, and inflammasome activation. Induction of MARCO, a phagocytic receptor for a range of bacteria, represents an adaptation of the innate immune response to microbial contact Analysis of the actions of individual cytokines (IFN*γ*, IL-10, IL-4/13) on defined Mø targets (murine peritoneal Mø and

human monocyte-derived Mø) reveals three characteristic and distinctive in vitro phenotypes across a spectrum of activation.[124,125] IFN*γ* and its production and amplification via IL-12, IL-23, or IL-18 play a central role in MHC II induction, enhanced antimicrobial resistance, and proinflammatory cytokine production, characteristic of Th1-type (M1-type) responses; conversely, IL-10 suppresses markers of activation while inducing selective expression of other Mø genes. A comparable link between Mø/APC and the induction of Th2-type responses has proved elusive to identify. IL-4/13 have closely overlapping functions and induce an alternative, M2-type activation phenotype in Mø consistent with increased APC function and humoral responses in allergy and parasitic infection as well as giant cell formation (see Table 19.3).[126] It is important to distinguish modulation of Mø immunologic properties by IL-4/13 from marked deactivation and inhibition of proinflammatory and cytotoxic

functions by IL-10 and glucocorticosteroid. Immune complexes are also able to induce an analogous, regulatory alternative activation pathway, which overlaps with, but differs from IL-4/13- and IL-10-induced phenotypes.[127] By extension, GM-CSF, glucocorticosteroids, TGF-β, and type I IFN all modulate Mø gene expression with individual signatures.

The interplay of cytokines derived from Mø themselves, from activated T- and B-lymphocytes, and from other cells (eg, natural killer cells, endothelial cells) results in reciprocal positive or negative interactions and time-dependent changes in activating and inhibitory signals. Some predictions from in vitro studies can be extended to the intact host. For example, IFNγ, IL-12, and IL-23 deficiency results in inability to restrict opportunistic organisms in murine models and in humans, and inducible nitric oxide synthase is important for resistance to a range of infectious agents.[128] IL-10 deficiency, on the other hand, results in overactive Th1-dependent inflammation, for example, in gut. IL-4 deficiency by itself has little effect on Mø phenotype in vivo because IL-13 mimics many of its actions. These cytokines share a common receptor subunit, and its targeted genetic ablation makes it possible to study Mø that lack the ability to respond to both IL-4 and IL-13.

The foregoing analysis is oversimplified. Combinations of cytokines in vitro have different effects on Mø than the sum of the parts. For example, the combination of IL-4 and GM-CSF induces differentiation of human monocytes into immature DCs, whereas each alone induces cells with distinctive Mø properties. Furthermore, a particular “Th2-type” cytokine such as IL-10 can display radically different effects on antimicrobial (inducible nitric oxide synthase dependent) killing, which is markedly suppressed, and anti-HIV activities of Mø, which are enhanced. Whereas IFNγ and IL-4 may have opposing actions on MR expression and phagocytosis of yeast, in combination they synergize to enhance uptake markedly. Other combinations of cytokines, such as IFN$\alpha\beta$ and IFNγ, can antagonize each other, presumably by competition for signaling pathways. Selected pathogens such as *Francisella tularensis* are able to modulate macrophage responses to infection from an M1- to an M2-type, thus facilitating their survival.[129] Finally, alternative activation of macrophages has recently been implicated in thermoregulation.[130] A great deal remains to be learned about Mø behavior in physiology as well as disease.

CONCLUSION AND SOME REMAINING ISSUES

Mø influence and respond to all other cells involved in immunity, during both the afferent and efferent limbs. Many of the molecules that mediate particular functions are now defined, but their role within the Mø and in intercellular interactions is often poorly understood. Mø developed during the evolution of multicellular organisms before immunologically specific, clonotypic responses of B- and T-lymphocytes emerged. A recent report of a rearranging, TCR-like receptor in macrophages needs confirmation.[131] Mø themselves diversified in parallel with T-helper lymphocytes, generating DCs as specialized APCs for naïve T-lymphocytes and yielding a range of effector cell phenotypes in response to diverse activated T cells, both CD4+ and CD8+. Mø and their derivatives cluster with differentiating hemopoietic cells in fetal liver and bone marrow, with developing thymocytes, with naïve CD4+ T lymphocytes and antigen during immune induction, and with activated T cells and microbial pathogens in granuloma formation (see Fig. 19.2). In addition, they associate with antigen-stimulated B-lymphocytes during cell expansion, diversification, and apoptosis. A major challenge will be to define the role of specific and accessory surface molecules by which Mø discriminate between live and dying cells and to uncover the intrinsic and extrinsic factors that control Mø activities within these diverse immune cell interactions.

Our understanding of the multiple roles of Mø and DCs in immunoregulation is also evolving as we better appreciate their specializations and adaptations. Central issues in the immunobiology of Mø remain interesting topics for further investigation. These include the following:

Mø display broad functions in homeostasis, beyond host defense and immunity, which may be special instances of a more general role in preserving host integrity, comparable to that of

the central nervous and endocrine systems. Their dispersion, plasticity, and responsiveness raise obvious questions for the biologist. In particular, what are their roles in development, in trophic interactions within different organs, in angiogenesis,[132] repair, and fibrosis[133]?

The Mø lie at the heart of the classic immunologic question of recognition of altered or non-self, especially of particulates. What are the actual exogenous and endogenous[134] ligands recognized by the diverse range of plasma membrane receptors capable of direct detection, and what determines whether uptake of a target is immunologically silent or productive? How can this information be harnessed for vaccine development?

The delineation of further subsets of CD4+ T lymphocytes (TH17, regulatory T cells) suggests that it will be useful to define the effects on the Mø phenotype of contact-and cytokine-dependent interactions with these cells. It is likely that further distinctive type 2 activation pathways of Mø will be discovered by microarray and protein analysis.

Once activated, Mø change their ability to recognize and destroy targets, directly or in concert with antibody, complement, and other less-defined opsonins. Can Mø directly kill virus-infected and other immunologically activated cells? If so, do they use MHC matching, even in a limited way, and do they contribute to tolerance and, by implication, autoimmunity by failure to perform such a suppressive function?

A special case in which Mø are present in large numbers at a site of “failure” to respond immunologically is the fetoplacental unit. CSF-1 is produced locally at high levels; does this deactivate Mø or make them switch to perform a trophic role? Do tumors that are rich in Mø adopt a similar strategy[135,136,137]? Catabolism of tryptophan by Mø enzymes has been put forward as another mechanism for preventing local destruction of an allogeneic fetus.[138] This is but one example of a growing interest in immunometabolism.[139]

Although Mø express a large number of genes involved in household functions and share expression of others with a limited range of cell types, they also express highly restricted molecules responsible for unique functions. Can these be harnessed for Mø-specific gene targeting at selected microanatomic sites to deliver functionally precise signals at predetermined times? Techniques are becoming available for at least part of this fantasy, and they should provide new insights into the multiple roles of the Mø in immunity.

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REFERENCES

1. Gordon S, ed. *Cells of the Innate Immune System: Role in Health and Disease* . London, United Kingdom: Biomedical and Life Sciences Collection, Henry Stewart Talks Limited; 2012.

2. Gordon S, ed. *Macrophage Heterogeneity and Function*. London, United Kingdom: Biomedical and Life Sciences Collection, Henry Stewart Talks Limited; 2012.

3. Cavaillon JM. The historical milestones in the understanding of leukocyte biology initiated by Elie Metchnikoff. *J Leukoc Biol* . 2011;90(3): 413-424.

4. Gordon S. The macrophage: past, present and future. *Eur J Immunol* . 2007;37(suppl 1):S9-S17.

5. Gordon S, ed. *Innate Immunity: Host Recognition and Response in Health and Disease*. London, United Kingdom: Biomedical and Life Sciences Collection, Henry Stewart Talks Limited; 2009.

6. Medzhitov R, Shevach EM, Trinchieri G, et al. Highlights of 10 years of immunology in Nature Reviews Immunology. *Nat Rev Immunol* . 2011;11(10):693-702.

7. Metchnikoff E. *Immunity in Infective Disease*. Cambridge: Cambridge University Press; 1905.

8. Russell DG, Gordon S, eds. *Phagocyte-Pathogen Interactions: Macrophages and the Host Response to Infection.* Washington, DC: American Society of Microbiology; 2009.

9. Beutler B. *Nobel Lectures*. 2012.

10. Hoffman J. *Nobel Lecture.* 2012.

11. Moberg CL. An appreciation of Ralph Marvin Steinman (1943-2011). *J Exp Med*. 2011;208:2337-2342.

12. Steinman RM, Moberg CL. Zanvil Alexander Cohn 1926-1993. *J Exp Med*. 1994;179(1):1-30.

13. Wiktor-Jedrzejczak W, Gordon S. Cytokine regulation of the macrophage (M phi) system studied using the colony stimulating factor-1-deficient op/op mouse. *Physiol Rev*. 1996;76(4):927-947.

14. Geissmann F, Gordon S, Hume DA, Mowat AM, Randolph GJ. Unravelling mononuclear phagocyte heterogeneity. *Nat Rev Immunol* . 2010;10(6):453-460.

15. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* . 2005;5(12):953-964.

16. Morris L, Graham CF, Gordon S. Macrophages in haemopoietic and other tissues of the developing mouse detected by the monoclonal antibody F4/80. *Development*. 1991;112(2):517-526.

17. Chorro L, Geissmann F. Development and homeostasis of ‘ resident’ myeloid cells: the case of the Langerhans cell. *Trends Immunol*. 2010;31(12):438-445.

18. Chow A, Brown BD, Merad M. Studying the mononuclear phagocyte system in the molecular age. *Nat Rev Immunol* . 2011;11(11):788-798.

19. Jenkins SJ, Ruckerl D, Cook PC, et al. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science*. 2011;332(6035):1284-1288.

20. Auffray C. Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu Rev Immunol* . 2009;27:669-692.

21. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*. 2003;19(1):71-82.

22. Geissmann F, Manz MG, Jung S, Sieweke S, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. *Science*. 2010;327(5966):656-661.

23. Ziegler-Heitbrock L, Ancuta P, Crowe S, et al. Nomenclature of monocytes and dendritic cells in blood. *Blood*. 2010;116(16):e74-80.

24. Hume DA. Applications of myeloid-specific promoters in transgenic mice support in vivo imaging and functional genomics but do not support the concept of distinct macrophage and dendritic cell lineages or roles in immunity. *J Leukoc Biol*. 2010;89(4):525-538.

25. Fogg DK, Sibon C, Miled C, et al. A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science*. 2006;311(5757):83-87.

26. Collin M, Bigley V, Haniffa M, Hambleton S. Human dendritic cell deficiency: the missing ID? *Nat Rev Immunol*. 2011;11(9):575-583.

27. Gautier EL, Jakubzick C, Randolph GJ. Regulation of the migration and survival of monocyte subsets by chemokine receptors and its relevance to atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2009;29(10):1412-1418.

28. Swirski FK, Nahrendorf M, Erzrodt M, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science*. 2009;325(5940):612-616.

29. Ishii M, Egen JG, Klauschen F, et al. Sphingosine-1-phosphate mobilizes osteoclast precursors and regulates bone homeostasis. *Nature*. 2009;458(7237):524-528.

30. Zhu FG, Reich CF, Pisetsky DS. The role of the macrophage scavenger receptor in immune stimulation by bacterial DNA and synthetic oligonucleotides. *Immunology*. 2001;103(2):226-234.

31. Yona S, Jung S. Monocytes: subsets, origins, fates and functions. *Curr Opin Hematol*. 2009;17(1):53-59.

32. Macrophages.com. Homepage. www.macrophages.com.

33. Gordon S, Hamman J, Lin HH, Stacey M. F4/80 and the related adhesion-GPCRs. *Eur J Immunol*. 2011;41(9):2472-2476.

34. Taylor PR, Martinez-Pomares L, Stacey M, Lin HH, Brown GD, Gordon S. Macrophage receptors and immune recognition. *Annu Rev Immunol*. 2005;23:901-944.

35. Chihara T, Suzu S, Hassan R, et al. IL-34 and M-CSF share the receptor Fms but are not identical in biological activity and signal activation. *Cell Death Differ*. 2010;17(12):1917-1927.

36. Den Haan JMM, Kraal G. Innate immune functions of macrophage subpopulations in the spleen. *J Innate Immun*, In Press.

37. Martinez-Pomares L, Gordon S. CD169+ macrophages at the crossroad of antigen presentation. *Trends in Immunology*, In Press.

38. Lin HH, Faunce DE, Stacey M, et al. The macrophage F4/80 receptor is required for

the induction of antigen-specific efferent regulatory T cells in peripheral tolerance. *J Exp Med*. 2005;201(10):1615-1625.

39. Taylor PR, Gordon S, Martinez-Pomares L. The mannose receptor: linking homeostasis and immunity through sugar recognition. *Trends Immunol*. 2005;26(2):104-110.

40. Ganz T. Hepcidin and iron regulation, 10 years later. *Blood*. 2011; 117(17):4425-4433.

41. Kurotaki D, Kon S, Bae K, et al. CSF-1-dependent red pulp macrophages regulate CD4 T cell responses. *J Immunol*. 2011;186(4):2229-2237.

42. Cyster J. Lymph node macrophages. *J Innate Immun*, In Press.

43. Bain CC, Mowat AM. Intestinal macrophages - specialised adaptation to a unique environment. *Eur J Immunol* . 2011;41(9):2494-2498.

44. Ivanov II, Littman DR. Modulation of immune homeostasis by commensal bacteria. *Curr Opin Microbiol* . 2011;14(1):106-114.

45. Graham LM, Brown GD. The Dectin-2 family of C-type lectins in immunity and homeostasis. *Cytokine*. 2009;48(1-2):148-155.

46. Perry VH, Andersson PB, Gordon S. Macrophages and inflammation in the central nervous system. *Trends Neurosci*. 1993;16(7):268-273.

47. Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol*. 2011;11(11):762-774.

48. Rosen H, Gordon S. Monoclonal antibody to the murine type 3 complement receptor inhibits adhesion of myelomonocytic cells in vitro and inflammatory cell recruitment in vivo. *J Exp Med*. 1987;166(6):1685-1701.

49. Liddiard K, Rosas M, Davies LC, Jones SA, Taylor PR. Macrophage heterogeneity and acute inflammation. *Eur J Immunol* . 2011;41(9): 2503-2508.

50. Casanova JL, Abel L, Quintana-Murci L. Human TLRs and IL-1Rs in host defense: natural insights from evolutionary, epidemiological, and clinical genetics. *Annu Rev Immunol*. 2011;29: 447-491.

51. Rosenzweig SD, Holland SM. Recent insights into the pathobiology of innate immune deficiencies. *Curr Allergy Asthma Rep* . 2011;11(5): 369-377.

52. Ford JW, McVicar DW. TREM and TREM-like receptors in inflammation and disease. *Curr Opin Immunol* . 2009;21(1):38-46.

53. Mukhopadhyay S, Pluddemann A, Hoe JC, et al. Immune inhibitory ligand CD200 induction by TLRs and NLRs limits macrophage activation to protect the host from meningococcal septicemia. *Cell Host Microbe*. 2010;8(3):236-247.

54. Cassel SL, Rothman PB. Chapter 3: role of SOCS in allergic and innate immune responses. *Adv Immunol*. 2009;103:49-76.

55. Dimitriou ID, Clemenza L, Scotter AJ, Chen G, Guerra FM, Rottapel R. Putting out the fire: coordinated suppression of the innate and adaptive immune systems by SOCS1 and SOCS3 proteins. *Immunol Rev*. 2008;224:265-283.

56. Kindler V, Sappino AP, Grau GE, Piquet PF, Vassalli P. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. *Cell*. 1989;56(5):731-740.

57. Keshav S, Chung P, Milon P, Gordon S. Lysozyme is an inducible marker of macrophage activation in murine tissues as demonstrated by in situ hybridization. *J Exp Med*. 1991;174(5):1049-1058.

58. Egen JG, Rothfuchs AG, Feng CG, Winter N, Sher A, Germain RN. Macrophage and T cell dynamics during the development and disintegration of mycobacterial granulomas. *Immunity*. 2008;28(2):271-284.

59. Rose CD, Martin TM, Wouters CH. Blau syndrome revisited. *Curr Opin Rheumatol* . 2011;23(5):411-418.

60. Smith AM, Rahman FZ, Hayee B, et al. Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease. *J Exp Med*. 2009;206(9):1883-1897.

61. Hunn JP, Howard JC. The mouse resistance protein Irgm1 (LRG-47): a regulator or an effector of pathogen defense? *PLoS Pathog*. 2010;6(7):e1001008.

62. Hunn JP, Feng CG, Sher A, Howard JC. The immunity-related GTPases in mammals: a fast-evolving cell-autonomous resistance system against intracellular pathogens. *Mamm Genome*. 2010;22(1-2):43-54.

63. Alam MM, O'Neill LA. MicroRNAs and the resolution phase of inflammation in macrophages. *Eur J Immunol* . 2011;41(9):2482-2485.

64. Yona S, Gordon S. Inflammation: glucocorticoids turn the monocyte switch. *Immunol Cell Biol*. 2007;85(2):81-82.

65. Chapman KE, Odermatt A. Steroids: modulators of inflammation and immunity. *J Steroid Biochem Mol Biol*. 2010;120(2-3):67-68.

66. Meintjes G, Rabie H, Wilkinson RJ, Cotton MF. Tuberculosisassociated immune reconstitution inflammatory syndrome and unmasking of tuberculosis by antiretroviral therapy. *Clin Chest Med*. 2009;30(4):797-810, x.

67. Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol*. 2002;20:197-216.

68. Nimmerjahn F, Ravetch JV. FcgammaRs in health and disease. *Curr Top Microbiol Immunol*. 2010;350:105-125.

69. Carroll MC. Complement and humoral immunity. *Vaccine*. 2008;26(suppl 8):I28-33.

70. Gollins SW, Porterfield JS. A new mechanism for the neutralization of enveloped viruses by antiviral antibody. *Nature*. 1986;321(6067):244-246.

71. Peiris JS, Porterfield JS. Antibody-mediated enhancement of Flavivirus replication in macrophage-like cell lines. *Nature*. 1979;282(5738): 509-511.

72. Pluddemann A, Mukhopadhyay S, Gordon S. Innate immunity to intracellular pathogens: macrophage receptors and responses to microbial entry. *Immunol Rev*. 2011;240(1):11-24.

73. Gazi U, Martinez-Pomares L. Influence of the mannose receptor in host immune responses. *Immunobiology*. 2009;214(7):554-561.

74. Brown GD. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat Rev Immunol*. 2006;6(1):33-43.

75. Ruland J. CARD9 signaling in the innate immune response. *Ann N Y Acad Sci* . 2008;1143:35-44.

76. Cheng SC, van de Veerdonk FL, Lenardon M, et al. The dectin-1/inflammasome pathway is responsible for the induction of protective T-helper 17 responses that discriminate between yeasts and hyphae of Candida albicans. *J Leukoc Biol*. 2011;90(2):357-366.

77. Netea MG, van de Veerdonk FL, van Deuren M, van der Meer JW. Defects of pattern recognition: primary immunodeficiencies of the innate immune system. *Curr Opin Pharmacol*. 2011;11(4):412-422.

78. Robinson MJ, Osorio F, Rosas M, et al, Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *J Exp Med*. 2009;206(9):2037-2051.

79. Neyen C, Pluddemann A, Roversi P, et al. Macrophage scavenger receptor A mediates adhesion to apolipoproteins A-I and E. *Biochemistry*. 2009;48(50):11858-11871.

80. Bowdish DM, Sakamoto K, Kim MJ, et al. MARCO, TLR2, and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate and Mycobacterium tuberculosis. *PLoS Pathog*. 2009;5(6):e1000474.

81. Schoenen H, Bodendorfer B, Hitchens K, et al. Cutting edge: mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate. *J Immunol*. 2010;184(6):2756-2760.

82. Ravichandran KS. Beginnings of a good apoptotic meal: the find-me and eat-me signaling pathways. *Immunity*. 2011;35(4):445-455.

83. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity*. 2011;34(5): 637-650.

84. Stuart LM, Ezekowitz RA. Phagocytosis: elegant complexity. *Immunity*. 2005;22(5):539-550.

85. Flannagan S, Jaumouille V, Grinstein S. The cell biology of phagocytosis. *Ann Rev Pathol Mech Dis*. 2012;7:49-86.

86. Heinsbroek SE, Taylor PR, Martinez FO, Martinez-Pomares L, Brown GD, Gordon S. Stage-specific sampling by pattern recognition receptors during Candida albicans

phagocytosis. *PLoS Pathog*. 2008;4(11):e1000218.

87. Goodridge HS, Reyes CN, Becker CA, et al. Activation of the innate immune receptor Dectin-1 upon formation of a “phagocytic synapse.” *Nature*. 2011;472(7344):471-475.

88. De Chastellier C. Electron microscopy. In: Cossart P, Boquet P, Normark S, eds. *Cellular Microbiology*. 2nd ed. Washington, DC: American Society of Microbiology Press; 2005:451.

89. Flannagan RS, Cosio G, Grinstein S. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat Rev Microbiol*. 2009;7(5):355-366.

90. Dorhoi A, Reece ST, Kaufmann SH. For better or for worse: the immune response against Mycobacterium tuberculosis balances pathology and protection. *Immunol Rev*. 2011;240(1):235-251.

91. Russell DG. Mycobacterium tuberculosis and the intimate discourse of a chronic infection. *Immunol Rev*. 2011;240(1):252-268.

92. Woodward JJ, Iavarone AT, Portnoy DA. c-di-AMP secreted by intracellular Listeria monocytogenes activates a host type I interferon response. *Science*. 2010;328(5986):1703-1705.

93. Stavru F, Archambaud C, Cossart P. Cell biology and immunology of Listeria monocytogenes infections: novel insights. *Immunol Rev*. 2011;240(1):160-184.

94. Mougneau E, Bihl F, Glaichenhaus N. Cell biology and immunology of Leishmania. *Immunol Rev*. 2011;240(1):286-296.

95. Franchi L, Munoz-Planillo R, Reimer T, Eigenbrod T, Nunez G. Inflammasomes as microbial sensors. *Eur J Immunol*. 2010;40(3):611-615.

96. Philpott DJ, Girardin SE. Nod-like receptors: sentinels at host membranes. *Curr Opin Immunol*. 2010;22(4):428-434.

97. Schroder K, Tschopp J. The inflammasomes. *Cell*. 2010;140(6): 821-832.

98. Rathinam VA, Jiang Z, Waggoner SN, et al. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat Immunol*. 2010;11(5):395-402.

99. Vance RE, Isberg RR, Portnoy DA. Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. *Cell Host Microbe*. 2009;6(1):10-21.

100. Burdette DL, Monroe KM, Sotelo-Troha K, et al. STING is a direct innate immune sensor of cyclic di-GMP. *Nature*. 2011;478:515-518.

101. Harris J. Autophagy and cytokines. *Cytokine*. 2011;56(2):140-144.

102. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. *Nature*. 2011;469:323-335.

102. Amer AO, Swanson MS. A phagosome of one's own: a microbial guide to life in the macrophage. *Curr Opin Microbiol* . 2002;5(1):56-61.

103. Clemens DL, Horwitz MA. Uptake and intracellular fate of Francisella tularensis in human macrophages. *Ann N Y Acad Sci* . 2007;1105:160-186.

104. Celli J. Surviving inside a macrophage: the many ways of Brucella. *Res Microbiol*. 2006;157(2):93-98.

105. Roy CR, Salcedo SP, Gorvel JP. Pathogen-endoplasmic-reticulum interactions: in through the out door. *Nat Rev Immunol* . 2006;6(2):136-147.

106. Sibley LD. Invasion and intracellular survival by protozoan parasites. *Immunol Rev*. 2011;240(1):72-91.

107. Gozzelino R, Jeney V, Soares MP. Mechanisms of cell protection by heme oxygenase-1. *Annu Rev Pharmacol Toxicol* . 2010;50:323-354.

108. Yang D, Tewary P, de la Rosa G, Wei F, Oppenheim JJ. The alarmin functions of high-mobility group proteins. *Biochim Biophys Acta*. 2010;1799(1-2):157-163.

109. Van Eden W, Wick G, Albani S, Cohen I. Stress, heat shock proteins, and autoimmunity: how immune responses to heat shock proteins are to be used for the control of chronic inflammatory diseases. *Ann N Y Acad Sci* . 2007;1113:217-237.

110. Leclerc E, Heizmann CW. The importance of Ca2+/Zn2 + signaling S100 proteins and RAGE in translational medicine. *Front Biosci (Schol Ed)* . 2011;3:1232-1262.

111. Shi Y, Evans JE, Rock KL. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature*. 2003;425(6957):516-521.

112. Nizet V, Johnson RS. Interdependence of hypoxic and innate immune responses. *Nat Rev Immunol* . 2009;9(9):609-617.

113. Areschoug T, Plüddemann A, Gordon S. Innate immunity against bacteria. In: Kaufmann SH, Rouse T, Sacks DL, eds. *The Immune Response to Infection*,. Washington, DC: American Society of Microbiology Press; 2011: 209-223.

114. Ivashkiv LB. Inflammatory signaling in macrophages: transitions from acute to tolerant and alternative activation states. *Eur J Immunol* . 2011;41(9):2477-2481.

115. Vallabhapurapu S, Karin M. Regulation and function of NF-kappaB transcription factors in the immune system. *Annu Rev Immunol* . 2009;27:693-733.

116. Glass CK, Saijo K. Nuclear receptor transrepression pathways that regulate inflammation in macrophages and T cells. *Nat Rev Immunol* . 2010;10(5):365-376.

117. Ostuni R, Natoli G. Transcriptional control of macrophage diversity and specialization. *Eur J Immunol* . 2011;41(9):2486-2490.

118. Sarrazin S, Sieweke M. Integration of cytokine and transcription factor signals in hematopoietic stem cell commitment. *Semin Immunol* . 2011;23(5):326-334.

119. Savitsky D, Tamura T, Yanai H, Taniguchi T. Regulation of immunity and oncogenesis by the IRF transcription factor family. *Cancer Immunol Immunother* . 2010;59(4):489-510.

120. Krausgruber T, Blazek K, Smallie T, et al. IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nat Immunol* . 2011;12(3):231-238.

121. Satoh T, Takeuchi O, Vandenbon A, et al. The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. *Nat Immunol* . 2010;11(10):936-944.

122. Barclay AN, Brown MH. The SIRP family of receptors and immune regulation. *Nat Rev Immunol* . 2006;6(6):457-464.

123. Lemke G, Rothlin CV. Immunobiology of the TAM receptors. *Nat Rev Immunol* . 2008;8(5):327-336.

124. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. *Immunity*. 2010;32(5):593-604.

125. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol* . 2009;27:451-483.

126. Helming L, Gordon S. Molecular mediators of macrophage fusion. *Trends Cell Biol* . 2009;19(10):514-522.

127. Fleming BD, Mosser DM. Regulatory macrophages: setting the threshold for therapy. *Eur J Immunol* . 2011;41(9):2498-2502.

128. Bogdan C. Regulation and antimicrobial function of inducible nitric oxide synthase in phagocytes. In: Russell DG, Gordon S, eds. *Phagocyte-Pathogen Interactions: Macrophages and the Host Response to Infection*. Washington, DC: American Society of Microbiology Press; 2009: 367-378.

129. Shirey KA, Cole LE, Keegan AD, Vogel SN. Francisella tularensis live vaccine strain induces macrophage alternative activation as a survival mechanism. *J Immunol* . 2008;181(6):4159-4167.

130. Nguyen KD, Qiu Y, Cui X, et al. Alternatively activated macrophages produce catecholamines to sustain adaptive thermogenesis. *Nature*. 2011;480(7375):104-108.

131. Beham AW, Puellmann K, Laird R, et al. A TNF-regulated recombinatorial macrophage immune receptor implicated in granuloma formation in tuberculosis. *PLoS Pathogens*. 2011;7:1-16.

132. Nucera S, Biziato D, De Palma M. The interplay between macrophages and angiogenesis in development, tissue injury and regeneration. *Int J Dev Biol* . 2011;55(4-5):495-503.

133. Barron L, Wynn TA. Macrophage activation governs schistosomiasis-induced inflammation and fibrosis. *Eur J Immunol* . 2011;41(9): 2509-2514.

134. Garcia-Vallejo JJ, van Kooyk Y. Endogenous ligands for C-type lectin receptors: the true regulators of immune homeostasis. *Immunol Rev*. 2009;230(1):22-37.

135. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol*. 2010;11(10):889-896.

136. Egeblad M, Nakasone ES, Werb Z. Tumors as organs: complex tissues that interface with the entire organism. *Dev Cell*. 2010;18(6): 884-901.

137. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell*. 2010;141(1):39-51.

138. Mellor AL, Sivakumar J, Chandler P, et al. Prevention of T cell-driven complement activation and inflammation by tryptophan catabolism during pregnancy. *Nat Immunol*. 2001;2(1):64-68.

139. Chawla A, Nguyen KD, Goh YP. Macrophage-mediated inflammation in metabolic disease. *Nat Rev Immunol*. 2011;11(11):738-749.

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Chapter 20

Granulocytes and Mast Cells

Patrizia Scapini

Nicola Tamassia

Carlo Pucillo

Marco A. Cassatella

INTRODUCTION

Polymorphonuclear leukocytes or granulocytes are hematopoietically derived blood cells that typically act at the frontline of innate defense in host response to foreign microorganisms. Granulocytes contain heterogeneous cytoplasmic granules, which are storage pools for cell-specific intracellular enzymes, preformed receptors, cationic proteins, and other cell-specific molecules. According to their granular staining properties, polymorphonuclear leukocytes are classified into three different populations: neutrophils, eosinophils (Eos), and basophils (Bas). Neutrophil granules stain preferentially with neutral dyes, eosinophilic granules stain with acidic colorants such as eosin, and Ba granules stain with basic dyes.

Mast cells (MCs), similarly to polymorphonuclear leukocytes, represent another crucial effector cell type of the innate immune system that also stores elevated amounts of preformed inflammatory mediators within their cytoplasmic granules. However, while polymorphonuclear leukocytes are mainly peripheral blood-circulating cells, MCs are tissue resident cells distributed throughout the vascularized tissues or serosal cavity. Granulocytes and MCs differ in their functions and roles during the inflammatory process, MCs, Bas, and Eos being, for instance, essential components of allergic inflammation. Interestingly, recent data have revealed that granulocytes and MCs may also play key roles in orchestrating the transition from innate to adaptive immunity. These latter observations have caught the attention of immunologists who are currently reevaluating the importance of granulocytes and MCs, and very intensively working to clarify their multifaceted aspects in immunity.

NEUTROPHILS

Neutrophils are well known to function as the first line of defense against invading pathogens, principally bacteria and fungi, but also viruses.[1] These cells, together with monocytes, macrophages, and dendritic cells (DCs), feature the characteristic properties of the “professional” phagocytes and utilize several effector mechanisms to destroy pathogens, including the generation of massive amounts of reactive oxygen species (ROS) in combination with the discharge of many potent antimicrobial enzymes or factors.[1] Because of their powerful microbicidal equipment, neutrophils are often depicted as harmful cells that can

cause damage to the surrounding tissues during acute inflammation (as observed in those inflammatory diseases dominated by neutrophils).[1] Nonetheless, extensive research performed in the last 20 years has recognized neutrophils as highly versatile and sophisticated cells displaying a significant synthetic capacity as well as an important role in linking the innate and adaptive arms of the immune response.[2,3]

Neutrophil Generalities

Neutrophils are the most abundant (40% to 70%) circulating leukocyte type in human blood, normally present at 2.5 to 7.5 × 10^9 cells/L.[4] Morphologically, these cells can be identified by the peculiar shape of their nucleus, which is polymorphous and usually consists of three to five sausage-shaped masses of chromatin connected by fine threads (Fig. 20.1). Mature neutrophils are terminally differentiated, nondividing cells that develop and mature in the bone marrow from pluripotent CD (cluster of differentiation)34-positive (CD34+) stem cells, under the regulatory effects of several colony-stimulating factors (CSFs), including granulocyte-macrophage CSF (GM-CSF), granulocyte CSF (G-CSF), interleukin (IL)-3, and IL-6.[4] Neutrophils can be cytofluorimetrically identified by their characteristic morphology (high side scatter) and expression pattern of plasma membrane proteins such as CD66b, CD11b, CD15, and CD16, in conjunction with the lack of expression of CD2 and CD19. In humans, circulating polymorphonuclear leukocytes are 10 to 20 μm in diameter, display a half-life previously thought to correspond to 7 to 12 hours, but more recently reevaluated and extended to up to 90 hours,[5] and exist in a dynamic equilibrium with a "marginated" pool that is sequestered within the microvasculature of many organs.[1,4] In the resting uninfected host, the peripheral neutrophil population is maintained within a constant number by several mechanisms. One of them consists in programming neutrophils to spontaneously undergo apoptosis[1,3] to be, in turn, cleared by tissue macrophages located in the bone marrow, spleen, and liver.[6] In this latter context, a feedback loop involving an IL-23/IL-17/G-CSF axis, crucial for the regulation of neutrophil production, has been recently identified in mice.[7] According to this model, the uptake of apoptotic neutrophils by macrophages and DCs would determine a downregulation of their IL-23 production. Consequently, the Th17 subset of proinflammatory T-lymphocytes would be poorly sustained and thus much less IL-17A would be generated. As IL-17A positively regulates fibroblast- and endothelial cell-derived G-CSF, which is essential for controlling both granulopoiesis and neutrophil survival, the final outcome of this circuit—triggered by the massive neutrophil

apoptosis at peripheral sites—would consist in a decrease in the levels of neutrophils released from the bone marrow.[7] On the other hand, the number of circulating neutrophils can dramatically increase (even up to 10-fold) under acute inflammatory conditions (eg, during a bacterial infection), from accelerated neutrophil production and release from the bone marrow.[4] Moreover, even the lifespan of neutrophils is significantly extended under inflammatory conditions, as various host- and pathogen-derived mediators such as G-CSF, GM-CSF, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, lipopolysaccharide, and nucleic acids inhibit neutrophil apoptosis and hence prolong their survival.[8]

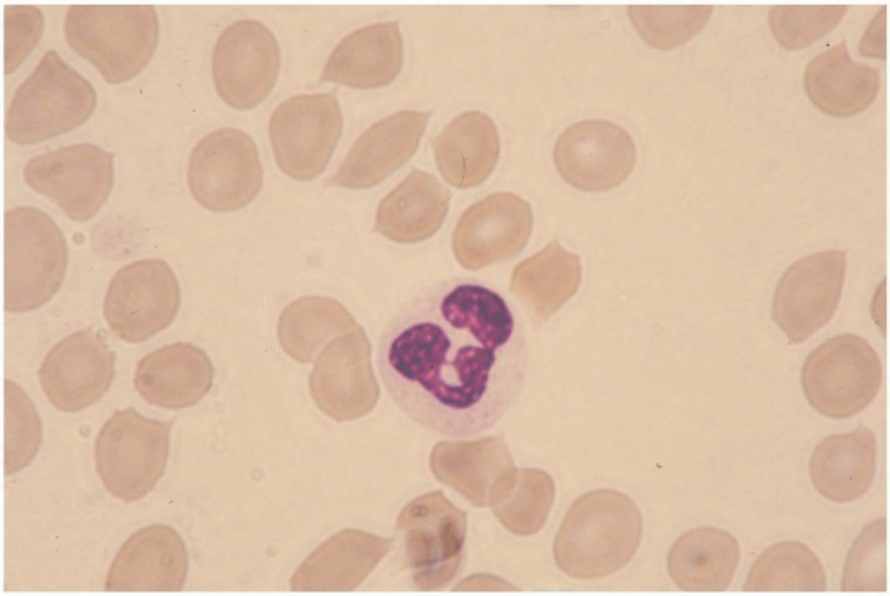

FIG. 20.1. A Polymorphonuclear Neutrophil Circulating in Peripheral Blood. From *Anderson's Atlas of Hematology* ; Anderson, Shauna C., PhD. Copyright 2003, Wolters Kluwer Health/Lippincott Williams & Wilkins.

During an acute inflammatory response, neutrophils are rapidly recruited to the site of injury by a coordinated sequence of events that begin with the elaboration of various mediators able to specifically promote their migration from the intravascular compartment. Mediators derive from numerous sources (tissue macrophages, endothelial cells, activated plasma components) and include vasoactive amines, proinflammatory lipids, small polypeptides, chemotactic factors, and cytokines such as TNFα, IL-1β, or IL-17A (the latter being one of the most abundant products of Th17 cells).[1,3] Chemotactic factors, in particular, are generated in temporally distinct waves and include C5a, leukotriene-B_4 (LTB_4), formyl-Met-Leu-Phe (fMLF), as well as neutrophil specific chemokines, such as CXCL8/IL-8, CXCL1/GROα, CXCL5/epithelial cell neutrophil-activating protein-78, etc.[1,3] Once recruited at an inflammatory site, neutrophils function as mobile arsenals that recognize, phagocytose, and ultimately destroy their targets. If the acute inflammatory response correctly subsides, then neutrophils may actively participate in its resolution (see following discussion). If not, an uncontrolled and continuous release of the proinflammatory cargo (ROS and proteases) by neutrophils recruited at the site of infection/injury may eventually lead to destruction of bystander tissue, and thus to exacerbation of the ongoing inflammation, ultimately provoking the onset of chronic inflammatory/autoimmune diseases.[9]

Neutrophil Microbicidal Mechanisms

To destroy and eliminate invading pathogens, neutrophils essentially utilize two fundamental mechanisms[10]: an oxygen-dependent process that is mediated by the generation of ROS, which include O_2^- (superoxide anion), hydrogen peroxide, singlet oxygen, and other products derived from the metabolism of hydrogen peroxide; and an oxygen-independent process consisting in the release into the phagocytic vacuole of lytic enzymes; and antimicrobial

polypeptides stored in their intracellular granules.[10] The oxygen-dependent process, also referred to as the “respiratory burst,” is defined as an increase of a mitochondrial independent oxidative metabolism that leads to the generation of O_2^-, which occurs through the activation of the phagocytic NADPH oxidase, an enzymatic system that is unique to phagocytes (neutrophils, monocytes, macrophages, DCs, and also Eos). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a multiprotein complex formed by a flavocytochrome-b_{558} (a heterodimer of gp91*phox* and p22*phox* chains, where *phox* stands for phagocyte oxidase), three cytoplasmic components (namely p40*phox*, p47*phox*, p67*phox*) and either Rac1 or Rac2 from the Rho family of low-molecular-weight GTPases (Fig. 20.2). Upon cell stimulation, the cytosolic components of the complex become phosphorylated and assemble together with the cytochrome and Rac1/2 on the plasma membrane, thus forming the active enzyme that produces superoxide anion radicals, by catalyzing the transfer of electrons from NADPH to molecular oxygen (see Fig. 20.2). O_2^- is converted by superoxide dismutase into hydrogen peroxide, which, in the presence of myeloperoxidase and halogens, is then metabolized into hypochlorous acid. The latter represents one of the neutrophil's

major weapons against microbes, as it also synergizes with granule proteins to kill pathogens in the neutrophil phagolysosome. O_2^- can also reacts with other cellular radicals, such as nitric oxide, to form different species of cytotoxic oxidant, such as peroxynitrite. The critical role of NADPH oxidase and its products in host defense is best illustrated by the plight of patients with chronic granulomatous disease, in which mutations in any of the NADPH oxidase complex subunits (gp91*phox*, p22*phox*, p40*phox*, p47*phox*, and p67*phox*) leads to a severe immunodeficiency characterized by defective killing of phagocytosed pathogens for the lack of ROS generation.[10] These infections typically involve microorganisms for which oxidant-mediated killing is particularly critical for effective host defense, such as *Staphylococcus aureus*, *Aspergillus* spp., *Nocardia*, and a variety of gramnegative enteric bacilli.

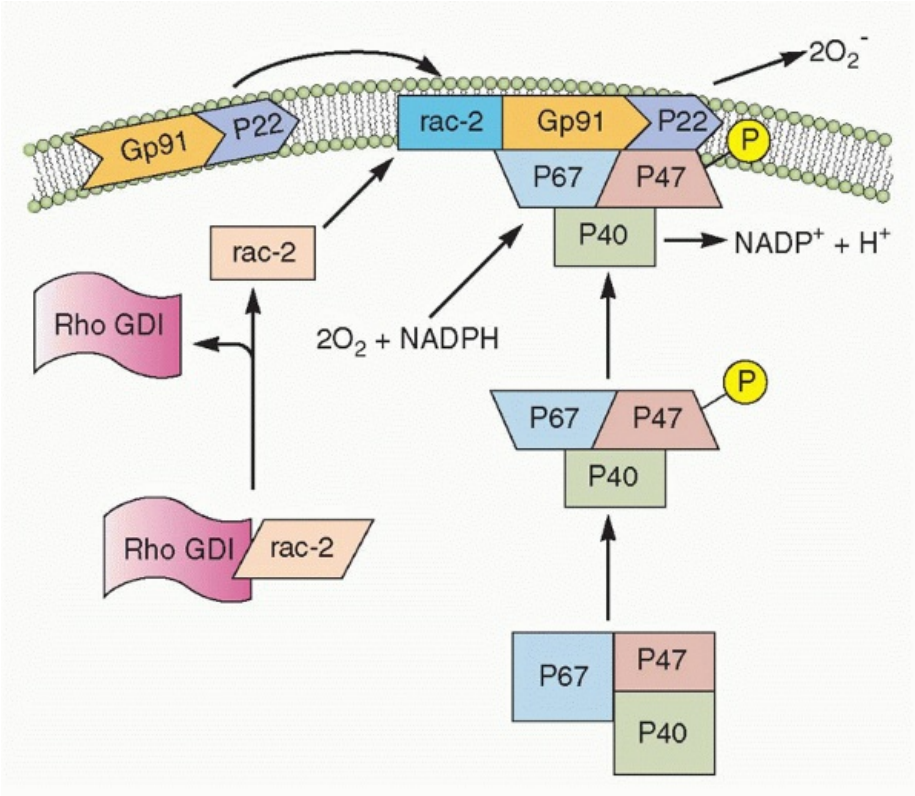

FIG. 20.2. Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase Assembly. In the resting neutrophil, the cytochrome b subunits gp91-phox and p22-phox are tightly bound in the membrane. p47-phox, p67-phox, and rac-s complex are in the cytosol. Upon activation, Rho GDP-dissociation inhibitor (GDI) releases rac-2, and p47-phox becomes phosphorylated. This causes translocation of rac-2, p47-phox, and p67-phox to the membrane and complex formation with the cytochrome components, thereby completing the assembly of the active oxidase. (After Burg ND, Pillinger MH. *Clin Immunol*. 2001;1:7-17.)

The release of potent proteolytic enzymes contained in their granules represents the other crucial mechanisms utilized by neutrophils to eliminate pathogens following phagocytosis.[10] Neutrophil granules are subdivided into peroxidase-positive granules (based on the presence of myeloperoxidase, their marker), also called primary or azurophil granules (owing to their affinity for the basic dye azure A), and peroxidase-negative granules that include the specific (secondary) granules, the gelatinase (tertiary) granules, and the secretory vesicles[11] (Fig. 20.3). The different types of granules appear at progressive stage of neutrophil development,[11] with the primary granules, as suggested by the name, being the first ones to appear during hematopoiesis at the promyelocyte stage. As highlighted in Figure 20.3, granules are released in a hierarchical order and under separate control by mature neutrophils, depending on the type of stimulus.[11]

Azurophil or primary granules undergo limited exocytosis in response to stimulation and are packaged with acidic hydrolases and antimicrobial proteins that contribute primarily to the killing and degradation of engulfed microorganisms into the phagolysosome.[11,12] These granules contain myeloperoxidase, an enzyme that catalyzes the formation of hypochlorous

acid, hydrolases, lysozyme, matrix metalloproteinases, and three structurally related serprocidins (serine proteases with microbicidal activity): proteinase-3, cathepsin G, and elastase. The latter proteins can degrade a variety of extracellular matrix components, including elastin, fibronectin, laminin, type IV collagen, and vitronectin. Azurophil granules also contain antimicrobial molecules such as bactericidal/permeability-increasing protein (which is important for killing gram-negative bacteria) and α-defensins, a family of small cysteine-rich antibiotic peptides with broad antimicrobial activity against bacteria, fungi, and certain enveloped viruses.[11,12]

Specific (or secondary) granules, which are formed at the myelocytic stage, are smaller and less dense than the azurophil ones, and contain unique constituents, such as collagenase, haptoglobin, vitamin B_{12}-binding protein, as well an extensive array of membrane-associated proteins including cytochromes, signaling molecules, and receptors.[11,12] Secondary granules also contain an arsenal of antimicrobial substances, such as lactoferrin, neutrophil gelatinase-associated lipocalin, lysozyme, hCAP18 (cathelicidin human cationic antimicrobial protein of 18 kDa, the proform of LL-37), and pentraxin-3. The inhibitory effect of antimicrobial proteins include depriving ions essential for microbial survival, degrading structural components of microorganisms (eg, peptidoglycan), and disrupting the integrity of target cell membrane by punching pores in the membrane or by perturbing membrane integrity. Neutrophil-specific granules also contain an important family of soluble proteinases, known as matrix metalloproteinases (MMPs), which include neutrophil collagenase-2 (MMP-8), gelatinase-B (MMP-9), and leukolysin (MMP-25). These proteinases are generally stored as inactive proenzymes and undergo proteolytic activation following granule fusion and interaction with azurophilic granule contents. Neutrophil MMPs disrupt major structural components of bacteria and/or extracellular membranes, and are therefore crucial not only for bacterial killing, but also for neutrophil extravasation and migration.[11,12]

Tertiary granules are produced at the metamyelocyte stage of differentiation and are smaller, lighter, and more easily exocytosed than the other granule classes.[11,12] These granules are indeed important primarily as a reservoir of matrixdegrading enzymes and membrane receptors needed during polymorphonuclear leukocyte extravasation and diapedesis. The primary constituent of tertiary granules is gelatinase, a latent metalloenzyme with the capacity for tissue destruction. Finally, the secretory vesicles are smaller than the other granules, are generated by endocytosis during the late stage

of nuclear neutrophil segmentation in the bone marrow, and are the most readily mobilizable.[11,12] These vesicles are preferentially directed to the plasma membrane, as reflected in the density of vesicle-associated membrane protein (VAMP), a fusogenic protein associated with the granule membrane. Secretory vesicles do not contain toxic substances, but mainly plasma proteins like albumin and receptors (including β_2-integrins, the complement receptor [CR]1, receptors for formylated bacterial peptides [fMLF-R], CD14, the Fc portion of γ-immunoglobulins (Igs) [FcγRIII/CD16], and the metalloprotease leukolysin). Heparin-binding protein (also known as CAP37 or azurocidin), whose release is essential for the polymorphonuclear leukocyte-induced increase in vascular permeability at the initial stage of extravasation, is also stored in the secretory vesicles.

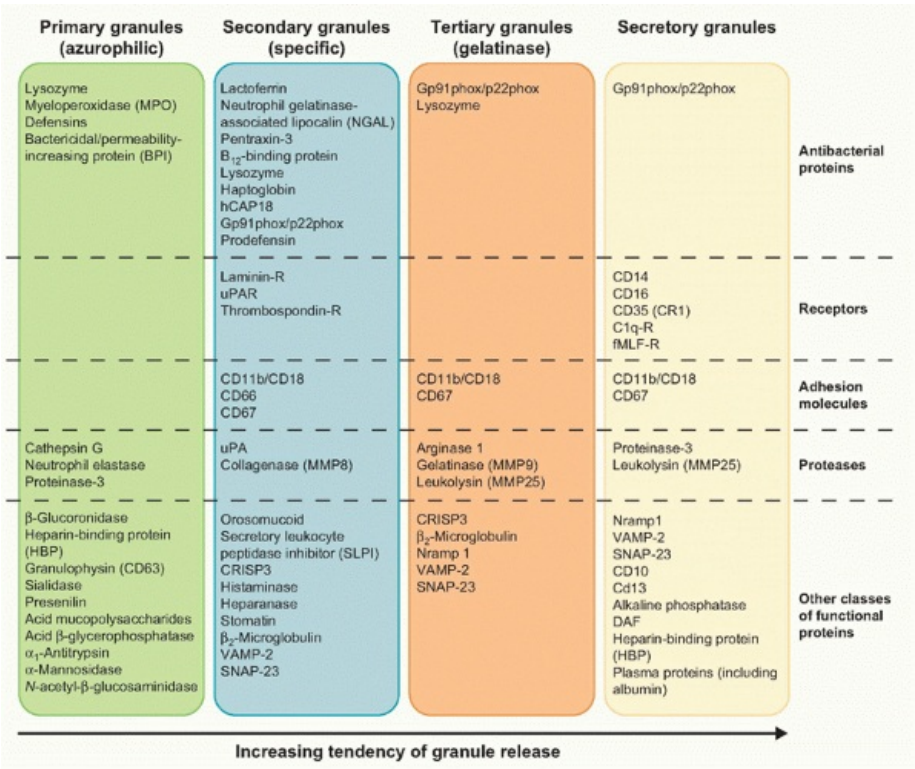

FIG. 20.3. Main Constituents of Neutrophil Granules.

An additional nonphagocytic microbicidial mechanism used by neutrophils to capture and destroy microbes in the extracellular space consists in the ability of neutrophils to form so-called neutrophil extracellular traps (NETs).[13] The latter structures consist of nuclear chromatin decorated with antimicrobial peptides and enzymes (eg, bactericidal/permeability-increasing protein, elastase, pentraxin3, cathepsin G, and many others) that lacks membranes and cytosolic markers.[13] NETtosis, a novel type of neutrophil death mechanism that occurs under settings of extreme neutrophil stimulation (different from necrosis, apoptosis, and also independent from caspase activation), underlies the generation of NETs.[14] Accordingly, the nuclear envelope, granules, and cell membranes gradually dissolve during NETtosis, allowing the nuclear contents to mix and condense in the cytoplasm before being released into the extracellular space.[14] NETs, in turn, bind to various grampositive and gram-negative bacteria (such as *Staphylococcus aureus*, *Salmonella typhimurium*, *Streptococcus pneumoniae*,

and group A streptococci), as well as to pathogenic fungi (such as *Candida albicans*). Similarly to what happens in the phagolysosome, the high local concentration of antimicrobial peptides and enzymes is responsible for the killing of the pathogens trapped by NETs.[13] The observation that neutrophils from patients with chronic granulomatous disease do not form NETs has suggested, on the one hand, that ROS-mediated signaling/cascades are involved in NET generation, and, on the other hand, that the lack of NETs might contribute to the pathogenesis of chronic granulomatous disease.[3,14] Whatever the case, it is noteworthy to remark that both the oxygen-dependent and -independent effector mechanisms in host

defense toward pathogens are also utilized by neutrophils for their cytotoxic and tumoricidal activities.

Neutrophil Receptors

Under inflammatory conditions, neutrophils sense a wide range of extracellular ligands that, through the interaction with specific receptors, subsequently trigger a number of effector functions, including adhesion, migration, phagocytosis, survival, cell activation, gene expression modulation, target cell killing, and mediator production and release.[1,3,15] For instance, agonist-stimulated neutrophils may trigger not only degranulation, but also the release of arachidonic acid and/or other eicosanoids (eg, prostaglandin [PG]E_2), via the activation and/or the upregulation of PLA_2 and COX-2, respectively. Upon appropriate stimulation, neutrophils can also generate LTA_4 through the action of 5-lipoxygenase, as well as LTB_4 by converting LTA_4 via the action of LTA_4 hydrolase.[15] A nonexhaustive list of neutrophil receptors includes 1) receptors for proinflammatory mediators (eg, the anaphylotoxin complement component C5a, LTB_4, platelet-activating factor [PAF], substance P, and fMLF); 2) receptors for cytokines, such as IFNγ, IL-1, IL-4, IL-6, IL-10, IL-13, IL-15, IL-18, TNFα, G-CSF, GM-CSF, and many others; 3) receptors for chemokines, including CXCR1 and CXCR2; 4) receptors/adhesion molecules for the endothelium; 5) receptors for tissue matrix proteins; and 6) opsonin receptors, such as FcγRs and those for the major cleavage fragments of the complement system (see following discussion). Neutrophils also express a variety of pattern recognition receptors (PRRs), including all toll-like receptors (TLRs), with the exception of TLR3, cytoplasmic ribonucleic acid helicases involved in viral ribonucleic acid recognition such as MDA5 and RIGI, and deoxyribonucleic acid binding cytoplasmic proteins (IFI16 and LRRFIP1).[3]

The Role of Neutrophils in Acute Inflammation

In order to carry out their prototypical defensive role in acute inflammation, bloodstream neutrophils must extravasate. To do so, they attach to activated endothelium, transmigrate through postcapillary venules (diapedesis), and then migrate toward a corresponding chemotactic gradient to the injury site where they recognize their target, engulf, and finally destroy it.

Neutrophil Extravasation

In general, leukocyte recruitment during inflammation, also called extravasation, is a multistep and highly complex phenomenon characterized by a number of predetermined steps occurring in the vessel lumen, known as leukocyte capture (or tethering), rolling, activation, and firm adhesion (arrest)[16] (Fig. 20.4). These latter steps are not discrete phases of inflammation; rather, they simply represent a sequence of events from the perspective of each leukocyte. Moreover, each of these steps appears to be necessary for the effective leukocyte recruitment; blocking any of them can severely reduce leukocyte accumulation in the tissue.[17] Adhesion of blood leukocytes to the endothelium during inflammation requires specific leukocyte-endothelial interactions involving different families of adhesion molecules. The latter include members of the selectin family and their cognate carbohydrate and glycoprotein ligands, which mediate the initial leukocyte deceleration along the vessel wall (a

process called “rolling”), as well as members of the integrin family and their cognate Ig superfamily ligands, which mediate the subsequent high-affinity adhesion and arrest of leukocytes to the venules[16,17] (Table 20.1).

Under noninflammatory conditions, neutrophils (as well as other leukocytes) travel primarily through the center of the blood vessel lumen, where the flow is fastest. In response to proinflammatory signals, however, both the neutrophils and the blood vessels undergo a series of changes. As a consequence of vascular dilatation, blood flow first increases and then slows, thus facilitating the interactions between leukocytes and the endothelial cells. The process of capture/tethering and rolling then ensues, in which L-selectins on neutrophils, and P- or E-selectins on endothelia, interact with sialyl-Lewisx moieties or PSGL-1 on their respective cell partners. Although these interactions are reversible and transient, they prepare neutrophils for a tighter binding, integrin-mediated step.[16,17] The most important neutrophil integrins are four, each one composed of an identical β-subunit β_2, known as CD18) noncovalently linked to different α-subunits: CD11a/CD18 ($\alpha_L\beta_2$, lymphocyte function antigen), CD11b/CD18 ($\alpha_M\beta_2$, CR3), CD11c/CD18 ($\alpha_X\beta_2$, CR4), and CD11d/CD18.[18] When neutrophils encounter their specific chemoattractants (eg, CXCL8), displayed bound to glycosaminoglycans on the vessel wall, neutrophil integrins are converted from an inactive to an active conformation[16,17] (see Fig. 20.4). Activated integrins can interact with their counterreceptors on the surface of the TNFα and/or IL-1β-activated endothelium (eg, intercellular adhesion molecule-1 [ICAM-1] and intercellular adhesion molecule-2), leading to the strong adhesion and arrest of the cells. Neutrophils then flatten and transmigrate between and through the endothelial cells of postcapillary venules into the surrounding tissue (see Fig. 20.4), also secreting a broad range of MMPs that degrade the basement membrane. Transmigration involves homophilic interaction of CD31/PECAM-1 and JAM-A on neutrophils and endothelial cells, where CD31/PECAM-1 and JAM-A act sequentially to mediate neutrophil migration through the venular walls.[17,18] Once in the interstitial compartment, neutrophils migrate along the chemotactic gradient toward the site of

injury or infection and, once arrived, begin to react with the etiopathogenic agent.

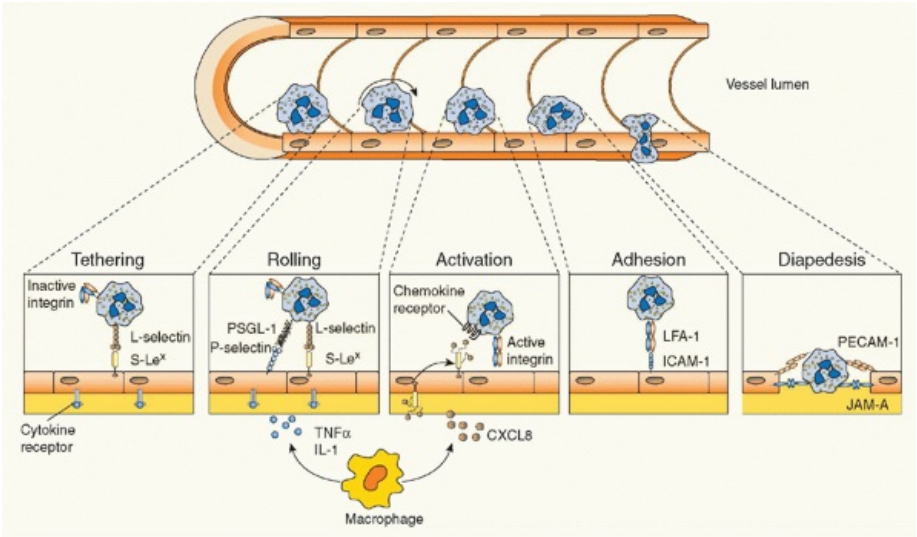

FIG. 20.4. Leukocyte Transmigration. Following an inflammatory stimulus, tissue-resident macrophages and other cells release inflammatory mediators such as tumor necrosis factor α and interleukin-1, which induce the rapid expression of preformed P-selectin (and transcription-dependent E-selectin expression) on the endothelium. The interaction between selectins and their glycoprotein ligands initiates leukocyte tethering and rolling. Activation by chemokines—and other leukocyte activators (eg, leukotriene-B_4 or platelet-activating factor)—presented on endothelial cells causes leukocyte integrin activation, thus resulting in transition from cell rolling to cell firm adhesion, in view of the strength of integrin-mediated binding with endothelial immunoglobulin superfamily members. Leukocytes can then transmigrate through the endothelial monolayer and chemotactically move toward the inflammatory stimulus. Examples of adhesion molecules involved in each step are depicted.

The fundamental importance of adhesive glycoproteins in vivo is testified by those individuals affected leukocyte adhesion deficiency (LAD) disease, who display an abnormally high susceptibility to bacterial infections.[19] Several types of LAD disease have been identified. In LAD-I, mutations of the β2 integrin typically eliminate the expression of all four integrin complexes. Because of the multiple defects in adhesion-related functions, patients with LAD-I develop recurrent bacterial and fungal infections, mostly with *Staphylococcus aureus* or gram-negative enteric microbes. Neutrophilia with paucity of neutrophils at inflamed or infected sites is characteristic of LAD-I, while typical clinical features include frequent skin and periodontal infections, delayed separation of the umbilical cord and omphalitis, and deep tissue abscesses. LAD-II is caused by mutations in the membrane transporter for fucose and thus is associated with loss of expression of fucosylated glycans on the cell surface. Fucosylated proteins such as sialyl-Lewis X (CD15s) are ligands for endothelial selectins and are important for the rolling phases of leukocyte extravasation. Patients with LAD-II also have leukocytosis and form pus poorly, although infections tend to be less severe in patients with LAD-II than in patients with LAD-I. Finally, LAD-III has been recently described. LAD-III is an autosomal recessive disorder caused by mutations in the human kindlin-3 gene, which codes for a protein essential for integrin activation. Consequently, LAD-III is characterized by impaired adhesion of leukocytes to the endothelium of inflamed tissues and by severe bleeding. Curiously, a number of LAD-III patients additionally suffer from osteopetrosis.

Neutrophil-Mediated Phagocytosis

Neutrophils play a critical role in host protection as they eliminate microorganisms through phagocytosis (the cellular process of engulfing particles larger than 0.5 μm). While many cells in our body are capable of phagocytosis, neutrophils do it to an extent sufficient to be considered “professional phagocytes” (eg, a single neutrophil can engulf up to 10 to 12 particles [eg, bacteria]).[20] Phagocytosis is triggered either through receptor (eg, mannose receptor or Dectin-1) recognition of certain polysaccharides present on the surface of some yeast cells and/or upon the binding of opsonized microorganisms through, for instance, FcγRs and CRs.[20] Neutrophils constitutively express the low-affinity

FcγRs (FcγRIIA/CD32A and FcγRIIIA/CD16A), and, when exposed to IFNγ or G-CSF, the high-affinity FcγR (FcγRI/CD64) as well.[3] CRs expressed by neutrophils are CR1 (also known as

CD35), which binds to complement components C1q, C4b, C3b, and mannan-binding lectin; CR3, which binds to iC3b, intercellular adhesion molecule-1, and some microbes; and CR4, which binds to iC3b. By expressing these latter receptors, neutrophils are able to recognize and bind, in a cooperative manner, IgG-opsonized particles and/or complement-opsonized microbes, and then activate their phagocytosis. During the phagocytic process, the foreign particle is internalized, initially through membrane recruitment to the site of particle contact, and then via membrane extensions outward to surround the particle and form a new vesicle called a cytoplasmic phagosome.[20] (Fig. 20.5). The phagosome then undergoes fusion with neutrophil granules to form a phagolysosome, a protected space in which proteolytic enzymes and other bactericidal components are discharged and pathogen degradation occurs. At the same time, NADPH oxidase assembles on the phagosomal membrane after phagocytosis and starts to generate ROS into the phagolysosome to kill bacteria by oxidizing microbial proteins and lipids. The activity of NADPH oxidase also leads to the acidification of the phagosome, which enhances the effectiveness of pH-sensitive antimicrobial compounds. Thus, neutrophil mechanisms of pathogen destruction within the phagosome are multiple and involve granule fusion, toxic oxygen radical production, activation of latent proteolytic enzymes, and the activity of antibacterial proteins (see Fig. 20.5). Remarkably, an activation of gene transcription and a selected generation of cytokines also occur during phagocytosis, a feature that neutrophils utilize for boosting a more effective innate immune response. For instance, recruited neutrophils that phagocytose

a pathogen also respond by producing chemokines, in particular CXCL8, to amplify their own recruitment, but also CCL3, CCL4, and CCL19 that serve to recruit monocytes and DCs.[21]

TABLE 20.1 Main Adhesion Molecules Involved in Leukocyte-Endothelial Cell Interaction

Adhesion Molecule	Distribution	Ligands and Counterreceptors	Function
E-selectin (CD62E)	Endothelial cells	PSGL-1, ESL-1, CD44[a], CD43[a]	Rolling
P-selectin (CD62P)	Endothelial cells, platelets	PSGL-1, PNAd	Rolling
L-selectin (CD62L)	All leukocytes except effector and memory effector T cells	PNAd, MAdCAM-1, PSGL-1, E-selectin, P-selectin	Rolling
Selectin ligands			

PSGL-1	All leukocytes	All selectins (essential for P-selectin)	Rolling
sLe^x	Myeloid cells, some memory T cells, HEVs	All selectins	Rolling
PNAd	HEV, some sites of chronic inflammation	L-selectin, P-selectin	Rolling
Integrins			
$\alpha_M\beta_2$(MAC-1; CD11b/CD18)	Granulocytes, monocytes, some activated T cells	ICAM-1, fibrinogen, C3b, JAM-C	Adhesion, transmigration
$\alpha_L\beta_2$(LFA-1; CD11a/CD18)	All leukocytes	ICAM-1, ICAM-2, JAM-A	Adhesion, transmigration
$\alpha_D\beta_2$(CD11d/CD18)	Monocytes, macrophages, eosinophils	ICAM-1, VCAM-1	Adhesion
$\alpha_x\beta_2$(p150,95; CD11c/CD18)	DCs	Fibrinogen, C3b	Adhesion
$\alpha_4\beta_1$(VLA-4)	Most leukocytes	VCAM-1, fibrinogen, JAM-B	Rolling, adhesion
$\alpha_4\beta_7$(LPAM-1)	Lymphocytes, NKCs, mast cells, monocytes	MAdCAM-1, fibronectin, VCAM-1	Rolling, adhesion
Immunoglobulin superfamily			
ICAM-1 (CD54)	Most types of cells	LFA-1 Mac-1, fibrinogen	Adhesion, transmigration
ICAM-1 (CD102)	Endothelial cells, platelets	LFA-1 Mac-1	Adhesion, transmigration
VCAM-1 (CD106)	Endothelial cells	VLA-4, $\alpha_4\beta_7\alpha_D\beta_2$	Rolling,

			adhesion
MAdCAM-1	HEVs in PP and MLN	$\alpha_4\beta_7$, L-selectin	Rolling
PECAM-1	Endothelial cells, platelets, leukocytes	PECAM-1	Transmigration
JAM-A	Endothelial cells, platelets, most leukocytes	JAM-A	Transmigration
JAM-B	Endothelial cells, HEVs	JAM-B, JAM-C	Transmigration
JAM-C	Endothelial cells, HEVs, platelets, monocytes, DC, some T cells	JAM-C, JAM-B	Transmigration

CD, cluster of differentiation; DC, dentritic cell; ESL, HEV, high endothelial venule; ICAM, intercellular adhesion molecule; JAM, LFA, MAdCAM, mucosal addressin cell adhesion molecule; NKC, PECAM, PNAd, PSGL, sLe^X, VCAM, VLA.

[a] CLA decorated.

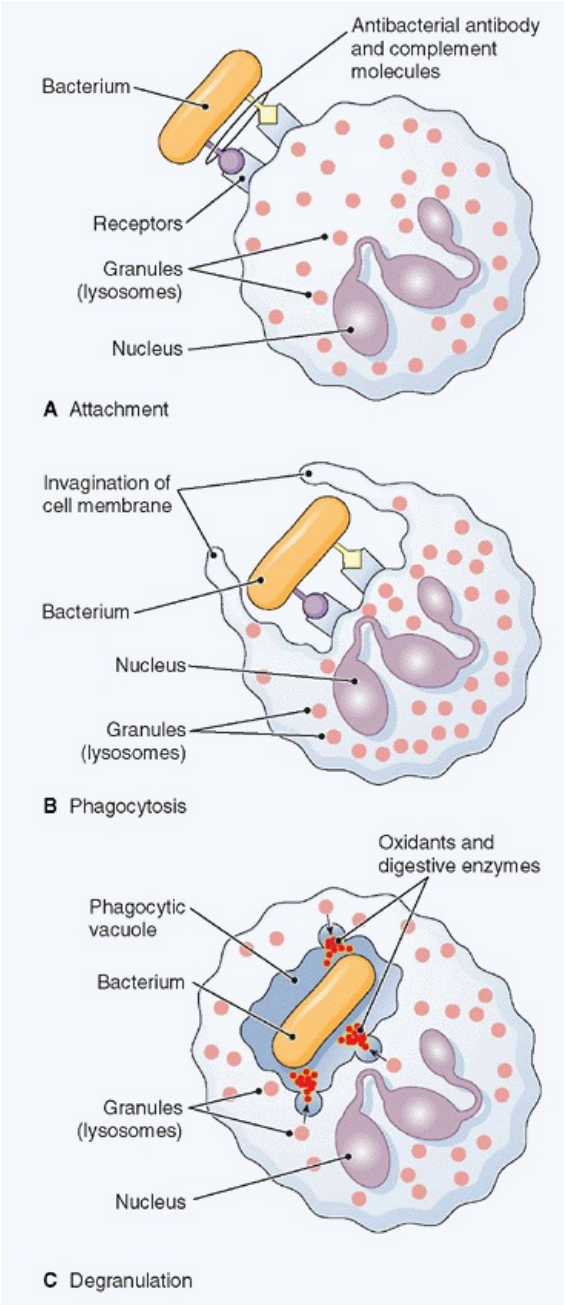

FIG. 20.5. Phagocytosis. The figure shows ingestion, digestion, and destruction of foreign particulate matter (a bacterium, in this example) by a neutrophil. **A:** Cell membrane receptors bind to antibody and complement molecules previously attached to the bacterial surface. **B:** The cell membrane creeps around the bacterium and envelopes it. **C:** The bacterium is trapped in a special space, the phagocytic vacuole, into which lysosomes discharge proteases, which together with oxidants kill it. Then, digestive enzymes dissolve it. (Thomas H. McConnell, *The Nature Of Disease Pathology for the Health Professions*, Philadelphia: Lippincott Williams & Wilkins, 2007.)

Role in the Resolution Phase of Inflammation

Locally activated neutrophils not only amplify the inflammatory process, but, surprisingly, actively participate in its resolution phase.[22] To do so, during the late, final phases of a

resolving, acute inflammatory reaction neutrophils, for instance, switch their eicosanoid biosynthesis potential from LTB_4 to lipoxins, with profound modifications of their effector functions. In fact, lipoxin A_4 (LXA_4) and LXB_4 stop neutrophil chemotaxis, adhesion, and transmigration through endothelium (by decreasing P-selectin expression), inhibit Eo recruitment, stimulate vasodilation (by inducing synthesis of PGI_2 and PGE_2), inhibit LTC_4- and LTD_4- stimulated vasoconstriction, inhibit LTB_4 inflammatory effects, and inhibit the function of natural killer (NK) cells.[22] Neutrophils also contribute to the biosynthesis of resolvins (such as resolvin E1, resolvin E2, resolvin D1, and resolvin D2) and protectin D1, which all inhibit neutrophil transendothelial migration and tissue infiltration, as well as stimulate resolution and reduce the magnitude of the inflammatory response in vivo.[22] Furthermore, neutrophils might also serve as major producers of anti-inflammatory cytokines such as transforming growth factor (TGF)β and IL-1 receptor antagonist, the latter being an endogenous inhibitor of IL-1β signaling and mediated effects.[21] Finally, neutrophils must be cleared from the inflammatory site as inflammation resolves. Indeed, neutrophils undergo apoptosis and are engulfed by tissue macrophages, which then reprogram into the M2 phenotype and start to generate antiinflammatory cytokines such as TGFβ and IL-10.[6,23]

Novel Neutrophil Effector Functions

The functions described previously for neutrophils in host defense are fundamental for combating infectious diseases. However, more recent discoveries on neutrophils as source of a variety of cytokines[21] have revealed that these cells are not only key components of the inflammatory response, but also crucial effectors of innate and adaptive immune regulatory networks.[23]

Neutrophil-Derived Cytokines and Chemokines

Numerous in vitro and in vivo studies, focusing on novel aspects of the neutrophil biology and function, have recently shed new light on the potential role that neutrophils can exert in the modulation of innate and adaptive immune responses.[23] It is now unequivocal that neutrophils are not, as for a long time thought, terminally differentiated cells “devoid of transcriptional and protein synthesis activity.”[23a] In fact, besides the several preformed or rapidly generated inflammatory mediators described previously, neutrophils display the capacity to de novo synthesize and release also several chemokines and cytokines with immunoregulatory properties.[21,23] It is, however, important to mention that, at least in vitro, neutrophils usually produce, on a per-cell

basis, fewer molecules of a given cytokine than mononuclear leukocytes.[21] However, considering that neutrophils clearly predominate over other cell types under inflammatory conditions in vivo, it becomes obvious that the contribution of neutrophil-derived cytokines can be of foremost importance. To date, a wide range of stimuli able to induce characteristic signatures of chemokine and cytokine synthesis by neutrophils have been identified. Among these, cytokines themselves, chemotactic factors (fMLF, LTB_4, PAF, C5a, and CXCL8), phagocytic particles, microorganisms (such as fungi, viruses, and bacteria), and PRR ligands can all induce the synthesis and release of chemokines and cytokines by neutrophils.[21]

Considering that neutrophils usually represent the first cell type infiltrating at the site of infections, a stimulus-specific response of neutrophils in terms of cytokine production might direct the evolution of certain types of inflammatory and immune reactions to support the transition from innate to adaptive immunity.

Table 20.2 lists all the cytokines that, to date, have been shown to be released by neutrophils in vitro, either constitutively or following appropriate stimulation, or in vivo. Numerous in vivo observations, in fact, not only have confirmed and reproduced the in vitro findings, but often have clarified their biological meaning and implications. As outlined in Table 20.2, neutrophils can produce proinflammatory, anti-inflammatory, immunoregulatory, angiogenic, and fibrogenic cytokines, chemokines, and ligands belonging to the TNF superfamily. The role of these molecules in mediating various neutrophil-dependent immunoregulatory functions is partially described in the following chapter. For instance, chemokines are particularly represented among the cytokines produced by neutrophils and include those primarily chemotactic for neutrophils themselves, monocytes, DCs, NK cells, and T helper (Th)1 and Th17 cells. It follows that a role for neutrophils in orchestrating the sequential recruitment to, and activation of, distinct leukocyte types in the inflamed tissue is plausible, as already demonstrated to occur in several experimental models.[21,23]

Neutrophils in Immunoregulation

There is now wide experimental evidence that neutrophils have the capacity to modulate the migration, maturation, and function of several leukocyte types including DCs, T cells, and B cells.[23] Regarding DCs, it is noteworthy to mention that neutrophils have been shown to produce biologically active CCL20 and CCL19, two structurally related CC-chemokines that have been suggested to play a fundamental role in trafficking of, respectively, immature and mature DCs to mucosal surfaces and lymphoid organs. Likewise, neutrophils release several antimicrobial compounds, such as lactoferrin, LL-37, and cathepsin G, that have been found to act as chemoattractants for immature DCs. In addition, neutrophils can proteolytically activate prochemerin to generate chemerin, one of the few chemokines that attracts both immature DCs and plasmacytoid DCs.[3] Neutrophils can also modulate DC maturation and function either through the release of several mediators or through direct physical interaction between Mac-1 (CD11b/CD18) and DC-specific intercellular adhesion molecule.[24] Neutrophils also act as transport vehicle for pathogens and, in turn, deliver antigens to DCs, thus playing an important role in the activation of T-cell immune responses controlled by DCs.[24]

TABLE 20.2 Cytokines Expressed in Resting or Activated Neutrophils

C-X-C Chemokines	**Proinflammatory Cytokines**	**TNF Superfamily Members**
GROα/CXCL1	**TNFβ**	**FasL**
CINC-2α/GROχ/ MIP-2α/**CXCL2**	IL-1α, IL-1β	CD30L
CINC-2β/GROγ/MIP-2β/CXCL3	**IL-6**(?),IL-7, IL-9	TRAIL
PF4/CXCL4	IL-16(?), **IL-17A/F**(?)	
ENA-78/CXCL5		

GCP-2/CXCL6
IL-8/CXCL8
MIG/CXCL9 IP-10/CXCL10 I-TAC/CXCL11
C-C Chemokines
MCP-1/CCL2MIP-1α, CCL3
MIP-1β, CCL4
TARC/CCL17
PARC/CCL18
MIP-3α, CCL19
MIP-3β, CCL20
MDC/CCL22

IL-18
MIF
Anti-inflammatory Cytokines
IL-1ra
IL-4(?), **IL-10**(?)
TGFβ$_1$, TGβ$_2$
Immunoregulatory Cytokines
IFNα, IFNβ, **IFNγ**(?)
IL-12
IL-23(?)
Other Cytokines
Oncostatin M
GDF (?)
NGF, BDNF, NT4
PBEF/visfatin/NAMPT
amphiregulin

LIGHT[a]
Lymphotoxin-β
APRIL,
BAFF/BLyS
RANKL
Colony Stimulating Factors
G-CSF
M-CSF(?)
GM-CSF(?)
IL-3(?)
SCF[a](?)
Angiogenic and Fibrogenic Factors
VEGF
BV8/Prokineticin-2
HB-EGF
FGF-2
TGFβ
HGF

?, requires definitive corroboration; GRO, growth regulated oncogene; CXCL, CXC chemokine ligand; CCL, CC chemokine ligand; MIP, macrophage inflammatory protein; CINC, Cytokine induced neutrophil chemoattractant; PF4, Platelet factor-4; ENA-78, epithelial-derived neutrophil-activating peptide 78; GCP-2, granulocyte chemotactic protein-2; IL-, interleukin-; MIG, Monokine induced by gamma interferon; I-TAC, Interferon-inducible T-cell alpha chemoattractant; IP-10, Interferon gamma-induced protein 10; MCP, monocyte chemotactic protein; TARC, Thymus and activation regulated chemokine; PARC, pulmonary and activationregulated chemokine; MDC, Macrophage-derived chemokine; TNF, tumor necrosis factor; MIF, macrophage inhibitory factor; IL-1ra, IL-1 receptor antagonist; TGF, Transforming growth factor; IFN, interferon; GDF, Growth Differentiation factor; NGF, nerve growth factor; BDNF, Brain derived neurotrophic factor; NT4, Neurotrophin-4; PBEF, pre-B-cell colony-enhancing factor; NAMPT, Nicotinamide phosphoribosyltransferase; TRAIL, TNF-related apoptosis-inducing ligand; APRIL, a proliferation-inducing ligand; BAFF/BLyS, B-cell activating factor/B lymphocyte stimulator; RANKL, Receptor activator of nuclear factor kappa-B ligand; G-CSF, macrophage colony stimulating factor; M-CSF, macrophage colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; SCF, stem cell factor; HB-EGF, heparin binding-like epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor.

Cytokines in **bold** refer to neutrophil studies in animal models that confirm human

findings.

[a] Messenger ribonucleic acid only.

Concerning the interactions between neutrophils and B cells, of particular interest are the findings that neutrophils produce significant amounts of BLyS/BAFF (B-lymphocyte stimulator/B-cell activating factor) and APRIL (a proliferation-inducing ligand), two related members of the TNF

family that are well known to be essential for B-lymphocyte homeostasis.[25] Therefore, it is plausible to assume a role of neutrophils not only in sustaining B and plasma cell antibody production and survival, but also in promoting B-cell-dependent autoimmune diseases and tumors, as already elegantly demonstrated in the case of B-cell lymphoma.[25]

Cross-talk between neutrophils and T cells has been repeatedly described to occur during infections or other inflammatory responses and diseases. Current evidence now indicates that neutrophils exhibit a significant chemotactic effect toward Th1 or Th17 cell subsets, through the release of CCL2, CXCL9, and CXCL10, or CCL2 and CCL20, respectively.[26] Neutrophils have also a role in directing T-cell polarization, for instance through their capacity to produce the Th1-inducing cytokine, IL-12. The latter has been clearly demonstrated in mouse models, in which strong Th1-dependent T-cell responses that result in pathogen clearance are elicited upon infection with *Candida albicans*, *Helicobacter pylori* , or *Legionella pneumophila*. Strikingly, depletion of neutrophils reverses the Th1 responses into a predominant Th2-response, therefore making the mice susceptible to infection. Besides the neutrophil's ability to modulate T-cell functions through the production of chemokines and cytokines, recent reports suggest that neutrophils travel to the lymph nodes during infections and express both major histocompatibility complex (MHC) II and costimulatory molecules.[27] However, whether neutrophils directly acquire antigen-presenting functions or transmit signals to naïve T cells remains still puzzling.

Neutrophils have also been shown to modulate the maturation, activation, and functions of NK cells, either by themselves or in cooperation with other cell types.[28] In this context, it is worth mentioning that neutrophils, by interacting with specific subsets of peripheral blood myeloid DC (eg, 6-sulpho LacNAc+ DC, also known as slanDC) can strongly potentiate IFNγ release by NK cells.[29] Importantly, the potential pathophysiologic relevance of a cell network among neutrophils, slanDC, and NK cells has been suggested by immunohistochemical studies that have revealed their colocalization in several chronic inflammatory pathologies, such as Crohn disease and psoriasis.[29] Finally, it has also been proposed that mature postmitotic neutrophils can also “transdifferentiate” into much-longer-lived cells with macrophage- or DC-like characteristics, which might constitute a further manner for neutrophils to act as regulatory cells of the adaptive immune response.[3,23]

It is important to mention that despite of all experimental observations in vivo that strongly suggest that neutrophils could potentially act as important players in the orchestration of immune responses, additional reevaluations and validations are required. Indeed, in order to

investigate the role of neutrophils in vivo, an antigranulocyte receptor-1 monoclonal antibody, RB6-8C5, has been extensively used to deplete mice of neutrophils.[30] However, RB6-8C5 not only binds to Ly6G, which is present on neutrophils, but also to Ly6C, which is expressed on neutrophils, DCs, and subpopulations of lymphocytes and monocytes. Therefore, it has recently been shown that in vivo administration of RB6-8C5 depletes not only neutrophils but also other granulocyte receptor-1+ (Ly6C+) cells. Luckily, a more specific anti-Ly6G monoclonal antibody (1A8) has been raised; it is now preferentially used to deplete neutrophils in vivo under different experimental settings.[31] Obviously, it will take some time prior to controlling and, eventually, revising all data on neutrophil depletion generated by using RB6-8C5. More importantly, the future availability of conditional knockout mice, selectively targeting, one by one, neutrophil function such as survival, migration, or activation, will help to finally clarify the specific contributions of neutrophils under different inflammatory/immune settings.

Role of Neutrophils in Angiogenesis and Tumor Growth

There is no longer doubt that, in addition to macrophages, neutrophils may positively or negatively influence the angiogenic process and tumor growth.[32] In the former case, it has been observed that neutrophils, via elastase release, may indirectly generate massive amounts of bioactive, angiostatin-like fragments, and thus inhibit fibroblast growth factor (bFGF) plus vascular endothelial growth factor (VEGF)-induced endothelial cell proliferation.[33] However, as described for macrophages, neutrophils can also favor malignant growth and progression, in relation to the type of tumor environment in which they reside, for instance via a remarkable production of proangiogenic molecules such as VEGF and CXCL8.[34] On the other hand, experimental studies of tumor cure and prevention have suggested that, at least in some models, engagement of neutrophil functions can be crucial for the establishment of an effective antitumoral immune response and immune memory reactions.[35] Indeed, neutrophils can produce several cytotoxic mediators for tumor and endothelial cell killing, including TNFα, defensins, proteases (such as elastase and cathepsin G), ROS, nitric oxide, and angiostatic chemokines (CXCL9, CXCL10, and CXCL11).[35] It has been recently found that neutrophils exposed to IFNs express and produce TNF-related apoptosis-inducing ligand,[36] another TNF superfamily member that selectively stimulates tumor cell killing. More recently, in vivo evidence proving that, similarly to M1 and M2 macrophages, neutrophils also polarize from an N1, proinflammatory and antitumoral phenotype, to an N2 anti-inflammatory and protumoral phenotype, has been provided,[37] thus supporting the notion that the tumor environment can profoundly shape the functional status of neutrophils. Furthermore, it is now well established that myelopoiesis can be profoundly modified during inflammation and cancer, releasing altered mature myelocytes and myeloid-derived suppressor cells (MDSCs) that exert immunosuppressive and protumoral activity, mainly by inhibiting T-cell functions.[38] Although mature human neutrophils do not seem to be a major component of such MDSC population, several mouse tumor models have revealed the existence of a granulocytic MDSC population with potent T-cell suppressing activity.[38] Nonetheless, lowdensity granulocytes (so called because of their abnormal behavior upon density centrifugation) able to inhibit T-cell activation and function have been found in human

patients with cancer.[39] Further research is now needed to better understand the origin, phenotype, and relationship to mature

neutrophils of all these immunosuppressive granulocytic populations. Such studies will better clarify the real role of neutrophils in cancer as well as in other inflammatory/autoimmune diseases (such as infections, psoriasis, and lupus), in which the presence of MDSC- or low-density granulocyte -like cells have been also described.[40]

Neutrophils in Diseases

Previous sections have already summarized some of the most common inherited disorders of neutrophil function that impair critical responses for host defense. Dramatic clinical consequences may also be observed as a consequence of acquired neutropenia (eg, a susceptibility to infectious diseases when neutrophil counts fall below 500 cells/μL[4]). Neutropenia can be due to depressed production (ie, hereditary neutropenias) or increased peripheral destruction.[4] Apart from the disorders associated with genetic dysfunctions or quantitative alterations of neutrophils, there are other pathologic situations in which neutrophils themselves become the predominant contributors to tissue injury, especially when the mechanisms supposed to control and inactivate their hypothetical beneficial and protective effector functions are deregulated. Examples of such pathologies, tentatively classified according to the major neutrophil-activating event,[1] are 1) diseases caused by ischemia reperfusion injury (ie, myocardial infarction); 2) bacterial infections (endotoxic shock, osteomyelitis, adult respiratory distress syndrome,); 3) cytokine-mediated diseases (rheumatoid arthritis, inflammatory bowel diseases); 4) diseases caused by crystal deposition (gout); 5) antineutrophil cytoplasmic antibody-associated vasculitis (Wegener granulomatosis, pauci-immune necrotizing crescentic glomerulonephritis); and 6) airway diseases (chronic obstructive pulmonary disease, bronchiectasis, bronchiolitis, cystic fibrosis, and even certain forms of asthma are characterized by neutrophil infiltration of the airway wall). One of the key challenges in neutrophil-dominated conditions is how to manipulate neutrophil function to abolish their destructive potential in a way that does not compromise their antibacterial and antifungal capacity. This has been difficult to achieve, as successful treatments in animal models have frequently proven ineffective or limited by side effects when used in human inflammatory diseases.

EOSINOPHILS, BASOPHILS, AND MAST CELLS

Eos, Bas, and MCs are critical effector cells not only in allergic inflammation, but also in innate and adaptive immunity. In addition, they have a crucial role in the surveillance of epithelial tissues, especially of the mucosa of the gastrointestinal, respiratory, and urogenital tracts: MCs as tissue resident sentinel cells, while Eos and Bas principally upon recruitment from the bloodstream. The microbicidal activity of these cells can be induced either after direct microorganism recognition or after activation by complement-or other leukocyte-derived products. Whereas neutrophils destroy internalized microorganisms by delivering cytotoxic intracellular compartments to them, Eos, Bas, and MCs are mainly involved in host defense against large parasites that cannot be internalized by other professional phagocytes. Eos can directly kill the parasites, whereas Bas and MCs preferentially release the contents of their granules into the external milieu upon activation, thus creating an environment hostile to

invading organisms. Furthermore, the unique property of Bas and MCs to express the high-affinity Fc receptor for IgE (FcεRI) that, after activation by antigenspecific IgE, induces rapid discharge of potent inflammatory mediators (histamine being one of them), is central to the initiation and propagation of immediate hypersensitivity reactions. Coughing, sneezing, and vomiting, all expulsive responses that typically are caused by basophil- and MCderived mediators and accompany allergic and pathologic inflammatory diseases, may actually reflect mechanisms that evolved to expel parasites. The properties and roles of Eos, Bas, and MCs in pathologic conditions such as allergy and autoimmunity are described in detail Chapter 45. Herein, we focus on the specialized properties that highlight their role in the innate and acquired immune response.

Eosinophils

Eosinophil Generalities

Eos are end-stage, multifunctional leukocytes involved in protection against parasitic helminths and bacterial and viral infections, that are also implicated in the pathogenesis of numerous Th2-type inflammatory processes and tissue injury.[41] These granulocytic, bilobed, nucleated cells were named as Eos by Paul Ehrlich in 1879, because of their intense staining with eosin, an acid aniline dye (Fig. 20.6). Eos are approximately 12 to 17 μm in diameter and represent 1% to 6 % of the total blood leukocyte population. Besides circulating and massively recruited at sites of Th2-dominated inflammation, Eos also reside in various organs, such as the gastrointestinal tract, mammary glands, and bone marrow.[41,42] Eos derive from bone marrow, CD34+ myeloid-committed progenitors, and upon maturation can be cytofluorimetrically identified by their characteristic morphology/side scatter and expression pattern of plasma membrane proteins such as CD66b, CCR3, IL-5Ra in conjunction

with the lack of expression of CD16 (to better distinguish them from neutrophils), and other lineage markers (such as CD2, CD14, and CD19).[41,42,43] Eosinophil differentiation and expansion from progenitor cells mainly occurs in response to three cytokines, namely IL-3, IL-5, and GMCSF, which provide permissive proliferative and differentiation signals through GATA-1, PU.1, C/EBPα, and C/EBPε transcription factors.[44] IL-5 is, however, the most specific factor for Eo development, differentiation, and release from the bone marrow into the peripheral circulation, especially during helminthic infection or allergic inflammation.[41,42,43] Also, allergen challenge or the experimental administration of CCL11/eotaxin-1 (acting through the CCR3 receptor) can cause bone marrow release of Eo precursors and mature Eos.[45,46] However, basal levels of Eo development can also occur in the absence of IL-5 as in antigen-induced tissue eosinophilia, as suggested by the presence of tissue Eos in asthmatic patients treated with anti-IL-5-neutralizing antibodies.[45,46] Once released from the bone marrow, Eos enter the circulation (with a half-life of 8 to 18 hours) and then traffic to tissues, at mucosal surfaces and/or sites of allergic inflammation, in response to a variety of different chemoattractants including CCL11, CCL24/eotaxin-2, CCL26/eotaxin-3, CCL5/RANTES, or LTB_4, LTD_2, PAF, and C5a. IL-4 and IL-13 play a central role in promoting Eo trafficking to mucosal tissue by upregulating eotaxins (CCL11 and CCL26) and endothelial expression of vascular cell adhesion molecule (VCAM)-1, the counterreceptor for VLA-4,

which is expressed on the surface of Eos.[45,47] In addition, GM-CSF, IL-3, and IL-5, as well as IL-33 and IFNγ, are important for promoting long-term Eo survival and activation in vitro and in tissues.[41,42,43,46]

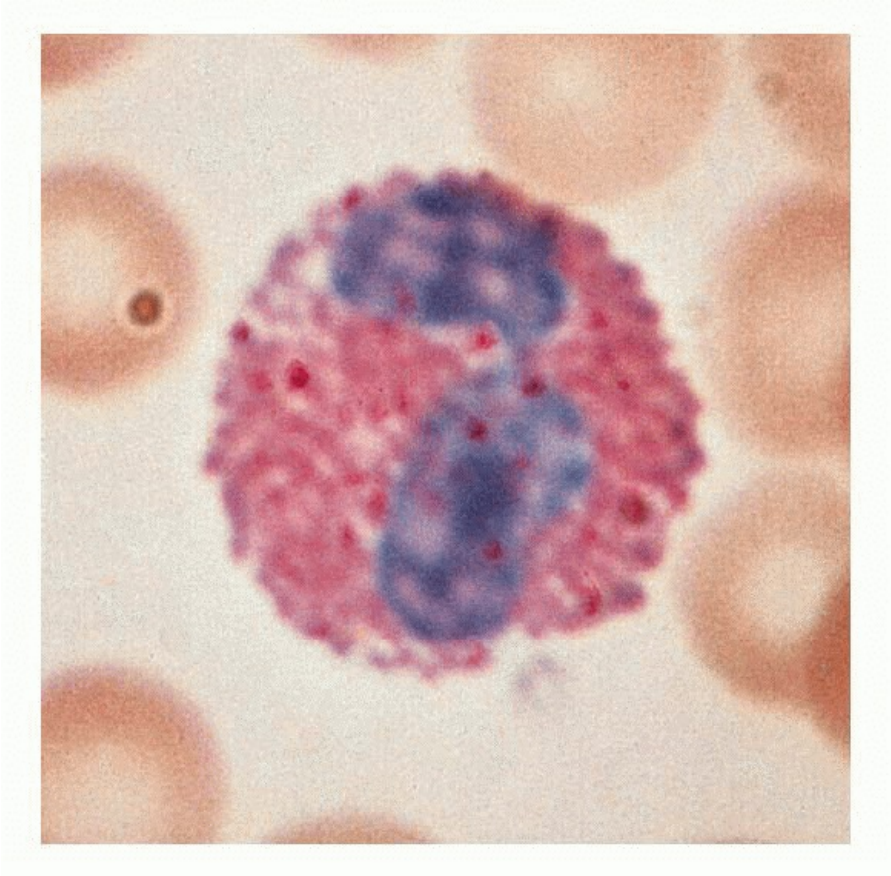

FIG. 20.6. A polymorphonuclear eosinophil circulating in peripheral blood. (Reprinted with permission from Cohen BJ, Wood DL. *Memmler's The Human Body in Health and Disease*. 9th Ed. Philadelphia: Lippincott Williams & Wilkins, 2000.)

Eosinophil Granules

Human Eos have a bilobed nucleus with highly condensed chromatin and contain up to four different populations of secretory organelles: primary granules, secondary/specific/crystalloid granules, small amorphous granules (containing arylsulfatase and acid phosphatase), and secretory vesicles.[48] These four organelles, along with vesiculotubular structures and small vesicles involved in transport and secretion by the activated cell, serve as the major subcellular sites for the eosinophil armamentarium of preformed cytotoxic and inflammatory mediators.[48] Eos also contain lipid bodies, which are non-membrane-bound lipid-rich organelles that are the major site of eicosanoid synthesis as they contain eicosanoid synthetic enzymes including 5-lipoxygenase, leukotriene C_4 synthase, and cyclooxygenase.[41,42,43,48,49] Eos contain greater numbers of lipid bodies than neutrophils, which even increase during eosinophil activation in vitro or engagement in inflammatory

reactions in vivo.[41,42,43,48,49]

Primary granules are similar to those found in other granulocyte lineages, are formed early in Eo development, and are enriched in Charcot-Leyden crystal protein (which represent less than 5% of total granules).[48,49] Charcot-Leyden crystal/galectin-10 is a hydrophobic protein of unknown function that is produced and released in high levels by activated Eos. Its characteristic hexagonal bipyramidal crystals can be detected as a hallmark of Eo involvement in host allergic and other immune responses, for instance in the stool or sputum of patients with gastrointestinal or respiratory eosinophilia.[48,49] Specific granules have a distinctive ultrastructural appearance with an electron-dense core and contain cationic proteins that give Eos their unique staining properties. The major cationic proteins in the specific granules are major basic protein (MBP, composed of two related cationic proteins, MBP-1 and MBP-2), Eo peroxidase (EPO), Eo cationic protein (the Eo's two ribonucleases RNase2 and RNase3, respectively), and Eo-derived neurotoxin (EDN). MBP is the major component of the crystalloid cores of specific granules and accounts for more than 50% of the Eo granule protein mass. MBP is highly cationic, it lacks enzymatic activity, and its toxicity is mediated by enhanced membrane permeability resulting from interactions of the cationic protein with the plasma membrane. MBP has in vitro activity toward parasites, including schistosomes and other helminths. In patients with asthma, serum and bronchoalveolar lavage fluid MBP concentration correlates with bronchial hyperresponsiveness. EDN and Eo cationic protein demonstrate in vitro toxicity to parasites and single-stranded ribonucleic acid pneumoviruses, including respiratory syncytial virus. EPO is another highly cationic protein that makes up approximately 25% of granule proteins and that catalyzes the oxidation of halides, pseudohalides, and nitric oxide to oxidant products that are toxic to microorganisms and host cells. It has been shown that, depending on the activation stimulus, Eo granule cationic proteins can be selectively secreted or released into tissues by a number of different secretory pathways, ranging from classical granule fusion and exocytosis (eg, in killing parasitic helminths), piecemeal degranulation (eg, vesicular transport from the granules in the absence of classical exocytosis), and cytolytic degranulation (release of intact membrane-bound granules directly into the tissue upon eosinophil apoptosis/cell death).[41,42,43,48,49] Studies in the past 15 to 20 years of the biochemistry, functions, and localization in tissues of the unique enzymatic and nonenzymatic cationic proteins present in Eo granules have provided compelling evidence supporting a pathologic proinflammatory and effector role for the Eo in directly inducing tissue damage.

Eosinophil Mediators

In addition to containing numerous highly basic and cytotoxic granule proteins that are released upon activation, Eos can also synthesize an arsenal of lipid mediators, ROS, and inflammatory/hematopoietic cytokines that mediate the pathophysiologic role of this granulocyte in health and disease (Table 20.3). Lipid-derived mediators generated by Eos include PGE_2, thromboxane, LTC_4, and PAF, which are all considered as responsible for many of the Eo-triggered proinflammatory activities, namely the increase in leukocyte trafficking, endothelial adhesion, smooth muscle contraction, vascular permeability, and mucus secretion.[41,42,43] Eo-derived cytokines (including TGFβ, GM-CSF, IL-3, IL-4, IL-5, CXCL8, IL-10, IL-12, IL-13, IL-16, IL-18, TNFα, CCL5, stem cell factor [SCF], NGF, and CCL11) are in part stored as preformed in granules and thus can be rapidly released upon

degranulation (similarly to MCs).[41,42,43,50,51,52]

Interestingly,GM-CSF is among the cytokines produced in greatest quantities by Eos.[53]

TABLE 20.3 Major Eosinophil-Derived Mediators

Class	Mediators	Physiologic Effects
Cationic granule proteins	MBP, ECP, EDN, EPO	Cytotoxic and bactericidal properties, smooth muscle cells contraction, mast cell activation and survival
Lipid mediators	LTD_4, LTE_4, PAF, PGE_1, PGE_2, 15-HETE	Leukocyte trafficking, endothelial adhesion, smooth muscle cells contraction, vascular permeability, inflammation
Cytokines	TNFα, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-11, IL-12, IL-13, IL-16, IL-17, IL-25	Inflammation, immunoregulation
Chemokines	CXCL1, CXCL5, CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL3, CCL5, CCL7, CCL11, CCL13	Leukocyte chemoattraction and tissue infiltration of leukocytes
Growth factors	HB-EGF, LBP, NGF, GM-CSF, TGFα, TGFβ, SCF, VEGF, APRIL	Growth of various cell types

ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EPO, eosinophil peroxidase; IFN, interferon; IL, interleukin; LTD, leukotriene D; LTE, leukotriene E; MBP, major basic protein; PAF, platelet-activating factor; PGE, prostaglandin E; TNF, tumor necrosis factor.

Eos are functionally activated via an array of cell surface molecules, including Ig receptors for IgG (FcγRII/CD32) and IgA (FcαRI/CD89), receptors for complement-derived fragments (CR1/CD35, CR3, and CD88), cytokines (IL-3R, IL-5R, GM-CSF, as well as IL-1αR, IL-2R, IL-4R, IFNαR, and TNFRI), chemokines (CCR1 and CCR3), adhesion molecules (VLA4, α4β7), leukotrienes (CysLT1R and CysLT2R; LTB_4-R), prostaglandins (PGD_2 type 2R), PAF, and ligands for TLRs (TLR1, TLR4, TLR7, TLR9, and TLR10, the TLR7/8 representing an

important mechanism for host defense against viral infections).[41,42,43]

Eosinophil Effector Functions

Eos have historically been considered as end-stage effector cells, causing damage to parasitic pathogens during helminthic infections or to host tissues in allergic diseases. However, as outlined in the following, accumulating evidence suggest that Eos can perform various immune regulatory functions, pointing to a more complex role of these cells not only in regulating inflammation and bridging innate and adaptive immunity, but also in maintaining epithelial barrier functions and affecting tissue remodeling.[41,42,43]

Eosinophils in Host Defense. The earliest recognized Eo effector functions were those associated to their role in host defense against multicellular helminthic parasites, although it is now clear that Eos are also involved in host defense against viral and bacterial infections.[54] Among the several mechanisms through which Eos can exert defensive functions, the best described consist in their release of cytotoxic cationic proteins (such as MBP, Eo cationic protein, EDN, and EPO), production of ROS, antibody- and complement-mediated killing, and the expulsion of extracellular deoxyribonucleic acid traps.[43,54] Despite substantial in vitro data, there is a lack of convincing evidence of the effective role of Eos in host defense in vivo, in particular during helminthic infections. Indeed, the only human Eo-specific condition, known as hereditary EPO deficiency, has not been related to increased susceptibility to helminthic infections. Furthermore, the results from infection studies carried out in mouse models remain unclear and controversial.[55,56] Considering that many of these experiments were performed using human pathogens, which do not naturally infect rodents, and that there are several phenotypic and functional differences between human and murine Eos, the relevance of these data should be reconsidered.[55,56] It is noteworthy to mention that, as described subsequently, some of the mechanisms used by Eos in host defense against pathogens may also produce detrimental effects on the host.[42,54]

Eosinophils in Immunoregulation. Accumulating evidence suggest that Eos can perform discrete immune regulatory functions mainly through production and release of cytokines and other immunomodulatory molecules, or via antigen presentation. For instance, Eos produce cytokines that are able to act on Eos themselves, the so-called autocrine cytokines, including IL-3 and GM-CSF, which function, in part, to prevent apoptosis and prolong Eo survival, once these cells are recruited into sites of tissue inflammation, such as the lung in asthma.[41,42,43] Eos can regulate their own recruitment, as well as that of DCs and T cells, through the secretion of CCL3, CCL5, and CCL11. Eo-derived cytokines may also influence the functions of other immune cells. For instance, Eos play an important role in the regulation of MC functions, including activation, differentiation, maturation, and survival, mainly via the release of SCF.[50,51,52] Eos have also been implicated in the regulation of B-cell function, not only under homeostatic conditions, but also during immunization responses with alum.[57] Eos seem indeed able to modulate early stages of B-cell activation and IgM production.[57] The best characterized role of Eos in immune regulation is their ability to initiate Th2-type responses by modulating DC and T-cell recruitment and activation. Eo-derived EDN can recruit and activate DCs and, in turn, skew them to a Th2-promoting phenotype.[58] In several murine models of parasitic infections and airway allergen challenge, Eos have been shown

not only to precede Th2 cells arrivals in tissues, but also to be able to migrate to draining lymph nodes and polarize Th2 immune responses through the secretion of cytokines such as IL-4 and IL-25.[42,58] However, human Eos also express indoleamine 2,3-dioxygenase which, through kynurenine production, inhibits Th1 effector functions.[59]

Another important role of Eos in immune regulation is their function as antigen-presenting cells. Eos possess the capacity to internalize, process, and present antigenic peptides within the context of surface expressed MHC II, to provide costimulatory signals to T cells through surface expression of molecules such as CD80, CD86, and CD40, and to physically interact with CD4+ T cells.[60] In humans, although circulating Eos from healthy donors are generally devoid of surface MHC class II expression, they can be induced to express MHC class II and costimulatory molecules upon appropriate cytokine stimulation and after transmigration through endothelial cell monolayers.[42,60] Human Eos also constitutively express a Jagged1, a Notch ligand, suggesting a capacity of Eos to provide a polarization signal to naive CD4+ T cells.[61]

Eosinophil Homeostatic Functions. As mentioned, at baseline conditions Eos are present in tissues such as the gut, mammary gland, uterus, thymus, and bone marrow, via recruitment by the CCL11/CCR3 axis. Although their homeostatic role in all these tissues is not fully understood, Eos seem to be mainly involved in regulating the morphogenesis and maintenance of mucosal organs, as well as the immune homeostasis of the thymus and bone marrow.[41,42,43] Thymic Eos, for instance, are thought to be involved in the MHC class I-restricted negative selection of double positive thymocytes. In the bone marrow, instead, Eos colocalize with plasma cells, and by secreting a proliferation inducing ligand (APRIL) and IL-6, they play a crucial role in long-term maintenance and survival of the same plasma cells.[41,43,62]

Eosinophils in Tissue Remodeling. A growing body of evidence has proven the ability of Eos to influence tissue remodeling and fibrosis in many Eo-associated diseases, mainly due to their ability of secreting high levels of TGFβ. Human Eos also produce other profibrotic and angiogenic factors, such as osteopontin, VEGF, and MMPs.[50,51,52] Furthermore, Eos produce and release NGF and promote the extension of neurites in nerve cells.

Eosinophils in Diseases

Eos are currently thought to participate in the pathogenesis of the chronic phases of allergic diseases such as asthma, allergic rhinitis, atopic dermatitis, and hypereosinophilic syndromes.[41,42,43] Allergic diseases are associated with a mild peripheral blood eosinophilia, although in tissues and in the nasal secretions, sputum, and BAL fluid, Eos can be more significantly elevated. Studies in murine models support a role for Eos in airway remodeling, airway hyperactivity, and mucous production. Elevation of peripheral blood Eos is also observed in drug reactions, helminthic infections, as well as in specific primary immunodeficiency diseases, most notably Omenn syndrome and hyper-IgE syndrome. Eosinopenia is typically seen in acute bacterial or viral infections and with systemic corticosteroid treatment. Finally, the use of Eo-specific antibodies has demonstrated that almost all human or mouse cancers are associated with an important Eo infiltrate at some

point in tumor growth.[43]

Mast Cells and Basophils

Mast Cell Generalities

MCs were identified as granular cells in the mesentery of the frog by Dr. Von Recklinghausen in 1863 and were named “Mastzellen” by Dr Paul Ehrlich in 1878. Initial studies focused on their histologic characteristics, distribution, and abundance in health and disease. The discovery of histamine in 1910, slow-reacting substance of anaphylaxis (now leukotrienes) in 1938, and IgE in 1966 provided initial insights into the role of MCs in allergic reactions. MCs are tissue-dwelling inflammatory cells widely distributed throughout the body and are common at perivascular sites and at mucosal surfaces, particularly in those tissues that form interfaces with the external environment, such as skin, conjunctivae, and intestinal and airway mucosa.[63,64,65] Thus, MCs are strategically placed so as to function in a first line of host defense.[66,67] Morphologically, MCs in tissues appear as round, spindle-shaped, or spider-like cells, with round or oval nuclei, ranging between 7 and 20 μm in diameter, and readily identified using cationic dyes such as toluidine blue or methylene blue.[68] These dyes impart a blueto-purple change in color, known as “metachromasia,” which occurs as a result of their abundant intragranular content of sulfated proteoglycans (eg, heparin and chondroitin sulfates).[68] Of all other hematopoietic cells, only Bas share this staining feature with MCs, along with other properties (see Table 20.4

for the major similarities and differences between MCs and Bas). MCs can be cytofluorimetrically identified by their characteristic expression pattern of plasma membrane proteins serving as markers, including FcεRI (as Bas and a subpopulation of DCs), CD23, CD117, and CD203, in conjunction with the lack of expression of lineage markers (such as CD2, CD14, and CD19).[69,70] Furthermore, MCs can be identified either by biochemical reagents that detect their intracellular proteases for their enzymatic properties or by immunostaining using antibodies toward tryptase (Fig. 20.7) or chymase (the latter representing the primary method of choice for identifying human MCs in tissues).[71]

TABLE 20.4 Major Features of Mast Cells and Basophils

	Mast Cells	Basophils
Origin	Hematopoietic stem cells	Hematopoietic stem cells
Lifespan	Months	Days
Primary location	Tissues	Intravascular circulation
Site of	Connective tissues	Bone marrow

maturation		
Differentiation factors	Stem cell factor	Interleukin-3
Histamine content	1 to 15 pg/cell	1 to 2 pg/cell
Size	7 to 20 μm	5 to 10 μm
Nucleus	Oval or round	Segmented
Granules	Smaller and more numerous compared with basophils	Larger and fewer compared with mast cells
Peptidoglycans	Heparin and chondroitin sulfates	Predominantly chondroitin sulfates
Chymase content	High	Absent
Tryptase content	High	Low
Lipid mediators	PGD_2, LTB_4, LTC_4, LTD_4, LTE_4, PAF	LTC_4, LTD_4, LTE_4
Activation by Substance P	Activation	No effect
Morphine	Activation	No effect
fMLF	No effect	Activation

fMLF, formyl-Met-Leu-Phe; LTB,leukotriene B; LTC, leukotriene C; LTD, leukotriene D; LTE, leukotriene E; PAF, platelet-activating factor; PGD, prostaglandin D.

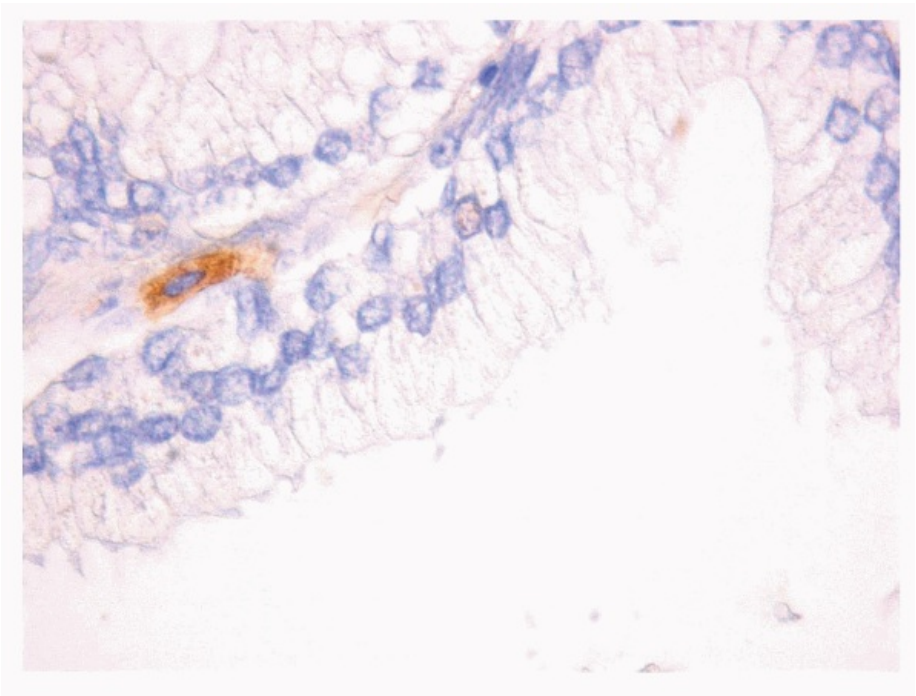

FIG. 20.7. Formalin-fixed, paraffin-embedded human prostate tissue stained with an anti-mast cell tryptase antibody.

Human MCs originate from CD34+/CD117+/CD13+ multipotent hematopoietic progenitors in bone marrow, and then migrate through blood to tissues where they mature.[72,73] Details of their differentiation and phenotypic diversification have not been definitively identified, but are likely included within the CD34+/KIT+ cell subpopulation and are clearly distinct from the basophil lineage (which is KIT/CD117- but IL-3R+).[74] In mice, a hematopoietic stem cell progresses to a multipotent progenitor, a common myeloid progenitor and a granulocyte/monocyte progenitor.[75,76] A monopotent MC progenitor is found in bone marrow and intestine, and a common basophil/MC progenitor is also found in mouse spleen, observations that await study in human. After their homing in the tissues, maturation of the MC precursors is dependent on SCF expressed on the surface of fibroblasts, stromal cells, and endothelial cells.[68,77] Accordingly, in vitro studies using mouse and human MCs confirm that soluble SCF protects MCs from apoptosis and induces their proliferation, chemotaxis, and also some degree of activation and secretion.[68] Consistently, mutant mice lacking KIT- or SCFmediated functions are often used as experimental tools to implicate a biologic role of MCs in given models.[68] While there are several studies on the mechanisms of localization of MCs to different tissues, there is little information about specific molecules that could regulate MC-progenitor trafficking to tissues, movement, and incremental recruitment upon inflammation.[63,72] Accordingly, while a range of chemokines are active on human and mouse MCs in vitro, no MC-selective chemokine has been identified yet.

MC localization to small intestine is reliant on adhesive interactions controlled by $\alpha_4\beta_7$ integrin, VCAM-1, and mucosal addressin cell adhesion molecule-1 (MAdCAM-1).[63] CXCR2 ligands also play a critical role for the constitutive localization of MC progenitors to the

intestine.[63,78] In contrast to the small intestine, MC progenitors are not abundant in normal lung. However, MCs are detected in the bronchial epithelium and airway smooth muscle, associated with pulmonary inflammation and abundant in human asthma.[79] VCAM-1 interactions with both $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins, but not mucosal addressin cell adhesion molecule-1, are essential for the trafficking of MC progenitors to the lung during antigen-induced pulmonary inflammation.[79] It is interesting to note that in a model of *Aspergillus fumigatus* extract-induced allergic pulmonary inflammation, IgE can influence the number and function of mature MCs, but not MC progenitor recruitment.[80]

On the basis of their location, histochemical staining, content of proteases, and reactivity to selected secretagogues and antiallergic drugs, two major subtypes of MCs have been described in rodents: mucosal-type MCs, which express MC protease-1 and -2; and connective tissue-type MCs, which are positive for MC protease-4, -5, -6, and carboxypeptidase A.[81] In humans, mucosal MCs preferentially express mouse MC protease-1 and -2, whereas connective tissue MCs express MC protease-4, -5, -6, and carboxypeptidase A.[81,82] Human MCs also exhibit heterogeneity and are thus classified by their content of serine proteases as tryptase-only MC, chymase-only MC, or both tryptase- and chymase-positive MC.[81,82] Both in humans and mice, these MC phenotypes are reversible in certain microenvironmental conditions, and transdifferentiation between the phenotypes has been shown.[81,82] Each MC subtype predominates in different locations: tryptase-only MC cells are prominent within the mucosa of the respiratory (nose, lung) and gastrointestinal tracts, and increase upon mucosal inflammation, whereas tryptase- and chymase-positive MC cells are prominent within connective tissues such as the dermis, submucosa of the gastrointestinal tract, heart, conjunctivae muscularis of the uterus, and perivascular tissues.[81] In light of murine studies highlighting a “fine tuning” on the MC functions and effector properties by cytokines or matrix proteins, it seems likely that human MCs will prove to be even more heterogeneous than currently thought. Accordingly, MC phenotype, behavior, and responsiveness may be dramatically altered by cytokines, including IL-4 (which upregulates the expression of FcεRI and is particularly pivotal in regulating functional responses of MCs in mucosal inflammation), IL-5 (which promotes MC proliferation in the presence of SCF), and IFNγ (which induces the expression of the high-affinity activating FcγRI/CD64 and also decreases MC numbers).[68] Furthermore, MCs express an array of adhesion and immune receptors that may assist in the recognition of invading pathogens and in the sampling of different stimuli coming from the microenvironment.[67] This versatility is reflected in the numerous

IgE-dependent and -independent activation pathways that modulate the quality and magnitude of MC responses and cytokine release. MCs are endowed with a range of potent proinflammatory effector molecules that may be selectively released not only through FcεR1 activation, but also in response to a variety of other receptors including C3aR and C5aR, FcγRIIa/CD32a, KIT/CD117/SCF-R, IL-3R, IL-4R, IL-5R, IL-9R, IL-10R, GM-CSFR, IFNγR, CCR3, CCR5, CXCR2, CXCR4, NGFR/TRKA, the type 1 receptor for cys-LTs (CysLT1R), the adenosineA3 receptor, and many TLRs, among others.[68] Depending on the type, property, strength, and combination of the stimuli that they receive, MCs secrete a diverse and wide

range of biologically active products (Table 20.5) that can trigger, direct, or suppress the immune response.[68,83,84]

TABLE 20.5 Major Basophil and/or Mast Cell-Derived Mediators

Class	Mediators	Physiologic Effects
Preformed mediators	Histamine	Vasodilation, angiogenesis, mitogenesis, suppressor of T-cell activation
	5-HT	Leukocyte regulation, vasoconstriction, pain
	Chymase	Tissue damage, pain, angiotensin II synthesis
	Tryptase	Activation of PAR, inflammation, pain, tissue damage, degradation of antigens and peptides
	kininogenases	Synthesis of kinins, pain
	Nitric oxide synthase	Nitric oxide production
	Carboxypeptidase A	Degrades enzymes
	CRH	Inflammation, vasodilation, mast-cell VEGF release
	Endothelin	Sepsis
	Kinins	Inflammation, pain, vasodilation, mast cell trigger
	Somatostatin	Anti-inflammatory effects, mast cell trigger
	VEGF	Neovascularization, vasodilation
	Chondroitin sulfate	Connective tissue

		component, anti-inflammatory, mast cell inhibitor
	Heparin	Angiogenesis, NGF stabilization, mast cell inhibitor
Lipid mediators	LTB_4, LTC_4, PAF, PGD_2	Leukocyte chemotaxis, vasoconstriction, pain, platelet activation, vasodilation, inflammation, bronchoconstriction
Cytokines	TNFα, TGFβ, IFNα, IFNβ, IFNγ, IL-1α, IL-1β, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-18, IL-25, SCF, MIF	Inflammation, leucocyte migration/proliferation
Chemokines	CXCL8, CCL3, CCL2, CCL7, CCL13, CCL5, CCL11, CCL19	Chemoattraction and tissue infiltration of leukocytes
Growth factors	CSF, GM-CSF, bFGF, VEGF, NGF, LIF	Growth of various cell types
Antimicrobic products	Nitric oxide, superoxide, antimicrobial peptides	Microbial killing

bFGF, fibroblast growth factor; CRH, corticotropin-releasing hormone; CSF, colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; LIF, leukemia inhibitory factor; LTB, leukotriene B; LTC, leukotriene C; MIF, migration inhibitory factor; NGF, nerve growth factor; PAF, platelet-activating factor; PAR, proteinase activated receptor; PGD, prostaglandin D; SCF; stem cell factor; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Basophil Generalities

Bas are the rarest of the granulocytes (typically constituting 0.5% to 1.5% of peripheral blood leukocytes), are 5 to 10 μm in diameter, and exhibit a bean-shaped or bilobed condensed nucleus.[85] Compared to MCs, they have little proliferative capacity and possess fewer, but larger (up to 1.2 μm) round metachromatic granules (Fig. 20.8) enriched in histamine (stored at about 1 to 2 pg per cell).[86,87] Bas develop from CD34+ progenitors, differentiate and mature in the bone marrow in response to IL-3, and circulate in the periphery with a half-life

thought to be of a few days.[85,88] Although not predominantly tissue-dwelling cells, Bas are able to infiltrate inflamed tissues, particularly at sites of allergic inflammation within several hours after exposure to allergens, and are often accompanied by a simultaneous influx of eosinophilic granulocytes and Th2 lymphocytes.[82,85,89] Bas can be cytofluorimetrically identified by their selective pattern of plasma membrane proteins serving as markers, including FcεRI (like MCs), CD123 (the α chain of the IL-3 receptor), CD11b, and CD13, in conjunction with the absence of other lineage markers (such as CD2, CD14, CD16, CD19, and MHC class II).[89] They also express, and dynamically respond to, ligands for a variety of functional cytokine/chemokine receptors (eg, IL-5R, GM-CSFR, CCR2, and CCR3), complement receptors (CD11b, CD11c, CD35, and CD88), prostaglandin receptors (CRTH2), Ig Fc receptors (FcεRI and FcγRIIb), and TLRs. For example, following FcεRI activation (which typically triggers granule exocytosis and mediator release), a number of basophil cell surface markers, including CD13, CD63, CD107a, CD164, and CD203C, may increase.[85,89,90]

Mast Cell and Basophil Mediators

The mediators produced by MCs and Bas are schematically divided into preformed mediators, newly synthesized lipid mediators, and cytokines/chemokines (see Table 20.5).[82,86,89]

Such subdivision is not exclusive as TNFα, for instance, occurs as both preformed and as a newly synthesized molecule.[91] Preformed mediators, including histamine or MC neutral proteases (tryptase, chymase, cathepsin G, and carboxypeptidase) are stored in cytoplasmic granules and form complexes with the negatively charged, very abundantly expressed, sulfated proteoglycans.[82,92] Upon MC/Ba activation, the granules fuse with the plasma membrane and, within minutes, release their content into the extracellular environment where the various mediators dissociate from proteoglycans.[68,85] Other than being secreted upon stimulation, some molecules are also constitutively released, as in the case of MC tryptase. Consequently, tryptase levels are considered as a parameter that reflects the MC burden, and, in fact, they significantly increase in systemic mastocytosis.[82,92] Curiously, the function of tryptase in vivo is still unknown, even though, in vitro, it digests fibrinogen, fibronectin, prourokinase, proMMP-3, protease-activated receptor-2, and complement component C3; it activates fibroblasts as well. The functions of MC/Ba-derived proteoglycans and proteases are less well understood, but proteases provide some protection against snake and insect venoms.[82,92] Differently from MCs, humans Bas do not express chymase, appear to contain very low levels of heparin and tryptase (with some exceptions), but contain MBP and Charcot-Leyden crystal (which are typical eosinophil products). Activated Bas have been shown to also contain granzyme B and to directly kill target cells, at least in part by this cytotoxic serine protease.[82,90,93] More importantly, Bas, together with neutrophils, seem to be the exclusive peripheral blood source of histamine.

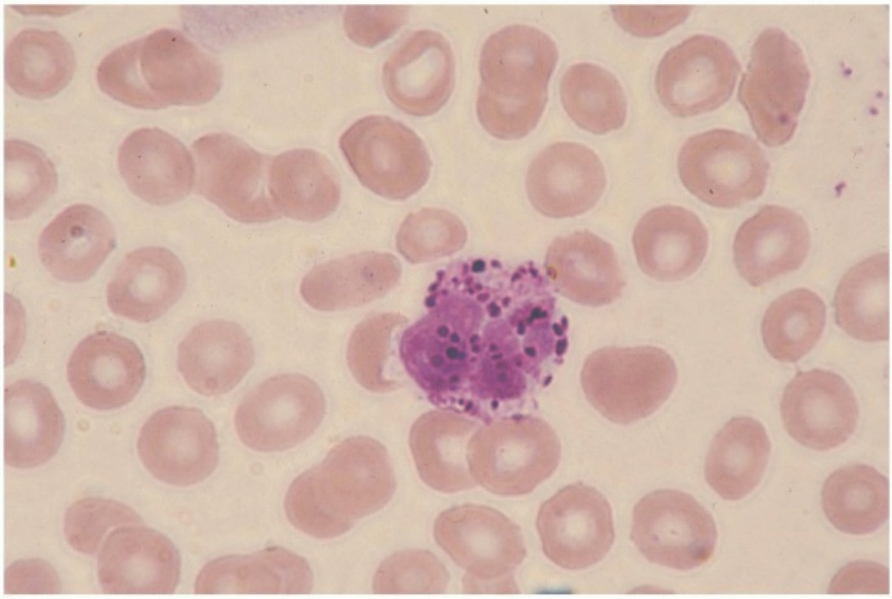

FIG. 20.8. A Polymorphonuclear Basophil Circulating in Peripheral Blood. From *Anderson's Atlas of Hematology* ; Anderson, Shauna C., PhD. Copyright 2003, Wolters Kluwer Health/Lippincott Williams & Wilkins.

Newly synthesized lipid mediators by FcεRI-activated MCs are PAF, PGD_2, and LTA_4. The latter is then converted to LTB_4 or conjugated with glutathione to form LTC_4 (the parent compound to the cysteinyl leukotrienes, which also include LTD_4 and LTE_4).[86,92] Activated Bas can produce the same lipid mediators but PGD_2 (as they lack the PGD_2 synthase).[82,86,93] PGD_2 functions as bronchoconstrictor and attracts both Eos and Bas, LTB_4 attract neutrophils and effector T-cells, while cysteinyl leukotrienes attract Eos and work as potent bronchoconstrictors, in addition to promote vascular permeability and to induce mucus production.

MCs produce several cytokines, including IL-3, IL-4, GM-CSF, IL-5, IL-6, IL-10, and IL-13, growth factors, including bFGF, SCF, VEGF, and several chemokines, including CXCL8 and CCL3.[82,86,92] In addition, MCs are considered the only cells storing very abundant levels of preformed TNFα, whose critical role in host defense against bacterial infections has been widely highlighted: in fact, specific experimental animal models lacking MC-derived TNFα result in drastically reduced neutrophil influx and significantly increased mortality.[91] Bas are a major source of GM-CSF and VEGF, and release significant amount of chemokines (CXCL8, CCL3, CCL11) in response to IgE cross-linking. Chief among the cytokines produced by Bas are IL-4 and IL-13, which are released in response to FcεRI or C5a stimulation within the range of 20 to more than 600 pg/million and < 20 to 2000 pg/million cells, respectively.[82,86,93]

Mast Cell and Basophil Effector Functions

MCs and Bas, although derived from distinct progenitors, have been recognized to express partially overlapping functions in many aspects of natural and acquired immunity.[86,87]

Traditionally, MCs and Bas, together with Eos, are considered essential components of IgE-mediated classic type 1 hypersensitivity (allergic) reactions and allergic inflammation (see Chapter 45). Nonetheless, MCs and Bas have also the ability to release and synthesize highly bioactive, proinflammatory, and cytotoxic substances, independent of signaling through FcεRI. Therefore, these cells, besides

being important modulators of allergic reaction, can also contribute to leucocyte recruitment, stromal and tissue cell activation, modulation of immune reactions, tissue remodelling, and angiogenesis.[84,89,90,94]

Mast Cells and Basophils in Allergy. Allergen-specific IgE production, with subsequent fixation of IgE to FcεRI receptors on MCs and Bas, followed by rapid degranulation and release of histamine and specific eicosanoids (eg, LTC_4), is central to the initiation and propagation of immediate hypersensitivity reactions.[65,82] It has been widely accepted that MCs contribute significantly to acute inflammatory reactions to antigens/allergens toward which the host bears antibodies of the IgE class.[95] MCs are responsible for virtually all of the increased vascular permeability and tissue swelling early in the IgE-dependent passive cutaneous anaphylactic response.[95,96] If the stimulation is of more persistent or of more severe nature, acute response may undergo transition into a late-phase reaction, which, except for the time scale ranging from a few to several hours from initial antigen challenge, is characterized by new recruitment of leukocytes (Eos, Bas, and Th2 lymphocytes) to the site of inflammation.[65] Bas play a crucial role in sustaining allergic chronic inflammation upon their entering the affected tissue, not only due to the release of stored and newly synthesized mediators, such as histamine and LTC_4, but also via the release of high amounts of the cytokines IL-4 and IL-13.[89,90,97]

Mast Cells and Basophils in Innate Immunity and Host Defense. Recent studies have demonstrated that MCs express many PRRs, including TLRs 1 to 10 and nucleotide oligomerization domain-like Receptors (NLR) (including nucleotide oligomerization domainreceptors and the NLRP3). Expression of various PRRs thus permits MCs to respond to both pathogen-associated molecular and “danger signals” resulting from cell stress or injury. Several MC functions triggered by PRR have been implicated in host defense.[84,94,98] These latter include enhancement of the recruitment or function of granulocytes, phagocytosisdependent bactericidal activities, and proteolytic degradation of endogenous mediators, which would otherwise be elevated to toxic levels, such as endothelin-1 and neurotensin. Secretion of proteases/enzymes and formation of extracellular traps that contain antimicrobial peptides, histone, deoxyribonucleic acid, and tryptase have also been proposed as potential mechanisms by which MCs exert protective functions.[94,98] In addition to their role in bacterial infections, MCs can promote host resistance to certain parasite and virus infections. However, the mechanisms involved in these functions have not been fully elucidated.[94,98] Differently from MCs, the role of Bas in host defense is less studied, especially in bacterial or viral infections. Some evidence for a role of Bas in host defense against parasitic infections and tick infestations has been reported, but the molecular mechanisms underlying the Ba-mediated protection in these processes remain to be determined.[89,99,100]

Mast Cells and Basophils in Immunoregulation. MCs and Bas manifest immunoregulatory functions in IgE-dependent and -independent inflammatory reactions, mainly by secreting soluble mediators and cytokines or by functioning as antigen-presenting cells. These cells contribute to the initiation of acquired immunity by orchestrating DC migration, maturation, and function and by interacting with T and B cells.[84,89,90,94] MCs have the potential to influence DC functions, mainly through the release of histamine, PGD_2, PGE_2, LTB_4, or cytokines such as TNF, IL-1, IL-16, IL-18, and CCL5. Generally, both in vitro and in vivo evidence indicate that MCs predominantly inhibit IL-12p70 production and induce a Th2-promoting phenotype in DCs.[94,101] MCs can also modulate B-lymphocyte functions by supporting their survival, proliferation, and IgA production, mainly through the expression of CD40L and IL-6.[94,102] Evidence of cell-contact interactions between MCs and B cells in vivo have been found to occur in secondary lymphoid organs and inflamed tissues.[94,103]

MC surface molecules and secreted products can also influence various aspects of the biology of T cells, for example, by polarizing naive T cells to Th1, Th2, Th17, and regulatory T (T_{reg}) cells or by modulating the functions of distinct T-cell subsets.[94,101] Interestingly, MCs through IL-6 secretion and via OX40/OX40L contact have recently been shown to counteract T_{reg}-mediated suppression, thus leading to the establishment of Th17-mediated inflammatory responses.[104] MCs can also induce antigen-specific T-cell proliferation and activation (of both CD8+ and CD4+ T cells) due to their expression of MHC class I and II molecules, of costimulatory molecules of the B7 family (such as ICOS-L, PD-L1, CD80, and CD86), and of members of the TNF/TNF receptor families (such as OXOL and CD40L).[84,94,101] Finally, MCs produce several mediators with anti-inflammatory activities, including TGFβ, IL-4, IL-10, and IL-9, and have been shown to play a crucial role in transplantation engraftment, for instance toward allogeneic skin graft.[94,101,105] Most likely, MCs manifest predominantly proinflammatory effects in the earlier phase of the immune response, while the anti-inflammatory effects of these cells are more pronounced at the later phase of the response to limit ongoing inflammation.[105]

Bas have the ability to regulate the acquired immune response, mainly by potentiating humoral responses and promoting Th2 polarization.[86,89] Bas can enhance B-cell survival and proliferation, and Ig production mainly through the release of IL-4, IL-6, BAFF, and APRIL.[89,106,107] The ability of Bas to polarize Th2 immune responses is dependent on their function as antigen-presenting cells and on IL-4 secretion. These Th2-promoting functions of Bas have been demonstrated in responses to protease allergens, helminthic parasites, or antigen-IgE complex in vivo.[108,109,110] Recently, the Th2-promoting functions of Bas have been linked to the development of systemic lupus erythematosus in mice deficient of the Src family protein tyrosin kinase Lyn, and in human patients.[111]

Mast Cells and Basophils in Disease

The most striking increase in MCs occurs during parasitic diseases and mastocytosis (the latter being a pathologic excess of MCs, most notably in the skin, bone marrow, gastrointestinal tract, spleen, liver, and lymph nodes, that is

usually caused by gain-of-function mutations of KIT).[68,112] The importance of MCs in IgE-allergic reactions (including rhinitis, urticaria, and asthma) is emphasized by the increased numbers of these cells seen in affected tissues in both mice and humans.[68,95] MC hyperplasia and increased MC products at sites of tissue injury have also been observed in chronic inflammatory and autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis. A pathologic role of MCs in these diseases has been suggested mainly by the utilization of the K/BxN mouse model of antibodyinduced arthritis and the experimental autoimmune encephalomyelitis mouse model of multiple sclerosis.[94,112] Moreover, studies in mice indicate that activation of MCs via the NLRP3 can contribute to IL-1β overproduction and chronic urticarial rash in subjects with cryopyrin-associated periodic syndrome, a disorder associated with NLRP3 mutations.[113] There is evidence for MCs in promoting, but also in protecting against, tumor growth. The accumulation of MCs at the periphery of tumors has been observed in both rodent models and in a diverse array of tumors in humans. It is generally thought that MCs promote early angiogenesis events in tumor development through the release of mediators such as VEGF, CXCL8, angiopoietin-1, bFGF, heparin, and proteases.[114] Finally, MCs could also contribute to the pathogenesis of acute or chronic vascular events: accordingly, acute myocardial infarctions show elevations of both histamine and mature β-tryptase, suggesting that MC activation occurs concomitantly with ischemia.[94,112]

As far as Bas, it has remained obscure whether these cells actually play a crucial role or are just redundant with MCs in allergic reactions in vivo.[89,97] Human Bas have been shown to release IL-1β and TNFα upon the crosslinking of surface-bound IgD, but not IgE, suggesting a possible role of these cells in autoinflammatory disorders such as hyper-IgD syndrome.[115] Bas may also contribute to the production of autoantibodies that cause lupus nephritis.[116]

CONCLUSION

In the last few years, our perception of granulocytes and MCs has changed dramatically. In fact, there has been mounting evidence that the function of these cells is not limited to acting as first line of defense against invading pathogens, but is extended to perform additional and unexpected activities in strict collaboration with adaptive immune and other nonimmune cells. Thus, cells of innate and adaptive immunity together orchestrate complex functional programs to promote host defense, control the development of self-tolerance, and avoid autoimmunity. In this context, the gene expression pattern and phenotype of the cells discussed in this chapter must rapidly change in a coordinate, time-dependent manner in response to microenvironmental soluble and cellular signals.[117] Granulocytes and MCs, in view of their wide variety of membrane receptor are able to mediate delivery of costimulatory signals, their broad array of molecules involved in cell-extracellular-matrix adhesion and in cell-cell contacts, their extensive assortment of soluble pro- and anti-inflamatory mediators, may profoundly influence the development, intensity, and duration of adaptive immune responses that ultimately serve for host defense, allergy, and autoimmunity. A representative example of this aptitude is the plasticity that MCs manifest as a result of their reciprocal interactions with the T-cell populations exerting regulatory or activatory functions in normal and/or pathologic immune responses.[101,105] In this context, it has been demonstrated that

CD4+CD25+ T_{reg} cells establish a cell-cell contact with MCs through the OX40:OX40L axis, which regulates the MC degranulation threshold and contributes to tissue tolerance.[104,118] However, in an inflammatory environment, this MCs/CD4+CD25+ T_{reg} cell interaction induces a Th17 switch by T_{reg} cells.[104,118] On the same line, it is remarkable that, under particular settings, granulocytes and MCs display functions that in the past were ascribed only to immune adaptive cells. For instance, it has been recently demonstrated that neutrophils, among their various immunoregulatory functions,[23] can also display a B-cell-helper function that promotes Ig class switching, Ig somatic hypermutation, and antibody production by activating marginal zone B cells through BAFF, APRIL, and IL-21 secretion.[119] Similarly to neutrophils, MCs are also able to provide costimulatory signals that sustain B-cell expansion and drive the development of IgA-oriented humoral immune responses,[102] thus ascribing a B-cell-helper function that in the past was retained exclusive of T cells.

Novel activities have been described for myeloid cells also in the context of host defense, such as for instance in the case of Bas that, during primary and secondary exposure to parasites, might display different roles.[93,120] Indeed, in a murine model of primary helminthes infection,[121] Bas often accumulate in affected tissues where they produce large quantities of Th2 cytokines that not only regulate the eosinophil recruitment to the lung and the IgE production, but also favor mucosal MC infiltration.[121] However, their major defensive role occurs during the secondary infection, in which worm expulsion is more rapid (by 5 days) than in the primary one (by 10 days postinfection), as revealed by Ba depletion studies that cause an impaired worm expulsion by mice, regardless of the presence or the absence of MCs.[122]

In view of the continuously emerging findings in the field, it is predictable that in the next years there will be the discovery of additional, unsuspected biologic features that granulocytes and MCs possess.

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REFERENCES

1. Witko-Sarsat V, Rieu P, Descamps-Latscha B, et al. Neutrophils: molecules, functions and pathophysiological aspects. *Lab Invest*. 2000;80:617-653.

2. Nathan C. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* . 2006;6:173-182.

3. Cassatella MA. The neutrophil (II). In: Serhan CN, Ward PA, Gilroy DW, eds. *Fundamentals of Inflammation* . Cambridge: Cambridge University Press; 2010:49-64.

4. Holland SM, Gallin JI. Disorders of granulocytes and monocytes. In: Longo DL, Fauci

AS, Kasper DL, Hauser SL, Jameson JL, Loscalzo J, eds. *Harrison's Principles of Internal Medicine*. 17th ed. New York, NY: McGraw-Hill Professional; 2008:375-385.

5. Pillay J, den Braber I, Vrisekoop N, et al. In vivo labeling with $2H_2O$ reveals a human neutrophil lifespan of 5.4 days. *Blood*. 2010;116: 625-627.

6. Fox S, Leitch AE, Duffin R, et al. Neutrophil apoptosis: relevance to the innate immune response and inflammatory disease. *J Innate Immun*. 2010;2:216-227.

7. Stark MA, Huo Y, Burcin TL, et al. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity*. 2005;22:3285-3294.

8. Witko-Sarsat V, Pederzoli-Ribeil M, Hirsch E, et al. Regulating neutrophil apoptosis: new players enter the game. *Trends Immunol*. 2011;32:117-124.

9. Wright HL, Moots RJ, Bucknall RC, et al. Neutrophil function in inflammation and inflammatory diseases. *Rheumatology (Oxford)*. 2010;49:1618-1631.

10. Segal AW. How neutrophils kill microbes. *Annu Rev Immunol*. 2005;23:197-223.

11. Borregaard N, Sørensen OE, Theilgaard-Mönch K. Neutrophil granules: a library of innate immunity proteins. *Trends Immunol*. 2007;28: 340-345.

12. Berton G. Degranulation. In: Gallin JI, Snyderman R, eds. *Inflammation: Basic Principles and Clinical Correlates*. 3rd ed. Philadelphia, PA: Lippincott Williams & Wilkins; 1999:703-720.

13. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. *Science*. 2004;303:1532-1535.

14. Fuchs TA, Abed U, Goosmann C, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol*. 2007;176:231-241.

15. Scher JU, Abramson SB, Pillinger MH. The neutrophil (I). In: Serhan CN, Ward PA, Gilroy DW, eds. *Fundamentals of Inflammation*. Cambridge: Cambridge University Press; 2010:39-48.

16. Ley K, Laudanna C, Cybulsky MI, et al. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*. 2007;7:678-689.

17. Norling LV, Leoni G, Cooper D, et al. Cell adhesion molecules. In: Serhan CN, Ward PA, Gilroy DW, eds. *Fundamentals of Inflammation*. Cambridge: Cambridge University Press; 2010:208-216.

18. Schmidt EP, Lee WL, Zemans RL, et al. On, around, and through: neutrophil-endothelial interactions in innate immunity. *Physiology*. 2011;26:334-347.

19. Etzioni A. Genetic etiologies of leukocyte adhesion defects. *Curr Opin Immunol* . 2009;21:481-486.

20. Flannagan RS, Jaumouillé V, Grinstein S. The cell biology of phagocytosis. *Annu Rev Pathol*. 2012;7:61-98.

21. Cassatella MA. Neutrophil-derived proteins: selling cytokines by the pound. *Adv Immunol*. 1999;73:369-509.

22. Serhan CN, Brain SD, Buckley CD, et al. Resolution of inflammation: state of the art, definitions and terms. *FASEB J*. 2007;21:325-332.

23. Mantovani A, Cassatella MA, Costantini C, et al. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* . 2011;11:519-531.

23a. MA. The neutrophil: an emerging regulator of inflammatory and immune response. In: Cassatella MA, ed. *Chemical Immunology and Allergy* . Basel, Switzerland: Karger; 2003;83;11.

24. Boudaly S. Activation of dendritic cells by polymorphonuclear neutrophils. *Front Biosci*. 2009;14:1589-1595.

25. Scapini P, Bazzoni F, Cassatella, MA. Regulation of BAFF/BLyS expression in human neutrophils. *Immunol Lett*. 2008;116:1-6.

26. Pelletier M, Maggi L, Micheletti A, et al. Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood*. 2010;115:335-343.

27. Abadie, V, Badell E, Douillard P, et al. Neutrophils rapidly migrate via lymphatics after Mycobacterium bovis BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. *Blood*. 2005;106: 1843-1850.

28. Costantini C, Cassatella MA. The defensive alliance between neutrophils and NK cells as a novel arm of innate immunity. *J Leukoc Biol* . 2011;89:221-233.

29. Constantini C, Calzetti F, Perbillini O, et al. Human neutrophils interact with both 6-sulfo LacNAc+ DC and NK cells to amplify NKderived IFN{gamma}: role of CD18, ICAM-1, and ICAM-3. *Blood*. 2011;117:1677-1686.

30. Daley JM, Thomay AA, Connolly MD, et al. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J Leukoc Biol* . 2008;83:64-70.

31. Bao Y, Cao X. Revisiting the protective and pathogenic roles of neutrophils: Ly-6G is key! *Eur J Immunol* . 2011;41:2535-2538.

32. Noonan DM, De Lerma Barbaro A, Vannini N, et al. Inflammation, inflammatory cells and angiogenesis: decisions and indecisions. *Cancer Metastasis Rev* . 2008;27:31-40.

33. Scapini P, Nesi L, Morini M, et al. Generation of biologically active angiostatin kringle 1-3 by activated human neutrophils. *J Immunol* . 2002;168:5798-5804.

34. Gregory AD, Houghton AD. Tumor-associated neutrophils: new targets for cancer therapy. *Cancer Res* . 2011;71:2411-2416.

35. Di Carlo E, Forni G, Lollini P, et al. The intriguing role of polymorphonuclear neutrophils in antitumor reactions. *Blood*. 2001;97:339-345.

36. Cassatella MA. On the production of TNF-related apoptosis-inducing ligand (TRAIL/Apo-2 ligand) by human neutrophils. *J Leukoc Biol* . 2006;79:1140-1149.

37. Fridlender ZG, Sum J, Kim F, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: “N1” versus “N2” TAN. *Cancer Cell* . 2009;16:183-194.

38. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* . 2011;11:693-702.

39. Müller I, Munder M, Kropf P, et al. Polymorphonuclear neutrophils and T lymphocytes: strange bedfellows or brothers in arms? *Trends Immunol*. 2009;30:522-530.

40. Kaplan MJ. Neutrophils in the pathogenesis and manifestations of SLE. *Nat Rev Rheumatol*. 2011;7:691-699.

41. Fillon S, Ackerman SJ, Furuta GT. Eosinophils. In Serhan CN, Ward PA, Gilroy DW, eds. *Fundamentals of Inflammation* . Cambridge: Cambridge University Press; 2010:86-94.

42. Kita H. Eosinophils: multifaceted biological properties and roles in health and disease. *Immunol Rev*. 2011;242:161-177.

43. Hogan SP, Rosenberg HF, Moqbel R, et al. Eosinophils: biological properties and role in health and disease. *Clin Exp Allergy*. 2008;38:709-750.

44. McNagny K, Graf T. Making eosinophils through subtle shifts in transcription factor expression. *J Exp Med*. 2002;195:43-47.

45. Collins PD, Marleau S, Griffiths-Johnson DA, et al. Cooperation between interleukin-5

and the chemokine eotaxin to induce eosinophil accumulation in vivo. *J Exp Med*. 1995 182:1169-1174.

46. Sanderson CJ. Interleukin-5, eosinophils, and disease. *Blood*. 1992; 79:3101-3109.

47. Wardlaw AJ, Walsh GM, Symon FA. Mechanisms of eosinophil and basophil migration. *Allergy*. 1994;49:797-807 .

48. Egesten A, Calafat J, Janssen H, et al. Granules of human eosinophilic leucocytes and their mobilization. *Clin Exp Allergy*. 2001;31:1173-1188.

49. Neves JS, Weller PF. Functional extracellular eosinophil granules: novel implications in eosinophil immunobiology. *Curr Opin Immunol* . 2009;21:694-699.

50. Spencer LA, Szela CT, Perez SA, et al. Human eosinophils constitutively express multiple Th1, Th2, and immunoregulatory cytokines that are secreted rapidly and differentially. *J Leukoc Biol* . 2009;85:117-123.

51. Moqbel R, Lacy P. New concepts in effector functions of eosinophil cytokines. *Clin Exp Allergy*. 2000;30:1667-1671.

52. Lamkhioued B, Aldebert D, Gounni AS, et al. Synthesis of cytokines by eosinophils and their regulation. *Int Arch Allergy Immunol* . 1995;107:122-123.

53. Kita H, Ohnishi T, Okubo Y, et al. Granulocyte/macrophage colonystimulating factor and interleukin 3 release from human peripheral blood eosinophils and neutrophils. *J Exp Med*. 1991;174:745-748.

54. Shamri R, Xenakis JJ, Spencer LA. Eosinophils in innate immunity: an evolving story. *Cell Tissue Res*. 2011;343:57-83.

55. Meeusen EN, Balic A. Do eosinophils have a role in the killing of helminth parasites? *Parasitol Today*. 2000;16:95-101.

56. Behm CA, Ovington KS. The role of eosinophils in parasitic helminth infections: insights from genetically modified mice. *Parasitol Today*. 2000;16:202-209.

57. Wang HB, Weller PF. Pivotal advance: eosinophils mediate early alum adjuvant-elicited B cell priming and IgM production. *J Leukoc Biol* . 2008;83:817-821.

58. Spencer LA, Weller PF. Eosinophils and Th2 immunity: contemporary insights. *Immunol Cell Biol* . 2010;88:250-256.

59. Odemuyiwa SO, Ghahary A, Li Y, et al. Cutting edge: human eosinophils regulate T cell subset selection through indoleamine 2,3-dioxygenase. *J Immunol* . 2004;173:5909-

5913.

60. Akuthota P, Wang HB, Spencer LA, et al. Immunoregulatory roles of eosinophils: a new look at a familiar cell. *Clin Exp Allergy*. 2008;38: 1254-1263.

61. Radke AL, Reynolds LE, Melo RC, et al. Mature human eosinophils express functional Notch ligands mediating eosinophil autocrine regulation. *Blood*. 2009;113:3092-3101.

62. Chu VT, Fröhlich A, Steinhauser G, et al. Eosinophils are required for the maintenance of plasma cells in the bone marrow. *Nat Immunol* . 2011;12:151-159.

63. Gurish MF, Boyce JA. Mast cells: ontogeny, homing, and recruitment of a unique innate effector cell. *J Allergy Clin Immunol* . 2006;117:1285-1291.

64. Bischoff SC. Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data. *Nat Rev Immunol* . 2007;7:93-104.

65. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. *Nature*. 2008;454:445-454.

66. Janssens AS, Heide R, den Hollander JC, et al. Mast cell distribution in normal adult skin. *J Clin Pathol* . 2005;58:285-289.

67. Marshall, JS. Mast-cell responses to pathogens. *Nat Rev Immunol* . 2004;4:787-799.

68. Boyce JA. Mast cells as sentinels of inflammation. In: Serhan CN, Peter A, Ward PA, Gilroy DW, eds. *Fundamentals of Inflammation* . 1st ed. Cambridge: Cambridge University Press, 2010:65-73.

69. Escribano L, Orfao A, Villarrubia J, et al. Immunophenotypic characterization of human bone marrow mast cells. A flow cytometric study of normal and pathological bone marrow samples. *Anal Cell Pathol* . 1998;16:151-159.

70. Beil WJ, Füreder W, Wiener H, et al. Phenotypic and functional characterization of mast cells derived from renal tumor tissues. *Exp Hematol* . 1998;26:158-169.

71. Irani AM, Bradford TR, Kepley CL, et al. Detection of MCT and MCTC types of human mast cells by immunohistochemistry using new monoclonal anti-tryptase and anti-chymase antibodies. *J Histochem Cytochem*. 1989;37:1509-1515.

72. Hallgren J, Gurish MF. Mast cell progenitor trafficking and maturation. *Adv Exp Med Biol* . 2011;716:14-28.

73. Collington SJ, Williams TJ, Weller CL. Mechanisms underlying the localisation of mast cells in tissues. *Trends Immunol*. 2011;32:478-485.

74. Franco CB, Chen CC, Drukker M, et al. Distinguishing mast cell and granulocyte differentiation at the single-cell level. *Cell Stem Cell*. 2010;6:361-368.

75. Chen, CC, Grimbaldeston, MA, Tsai, M, et al. Identification of mast cell progenitors in adult mice. *Proc Natl Acad Sci U S A* . 2005;102: 11408-11413.

76. Arinobu Y, Iwasaki H, Gurish MF, et al. Developmental checkpoints of the basophil/mast cell lineages in adult murine hematopoiesis. *Proc Natl Acad Sci U S A* . 2005;102:18105-18110.

77. Jamur MC, Oliver C. Origin, maturation and recruitment of mast cell precursors. *Front Biosci (Schol Ed)*. 2011;3:1390-1406.

78. Abonia JP, Austen KF, Rollins BJ, et al. Constitutive homing of mast cell progenitors to the intestine depends on autologous expression of the chemokine receptor CXCR2. *Blood*. 2005;105:4308-4313.

79. Abonia JP, Hallgren J, Jones T, et al. Alpha-4 integrins and VCAM-1, but not MAdCAM-1, are essential for recruitment of mast cell progenitors to the inflamed lung. *Blood*. 2006;108:1588-1594.

80. Mathias CB, Freyschmidt EJ, Caplan B, et al. IgE influences the number and function of mature mast cells, but not progenitor recruitment in allergic pulmonary inflammation. *J Immunol*. 2009;182:2416-2424.

81. Zhao W, Schwartz LB. Mast cells. In: Greaves M, Kaplan AP, eds. *Urticaria and Angioedema*. New York, NY: Marcel Dekker; 2009:31-52.

82. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol*. 2010;125:S73-S80.

83. Frossi B, De Carli M, Pucillo C. The mast cell: an antenna of the microenvironment that directs the immune response. *J Leukoc Biol*. 2004;75:579-585.

84. Shelburne CP, Abraham SN. The mast cell in innate and adaptive immunity. *Adv Exp Med Biol*. 2011;716:162-185.

85. Arm J, Sloane D. Basophils. In: Serhan CN, Ward PA, Gilroy DW, eds. *Fundamentals of Inflammation*. 1st ed. Cambridge: Cambridge University Press; 2010:74-85.

86. Knol EF, Olszewski M. Basophils and mast cells: underdog in immune regulation? *Immunol Lett*. 2011;138:28-31.

87. Crivellato E, Nico B, Ribatti D. The history of the controversial relationship between mast cells and basophils. *Immunol Lett*. 2011;141:10-17.

88. Valent P, Dahinden CA. Role of interleukins in the regulation of basophil development and secretion. *Curr Opin Hematol* . 2010;17:60-66.

89. Karasuyama H, Mukai K, Obata K, et al. Nonredundant roles of basophils in immunity. *Annu Rev Immunol*. 2011;29:45-69.

90. Sullivan BM, Locksley RM. Basophils: a nonredundant contributor to host immunity. *Immunity*. 2009;30:12-20.

91. Gordon JR, Galli SJ. Mast cells as a source of both preformed and immunologically inducible TNF-alpha/cachectin. *Nature*. 1990;346: 274-276.

92. Hsu F, Boyce JA. Biology of mast cells and their mediators. In: Adkinson N, Bochner BS, Busse WW, et al., eds. *Middleton's Allergy: Principles and Practice*. 7th ed. St. Louis, MO: Mosby Elsevier; 2009: 311-325.

93. Karasuyama H, Mukai K, Tsujimura Y, Obata K. Newly discovered roles for basophils: a neglected minority gains new respect. *Nat Rev Immunol* . 2009;9:9-13.

94. Tsai M, Grimbaldeston M, Galli SJ. Mast cells and immunoregulation/immunomodulation. *Adv Exp Med Biol* . 2011;716:186-211.

95. Amin K. The role of mast cells in allergic inflammation. *Respir Med* . 2012;106:9-14.

96. Hakim-Rad K, Metz M, Maurer M. Mast cells: makers and breakers of allergic inflammation. *Curr Opin Allergy Clin Immunol* . 2009;9:427-430.

97. Schroeder JT. Basophils: emerging roles in the pathogenesis of allergic disease. *Immunol Rev*. 2011;242:144-160.

98. Abraham SN, St John AL. Mast cell-orchestrated immunity to pathogens. *Nat Rev Immunol*. 2010;10:440-452.

99. Falcone FH, Pritchard DI, Gibbs BF. Do basophils play a role in immunity against parasites? *Trends Parasitol*. 2001;17:126-129.

100. Voehringer D. The role of basophils in helminth infection. *Trends Parasitol*. 2009;25:551-556.

101. Galli SJ, Grimbaldeston M, Tsai M. Immunomodulatory mast cells: negative, as well

as positive, regulators of immunity. *Nat Rev Immunol* . 2008;8:478-486.

102. Merluzzi S, Frossi B, Gri G, et al. Mast cells enhance proliferation of B lymphocytes and drive their differentiation toward IgA-secreting plasma cells. *Blood*. 2010;115:2810-2817.

103. McLachlan JB, Shelburne CP, Hart JP, et al. Mast cell activators: a new class of highly effective vaccine adjuvants. *Nat Med*. 2008;14:536-541.

104. Piconese S, Gri G, Tripodo C, et al. Mast cells counteract regulatory T-cell suppression through interleukin-6 and OX40/OX40L axis toward Th17-cell differentiation. *Blood*. 2009;114:2639-2648.

105. Frossi B, Gri G, Tripodo C, Pucillo C. Exploring a regulatory role for mast cells: “MCregs”? *Trends Immunol*. 2010;31:97-102.

106. Denzel A, Maus UA, Rodriguez Gomez M, et al. Basophils enhance immunological memory responses. *Nat Immunol* . 2008;9:733-742.

107. Chen K, Xu W, Wilson M, et al. Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cellstimulating programs in basophils. *Nat Immunol*. 2009;10:889-898.

108. Perrigoue JG, Saenz SA, Siracusa MC, et al. MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. *Nat Immunol* . 2009;10:697-705.

109. Sokol CL, Chu NQ, Yu S, et al. Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nat Immunol* . 2009;10:713-720.

110. Yoshimoto T, Yasuda K, Tanaka H, et al. Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. *Nat Immunol* . 2009;10:706-712.

111. Charles N, Hardwick D, Daugas E, et al. Basophils and the T helper 2 environment can promote the development of lupus nephritis. *J Nat Med* . 2010;16:701-707.

112. Weller CL, Collington SJ, Williams T, et al. Mast cells in health and disease. *Clin Sci (Lond)*. 2011;120:473-484.

113. Nakamura Y, Kambe N, Saito M, et al. Mast cells mediate neutrophil recruitment and vascular leakage through the NLRP3 inflammasome in histamine-independent urticaria. *J Exp Med*. 2009;206:1037-1046.

114. Khazaie K, Blatner NR, Khan MW, et al. The significant role of mast cells in cancer.

Cancer Metastasis Rev . 2011;30:45-60.

115. Chen K, Xu W, Wilson M, et al. Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cellstimulating programs in basophils. *Nat Immunol* . 2009;10:889-898.

116. Charles N, Rivera J. Basophils and autoreactive IgE in the pathogenesis of systemic lupus erythematosus. *Curr Allergy Asthma Rep* . 2011;11: 378-387.

117. Galli SJ, Borregaard N, Wynn TA. Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. *Nat Immunol* . 2011;12:1035-1044.

118. Gri G, Piconese S, Frossi B, et al. CD4+CD25+ regulatory T cells suppress mast cell degranulation and allergic responses through OX40-OX40L interaction. *Immunity* . 2008;29(5):771-781.

119. Puga I, Cols M, Barra CM, et al. B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. *Nat Immunol* . 2011;13(2):170-180.

120. Karasuyama H, Wada T, Yoshikawa S, et al. Emerging roles of basophils in protective immunity against parasites. *Trends Immunol* . 2011;32:125-130.

121. Ohnmacht C, Schwartz C, Panzer M, et al. Basophils orchestrate chronic allergic dermatitis and protective immunity against helminths. *Immunity* . 2010;33:364-374.

122. Ohnmacht C, Voehringer D. Basophils protect against reinfection with hookworms independently of mast cells and memory Th2 cells. *J Immunol* . 2010;184:344-350.

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Chapter 21

The Major Histocompatibility Complex and Its Proteins

David H. Margulies

Kannan Natarajan

Jamie Rossjohn

James McCluskey

INTRODUCTION

The extended family of glycoproteins known as major histocompatibility complex (MHC) and MHC-like molecules is now recognized to comprise a class of receptors, usually expressed at the surface of somatic cells of vertebrates, which confer a wide range of functions in the regulation of the immune response. Their cumbersome moniker, MHC, derives from the early determination that the genes that encode these molecules control one of the fundamental manifestations of an immune response, skin graft rejection, but contemporary immunologists, virologists, and tumor biologists rarely think about these molecules with allogeneic (ie, genetically distinct) skin transplantation in mind. The name MHC is a general label, and specific immunologic functions or molecular structures invoked when a particular MHC is mentioned are dependent on biologic context. In recent years, our understanding of the structure, evolution, and molecular basis of MHC-based interactions has blossomed, and our ability to exploit engineered forms of these molecules has also expanded enormously. Our blueprint for this chapter is to give an overview of MHC-regulated immunologic function and then to summarize the explosive growth in understanding of the molecular structures of MHC molecules and their ligands. Of necessity, we discuss briefly some aspects of the cell biology and cellular maturation of MHC molecules but leave the detailed description of these essential aspects of the classic MHC molecules to the chapter on antigen presentation. Basic aspects of the genetics of MHC molecules not only descriptions of the genetic loci that encode them but also discussion of what we know of the evolution of their encoding genes will be raised.

Functional glycoproteins are molecular machines and their cellular and immunologic activities depend on the shape of these macromolecules, their surface charge and ability to interact with solvent, as well as the flexibility and relationship of their structured domains and unstructured regions. To understand molecules of the MHC in a rational functional context, we consider it crucial to describe their overall structures as well as their structural interactions with bound ligands and their binding to their cognate receptors found on T cells and natural killer (NK) cells. Despite a large and ever increasing database of MHC and MHC-like structures, a number of general questions remain unanswered. We will present some of the unsolved mysteries of the evolution and function of the MHC-like molecules and their viral homologs.

The importance of MHC molecules is underscored by two general characteristics: the enormous degree of polymorphism of genes of the *Mhc* and the identification of a large number of human diseases that are profoundly influenced by either defects of MHC expression or by polymorphic variants. Every student of immunology must be acquainted with the basic biology of MHC-encoded molecules, and the student's comprehension of the regulation of the immune response, encompassing the inflammatory, NK, T-cell, and B-cell responses, will be lacking unless the function and ongoing evolution of the MHC is understood.

We stress that the evolution of the MHC is *ongoing* because for no other set of genetic markers is there such a large database cataloguing genetic polymorphism; as a result, it is apparent that not only are new alleles being identified, but that many of these new alleles have arisen recently. Dramatic examples of the ongoing evolution of MHC molecules are seen not only in new variants of the deoxyribonucleic acid (DNA) sequences observed by those that perform the necessary typing of *Mhc* genotypes for transplantation but also in surveys of the sequences of viruses, particularly the cytomegaloviruses (CMVs), which

appear to have purloined host *Mhc* genes for immunoevasion. In sequence gazing, we observe the interplay between host and pathogen, as the pathogenic viruses, exploiting their rapid generation times, develop new variations on the MHC theme for distinct purposes.

The prototypes of MHC molecules are the MHC class I (MHC-I) and class II (MHC-II) molecules, obligate cellsurface heterodimers that bind and display self- or foreign peptides at the cell surface so that T-cell receptors (TCRs) or NK cell receptors can interact with the molecular complexes in an MHC- and peptide-dependent manner.

MHC molecules are crucial for both TCR- and NK- mediated interactions. Our efforts will be to preserve some sense of the historical development of this exciting field of study and also to focus on paradigmatic genetic, structural, and functional features that unify this extensive gene/protein family. Finally, the *Mhc* provides a genetic link from immune responsiveness to autoimmune disease—those well-known strong associations of particular *Mhc* genes to particular human diseases—and we will provide an outline of the molecular basis for such associations.

Mhc Nomenclature—Dialects of Mhc

The names of the genes and proteins that are critical to understanding the *Mhc* reflect the historical discovery of their functions and differ among different species. Often confusing to students of immunology, the nomenclature of

the *Mhc* differs for different species and must deal not only with a number of distinct, but usually linked, genetic loci but also with their encoded molecules, many of which are heterodimeric glycoproteins. Because the first genes of the *Mhc* identified were those that encoded cell surface molecules that could be detected by antibodies or by transplantation responses, these are the ones that are referred to as *Mhc* genes. Now we know of more than 400 genes that map to the human or mouse *Mhc*, although technically they are all "*Mhc*" genes, the "MHC" molecules refer specifically to the MHC-I or MHC-II molecules that are related in structure and function. (Genes for several complement components and related molecules also map here and are occasionally referred to as MHC class III.) Other *Mhc*-encoded molecules with distinct structure and function are usually referred to by their specific names. Molecules that exhibit structural similarity with MHC-I molecules, whether they map to the *Mhc* or not, may be called as a group "MHC-Ib" to distinguish them from the "classical" MHC-I molecules, also referred to as "MHC-Ia." Another subset of molecules that exhibit the MHC-I fold but that are expressed by viruses may be called "MHC-Iv" molecules.[1,2]

By convention, genetic loci are indicated by designations in italics, and the encoded protein products or phenotypic descriptions are shown in a standard font. The extended genetic region is referred to as the complex; thus the general term used for all species is the *Mhc* or *MHC.* The mouse *Mhc* is referred to as *H2* (previously called *H-2)* because it was the second genetic locus involved in control of expression of erythrocyte antigens identified by Gorer.[3,4] The *Mhc* in the rat is known as *RT1*, the human locus is known as *HLA* (for human leukocyte antigen), *DLA* for the dog, *GPLA* for the guinea pig, *SLA* for the swine, and *RLA* for the rabbit. For other species, based on a suggestion by Klein,[5] the taxonomic name forms the basis for the designation, contributing the first two letters of the genus and the first two of the species to name the locus. Thus, we have *Patr* for the chimpanzee, *Pan troglodytes*; *Gogo* for the Gorilla, *Gorilla gorilla*; *Mamu* for the Rhesus macaque, *Macaca mulatta*; *Mane* for the pig-tailed macaque, *Macaca nemestrina, Mafa* for the cynomolgous monkey, *Macaca fascicularis*; and *Papa* for the Bonobo, *Pan paniscus*. A single site for curated sequences of many species of MHC molecules is the IPD-MHC Database at www.ebi.ac.uk/ipd/mhc/.[6] The most important and widely studied models are those of the human, the mouse, and the rat. For the mouse and rat *Mhc*, the search engine on the Jackson Laboratory Web site (www.informatics.jax.org/mgihome/nomen) is the most comprehensive.

The naming of new HLA genes and alleles and their quality control are governed by the World Health Organization Nomenclature Committee for Factors of the HLA System. Software conversion tools to assist in gene/protein identification are to be found at the IMGT/HLA database (www.ebi.ac.uk/imgt/hla/dictionary.html). Regular updates on HLA nomenclature featuring new alleles can be found in the journal *Tissue Antigens* and via http://hla.alleles.org/nomenclature/nomenc_updates.html. The standardization of serologically defined HLA antigens has been achieved by the exchange of typing reagents and cells in the International Histocompatibility Workshops. The *HLA Dictionary* is a summary of HLA-A,

HLA-B, HLA-C, HLA-DRB1/3/4/5, and HLA-DQB1 alleles and their association with serologically defined HLA-A, -B, -C, -DR, and -DQ antigens, respectively, available free online at the IMGT/HLA database site.[7] As defined by the Nomenclature Committee (http://hla.alleles.org/nomenclature/naming.html), each HLA allele name has a unique number corresponding to up to four sets of digits separated by colons (Table 21.1 and Fig. 21.1). The digits before the first colon describe the type, which often corresponds to the serologic antigen carried by an allotype (a genetically distinguishable form of the molecule within the same species). The next set of digits lists the subtypes, numbers being assigned in the order in which DNA sequences have been determined. Longer names are assigned only when necessary. Precise designation of human genes is by a nomenclature including a number following the locus (eg, HLA-A*01:01 and HLA-DRB1*01:01). Summaries of the hundreds of human alleles that have been identified are in Tables 21.2 and 21.3. Comprehensive databases of human *Mhc* genes are maintained at the IMGT/HLA database (see previous link)[8,9] where, at the time of this writing, more than 7000 HLA class I and class II alleles have been tabulated.

TABLE 21.1 Nomenclature of HLA Loci and Alleles

Nomenclature	Definition
HLA	The HLA region and prefix for HLA gene
HLA-DRB1	Particular HLA genetic locus
*HLA-DRB1*13*	Group of alleles encoding DR13 antigen or with sequence similarity to other DRB1*13 alleles
*HLA-DRB1*13:01*	A specific HLA allele
*HLA-DRB1*13:01:02*	An allele that differs by a synonymous mutation from DRB1*13:01:01 (ie, DNA sequence difference but no amino acid sequence difference)
*HLA-DRB1*13:01:01:02*	An allele that contains a mutation outside the coding region distinguishing it from DRB1*13:01:02
*HLA-A*24:09N*	A "null," unexpressed allele
*HLA-A*30:14L*	An allele encoding reduced or "low" cell surface expression
*HLA-A*24:02:01:02L*	An allele encoding a reduced or "low" cell surface expression, where the mutation is outside the coding region
*HLA-B*44:02:01:02S*	An allele encoding a protein that is expressed as a "secreted" molecule only
*HLA-A*32:11Q*	An allele for which a previous effect on surface expression has not been confirmed and is considered "questionable"

DNA, deoxyribonucleic acid; HLA, human leukocyte antigen.

Source: Adapted from the designations summarized by the *WHO Nomenclature Committee For Factors of the HLA System*, Stephen G.E. Marsh, http://hla.alleles.org. Also available at the IMGT website: www.ebi.ac.uk/imgt/hla/nomenclature/index.html.

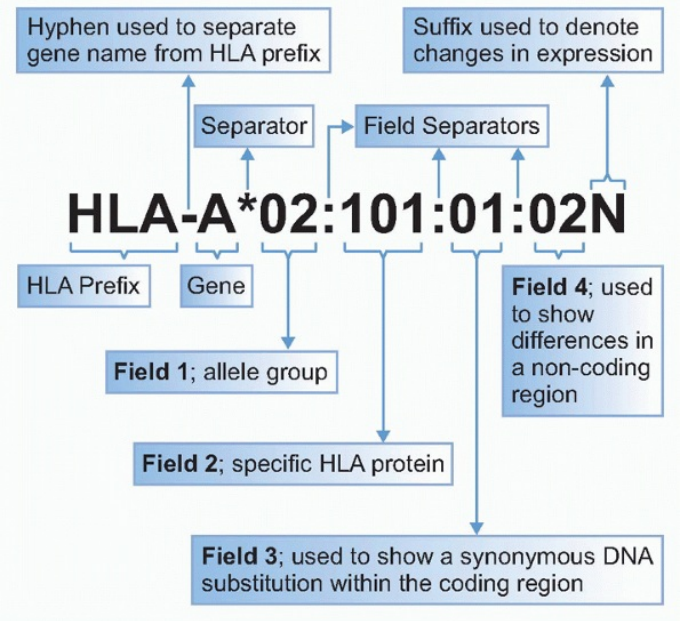

FIG. 21.1. Summary of Current HLA Gene Nomenclature. This illustration explains the nomenclature for HLA alleles as described in http://hla.alleles.org/nomenclature/naming.html.[164]

The commonly used term "haplotype" refers to the linkage of particular alleles at distinct loci that occur as a group on a parental chromosome.[10] The concept of haplotype is important in typing the *HLA* loci in the human where the linked *Mhc* genes (those genes in "linkage disequilibrium") of one chromosome of one parent will generally segregate as a group to the children. Individual haplotypes of the *Mhc* in the mouse are referred to by a lowercase letter superscript as $H2^b$, $H2^d$, or $H2^k$. Thus, the $H2^k$ haplotype refers to the full set of linked genes, *H2-K*^{k}, *H2-IA*^{k}, *H2-IE*^{k}, *H2-D*^{k}, and extends to the genes of the *Q* and *T* regions as well.[11,12] (Some haplotype designations, such as $H2^a$, refer to natural recombinants and thus have some of the linked genes from one haplotype and some from another.) A complication that demands the precise use of gene and encoded protein names is that the number of genes in a particular homologous genetic region may differ between strains or between individuals. In the mouse, although some strains have only a single gene at the *D* locus (*H2-D*^{b} for instance), other strains may have as many as five genes in homologous region (*H2-D*^{d}, *H2-D2*^{d}, *H2-D3*^{d}, *H2-D4*^{d}, and *H2-L*^{d}).[13,14]

Table 21.4 summarizes the haplotypes of common mouse strains. Included in this listing are a number of congenic inbred mouse strains, strains that contain the *Mhc* derived from one strain, and the remaining background genes from another. A regularly updated and comprehensive listing can be obtained at http://jaxmice.jax.org/literature/catalog/mhc_h2_haplotypes.pdf. An ongoing project, the "Collaborative Cross," aims to establish a panel of mouse recombinant inbred lines derived from eight diverse founder mouse strains as a resource for mammalian genetics. These lines, when their breeding is completed, will provide an opportunity for mapping a wide variety of quantitative trait loci with respect to measurable phenotypes and can be expected to play an important role in studies of immune recognition.[15]

The Immunologic Function of MHC Molecules

MHC molecules are a molecular reflection of the health status of either the cell that synthesizes them (for MHC-I molecules) or of the local environment in which the cell resides (for MHC-II). The structure of the MHC molecule depends not only on the amino acid sequence of the two polypeptide chains (α and β for MHC-II; heavy [or α] and β2m [for the light chain] for MHC-I) that form the core of the complex but also of the variable bound peptide that forms an integral part of the trimer. The MHC molecule, governed by the sequence of the encoding structural genes for the *Mhc-I* heavy chain and the *Mhc-II* α and β chains, as well as other genes involved in antigen processing and presentation, must satisfy

at least two distinct recognition functions: the binding of peptides or in some cases nonpeptidic molecules and the interaction with either T or NK cells via their respective receptors. The TCR may augment its interaction with the MHC molecule by virtue of interaction with a T cell-expressed coreceptor (cluster of differentiation [CD]8 for MHC-I and CD4 for MHC-II). Some NK receptors may also serve as coreceptors when expressed on T cells.[16] The binding of peptides by an MHC-I or MHC-II molecule is the initial selective event that permits the cell expressing the MHC molecule (the antigenpresenting cell [APC] or when this cell is to be the recipient of a cytolytic signal, the target cell) to sample fragments derived either from its own proteins (for MHC-I-restricted antigen presentation) or from those proteins ingested from the immediate extracellular environment (for the case of MHC-II). The biochemical steps involved in the production of antigen fragments from large molecules are collectively known as "antigen processing," whereas those that concern the binding of antigen fragments by MHC molecules and their display at the cell surface are known as "antigen presentation."

Specifically, MHC-I glycoproteins gather from the cell's biosynthetic pathway fragments of proteins derived from infecting viruses, intracellular parasites, or self-molecules, either expressed normally or in a dysregulated fashion as a result of tumorigenesis, and then display these molecular fragments, in complex with the mature MHC-I molecule, at the cell surface.[17,18,19,20,21] The cell-bound MHC-I/β2m/peptide complex on the APC is exposed to the extracellular milieu and is available for interaction with either T cells or NK cells. The T cell bearing an αβ receptor recognizes the particular MHC/peptide complex by virtue of a specific physical binding interaction. Each T cell is representative of a clonal population and bears a unique TCR encoded by somatically rearranged TCR genetic elements. T cells bearing αβ receptors undergo a complex selective process in the thymus. Only a small proportion of T cells that enter the thymus ultimately reach peripheral lymphoid organs, such as lymph nodes and spleen. A particular TCR can only bind a very limited selection of MHC/peptide complexes. The recognition by T cells is considered "MHC restricted" in that only a limited set of MHC molecules can bind a particular TCR and is also

termed "antigen specific" in that a particular T cell identifies a particular peptide. For any given T-cell clone, single amino acid substitutions of either the MHC or the peptide may severely diminish, obliterate, or even augment the functional interaction of the TCR with the MHC-II/peptide complex. The MHC-I system draws its spectrum of peptides from proteins in the cytosol that are degraded by the proteolytic proteasome complex to peptides that are transported from the cytosol to the endoplasmic reticulum (ER) with the aid of the intrinsic membrane peptide transporter, the transporter associated with antigen processing (TAP), are then trimmed at their amino termini[22] and are cooperatively folded as an intrinsic component of the newly synthesized MHC-I molecule.[23] Interactions with the peptide-loading complex (PLC), which includes TAP, the chaperone tapasin,[24] and Erp57,[25] are crucial to MHC-I loading. MHC-I molecules are unique among proteins in that their three-dimensional structure and thermal stability are exquisitely dependent on the heavy/light chain heterodimer being bound by an appropriate peptide.[26,27] In mutant cells that lack the necessary apparatus for generating and transporting peptides to the ER where peptide loading takes place, MHC-I molecules are expressed poorly and are inherently unstable.[28] MHC-II/peptide loading is controlled in part by the multifunctional chaperone/groove protector, Ii, as well as the important catalytic machinery of the endosomes, molecules known as HLA-DM and -DO in the human, and H2-M and H2-O in the mouse.[24] Although there remains some controversy as

to the precise site of interaction of HLA-DM with HLA-DR, evidence supports the view that this interaction catalyzes peptide interchange, leading to the selection of high-affinity antigenic peptides.[29]

TABLE 21.2 Listing of HLA Class I Alleles [a]

HLA-A Alleles	HLA-B Alleles	HLA-C Alleles

A*01:01:01:01—A*01:104	B*07:02:01—B*07:144	C*01:02:01—C*01:61
A*02:01:01:01—A*02:336	B*08:01:01—B*08:80	C*02:02:01—C*02:55
A*03:01:01:01—A*03:135	B*14:01:01—B*:14:28	C*03:02:01—C*03:139
A*11:01:01—A*11:112	B*15:01:01:01—B*15:238	C*04:01:01:01—C*04:107
A*23:01:01—A*23:50	B*18:01:01—B*18:68	C*05:01:01:01—C*05:72
A*24:02:01:01—A*24:190	B*27:01—B*27:86	C*06:02:01:01—C*06:69
A*25:01:01—A*25:16	B*35:01:01:01—B*35:186	C*07:01:01—C*-7:220
A*26:01:01—A*26:72	B*37:01:01—B*37:31	C*08:01:01—C*08:56
A*29:01:01:01—A*29:32	B*38:01:01—B*38:37	C*12:02:01—C*12:68
A*30:01:01—A*30:58	B*39:01:01:01—B*39:68	C*14:02:01-C*14:34
A*31:01:02—A*31:59	B*40:01:01—B*40:180	C*15:02:01—C*15:56
A*32:01:01—A*32:37	B*41:01—B*41:19	C*16:01:01—C*16:44
A*33:01:01—A*33:54	B*42:01:01—B*42:16	C*17:01:01:01—C*17:11
A*34:01:01—A*34:09	B*44:02:01:01—B*44:139	C*18:01—C*8:05
A*36:01—A*36:05	B*45:01—B*45:13	
A*43:01	B*46:01:01—B*46:30	
A*66:01—A*66:16	B*47:01:01:01—B*47:08	
A*68:01:01:01—A*68:85	B*48:01:01—B*48:27	
A*74:01—A*74:15	B*49:01:01—B*49:20	
A*80:01—A*80:02	B*50:01:01—B*50:15	
	B*51:01:01—B*51:126	
	B*52:01:01:01—B*52:27	
	B*53:01:01—B*53:27	
	B*54:01:01—B*54:24	
	B*55:01:01—B*55:54	
	B*56:01:01—B*56:32	

B*57:01:01—B*57:52

B*58:01:01—B*48:36

B*59:01:01:01—B*59:05

B*67:01:01—B*67:03

B*73:01—B*73:02

B*78:01:01—B*78:07

B*81:01—B*81:05

B*82:01—B*82:03

B*83:01

[a]This summarizes HLA alleles as of January, 2012, as described by Robinson et al.8,9 and available at http://www.imgt.org or at www.ncbi.nlm.nih.gov/projects/gv/mhc/main.fcgi?cmd-init. HLA-Bw4 alleles are generally agreed to include B5, B5102, B5103, B13, B17, B27, B37, B38(16), B44(12), B47, B49(21), B51(5), B52(5), B53, B57(17), B58(17), B59, B63(15), B77(15), and A9, A23(9), A24(9), A2403, A25(10), A32(19) (http://hla.alleles.org/antigens/bw46.html). Similarly, HLA-Bw6 alleles include B7, B703, B8, B14, B18, B22, B2708, B35, B39(16), B3901, B3902, B40, B4005, B41, B42, B45(12), B46, B48, B50(21), B54(22), B55(22), B56(22), B60(40), B61(40), B62(15), B64(14), B65(14), B67, B70, B71(70), B72(70), B73, B75(15), B76(15), B78, B81, and B82.

HLA, human leukocyte antigen.

TABLE 21.3 Listing of HLA Class II Alleles [a]

HLA-DR Alleles	HLA-DQ Alleles	HLA-DP Alleles	HLA-DM Alleles	HLA-DO Alleles
α chain	α chain	α chain	α chain	α chain
DRA	DQA1	DPA1	DMA	DOA
DRA*01:01:01—DRA*01:02	DQA1*01:01:01—DQA1*01:09	DPA1*01:03:01:01—DPA1*01:10	DMA*01:01:01:01—DMA*01:04	DOA*01:01:01—DOA*01:04N
	DQA1*02:01	DPA1*02:01:01—DPA1*02:04		
	DQA1*03:01:01—DQA1*03:03:02	DPA1*03:01—DPA1*03:03		
	DQA1*04:01:01—DQA1*04:04	DPA1*04:01		
	DQA1*05:01:01:01—DQA1*05:11			
	DQA1*06:01:01			

	—DQA1*06:02			
β chain	β chain	β chain	β chain	β chain
DRB1	DQB1	DPB1	DMB	DOB
DRB1*01:01:01 —DRB1*01:45	DQB1*05:01:01:01 —DQB1*05:14	DPB1*01:01:01 —DPB1:01:03	DMB*01:0101:01 —DMB*01:07	DOB*01:01 —DOB*01:05
DRB1*03:01:01:01 —DRB1*03:77	DQB1*06:01:01 —DQB1*06:47	DPB1*02:01:02 —DPB1*02:02		
DRB1*04:01:01 —DRB1*04:107	DQB1*02:01:01 —DQB1*02:06	DPB1*03:01:01 —DPB1*03:01:02		
DRB1*07:01:01:01 —DRB1*07:22	DQB1*03:01:01:01 —DQB1*03:39	DPB1*04:01:01:01 —DPB1*04:02:01:02		
DRB1*08:01:01 —DRB1*08:49	DQB1*04:01:01 —DQB1*04:08	DPB1*05:01:01 —DPB1*05:01:02		
DRB1*09:01:02 —DRB1*09:17		DPB1*06:01		
DRB1*10:01:01 —DRB1*10:04		DPB1*08:01		
DRB1*11:01:01 —DRB1*11:121		DPB1*09:01		
DRB1*12:01:01 —DRB1*12:35		DPB1*10:01		
DRB1*13:01:01 —DRB1*13:135		DPB1*11:01:01 —DPB1*11:01:02		
DRB1*14:01:01 —DRB1*14:122		DPB1*13:01 —DPB1*19:01		
DRB1*15:01:01:02 —DRB1*15:69		DPB1*20:01:01 —DPB1*20:01:02		
DRB1*16:01:01 —DRB1*16:19		DPB1*20:01:01 —DPB1*35:01:01		
DRB2		DPB1*35:01:01 —DPB1*35:01:02		
DRB2*01:01				
DRB3		DPB1*35:01:01 —DPB1*41:01:01		
DRB3*01:01:02:01 —DRB3*03:03				
DRB4		DPB1*41:01:01 —DPB1*41:01:02		

	DRB4*01:01:01:01 —DRB4*01:08	
DRB5		DPB1*44:01 —DPB1*136:01
	DRB5*01:01:01 —DRB5*02:05	
DRB6		
	DRB6*01:01 —DRB6*02:02	
DRB7		
	DRB7*01:01:01 —DRB7*01:01:02	
DRB8		
	DRB8*01:01	
DRB9		
	DRB9*01:01	

[a]This table is based on a listing of alleles maintained by the World Health Organization Nomenclature Committee for Factors of the HLA System, as of January 2012. All new and confirmatory sequences are generally submitted directly to the committee via the IMGT/HLA database using the sequence submission tool provided. The IMGT/HLA database may be accessed at www.ebi.ac.uk/imgt/hla. Serologic assignment of HLA-DR molecules is largely determined by the DRB1 gene product, whereas assignment of DQ molecules reflects serologic contributions from both DQA1 and DQB1 gene products.

HLA, human leukocyte antigen.

TABLE 21.4 Commonly Used Mouse Strains: *H-2* Haplotypes[a]

		***H-2* Complex**							
Strain	**Haplotype**	***KK***	***Ab***	***Aa***	***Eb***	***Ea***	***D***	***Q***	***T***
Common strains									
129/J	*bc*	*b*	*b*	*b*	*b*	-	*b*	*b*	*f*
AKR/J	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*b*	*b*
ASW/Sn	*s*	*s*	*s*	*s*	*s*	-	*s*	*b*	*b*
BALB/c	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*b*	*c*
C3H/HeJ	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*b*	*b*
CBA/J	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*b*	*b*
C57BL/6	*b*	*b*	*b*	*b*	*b*	-	*b*	*b*	*b*

C57BL/10	*b*	*b*	*b*	*b*	*b*	-	*b*	*b*	*b*
C57BR	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*a*	*a*
DBA/2J	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*b*	*c*
NOD/LtJ	*g7*	*d*	*g7*	*d*	-	-	*b*		
NON/LtJ	*nb1*	*b*	*nb1*	*?*	*k*	*k*	*b*		
NZB/BINJ	*d2*	*d*	*d*	*d*	*d*	*d*	*d*	*a*	*a*
NZW/LacJ	*z*	*u*	*u*	*u*	*u*	*u*	*z*		
P/J	*p*	*p*	*p*	*p*	*p*	*p*	*p*	*a*	*e*
PL/J	*u*	*u*	*u*	*u*	*u*	*u*	*d*		
RIII	*r*	*r*	*r*	*r*	*r*	*r*	*r*	*c(r)*	*b*
SJL	*s2*	*s*	*s*	*s*	*s*	-	*s*	*a*	*a*
Congenic strains									
B10.BR	*k2*	*k*	*k*	*k*	*k*	*k*	*k*		
B10.D2	*d*	*d*	*d*	*d*	*d*	*d*	*d*		
B10.S	*s*	*s*	*s*	*s*	*s*	-	*s*		
BALB.B	*b*	*b*	*b*	*b*	*b*	-	*b*		
BALB.K	*k*	*k*	*k*	*k*	*k*	*k*	*k*		
C3H.SW	*b*	*b*	*b*	*b*	*b*	-	*b*		
Recombinant strains									
A	*a*	*k*	*k*	*k*	*k*	*k*	*k*		
A.TL	*tl*	*s*	*k*	*k*	*k*	*k*	*d*		
B10.A	*a*	*k*	*k*	*k*	*k*	*k*	*d*		
B10.A(1R)	*h1*	*k*	*k*	*k*	*k*	*k*	*b*		
B10.A(2R)	*h2*	*k*	*k*	*k*	*k*	*k*	*b*		
B10.A(3R)	*I3*	*b*	*b*	*b*	*b/k*	*k*	*d*		
B10.A(4R)	*h4*	*k*	*k*	*k*	*k/b*	-	*b*		
B10.A(5R)	*I5*	*b*	*b*	*b*	*b/k*	*k*	*d*		
B10.T(6R)	*y2*	*q*	*q*	*q*	*q*	-	*d*		
B10.S(7R)	*t2*	*s*	*s*	*s*	*s*	-	*d*		

B10.S(8R)	*as1*	*k*	*k*	*k*	*k/s*	-	*s*
B10.S(9R)	*t4*	*s*	*s*	*s*	*s/k*	*k*	*d*
B10.HTT	*t3*	*s*	*s*	*s*	*s/k*	*k*	*d*

*Adapted from Kruisbeek561 and www.imgt.org/IMGTrepertoireMHC/Polymorphism/haplotypes/mouse/MHC/Mu_haplotypes.html#polymorphism. A dash indicates abnormal gene expression, although precise mechanism may differ in different strain. Blanks indicate insufficient data for characterization. Additional strains may be identified at http://jaxmice.jax.org/findmice/index.html.

NOD, nonobese diabetic.

MHC-II molecules, in contrast to MHC-I, are expressed on a more limited set of somatic cells —B cells, macrophages, dendritic cells, activated but not resting T cells in the human—and have a somewhat more specific function in peptide selection and presentation. In general, they bind peptides derived from the degradation of proteins ingested by the APC, and they sort their MHC-II molecules into cellular compartments where the degraded peptides are generated and catalytically transferred to the binding site of the MHC-II. The MHC-II antigen-presentation pathway is based on the initial assembly of the MHC-II αβ heterodimer with a dual function molecule, the invariant chain (Ii),

which serves as both a chaperone to direct the αβ heterodimer to an endosomal, acidic, protein processing location where it encounters antigenic peptides and also serves to protect the antigen-binding site of the MHC-II molecule so that it preferentially will be loaded with antigenic peptides in this location.[30,31,32,33] The loading of the MHC-II molecule with antigenic peptide, a process dependent on the release of the Ii derived "CLIP" peptide, in part dependent on the MHC-II-like molecule, HLA-DM in the human,[34,35] H2-M in the mouse,[36] then leads to the cell surface expression of MHC-II/peptide complexes. The MHC-II-recognizing T cells then secrete cytokines and may also be induced to proliferate or to undergo programmed cell death. Such MHC-restricted cytokine production that facilitates and augments the recruitment of additional inflammatory cells as well as APCs and antibody-producing cells is a contemporary explanation for what was historically referred to as "T-cell help." Under some physiologic circumstances, particularly during viral infection, antigens incorporated into antigen-presenting dendritic cells via an "outside in" pathway may alternatively enter the MHC-I presentation pathway. This phenomenon is known as "cross-presentation."[37,38]

MHC molecules perform a crucial role in the thymus by shaping the TCR repertoire as T-cell precursors mature into cells that eventually emigrate from the thymus and populate the spleen and other secondary lymphoid organs. In a complex, multistep process termed "thymic education,"[39] developing T cells expressing randomly rearranged TCR αβ receptors are first subjected to positive selection on MHC-I and MHC-II molecules expressed by cells of the thymic cortex in order to select for further maturation of only those cells capable of recognizing peptides in the context of self-MHC molecules. Positive selection may be viewed as setting the minimum threshold for T-cell activation. T cells surviving positive selection then migrate to the thymic medulla where those T cells with higher-than-average affinity for self-MHC-II/peptide complexes, and which therefore pose the threat of autoimmune disease should they be allowed to emigrate to secondary lymphoid organs, are deleted by negative selection. MHC-I and MHC-II molecules thus play a critical role in shaping the peripheral TCR repertoire. Indeed, mice engineered to lack MHC-I or MHC-II expression are also deficient in T cells.[36,40]

Distinct from the recognition of MHC molecules by TCRs, a number of NK-cell receptors, both activating and inhibitory, bind MHC-I molecules, and several NK receptors interact with MHC-I-like molecules encoded by CMVs.[2,41] In general, the NK/MHC-I interaction, as compared to the TCR/MHC interaction, shows considerably less peptide specificity, although the interaction is peptide dependent, and in some cases may exhibit clear-cut peptide

preferences.[42,43,44] The functional purpose of the MHC-I or MHC-I-like molecule in NK-cell recognition appears to be more subtle than that in T-cell recognition. The NK cell is tuned to a balance of inhibitory and activating signals conveyed to it via MHC interaction. In its resting state, the inhibitory signals predominate. MHC-I is a sensor of the biosynthetic and metabolic state of the cell in which it is synthesized. When the level of MHC-I is dysregulated by tumorigenesis or viral infection, this change can be detected by the NK cell. This ability of the NK cell to sense altered levels of MHC-I on target cells is the basis of the "missing self hypothesis," which explains that NK cells detect and lyse those cells defective in MHC-I expression due to the loss of the inhibitory signal that results from engagement of NK receptors by MHC-I.[45,46,47] The prototype NK receptor in the mouse is the NK inhibitory receptor, Ly49A, a C-type lectin-like molecule that signals its interaction with a normally expressed MHC-I molecule, such as H2-D^d.[48] Distinct clones of NK cells differ in the combinatorial expression of different NK receptors that have different MHC preferences. Thus, in the mouse, each distinct NK clone may express a different combination of NK inhibitory receptors such as Ly49A, Ly49C, Ly49G2, and Ly49I.[49] Because each inhibitory molecule may exhibit slight differences in its MHC-I and/or peptide preference and specificity, this kind of combinatorial expression of NK activity offers a breadth of specificity toward different potential target cells. Recently, it has been shown that developing NK cells within the thymus are also subject to an education process, known as "licensing," which prevents improper activation of their cytolytic function in peripheral tissues.[50,51,52] NK cells that develop in MHC-I-deficient humans and mice fail to kill target cells that lack MHC-I expression. Such targets are readily killed by NK cells that develop in MHC-I-sufficient thymi. Human NK cells seem to go through a similar selective/educating process, but the relevant NK receptors on human cells are generally of the killer cell immunoreceptor (KIR) inhibitory receptor family.[53]

In addition to showing preference for distinct pathways of antigen presentation, the MHC-I and MHC-II molecules also show preferential restriction to T cells of the CD8- or CD4-bearing lineages. This results from the interaction of CD8 with the nonpolymorphic α3 domain of MHC-I molecules,[54,55,56,57,58] whereas CD4 binds to membrane proximal domains of MHC-II.[59,60,61,62,63,64] The CD8 and CD4 molecules serve as "coreceptors" on the surface of T lymphocytes, providing both adhesion (increase in avidity) and specific activating signals, mediated through the kinase, lck, which modulate the avidity of the T cell in a time-dependent manner.[65,66,67,68] Additional complexities arise from the interaction of CD8 with MHC-I. CD8 is expressed as either the CD8αα homodimer or the CD8αβ heterodimer, which are expressed in a developmentally regulated fashion and seem to have distinct functions.[69] The numerous interactions of MHC molecules with other cellular components as well as with the wide variety of peptides and of various immunologic receptors reflect the robust potential of the MHC structure as a molecular sensor and as a master regulator of immune responses. These molecular interactions then read out in different cell trafficking and signaling functions.

THE MAJOR HISTOCOMPATIBILITY COMPLEX

Mhc Genetics

The *Mhc* is an extended region of the genome that spans some 4 million basepairs (Mb) on the short arm of human chromosome 6 in the region 6p21.3. The *Mhc* in the human may be considered to map from HLA-F to the gene encoding the MHC chaperone tapasin (TAP binding protein), a distance of some 2 Mb, but the "extended" human

Mhc (also known as the xMHC) covers some 7.6 Mb from the HIST1H2AA locus to SYNGAP1.[70,71,72] In the mouse, the *Mhc* encompasses almost 3.5 Mb on chromosome 17, extending from the *H2-Ke1* gene at basepair position 34056423 to the *H2-M2* gene at basepair position 37620474 (www.imgt.org/IMGTrepertoireMHC/LocusGenes/index.php?repertoire=listIG_TR&species=mouse&group=MHC). Although *Mhc* genes were among the first to be mapped here, it is now clear that a large number of genes with function unrelated to immune recognition also reside in this region. The interested reader is referred to the regularly updated maps and linkages available at various Web sites including www.ebi.ac.uk/imgt/hla, and the MHC haplotype project at www.sanger.ac.uk/HGP/Chr6/.

A schematic of the human *Mhc* is shown in Figure 21.2. For a rough guide, we also provide a

simple illustrative map comparing human, mouse, and rat *Mhc* regions (Fig. 21.3) and refer the reader to the National Center for Biotechnology Information homology Web site: www.ncbi.nlm.nih.gov/projects/homology/maps/ and National Center for Biotechnology Information Web viewer for access to detailed maps and sequences.

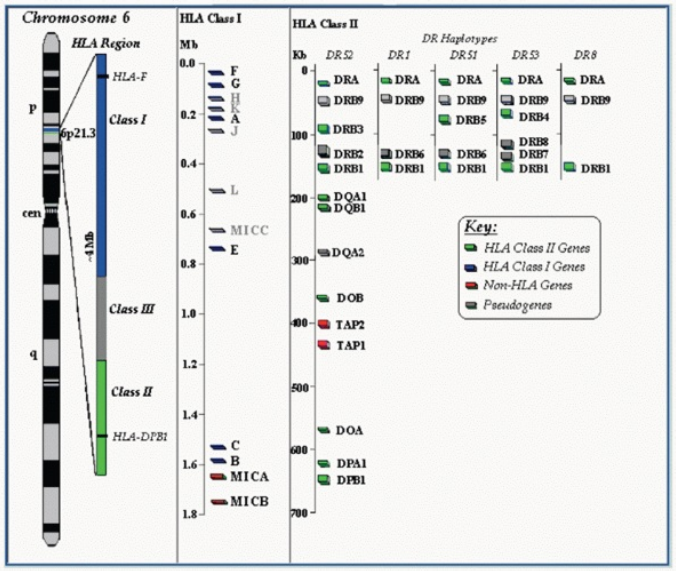

FIG. 21.2. Map of the Human MHC (HLA Region) on the Short Arm of Chromosome 6. A variety of online tools for seeking and manipulating MHC sequences can be found at www.ncbi.nlm.nih.gov/gv/mhc/main.cgi?cmd=init.

The human *Mhc* map reveals clusters of genes grouped roughly into an Mhc-II region covering about 1000 kb, an Mhc-III of about 1000 kb, and an *Mhc* class I region spanning 2000 kb (see Fig. 21.2). *HLA-DP* genes (*DPA* encoding the α chain and *DPB* encoding the β chain) are proximal to the centromere on the short arm of the chromosome and are linked to the genes encoding the related HLA-DM molecule (*DMB* and *DMA*). Between these and the *DQ* genes lie *LMP* (for low molecular weight proteins[73,74,75,76]) and *TAP*[77,78,79,80,81] genes that encode molecules that are involved in peptide generation in the cytosol and peptide transport across the ER membrane, respectively. Low molecular weight proteins

are subunits of the catalytic proteolytic proteasome complex that regulate the specificity of cleavage of proteins, and thus modulate the repertoire of peptides available for MHC-I-restricted antigen presentation.[82,83,84,85] The *TAP* genes encode a two-chain intrinsic membrane protein that resides in the ER of all cells and functions as an adenosine triphosphate-dependent transporter that pumps peptides generated in the cytosol into the lumen of the ER.[86,87] The selective transport of cytoplasmically generated peptides by different TAP proteins in the rat demonstrates that the spectrum of MHC/peptide complexes expressed at the cell surface can be significantly altered by differences in the antigen presentation pathway,[88,89,90] although there is little evidence for this phenomenon in humans.[91] Nevertheless, TAP deficiency syndromes in humans[92,93,94,95] emphasize the importance of peptide delivery in the antigen presentation pathway for normal immune function.

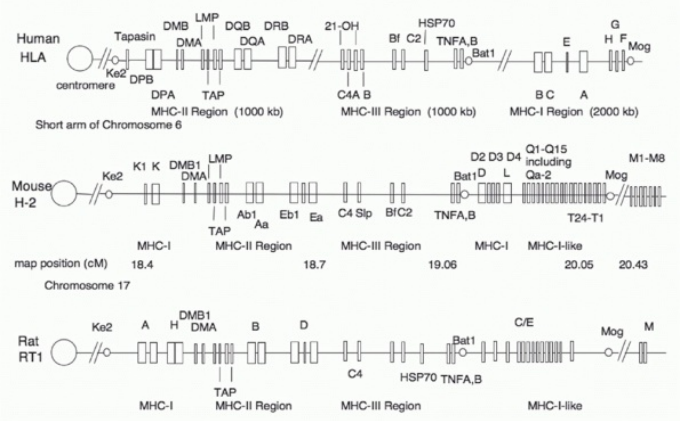

FIG. 21.3. Comparative Maps of Human, Mouse, and Rat *Mhc* Regions. This schematic map is not complete nor drawn to scale and is derived from maps available at www.ncbi.nlm.nih.gov/projects/homology/maps/.

The major *Mhc-II* genes of the human are *HLA-DRA* and *HLA-DRB* that encode the chains that form the HLA-DR molecule, a major antigen-presentation element. Genetic mapping of the human DRB region indicates that several alternative arrangements of *DRB* loci explain the varied serotypes and genotypes observed among different individuals (see Fig. 21.2). In particular, note that several HLA-DR "haplotypes," originally defined serologically as DR52, DR1, DR51, DR53, and DR8, differ not only in particular allelic genes at the *DR* locus but also in the number and precise location of several of the *DRB* genes and pseudogenes. The *Mhc-III* region is important in immunologic terms for several reasons—the structural genes for several complement components map here, as well as the structural genes for 21-hydroxylase (CYP21A2),[96,97] an enzyme critical in the biosynthesis of glucocorticoids, a deficiency of which can lead to the genetic disease congenital adrenal hyperplasia. Also located in the *Mhc-III* region are the structural genes for tumor necrosis factor (TNF) A (also known as lymphotoxin α) and B, which are cytokines made by activated T cells.[98,99,100]

The more distal region of the *Mhc* encodes other MHC molecules. In humans, the cluster of the major *Mhc-I* genes lies here, spanning two Mb, including the genes encoding HLA-B, HLA-C, HLA-E, and HLA-A, as well as HLA-H, HLA-G, and HLA-F. HLA-A, HLA-B, and HLA-C are the major MHC-I molecules of man. (A summary of these is in Table 21.2.) Serologic identification of HLA-C molecules has been difficult and imprecise. However, HLA-C molecules interact directly with NK receptors of the KIR2D family. Direct binding studies have analyzed the kinetics of the interaction of the KIR2 immunoglobulin (Ig)-like NK receptors,[101,102,103] and three-dimensional structures of KIR2DL2 in complex with HLA-Cw3 and KIR2DL1 complexed with HLA-Cw4 have been published.[104,105] Recently, the structure of a KIR3DL1 molecule in complex with HLA-B*57:01 emphasizes the unique nature of different KIR/MHC interactions.[106] The precise functions of HLA-E, HLA-F, and HLA-G are not yet clear. HLA-E and its murine analog Qa-1 bind hydrophobic leader peptides derived from some MHC-I

molecules, forming a complex recognized by the C-type lectin-like NK receptor CD94/NKG2.[107,108,109,110,111] This implies an important function for HLA-E because these molecules are expressed on placental trophoblast cells and would be expected to bind the inhibitory NK receptor, CD94/NKG2A, preventing NK-mediated rejection of the fetus.[112] HLA-E may serve as a recognition element for some T cells as well, so it also seems capable of a classical role.[113,114] Additional evidence now supports an antigen presentation function of HLA-F and HLA-G,[115,116] and the tissue restricted expression of HLA-G as well as the observation that a soluble form leads to apoptosis of CD8+ T cells suggest that this molecule may be involved in the mother's immunologic tolerance of the fetus.[117] *HLA-H* is a pseudogene mapping to this region.[118] This should not be confused with the more distantly related *HLA-HFE*, an *Mhc-Ib* gene erroneously called *HLA-H* by some authors,[118,119] which controls hereditary hemochromatosis by virtue of the interaction of its encoded protein with

the transferrin receptor.[120,121,122,123,124,125] The observations that extravillous trophoblast cells express an unusual combination of HLA-C, HLA-E, and HLA-G molecules; that uterine NK cells express NK inhibitory receptor (KIR) molecules known to interact with HLA-C allelic products; and that complex genotypes predispose to preeclampsia raise the possibility that important aspects of fetal rejection and clinical infertility may be related to MHC recognition by NK cells in the placenta.[126]

Comparison of the mouse, rat, and human *Mhc* maps (Fig. 21.3) reveals several interesting differences.[5,127,128,129] The *Mhc* genes proximal to the centromere of the mouse and rat belong to the *Mhc-I* family, rather than to the *Mhc-II* family, as they do in the human. This mapping has suggested that an intrachromosomal recombination event that occurred in some common rodent ancestor relocated some of the *Mhc-I* genes from a more distal location to the proximal site.[130] Inspection of the current human, mouse, and rat maps clearly indicates similarities in the relative locations and organization of *Mhc-II*, *Mhc-III*, and the distal *Mhc-I* genes.[128] Various genetic expansions and contractions[131] are obvious as well. In particular, the mouse *Q* and *T* regions have expanded the pool of *Mhc-I* genes, which are relatively few in the human and the rat. Early studies of congenic mouse strains mapped multiple genes to the *Q* and *T* regions,[132,133,134] and recent evidence suggests significant differences in the number of genes of this region in different strains. The mouse has some *MHC-Ib* genes that seem to be relatively unique in function. In particular, the *H2-M3* gene, which maps distal to the *Q* and *T* regions, encodes a protein that exhibits a preference for binding peptides that have N-formyl amino terminal modifications. This antigen presentation function may be geared to bacterial, protozoal, and mitochondrial antigens.[135,136,137] Rat homologues of the mouse *H2-M3* and *H2-M2* genes have also been identified.[138,139]

Mhc Polymorphism

The *Mhc's* function in immune responsiveness is also reflected in its genetic polymorphism. Polymorphism is the presence at any given time of a larger than expected number of genetic variants in a population. As populations change and evolve, we expect that genetic variants should arise but because of the selection exerted on most gene products, relatively few of these genetic variants will persist. A genetic locus that exhibits variant alleles at a frequency of more than 1% is considered "polymorphic."[5] A genetic locus that is relatively invariant is often referred to as "monomorphic," even if more than one allele is known. *HLA* genes exhibit a high degree of polymorphism; a number of different mechanisms may contribute to the generation and maintenance of polymorphism. Among these are the selective advantage of a heterozygous pool of antigen-presenting elements in a given individual that might allow the binding and presentation of antigenic peptides derived from a wide variety of environmental pathogens. Limited polymorphism would make the entire population susceptible to a chance infectious agent for which all individuals would be unable to respond, whereas widespread polymorphism would be expected to allow the APCs of at least a proportion of the population to effectively bind and present antigens derived from invading pathogens.[140] Although such a view was originally based on HLA molecules as presenting elements for pathogen-derived peptide fragments to T cells and their antigen-specific TCRs, studies suggest an additional role for *Mhc-I*-related resistance to viral infection via NK cell-mediated recognition[141,142] and altered antigen presentation pathway.[143] Additionally, the presence of a high degree of polymorphism also implies that there is little or no selective disadvantage to the expression of new variant MHC molecules.

The human *Mhc-I* and *Mhc-II* genes are clearly polymorphic, with more than 5000 alleles at the *Mhc-I* loci and some 2000 alleles at the *Mhc-II* loci known (see Tables 21.2 and 21.3) (www.ebi.ac.uk/imgt/hla/index.html).[8,9] In experimental animals, it is more difficult to demonstrate polymorphism in terms of population genetics, although typing of wild mice has confirmed the existence of natural polymorphism predicted from the analysis of inbred strains and their mutants.[144,145] The polymorphism of *Mhc-I* and *Mhc-II* genes, so evident in man and mouse, has also been documented in analyses of cichlid fishes—animals that diverged from the line leading to mammals at least several hundred million years ago.[146,147,148,149]

Genetic Mechanisms for Mhc Evolution

As both an extended genetic region and a group of genes with many belonging to the Ig supergene family,[150,151] the *Mhc* has served as a prototype for elucidating mechanisms that

contribute to the evolution of a multigene family and that add to the polymorphism that is such a dominant characteristic of the classical MHC molecules.[152] The analysis of mutations in the mouse, mostly those of *Mhc-I* genes, has led to the understanding of the mechanisms that give rise to polymorphism. Both induced and spontaneous mutations affecting skin graft acceptance or rejection have been identified, and many of these have been mapped to the *Mhc.* Gross recombinational events have been documented in the *Mhc*,[153,154] as well as more subtle mutations, many of which are multiple amino acid substitutions in a relatively small part of the protein that seem to derive from nonreciprocal recombinational events. Such recombination that occurs over short sequences

is known as "gene conversion" because of its similarity to the phenomenon that occurs in yeast.[153,154,155,156,157,158,159,160,161] Convincing evidence for interlocus gene conversion has been documented in for the *Mhc-II* genes of teleost fish[162] and by deep sequencing of *Mhc-I* genes in recently founded bird populations.[163]

Some of the polymorphisms of *Mhc* genes that have been identified clearly reflect point mutations.[164] Structural studies have shown that the profound immunologic effects of mutations of the *H2* genes $H2K^{bm1}$ and $H2K^{bm8}$ result from minimal detectable changes that may affect thermostability.[165] In addition to such mouse mutants, a number of somatic cell variants and mutants, some due to major deletions or regulatory defects, others clearly point mutants of structural genes, have been described.[166]

The Mhc and Transplantation

Although the early description of the genes of the *Mhc* was based on identification of loci involved in tumor and allograft rejection, and although these genes clearly play a role in such complex phenomena, a contemporary understanding of the function of *Mhc* genes in immunology requires little understanding of the rules of transplantation. The early history of transplantation is chronicled extensively in several books[5,167] and reflects a developing interest in tumor immunology and congenic mouse strains. The mouse has served as the model system for understanding the genetic underpinnings of skin, tumor, and organ transplantation, so a brief description of some relevant principles is in order. Comprehensive manuals and reviews are available.[168] Propagation of a mouse strain by repeated matings of brothers and sisters leads to the establishment of an inbred strain, a group of animals that is genetically identical at all loci. More complete descriptions of the process by which brother-sister mating leads to homozygosity at all loci are given elsewhere.[5,167]

"Congenic" mouse strains, also known as "congenic resistant" or "CR" strains, are those derived by first crossing two inbred strains that differ in a histocompatibility phenotype such as resistance to a transplantable tumor or ability to reject a skin graft. These are then successively backcrossed to one parental strain, and the resistance phenotype is preserved. Following at least 10 backcross generations (N10), a point at which $(1/2)^9 = 0.002$ of the genes of the selected strain should be present, the new strain is propagated by brother-sister mating. Several relevant inbred and congenic mouse strains are listed in Table 21.4 along with their *H2* designations.

The early rules of transplantation were determined by observation of the ability of either transplantable tumors or allografts (usually from skin) to survive in a particular inbred mouse strain host. The graft rejection phenomenon is an extremely sensitive and specific bioassay that permits the detection of genetic differences as small as a single amino acid in an MHC protein. It has been particularly valuable in assessing spontaneous and induced mutants (see previous discussion) and remains the absolute experimental discriminator of "histocompatibility."

In addition to the *Mhc* genes, we should not overlook the genes that encode minor histocompatibility antigens. In the mouse, these were originally identified as genetic loci responsible for graft rejection after extended periods of time. More recently, several minor histocompatibility loci have been identified as those that encode polymorphic proteins that give rise to peptides presented by MHC molecules,[169,170,171,172,173] and we now can understand the complexities of transplantation tolerance in terms not only of *Mhc* genes but also in terms of numerous proteins that may give rise to variant peptides for T-cell recognition. Not only can peptides derived from polymorphic genes throughout the genome serve as minor histocompatibility antigens but also defective translation products, or peptides

resulting from transcription of introns or noncoding strands of DNA may also produce immunologically significant peptides, which, bound by self-MHC molecules may stimulate T cells.[174,175] Whether such defective, newly synthesized proteins serve as a major source of MHC-I-bound peptides remains controversial.[176] Minor antigens that are confined to hematopoietic cells can be recognized as targets by antitumor cytolytic cells and may explain the lower incidence of relapse in hematopoietic stem cell (HSC) transplant recipients who experience graft-versus-host disease (GVHD).[177]

The Mhc and Clinical Transplantation

Processed foreign antigen complexed to HLA class I or class II molecules is recognized by a specific clonally distributed TCR for antigen on the surface of T lymphocytes. The T cell bearing an αβ receptor is capable of recognizing the unitary structure of the HLA molecule itself coordinately with the exposed parts of the peptide antigen. Corecognition of HLA and peptide antigen means that TCRs are highly specific and genetically restricted to recognizing HLA molecules of the individual from which they were derived. Thus, a killer (cytotoxic) T cell raised against an influenza virus peptide in an individual expressing HLA-A2 will not recognize influenza-infected HLA-A1. This concept is known as “MHC restriction” and was described by Shevach and Rosenthal for recognition of amino acid polymers,[178] by Zinkernagel and Doherty for recognition of viral antigens,[179,180] and by Shearer et al. for recognition of altered self-ligands.[181] Given that T cells are MHC-restricted, it is difficult to understand why they should ever recognize a foreign HLA type. However, in practice they do, data indicate that such alloreactive T cells arise with remarkably high frequency. It is estimated that between 1/10 to 1/1000 activated clonally distinct T cells are capable of responding to any random allogeneic HLA molecule.[182,183,184,185] Given the number of T cells in the human lymphoid system, this represents a striking tendency for T cells that are normally restricted to recognizing self-HLA molecules complexed to foreign peptides to cross-react on allogeneic HLA molecules. This cross-reaction can arise from direct recognition of the allogeneic HLA/peptide complex, which usually depends on the peptide antigen as well as the allogeneic HLA molecule. Alternatively, allorecognition by T cells can occur indirectly.[186,187] In such cases, peptides derived from the allogeneic HLA molecules are presented as nominal antigen after processing by the host cells bearing self-HLA molecules. In the normal course of

events, T-cell alloreactivity is an in vitro curiosity, although it is still not entirely clear why the fetal “allograft” does not stimulate the maternal immune system. However, it is the clinical transplantation of organs and hematopoietic stem cells across HLA compatibility barriers that produces graft rejection or GVHD due to T-cell alloreactivity. Fully allogeneic transplants theoretically expose the recipient immune system to up to 12 non-self-HLA allele products expressed by the allograft. Moreover, the “self-peptides” constitutively presented by allogeneic HLA molecules are likely to be quite distinct from those presented by syngeneic HLA molecules because the polymorphisms of the peptide antigen-binding cleft of the MHC-I molecule that distinguish HLA alleles alter the spectrum of selected peptides. In the presence of appropriate costimuli, allogeneic HLA/peptide complexes probably stimulate powerful T-cell responses because of the high density of unusual determinants and the diversity of new peptide ligands presented by the allogeneic HLA/peptide complexes. Because there are many MHC-linked genes encoding a host of proteins, many lacking known immunologic function, it is likely that polymorphisms in these molecules contribute significantly to the alloresponse (see previous discussion of *Mhc* genetic maps and *Mhc* alleles). Accordingly, many studies have demonstrated an incremental improvement in long-term graft survival with progressively higher levels of HLA matching at HLA-A, -B, and -DR loci. For this reason, HLA matching is essential in allogeneic HSC transplantation and highly desirable in solid-organ transplantation. The degree of HLA matching usually required for renal transplantation is shown in Figure 21.4 and for bone marrow transplantation in Table 21.5. Survival of HSC transplants varies according to the nature of the disease, disease stage, and age of the patient but can be >70% in some cases (see Centre for International Blood and Marrow Transplant Research at www.cibmtr.org/pages/index.aspx). The effect of HLA matching on solid-organ transplantation has been monitored by the Collaborative Transplant Study since 1982 (www.ctstransplant.org/).

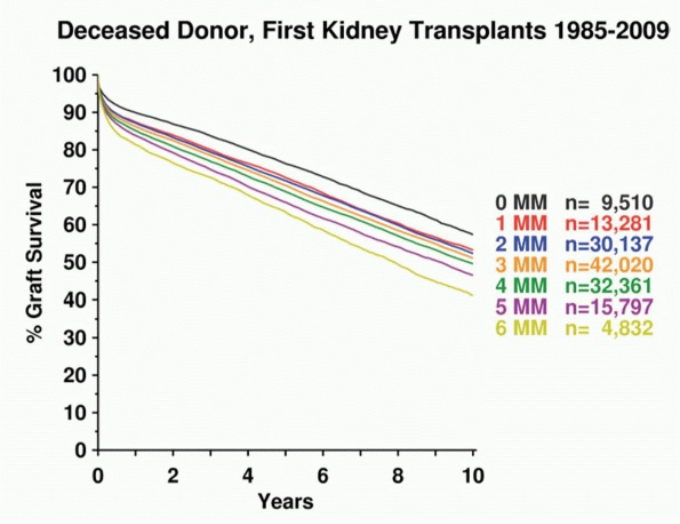

FIG. 21.4. Effect of HLA Matching on First Deceased Donor Kidnery Graft Survival. Survival of first kidney transplants based on number of HLA-A, -B, and -C mismatches as a function of time. Courtesy of Collaborative Transplant Study, G. Opelz, University of Heidelberg (www.ctstransplant.org/public/graphics.shtml).

TABLE 21.5 MHC Matching versus Success of Bone Marrow Transplantation

MHC Compatibility	Risk of Graft Rejection (%)	Risk of Acute Graftversus-Host Disease (%)	Survival (3 Years) (%)
Share two haplotypes (HLA identical sibling)	2	40	50
Share one haplotype plus phenotypically identical	7	40	50
1 HLA mismatch	9	70	50
>2 HLA mismatches	21	80	15
Share zero haplotypes (unrelated)			
“Matched”	3	80	35
“Mismatched”	5	95	35

HLA, human leukocyte antigen; MHC, major histocompatibility complex.

Reprinted from Christiansen FT, Witt CS. Allogeneic bone marrow transplantation. In: Bradley J, McCluskey J, eds. *Clinical Immunology*. Oxford: Oxford University Press; 1997:445, with permission.

In addition to the allogeneic cellular response, the antibody response to HLA molecules and ABO blood groups can also cause rejection of certain grafts, especially where these antibodies are preformed and, therefore, present at the time of organ transplantation.

Antibodies to ABO blood-group antigens react with these determinants on vascular endothelium, and, therefore, ABO-incompatible solid organs can be rapidly rejected by humoral mechanisms. In patients who have been transfused or previously transplanted, or in multiparous females, exposure to allogeneic HLA molecules can also result in the production of anti-HLA class I antibodies. These preformed antibodies can lead to acute and hyperacute rejection of grafts expressing the particular HLA molecules recognized by these antibodies. Renal graft survival improves with fewer HLA mismatches. In Figure 21.4, cumulative data for graft survival are plotted as a function of time. Curves represent those groups with the indicated number of mismatches. Therefore, for solid-organ transplants, individuals are not only matched as closely as possible for their HLA types to avert cellular rejection but, it is also necessary to ensure ABO compatibility and to exclude preformed antidonor HLA antibodies in the host. Paradoxically, some patients who have received multiple blood transfusions prior to transplantation appear to develop some form of T-cell tolerance to allogeneic donor HLA alleles; renal graft survival is actually enhanced in these individuals. This is known as the "transfusion effect," and in some centers, pretransplant transfusion and even donor-specific transfusions are routinely carried out.

Transfusion of potential renal transplant recipients carries the risk, however, of inducing undesirable anti-HLA

antibodies in the patient. Testing for anti-HLA antibodies is known as the "crossmatch." In practice, many laboratories crossmatch only for anti-HLA class I antibodies. Crossmatch compatibility to exclude anti-HLA class I antibodies is essential in renal transplantation and is widely practiced in heart/lung transplantation. Crossmatching for liver transplantation is practiced at only some centers, and the evidence that a positive crossmatch predicts allogeneic liver graft rejection has not convinced everyone of its importance in routine matching. Patients awaiting renal transplantation are usually monitored regularly for anti-HLA class I antibodies because the level and specificity of these antibodies can change with time. This monitoring involves regular crossmatching of patient serum against a panel of randomly selected cells bearing different HLA types. The percentage of positively reacting cells is known as the "panel reactivity." When carrying out a crossmatch between a patient's serum and donor cells, many centers test the current as well as "historical peak" serum from the patient. The historical peak is defined as the patient serum sample giving the highest panel reactivity throughout the monitoring period and is thought to be a reflection of previous HLA sensitization. Most centers now prescreen patients on transplant waiting lists for anti-HLA antibodies using MHC-coated beads as a source of antigen and a highly sensitive flow cytometry technology for their detection. In highly sensitized patients with multiple host antidonor antibodies, these unwanted antibodies can sometimes be functionally eliminated using B-cell ablation therapies (anti-CD20 monoclonal antibodies), plasmapharesis, and intravenous gammaglobulin infusions, thus allowing successful transplantation. Similar approaches, coupled with plasmapharesis, are now taken with increasing frequency in transplanting kidneys across the ABO barrier. The role of antibody crossmatching in allogeneic HSC transplantation is less clear, and many centers do not take the class I or class II crossmatch into account when identifying a bone marrow transplant donor. On the other hand, some large centers place considerable importance on a positive crossmatch as a predictor of bone marrow rejection, and it is therefore advisable to crossmatch bone marrow donor-recipient pairs when there is a high risk of rejection (eg, aplastic anemia). Crossmatching is also used to detect anti-HLA antibodies that may cause refractoriness to platelet transfusion with random platelets.

Family Studies in Histocompatibility Testing

The linkage of HLA loci on chromosome 6 means that individuals will usually inherit a set of nonrecombined HLA alleles encoded at linked HLA loci from each parent. This set of genes (the haplotype) is often identifiable in family studies, where all the alleles present on one chromosome cosegregate. In identifying donors for HSC transplantation, testing of family members is essential to determine haplotypes accurately.[188,189] This is because sharing of HLA antigens from different haplotypes is quite common in families, so mismatches within HLA subtypes (ie, allele-level mismatches) are easily overlooked as a result of mistaken haploidentity of siblings or other family members. Because unrecognized HLA mismatching is poorly tolerated in HSC transplantation, high-resolution sequence-based DNA matching (or its equivalent) is required to avoid GVHD.[190,191,192] The impact of HLA on the outcome of HSC transplants has been studied in unrelated donor-recipient pairs by several groups,[193,194] and guidelines for matching have been published by the U.S. National

Marrow Donor Panel.[195] These data show that unrelated matched transplants can be as effective as sibling matched donor-recipient transplants in donor survival. Mismatching beyond a single, or at most two, HLA loci is associated with increased GVHD and inversely with a lower rate of leukemic relapse. Although allogeneic HSC transplantation can be carried out across single-locus mismatches, there is little correlation with the magnitude of a given mismatch (ie, number of polymorphisms between alleles) and the subsequent immune response. Donor-recipient MHC differences of just a single amino acid can provoke strong alloresponses comparable to reactions between vastly different MHC products.[191] An example of haplotyping in a family study is shown in Figure 21.5. Matching for HLA-DP in allogeneic stem cell transplantation appears to improve graft success in both stem cell and renal transplantation,[196,197] but testing for this locus is not routinely carried out clinically for renal transplants and only for HSC when several donors are available and a rational choice of the best donor has to be made.[198,199] HLA-C matching is also important for chronic myeloid leukemia.[200] Typically, an HLA typing laboratory will test for HLA-A, -B, -DRB1, -DRB3, -DRB4, -DRB5, and -DQ loci for HSC transplant matching.[198] Many centers will also insist on HLA-C testing. In the family study shown in Figure 21.5, the mother and father are mismatched at both haplotypes. Among the children, John and Andrew are haploidentical (and therefore phenotypically identical). Jane and Jim share a single haplotype, as do Tom and Jim. Jane's paternal haplotype is a recombinant involving a crossover event between HLA-A and HLA-B. Recombination is observed between HLA-A/B and HLA-B and HLA-DR in about 1% of meiotic events. The implications of this family study are that Andrew and John would be ideal bone marrow donors for each other. However, none of the other siblings would be suitable as a donor for these brothers. Even though there is sharing of a single haplotype between Tom and both Andrew and John, the complete mismatch in the second haplotype would make Tom unsuitable as a donor for HSC transplantation, which requires very close matching of HLA. On the other hand, haplotype mismatching is common in renal transplantation, in which perfect HLA matching is not absolutely required or routinely achievable. However, for renal and other solid organ transplantation, ABO blood group compatibility is usually essential because these determinants are expressed on vascular endothelium where recognition by isohemagglutinins leads to rapid intravascular coagulation and organ failure. When a matched sibling donor does not exist for a patient requiring allogeneic HSC transplantation (70% of cases), searching of the extended family or unrelated bone marrow registries is indicated. The National Marrow Donor

Panel (www.marrow.org) has several million potential donors suitable for unrelated stem cell transplantation; these donors are used in the United States and worldwide. Bone marrow donor registries also exist in Europe, Australia, Hong Kong, and Japan; these registries often provide donors for patients in other parts of the world. Mobilization of stem cells in the blood following administration of hematopoietic growth factors is now widely used to avoid the need for marrow collections from donors.

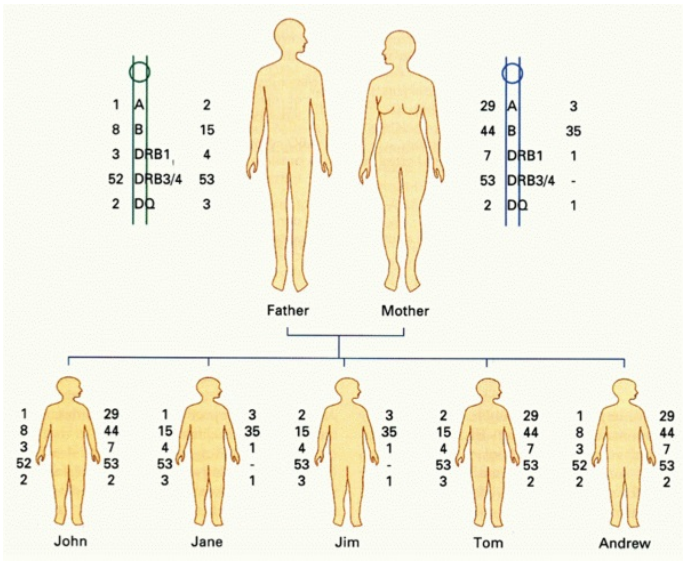

FIG. 21.5. Segregation of Haplotypes in a Family. From McCluskey,[558] with permission.

In the last decade, many HSC transplant protocols have sought to reduce the pretransplant conditioning of patients. There have also been controversial reports indicating dramatic outcomes of HSC transplantation where deliberate KIR-ligand mismatching leads to donor-versus-recipient NK-cell alloreactivity toward cells of hematopoietic origin.[201,202,203,204] The Velardi group in Perugia has championed this approach providing evidence that in the absence of GVHD, a high frequency of donor NK cells in KIR-ligand mismatched transplants can remove recipient's target cells.[202] In particular, HLA haplotype-mismatched hematopoietic transplants can be beneficial in adult acute myeloid leukemia and childhood acute lymphoblastic leukemia leading to the development of adoptive NK-cell transfers as an experimental therapy for graft-versus-leukemia treatment.[205] However, these transplants must be rigorously T-cell depleted, and variable results appear to reflect different transplant protocols, disease parameters, and KIR-mismatching assignment.[203,204,206]

Cord blood banks have also been established around the world. However, cord blood donation of stem cells is often unsuitable for adult transplantation because of limitations in the volume of cord blood collections providing inadequate numbers of stem cells. Many centers will combine different cord bloods that are matched with the recipient as much as feasible to increase the stem cell number. Cord bloods offer the advantage of finding donors faster than adult unrelated registries[197] and theoretically providing banked stem cells from ethnic minority groups that are not well represented in bone marrow donor registries.[198] Cord blood transplants induce less GVHD than bone marrow or peripheral blood stem cell transplants, but posttransplant engraftment is

slower.[196,207] Cord transplants may also tolerate greater levels of HLA mismatching than unrelated bone marrow transplant, but this is dependent on the transfer of sufficient CD34+ stem cells.[208] The results of transplants using bone marrow or peripheral blood stem cells are now considered comparable clinically,[207] but the impact of HLA matching in patients transplanted at early stage in their disease appears to be more marked. Functional tests of HLA compatibility testing for HLA identity at all HLA loci is a daunting task for most laboratories because of the very large number of alleles present in the population. Moreover, in renal transplantation, some mismatches appear to be well tolerated and are associated with long-term graft survival, whereas other mismatches of similar genetic disparity are poorly tolerated and are associated with early rejection.[209,210] Reliable methods for predicting these “taboo” mismatches are not readily available. Similarly, high-resolution HLA typing does not predict all GVHD when selecting suitable unrelated donors for HSC transplantation.[190,211] Therefore, there has been a great deal of interest in developing functional or in vitro cellular tests of overall donor-recipient compatibility. Unfortunately, none of the tests so far developed provides convincing predictability of impending graft rejection or, more importantly, GVHD.

Among the tests used historically for assessing functional compatibility are the mixed lymphocyte reactivity (MLR) test and allogeneic T-helper or cytotoxic T-lymphocyte precursor studies. The MLR or MLC (C = culture) involves measuring T-cell proliferation of host T cells in response to donor lymphocytes and vice versa. In a one-way MLR, the stimulating lymphocytes are irradiated to prevent their proliferation; in the two-way MLR, both stimulator and responder cells are allowed to proliferate. In MLR studies, it is necessary to include controls showing that responder cells can all respond and that stimulator cells can all stimulate across an appropriate barrier such as third-party donor cells. Responses can vary widely, and individual laboratories use their own cutoff values to define negative (ie, nonreactive) and positive (ie, reactive) MLR results.[211] Unfortunately, known HLA mismatches can be present in a negative MLR, and a positive MLR can be obtained between phenotypically HLA-identical transplant pairs. Because the MLR is biased toward measuring HLA class II discrepancies and is notoriously irreproducible,[211] most laboratories have abandoned this test in favor of implementation of high-resolution polymerase chain reaction-based HLA class II typing. Measurement of allogeneic cytotoxic T-lymphocyte or helper T-cell precursor frequencies is carried out at specialized bone marrow transplant centers but is not

universally accepted as being predictive of GVHD.[212,213] The test is labor intensive and requires a skilled technician for reproducibility. Precursor frequencies are estimated by limiting dilution analysis or ELISpot of donor-versus-host lymphocytes (ie, T cells expected to cause GVHD). High precursor frequencies (up to 1 in 10^4 cells) are thought to be associated with a greater risk for acute GVHD.[214] It is possible that precursor studies detect major and minor incompatibilities, and so, theoretically, they might give a broad measure of the transplant barrier, but technical improvements will be required before this test is widely adopted in clinical practice.[215]

The Mhc and Disease

In addition to the control of transplant acceptance or rejection and immune responsiveness, the *Mhc* in the human plays an important role in the etiology of a number of diseases, many of which are autoimmune in nature.[123,216,217,218] Several human diseases are associated with the *Mhc-III* genes because some of the structural genes for enzymes involved in the adrenal steroid biosynthetic pathway (ie, 21-hydroxylase, CYP21A2) map to this region. As many as 200 diseases have well-established genetic linkages to the *Mhc*,[217,219,220,221,222] the most important of which are summarized in Tables 21.6 and 21.7. Recent genome-wide association studies confirm the importance of the HLA-DRB1 locus in rheumatoid arthritis and type 1 diabetes,[71,223,224,225] and fine mapping analysis suggests that a total of five amino acid polymorphisms (three in HLA-DRB1, one in HLA-B, and one in HLA-DPB1), all located in peptide-binding grooves, almost completely explain the MHC association with the risk of rhuematoid arthritis.[226] The precise mechanisms underlying the association of most of these diseases with the particular *Mhc* haplotypes are unknown, but several models have been proposed, including the cross-reactivity of antimicrobial antibodies and the molecular mimicry of

viral antigens that might induce T-cell responses to selfantigens.[227,228,229] The very high incidence of some diseases associated with certain HLA genes assists in the diagnosis as well as the counseling of patients and their families. Several of these diseases are of particular note. Because virtually 100% of patients with narcolepsy have HLA-DQB1*06:02 (associated with HLA-DR2),[230,231] HLA typing can be used as a test of disease exclusion. Thus, a diagnosis of narcolepsy can be excluded with reasonable certainty if the patient does not have HLA-DQB1*06:02. On the other hand, the presence of HLA-DQB1*06:02 is of little predictive value in diagnosis of narcolepsy because this HLA type is relatively common in many populations and occurs frequently in the absence of disease.

TABLE 21.6 Some HLA Disease Associations

Disease	MHC-I	Strength of Association
Ankylosing spondylitis	HLA-B27	+++
Reiter disease	HLA-B27	++
Psoriasis	HLA-C*06	+
Abacavir drug hypersensitivity	HLA-B*57:01	+++
Behcet disease	HLA-B*51	+
Birdshot retinopathy	HLA-A*29	+++
	MHC-II	
Narcolepsy	HLA-DQB1*06:02	++

Insulin-dependent diabetes mellitus	HLA-DQ8	++
	HLA-DQ2	+
	HLA-DR2	-
Rheumatoid arthritis	HLA-DR4	+
Celiac disease	HLA-DQ2	+++
	HLA-DQ8	+
Multiple sclerosis	HLA-DR2	+

HLA, human leukocyte antigen; MHC, major histocompatibility complex

+++ = very strong association that is clinically useful as a diagnostic tool

++ = strong association with likely primary involvement in disease pathogenesis

+ = clear association with likely role in disease pathogenesis

- = negative or protective influence on disease probability

For a more detailed summary of MHC and disease associations see Shiina et al.220

TABLE 21.7 HLA Disease Associations and Relative Risk[a]

Disease	Antigen	Estimated or Relative Risk
Graves disease or myasthenia gravis	B*08:01 DRB1*03:01 DQA1*05:01 DQB1*02:01	4 or 2.5
Multiple sclerosis	DRB1*15:01 DQB1*06:02	4
Multiple sclerosis	DQA1*01:02	4
Psoriasis	C*06:02	5
Celiac disease	DQA1*02:01/DQB* 02:02 (DQ2.2)	1
	DQA1*05:01/DQB1* 02:01 (DQ2.5)	7
SLE	DRB1*15:01	2
Type 1 diabetes or SLE	DRB1*03:0101	4.5

[a]These examples were taken from single nucleotide polymorphism associations reported by de Bakker et al.,71 where the tag single nucleotide polymorphisms and coefficients of determination ($r2$) are also reported. For celiac disease, DQ2.2 only has an effect when in combination with DQ2.5. The relative risk of DQ2.5 changes

depending on the coordinate presence of other DQ types.

SLE, systemic lupus erythematosus.

Ankylosing spondylitis is so strongly associated with the *Mhc-I* allele HLA-B27 and the presence of some bacterial pathogens that it is a popular hypothesis that ankylosing spondylitis is due to the stimulation of particular T cells by HLA-B27-presented bacterial antigens that cross-react on self-tissues. These T cells are then thought to initiate an inflammatory cascade. Despite the strong association of HLA with spondyloarthropathy, critical evaluation of the literature brings a postinfectious etiology into question and certainly more studies are indicated.[232,233] Recent genome-wide studies strongly indicate that a genetic interaction between an enzyme involved in processing MHC-I-associated peptides, ERAP1, and HLA-B27 contributes to disease susceptibility.[234] The tendency of HLA-B27 to form disulfide-linked covalent dimers raises the question whether the resulting cellular pathology related to poor cell surface expression of this MHC-I molecule may be related to the inflammation of joints that is characteristic of this disease.[235,236]

Hereditary hemochromatosis is one of the most common genetic disorders in Caucasian populations (with a prevalence of 1/300 to 1/400). The gene controlling this condition (*HFE*) is *Mhc* linked, mapping approximately three Mb telomeric to the HLA-A locus.[122] The HFE protein is a class I-like molecule, the structure of which has been determined.[237,238] HFE assembles with β2m and is expressed in the intestinal mucosa and placenta, where it plays a role in regulating iron uptake and transport.[239,240] Mice that are homozygous for an induced defect of β2m as well as those with targeted inactivation of the HFE gene suffer from iron overload or hemochromatosis.[241,242,243,244] Mutations at loci other than β2m or HFE also lead to the same disease phenotype.[245] HFE regulates the affinity of the receptor for transferrin, resulting in an alteration of the efficiency of iron transport. The most common molecular defect associated with hereditary hemochromatosis involves a point mutation that results in a Cys282Tyr substitution in the α3 domain of this class I-like molecule.[122] This mutation accounts for >80% of patients with hereditary hemochromatosis.[246] The disruption of the disulfide bond in the α3 domain at this site prevents efficient folding of the molecule and impairs assembly with β2m, resulting in improper HFE protein expression. This leads to a failure to downregulate the affinity of the transferrin receptor for its ligand, transferrin, presumably causing increased iron uptake by cells and tissue damage as a result of iron overload. A second common HFE mutation, 187G, results in a His63Asp substitution and a very slight increase in susceptibility to developing hereditary hemochromatosis, depending on the genotype of the individual.[247] Incomplete penetrance of even the high-risk Cys282Tyr *HFE* genotypes can be partly explained by natural iron deficiency from limited dietary intake and menstrual losses in women.

As summarized in Tables 21.6 and 21.7 and discussed previously, there are a number of autoimmune diseases associated with particular alleles of HLA class II loci, especially with DR and DQ.[221] These diseases include type 1 (insulin-dependent) diabetes mellitus, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, thyrogastric autoimmunity, Sjögren syndrome, and many others. Rheumatoid arthritis is strongly associated with HLA-DR4 subtypes that share a common sequence motif within the DRβ chain,[248] suggesting antigen presentation of self-epitopes by these molecules. The relative risk of severe rheumatoid arthritis is increased in DR4 homozygotes and particularly in compound heterozygotes with high-risk alleles,[249,250] indicating a gene dose effect in susceptibility to autoimmune inflammation.

Although the number of different *HLA* class I and class II alleles that are associated with type 1 diabetes clearly indicates that this relatively common disease has a complex etiology, the identification of a novel *Mhc-II* haplotype in the mutant nonobese diabetic mouse,[251,252,253,254,255] and the recognition that particular TCRs can mediate disease,[256] suggest that a cross-reactive response to a common self- or environmental antigen may play an important role in the etiology of this disease as well. In human type 1 diabetes, the incidence of disease is significantly increased in Caucasians with HLA-DR3-DQ2

and DR4-DQ8 haplotypes.[257,258,259] These haplotypes impart a synergistically increased relative risk when they occur as a heterozygous combination compared to the risk of disease conferred by either haplotype alone.[258,259,260] This raises the possibility of transcomplementation of HLA-DQ gene products producing new molecules involved in antigen presentation.[261] Analysis of large cohorts of patients in genome-wide marker studies offers a promising approach to identify additional genetic factors that act synergistically with HLA allelic linkages in raising the odds of disease susceptibility.[262] Online genomic maps and correlations of associations with particular loci further augments our ability to understand the relative contributions of MHC-linked and MHC-unlinked genes in autoimmune diseases such as type 1 diabetes.

Celiac disease is an underdiagnosed inflammatory disorder affecting the small intestine[263] but resulting from a Th1 hypersensitivity to wheat gluten and related proteins present in barley and rye. Normally manifest in childhood as a failure to thrive, the disease may affect adults quite later in life leading to malabsorption and wasting.[264] Celiac disease can be subclinical, presenting with anemia, osteoporosis, or neurologic symptoms.[263] This disease is strongly associated with HLA-DQ2 and/or HLA-DQ8 (DQ3)[265] as well as with autoantibodies to the enzyme tissue transglutaminase (TG2). Deamidated gluten peptides are presented to CD4+ T cells by HLA-DQ2 or HLADQ8 molecules via macrophages, dendritic cells, and B cells.[266,267] HLA-DQ2 preferentially binds peptides that contain glutamic acid residues at P4 or P6/P7. The dominant gluten peptide is thought to have a low affinity for HLA-DQ2 but this is improved if P6 glutamine is deamidated first.[266,267] HLA-DQ2 forms a network of hydrogen bonds that bind a dominant deamidated gliadin determinant.[268] This leads to enhanced Ag presentation and T-cell recognition with intestinal inflammation in susceptible patients.[266,267,269] The protease resistance of the dominant DQ2-restricted gliadin peptides contrasts with DQ8-associated disease where the dominant gliadin peptides are protease sensitive. The HLA-DQ2-associated and HLA-DQ8-associated forms of celiac disease have their own epitope patterns of deamidation dependence leading to T-cell reactivity. The DQ2-linked peptides possess single residue deamidation at P6 as compared with the preferred deamidation at positions P1 and P9 for DQ8-associated celiac disease.[270] Deamidation of both P1 and P9 to form glutamate residues engenders peptide salt-bridges with HLA-DQ8.[271] Whereas the mechanism of immunopathology of celiac disease is well defined, the exact combination of factors that trigger celiac pathology is still mysterious.[272]

TABLE 21.8 HLA-Linked Drug Hypersensitivity Reactions

	Drug	Adverse Drug Reaction	HLA Association
MHC class I associations	Abacavir	HSS	B*57:01
	Allopurinol	SJS/TEN and HSS	B*58:01
	Carbamazepine	SJS/TEN	B*15:02
	Feprazone	Fixed drug eruption	B22
	Flucloxacillin	Hepatitis	B*57:01
	Sulfamethoxazole	Fixed drug eruptions	A30, B13, Cw6
	Sulfonamides	TEN	A29, B12, DR7

	(including sulfamethoxazole)		
	Levamisole	Agranulocytosis	B27
	Oxicam	SJS/TEN	A2, B12
	Phenytoin	SJS/TEN	B*15:02
MHC class II associations	Aspirin	Asthma	DPB1*03:01
		Urticaria	DRB1*13:02-DQB1*06:09
	Hydralazine	Systemic lupus erythematosus	DR4
	Lapatinib	Hepatotoxicity	DRB1*07:01-DQA1*02:01-DQB1*0202/0203
	NSAIDs	Anaphylactoid and cutaneous reactions	DR11
	Ximelagatran	Elevated alanine aminotransferase	DRB1*07, DQA1*02

HLA, human leukocyte antigen; HSS, allopurinol hypersensitivity syndrome; MHC, major histocompatibility complex; NSAID, nonsteroidal anti-inflammatory drug; SJS, Steven-Johnson syndrome; TEN, toxic epidermal necrolysis.

Adapted from Bharadwaj et al.273

HLA and Drug Reactions

Recently, a number of adverse drug reactions (ADRs) have revealed strong associations with particular HLA types[273] (Table 21.8). The most notable HLA associations are HLA-B* 57:01 with abacavir hypersensitivity syndrome in the Caucasian population,[274,275,276] HLA-B*15:02 with carbamazepine induced Stevens-Johnson syndrome and toxic epidermal necrolysis in Asian populations,[277,278,279] and HLA-B*58:01 and allopurinol hypersensitivity syndrome and Stevens-Johnson syndrome/toxic epidermal necrolysis.[280,281,282] Odds ratios or relative risks of these associations are greater than 500, 1000, and 800, respectively. These ADRs are all associated with HLA class I allotypes, with evidence of T-cell involvement.

The kinetics of the syndromes are also consistent with T-cell-mediated drug hypersensitivity.[276,283,284,285,286] The systemic features of many HLA-linked ADRs suggest that general mechanisms might mediate these reactions. Abacavirpulsed APCs expressing HLA-B*57:01, but not closely related allotypes, stimulate CD8-positive T cells that are drug-specific.[287] It is now known that abacavir specifically binds the HLA-B*57:01 Ag-binding cleft straddling the central pockets and occupying the F-pocket which normally accommodates a tryptophan anchoring the carboxyl terminus of the peptide.[287a] Consequentially, the chemical environment of the cleft is changed which in turn alters the range of self-peptides that now bind HLA-B*57:01. Accordingly, abacavir induces a dramatic shift in the endogenous peptides selected for binding by HLA-B*57:01. This leads to a state

of “altered self” that stimulates a broad range of CD8+ T cells reactive in a GVHD-like manner, explaining the clinical manifestations of the drug hypersensitivity.[287] The mode of abacavir binding provides a basis for understanding its exquisite specificity in binding to HLA-B*57:01 and not to closely related allotypes with polymorphisms in the C, D, E, or F pockets. It will be interesting to learn whether this represents a general mechanism for HLA-linked drug reactions applicable to other small molecule drugs that might bind specifically to HLA antigen-binding clefts. HLA testing before administration of these drugs is now recommended by many physicians and the U.S. Food and Drug Administration in order to avoid life-threatening ADRs.[273,288] The very high relative risks of abacavir, carbamazepine, and allopurinol ADRs and HLA class I allotypes suggests a very high level of specificity in HLA binding, either directly or through a drug-peptide conjugate(s) that only binds the associated HLA allotype. Interestingly, such high relative risks are not commonplace in HLA allotypes and protective immunity, perhaps because it is unusual for any single determinant or HLA allotype to control protective phenotypes.

In addition to recent progress in recognizing *Mhc* associations with adverse reactions to small molecule drugs and a variety of autoimmune and immune deficiency conditions, further analysis has identified relationships of HLA-linked markers with susceptibility and prognosis in a number of infectious diseases. Among the most striking has been the recognition that delayed progression of human immunodeficiency virus (HIV) infection to acquired immunodeficiency syndrome correlates with possession of HLA alleles that express a molecule with a “HLA-Bw4 motif” (see Table 21.2) containing isoleucine at position 80 along with a gene for the NK-cell receptor KIR3DS1. This observation suggested that KIR3DS1 offered protection early in HIV infection for those patients bearing the appropriate Bw4 allele.[289] More recent observations indicate that patients with such a KIR3DS1/B*04 ile80 genotype also exhibit resistance to late opportunistic infections.[290] Other studies now show that particular *HLA-DRB* alleles as well as *MHC-I* alleles significantly influence hepatitis B and C virus susceptibility, persistence, and response to treatment.[291,292,293]

Mutations in the H2 Region

The rapid pace of improvements in deriving mouse and other models for genetic disease based on the engineering of transgenic, targeted deletion and mutation, and conditional knockout mice emphasizes the importance of understanding the valuable reservoir of *Mhc-I* and *Mhc-II* mutants originally derived by laborious methods and still available for precise studies of the role of the *Mhc* in immune responses. Mutations at the *H2* locus have been identified in animals screened by skin grafting in extensive experiments carried out over a 25-year period.[294,295] By grafting tail skin of siblings to and from each other, spontaneous or induced mutant animals that displayed either “gain,” “loss,” or “gain plus loss” transplantation phenotype were identified. Gain mutants are those that express a new transplantation antigen—thus their skin is rejected by their nonmutant siblings; loss mutants have lost a transplantation antigen—thus they recognize the skin of their siblings as foreign and reject that graft. Gain plus loss mutations give effects in both directions—they reject the skin of their siblings, and their skin is rejected by their siblings as well. In classic studies, Melvold, Kohn, and colleagues screened a large number of mouse progeny.[294,295] Homozygous inbred and F1 animals were examined, yielding a total of 25 *H2* mutations identified at *K*, *D*, *L*, and *Ab* loci, and an additional 80 mutations of non-*H2* histocompatibility genes. Although earlier studies suggested that all *H2* genes might be hypermutable, a more complete retrospective evaluation of the available data suggests that with the exception only of the *H2-K*^{b} gene, the spontaneous mutation rate for *H2* genes was comparable to that for non-*H2* genes. The characterization of these mutant animals, first based on peptide maps and amino acid sequences of the H2 proteins,[296,297,298] and later based on the nucleotide sequences of the cloned complementary DNAs or genes,[159] provided some of the basic biochemical information on which further studies of structure and function and mechanism of gene evolution were based. X-ray structure determination of the H2-K^{bm1} and H2-K^{bm8} mutant proteins suggests explanations for the differences in T-cell recognition that result from what might appear to be subtle amino acid substitutions.[165]

Control of Expression of MHC Molecules

MHC molecules, synthesized in the ER and destined for cell surface expression, are controlled at many checkpoints before their final disposition as receptors available for

interaction with either T cells or NK cells. The classical MHC-I molecules are trimers, consisting of the polymorphic H2 or HLA heavy chain, the light chain β2m, and the assembled self-peptide. Thus, the cell surface expression of the assembled trimer may be reduced by a defect in the synthesis, steady state, or assembly of any of these requisite protein/peptide chains. The first level of MHC-I expression control is transcriptional; interferon-γ-dependent regulation is particularly important.[299] For the most part, MHC-I molecules are ubiquitously expressed. Their expression is dependent on a complex regulatory process that coordinately controls the transcription of both MHC-I and β2m.[300,301] The basis of the

more limited tissue specific expression of MHC-Ib molecules is of interest because of the potential importance in the role of some of the MHC-Ib molecules in tolerance to the placenta. HLA-E and HLA-G, expressed on placenta, and HLA-F, another MHC-Ib with limited tissue specific expression, have been examined in considerable detail.[116,302,303,304,305] Another critical level of control of MHC-I expression is genetic, that is, the genes for a particular chain or key components of the PLC must be present for the trimer to be assembled and then expressed at the cell surface. A decrease in heavy chain synthesis as a result of a structural mutation in a coding (such as HLA-A*30:14L [see Table 21.1]) or in a noncoding region (such as HLA-A*24:02:01:02L) would reveal the simplest level of control. In addition, the light chain is absolutely required for cell surface expression of most MHC-I molecules. Deficiency of β2m in various tumors is a relatively common occurrence.[306,307,308] Induced β2m defective animals (*B2m*$^{o/o}$, "β2m knockouts") lack normal levels of MHC-I expression because of this requirement for cell surface expression.[309,310] In addition, polymorphism of the MHC-I heavy chain may influence the efficiency with which the molecule can interact with the PLC or with which it binds self-peptides, and thus some alleles, because of a decreased ability to be loaded with self-peptides, may not be well expressed at the surface. The rest of the MHC-I biosynthetic pathway is dependent on proper generation of cytosolic peptides by the proteasome and delivery to the ER by TAP, appropriate core glycosylation in the ER, transport through the Golgi, and arrival at the plasma membrane.[311] A number of persistent viruses have evolved mechanisms for subverting this pathway of expression.[312] The herpes simplex virus encodes a protein, infected cell peptide 47,[313,314,315] that blocks the activity of the peptide transporter TAP.[316] Cowpox virus encodes two proteins, CPXV12 and CPXV203, which contribute to the downregulation of host MHC-I, CPXV12 inhibits TAP, and CPXV203 retains MHC-I in the ER.[317,318] The human disease bare lymphocyte syndrome, type I, manifested by chronic lung disease in late childhood, results from genetic lesions in either TAP1, TAP2, or tapasin, also known as TAP-binding protein.[93,319,320,321,322,323] Several proteins encoded by the human CMV, unique short region proteins 2 and 11 (US2 and US11), cause rapid protein degradation of MHC-I molecules.[324] Murine CMV encodes an MHC-I-like protein, m152/gp40, which inhibits MHC-I expression by diverting MHC-I molecules to rapid degradation in the endosome.[325,326]

Other molecules that assist the large DNA viruses in evading either the T-cell or NK-cell immune response include human CMV UL142.[327] Murine CMV[328] encodes a number of open reading frames considered MHC-I homologues that may function to deceive NK cells into the perception of normal MHC-I expression.[2,329,330,331] The adenovirus 2 protein, E3/19K, also functions to block the transfer of folded assembled MHC-I molecules from the ER to the Golgi.[332,333,334,335]

MHC-II molecules are also susceptible to regulation at multiple steps. The clear-cut tissue dependence of MHC-II expression—MHC-II molecules are generally found on cells, which have specific antigen presentation functions such as macrophages, dendritic cells, Langerhans cells, thymic epithelial cells, and B cells, but can also be detected on activated T cells of the human and rat—suggests that transcriptional regulation plays an important role. Extensive studies of the promoter activities of *Mhc-II* genes have defined a number of specific transcriptional regulatory sequences.[336] One transcription factor, MHC class II transcriptional activator (CIITA) is clearly crucial.[300,336,337,338] The expression of CIITA is regulated in a complex fashion, possessing at least three distinct promoters that function in a cell type-specific manner.[339] MHC-II deficiency diseases categorized as bare lymphocyte syndrome, type II, result from lesions in at least four different complementation groups, implicating defects in CIITA, RFX5, RFXAP, or RFXANK, all encoding proteins regulating

MHC-II transcription.[338,340,341,342,343,344] Polymorphisms in MHC-II and the resulting differences in tissue and lineagespecific expression of MHC-II suggest a role in the control of autoimmune disease.[145,345,346,347]

A unique aspect of MHC-II regulation is the need to protect its peptide-binding site from loading with self-peptides in the ER and the requirement to traffic to an acidic endosomal compartment where antigenic peptides, the products of proteolytic digestion of exogenous proteins, can be obtained. These two functions are controlled by the type II membrane protein, invariant chain, Ii,[30,348,349,350] which forms a nine subunit complex consisting of three Ii and three αβ MHC-II heterodimers. The region of Ii that protects the MHC-II peptide-binding groove, the class II-associated invariant chain peptide (CLIP), is progressively trimmed from Ii and is ultimately released from the MHC-II by the catalytic action of HLA-DM in the endosome to allow exchange for peptides generated there. HLA-DO, another MHC-II-like molecule, regulates the repertoire of bound peptides, presumably by modulating the catalytic activity of the DM exchange reaction. The important role of Ii in regulating MHC-II expression has been emphasized by the behavior of induced mutant mice lacking normal Ii[351,352,353] that exhibit a profound defect in MHC-II function and expression.

STRUCTURE OF MHC AND MHC-LIKE MOLECULES

"There is nothing that living things do that cannot be understood from the point of view that they are made of atoms acting according to the laws of physics."

—Richard Feynman[354]

"Love hides in molecular structures, yeah, Love is the answer."

—Jim Morrison[355]

So central are *Mhc* genes and their encoded molecules to both the regulation and the effector function of the immune system that it has been apparent almost since their discovery that an understanding of their structure and structural interactions would be fundamental to a comprehension of their physiologic effects. Structural relationships of MHC molecules came first from understanding serologic differences, then from analysis of cellular immune responses, and subsequently from biochemical studies of the MHC-I and MHC-II chains. Amino acid sequence comparisons

suggested a domain structure for the MHC-I molecules, and an exon/domain correspondence was apparent with the initial identification of genomic and complementary DNA clones.[356] Shortly thereafter, a large number of sequences were determined leading to their alignment and comparison. Databases of these sequences are maintained and updated as indicated in previous sections of this chapter, and searches of standard sequence databases may find *Mhc* gene and protein sequences of numerous species. Expression of recombinant clones encoding MHC molecules in several systems: mammalian cells, insect cells, and bacteria, then permitted the accumulation and purification of molecules for functional, binding and ultimately x-ray structural studies.

A current search of the protein data bank (www.rcsb.org/pdb) for the keyword "histocompatibility" yields threedimensional coordinates of more than 500 MHC/peptide, MHC/peptide/TCR, MHC/peptide/coreceptor, and MHC/peptide/NK receptor complexes that allow an understanding of the function of these molecules in a detailed structural context.[357] However, these structures also pose a number of questions that may only be addressed by additional functional experiments in whole animals complemented by biophysical methods applied in vitro. The molecular biologic, functional, and structural studies have led to the development of the use of MHC multimers as extremely powerful tools for imaging specific T cells or NK cells. The goal of this section of this chapter is to summarize these developments with an eye toward explanation of function by structure and in hopes of revealing some of the current quandaries that continue to confound our understanding of the function of the *Mhc.*

Amino Acid Sequences—Primary Structure

Before the cloning of *Mhc* genes, the biochemical purification and amino acid sequence determination of the HLA-A2, HLA-B7, and H2-K^b MHC-I molecules[358,359] indicated that the MHC molecules had unique amino terminal "domains" and showed similarities to Igs in their membrane proximal regions. Early concerns were to identify the differences between allelic gene products as well as the differences between MHC proteins encoded at different loci.

With the cloning of complementary DNAs and genomic clones for MHC-I molecules[156,360,361,362] and shortly thereafter for MHC-II molecules,[151,363,364,365] the encoded amino acid sequences of a large number of MHC molecules of a number of species quickly became available. The comparison of gene and complementary DNA sequences gave an indication of the exon/intron organization of the genes and explained the evolution of the MHC molecules as having been derived from primordial single domain structures of a unit size of a single Ig-domain (such as the light chain β2m) that duplicated to form the basic unit of the MHC-II chain (two extracellular domains) and the MHC-I chain (three extracellular domains).[129] The canonical MHC-I molecule has a heavy chain (also known as the α chain), which is an intrinsic type I integral membrane protein with amino terminal domains called α1, α2, and α3, is embedded in the cell membrane by a hydrophobic transmembrane domain and extends into the cytoplasm of the cell with a short carboxyl-terminal tail. The light chain of the MHC-I molecule, β2m, is a single domain soluble molecule that is encoded elsewhere in the genome.

The MHC-II molecule consists of two chains embedded in the membrane as type I proteins, an α chain and a β chain. The α and β chains both consist of two major extracellular domains, α1 and α2, and β1 and β2, respectively, each linked to a transmembrane domain and cytoplasmic sequences. An initial analysis of both MHC-I and MHC-II molecules suggests that they are noncovalently assembled heterodimers consisting of four extracellular domains, the two membrane proximal domains (α3 and β2m for MHC-I and α2 and β2 for MHC-II) of each molecule are Ig-like, while the two amino terminal domains (α1 and α2 of MHC-I and α1 and β1 of MHC-II) possess what is known as the MHC-fold. The α1 domains of both MHC-I and MHC-II lack the intradomain disulfide bond characteristic of the other extracellular domains. The cytoplasmic domain of MHC-I molecules can be regulated by splicing and differential phosphorylation or other modification, and is likely to play a role in cell surface stability and cycling between the cell surface and other intracellular compartments.[366,367,368,369,370,371,372] However, analysis of directed mutants of MHC-I in some systems indicates that the cytoplasmic domain is not required for cytoskeletal association or surface recycling.[373] (The cytoplasmic domain of MHC-I molecules is the target for the Nef protein of HIV, and the Nef/MHC interaction results in MHC-I downregulation in HIV-infected cells.[374,375] The MHC-II transmembrane and cytoplasmic domains have clear effects on the level of cell surface expression, the efficiency of antigen presentation, and the rate of lateral diffusion of the molecules in the cell membrane.[376,377] Single particle tracking of MHC-II molecules indicates that such lateral diffusion is governed, at least in part, by interaction with the actin-based membrane skeleton.[378] Amino acid sequence alignments of MHC-I and MHC-II proteins, particularly of the human molecules, are available in a number of databases such as www.ebi.ac.uk/imgt/hla/ and http://www.ebi.ac.uk/ipd/mhc/.

Identification of Peptides Bound by MHC Molecules

Although initial understanding of MHC molecules indicated that they are heterodimers, a complete understanding of their structure and resulting function requires the appreciation that they are, in fact, heterotrimers, in which the third component is a short peptide ranging from eight to roughly 15 amino acids in length. This characteristic of mature MHC-I and MHC-II molecules makes them a unique class of protein molecules because an integral part of their peptide composition and resulting structure derive from a heterogeneous array of peptides delivered to the assembling molecules during their maturation. Many different lines of evidence coalesced over a short period of time to demonstrate that MHC molecules function by binding peptides. From functional experiments, MHC-II-restricted T-cell responses to protein antigens were shown to be dependent on peptide fragments.[379] The first direct evidence of MHC/peptide interactions was that purified MHC-II proteins could

bind synthetic peptides in a specific, saturable, and stable manner[380,381] with measurable affinity and a remarkably slow dissociation rate.[381] For MHC-I molecules, the results were at first less clear, but the realization that some cell lines defective in MHC-I surface expression could be induced to express higher levels of surface MHC-I molecules by exposure to the appropriate peptides[382] led the way for direct measurement of MHC-I peptide binding.[383]

Several laboratories succeeded in developing methods for the partial purification and identification of the peptides that copurified with MHC molecules. A viral, antigenic peptide

was identified bound to the MHC-I molecule H2-K^b by recovering MHC molecules from virus infected cells, fractionating the bound peptides chromatographically, identifying a peak of functional biologic activity in a cytotoxic T-cell assay, and determining the amino acid sequence of the recovered peptide by radiochemical techniques.[384] Another method that was applied to the identification of both virusderived peptides as well as the "motif" of self-peptides by particular MHC-I molecules involved first the isolation of a large amount of detergent solubilized MHC-I using appropriate antibodies, the elution of the bound peptides, their partial purification as pools by reverse phase high pressure liquid chromatography, and the determination of the amino acid sequence of the bound peptides by classic amino terminal sequencing.[385,386,387] The unpredicted and surprising result obtained from these studies of MHC-I-derived peptides was that specific amino acid residues were favored at particular positions of the sequence, depending on the MHC-I molecule from which the peptides were obtained, and that the length of the bound peptides was well defined and short, ranging from 8 to 10 amino acids. From such experiments, a number of peptide "motifs" of peptides bound to particular MHC-I molecules and allelic products were identified. Often, specific amino acids were identified at particular Edman degradation steps, indicating a common, highly preferred residue at the same spacing from the amino terminus of the peptide. Thus, the peptide "motif" could be determined even from heterogeneous pools of peptides eluted from particular MHC-I molecules. Further refinements in methodology have included the application of mass spectrometry to the identification and sequencing of individual peptides.[388,389] Alternative approaches for identifying peptide motifs include the use of soluble analogs of MHC-I molecules to ease the purification[390,391] or the use of peptide display libraries to identify those peptides that can bind the MHC.[392,393] With steady improvements in technology, including the use of transfected soluble versions of MHC-I molecules, immunoaffinity purification of the MHC-I molecules of interest, tandem reverse phase high pressure liquid chromatography and further identification and sequencing by tandem mass spectrometry, particularly MHC-I motifs, as well as tumor antigen peptides, may now be identified routinely.[394] A summary of MHC-I peptide motifs is given in Table 21.9. Regularly maintained online databases of MHC-I peptide motifs may be found at several sites (www.imtech.res.in/raghava/mhcbn/links.html and www.syfpeithi.de).[395] Antigenic peptides that represent those observed as tumor antigens

and available for recognition by T cells are summarized at www.cancerimmunity.org/peptidedatabase/differentiation.htm. In addition, the immune epitope database (www.immuneepitope.org)[396] offers a regularly updated and curated Web site that describes MHC motifs, allows evaluation of new proteins for MHC epitopes, and provides new computational tools for such evaluation. An algorithm that allows the prediction of candidates for MHC-I-restricted peptides based on the amino acid sequence of the protein of interest is also available (www-bimas.cit.nih.gov/molbio/hla_bind/).[397] The distinction between "motif" residues of an MHC-restricted peptide and "anchor" residues is an important one. Motif refers to those amino acid residues that are identified based on the sequences of self-peptide or antigenic peptides that have been demonstrated to bind or copurify with a particular MHC molecule. Anchor implies a biophysical function of the particular amino acid residue as specifically interacting with a particular part of the MHC molecule itself. The designation of a residue as an anchor residue may be inferred by analysis of binding to peptide variants in the context of the parental peptide. Alternatively, anchors can be defined from a knowledge of the x-ray structure of the peptide complexed to the MHC-I molecule, and predictions of likely peptide motifs for MHC molecules whose sequence is known but whose structure has not yet been determined can be made by comparison to known structures.

TABLE 21.9 Peptide-Binding Motifs for Some MHC Class I Molecules[a]

Position	1	2	3	4	5	6	7	8	9 (or C-term)
MHC-I molecule									

HLA-A*01		(T)	D			(L)	Y
HLA-A*02:01		L(M)			(V)		V(L)
HLA-A*03		L(V,M)	(F,Y)		(I,M,F,V,L)	(I,L,M,F)	K,Y,F
HLA-B*07		P	(R)				L,F
HLA-B*08:01			K	K,R			L
HLA-B*27:05		R					L,F (Y,R,H,K)
HLA-B*35:01		P					Y,F,M,L,I
HLA-B*53:01		P					W,F,L
H2-K^b			Y	F (Y)		L (M,I,V)	*prefers 8-mers
H2-D^b				N			M(I,L)
H2-K^d		Y(F)					I(L,V)
H2-D^d		G	P	(R)			I(L,F)
H2-L^d		P,S					F,L,M
H2-K^k		E				I(V)	*prefers 8-mers
H2-D^k	Motif uncertain	(R)					
Qa-2		(M,L,Q)	(N,I,L)	(V,I)	(K,M,I)	H	L,I,F
H2-M3	formyl-M						
RT1.A1		(A,S,V)	F,Y				Y,F,L,M

[a]Peptide-binding motifs for the indicated MHC class I molecules are shown in the single amino acid code. Position refers to the amino acid position of the peptide from the amino terminus. Only the most common residues are shown. Assignments in parenthesis are less common than the others. These motifs are taken from the more extensive summary of www.syfpeithi.de/, www.imtech.res.in/raghava/mhcbn/links.html, and Biddison and Martin.562 Full sequences of proteins may be queried for MHC peptides presented by particular alleles with http://bimas.dcrt.nih.gov/molbio/hla_bind/, www.immuneepitope.org/, or www.mpiib-berlin.mpg.de/MAPPP/expertquery.html.

HLA, human leukocyte antigen; MHC, major histocompatibility complex.

The identification of MHC-II-bound self-peptide-or antigenic peptides by biochemical methods similar to those employed for MHC-I molecules has been proved more difficult because the MHC-II molecules do not have the rigorous requirements for a defined amino terminus or restricted length. Whereas MHC-I molecules bind peptides with a particular motif residue at a specific position as defined by the amino terminus, resulting in the ability to identify the dominant residue at a particular step in the Edman degradation even amidst a pool of peptides, MHC-II molecules bind peptides with "ragged ends," and little information is obtained from the standard methods of sequencing of pools of peptides.[398,399,400,401] Thus, more precise fractionation of the peptides released from purified MHC-II molecules is required. Identification of MHC-II/peptide-binding motifs by bacteriophage display is also possible.[402] In accord with the view that MHC-II molecules present peptides derived from an "outside in" pathway, many of the peptides that copurify with MHC-II molecules represent molecules derived from the extracellular milieu or the medium in which the cells were grown. Analysis of MHC-II/peptide complexes with cloned T cells and monoclonal antibodies with MHC/peptide specificity reveals that, in part because of the ability of MHC-II molecules to accommodate peptides with extensions at their amino and carboxyl-termini, and in part because of the smaller role that anchor residues seem to play in peptide binding by MHC-II molecules, occasionally even a unique peptide can bind a particular MHC-II molecule in more than one frame.[403,404,405,406] As a result of structural studies (summarized in the following) and compilation of sequences of those peptides bound by particular MHC-II molecules, the general conclusion is that MHC-II molecules all have binding pockets identifiable for the particular allelic product. For some alleles, such as HLA-DR1 and IA^d, these pockets are spaced at position P1, P4, P6, and P9 (or i, i + 3, i + 5, i + 9). (Some analyses, borne out in part by structural studies, suggest that a pocket at P7 [i + 6] may also contribute to the energy of binding.) Because of the complexity and potential degeneracy of peptide motifs for MHC-II molecules, more elaborate schemes have been devised for predicting those peptides that may bind to particular MHC-II molecules.[407] A summary of identified peptide-binding motifs for MHC-II molecules is in Table 21.10, but for some alleles, the analysis of MHC-II binding peptides is not straightforward and demands sophisticated algorithms for identifying potential antigenic peptides from new proteins. The site www.imtech.res.in/raghava/mhcbn/links.html tabulates a number of links to various servers for predicting likely peptide epitopes for particular MHC-I and MHC-II molecules. Implicit in the description of MHC-I and MHC-II peptide motifs is that the bound peptides bind to the MHC molecule only in an amino to carboxyl-terminal orientation, that is, that all peptides

bound to MHC molecules bind with the amino terminus at the lefthand side of the peptide-binding groove, in the canonical structural representation that will be described in further detail subsequently. However, a recent analysis employing both x-ray crystallography and nuclear magnetic resonance indicates that the CLIP peptide may undergo orientational inversion, binding dynamically in the carboxyl to amino as well as the amino to carboxyl direction.[408]

TABLE 21.10 Peptide-Binding Motifs for Some MHC Class II Molecules[a]

Position	i (P1)	i + 1	i + 2	i + 3 (P4)	i + 4	i + 5 (P6)	i + 6	i + 7	i + 8 (P9)
Allele									
DRB1*01:01	YVLFIAMW			LAIVMNQ		AGSTCP			LAIVNFY
DRB1*03:01	LIFMV			D		KREQN			YLF
DRB1*04:01	FYWILVM			PWILVADE		STQHR	DEHKNQR..		DEHKNQR..
DRB1*04:05	FYWVILM			VILMDE		NSTQKD			DEQ
DQA1*05:01/B1*03:01	WYAVM			A		AIVTS			QN

H2-IA^b (undefined)				
H2-IA^d	STYEVWMLI..	VLIA	AV	
H2-IA^{g7}	KHSAV	L	VA	DSE
H2-IA^k	DN	IVLN	EQ	
H2-IA^s (undefined)				
H2-IE^b	WFYILV	LIFSA	QNASTHRE	KR
H2-IE^d	WFYILV	KRIV	ILVG	KR
H2-IE^{g7}	ILVFWM	DESMV	QNASTED	RKMF

[a]Major histocompatibility complex class II peptide-binding motifs are summarized from those of Biddison and Martin562 and www.syfpeithi.de/home.htm.

High-Resolution Structures

MHC-I Molecules

A most graphic description of the relationship of form to function of the MHC molecules was made by Bjorkman et al.[409,410] who determined the three-dimensional structure of the human MHC-I molecule, HLA-A*02:01, by x-ray crystallography. For these studies, the extracellular, soluble portion of the type I membrane-associated molecule was purified by papain cleavage from the surface of tissue culture cells. At the time, there was not a clear appreciation of the role of peptide either in the assembly of the molecule or of the nature of the recognition of the MHC molecule by TCRs or, for that matter, NK receptors. Despite the fact that the first purified HLA-A2 molecules possessed a heterogeneous mixture of bound peptides, protein from these preparations crystallized readily, and electron density maps calculated from the diffraction data were interpretable, allowing modelling of the backbone molecular structure. The most important insight in the interpretation of the derived electron density map was that part of the electron density, and thus part of the structure, was due to this mixture of peptides bound tightly by the molecule, and that this density could not be modeled based on the known amino acid sequence of HLA-A2.

This first MHC-I structure clarified several important aspects of the mechanism by which the MHC-I molecule carries out its peptide binding function. The amino terminal domains (α1 and α2) form a unitary binding site for peptide. This domain unit (also called a “superdomain”) consists of a floor of eight strands of antiparallel β-pleated sheet that supports two α-helices, one contributed from the α1 domain and one from the α2 domain, aligned in an antiparallel orientation. The membrane proximal α3 domain has an Ig C-type fold and pairs asymmetrically with the other Ig domain of the molecule contributed by β2m. The nature of recognition by T-cells was suggested by comparing the location of those amino acid residues that had been characterized as being strong elements in T-cell recognition, residues that distinguished closely related allelic gene products, and amino acid residues that had been identified as those that were responsible for the transplant rejection of the mutants of the *H2-K^b* series.[409] Amino acid residues of the MHC-I molecule responsible for T-cell recognition were clearly classified into one of two categories or an overlapping set: those residues that were “on the top of the molecule,” exposed to solvent and available for direct interaction with the TCR, and those residues whose side chains pointed into the peptide binding groove and might be considered crucial in the peptide-binding specificity of the particular MHC molecule. The original publications, based on a structure determined to a resolution of 3.5 Å, focused mainly on the general structural outline of the molecule. More recently, structures of a wide variety of MHC-I molecules of different species including human, mouse, rat, and chicken, complexed with specific peptides, have been determined. Ribbon diagrams of HLA-A2 as

seen from the side (Fig. 21.6A) and from the top (Fig. 21.6B), complexed with a unique synthetic peptide, indicate the design of the entire molecule: the peptide-binding site is supported by the β-sheet floor, and the floor in turn is supported by the two Ig-like domains, the α3 domain of the heavy chain and the β2m light chain. (In the canonical view of the MHC-I, HLA-A2 molecule shown in Fig. 21.6B through D, the amino terminal residue of the bound peptide lies to the left, and the carboxyl terminal residue lies to the right.)

The comparison of this structure with that of the closely related human MHC-I molecule, HLA-A*68:01 (see Table 21.2), suggested that surface depressions in the groove of the MHC-I molecule, now designated pockets A through F, would be available for interactions with some of the side chains of the bound peptide.[411,412] These six pockets are illustrated in Fig. 21.6C and with bound peptide in Fig. 21.6D. Concomitant with the determination of the x-ray structure of the human MHC-I molecule, HLA-B*27:05,[413,414] the motif of the peptides that were recovered from this molecule was determined, permitting the more precise modeling of the bound peptide in the cleft of the MHC-I. For HLA-B27, this was of particular interest because the bound peptides had a strong overrepresentation of arginine at position 2,

and scrutiny of the HLA-B27 structure suggested that the amino acid residues lining the B pocket, particularly glutamic acid at position 45 as well as cysteine 67, were complementary to the long, positively charged arginine side chain of the peptide amino acid at that position. Structural studies supported a view of MHC-I/peptide binding in which the side chain of the carboxyl-terminal residue of the bound peptide sits deep in the F pocket. In addition, the amino terminal amino group of the peptide forms hydrogen bonds with the hydroxyl groups of conserved amino acids tyrosine 59 and tyrosine 171 that line the A pocket. A hydrogen bond from the amino group of conserved tryptophan 147 to the backbone carbonyl oxygen of the penultimate peptide amino acid (usually position 8) also seems important, as do charge interactions and hydrogen bonds of the free carboxyl group at the carboxyl terminus of the peptide with tyrosine 84, threonine 143, and lysine 146.

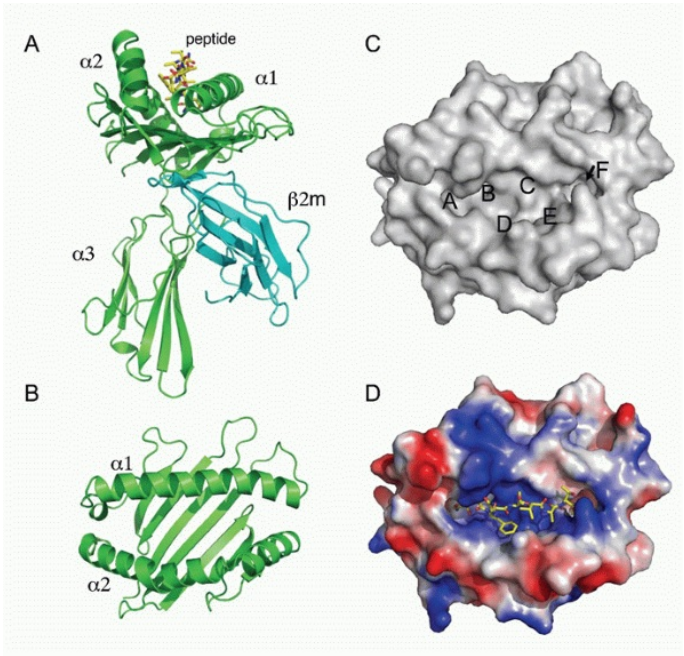

FIG. 21.6. Structure of HLA-A*02:01. A: Ribbon representation of HLA-A2 heavy chain *(green)*, β2m light chain *(cyan)*, and bound peptide *(yellow)*. **B:** Ribbon representation of the peptide-binding groove. **C:** Surface representation of the binding groove with pockets labeled. **D:** Surface representation of binding groove colored by electrostatic charge (*red*, acidic; *blue*, basic) with peptide shown in stick illustration. Figure generated from protein data bank[559] structure 2BSV using PyMOL.[560]

Other structures of MHC-I molecules were determined involving complexes produced with homogeneous peptide, assembled either in vitro from bacterially expressed proteins with

synthetic peptide[415] or exploiting MHC proteins expressed in insect cells.[416,417] The structures determined with homogeneous peptide confirmed the impression obtained from the structures obtained from molecules with heterogeneous self-peptides. Examination of the H2-K^b molecule complexed with synthetic, known antigenic peptides derived either from Sendai virus, vesicular stomatitis virus, or chicken ovalbumin revealed that the same MHC molecule can bind peptides of different sequence, length, and structure by virtue of their conserved motif residue side chains. Although small conformational changes of the MHC are detectable on binding the different peptides, the main distinction in the recognition of different peptides bound by the same MHC molecule is due to the location, context, size, and charge of amino acid side chains that are anchored in the MHC pockets. Conversely, solvent-exposed side chains contribute to the unique structure of the surface of the molecule available for interaction with TCR and other receptors.

The most consistent rule learned from the first x-ray structures and complemented by peptide recovery and early binding studies was that the carboxyl-terminal amino acid side chain of the MHC-I-bound peptide was embedded in the F pocket. A recent study revealing that the MHC-I molecule H2-D^b binds a pentapeptide well anchored in the F pocket, but lacking amino terminal interactions, is consistent with this view.[418] However, other studies indicate that MHC-I molecules may bind longer peptides that extend beyond the residue anchored in the F pocket,[419] an observation confirmed by a crystallographic structure.[420] More recently, the lack of an absolute requirement for a free C-terminal amino acid has been exploited in the engineering of single chain molecules in which peptide, β2m, and the MHC-I heavy chain are covalently linked. These molecules have unusual thermal stability and are effective MHC/peptide immunogens.[421] An additional variation on the theme of MHC-I binding peptides based on particular anchor residues includes the demonstration that glycopeptides, bound to MHC-I via amino acid side chains and termini, can expose their carbohydrate moieties to solvent and be available for TCR interaction.[422,423,424] An example of a 13-residue peptide bound to its MHC-I-presenting element, the rat MHC-I molecule, RT1-Aa, was crystallized and shown to produce a peptide/MHC complex with a large central bulge. Two different complexes consisting of the same MHC and peptide reveal significantly different conformations in this central bulge region.[425] "Bulged" viral peptides have also been characterized in complex with human MHC-I structures of HLA-B35 allotypes complexed with 13-mer and 11-mer peptides.[426,427] TCR recognition of such bulged peptides can involve conformational adjustments of the TCR in recognizing a fairly rigid peptide[426] or "crumplings" of the bound peptide by a largely rigid face of the TCR upon binding the MHC-I/peptide complex.[428] Another set of modified peptides that can be bound by MHC-I molecules and presented to TCR includes self-peptides modified by phosphorylation.[429,430] Although the fundamental topology of MHC-I structure and its interactions with antigenic peptide have been solved by the classic structures mentioned previously, many more MHC-I/peptide complexes have been studied crystallographically in order to define the details of particular interactions with antigenic peptides. A number of structures at a resolution of 1.40 Å or better have been deposited in the protein database. Structure determination of representative single-chain peptide-β2m-MHC-I,[431] and of MHC-I molecules containing photolabile peptides that can be exchanged with other peptides easily,[432] reinforce our basic understanding of the structure of MHC-I/peptide complexes.

MHC-II Molecules

Before any MHC-II structure had been determined experimentally, a model was constructed based on the alignment of amino acid sequences and the available MHC-I three-dimensional structure.[433] This model made several valid predictions that were borne out by the subsequent x-ray structure determination of HLA-DR1.[434] MHC-II clearly showed similarity to MHC-I and formed its binding groove by the juxtaposition of the α1 and β1 domains. The position of the electron density representing the heterogeneous peptide that copurified with the HLA-DR1 was identified. Figure 21.7A and C show a ribbon diagram of HLA-DR1 with a homogeneous bound peptide. In comparison to the MHC-I structure (see Fig. 21.6), the peptides bound to the MHC-II molecule extend through the binding groove rather than being anchored at both ends.

A comparison of the α-carbon backbone of the peptide-binding region of an MHC-I structure with that of an MHC-II structure is shown in Figure 21.8. The structures are very similar

although the binding domain is built of the α1 and α2 domains from the same chain (for MHC-I) or of the α1 and β1 domains that derive from two chains (for MHC-II). The location of polymorphic residues can be determined by variability plots based on multiple sequence alignments, as originally suggested by Wu and Kabat.[435] The current bioinformatics approach to variability prefers to calculate *Shannon entropy*, a function that describes the degree of unpredictability of an event.[436,437] For protein sequence

alignments, Shannon entropy provides a sensitive measure of the diversity of particular amino acid positions.[438] Several online tools are available for implementing and displaying the results of such calculations (http://bio.dfci.harvard.edu/Tools/svs_help.html; http://imed.med.ucm.es/Tools/svs_help.html; and http://consurfdb.tau.ac.il/). Comparative ribbon diagrams (Fig. 21.9), in which the location of the amino acid residues that are polymorphic for the human MHC-I and MHC-II chains are indicated with a color map, show that the bulk of MHC polymorphism derives from amino acid variability in regions that line the peptidebinding groove. This suggests that MHC polymorphism is required to allow the MHC molecules, and as a result, the organism and its species, to respond to a changing antigenic environment.

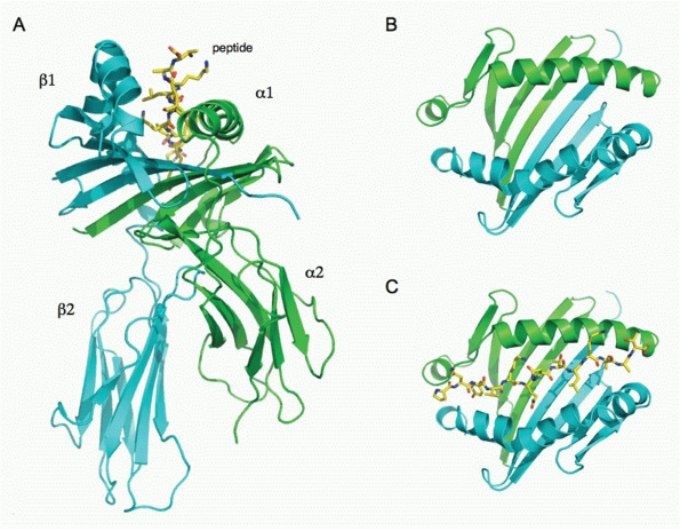

FIG. 21.7. Color Ribbon Representation of HLA-DR1. A: Side view. α chain is in *green;* β chain is in *blue;* peptide in stick representation in *yellow*. **B:** Top view. **C:** Top view with bound peptide (PKYVKQNTLKLAT) visualized. The illustration was made with PyMol[560] based on the protein data bank[559] coordinates of 1DLH.

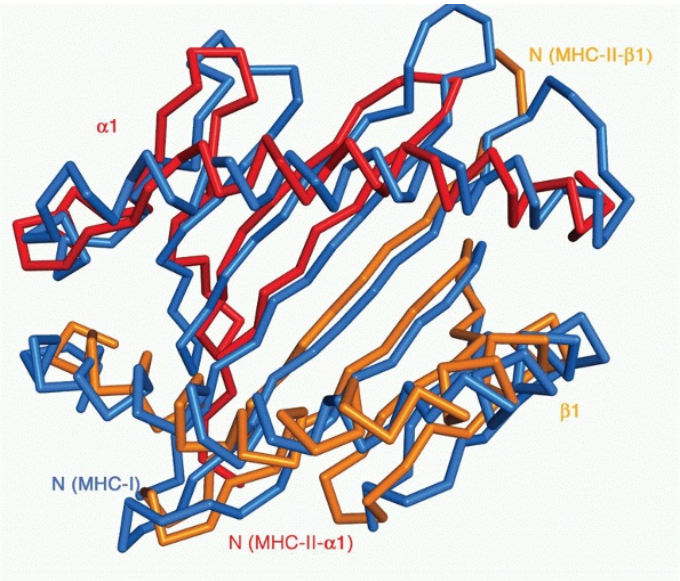

FIG. 21.8. Comparison of α-Carbon Backbone of MHC-I and MHC-II molecules. The α1α2 domains of MHC-I (human leukocyte antgien [HLA]-A2, PDB 3HLA) and the α1 and β1 domans of MHC-II (HLA-DR1, PDB 1DLH) were superposed with PyMOL[560] and displayed as a Cα carbon representation. MHC-I is *blue*, the α1 domain of MHC-II is *red*, and the β1 domain of MHC-II is *orange*.

As with MHC-I, a further understanding of the details of the interactions of peptides with the MHC-II molecule came from crystallographic studies of molecules prepared with

homogeneous peptide—in the first case HLA-DR1 complexed with an antigenic peptide derived from the hemagglutinin of influenza virus.[439] Based on this structure, a set of pockets was initially designated, numbered for the peptide position that is bound. For the influenza peptide studied in this example, the major interactions were from peptide positions 1, 4, 6, 7, and 9 that are indicated in Figure 21.10. The deep P1 pocket accommodates the tyrosine (the third position of the peptide PK**Y**VK**Q**N**T**L**K**L**A**T) and the pockets indicated by 4, 6, 7, and 9 fit the Q, T, L, and L residues, respectively. MHC-II exploits a similar mode of binding as compared with MHC-I, but there are key differences. MHC-II lacks the requirement for free amino and carboxyl termini of the peptide, the peptide conformation is relatively extended (like that of a type II polyproline helix), and the MHC-II forms number of hydrogen bonds between conserved amino acids that line the binding cleft and the main chain (ie, amino nitrogen and carbonyl oxygen) atoms of the peptide. Although the pocket designations refer specifically to the features of the MHC-II molecule surface, occasionally the cognate peptide positions that reside in such pockets are given the same names.

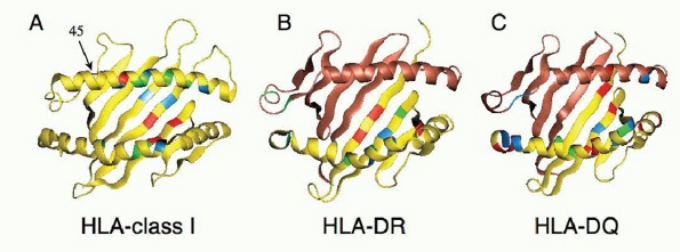

FIG. 21.9. Location of Polymorphic Amino Acid Residues in MHC-I and MHC-II molecules. Variability plots were calculated as described by Kabat and Wu[435] and level of variability illustrated on ribbon diagrams (generated in QUANTA 2000 [Accelrys] of 3HLA[412] [HLA-class I], 1DLH[439] [HLA-DR], and 1JK8 [HLA-DQ]) where greatest variability is *red*, intermediate is *green*, and least is *blue*.

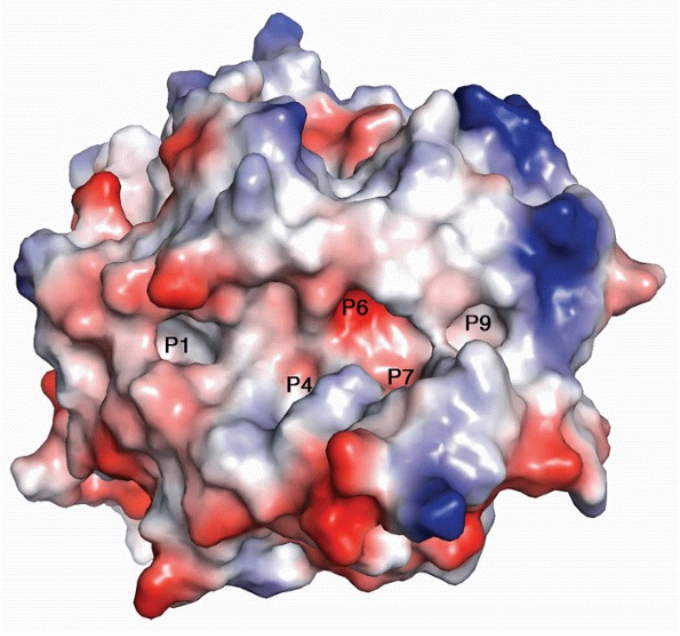

FIG. 21.10. Location of Pockets in HLA-DR1 Based on the Cocrystal of HLA-DR1 with a Peptide Derived from the Influenza Hemagglutinin. An electrostatic surface representation of HLA-DR1 (1DLH) generated in PyMOL[560] is shown with the positions of pockets P1, P4, P6, P7, and P9 indicated.

Among the most provocative observations from the first MHC-II structures was that the molecule was visualized as a dimer of dimers, and this moved a number of investigators to consider the possibility that activation of the T cell via its receptor might require the dimerization or multimerization of the TCR, an event thought to be dependent on the propensity of the MHC/peptide complex to self-dimerize. The simple elegance of this dimer of dimers is illustrated in Figure 21.11. Several arguments support the dimerization hypothesis: the finding of a dimer of dimers in the crystals of HLA-DR that formed in several different space groups,[434,439] the observation that a TCR Vα domain formed tight dimers and in its crystals formed dimers of the dimers,[440] the demonstration of the ability to immunoprecipitate MHC-II dimers from B cells,[441] the apparent requirement for purified MHC-I dimers for stimulation of a T cell in an in vitro system,[442] and the finding that MHC-II/peptide/TCR complexes could form higher order multimers in solution as detected by quasielastic light scattering.[443] However, a number of strong counterarguments draw this hypothesis into question. Many MHC-II molecules other than HLA-DR1 that have been crystallized do not seem to form the same kind of

dimer of dimers in their crystals.[444,445] Modes of MHC-II dimerization with distinct topology that would preclude simultaneous interaction with multiple TCRs on the same T cell have been observed. None of the MHC-I molecules that have been examined by x-ray crystallography show dimers in the same orientation as the MHC-II ones reported. A different Vα domain fails to dimerize even at high concentration.[446] Many reported x-ray structures of MHC/peptide/TCR complexes[447,448,449,450] fail to show dimerization. Despite the simple elegance of the dimer hypothesis, it is clear that additional experimentation will be required to understand the topologic requirements for T-cell activation through the αβ TCR.

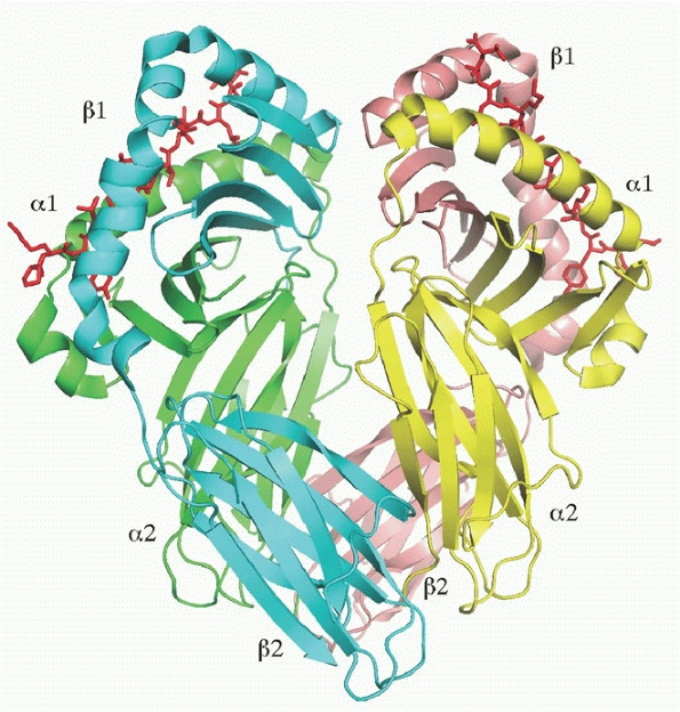

FIG. 21.11. Ribbon Diagram of the Structure of HLA-DR1 showing the dimer of dimers and the individual domains of the protein. α chains are in *green* and *yellow;* β chains are in *blue* and *salmon*. Peptide in stick representation is in *red*. The illustration was generated from PDB 1DLH with PyMOL.

Recent additions to the library of MHC-II structures include the I-A^{97} molecule, a unique MHC-II that provides one link in the susceptibility to insulin-dependent diabetes in the mouse model.[451,452] Although the structure fails to provide direct evidence to explain the linkage to diabetes, it suggests that the novel repertoire of peptides bound by this MHC-II molecule reveals unique features of a wider peptide binding groove and resulting relatively low-affinity interaction with peptide. Until recently, all of the MHC-II/peptide structures revealed bound peptide in a canonical left to right, amino to carboxyl orientation. A detailed analysis of HLA-DR1 molecules bound to the CLIP 106 to 120 peptide revealed crystals in two different forms, which on solution and refinement indicated that this peptide could interact in either of two distinct orientations (Fig. 21.12).[408] This exception to the general rule of left to right, amino to carboxyl orientation, may reflect the unique role that the CLIP peptide must play in protecting the MHC-II-binding site while simultaneously being available for DM-mediated peptide exchange in the endosome.

MHC-Ib Molecules

H2-M3

To this point, our description of MHC-I molecules has focused on the classical MHC-Ia molecules, represented by HLA-A, HLA-B, and HLA-C in the human, and by H2-K, H2-D, and H2-L in the mouse. Several MHC-Ib molecules for which three-dimensional structures have been determined are of particular interest, the CD1 molecules,[453,454] H2-M3,[137,455] MICA,[456,457] FcRn,[458] and Rae-1.[459] Other molecules that are thought to show structural relatedness to the MHC-I fold but whose x-ray structures have not yet been reported include the stress-induced molecules H-60[173] and MULT1,[460] and the mucosally expressed MR1 molecule that selects invariant T cells in the gut.[461] H2-M3 is of particular note because of its ability to bind and present peptide antigens that contain amino terminal N-formyl groups. H2-M3 was originally identified as the MHC-Ib molecule that presents an endogenous peptide derived from the mitochondrially encoded protein ND1 known as maternally transmitted factor.[137,462] Thus, it was of interest to understand in structural terms how this molecule binds such N-formylated peptides.[455,463] The crystal structure of H2-M3 complexed with an

N-formylated nonamer peptide, fMYFINILTL, revealed that the structure of the A pocket, highly conserved among MHC-Ia molecules, which have tyrosine 7, tyrosine 59, tyrosine 159, tryptophan 167, and tyrosine 171, is quite different so that it can accommodate the N-formyl group in the A pocket. In particular, H2-M3 has an A pocket reduced in size and lined by hydrophobic residues, leucine at 167 and phenylalanine at 171, and leucine 16. These structural features cause the amino terminal nitrogen of the formylated peptide to be positioned where the peptide position 2 amino nitrogen would lie in a MHC-Ia molecule. Thus, the unique peptide selectivity of H2-M3 is explained in structural terms.

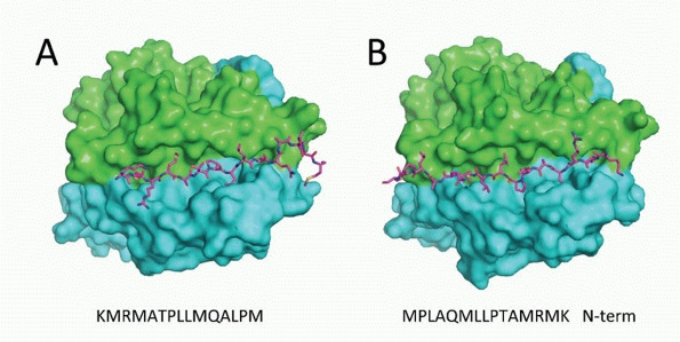

FIG. 21.12. MHC-II Binds Class II-Associated Invariant Chain Peptide in Either N to C or C to N Orientation. The structures of HLA-DR1 bound to the CLIP 106 to 120 peptide in either the N to C **(A)** or C to N **(B)** orientation is illustrated. α1 domain is in *green*; β1 domain is in *cyan*. Peptide sequences are shown at the bottom.

CD1

Another MHC-Ib molecule of great interest is CD1, representative of a class of MHC-I molecules that map outside of the MHC, that have limited tissue-specific expression, and that are capable of interaction with both αβ and γδ T cells.[464] In the human, there are two clearly distinct groups of CD1 molecules: one consisting of CD1a, CD1b, CD1c, and CD1e; and another of CD1d alone.[465] In the mouse and rat, only CD1d is expressed.[466] As a group, these are β2m-associated chains that bind hydrophobic antigens, primarily glycolipids, with the lipid moiety embedded in the CD1 heavy chain and the carbohydrate portion exposed to solvent. CD1a, CD1b, and CD1c are capable of binding and presenting various nonpeptidic mycobacterial cell wall components such as mycolic acid containing lipids and lipoarabinomannan lipoglycans.[467,468] NK T cells, a subset of αβ-TCR-bearing T cells, of independent lineage and defined by the expression of the NK1.1 marker, are restricted to CD1 recognition.[469] The crystal structures of both mouse and human CD1d1 have been determined,[453,470] revealing classic MHC-I structures with the basic protein fold and β2m association quite similar to that of the MHC-Ia molecules. Consistent with its apparent biologic function of binding hydrophobic lipid-containing molecules, its binding groove is somewhat narrower and deeper than that of either MHC-Ia molecules or MHC-II molecules. The backbone configuration of the α1α2 domain structure of CD1 is shown in Figure 21.13, where it is compared to the homologous region of H2-K^b, HLA-DR1, and another MHC-Ib molecule, a neonatal Fc receptor (FcRn). The structure of a number of CD1-glycolipid complexes

have been determined, and human and mouse CD1 molecules bound to different glycolipids have been compared.[466] The depth of the groove of CD1 results from the merging of pockets to form what have been termed the *A′ and F′ pockets* in place of the MHC-Ia A through F pockets. This A′ pocket is about the size of the binding site of a nonspecific lipid binding protein.

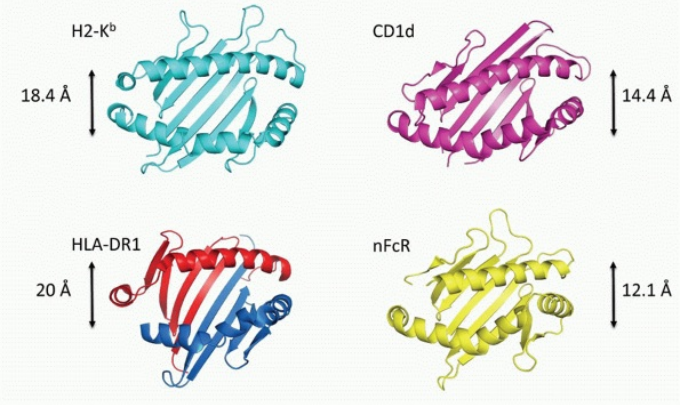

FIG. 21.13. Comparison of the Ribbon Representations and Size of the Potential Peptide-Binding Cleft. MHC-I molecule H2-K^b (2vaa), MHC-II HLA-DR1 (1DLH), and MHC-Ib molecules CD1d (1Z5L) and FcRn (1EXU) are compared. Approximate maximal distances separating the two helices are indicated.

In an effort to understand more precisely how CD1 molecules bind lipid antigens, Gadola et al.[454] crystallized human CD1b complexed with either phosphatidylinositol or ganglioside GM2 and determined their x-ray structures. The structures were essentially identical for the CD1b heavy chain and β2m in the two complexes, and revealed a network of four hydrophobic channels at the core of the α1α2 domain, which accommodate four hydrocarbon chains of length from 11 to 22 carbon atoms. These channels are called A', C', and F' for the three analagous to the A, C, and F pockets of the MHC-Ia molecules, and a fourth, termed *T'* which is a distinct tunnel. Illustrations of the binding groove with the bound alkyl chains is shown in Figure 21.14, which shows views of the binding pockets of CD1a, b, and d with different ligands. These structures illustrate how the

binding site of a classical MHC-I molecule may have evolved from (or to) the binding site of molecule like CD1 to provide antigen selectivity for a distinct set of molecules that would be common to a set of important mycobacterial pathogens. Complexes of CD1 with their cognate NK TCR have been reported and will be discussed subsequently.[471,472,473,474]

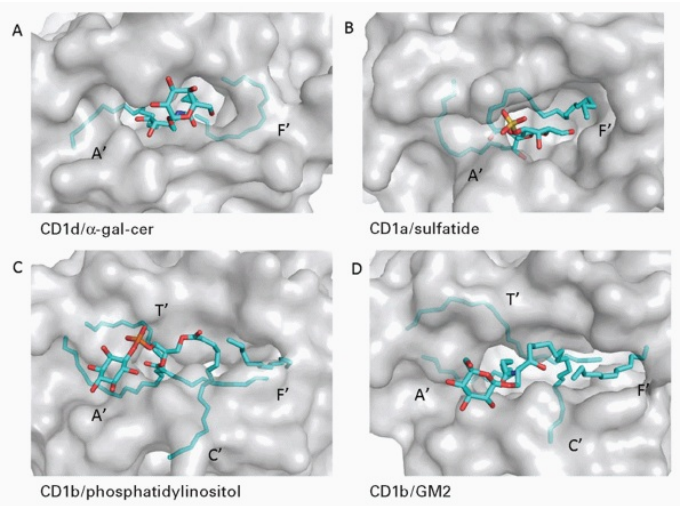

FIG. 21.14. Tunnels and Pockets of CD1 Molecules. Structures of **(A)** CD1d with α-galactosyl ceramide (1Z5L), **(B)** CD1a with sulfatide (1ONQ), **(C)** CD1b with phosphatidylinositol (1GZQ), and **(D)** CD1b with ganglioside GM2 are shown, with pockets A', C', and F' indicated and tunnel T'.

Neonatal Fc Receptor

Another example of an MHC-Ib molecule, noteworthy because it exemplifies a novel function of MHC molecules, is the FcRn. Originally described in the rat as a molecule of the intestinal epithelium that is involved in the transport of colostral Ig from the lumen to the bloodstream,[475,476] homologues in the mouse and human have also been described,[477,478,479] and the structure of the rat molecule has been determined crystallographically[458] (Fig. 21.15). As suggested by the amino acid sequence similarity of the FcRn to MHC-I proteins, the three-dimensional structure revealed considerable similarity to MHC-Ia molecules.[458] Specifically, α1 and α2 domains have similar topology to the MHC-I molecule, although, as discussed previously, what would be the peptide-binding groove in the MHC-Ia molecules is closed tightly and lacks space sufficient for a ligand. The most provocative feature of the structure of the FcRn/Fc complex is that the MHC-I-like FcRn interacts with the Fc through contacts from the α2 and β2m domains to interact with the Fc $C_\gamma2$-$C_\gamma3$ interface. As compared to the structure of the unliganded Fc, the complex reveals both conformational changes in the Fc and the presence of several titratable groups in the interface that must play a role in the pH-dependent binding and release of Ig molecules from the FcRn. The FcRn has taken the MHC-I fold and diverted its function for an interaction with the Fc of the Ig. Amino acids at what would classically be considered the "righthand side" of the peptide-binding groove make contact with the Fc interface that lies between $C_\gamma2$ and $C_\gamma3$ domains. The FcRn serves as an excellent example of similar structures in the immune system being diverted for alternate purpose. The importance of the FcRn has been underscored by observations of differences in the serum half-life of Ig in animals that, as a result of an induced deletion of β2m, lack the normal expression FcRn as well and seem to metabolize serum Ig aberrantly.[480]

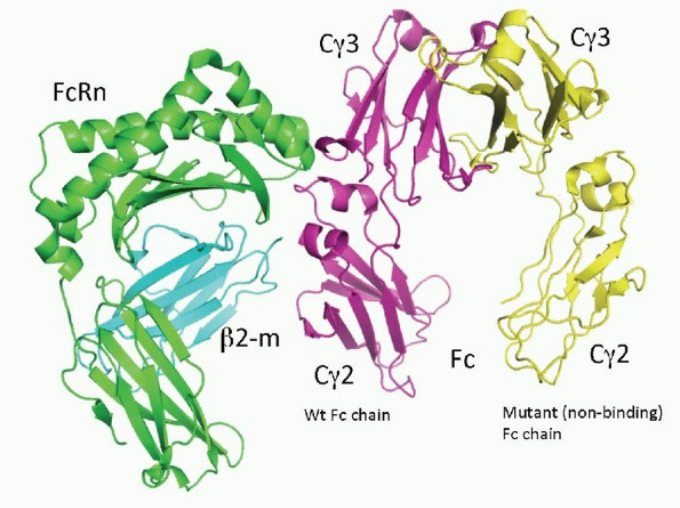

FIG. 21.15. Structure of the Rat Neonatal Fc Receptor Complexed with Fc. Ribbon diagram of rat neonatal Fc receptor complexes with the Fc heterodimer consisting of the wild-type and nonbinding chains. This is from PDB 1I1A.

MILL MHC-Like Molecules

A novel set of MHC-Ib-like genes designated *Mill* (*M*HC class *I*-*l*ike located near the *l*eukocyte receptor complex) has recently been identified in both mice and rats.[481,482,483] These encode MICA/B-like glycophosphatidylinsol-linked cell surface molecules that are associated with β2m and do not require TAP for cell surface expression. Their function remains unclear, although further examination indicates the presence of two family members, Mill1 and Mill2, observed on cycling thymocytes, proliferating smooth muscle cells and fibroblasts.[484]

M10 Proteins

The robust MHC fold seems to possess both the stability and flexibility requisite for the molecular function of binding ligands such as peptides, glycopeptides, phosphopeptides, and glycolipids as well as for subsequent interactions with macromolecular receptors such as NK

receptors and TCRs. Likely, these qualities have allowed its adaptation for distinct function related to a class of MHC-Ib molecules, the M1 and M10 families, some members of which are associated with the V2R mouse pheromone receptors. The structure determination of M10.5 revealed a β2m-associated MHC-I-like molecule with clear three-dimensional similarity to the MHC-I molecule H2-D^d.[485]

MHC-Iv Molecules

Because viruses are constantly engaged in a struggle for survival in their interplay with the immune system of their hosts, there is little surprise to note that pathogenic eukaryotic viruses can evolve numerous approaches to evade the immune response. MHC and structurally related molecules are produced not only by vertebrates but also by CMVs, large DNA viruses of the β-herpesvirus family, which have coevolved with their vertebrate hosts over millennia and are exquisitely adapted to persist in the face of host immune responses. 2 Viral MHC-I-like molecules have been identified bioinformatically from amino acid sequences of the open reading frames predicted from genome sequence data. A number of putative MHC-Iv molecules, including UL18 and UL142 of the human CMV,[327,486] members of the "m145 family" of the mouse CMV1[487,488] the 2L molecule of tanapox,[489] and the U21 molecule of human herpesvirus 7,[490] may modulate the immune response of the host. In some cases, such as the UL18 protein of human CMV and the m144 protein of mouse CMV, the sequence homology to bonafide MHC-I molecules is sufficiently strong to unambiguously identify them as MHC-I like. However, in most cases, such as with the m145 family of mouse CMV, the sequence homology to MHC-I is very weak, and relatedness to MHC-I was originally based on structure prediction algorithms alone. We term these virally encoded MHC-I-like molecules MHC-Iv

to reflect their distinct evolutionary history, their structural deviation from typical MHC-I molecules, and their role in evading host immune responses. A survey of DNA sequences of 11 genes obtained from 26 wild murine CMV isolates and laboratory strains indicated that several of the *MHC-Iv* genes (m144, m145, and m155) revealed significant sequence variation, consistent with the view that this variation may offer some immune-evasive benefit to the virus.[491]

Structural characterization of MHC-Iv molecules begins with an evaluation of the requirement for β2m and/or peptide for stable expression. The UL18 is associated with both β2m and peptide, whereas m144 may be associated only with β2m but does not require β2m for cell surface expression. The structure of m144 has been reported,[487] revealing the preservation of all structural elements of the MHC-I fold (Fig. 21.16). The cleft of m144 does not seem capable of binding peptides as the groove is narrow and critical tyrosine residues are not conserved, results consistent with earlier biochemical studies.[492] A unique disulfide anchors the α1 helix to the β sheet. The structure of m157, a CMV MHC-I-like immunoevasin that binds the Ly49H activation receptor of C57BL/6 mice and the Ly49I inhibitory receptor of 129/J mice, has been reported.[493] This β2m-independent, peptide-free MHC-Iv molecule reveals the major features of the MHC-I fold with some unique aspects, in particular a novel amino terminal α helix. How it binds its Ly49 ligands remains unclear. The m153 protein of mouse CMV, also a member of the m145 family, reveals novel adaptations of the MHC-I fold.[1] The m153 protein does not require β2m or peptide and, in contrast to other MHC-I molecules, is a noncovalent dimer. The monomers are associated in a head-to-tail fashion. An extended N-terminus contains a unique disulfide that anchors it to the α3 domain. The m153 structure hints tantalizingly of more surprises to come as structures of other MHC-Iv molecules become available. Other MHC-Iv proteins whose structure have been determined include the 2L tanapox virus protein that binds the inflammatory cytokine TNF-α[489] and the mouse CMV-encoded m152, which sequesters both classical MHC-I and the stressinduced NKG2D ligand, RAE-1, from the cell surface.[494]

In those cases where functions have been elucidated, MHC-1v molecules have been shown to act as immunoevasins that inhibit the NK cell to enable virus survival and persistence in the host. Both UL18 and UL142 inhibit human NK function, the former by binding to the inhibitory receptor LIR-1. The mode of action of UL142 remains to be determined.[327] The m157 binds to the inhibitory NK receptor Ly49I in mouse strains susceptible to viral infection but remarkably serves as a target for the activating NK receptor Ly49H expressed in resistant

strains. The m144 protein has also been shown to inhibit NK activation,[495,496] but the ligand remains to be identified.

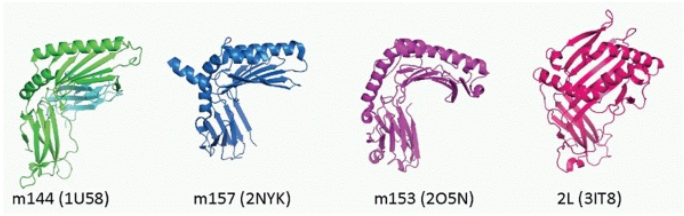

FIG. 21.16. Comparison of MHC-Iv Molecules. Structures of MHC-Iv chains of m144 (1U58), m153 (2O5N), m157 (2NYK), and the 2L protein of tanapox virus (3IT8) were superposed and are illustrated as ribbon diagrams.

Complexes of MHC Molecules with Ligands

The most recent and exciting developments in MHC biology include the detailed description of the interactions of MHC-I and MHC-II molecules with their receptors and coreceptors on cells of the innate and adaptive immune system. Our structure/function survey will be completed by brief descriptions of the interactions of MHC-I and MHC-II molecules with αβ TCRs, with the T-cell coreceptors CD8 and CD4, and of MHC-I molecules with NK receptors. Details of the interactions of a number of CD1 molecules with their glycolipid antigens and specific NK T-cell receptors shed light on the biology and evolution of these molecules. A brief description of interactions of MHC-II molecules with superantigen will follow. Each of these structural studies complements a host of biologic experiments that have led to an appreciation of the importance of understanding the structural basis of these immune reactions.

MHC/T-Cell Receptor Interactions

Second to the initial visualization of the structure of the MHC-I molecule HLA-A2 by crystallographic analysis, the images of TCR/MHC/peptide complexes initially reported some 15 years ago clarified the phenomenologic details of T-cell recognition, antigen specificity, and MHC restriction. The initial structures were determined in several systems,[447,448,449,497,498] and there remains ongoing interest in the details of particular TCR/MHC interactions. The first examples were of MHC-I-restricted TCR, one from the mouse[448] and one from the human.[447] The mouse MHC-I molecule H2-K^b was analyzed in complex with a self-peptide, dEV8, and a TCR known as 2C,[448,499] and the human

HLA-A2 complexed with the Tax peptide was studied with its cognate TCR derived from a cytolytic cell known as A6.[447] These structures offered a consistent first glimpse at the orientation of the TCR on the MHC/peptide complex, but additional structures, including that involving a murine MHC-II-restricted TCR suggest that more molecular variations may exist. As a canonical example of the MHC/peptide/TCR complex, we include an illustration of the H2-K^b/dEV8/2CTCR complex[499] (Fig. 21.17). This illustration shows that the complementarity determining regions (CDRs) of the TCR (labeled 1α, 2α, 3α for CDRs of Vα and 1β, 2β, and 3β, for the CDRs of Vβ, respectively) sit symmetrically on the MHC/peptide complexes. For the H2-K^b/dEV8/2CTCR complex, the region contacted by the CDRs of the Vα domain of the TCR lie to the left and that contacted by the CDRs of the Vβ domain lie to the right. The regions contacted by CDR3, labeled 3α and 3β, are at the center of the bound peptide, whereas the regions contacted by CDR1 and CDR2 of both Vα and Vβ lie peripherally. Footprints of other TCR have been reviewed elsewhere.[357]

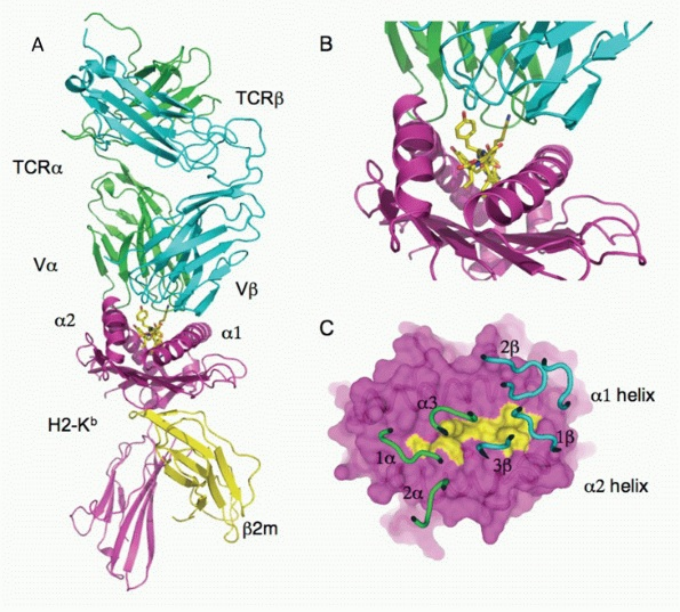

FIG. 21.17. Structure of an MHC-I/Peptide/T-Cell Receptor (TCR) Complex and the TCR Footprint on the MHC/Peptide. The structure of the H2-K^b/dEV8/2CTCR complex (2TCR) is displayed. **A:** The complex complex. **B:** A close-up of the MHC/peptide/TCR interface. **C:** The surface of the MHC *(magenta)*/peptide *(yellow)* complex with the complementarity determining region loop of the TCR Vα *(green)* and Vβ *(blue)* shown and labeled.

With the publication of additional MHC/peptide/TCR structures,[357] additional points have emerged: 1) There is considerable variability in the orientation of the TCR Vα and Vβ domains with respect to the MHC/peptide complex. Although the first MHC-II/peptide/TCR structure suggested that an orthogonal disposition, in which Vβ makes the great majority of contacts with the MHC-II α1 domain and the Vα predominantly interacts with the β1 domain, might be the preference for MHC-II/TCR interactions,[449] additional MHC-II/peptide/TCR structures[450,497] suggest that this disposition is not indicative of MHC-II as compared to MHC-I but rather reveals the wide variety of possibilities. 2) Considerable plasticity in the conformation of the CDR loops of the TCR, particularly long CDR3 loops, is observed in the comparison of TCR free or bound to their cognate MHC/peptide ligands.[499,500] A striking example of this is illustrated by the structure of the KB5-C20 TCR alone as compared to its complex with H2-K^b/peptide (Fig. 21.18).

Structural Insights into Alloreactivity

During T-cell development, TCRs destined to be useful to the host are selected for weak reactivity with one or more self-peptides complexed with cell surface host major histocompatibility (MHC/peptide) molecules. TCR selection is customized in each individual because of extensive polymorphism in MHC molecules that is designed to diversify peptide repertoire and optimize immune responsiveness. Thymic selection and MHC polymorphism conspire to generate antimicrobial T-cell responses that are genetically restricted to recognizing host MHC molecules while retaining Ag-specificity. MHC restriction has been a central paradigm of T-cell immunity and was the basis for the 1996 Nobel Prize awarded to Peter Doherty and Rolf Zinkernagel.

Unfortunately for transplant clinicians and their patients, the rule of MHC restriction is violated when T cells are exposed to allogeneic MHC/peptide complexes. Remarkably, up to 10% of naive T cells react strongly against allogeneic MHC/peptide in vitro (mixed lymphocyte reaction) and in vivo leading to allograft rejection and GVHD. This reaction, known as T-cell alloreactivity, is why MHC molecules were initially called transplantation or histocompatibility molecules and has puzzled immunologists for decades.

There are two main historical theories to explain the high frequency of alloreactive T cells. The first, proposed in 1977,[183] postulated that a single allogeneic MHC molecule

could give rise to multiple binary complexes with cell surface molecules, creating "neoantigenic determinants" recognized by clonally distinct T cells. This has been reinterpreted in a "peptide-centric" hypothesis whereby as a single MHC molecule presents disparate peptides recognized by multiple different T-cell clones. The "multiple binary complex" model implies the TCR interacts with a set of amino acids shared by self- and allogeneic MHC molecules, so the crossreaction depends crucially on the peptide antigen. However, it was later suggested that alloreactive T cells might focus on polymorphic residues exposed on the allogeneic MHC molecule itself, the so-called high determinant density model in which the TCR focus is "MHC centric," and the peptide is largely irrelevant.[501]

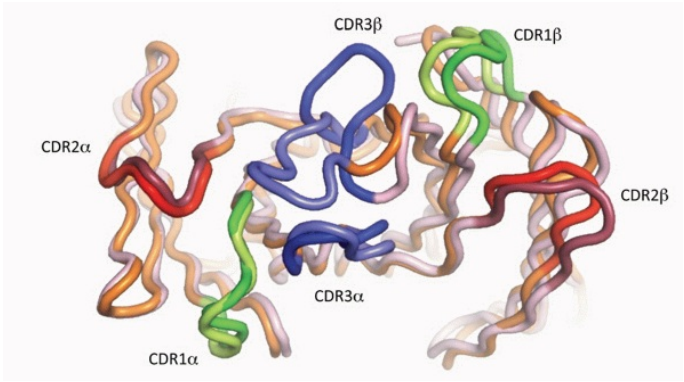

FIG. 21.18. Complementarity Determining Region (CDR)3 Plasticity. Backbone tracings of the KB5-C20 T-cell receptor CDR loops free (1KB5) or bound to the major histocompatibility/peptide complex (1KJ2) are superposed. Unliganded forms are in *darker colors*. The largest conformational difference is seen in the CDR3β loop.

Colf et al.[502] solved the structure of the 2C TCR in complex with its known allogeneic ligand, H2-L^d-QL9, and then compared it to the structure of 2C in complex with its positively selecting ligand, H2-K^b-dEV8, and thus were able to provide important insight into the structural basis of alloreactivity. These two TCR footprints were typical of known MHC/peptide/TCR structures but differed from each other in a number of ways. For instance, each chain of 2C made contacts with one MHC α helix of H2-L^d-QL9, whereas both chains contacted both α helices in H2-K^b-dEV8. The geometry of 2C adopted a more perpendicular orientation on H2-L^d-QL9, and there was a relative rotation of H2-K^b-dEV8 by 20 degrees. The alloreactive complex reveals that both peptide-centric and MHC-centric interactions underpin direct T-cell allorecognition by the 2C receptor but with a heavy emphasis on MHC-centric interactions. Most surprising, however, was the small number of shared contacts between the two structures, implying a limited role for mimicry between cognate and allogeneic MHC/peptide. The H2-K^b and H2-L^d have 31 amino acid differences, and there is no sequence similarity between the H2-K^b-restricted octamer self-peptide, dEV8, and the H2-L^d-restricted nonamer, QL9. Given these differences, it was a fair bet that the cross-reactivity of 2C on H2-K^b-dEV8 and H2-L^d-QL9 would depend upon plasticity in the CDR3 regions of the TCR as documented in comparisons of bound and free TCRs, including 2C. Surprisingly, this was not the case as the TCR actually adopted very similar conformations in the two structures. In contrast to the findings with the alloreactive murine, 2C TCR, alloreactivity of the natural human LC13 TCR is based on striking molecular mimicry between the cognate and alloantigens.[503] The LC13 recognizes an Epstein-Barr viral peptide complexed with self-HLA-B*08:01 but also alloreacts with B44 allotypes (HLA-B*44:02 and 44:05) bound to an endogenous allopeptide from an adenosine triphosphate-binding cassette protein ABCD3. HLA-B*08:01, and the closely related allotypes HLA-B*44:02 and HLA-B*44:05, are distinguished by numerous polymorphisms and the LC13-reactive viral and endogenous peptides are unrelated in their sequence. Nonetheless, the LC13 TCR bound the allogeneic and cognate virus-specific HLA-peptide structures in a very similar manner.[503] Moreover, there was mimicry of the viral peptide by the allopeptide. Hence, this study demonstrates that recognition of allogeneic and cognate HLA-peptide structures can occur via molecular

mimicry, even in the face of polymorphism and disparate peptide ligands.[503] Interestingly, another HLA-B*08:01-restricted TCR (CF34) with the identical Epstein-Barr virus-peptide specificity as LC13 lacks B44 reactivity because it arises when HLA-B44 is coinherited in trans with HLA-B8. Docking of the CF34 TCR focused on the N-terminus of the HLA-B*08:01-peptide while LC13 docked over the C-terminus of the viral peptide bound by HLA-B*08:01.[504] This corresponds with CF34 engagement of a polymorphic region distinguishing HLA-B8 from HLA-B44, revealing a topographical image of shifting TCR specificity to accommodate T cell self-tolerance.[504]

The role of molecular mimicry and the relative importance of peptide-centric versus MHC-centric bias in T-cell allorecognition is likely to vary in different systems. Hence, it is likely that the nature of the polymorphisms between cognate and allogeneic MHC allotypes will affect TCR focus. Thus, closely related MHC allotypes that differ by as little as one amino acid (eg, H2-K^b mutants in mice; HLA-A2, B44, and B27 families in humans) are set up for MHC mimicry to be a key component of T-cell allorecognition where *specificity* is likely to be peptide centric. For example, HLA-B*44:02 and B*44:03 allotypes differ by only a single amino acid and yet stimulate strong mutual allogeneic T-cell responses. Indeed, the potency of T-cell alloresponses between closely related MHC allotypes probably occurs because positive selection of host T cells is purposely designed to create a repertoire responsive to subtle changes in peptide display. Therefore, closely related MHC allotypes, with differences in both peptide repertoire and MHC/peptide conformation of a shared repertoire, play straight into nature's design for T-cell recognition.

Important questions concerning the nature of T-cell signaling mediated by engagement of the TCR by MHC/peptide complexes remain. What are the molecular and biophysical determinants of agonist as compared with antagonist activity of particular MHC/peptide ligands? Although it is clear that a number of parameters such as binding kinetics, half-life, and affinity (both two dimensional and three dimensional) as well as coreceptor contributions may play a role in the outcome of TCR engagement by MHC/peptide complexes, and the additional role of receptor clustering may contribute to such signaling, the precise contribution of each of these factors has not been established. A provocative approach to the question of the role of the geometry of docking of the TCR onto the MHC/peptide complex has been addressed in the analysis of the relationship of stimulatory parameters of four different peptides bound to H2-L^d with respect to the three-dimensional structure of the relevant MHC/peptide/TCR complex.[505] One of the four peptides failed to induce signaling, and remarkably, the TCR docked onto the MHC/peptide in a unique docking geometry. The authors interpreted these results to demonstrate the relationship between TCR/pMHC docking geometry, peptide cross-reactivity, and signaling as well as explaining the basis for germline bias in TCR/MHC interactions.

MHC/Coreceptor Complexes

The major coreceptors for recognition by αβ TCRs are CD8, which interacts with MHC-I molecules, and CD4, which interacts with MHC-II. Coreceptor function plays a role in signaling the T cell in addition to contributions the MHC/coreceptor interaction may provide in increasing apparent avidity between the MHC/peptide and the TCR. CD8, the coreceptor on MHC-I-restricted αβ T cells, exists as a cell surface homodimer of two α chains or a heterodimer of α and β chains and plays an important role both in the activation of mature peripheral T cells as well as in the thymic development of MHC-I-restricted lymphocytes.[506,507] Mature peripheral CD8 T cells express significant amounts of CD8αβ, whereas other lymphocytes, such as intraepithelial lymphocytes in the gut, predominantly express the CD8αα homodimer. The CD8αβ heterodimer is considered the relevant functional coreceptor on peripheral T cells. The three-dimensional structures of human and mouse MHC-I/CD8αα complexes have been described,[508,509] and more recently, a complex of mouse MHC-I/CD8αβ has been reported.[58] These structures localize the binding site of the CD8 Ig-like αα homodimer or CD8αβ heterodimer to a region on an exposed loop of the MHC-I α3 domain. This interaction is illustrated in Figure 21.19, which shows the flexible loop of residues 223 to 229 clamped into the CD8 combining site. This is one of relatively few examples of an non-Ig, Ig-like molecule exploiting an Ig-like heterodimer interface to bind another ligand. The structural identification of the CD8αα and CD8αβ on the MHC-I provides a context for understanding the contribution of CD8, which binds the kinase Lck through the cytoplasmic domain of its α chain to T-cell signaling.

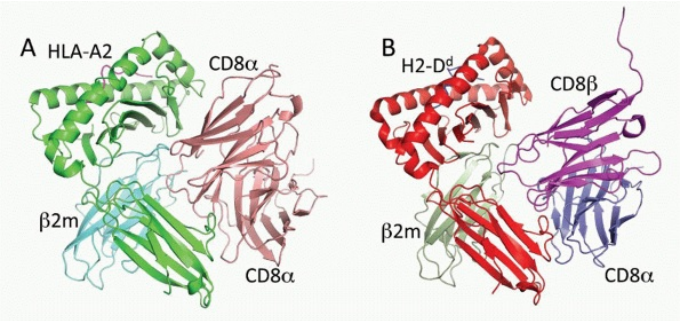

FIG. 21.19. CD8αα and CD8αβ Bind the Same Site on the MHC-Iα3 Domain. The MHC-I interactions with the CD8αα (PDB 1AKJ) homodimer **(A)** and CD8αβ (PDB 3DMM) heterodimer **(B)** are shown.

The T-cell coreceptor associated with cells restricted to MHC-II antigens, CD4, has also been the subject of detailed structural studies, in part, because of its role as a receptor for attachment and entry of HIV.[510] The x-ray structure determination of the complete extracellular portion of the molecule (domains D1 through D4) indicates a degree of segmental flexibility between domains D2 and D3, and both crystallographic and biochemical data suggest that dimerization of cell surface CD4 occurs.[511] These results have been interpreted to support a role for CD4-mediated MHC-II-dependent dimerization in facilitating TCR dimerization and signaling. Although a mixed species structure of human CD4 D1-D2 domains with a mouse MHC-II molecule was reported at low resolution some years ago,[63] only recently, employing an affinity matured human CD4 molecule have diffraction quality crystals of human MHC-II/human CD4 D1-D2 molecules been obtained[64] (Fig. 21.20). This recently determined human MHC-II/hCD4 structure is remarkably similar to the low-resolution mixed species structure determined more than 10 years earlier. Both structures reveal the focus of the CD4 interaction on a stretch of amino acid residues on the Ig-like β2 domain of the MHC-II molecule. Earlier mutagenesis and functional studies of CD4-dependent MHC-II-mediated T-cell activation

also implicated a region on the α2 domain of MHC-II.[61,512] Although the α2 domain residues do not directly interact with CD4 in these structures, it is conceivable that they play some indirect role in MHC multimerization that may contribute to signaling.

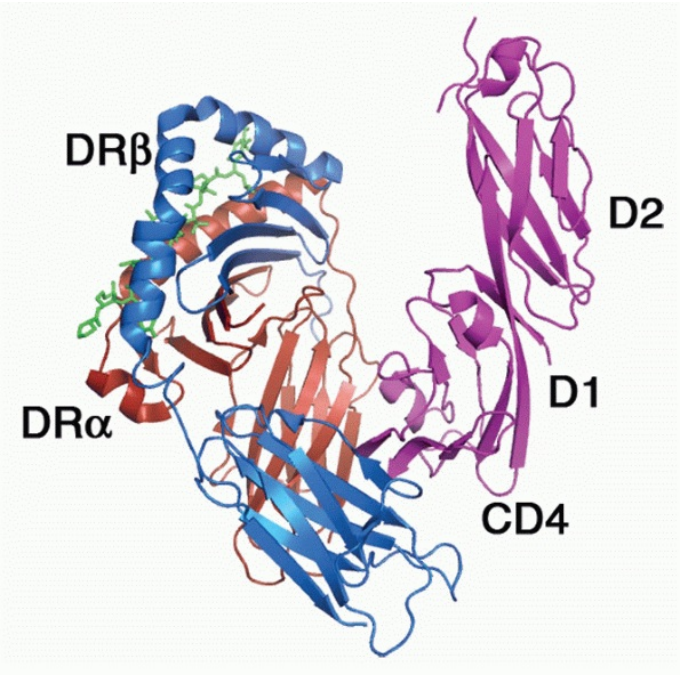

FIG. 21.20. CD4 Interacts Directly with the β2 Domain of MHC-II. A ribbon illustration of a complex of human CD4 with HLA-DR1 (PDB 3S5L) is shown. The HLA-DRα chain is in *firebrick*, HLA-DRβ chain in *blue*, peptide in *green*, and CD4 in *magenta*.

MHC/Natural Killer Receptor Complexes

NK cells provide a first line of defense against virus-infected and host cells, and their recognition of such targets is dependent on a balance of NK activating and inhibitory receptors.[513,514] In addition to their expression on NK cells, some of the NK receptors are also expressed on subsets of T cells and other hematopoietic cells. In general, each of these classes of NK receptors also falls into two different structural groups: the Ig-like receptors and the C-type lectin-like receptors. Because of their functional interactions with MHC-I and MHC-I-like molecules, and because several different systems have evolved to recognize MHC-I molecules differently, it is worthwhile to examine the recently determined structures of several MHC-I/NK receptor complexes.

In the human, the major NK receptors fall into several categories: those of the NKG2 family, the NKp molecules, and the KIRs. The NKG2 family molecules are C-type lectin-like molecules that recognize ligands grouped broadly as the NKG2D ligands, which include the MHC-like molecules, MICA and MICB, as well as the ULBP family of stress-induced molecules. NKp molecules include NKp30, NKp44, and NKp46, all members of the Ig superfamily. The KIR molecules, also Ig superfamily members, bind either HLA-C, or for the KIR3 subfamily, HLA-B molecules. Because the focus of this chapter is members of the MHC protein family, we will restrict our discussion of the human NK receptor/ligand interactions to the NKG2/MIC and ULBP complexes and to the KIR2D and KIR3D/MHC interactions.

The activating receptor, NKG2D, interacts with MICA, a stress-induced MHC-I-like molecule, allowing NK cells to eliminate virus-infected or tumor cells that are marked by the surface expression of MICA. The structure of the human NKG2D/MICA complex reveals that the homodimeric binding site of NKG2D recognizes the surface α-helical superdomain, in an orientation similar to that by which TCR see MHC-I molecules[456] (Fig. 21.21). This structure shows remarkable similarity to that of the hNKG2D bound to another MHC-Ib protein, ULBP3.[515] Similarly, the mouse NKG2D interacts, in a similar orientation, with the murine stress-induced molecule, RAE-1β.[459]

The short KIRs (eg, KIR3DS and KIR2DS molecules) are considered activating receptors because they have the potential to interact with the DAP12 signal-transducing molecule, and the long KIRs (eg, KIR3DL and KIR2DL) are inhibitory because they have cytoplasmic domains that contain immunoreceptor tyrosine-based inhibitory motifs. KIR2DL1 and KIR2DL2 have been studied extensively. They interact with the human MHC-I molecules HLA-C*04 and HLA-C* 03, respectively, and show some preferences for MHC-I molecules complexed with particular peptides. Among the polymorphic amino acid residues that distinguish HLA-C* 03 and HLA-C*04 are Asn80 of -C*03 and Lys80 of -C*04. Thus, it was of interest when the structures of HLA-C* 03/KIR2DL2[104] and HLA-C*04/KIR2DL1 complexes were reported.[105] The KIR2DL2/HLA-C*03 interaction is illustrated in Figure 21.22. The important conclusions from this structure and those of similar KIR2DL complexes is that the recognition of MHC-I is via amino acid residues of the

elbow bend joining the two Ig-like domains of the KIR, and that residues that vary among different KIRs determine the molecular specificity of the interaction with the particular allelic product of HLA-C. In addition, the interaction of the KIR with the HLA-C is also modulated by the particular bound peptide, explaining the results of binding/peptide specificity studies. Structural understanding of the KIR3 molecules, which have three extracellular Ig-like domains, eluded the field for many years. Recently, a molecular complex of HLA-B*57:01 with KIR3DL1 has been reported (see Fig. 21.22). This structure reveals the contribution of the D0 amino terminal domain of KIR3DL1 in recognizing a region of the MHC-I with limited polymorphism, the D1 domain accommodates a region with sequence variation, and the D2 domain shows high complementarity.

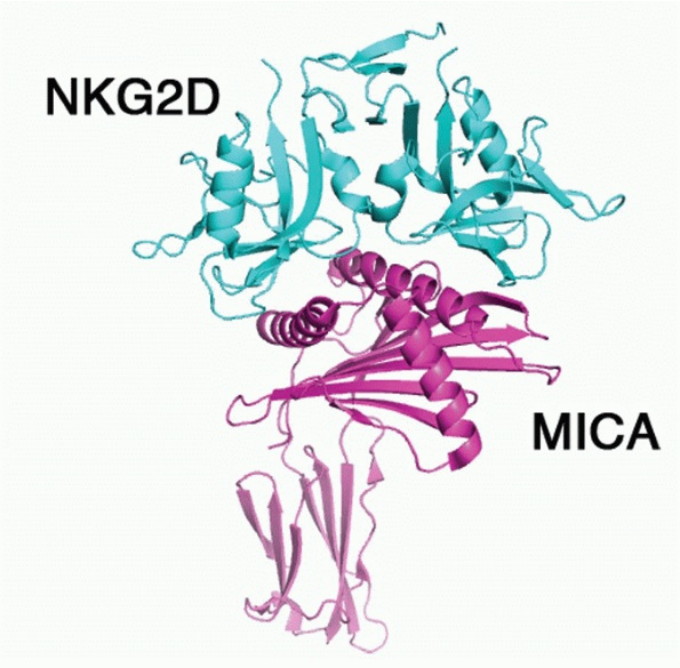

FIG. 21.21. The NKG2D sees the MHC-Ib MICAα-Helical Superdomain. The structure of the human NKG2D/MICA complex (PDB 1HYR) reveals the homodimer NKG2D sitting asymmetrically astride the MICA α-helical superdomain. The NKG2D domains are in *cyan* and MICA in *magenta*.

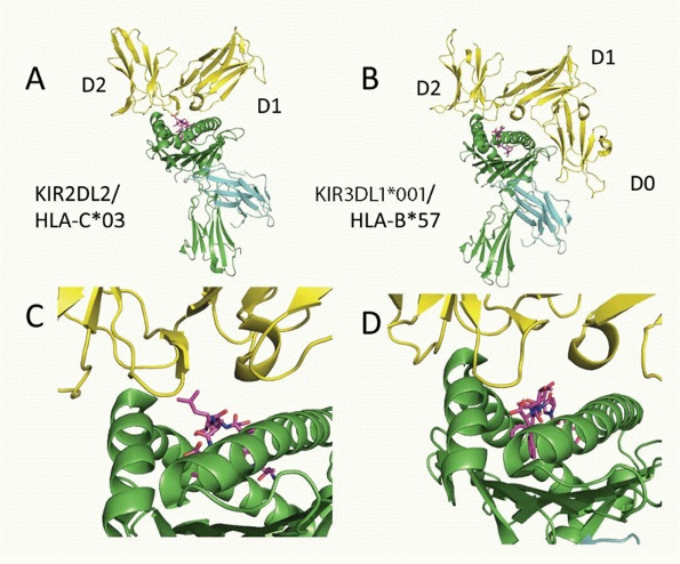

FIG. 21.22. KIR2DL2 and KIR3DL1 Interact Similarly with Their MHC-I/p Ligands. KIR2DL2 complexed with HLA-C*03 (PDB 1EFX) **(A,C)** and KIR3DL1 complexed with HLA-B*57:01 (PDB 3VH8) **(B,D)** are shown to illustrate the overall interactions **(A,B)** and close-ups of the contact with bound peptide **(C,D)**.

The mouse exploits a set of NK receptors of a different structural family to provide the same function. In particular, the predominant, and best studied, mouse NK receptors are those of the Ly49 family. Functional experiments had demonstrated that Ly49A interacts with the MHC-I molecule, H2-D^d, and also that for appropriate interaction, H2-D^d needs to be complexed with a peptide. In contrast to human NK recognition, however, surveys of H2-D^d-binding peptides revealed little if any peptide preference or specificity. This would explain the function of the NK inhibitory receptor in that they are at baseline chronically stimulated by normal

MHC-I on somatic cells, turning off the NK cell. When MHC-I is dysregulated by tumorigenesis or by pathogenic infection, the lower level of surface MHC-I diminishes the NKIR-mediated signal, and the NK cell is activated. The structure of the mouse Ly49A inhibitory receptor in complex with its MHC-I ligand, H2-D^d (Fig. 21.23), reveals several crucial features of the interaction: 1) the Ly49A C-type lectin-like molecule is a homodimer; 2) the Ly49A molecule makes no direct contact with residues of the MHC-bound peptide; and 3) in the x-ray structure, there are two potential sites for Ly49A interaction with the MHC molecule: site 1 at the end of the α1 and α2 helices, and site 2, an extensive region making contact with the floor of the peptide binding groove, the α3 domain of the MHC-I molecule, and the β2m domain as well. The ambiguity suggested by the x-ray structure has been resolved by extensive mutagenesis studies that are consistent with the view that site 2 is functionally significant.[516,517] Recently, several other NK receptors have been studied structurally. These include the Ly49I inhibitory receptor, which interacts functionally with H2-K^b. The Ly49I has been crystallized without a ligand, revealing a basic fold similar to that of Ly49A but with a somewhat different dimeric arrangement.[518] The Ly49C, another murine NK inhibitory receptor, has been examined in complex with its H2-K^b ligand.[519] Differences between H2-K^b/Ly49C and H2-D^d/Ly49A suggest different modes of NK receptor binding to MHC-I depending on whether the interaction is “cis” (ie, between the NK receptor and the MHC-I on the same NK cell) or “trans” (between the NK receptor on the NK cell and the MHC-I on its target).[520]

MHC-II Superantigen Complexes

Superantigens are molecules, frequently toxic products of bacteria, which bind MHC molecules on the cell surface and are then presented to a large subset of T cells, usually defined by the expression of a particular family of TCR V regions.[521] Most of the known superantigens bind MHC-II molecules, although one, the agglutinin from *Urtica dioica*, the stinging nettle, can be bound by both MHC-I and MHC-II molecules and presented to T cells of the Vβ8.3 family.[522] Its structure in complex with carbohydrate ligand has been reported.[523,524] MHC-II interactions with superantigens, such as those derived from pathogenic bacteria, are the first step in the presentation of the multivalent array of the APC-bound superantigen to T cells bearing receptors of the family or class that can bind the superantigen.[521] A number of structural studies examining the interaction

of superantigen both with their MHC-II ligands and with TCR have been reported.[525] Structural analysis of crystals derived from staphylococcal enterotoxin B complexed with HLA-DR1[526] and from toxic shock syndrome toxin-1 complexed with HLA-DR1[527] revealed that the two toxins bind to an overlapping site, primarily on the MHC-II α chain, and indicated that the staphylococcal enterotoxin B site would not be expected to be influenced by the specific peptide bound by the MHC, although the toxic shock syndrome toxin-1 site would. The view that superantigens exert their biologic effects by interaction with conserved regions of the MHC-II molecule as well as with conserved regions of the TCR has been challenged by the determination of two structures, that of *S. aureus* enterotoxin H complexed with HLA-DR1[528] and that of streptococcal pyrogenic toxin C in complex with HLA-DR2a.[529] Both of these studies indicate that these superantigens can interact with the MHC-II β chain through a zinc-dependent site that includes superantigen contacts to bound peptide. Our understanding of superantigen interactions has been augmented by the determination of ternary structures of MHC-II/superantigen/TCR complexes for two examples, that of the *Mycoplasma arthritidis* mitogen in complex with HLA-DR1 and TCR,[530] and that of *Staphylococcus aureus* enterotoxin H complexed with HLA-DR1 and TCR.[531] In both of these cases, the superantigen interacts with the MHC-II and with both TCR Vα and Vβ domains.

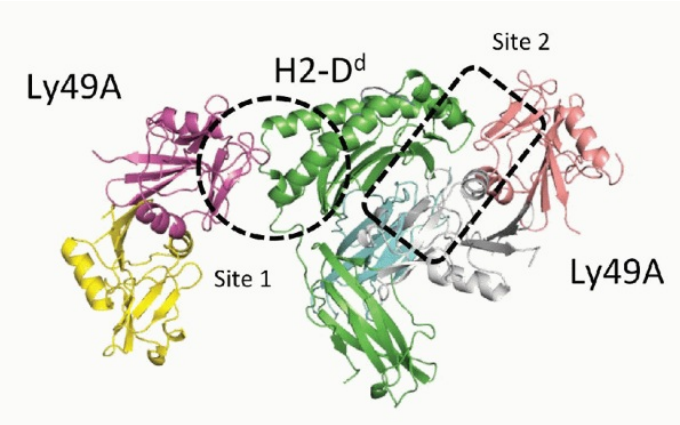

FIG. 21.23. Structure of the H2-D^d/Ly49A Complex Reveals an Alternate Site for MHC/Natural Killer Receptor Interaction. The structure of the complex (PDB 1QO3) is shown, illustrating both potential sites of interaction.

MOLECULAR INTERACTIONS OF MHC MOLECULES

Assays for Molecular Interactions

Whereas the crystal structures provide a vivid static illustration of the interactions of MHC molecules with their peptide, FcRn, CD8, CD4, superantigen, NK receptor, and TCR ligands, the dynamic aspects of these binding steps can be approached by a variety of biophysical methods.[532] It is important to note that affinities and kinetics of interaction of MHC/peptide complexes for TCR have been determined by several methods in a variety of systems.[533,534,535,536,537,538,539,540] In addition, MHC interactions with NK receptors[101,102,541,542] have been quantified by similar techniques. Although there are clear differences in the affinity and kinetics of binding of different TCR and NK receptors for their respective cognate MHC/peptide complexes, the generally consistent findings are that the affinities are low to moderate (ie, $K_d = 5 \times 10^{-5}$ to 10^{-7} M) and are characterized by relatively rapid dissociation rates (ie, $k_d = 10^{-1}$ to 10^{-3} sec^{-1}). Recently, attention has been drawn to the differences in measuring interactions with one component in solution (three-dimensional affinity) as compared with measurements made between multivalent displays of the interactions on two surfaces, such as might mimic the apposition of two cells. Methods exploiting microscopic techniques[543,544] or a micropipette adhesion frequency assay[545,546] suggest that the biologic readout of T-cell activation may be more readily correlated with twodimensional rather than three-dimensional measurements of MHC/TCR affinities.

Multivalent MHC/Peptide Complexes

A major development in the past several years has been the engineering and application of multivalent MHC/peptide complexes for the identification, quantification, purification, and functional modulation of T cells with particular MHC or MHC/peptide specificity. Two general approaches have been exploited: one based on the enzymatic biotinylation of soluble MHC/peptide molecules generated in bacterial expression systems that are then multimerized by binding of the biotinylated molecules to the tetravalent streptavidin,[547] and another based on the engineering of dimeric MHC/Ig fusion proteins.[548] These reagents can be used in flow cytometric assays that permit the direct enumeration of MHC/peptide-specific T cells taken directly ex vivo. In either of these methods, multivalent MHC/peptide complexes are generated, and the relatively weak intrinsic affinity of the MHC/peptide complex for its cognate TCR is effectively magnified by the gain in avidity obtained by the increase in valency. For MHC-I molecules, the technology has been so reliable in producing multivalent (tetrameric) molecules loaded homogeneously with synthetic peptides that a wide variety of specific MHC-I/peptide multimers are available either from a resource facility sponsored by the National Institute of Allergy and Infectious Diseases (www.niaid.nih.gov/reposit/tetramer/index.html) or from commercial suppliers that offer either

the tetramer, Ig multimer, or a pentamer preparation. The MHC-I/peptide multimers have also been exploited for identification of specific populations of NK cells and for assignment of various NK-receptor specificities.[549,550,551,552] For some MHC-II molecules, similar success has been achieved in the production of such multimers, using insect cell or mammalian cell expression systems for molecules produced by either the tetramer or Ig chimera strategy.[553,554,555,556,557] MHC-II/peptide tetramers can be effectively used for a variety of CD4 T-cell applications, but some TCRs (particularly those involved in autoimmune diseases) have an affinity for their cognate MHC-II/peptide that is beneath the threshold for detection.[545] Also, the potential for the same peptide binding to one MHC-II in multiple frames generates difficulties in the engineering and preparation of effective MHC-II tetramers.[406]

CONCLUSION

We have surveyed the *Mhc* as a genetic region and a source for molecules crucial to immune regulation and immunologic disease. These genes reflect the panoply of mechanisms involved in the evolution of complex systems and encode cell surface proteins that interact via complex orchestration with small molecules including peptides and glycolipids as well as with macromolecular receptors on T cells and NK cells. The MHC-I molecules provide the immune system with a window for viewing the biologic health of the cell in which they are expressed, and MHC-II molecules function as scavengers to taste and display the remnants of the cellular environment. Viruses and bacterial pathogens contribute enormously to the genetic dance—

they modulate and compete in the control of MHC expression, sometimes exploiting MHC mimics that they have acquired—and the host by adjusting its T-cell and NK-cell repertoire on the time scale of both the individual organism and the species, resisting the push to extinction. The immune system, dynamically, resourcefully, and creatively provides, through the concerted action of its MHC molecules, TCRs, NK receptors, and antibodies as well as a host of other regulatory molecules, an organ system vital not only to the survival of the individual but also to the success of the species. As we understand better the molecular functions of the MHC, we should better understand rational approaches to manipulating the immune system in the prevention, diagnosis, and treatment of immunologic and infectious diseases.

ACKNOWLEDGMENTS

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REFERENCES

1. Mans J, Natarajan K, Balbo A, et al. Cellular expression and crystal structure of the murine cytomegalovirus major histocompatibility complex class I-like glycoprotein, m153. *J Biologic Chem.* 2007;282(48): 35247-35258.

2. Revilleza MJ, Wang R, Mans J, et al. How the virus outsmarts the host: function and structure of cytomegalovirus MHC-I-like molecules in the evasion of natural killer cell surveillance. *J Biomed Biotechnol* . 2011;2011:724607.

3. Gorer PA. Studies in antibody response of mice to tumour inoculation. *Br J Cancer* . 1950;4(4):372-379.

4. Gorer PA. The detection of antigenic differences in mouse erythrocyte by the employment of immune sera. *Br J Exp Pathol* . 1936;17:42-50.

5. Klein J. *Natural History of the Major Histocompatibility Complex* . New York, NY: Wiley-Interscience; 1986.

6. Robinson J, Waller MJ, Stoehr P, et al. IPD—the immuno polymorphism database.

Nucleic Acids Res. 2005;33:D523-526.

7. Holdsworth R, Hurley CK, Marsh SG, et al. The HLA dictionary 2008: a summary of HLA-A, -B, -C, -DRB1/3/4/5, and -DQB1 alleles and their association with serologically defined HLA-A, -B, -C, -DR, and -DQ antigens. *Tissue Antigens*. 2009;73(2):95-170.

8. Robinson J, Mistry K, McWilliam H, et al. The IMGT/HLA database. *Nucleic Acids Res*. 2011;39:D1171-1176.

9. Robinson J, Malik A, Parham P, et al. IMGT/HLA database—a sequence database for the human major histocompatibility complex. *Tissue Antigens*. 2000;55(3):280-287.

10. Ceppellini R. *Histocompatibility Testing, 1967*. Copenhagen, Denmark: Munksgaard; 1967.

11. Yoshino M, Xiao H, Jones EP, et al. Genomic evolution of the distal Mhc class I region on mouse Chr 17. *Hereditas*. 1997;127(1-2): 141-148.

12. Yoshino M, Xiao H, Amadou C, et al. BAC clones and STS markers near the distal breakpoint of the fourth t-inversion, In(17)4d, in the H2-M region on mouse chromosome 17. *Mamm Genome*. 1998;9(3):186-192.

13. Duran LW, Horton RM, Birschbach CW, et al. Structural relationships among the H-2 D-regions of murine MHC haplotypes. *J Immunol* . 1989;142(1):288-296.

14. Hedley M, Forman J, Tucker PW. D2d, a D-End class I gene: tissue expression and alternative processing of the pre-mRNA. *Int Immunol* . 1990;2(1):25-32.

15. Aylor DL, Valdar W, Foulds-Mathes W, et al. Genetic analysis of complex traits in the emerging Collaborative Cross. *Genome Res*. 2011; 21(8):1213-1222.

16. Jamieson AM, Diefenbach A, McMahon CW, et al. The role of the NKG2D immunoreceptor in immune cell activation and natural killing. *Immunity*. 2002;17(1):19-29.

17. Germain RN, Margulies DH. The biochemistry and cell biology of antigen processing and presentation. *Ann Rev Immunol* . 1993;11:403-450.

18. York IA, Rock KL. Antigen processing and presentation by the class I major histocompatibility complex. *Ann Rev Immunol* . 1996;14: 369-396.

19. Yewdell JW, Bennink JR. Cell biology of antigen processing and presentation to major histocompatibility complex class I molecule-restricted T lymphocytes. *Adv Immunol* . 1992;52:1-123.

20. Androlewicz MJ. Peptide generation in the major histocompatibility complex class I antigen processing and presentation pathway. *Curr Opin Hematol* . 2001;8(1):12-16.

21. Watts C, Powis S. Pathways of antigen processing and presentation. *Rev Immunogen*. 1999;1(1):60-74.

22. Nguyen TT, Chang SC, Evnouchidou I, et al. Structural basis for antigenic peptide precursor processing by the endoplasmic reticulum aminopeptidase ERAP1. *Nat Structur Mol Biol*. 2011;18(5):604-613.

23. Heemels MT, Ploegh H. Generation, translocation, and presentation of MHC class I-restricted peptides. *Ann Rev Biochem*. 1995;64:463-491.

24. Brocke P, Garbi N, Momburg F, et al. HLA-DM, HLA-DO and tapasin: functional similarities and differences. *Curr Opin Immunol* . 2002; 14(1):22-29.

25. Zhang Y, Kozlov G, Pocanschi CL, et al. ERp57 does not require interactions with calnexin and calreticulin to promote assembly of class I histocompatibility molecules, and it enhances peptide loading independently of its redox activity. *J Biol Chem*. 2009;284(15):10160-10173.

26. Bouvier M, Wiley DC. Importance of peptide amino and carboxyl termini to the stability of MHC class I molecules. *Science*. 1994; 265(5170):398-402.

27. Bouvier M, Wiley DC. Structural characterization of a soluble and partially folded class I major histocompatibility heavy chain/beta 2m heterodimer. *Nat Struct Biol* . 1998;5(5):377-384.

28. Ljunggren HG, Stam NJ, Ohlen C, et al. Empty MHC class I molecules come out in the cold. *Nature*. 1990;346(6283):476-480.

29. Painter CA, Negroni MP, Kellersberger KA, et al. Conformational lability in the class II MHC 310 helix and adjacent extended strand dictate HLA-DM susceptibility and peptide exchange. *Proc Natl Acad Sci U S A* . 2011;108(48):19329-19334.

30. Cresswell P. Invariant chain structure and MHC class II function. *Cell*. 1996;84(4):505-507.

31. Amigorena S, Webster P, Drake J, et al. Invariant chain cleavage and peptide loading in major histocompatibility complex class II vesicles. *J Exp Med*. 1995;181(5):1729-1741.

32. Thery C, Amigorena S. The cell biology of antigen presentation in dendritic cells. *Curr Opin Immunol*. 2001;13(1):45-51.

33. Newcomb JR, Carboy-Newcomb C, Cresswell P. Trimeric interactions of the invariant chain and its association with major histocompatibility complex class II alpha beta dimers. *J Biol Chem*. 1996;271(39): 24249-24256.

34. Denzin LK, Hammond C, Cresswell P. HLA-DM interactions with intermediates in HLA-DR maturation and a role for HLA-DM in stabilizing empty HLA-DR molecules. *J Exp Med*. 1996;184(6):2153-2165.

35. Pierre P, Denzin LK, Hammond C, et al. HLA-DM is localized to conventional and unconventional MHC class II-containing endocytic compartments. *Immunity*. 1996;4(3):229-239.

36. Grusby MJ, Glimcher LH. Immune responses in MHC class II-deficient mice. *Ann Rev Immunol*. 1995;13:417-435.

37. Cresswell P, Ackerman AL, Giodini A, et al. Mechanisms of MHC class I-restricted antigen processing and cross-presentation. *Immunol Rev*. 2005;207:145-157.

38. Segura E, Villadangos JA. A modular and combinatorial view of the antigen cross-presentation pathway in dendritic cells. *Traffic*. 2011;12(12):1677-1685.

39. Gascoigne NR, Palmer E. Signaling in thymic selection. *Curr Opin Immunol* . 2011;23(2):207-212.

40. Lemonnier FA. The utility of H-2 class I knockout mice. *Virus Res*. 2002;82(1-2):87-90.

41. Lanier LL. NK cell receptors. *Ann Rev Immunol*. 1998;16:359-393.

42. Correa I, Raulet DH. Binding of diverse peptides to MHC class I molecules inhibits target cell lysis by activated natural killer cells. *Immunity*. 1995;2(1):61-71.

43. Malnati MS, Peruzzi M, Parker KC, et al. Peptide specificity in the recognition of MHC class I by natural killer cell clones. *Science*. 1995; 267(5200):1016-1018.

44. Orihuela M, Margulies DH, Yokoyama WM. The natural killer cell receptor Ly-49A recognizes a peptide-induced conformational determinant on its major histocompatibility complex class I ligand. *Proc Natl Acad Sci U S A* . 1996;93(21):11792-11797.

45. Karre K. How to recognize a foreign submarine. *Immunol Rev*. 1997;155:5-9.

46. Karre K. NK cells, MHC class I molecules and the missing self. *Scand J Immunol* . 2002;55(3):221-228.

47. Ljunggren HG, Karre K. In search of the "missing self": MHC molecules and NK cell recognition. *Immunol Today*. 1990;11(7):237-244.

48. Karlhofer FM, Ribaudo RK, Yokoyama WM. MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. *Nature*. 1992;358(6381):66-70.

49. Dorfman JR, Raulet DH. Acquisition of Ly49 receptor expression by developing natural killer cells. *J Exp Med*. 1998;187(4):609-618.

50. Kim S, Poursine-Laurent J, Truscott SM, et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature*. 2005;436(7051):709-713.

51. Brodin P, Karre K, Hoglund P. NK cell education: not an on-off switch but a tunable rheostat. *Trends Immunol*. 2009;30(4):143-149.

52. Orr MT, Lanier LL. Natural killer cell education and tolerance. *Cell*. 2010;142(6):847-856.

53. Anfossi N, Andre P, Guia S, et al. Human NK cell education by inhibitory receptors for MHC class I. *Immunity*. 2006;25(2):331-342.

54. Salter RD, Benjamin RJ, Wesley PK, et al. A binding site for the T-cell co-receptor CD8 on the alpha 3 domain of HLA-A2. *Nature*. 1990;345(6270):41-46.

55. Connolly JM, Potter TA, Wormstall EM, et al. The Lyt-2 molecule recognizes residues in the class I alpha 3 domain in allogeneic cytotoxic T cell responses. *J Exp Med*. 1988;168(1):325-341.

56. Norment AM, Salter RD, Parham P, et al. Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature*. 1988;336(6194):79-81.

57. Salter RD, Norment AM, Chen BP, et al. Polymorphism in the alpha 3 domain of HLA-A molecules affects binding to CD8. *Nature*. 1989;338(6213):345-347.

58. Wang R, Natarajan K, Margulies DH. Structural basis of the CD8 alpha beta/MHC class interaction: focused recognition orients CD8 beta to a T cell proximal position. *J Immunol*. 2009;183(4):2554-2564.

59. Doyle C, Strominger JL. Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature*. 1987;330(6145):256-259.

60. Doyle C, Shin J, Dunbrack RL Jr, et al. Mutational analysis of the structure and function of the CD4 protein. *Immunol Rev*. 1989;109:17-37.

61. Konig R, Fleury S, Germain RN. The structural basis of CD4-MHC class II interactions: coreceptor contributions to T cell receptor antigen recognition and oligomerization-dependent signal transduction. *Curr Topics Microbiol Immunol* . 1996;205:19-46.

62. Konig R. Interactions between MHC molecules and co-receptors of the TCR. *Curr Opin Immunol*. 2002;14(1):75-83.

63. Wang JH, Meijers R, Xiong, Y, et al. Crystal structure of the human CD4 N-terminal two-domain fragment complexed to a class II MHC molecule. *Proc Natl Acad Sci U S A* . 2001;98(19):10799-10804.

64. Wang XX, Li Y, Yin Y, et al. Affinity maturation of human CD4 by yeast surface display and crystal structure of a CD4-HLA-DR1 complex. *Proc Natl Acad Sci U S A* . 2011;108(38):15960-15965.

65. Kittipatarin C, Tschammer N, Khaled AR. The interaction of LCK and the CD4 co-receptor alters the dose response of T-cells to interleukin-7. *Immunol Lett*. 2010;131(2):170-181.

66. Trobridge PA, Forbush KA, Levin SD. Positive and negative selection of thymocytes depends on Lck interaction with the CD4 and CD8 coreceptors. *J Immunol*. 2001;166(2):809-818.

67. Luescher IF, Vivier E, Layer A, et al. CD8 modulation of T-cell antigen receptor-ligand interactions on living cytotoxic T lymphocytes. *Nature*. 1995;373(6512):353-356.

68. Arcaro A, Gregoire C, Bakker TR, et al. CD8beta endows CD8 with efficient coreceptor function by coupling T cell receptor/CD3 to raft-associated CD8/p56(lck) complexes. *J Exp Med*. 2001;194(10):1485-1495.

69. Gangadharan D, Cheroutre H. The CD8 isoform CD8alphaalpha is not a functional homologue of the TCR co-receptor CD8alphabeta. *Curr Opin Immunol* . 2004;16(3):264-270.

70. Horton R, Wilming L, Rand V, et al. Gene map of the extended human MHC. *Nat Rev Gen*. 2004;5(12):889-899.

71. de Bakker PI, McVean G, Sabeti PC, et al. A high-resolution HLA and SNP haplotype map for disease association studies in the extended human MHC. *Nat Gen*. 2006;38(10):1166-1172.

72. Horton R, Gibson R, Coggill P, et al. Variation analysis and gene annotation of eight MHC haplotypes: the MHC Haplotype Project. *Immunogenetics*. 2008;60(1):1-18.

73. Monaco JJ, McDevitt HO. H-2-linked low-molecular weight polypeptide antigens assemble into an unusual macromolecular complex. *Nature*. 1984;309(5971):797-799.

74. Martinez CK, Monaco JJ. Post-translational processing of a major histocompatibility complex-encoded proteasome subunit, LMP-2. *Mol Immunol*. 1993;30(13):1177-1183.

75. Monaco JJ, McDevitt HO. The LMP antigens: a stable MHC-controlled multisubunit protein complex. *Human Immunol*. 1986;15(4):416-426.

76. van Endert PM, Lopez MT, Patel SD, et al. Genomic polymorphism, recombination, and linkage disequilibrium in human major histocompatibility complex-encoded antigen-processing genes. *Proc Natl Acad Sci U S A* . 1992;89(23):11594-11597.

77. Suh WK, Mitchell EK, Yang Y, et al. MHC class I molecules form ternary complexes with calnexin and TAP and undergo peptide-regulated interaction with TAP via their extracellular domains. *J Exp Med*. 1996; 184(2):337-348.

78. Spies T, Cerundolo V, Colonna M, et al. Presentation of viral antigen by MHC class I molecules is dependent on a putative peptide transporter heterodimer. *Nature*. 1992;355(6361):644-646.

79. Powis SJ. Major histocompatibility complex class I molecules interact with both subunits of the transporter associated with antigen processing, TAP1 and TAP2. *Eur J Immunol*. 1997;27(10):2744-2747.

80. Hill A, Ploegh H. Getting the inside out: the transporter associated with antigen processing (TAP) and the presentation of viral antigen. *Proc Natl Acad Sci U S A* . 1995;92(2):341-343.

81. Yang Y, Sempe P, Peterson PA. Molecular mechanisms of class I major histocompatibility complex antigen processing and presentation. *Immunol Res*. 1996;15(3):208-233.

82. Driscoll J, Brown MG, Finley D, et al. MHC-linked LMP gene products specifically alter peptidase activities of the proteasome. *Nature*. 1993;365(6443):262-264.

83. Nandi D, Jiang H, Monaco JJ. Identification of MECL-1 (LMP-10) as the third IFN-gamma-inducible proteasome subunit. *J Immunol*. 1996;156(7):2361-2364.

84. Kuckelkorn U, Frentzel S, Kraft R, et al. Incorporation of major histocompatibility complex—encoded subunits LMP2 and LMP7 changes the quality of the 20S proteasome polypeptide processing products independent of interferon-gamma. *Eur J Immunol* . 1995;25(9):2605-2611.

85. Ehring B, Meyer TH, Eckerskorn C, et al. Effects of major-histocompatibility-complex-encoded subunits on the peptidase and proteolytic activities of human 20S proteasomes. Cleavage of proteins and antigenic peptides. *Eur J Biochem* . 1996;235(1-2):404-415.

86. Elliott T, Willis A, Cerundolo V, et al. Processing of major histocompatibility class I-restricted antigens in the endoplasmic reticulum. *J Exp Med*. 1995;181(4):1481-1491.

87. Velarde G, Ford RC, Rosenberg MF, et al. Three-dimensional structure of transporter associated with antigen processing (TAP) obtained by single Particle image analysis. *J Biol Chem*. 2001;276(49):46054-46063.

88. Powis SJ, Young LL, Joly E, et al. The rat cim effect: TAP allele-dependent changes in a class I MHC anchor motif and evidence against C-terminal trimming of peptides in the ER. *Immunity*. 1996;4(2):159-165.

89. Livingstone AM, Powis SJ, Diamond AG, et al. A trans-acting major histocompatibility complex-linked gene whose alleles determine gain and loss changes in the antigenic structure of a classical class I molecule. *J Exp Med*. 1989;170(3):777-795.

90. Livingstone AM, Powis SJ, Gunther E, et al. Cim: an MHC class II-linked allelism affecting the antigenicity of a classical class I molecule for T lymphocytes. *Immunogenetics*. 1991;34(3):157-163.

91. McCluskey J, Rossjohn J, Purcell AW. TAP genes and immunity. *Curr Opin Immunol* . 2004;16(5):651-659.

92. Caversaccio M, Bonel HM, Carter R, et al. TAP deficiency syndrome: chronic rhinosinusitis and conductive hearing loss. *Eur Arch Otorhinolaryngol*. 2008;265(10):1289-1292.

93. Dogu F, Ikinciogullari A, Fricker D, et al. A novel mutation for TAP deficiency and its possible association with Toxoplasmosis. *Parasitol Int*. 2006;55(3):219-222.

94. Parissiadis A, Dormoy A, Fricker D, et al. Unilateral necrotising toxoplasmic retinochoroiditis as the main clinical manifestation of a peptide transporter (TAP) deficiency. *Br J Ophthalmol* . 2005;89(12): 1661-1662.

95. Hanna J, Mussaffi H, Steuer G, et al. Functional aberrant expression of CCR2 receptor on chronically activated NK cells in patients with TAP-2 deficiency. *Blood*. 2005;106(10):3465-3473.

96. White PC, Chaplin DD, Weis JH, et al. Two steroid 21-hydroxylase genes are located in the murine S region. *Nature*. 1984;312(5993): 465-467.

97. White PC, New MI, Dupont B. HLA-linked congenital adrenal hyperplasia results from a defective gene encoding a cytochrome P-450 specific for steroid 21-hydroxylation. *Proc Natl Acad Sci U S A*. 1984; 81(23):7505-7509.

98. Lawton P, Nelson J, Tizard R, et al. Characterization of the mouse lymphotoxin-beta gene. *J Immunol* . 1995;154(1):239-246.

99. Browning JL, Ngam-ek A, Lawton P, et al. Lymphotoxin beta, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. *Cell*. 1993;72(6):847-856.

100. Iraqi F, Teale A. Polymorphisms in the Tnfa gene of different inbred mouse strains. *Immunogenetics*. 1999;49(3):242-245.

101. Vales-Gomez M, Reyburn HT, Erskine RA, et al. Differential binding to HLA-C of p50-activating and p58-inhibitory natural killer cell receptors. *Proc Natl Acad Sci U S A* . 1998;95(24):14326-14331.

102. Vales-Gomez M, Reyburn HT, Mandelboim M, et al. Kinetics of interaction of HLA-C ligands with natural killer cell inhibitory receptors. *Immunity*. 1998;9(3):337-344.

103. Fan QR, Garboczi DN, Winter CC, et al. Direct binding of a soluble natural killer cell inhibitory receptor to a soluble human leukocyte antigen-Cw4 class I major histocompatibility complex molecule. *Proc Natl Acad Sci U S A* . 1996;93(14):7178-7183.

104. Boyington JC, Motyka SA, Schuck P, et al. Crystal structure of an NK cell immunoglobulin-like receptor in complex with its class I MHC ligand. *Nature*. 2000;405(6786):537-543.

105. Fan QR, Long EO,Wiley DC. Crystal structure of the human natural killer cell inhibitory receptor KIR2DL1-HLA-Cw4 complex. *Nat Immunol* . 2001;2(5):452-460.

106. Vivian JP, Duncan RC, Berry R, et al. Killer cell immunoglobulin-like receptor 3DL1-mediated recognition of human leukocyte antigen B. *Nature*. 2011;479(7373):401-405.

107. Braud VM, Allan DS, Wilson D, et al. TAP- and tapasin-dependent HLA-E surface expression correlates with the binding of an MHC class I leader peptide. *Curr Biol* . 1998;8(1):1-10.

108. Braud V, Jones EY, McMichael A. The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. *Eur J Immunol* . 1997;27(5):1164-1169.

109. Aldrich CJ, DeCloux A, Woods AS, et al. Identification of a Tapdependent leader peptide recognized by alloreactive T cells specific for a class Ib antigen. *Cell*. 1994;79(4):649-658.

110. Soloski MJ, DeCloux A, Aldrich CJ, et al. Structural and functional characteristics of the class IB molecule, Qa-1. *Immunol Rev*. 1995;147:67-89.

111. Kurepa Z, Hasemann CA, Forman J. Qa-1b binds conserved class I leader peptides derived from several mammalian species. *J Exp Med*. 1998;188(5):973-978.

112. King A, Hiby SE, Gardner L, et al. Recognition of trophoblast HLA class I molecules by decidual NK cell receptors—a review. *Placenta*. 2000;21(suppl A):S81-85.

113. Deenik W, Janssen JJ, van der Holt B, et al. Efficacy of escalated imatinib combined with cytarabine in newly diagnosed patients with chronic myeloid leukemia. *Haematologica*. 2010;95(6):914-921.

114. Lowen LC, Aldrich CJ, Forman J. Analysis of T cell receptors specific for recognition of class IB antigens. *J Immunol*. 1993;151(11):6155-6165.

115. Le Bouteiller P, Solier C, Proll J, et al. Placental HLA-G protein expression in vivo: where and what for? *Hum Reprod Update*. 1999;5(3): 223-233.

116. Le Bouteiller P, Lenfant F. Antigen-presenting function(s) of the nonclassical HLA-E, -F and -G class I molecules: the beginning of a story. *Res Immunol*. 1996;147(5):301-313.

117. Fournel S, Aguerre-Girr M, Huc X, et al. Cutting edge: soluble HLA-G1 triggers CD95/CD95 ligand-mediated apoptosis in activated CD8+ cells by interacting with CD8. *J Immunol*. 2000;164(12):6100-6104.

118. Mercier B, Mura C, Ferec C. Putting a hold on “HLA-H.” *Nat Gen*. 1997; 15(3):234.

119. Bodmer JG, Parham P, Albert ED, et al. Putting a hold on “HLA-H.” The WHO Nomenclature Committee for Factors of the HLA System. *Nat Gen*. 1997;15(3):234-235.

120. Gerhard GS, Ten Elshof AE, Chorney MJ. Hereditary haemochromatosis as an immunological disease. *Br J Haematol* . 1998;100(2):247-255.

121. Gerhard GS, Levin KA, Price Goldstein J, et al. Vibrio vulnificus septicemia in a patient with the hemochromatosis HFE C282Y mutation. *Arch Pathol Lab Med*. 2001;125(8):1107-1109.

122. Feder JN, Gnirke A, Thomas W, et al. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat Gen*. 1996;13(4):399-408.

123. Mura C, Le Gac G, Raguenes O, et al. Relation between HFE mutations and mild iron-overload expression. *Mol Gen Metabol*. 2000;69(4): 295-301.

124. Hanson EH, Shue PM, Palm-Leis A, et al. An aviator with cardiomyopathy and genetic susceptibility to hereditary hemochromatosis: a case report. *Aviat Space Environ Med*. 2001;72(10):924-927.

125. Fleming RE, Ahmann JR, Migas MC, et al. Targeted mutagenesis of the murine transferrin receptor-2 gene produces hemochromatosis. *Proc Natl Acad Sci U S A* . 2002;99(16):10653-10658.

126. Moffett A, Hiby SE. How does the maternal immune system contribute to the development of pre-eclampsia? *Placenta*. 2007;28(suppl A): S51-56.

127. Vincek V, Nizetic D, Golubic M, et al. Evolutionary expansion of Mhc class I loci in the mole-rat, Spalax ehrenbergi. *Mol Biol Evol* . 1987;4(5):483-491.

128. Gunther E, Walter L. The major histocompatibility complex of the rat (Rattus norvegicus). *Immunogenetics*. 2001;53(7):520-542.

129. Lawlor DA, Zemmour J, Ennis PD, et al. Evolution of class-I MHC genes and proteins: from natural selection to thymic selection. *Ann Rev Immunol*. 1990;8:23-63.

130. Bodmer WF. HLA structure and function: a contemporary view. *Tissue Antigens*. 1981;17(1):9-20.

131. Shiina T, Shimizu S, Hosomichi K, et al. Comparative genomic analysis of two avian (quail and chicken) MHC regions. *J Immunol*. 2004;172(11):6751-6763.

132. Pease LR, Nathenson SG, Leinwand LA. Mapping class I gene sequences in the major histocompatibility complex. *Nature*. 1982;298(5872):382-385.

133. Steinmetz M, Minard K, Horvath S, et al. A molecular map of the immune response region from the major histocompatibility complex of the mouse. *Nature*. 1982;300(5887):35-42.

134. Margulies DH, Evans GA, Flaherty L, et al. H-2-like genes in the Tla region of mouse chromosome 17. *Nature*. 1982;295(5845):168-170.

135. Shawar SM, Vyas JM, Rodgers JR, et al. Antigen presentation by major histocompatibility complex class I-B molecules. *Ann Rev Immunol*. 1994;12:839-880.

136. Kurlander RJ, Shawar SM, Brown ML, et al. Specialized role for a murine class I-b MHC molecule in prokaryotic host defenses. *Science*. 1992;257(5070):678-679.

137. Lindahl KF, Byers DE, Dabhi VM, et al. H2-M3, a full-service class Ib histocompatibility antigen. *Ann Rev Immunol*. 1997;15:851-879.

138. Walter L, Gunther E. Physical mapping and evolution of the centromeric class I gene-containing region of the rat MHC. *Immunogenetics*. 2000;51(10):829-837.

139. Gunther E, Walter L. Comparative genomic aspects of rat, mouse and human MHC class I gene regions. *Cytogenet Cell Genet*. 2000;91(1-4): 107-112.

140. Moore CB, John M, James IR, et al. Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science*. 2002; 296(5572):1439-1443.

141. Michael NL. Host genetics and HIV—removing the mask. *Nat Med*. 2002;8(8):783-785.

142. Martin MP, Gao X, Lee JH, et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Gen*. 2002;31(4): 429-434.

143. Zernich D, Purcell AW, Macdonald WA, et al. Natural HLA class I polymorphism controls the pathway of antigen presentation and susceptibility to viral evasion. *J Exp Med*. 2004;200(1):13-24.

144. Figueroa F, Tichy H, McKenzie I, et al. Polymorphism of lymphocyte antigens-encoding loci in wild mice. *Curr Top Microbiol Immunol*. 1986;127:229-235.

145. Mitchison NA, Mayer W. A survey of H2 gene sequences, including new wild-derived genes. *Int J Immunogen*. 2007;34(1):3-12.

146. Sultmann H, Mayer WE, Figueroa F, et al. Zebrafish Mhc class II alpha chain-encoding genes: polymorphism, expression, and function. *Immunogenetics*. 1993;38(6):408-420.

147. Sato A, Klein D, Sultmann H, et al. Class I mhc genes of cichlid fishes: identification, expression, and polymorphism. *Immunogenetics*. 1997; 46(1):63-72.

148. Sato A, Dongak R, Hao L, et al. Mhc class I genes of the cichlid fish Oreochromis niloticus. *Immunogenetics*. 2006;58(11):917-928.

149. Murray BW, Shintani S, Sultmann H, et al. Major histocompatibility complex class II A genes in cichlid fishes: identification, expression, linkage relationships, and haplotype

variation. *Immunogenetics*. 2000;51(7):576-586.

150. Williams AF. Immunoglobulin-related domains for cell surface recognition. *Nature*. 1985;314(6012):579-580.

151. Hood L, Steinmetz M, Malissen B. Genes of the major histocompatibility complex of the mouse. *Annu Rev Immunol* . 1983;1:529-568.

152. Lawlor DA, Warren E, Ward FE, et al. Comparison of class I MHC alleles in humans and apes. *Immunol Rev*. 1990;113:147-185.

153. Sun YH, Goodenow RS, Hood L. Molecular basis of the dm1 mutation in the major histocompatibility complex of the mouse: a D/L hybrid gene. *J Exp Med*. 1985;162(5):1588-1602.

154. Burnside SS, Hunt P, Ozato K, et al. A molecular hybrid of the H-2Dd and H-2Ld genes expressed in the dm1 mutant. *Proc Natl Acad Sci U S A* . 1984;81(16):5204-5208.

155. Ohta T. Gene conversion vs point mutation in generating variability at the antigen recognition site of major histocompatibility complex loci. *J Mol Evol* . 1995;41(2):115-119.

156. Evans GA, Margulies DH, Camerini-Otero RD, et al. Structure and expression of a mouse major histocompatibility antigen gene, H-2Ld. *Proc Natl Acad Sci U S A* . 1982;79(6):1994-1998.

157. Schulze DH, Pease LR, Geier SS, et al. Comparison of the cloned H-2Kbm1 variant gene with the H-2Kb gene shows a cluster of seven nucleotide differences. *Proc Natl Acad Sci U S A*. 1983;80(7): 2007-2011.

158. Geliebter J, Zeff RA, Schulze DH, et al. Interaction between Kb and Q4 gene sequences generates the Kbm6 mutation. *Mol Cell Biol* . 1986; 6(2):645-652.

159. Nathenson SG, Geliebter J, Pfaffenbach GM, et al. Murine major histocompatibility complex class-I mutants: molecular analysis and structurefunction implications. *Annu Rev Immunol*. 1986;4:471-502.

160. Nathenson SG, Kesari K, Sheil JM, et al. Use of mutants to analyze regions on the H-2Kb molecule for interaction with immune receptors. *Cold Spring Harb Symp Quant Biol*. 1989;54(pt 1):521-528.

161. Hasenkrug KJ, Nathenson SG. Nucleic acid sequences of the H-2Ks and H-2Ksm1 genes. *Immunogenetics*. 1991;34(1):60-61.

162. Reusch TB, Schaschl H, Wegner KM. Recent duplication and inter-locus gene conversion in major histocompatibility class II genes in a teleost, the three-spined stickleback. *Immunogenetics*. 2004;56(6):427-437.

163. Spurgin LG, van Oosterhout C, Illera JC, et al. Gene conversion rapidly generates major histocompatibility complex diversity in recently founded bird populations. *Mol Ecol* . 2011;20(24):5213-5225.

164. Marsh SG. Nomenclature for factors of the HLA system, update September 2011. *Tissue Antigens*. 2011;78(6):482-484.

165. Rudolph MG, Speir JA, Brunmark A, et al. The crystal structures of K(bm1) and K(bm8) reveal that subtle changes in the peptide environment impact thermostability and alloreactivity. *Immunity*. 2001; 14(3):231-242.

166. Witte T, Smolyar A, Spoerl R, et al. Major histocompatibility complex recognition by immune receptors: differences among T cell receptor versus antibody interactions with

the VSV8/H-2Kb complex. *Eur J Immunol* . 1997;27(1):227-233.

167. Snell GD, Dausset J, Nathenson S. *Histocompatibility*. New York, NY: Academic Press; 1976:401.

168. Silver LM. *Mouse Genetics: Concepts and Applications.* New York, NY: Oxford University Press; 1995:362.

169. den Haan JM, Meadows LM, Wang W, et al. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. *Science*. 1998;279(5353):1054-1057.

170. Simpson E. Minor transplantation antigens: mouse models for human host-versus-graft, graft-versus-host and graft-versus-leukemia reactions. *Arch Immunol Ther Exp (Warsz)*. 1998;46(6):331-339.

171. Simpson E, Scott D, James E, et al. Minor H antigens: genes and peptides. *Transpl Immunol*. 2002;10(2-3):115-123.

172. Mendoza LM, Villaflor G, Eden P, et al. Distinguishing self from nonself: immunogenicity of the murine H47 locus is determined by a single amino acid substitution in an unusual peptide. *J Immunol*. 2001;166(7):4438-4445.

173. Malarkannan S, Shih PP, Eden PA, et al. The molecular and functional characterization of a dominant minor H antigen, H60. *J Immunol*. 1998;161(7):3501-3509.

174. Schwab SR, Li KC, Kang C, et al. Constitutive display of cryptic translation products by MHC class I molecules. *Science*. 2003;301(5638): 1367-1371.

175. Yewdell JW, Anton LC, Bennink JR. Defective ribosomal products (DRiPs): a major source of antigenic peptides for MHC class I molecules? *J Immunol*. 1996;157(5):1823-1826.

176. Eisenlohr LC, Huang L, Golovina TN. Rethinking peptide supply to MHC class I molecules. *Nat Rev Immunol* . 2007;7(5):403-410.

177. Goulmy E. Minor histocompatibility antigens: from transplantation problems to therapy of cancer. *Hum Immunol*. 2006;67(6):433-438.

178. Shevach EM, Rosenthal AS. Function of macrophages in antigen recognition by guinea pig T lymphocytes. II. Role of the macrophage in the regulation of genetic control of the immune response. *J Exp Med*. 1973;138(5):1213-1229.

179. Zinkernagel RM, Dunlop MB, Blanden RV, et al. H-2 compatibility requirement for virus-specific T-cell-mediated cytolysis. Evaluation of the role of H-2I region and non-H-2 genes in regulating immune response. *J Exp Med*. 1976;144(2):519-532.

180. Zinkernagel RM, Doherty PC. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature*. 1974;248(450):701-702.

181. Shearer GM, Rehn TG, Garbarino CA. Cell-mediated lympholysis of trinitrophenyl-modified autologous lymphocytes. Effector cell specificity to modified cell surface components controlled by H-2K and H-2D serological regions of the murine major histocompatibility complex. *J Exp Med*. 1975;141(6):1348-1364.

182. Lindahl KF, Wilson DB. Histocompatibility antigen-activated cytotoxic T lymphocytes. I. Estimates of the absolute frequency of killer cells generated in vitro. *J Exp Med*. 1977;145(3):500-507.

183. Matzinger P, Bevan MJ. Hypothesis: why do so many lymphocytes respond to major histocompatibility antigens? *Cell Immunol*. 1977;29(1):1-5.

184. Maryanski JL, MacDonald HR, Cerottini JC. Limiting dilution analysis of alloantigen-reactive T lymphocytes. IV. High frequency of cytolytic T lymphocyte precursor cells in MLC blasts separated by velocity sedimentation. *J Immunol*. 1980;124(1):42-47.

185. Dalchau R, Fangmann J, Fabre JW. Allorecognition of isolated, denatured chains of class I and class II major histocompatibility complex molecules. Evidence for an important role for indirect allorecognition in transplantation. *Eur J Immunol* . 1992;22(3):669-677.

186. Auchincloss H Jr, Sultan H. Antigen processing and presentation in transplantation. *Curr Opin Immunol* . 1996;8(5):681-687.

187. Tiercy JM. Molecular basis of HLA polymorphism: implications in clinical transplantation. *Transplant Immunol*. 2002;9(2-4):173-180.

188. Hansen JA, Yamamoto K, Petersdorf E, et al. The role of HLA matching in hematopoietic cell transplantation. *Rev Immunogen*. 1999;1(3): 359-373.

189. Mickelson EM, Petersdorf E, Anasetti C, et al. HLA matching in hematopoietic cell transplantation. *Human Immunol*. 2000;61(2):92-100.

190. Hansen JA, Petersdorf E, Martin PJ, et al. Impact of HLA matching on hematopoietic cell transplants from unrelated donors. *Vox Sanguinis*. 2000;78(suppl 2):269-271.

191. Petersdorf EW, Gooley T, Malkki M, et al. The biological significance of HLA-DP gene variation in haematopoietic cell transplantation. *Br J Haematol* . 2001;112(4):988-994.

192. Varney MD, Lester S, McCluskey J, et al. Matching for HLA DPA1 and DPB1 alleles in unrelated bone marrow transplantation. *Human Immunol*. 1999;60(6):532-538.

193. Morishima Y, Sasazuki T, Inoko H, et al. The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients receiving a marrow transplant from serologically HLA-A, HLA-B, and HLA-DR matched unrelated donors. *Blood*. 2002;99(11):4200-4206.

194. Petersdorf EW, Hansen JA, Martin PJ, et al. Major-histocompatibilitycomplex class I alleles and antigens in hematopoietic-cell transplantation. *N Engl J Med*. 2001;345(25):1794-1800.

195. Bray RA, Hurley CK, Kamani NR, et al. National marrow donor program HLA matching guidelines for unrelated adult donor hematopoietic cell transplants. *Biol Blood Marrow Transplant*. 2008;14(9) (suppl):45-53.

196. Gluckman E, Rocha V, Chastang C. Peripheral stem cells in bone marrow transplantation. Cord blood stem cell transplantation. Bailliere's best practice & research. *Clin Haematol*. 1999;12(1-2):279-292.

197. Barker JN, Krepski TP, DeFor TE, et al. Searching for unrelated donor hematopoietic stem cells: availability and speed of umbilical cord blood versus bone marrow. *Biol Blood Marrow Transplant* . 2002;8(5): 257-260.

198. Rubinstein P. HLA matching for bone marrow transplantation—how much is enough? *N Engl J Med* . 2001;345(25):1842-1844.

199. Hakenberg P, Kogler G, Wernet P. NETCORD: a cord blood allocation network. *Bone Marrow Transplant*. 1998;22(suppl 1):S17-18.

200. Petersdorf EW, Anasetti C, Martin PJ, et al. Limits of HLA mismatching in unrelated hematopoietic cell transplantation. *Blood*. 2004; 104(9):2976-2980.

201. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. 2002;295(5562):2097-2100.

202. Ruggeri L, Capanni M, Casucci M, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood*. 1999;94(1):333-339.

203. Claas FH, Oudshoorn M. Role of NK cells in mismatched unrelated haematopoietic stem-cell transplantation: fact or fiction? *Tissue Antigens*. 2005;65(6):515-518.

204. Willemze R, Ruggeri A, Purtill D, et al. Is there an impact of killer cell immunoglobulin-like receptors and KIR-ligand incompatibilities on outcomes after unrelated cord blood stem cell transplantation? *Best Pract Res Clin Haematol* . 2010;23(2):283-290.

205. Velardi A, Ruggeri L, Mancusi A, et al. Natural killer cell allorecognition of missing self in allogeneic hematopoietic transplantation: a tool for immunotherapy of leukemia. *Curr Opin Immunol* . 2009;21(5): 525-530.

206. Brunstein CG, Wagner JE, Weisdorf DJ, et al. Negative effect of KIR alloreactivity in recipients of umbilical cord blood transplant depends on transplantation conditioning intensity. *Blood*. 2009;113(22): 5628-5634.

207. Gluckman E, Rocha V, Chevret S. Results of unrelated umbilical cord blood hematopoietic stem cell transplant. *Transfus Clin Biol*. 2001; 8(3):146-154.

208. Wagner JE, Barker JN, DeFor TE, et al. Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. *Blood*. 2002;100(5):1611-1618.

209. Doxiadis II, Smits JM, Schreuder GM, et al. Association between specific HLA combinations and probability of kidney allograft loss: the taboo concept. *Lancet*. 1996;348(9031):850-853.

210. Doxiadis II, Smits JM, Stobbe I, et al. Taboo HLA mismatches in cadaveric renal transplantation: definition, analysis, and possible implications. *Transplant Proc*. 1996;28(1):224.

211. Mickelson EM, Longton G, Anasetti C, et al. Evaluation of the mixed lymphocyte culture (MLC) assay as a method for selecting unrelated donors for marrow transplantation. *Tissue Antigens*. 1996;47(1):27-36.

212. Pei J, Akatsuka Y, Anasetti C, et al. Generation of HLA-C-specific cytotoxic T cells in association with marrow graft rejection: analysis of alloimmunity by T-cell cloning and testing of T-cell-receptor rearrangements. *Biol Blood Marrow Transplant* . 2001;7(7):378-383.

213. Wang XN, Taylor PR, Skinner R, et al. T-cell frequency analysis does not predict the incidence of graft-versus-host disease in HLA-matched sibling bone marrow transplantation. *Transplantation*. 2000;70(3): 488-493.

214. Kaminski E, Hows J, Man S, et al. Prediction of graft versus host disease by frequency analysis of cytotoxic T cells after unrelated donor bone marrow transplantation. *Transplantation*. 1989;48(4):608-613.

215. Oudshoorn M, Doxiadis II, van den Berg-Loonen PM, et al. Functional versus structural matching: can the CTLp test be replaced by HLA allele typing? *Human*

Immunol. 2002;63(3):176-184.

216. Nepom GT, Erlich H. MHC class-II molecules and autoimmunity. *Annu Rev Immunol*. 1991;9:493-525.

217. Thomson G. HLA disease associations: models for the study of complex human genetic disorders. *Crit Rev Clin Lab Sci*. 1995;32(2):183-219.

218. Baines M, Ebringer A. HLA and disease. *Mol Aspects Med*. 1992; 13(4):263-378.

219. Malkki M, Single R, Carrington M, et al. MHC microsatellite diversity and linkage disequilibrium among common HLA-A, HLA-B, DRB1 haplotypes: implications for unrelated donor hematopoietic transplantation and disease association studies. *Tissue Antigens*. 2005;66(2): 114-124.

220. Shiina T, Inoko H, Kulski JK. An update of the HLA genomic region, locus information and disease associations: 2004. *Tissue Antigens*. 2004;64(6):631-649.

221. Lechler R, Warrens A. *HLA in Health and Disease*. 2nd ed. San Diego, CA: Academic Press; 2000.

222. Tiwari JL, Terasaki PI. *HLA and Disease Associations*. New York, NY: Springer-Verlag; 1985.

223. Zhernakova A, Stahl EA, Trynka G, et al. Meta-analysis of genomewide association studies in celiac disease and rheumatoid arthritis identifies fourteen non-HLA shared loci. *PLoS Gen*. 2011;7(2):e1002004.

224. Barrett JC, Clayton DG, Concannon P, et al. Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes. *Nat Gen*. 2009;41(6):703-707.

225. Oldstone MB. Virus-induced autoimmunity: molecular mimicry as a route to autoimmune disease. *J Autoimmunity*. 1989;2(suppl): 187-194.

226. Raychaudhuri S, Sandor C, Stahl EA, et al. Five amino acids in three HLA proteins explain most of the association between MHC and seropositive rheumatoid arthritis. *Nat Gen*. 2012;44(3):291-296.

227. Oldstone MB, von Herrath M. Virus-induced autoimmune disease: transgenic approach to mimic insulin-dependent diabetes mellitus and other autoimmune diseases. *APMIS*. 1996;104(10):689-697.

228. Oldstone MB. Molecular mimicry and autoimmune disease. *Cell*. 1987;50(6):819-820.

229. Wucherpfennig KW. Structural basis of molecular mimicry. *J Autoimmunity*. 2001;16(3):293-302.

230. Mignot E, Tafti M, Dement WC, et al. Narcolepsy and immunity. *Adv Neuroimmunol*. 1995;5(1):23-37.

231. Ellis MC, Hetisimer AH, Ruddy DA, et al. HLA class II haplotype and sequence analysis support a role for DQ in narcolepsy. *Immunogenetics*. 1997;46(5):410-417.

232. Ringrose JH, Muijsers AO, Pannekoek Y, et al. Influence of infection of cells with bacteria associated with reactive arthritis on the peptide repertoire presented by HLA-B27. *J Med Microbiol*. 2001;50(4):385-389.

233. Ringrose JH. HLA-B27 associated spondyloarthropathy, an autoimmune disease

based on crossreactivity between bacteria and HLA-B27? *Ann Rheum Dis*. 1999;58(10):598-610.

234. Evans DM, Spencer CC, Pointon JJ, et al. Interaction between ERAP1 and HLA-B27 in ankylosing spondylitis implicates peptide handling in the mechanism for HLA-B27 in disease susceptibility. *Nat Gen*. 2011;43(8):761-767.

235. Kollnberger S, Bird LA, Roddis M, et al. HLA-B27 heavy chain homodimers are expressed in HLA-B27 transgenic rodent models of spondyloarthritis and are ligands for paired Ig-like receptors. *J Immunol* . 2004;173(3):1699-1710.

236. Bird LA, Peh CA, Kollnberger S, et al. Lymphoblastoid cells express HLA-B27 homodimers both intracellularly and at the cell surface following endosomal recycling. *Eur J Immunol* . 2003;33(3): 748-759.

237. Bennett MJ, Lebron JA, Bjorkman PJ. Crystal structure of the hereditary haemochromatosis protein HFE complexed with transferrin receptor. *Nature*. 2000;403(6765):46-53.

238. Lebron JA, Bennett MJ, Vaughn DE, et al. Crystal structure of the hemochromatosis protein HFE and characterization of its interaction with transferrin receptor. *Cell*. 1998;93(1):111-123.

239. Trinder D, Olynyk JK, Sly WS, et al. Iron uptake from plasma transferrin by the duodenum is impaired in the Hfe knockout mouse. *Proc Natl Acad Sci U S A* . 2002;99(8):5622-5626.

240. Waheed A, Grubb JH, Zhou XY, et al. Regulation of transferrin-mediated iron uptake by HFE, the protein defective in hereditary hemochromatosis. *Proc Natl Acad Sci U S A* . 2002;99(5):3117-3122.

241. Zhou XY, Tomatsu S, Fleming RE, et al. HFE gene knockout produces mouse model of hereditary hemochromatosis. *Proc Natl Acad Sci U S A* . 1998;95(5):2492-2497.

242. Santos M, Clevers HC, Marx JJ. Mutations of the hereditary hemochromatosis candidate gene HLA-H in porphyria cutanea tarda. *N Engl J Med* . 1997;336(18):1327-1328.

243. Santos M, Schilham MW, Rademakers LH, et al. Defective iron homeostasis in beta 2-microglobulin knockout mice recapitulates hereditary hemochromatosis in man. *J Exp Med*. 1996;184(5):1975-1985.

244. Rothenberg BE, Voland JR. beta2 knockout mice develop parenchymal iron overload: a putative role for class I genes of the major histocompatibility complex in iron metabolism. *Proc Natl Acad Sci U S A* . 1996;93(4):1529-1534.

245. Fleming RE, Migas MC, Zhou X, et al. Mechanism of increased iron absorption in murine model of hereditary hemochromatosis: increased duodenal expression of the iron transporter DMT1. *Proc Natl Acad Sci U S A* . 1999;96(6):3143-3148.

246. Consortium TUH. A simple genetic test identifies 90% of UK patients with haemochromatosis. *Gut*. 1997;41(6):841-844.

247. Lester S, Bardy P, McCluskey J. HFE genotypes and haemochromatosis: quantifying the risks of disease. *Tissue Antigens*. 1999;54(3):282-284.

248. Wordsworth P. Rheumatoid arthritis. *Curr Opin Immunol* . 1992; 4(6):766-769.

249. Wordsworth P, Pile KD, Buckely JD, et al. HLA heterozygosity contributes to susceptibility to rheumatoid arthritis. *Am J Hum Gen*. 1992; 51(3):585-591.

250. Hall FC, Weeks DE, Camilleri JP, et al. Influence of the HLA-DRB1 locus on susceptibility and severity in rheumatoid arthritis. *QJM*. 1996; 89(11):821-829.

251. Hattori M, Buse JB, Jackson RA, et al. The NOD mouse: recessive diabetogenic gene in the major histocompatibility complex. *Science*. 1986; 231(4739):733-735.

252. Acha-Orbea H, McDevitt HO. The first external domain of the nonobese diabetic mouse class II I-A beta chain is unique. *Proc Natl Acad Sci U S A* . 1987;84(8):2435-2439.

253. Atkinson MA, Leiter EH. The NOD mouse model of type 1 diabetes: as good as it gets? *Nat Med*. 1999;5(6):601-604.

254. Wicker LS, Miller BJ, Fischer PA, et al. Genetic control of diabetes and insulitis in the nonobese diabetic mouse. Pedigree analysis of a diabetic H-2nod/b heterozygote. *J Immunol*. 1989;142(3):781-784.

255. Quartey-Papafio R, Lund T, Chandler P, et al. Aspartate at position 57 of nonobese diabetic I-Ag7 beta-chain diminishes the spontaneous incidence of insulin-dependent diabetes mellitus. *J Immunol*. 1995; 154(10):5567-5575.

256. Tisch R, McDevitt H. Insulin-dependent diabetes mellitus. *Cell*. 1996; 85(3):291-297.

257. Noble JA, Valdes AM. Genetics of the HLA region in the prediction of type 1 diabetes. *Curr Diabetes Rep*. 2011;11(6):533-542.

258. Undlien DE, Thorsby E. HLA associations in type 1 diabetes: merging genetics and immunology. *Trends Immunol*. 2001;22(9):467-469.

259. Undlien DE, Lie BA, Thorsby E. HLA complex genes in type 1 diabetes and other autoimmune diseases. Which genes are involved? *TIG*. 2001;17(2):93-100.

260. Lechler R, Warrens A. *Handbook of HLA and Disease* . 2nd ed. London, United Kingdom: Academic Pres; 1998.

261. Morton NE, Green A, Dunsworth T, et al. Heterozygous expression of insulin-dependent diabetes mellitus (IDDM) determinants in the HLA system. *Am J Hum Gen*. 1983;35(2):201-213.

262. Brorsson C, Tue Hansen N, Bergholdt R, et al. The type 1 diabetes - HLA susceptibility interactome—identification of HLA genotype-specific disease genes for type 1 diabetes. *PloS One*. 2010;5(3):e9576.

263. Green PH, Cellier C. Celiac disease. *N Engl J Med*. 2007;357(17): 1731-1743.

264. Vilppula A, Kaukinen K, Luostarinen L, et al. Increasing prevalence and high incidence of celiac disease in elderly people: a populationbased study. *BMC Gastroenterol*. 2009;9:49.

265. Spurkland A, Celius EG, Knutsen I, et al. The HLA-DQ(alpha 1*0102, beta 1*0602) heterodimer may confer susceptibility to multiple sclerosis in the absence of the HLA-DR(alpha 1*01, beta 1*1501) heterodimer. *Tissue Antigens*. 1997;50(1):15-22.

266. Anderson RP, Degano P, Godkin AJ, et al. In vivo antigen challenge in celiac disease identifies a single transglutaminase-modified peptide as the dominant A-gliadin T-cell epitope. *Nat Med*. 2000;6(3):337-342.

267. Arentz-Hansen H, Korner R, Molberg O, et al. The intestinal T cell response to alpha-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. *J Exp Med*. 2000;191(4):603-612.

268. Kim CY, Quarsten H, Bergseng E, et al. Structural basis for HLA-DQ2-mediated presentation of gluten epitopes in celiac disease. *Proc Natl Acad Sci U S A* . 2004;101(12):4175-4179.

269. Tye-Din JA, Stewart JA, Dromey JA, et al. Comprehensive, quantitative mapping of T cell epitopes in gluten in celiac disease. *Sci Transl Med* . 2010;2(41):41ra51.

270. van de Wal Y, Kooy Y, van Veelen P, et al. Selective deamidation by tissue transglutaminase strongly enhances gliadin-specific T cell reactivity. *J Immunol* . 1998;161(4):1585-1588.

271. Henderson KN, Tye-Din JA, Reid HH, et al. A structural and immunological basis for the role of human leukocyte antigen DQ8 in celiac disease. *Immunity*. 2007;27(1):23-34.

272. Tye-Din J, Anderson R. Immunopathogenesis of celiac disease. *Curr Gastroenterol Rep*. 2008;10(5):458-465.

273. Bharadwaj M, Illing P, Theodossis A, et al. Drug hypersensitivity and human leukocyte antigens of the major histocompatibility complex. *Ann Rev Pharmacol Toxicol* . 2012;52:401-431.

274. Hetherington S, Hughes AR, Mosteller M, et al. Genetic variations in HLA-B region and hypersensitivity reactions to abacavir. *Lancet*. 2002;359(9312):1121-1122.

275. Mallal S, Nolan D, Witt C, et al. Association between presence of HLA-B* 5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reversetranscriptase inhibitor abacavir. *Lancet*. 2002;359(9308):727-732.

276. Martin AM, Nolan D, Gaudieri S, et al. Predisposition to abacavir hypersensitivity conferred by HLA-B*5701 and a haplotypic Hsp70-Hom variant. *Proc Natl Acad Sci U S A*. 2004;101(12):4180-4185.

277. Chung WH, Hung SI, Hong HS, et al. Medical genetics: a marker for Stevens-Johnson syndrome. *Nature*. 2004;428(6982):486.

278. Hung SI, Chung WH, Jee SH, et al. Genetic susceptibility to carbamazepine-induced cutaneous adverse drug reactions. *Pharmacogenet Genomics*. 2006;16(4):297-306.

279. Locharernkul C, Loplumlert J, Limotai C, et al. Carbamazepine and phenytoin induced Stevens-Johnson syndrome is associated with HLA-B* 1502 allele in Thai population. *Epilepsia*. 2008;49(12):2087-2091.

280. Hung SI, Chung WH, Liou LB, et al. HLA-B*5801 allele as a genetic marker for severe cutaneous adverse reactions caused by allopurinol. *Proc Natl Acad Sci U S A* . 2005;102(11):4134-4139.

281. Tassaneeyakul W, Jantararoungtong T, Chen P, et al. Strong association between HLA-B*5801 and allopurinol-induced Stevens-Johnson syndrome and toxic epidermal necrolysis in a Thai population. *Pharmacogenet Genomics*. 2009;19(9):704-709.

282. Kaniwa N, Saito Y, Aihara M, et al. HLA-B locus in Japanese patients with anti-epileptics and allopurinol-related Stevens-Johnson syndrome and toxic epidermal necrolysis. *Pharmacogenomics*. 2008;9(11):1617-1622.

283. Phillips EJ, Sullivan JR, Knowles SR, et al. Utility of patch testing in patients with hypersensitivity syndromes associated with abacavir. *AIDS*. 2002;16(16):2223-2225.

284. Nassif A, Bensussan A, Dorothee G, et al. Drug specific cytotoxic T-cells in the skin lesions of a patient with toxic epidermal necrolysis. *J Invest Dermatol* . 2002;118(4):728-733.

285. Hari Y, Frutig-Schnyder K, Hurni M, et al. T cell involvement in cutaneous drug eruptions. *Clin Exp Allergy*. 2001;31(9):1398-1408.

286. Le Cleach L, Delaire S, Boumsell L, et al. Blister fluid T lymphocytes during toxic epidermal necrolysis are functional cytotoxic cells which express human natural killer (NK) inhibitory receptors. *Clin Exp Immunol*. 2000;119(1):225-230.

287. Chessman D, Kostenko L, Lethborg T, et al. Human leukocyte antigen class I-restricted activation of CD8+ T cells provides the immunogenetic basis of a systemic drug hypersensitivity. *Immunity*. 2008; 28(6):822-832.

287a. Illing PT, Vivian JP, Dudek NL, et al. Immune self-reactivity triggered by drug-modified HLA-peptide repertoire. *Nature*. 2012;486:554-558.

288. Mallal S, Phillips E, Carosi G, et al. HLA-B*5701 screening for hypersensitivity to abacavir. *N Engl J Med*. 2008;358(6):568-579.

289. Carrington M, Martin MP, van Bergen J. KIR-HLA intercourse in HIV disease. *Trends Microbiol*. 2008;16(12):620-627.

290. Qi Y, Martin MP, Gao X, et al. KIR/HLA pleiotropism: protection against both HIV and opportunistic infections. *PLoS Pathog*. 2006;2(8):e79.

291. Zeniya M, Takahashi H. Characteristics of autoimmune hepatitis in the Asia-Pacific Region: historical review. *Hepatol Int*. 2011. Epub ahead of print.

292. Elfaramawy AA, Elhossiny RM, Abbas AA, et al. HLA-DRB1 as a risk factor in children with autoimmune hepatitis and its relation to hepatitis A infection. *Ital J Pediatr*. 2010;36:73.

293. Wawrzynowicz-Syczewska M, Underhill JA, Clare MA et al. HLA class II genotypes associated with chronic hepatitis C virus infection and response to alpha-interferon treatment in Poland. *Liver*. 2000;20(3):234-239.

294. Kohn HI, Melvold RW. Spontaneous histocompatibility mutations detected by dermal grafts: significant changes in rate over a 10-year period in the mouse H-system. *Mutat Res*. 1974;24(2):163-169.

295. Melvold RW, Wang K, Kohn HI. Histocompatibility gene mutation rates in the mouse: a 25-year review. *Immunogenetics*. 1997;47(1):44-54.

296. Yamaga KM, Pfaffenbach GM, Pease LR, et al.. Biochemical studies of H-2K antigens from a group of related mutants. I. Identification of a shared mutation in B6-H-2bm5 and B6-H-2bm16. *Immunogenetics*. 1983;17(1):19-29.

297. Pease LR, Ewenstein BM, McGovern D, et al. Biochemical studies on the H-2K mutant B6.C-H-2bm10. *Immunogenetics*. 1983;17(1):7-17.

298. Ewenstein BM, Uehara H, Nisizawa T, et al. Biochemical studies on the H-2K antigens of the MHC mutants bm3 and bm11. *Immunogenetics*. 1980;11(4):383-395.

299. Boehm U, Klamp T, Groot M, et al. Cellular responses to interferongamma. *Annu Rev Immunol*. 1997;15:749-795.

300. Gobin SJ, van den Elsen PJ. Locus-specific regulation of HLA-A and HLA-B expression is not determined by nucleotide variation in the X2 box promoter element. *Blood*. 2001;97(5):1518-1521.

301. van den Elsen PJ, Gobin SJ. The common regulatory pathway of MHC class I and class II transactivation. *Microbes Infect*. 1999;1(11):887-892.

302. Wainwright SD, Biro PA, Holmes CH. HLA-F is a predominantly empty, intracellular, TAP-associated MHC class Ib protein with a restricted expression pattern. *J Immunol*. 2000;164(1):319-328.

303. Houlihan JM, Biro PA, Harper HM, et al. The human amnion is a site of MHC class Ib expression: evidence for the expression of HLA-E and HLA-G. *J Immunol*. 1995;154(11):5665-5674.

304. McMaster MT, Librach CL, Zhou Y, et al. Human placental HLA-G expression is restricted to differentiated cytotrophoblasts. *J Immunol*. 1995;154(8):3771-3778.

305. Gobin SJ, van den Elsen PJ. Transcriptional regulation of the MHC class Ib genes HLA-E, HLA-F, and HLA-G. *Hum Immunol*. 2000; 61(11):1102-1107.

306. Hicklin DJ, Wang Z, Arienti F, et al. beta2-Microglobulin mutations, HLA class I antigen loss, and tumor progression in melanoma. *J Clin Invest*. 1998;101(12):2720-2729.

307. Hsieh CH, Hsu YJ, Chang CC, et al. Total HLA class I loss in a sarcomatoid renal carcinoma cell line caused by the coexistence of distinct mutations in the two encoding beta2-microglobulin genes. *Cancer Immunol Immunother* . 2009;58(3):395-408.

308. Chang CC, Ogino T, Mullins DW, et al. Defective human leukocyte antigen class I-associated antigen presentation caused by a novel beta2-microglobulin loss-of-function in melanoma cells. *J Biol Chem*. 2006;281(27):18763-18773.

309. Zijlstra M, Li E, Sajjadi F, et al. Germ-line transmission of a disrupted beta 2-microglobulin gene produced by homologous recombination in embryonic stem cells. *Nature*. 1989;342(6248):435-438.

310. Koller BH, Smithies O. Inactivating the beta 2-microglobulin locus in mouse embryonic stem cells by homologous recombination. *Proc Natl Acad Sci U S A* . 1989;86(22):8932-8935.

311. Wiertz EJ, Mukherjee S, Ploegh HL. Viruses use stealth technology to escape from the host immune system. *Mol Med Today*. 1997;3(3): 116-123.

312. Fruh K, Ahn K, Peterson PA. Inhibition of MHC class I antigen presentation by viral proteins. *J Mol Med*. 1997;75(1):18-27.

313. Easterfield AJ, Austen BM, Westwood OM. Inhibition of antigen transport by expression of infected cell peptide 47 (ICP47) prevents cell surface expression of HLA in choriocarcinoma cell lines. *J Reprod Immunol* . 2001;50(1):19-40.

314. Berger C, Xuereb S, Johnson DC, et al. Expression of herpes simplex virus ICP47 and human cytomegalovirus US11 prevents recognition of transgene products by CD8(+) cytotoxic T lymphocytes. *J Virol*. 2000; 74(10):4465-4473.

315. Furukawa L, Brevetti LS, Brady SE, et al. Adenoviral-mediated gene transfer of ICP47 inhibits major histocompatibility complex class I expression on vascular cells in vitro. *J Vasc Surg*. 2000;31(3):558-566.

316. Jugovic P, Hill AM, Tomazin R, et al. Inhibition of major histocompatibility complex class I antigen presentation in pig and primate cells by herpes simplex virus type 1 and 2 ICP47. *J Virol*. 1998;72(6):5076-5084.

317. Byun M, Verweij MC, Pickup DJ, et al. Two mechanistically distinct immune evasion proteins of cowpox virus combine to avoid antiviral CD8 T cells. *Cell Host Microbe*. 2009;6(5):422-432.

318. Alzhanova D, Edwards DM, Hammarlund E, et al. Cowpox virus inhibits the transporter associated with antigen processing to evade T cell recognition. *Cell Host Microbe*. 2009;6(5):433-445.

319. de la Salle H, Hanau D, Fricker D, et al. Homozygous human TAP peptide transporter mutation in HLA class I deficiency. *Science*. 1994; 265(5169):237-241.

320. de la Salle H, Houssaint E, Peyrat MA, et al. Human peptide transporter deficiency: importance of HLA-B in the presentation of TAP-independent EBV antigens. *J Immunol*. 1997;158(10):4555-4563.

321. de la Salle H, Saulquin X, Mansour I, et al. Asymptomatic deficiency in the peptide transporter associated to antigen processing (TAP). *Clin Exp Immunol*. 2002;128(3):525-531.

322. Zimmer J, Andres E. Comments on type I bare lymphocyte syndrome. *Immunol Lett*. 2012;143(2):218-219.

323. Shrestha D, Szollosi J, Jenei A. Bare lymphocyte syndrome: an opportunity to discover our immune system. *Immunol Lett*. 2012;141(2): 147-157.

324. Rehm A, Engelsberg A, Tortorella D, et al. Human cytomegalovirus gene products US2 and US11 differ in their ability to attack major histocompatibility class I heavy chains in dendritic cells. *J Virol*. 2002; 76(10):5043-5050.

325. Gold MC, Munks MW, Wagner M, et al. The murine cytomegalovirus immunomodulatory gene m152 prevents recognition of infected cells by M45-specific CTL but does not alter the immunodominance of the M45-specific CD8 T cell response in vivo. *J Immunol*. 2002;169(1): 359-365.

326. Kavanagh DG, Gold MC, Wagner M, et al. The multiple immuneevasion genes of murine cytomegalovirus are not redundant: m4 and m152 inhibit antigen presentation in a complementary and cooperative fashion. *J Exp Med*. 2001;194(7):967-978.

327. Wills MR, Ashiru O, Reeves MB, et al. Human cytomegalovirus encodes an MHC class I-like molecule (UL142) that functions to inhibit NK cell lysis. *J Immunol*. 2005;175(11):7457-7465.

328. Koszinowski UH, Reddehase MJ, Del Val M. Principles of cytomegalovirus antigen presentation in vitro and in vivo. *Semin Immunol*. 1992; 4(2):71-79.

329. Scalzo AA, Corbett AJ, Rawlinson WD, et al. The interplay between host and viral factors in shaping the outcome of cytomegalovirus infection. *Immunol Cell Biol*. 2007;85(1):46-54.

330. Rawlinson WD, Farrell HE, Barrell BG. Analysis of the complete DNA sequence of murine cytomegalovirus. *J Virol*. 1996;70(12):8833-8849.

331. Smith HR, Heusel JW, Mehta IK, et al. Recognition of a virus-encoded ligand by a natural killer cell activation receptor. *Proc Natl Acad Sci U S A*. 2002;99(13):8826-8831.

332. Burgert HG, Kvist S. An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class I antigens. *Cell*. 1985;41(3):987-997.

333. Burgert HG, Kvist S. The E3/19K protein of adenovirus type 2 binds to the domains of histocompatibility antigens required for CTL recognition. *EMBO J*. 1987;6(7):2019-2026.

334. Sester M, Koebernick K, Owen D, et al. Conserved amino acids within the adenovirus 2 E3/19K protein differentially affect downregulation of MHC class I and

MICA/B proteins. *J Immunol*. 2010;184(1):255-267.

335. Menz B, Sester M, Koebernick K, et al. Structural analysis of the adenovirus type 2 E3/19K protein using mutagenesis and a panel of conformation-sensitive monoclonal antibodies. *Mol Immunol*. 2008;46(1): 16-26.

336. Boss JM. Regulation of transcription of MHC class II genes. *Curr Opin Immunol*. 1997;9(1):107-113.

337. Fontes JD, Kanazawa S, Nekrep N, et al. The class II transactivator CIITA is a transcriptional integrator. *Microbes Infect*. 1999;1(11):863-869.

338. Jabrane-Ferrat N, Nekrep N, Tosi G, et al. Major histocompatibility complex class II transcriptional platform: assembly of nuclear factor Y and regulatory factor X (RFX) on DNA requires RFX5 dimers. *Mol Cell Biol*. 2002;22(15):5616-5625.

339. Zinzow-Kramer WM, Long AB, Youngblood BA, et al. CIITA promoter I CARD-deficient mice express functional MHC class II genes in myeloid and lymphoid compartments. *Genes Immun*. 2012;13(4):299-310.

340. Rohn WM, Lee YJ, Benveniste EN. Regulation of class II MHC expression. *Crit Rev Immunol*. 1996;16(3):311-330.

341. Reith W, Mach B. The bare lymphocyte syndrome and the regulation of MHC expression. *Annu Rev Immunol*. 2001;19:331-373.

342. Abdulkadir SA, Krishna S, Thanos D, et al. Functional roles of the transcription factor Oct-2A and the high mobility group protein I/Y in HLA-DRA gene expression. *J Exp Med*. 1995;182(2):487-500.

343. Hume CR, Shookster LA, Collins N, et al. Bare lymphocyte syndrome: altered HLA class II expression in B cell lines derived from two patients. *Hum Immunol*. 1989;25(1):1-11.

344. Mach B. MHC class II regulation—lessons from a disease. *N Engl J Med*. 1995;332(2):120-122.

345. Mitchison NA, Schuhbauer D, Muller B. Natural and induced regulation of Th1/Th2 balance. *Springer Semin Immunopathol*. 1999;21(3):199-210.

346. Mitchison NA, Roes J. Patterned variation in murine MHC promoters. *Proc Natl Acad Sci U S A*. 2002;99(16):10561-10566.

347. Guardiola J, Maffei A, Lauster R, et al. Functional significance of polymorphism among MHC class II gene promoters. *Tissue Antigens*. 1996;48(6):615-625.

348. Stumptner-Cuvelette P, Benaroch P. Multiple roles of the invariant chain in MHC class II function. *Biochim Biophys Acta*. 2002;1542(1-3):1-13.

349. Roche PA, Marks MS, Cresswell P. Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature*. 1991;354(6352):392-394.

350. Roche PA, Cresswell P. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature*. 1990; 345(6276):615-618.

351. Bikoff EK, Huang LY, Episkopou V, et al. Defective major histocompatibility complex class II assembly, transport, peptide acquisition, and CD4+ T cell selection in mice lacking invariant chain expression. *J Exp Med*. 1993;177(6):1699-1712.

352. Viville S, Neefjes J, Lotteau V, et al. Mice lacking the MHC class II-associated

invariant chain. *Cell*. 1993;72(4):635-648.

353. Bonnerot C, Marks MS, Cosson P, et al. Association with BiP and aggregation of class II MHC molecules synthesized in the absence of invariant chain. *EMBO J*. 1994;13(4):934-944.

354. Feynman R. The Feynman lectures on physics. In: Feynman R, Leighton R, Sands M, eds. *The Feynman Lectures on Physics*. Reading, MA: Addison-Wesley; 1963:5.

355. Morrison J. Love hides. In: *Absolutely Live*. Elektra; 1970.

356. Margulies DH, McCluskey J. Exon shuffling: new genes from old. *Surv Immunol Res*. 1985;4(2):146-159.

357. Rudolph MG, Stanfield RL, Wilson IA. How TCRs bind MHCs, peptides, and coreceptors. *Annu Rev Immunol*. 2006;24:419-466.

358. Orr HT, Lopez de Castro JA, Parham P, et al. Comparison of amino acid sequences of two human histocompatibility antigens, HLA-A2 and HLA-B7: location of putative alloantigenic sites. *Proc Natl Acad Sci U S A*. 1979;76(9):4395-4399.

359. Coligan JE, Kindt TJ, Uehara H, et al. Primary structure of a murine transplantation antigen. *Nature*. 1981;291(5810):35-39.

360. Steinmetz M, Frelinger JG, Fisher D, et al. Three cDNA clones encoding mouse transplantation antigens: homology to immunoglobulin genes. *Cell*. 1981;24(1):125-134.

361. Sood AK, Pereira D, Weissman SM. Isolation and partial nucleotide sequence of a cDNA clone for human histocompatibility antigen HLA-B by use of an oligodeoxynucleotide primer. *Proc Natl Acad Sci U S A*. 1981;78(1):616-620.

362. Ploegh HL, Orr HT, Strominger JL. Molecular cloning of a human histocompatibility antigen cDNA fragment. *Proc Natl Acad Sci U S A*. 1980;77(10):6081-6085.

363. Choi E, McIntyre K, Germain RN, et al. Murine I-A beta chain polymorphism: nucleotide sequences of three allelic I-A beta genes. *Science*. 1983;221(4607):283-286.

364. Mathis DJ, Benoist CO, Williams VE II, et al. The murine E alpha immune response gene. *Cell*. 1983;32(3):745-754.

365. Davis MM, Cohen DI, Nielsen EA, et al. Cell-type-specific cDNA probes and the murine I region: the localization and orientation of Ad alpha. *Proc Natl Acad Sc U S A*. 1984;81(7):2194-2198.

366. Lew AM, Margulies DH, Maloy WL, et al. Alternative protein products with different carboxyl termini from a single class I gene, H-2Kb. *Proc Natl Acad Sci U S A*. 1986;83(16):6084-6088.

367. Lew AM, McCluskey J, Maloy WL, et al. Multiple class I molecules generated from single genes by alternative splicing of pre-mRNAs. *Immunol Res*. 1987;6(1-2):117-132.

368. Handy DE, McCluskey J, Lew AM, et al. Signals controlling alternative splicing of major histocompatibility complex H-2 class I pre-mRNA. *Immunogenetics*. 1988;28(2):81-90.

369. Vega MA, Strominger JL. Constitutive endocytosis of HLA class I antigens requires a specific portion of the intracytoplasmic tail that shares structural features with other endocytosed molecules. *Proc Natl Acad Sci U S A*. 1989;86(8):2688-2692.

370. Balomenos D, Poretz RD. An acidic modification of the cytoplasmic domain

contributes to the charge heterogeneity of the MHC class I antigens. *Immunogenetics*. 1998;47(5):381-389.

371. Capps GG, Pine S, Edidin M, et al. Short class I major histocompatibility complex cytoplasmic tails differing in charge detect arbiters of lateral diffusion in the plasma membrane. *Biophys J*. 2004;86(5):2896-2909.

372. Fooksman DR, Edidin M, Barisas BG. Measuring rotational diffusion of MHC class I on live cells by polarized FPR. *Biophys Chem*. 2007;130 (1-2):10-16.

373. Gur H, Geppert TD, Lipsky PE. Structural analysis of class I MHC molecules: the cytoplasmic domain is not required for cytoskeletal association, aggregation and internalization. *Mol Immunol* . 1997;34(2):125-132.

374. Singh RK, Lau D, Noviello CM, et al. An MHC-I cytoplasmic domain/HIV-1 Nef fusion protein binds directly to the mu subunit of the AP-1 endosomal coat complex. *PloS One*. 2009;4(12):e8364.

375. Yi L, Rosales T, Rose JJ, et al. HIV-1 Nef binds a subpopulation of MHC-I throughout its trafficking itinerary and down-regulates MHC-I by perturbing both anterograde and retrograde trafficking. *J Biol Chem*. 2010;285(40):30884-30905.

376. St-Pierre Y, Nabavi N, Ghogawala Z, et al. A functional role for signal transduction via the cytoplasmic domains of MHC class II proteins. *J Immunol* . 1989;143(3):808-812.

377. Nabavi N, Freeman GJ, Gault A, et al. Signalling through the MHC class II cytoplasmic domain is required for antigen presentation and induces B7 expression. *Nature*. 1992;360(6401):266-268.

378. Umemura YM, Vrljic M, Nishimura SY, et al. Both MHC class II and its GPI-anchored form undergo hop diffusion as observed by singlemolecule tracking. *Biophys J*. 2008;95(1):435-450.

379. Schwartz RH. Immune response (Ir) genes of the murine major histocompatibility complex. *Adv Immunol* . 1986;38:31-201.

380. Babbitt BP, Allen PM, Matsueda G, et al. Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature*. 1985;317(6035): 359-361.

381. Buus S, Sette A, Colon SM, et al. Isolation and characterization of antigen-Ia complexes involved in T cell recognition. *Cell*. 1986;47(6): 1071-1077.

382. Townsend A, Ohlen C, Foster L, et al. A mutant cell in which association of class I heavy and light chains is induced by viral peptides. *Cold Spr Harb Symp Quant Biol* . 1989;54(pt 1):299-308.

383. Boyd LF, Kozlowski S, Margulies DH. Solution binding of an antigenic peptide to a major histocompatibility complex class I molecule and the role of beta 2-microglobulin. *Proc Natl Acad Sci U S A* . 1992; 89(6):2242-2246.

384. Van Bleek GM, Nathenson SG. Isolation of an endogenously processed immunodominant viral peptide from the class I H-2Kb molecule. *Nature*. 1990;348(6298):213-216.

385. Rotzschke O, Falk K, Deres K, et al. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature*. 1990;348(6298):252-254.

386. Rotzschke O, Falk K, Wallny HJ, et al. Characterization of naturally occurring minor histocompatibility peptides including H-4 and H-Y. *Science*. 1990;249(4966):283-287.

387. Rammensee HG, Falk K, Rotzschke O. Peptides naturally presented by MHC class I molecules. *Annu Rev Immunol*. 1993;11:213-244.

388. Arnott D, Shabanowitz J, Hunt DF. Mass spectrometry of proteins and peptides: sensitive and accurate mass measurement and sequence analysis. *Clin Chem*. 1993;39(9):2005-2010.

389. Hunt DF, Henderson RA, Shabanowitz J, et al. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science*. 1992;255(5049):1261-1263.

390. Corr M, Boyd LF, Frankel SR, et al. Endogenous peptides of a soluble major histocompatibility complex class I molecule, H-2Lds: sequence motif, quantitative binding, and molecular modeling of the complex. *J Exp Med*. 1992;176(6):1681-1692.

391. Corr M, Boyd LF, Padlan EA, et al. H-2Dd exploits a four residue peptide binding motif. *J Exp Med*. 1993;178(6):1877-1892.

392. Gavin MA, Dere B, Grandea AG III, et al. Major histocompatibility complex class I allele-specific peptide libraries: identification of peptides that mimic an H-Y T cell epitope. *Eur J Immunol* . 1994;24(9):2124-2133.

393. Gavin MA, Bevan MJ. Major histocompatibility complex allele-specific peptide libraries and identification of T-cell mimotopes. *Methods Mol Biol*. 1998;87:235-248.

394. Hendrickson RC, Cicinnati VR, Albers A, et al. Identification of a 17beta-hydroxysteroid dehydrogenase type 12 pseudogene as the source of a highly restricted BALB/c Meth A tumor rejection peptide. *Cancer Immunol Immunother* . 2010;59(1):113-124.

395. Rammensee H, Bachmann J, Emmerich NP, et al. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics*. 1999;50(3-4): 213-219.

396. Peters B, Sidney J, Bourne P, et al. The design and implementation of the immune epitope database and analysis resource. *Immunogenetics*. 2005;57(5):326-336.

397. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J Immunol*. 1994;152(1):163-175.

398. Rudensky A, Preston-Hurlburt P, Hong SC, et al. Sequence analysis of peptides bound to MHC class II molecules. *Nature*. 1991;353(6345): 622-627.

399. Chicz RM, Urban RG, Lane WS, et al. Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. *Nature*. 1992;358(6389):764-768.

400. Janeway CA Jr, Mamula MJ, Rudensky A. Rules for peptide presentation by MHC class II molecules. *Int Rev Immunol*. 1993;10(4):301-311.

401. Urban RG, Chicz RM, Vignali DA, et al. The dichotomy of peptide presentation by class I and class II MHC proteins. *Chem Immunol*. 1993; 57:197-234.

402. Hammer J, Takacs B, Sinigaglia F. Identification of a motif for HLA-DR1 binding peptides using M13 display libraries. *J Exp Med*. 1992;176(4):1007-1013.

403. Carrasco-Marin E, Petzold S, Unanue ER. Two structural states of complexes of peptide and class II major histocompatibility complex revealed by photoaffinity-labeled peptides. *J Biol Chem*. 1999;274(44): 31333-31340.

404. Gugasyan R, Velazquez C, Vidavsky I, et al. Independent selection by I-Ak molecules of two epitopes found in tandem in an extended polypeptide antigen. *J Immunol*. 2000;165(6):3206-3213.

405. Cease KB, Berkower I, York-Jolley J, et al. T cell clones specific for an amphipathic alpha-helical region of sperm whale myoglobin show differing fine specificities for synthetic peptides. A multiview/single structure interpretation of immunodominance. *J Exp Med*. 1986; 164(5):1779-1784.

406. Landais E, Romagnoli PA, Corper AL, et al. New design of MHC class II tetramers to accommodate fundamental principles of antigen presentation. *J Immunol*. 2009;183(12):7949-7957.

407. Chang KY, Suri A, Unanue ER. Predicting peptides bound to I-Ag7 class II histocompatibility molecules using a novel expectation-maximization alignment algorithm. *Proteomics*. 2007;7(3):367-377.

408. Gunther S, Schlundt A, Sticht J, et al. Bidirectional binding of invariant chain peptides to an MHC class II molecule. *Proc Natl Acad Sci U S A* . 2010;107(51):22219-22224.

409. Bjorkman PJ, Saper MA, Samraoui B, et al. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature*. 1987;329(6139):512-518.

410. Bjorkman PJ, Saper MA, Samraoui B, et al. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature*. 1987;329(6139): 506-512.

411. Garrett TP, Saper MA, Bjorkman PJ, et al. Specificity pockets for the side chains of peptide antigens in HLA-Aw68. *Nature*. 1989;342(6250): 692-696.

412. Saper MA, Bjorkman PJ, Wiley DC. Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 A resolution. *J Mol Biol* . 1991;219(2):277-319.

413. Madden DR, Gorga JC, Strominger JL, et al. The three-dimensional structure of HLA-B27 at 2.1 A resolution suggests a general mechanism for tight peptide binding to MHC. *Cell*. 1992;70(6):1035-1048.

414. Jardetzky TS, Lane WS, Robinson RA, et al. Identification of self peptides bound to purified HLA-B27. *Nature*. 1991;353(6342):326-329.

415. Zhang W, Young AC, Imarai M, et al. Crystal structure of the major histocompatibility complex class I H-2Kb molecule containing a single viral peptide: implications for peptide binding and T-cell receptor recognition. *Proc Natl Acad Sci U S A* . 1992;89(17):8403-8407.

416. Fremont DH, Matsumura M, Stura EA, et al. Crystal structures of two viral peptides in complex with murine MHC class I H-2Kb. *Science*. 1992;257(5072):919-927.

417. Fremont DH, Stura EA, Matsumura M, et al. Crystal structure of an H-2Kb-ovalbumin peptide complex reveals the interplay of primary and secondary anchor positions in the major histocompatibility complex binding groove. *Proc Natl Acad Sci U S A* . 1995;92(7):2479-2483.

418. Glithero A, Tormo J, Doering K, et al. The crystal structure of H-2D(b) complexed with a partial peptide epitope suggests a major histocompatibility complex class I assembly intermediate. *J Biol Chem*. 2006; 281(18):12699-12704.

419. Joyce S, Kuzushima K, Kepecs G, et al. Characterization of an incompletely assembled major histocompatibility class I molecule (H-2Kb) associated with unusually long peptides: implications for antigen processing and presentation. *Proc Natl Acad Sci*

U S A. 1994;91(10):4145-4149.

420. Collins EJ, Garboczi DN, Wiley DC. Three-dimensional structure of a peptide extending from one end of a class I MHC binding site. *Nature*. 1994;371(6498):626-629.

421. Lybarger L, Yu YY, Miley MJ, et al. Enhanced immune presentation of a single-chain major histocompatibility complex class I molecule engineered to optimize linkage of a C-terminally extended peptide. *J Biol Chem*. 2003;278(29):27105-27111.

422. Kastrup IB, Stevanovic S, Arsequell G, et al. Lectin purified human class I MHC-derived peptides: evidence for presentation of glycopeptides in vivo. *Tissue Antigens*. 2000;56(2):129-135.

423. Speir JA, Abdel-Motal UM, Jondal M, et al. Crystal structure of an MHC class I presented glycopeptide that generates carbohydrate-specific CTL. *Immunity*. 1999;10(1):51-61.

424. Glithero A, Tormo J, Haurum JS, et al. Crystal structures of two H-2Db/glycopeptide complexes suggest a molecular basis for CTL cross-reactivity. *Immunity*. 1999;10(1):63-74.

425. Speir JA, Stevens J, Joly E, et al. Two different, highly exposed, bulged structures for an unusually long peptide bound to rat MHC class I RT1-Aa. *Immunity*. 2001;14(1):81-92.

426. Miles JJ, Elhassen D, Borg NA, et al. CTL recognition of a bulged viral peptide involves biased TCR selection. *J Immunol*. 2005;175(6): 3826-3834.

427. Tynan FE, Borg NA, Miles JJ, et al. High resolution structures of highly bulged viral epitopes bound to major histocompatibility complex class I. Implications for T-cell receptor engagement and T-cell immunodominance. *J Biol Chem*. 2005;280(25):23900-23909.

428. Tynan FE, Reid HH, Kjer-Nielsen L, et al. A T cell receptor flattens a bulged antigenic peptide presented by a major histocompatibility complex class I molecule. *Nat Immunol*. 2007;8(3):268-276.

429. Mohammed F, Cobbold M, Zarling AL, et al. Phosphorylationdependent interaction between antigenic peptides and MHC class I: a molecular basis for the presentation of transformed self. *Nat Immunol*. 2008;9(11):1236-1243.

430. Petersen J, Wurzbacher SJ, Williamson NA, et al. Phosphorylated selfpeptides alter human leukocyte antigen class I-restricted antigen presentation and generate tumor-specific epitopes. *Proc Natl Acad Sci U S A* . 2009;106(8):2776-2781.

431. Mitaksov V, Truscott SM, Lybarger L, et al. Structural engineering of pMHC reagents for T cell vaccines and diagnostics. *Chem Biol*. 2007; 14(8):909-922.

432. Celie PH, Toebes M, Rodenko B, et al. UV-induced ligand exchange in MHC class I protein crystals. *J Am Chem Soc*. 2009;131(34):12298-12304.

433. Brown JH, Jardetzky T, Saper MA, et al. A hypothetical model of the foreign antigen binding site of class II histocompatibility molecules. *Nature*. 1988;332(6167):845-850.

434. Brown JH, Jardetzky TS, Gorga JC, et al. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature*. 1993;364(6432):33-39.

435. Wu TT, Kabat EA. An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. *J Exp Med*. 1970;132(2):211-250.

436. Shannon CE. The mathematical theory of communication. *Bell System Techn J*. 1948;27:379-423.

437. Shannon CE. The mathematical theory of communication. *Bell System Techn J*. 1948;27:623-656.

438. Strait BJ, Dewey TG. The Shannon information entropy of protein sequences. *Biophys J*. 1996;71(1):148-155.

439. Stern LJ, Brown JH, Jardetzky TS, et al. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature*. 1994;368(6468):215-221.

440. Fields BA, Ober B, Malchiodi EL, et al. Crystal structure of the V alpha domain of a T cell antigen receptor. *Science*. 1995;270(5243):1821-1824.

441. Schafer PH, Malapati S, Hanfelt KK, et al. The assembly and stability of MHC class II-(alpha beta)2 superdimers. *J Immunol*. 1998;161(5): 2307-2316.

442. Abastado JP, Lone YC, Casrouge A, et al. Dimerization of soluble major histocompatibility complex-peptide complexes is sufficient for activation of T cell hybridoma and induction of unresponsiveness. *J Exp Med*. 1995;182(2):439-447.

443. Reich Z, Boniface JJ, Lyons DS, et al. Ligand-specific oligomerization of T-cell receptor molecules. *Nature*. 1997;387(6633):617-620.

444. Fremont DH, Hendrickson WA, Marrack P, et al. Structures of an MHC class II molecule with covalently bound single peptides. *Science*. 1996;272(5264):1001-1004.

445. Fremont DH, Dai S, Chiang H, et al. Structural basis of cytochrome c presentation by IE(k). *J Exp Med*. 2002;195(8):1043-1052.

446. Plaksin D, Chacko S, McPhie P, et al. A T cell receptor V alpha domain expressed in bacteria: does it dimerize in solution? *J Exp Med*. 1996;184(4):1251-1258.

447. Garboczi DN, Ghosh P, Utz U, et al. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature*. 1996; 384(6605):134-141.

448. Garcia KC, Degano M, Stanfield RL, et al. An alphabeta T cell receptor structure at 2.5 A and its orientation in the TCR-MHC complex. *Science*. 1996;274(5285):209-219.

449. Reinherz EL, Tan K, Tang L, et al. The crystal structure of a T cell receptor in complex with peptide and MHC class II. *Science*. 1999; 286(5446):1913-1921.

450. Hennecke J, Carfi A, Wiley DC. Structure of a covalently stabilized complex of a human alphabeta T-cell receptor, influenza HA peptide and MHC class II molecule, HLA-DR1. *EMBO J*. 2000;19(21):5611-5624.

451. Corper AL, Stratmann T, Apostolopoulos V, et al. A structural framework for deciphering the link between I-Ag7 and autoimmune diabetes. *Science*. 2000;288(5465):505-511.

452. Latek RR, Suri A, Petzold SJ, et al. Structural basis of peptide binding and presentation by the type I diabetes-associated MHC class II molecule of NOD mice. *Immunity*. 2000;12(6):699-710.

453. Zeng Z, Castano AR, Segelke BW, et al. Crystal structure of mouse CD1: an MHC-like fold with a large hydrophobic binding groove. *Science*. 1997;277(5324):339-345.

454. Gadola SD, Zaccai NR, Harlos K, et al. Structure of human CD1b with bound

ligands at 2.3 A, a maze for alkyl chains. *Nat Immunol*. 2002; 3(8):721-726.

455. Wang CR, Lindahl KF, Deisenhofer J. Crystal structure of the MHC class Ib molecule H2-M3. *Res Immunol*. 1996;147(5):313-321.

456. Li P, Morris DL, Willcox BE, et al. Complex structure of the activating immunoreceptor NKG2D and its MHC class I-like ligand MICA. *Nat Immunol*. 2001;2(5):443-451.

457. Li P, Willie ST, Bauer S, et al. Crystal structure of the MHC class I homolog MIC-A, a gammadelta T cell ligand. *Immunity*. 1999;10(5): 577-584.

458. Burmeister WP, Gastinel LN, Simister NE, et al. Crystal structure at 2.2 A resolution of the MHC-related neonatal Fc receptor. *Nature*. 1994;372(6504):336-343.

459. Li P, McDermott G, Strong RK. Crystal structures of RAE-1beta and its complex with the activating immunoreceptor NKG2D. *Immunity*. 2002;16(1):77-86.

460. Carayannopoulos LN, Naidenko OV, Kinder J, et al. Ligands for murine NKG2D display heterogeneous binding behavior. *Eur J Immunol* . 2002;32(3):597-605.

461. Treiner E, Duban L, Bahram S, et al. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature*. 2003; 422(6928):164-169.

462. Loveland B, Wang CR, Yonekawa H, et al. Maternally transmitted histocompatibility antigen of mice: a hydrophobic peptide of a mitochondrially encoded protein. *Cell*. 1990;60(6):971-980.

463. Wang CR, Castano AR, Peterson PA, et al. Nonclassical binding of formylated peptide in crystal structure of the MHC class Ib molecule H2-M3. *Cell*. 1995;82(4):655-664.

464. Moody DB, Besra GS, Wilson IA, et al. The molecular basis of CD1-mediated presentation of lipid antigens. *Immunol Rev*. 1999;172:285-296.

465. Calabi F, Jarvis JM, Martin L, et al. Two classes of CD1 genes. *Eur J Immunol* . 1989;19(2):285-292.

466. Zajonc DM, Kronenberg M. CD1 mediated T cell recognition of glycolipids. *Curr Opin Struct Biol*. 2007;17(5):521-529.

467. Shamshiev A, Donda A, Carena I, et al. Self glycolipids as T-cell autoantigens. *Eur J Immunol*. 1999;29(5):1667-1675.

468. Rosat JP, Grant EP, Beckman EM, et al. CD1-restricted microbial lipid antigen-specific recognition found in the CD8+ alpha beta T cell pool. *J Immunol*. 1999;162(1):366-371.

469. Bendelac A, Rivera MN, Park SH, et al. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu Rev Immunol*. 1997; 15:535-562.

470. Koch M, Stronge VS, Shepherd D, et al. The crystal structure of human CD1d with and without alpha-galactosylceramide. *Nat Immunol*. 2005; 6(8):819-826.

471. Wun KS, Cameron G, Patel O, et al. A molecular basis for the exquisite CD1d-restricted antigen specificity and functional responses of natural killer T cells. *Immunity*. 2011;34(3):327-339.

472. Borg NA, Wun KS, Kjer-Nielsen L, et al. CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor. *Nature*. 2007; 448(7149):44-49.

473. Pellicci DG, Clarke AJ, Patel O, et al. Recognition of beta-linked self glycolipids mediated by natural killer T cell antigen receptors. *Nat Immunol* . 2011;12(9):827-833.

474. Girardi E, Yu ED, Li Y, et al. Unique interplay between sugar and lipid in determining the antigenic potency of bacterial antigens for NKT cells. *PLoS Biol* . 2011;9(11):e1001189.

475. Simister NE, Rees AR. Isolation and characterization of an Fc receptor from neonatal rat small intestine. *Eur J Immunol* . 1985;15(7):733-738.

476. Simister NE, Mostov KE. Cloning and expression of the neonatal rat intestinal Fc receptor, a major histocompatibility complex class I antigen homolog. *Cold Spring Harb Symp Quant Biol* . 1989;54(pt 1): 571-580.

477. Ahouse JJ, Hagerman CL, Mittal P, et al. Mouse MHC class I-like Fc receptor encoded outside the MHC. *J Immunol* . 1993;151(11): 6076-6088.

478. Story CM, Mikulska JE, Simister NE. A major histocompatibility complex class I-like Fc receptor cloned from human placenta: possible role in transfer of immunoglobulin G from mother to fetus. *J Exp Med* . 1994;180(6):2377-2381.

479. Simister NE. IgG Fc receptors that resemble class I major histocompatibility complex antigens. *Biochem Soc Trans*. 1993;21(4):973-976.

480. Israel EJ, Wilsker DF, Hayes KC, et al. Increased clearance of IgG in mice that lack beta 2-microglobulin: possible protective role of FcRn. *Immunology* . 1996;89(4):573-578.

481. Kasahara M, Watanabe Y, Sumasu M, et al. A family of MHC class I-like genes located in the vicinity of the mouse leukocyte receptor complex. *Proc Natl Acad Sci U S A*. 2002;99(21):13687-13692.

482. Watanabe Y, Maruoka T, Walter L, et al. Comparative genomics of the Mill family: a rapidly evolving MHC class I gene family. *Eur J Immunol* . 2004;34(6):1597-1607.

483. Kajikawa M, Baba T, Tomaru U, et al. MHC class I-like MILL molecules are beta2-microglobulin-associated, GPI-anchored glycoproteins that do not require TAP for cell surface expression. *J Immunol* . 2006;177(5):3108-3115.

484. Rabinovich BA, Ketchem RR, Wolfson M, et al. A role for the MHC class I-like Mill molecules in nutrient metabolism and wound healing. *Immunol Cell Biol* . 2008;86(6):489-496.

485. Olson R, Huey-Tubman KE, Dulac C, et al. Structure of a pheromone receptor-associated MHC molecule with an open and empty groove. *PLoS Biol* . 2005;3(8):e257.

486. Yang Z, Bjorkman PJ. Structure of UL18, a peptide-binding viral MHC mimic, bound to a host inhibitory receptor. *Proc Natl Acad Sci U S A* . 2008;105(29):10095-10100.

487. Natarajan K, Hicks A, Mans J, et al. Crystal structure of the murine cytomegalovirus MHC-I homolog m144. *J Mol Biol* . 2006;358(1): 157-171.

488. Zhi L, Mans J, Paskow MJ, et al. Direct interaction of the mouse cytomegalovirus m152/gp40 immunoevasin with RAE-1 isoforms. *Biochemistry* . 2010;49(11):2443-2453.

489. Yang Z, West AP Jr, Bjorkman PJ. Crystal structure of TNFalpha complexed with a poxvirus MHC-related TNF binding protein. *Nat Struct Mol Biol* . 2009;16(11):1189-1191.

490. Schneider CL, Hudson AW. The human herpesvirus-7 (HHV-7) U21 immunoevasin subverts NK-mediated cytoxicity through modulation of MICA and MICB. *PLoS Pathog* . 2011;7(11):e1002362.

491. Smith LM, Shellam GR, Redwood AJ. Genes of murine cytomegalovirus exist as a number of distinct genotypes. *Virology*. 2006;352(2): 450-465.

492. Chapman TL, Bjorkman PJ. Characterization of a murine cytomegalovirus class I major histocompatibility complex (MHC) homolog: comparison to MHC molecules and to the human cytomegalovirus MHC homolog. *J Virol* . 1998;72(1):460-466.

493. Adams EJ, Juo ZS, Venook RT, et al. Structural elucidation of the m157 mouse cytomegalovirus ligand for Ly49 natural killer cell receptors. *Proc Natl Acad Sci U S A* . 2007;104(24):10128-10133.

494. Wang R, Natarajan K, Margulies DH. Personal communication, August 6, 2012.

495. Cretney E, Degli-Esposti MA, Densley EH, et al. m144, a murine cytomegalovirus (MCMV)-encoded major histocompatibility complex class I homologue, confers tumor resistance to natural killer cell-mediated rejection. *J Exp Med* . 1999;190(3):435-444.

496. Kubota A, Kubota S, Farrell HE, et al. Inhibition of NK cells by murine CMV-encoded class I MHC homologue m144. *Cell Immunol* . 1999;191(2):145-151.

497. Hennecke J, Wiley DC. Structure of a complex of the human alpha/beta T cell receptor (TCR) HA1.7, influenza hemagglutinin peptide, and major histocompatibility complex class II molecule, HLA-DR4 (DRA*0101 and DRB1*0401): insight into TCR cross-restriction and alloreactivity. *J Exp Med* . 2002;195(5):571-581.

498. Reiser JB, Darnault C, Guimezanes A, et al. Crystal structure of a T cell receptor bound to an allogeneic MHC molecule. *Nat Immunol* . 2000; 1(4):291-297.

499. Garcia KC, Degano M, Pease LR, et al. Structural basis of plasticity in T cell receptor recognition of a self peptide-MHC antigen. *Science*. 1998;279(5354):1166-1172.

500. Reiser JB, Gregoire C, Darnault C, et al. A T cell receptor CDR3beta loop undergoes conformational changes of unprecedented magnitude upon binding to a peptide/MHC class I complex. *Immunity*. 2002; 16(3):345-354.

501. Crispe IN, Husmann LA, Bevan MJ. T cell receptor expression and receptor-mediated induction of clonal growth in the developing mouse thymus. High surface beta-chain density is a requirement for functional maturity. *Eur J Immunol* . 1986;16(10):1283-1288.

502. Colf LA, Bankovich AJ, Hanick NA, et al. How a single T cell receptor recognizes both self and foreign MHC. *Cell* . 2007;129(1):135-146.

503. Macdonald WA, Chen Z, Gras S, et al. T cell allorecognition via molecular mimicry. *Immunity*. 2009;31(6):897-908.

504. Gras S, Burrows SR, Kjer-Nielsen L, et al. The shaping of T cell receptor recognition by self-tolerance. *Immunity*. 2009;30(2):193-203.

505. Adams JJ, Narayanan S, Liu B, et al. T cell receptor signaling is limited by docking geometry to peptide-major histocompatibility complex. *Immunity*. 2011;35(5):681-693.

506. Devine L, Kavathas PB. Molecular analysis of protein interactions mediating the function of the cell surface protein CD8. *Immunol Res*. 1999;19(2-3):201-210.

507. Gao GF, Jakobsen BK. Molecular interactions of coreceptor CD8 and MHC class I: the molecular basis for functional coordination with the T-cell receptor. *Immunol Today*. 2000;21(12):630-636.

508. Gao GF, Tormo J, Gerth UC, et al. Crystal structure of the complex between human CD8alpha(alpha) and HLA-A2. *Nature*. 1997; 387(6633):630-634.

509. Kern PS, Teng MK, Smolyar A, et al. Structural basis of CD8 coreceptor function revealed by crystallographic analysis of a murine CD8alphaalpha ectodomain fragment in complex with H-2Kb. *Immunity*. 1998;9(4):519-530.

510. Maddon PJ, Dalgleish AG, McDougal JS, et al. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell*. 1986;47(3):333-348.

511. Wu H, Kwong PD, Hendrickson WA. Dimeric association and segmental variability in the structure of human CD4. *Nature*. 1997; 387(6632):527-530.

512. Shen X, Hu B, McPhie P, et al. Peptides corresponding to CD4-interacting regions of murine MHC class II molecules modulate immune responses of CD4+ T lymphocytes in vitro and in vivo. *J Immunol*. 1996;157(1):87-100.

513. Lanier LL. Activating and inhibitory NK cell receptors. *Adv Exp Med Biol*. 1998;452:13-18.

514. Natarajan K, Dimasi N, Wang J, et al. Structure and function of natural killer cell receptors: multiple molecular solutions to self, nonself discrimination. *Annu Rev Immunol*. 2002;20:853-885.

515. Radaev S, Rostro B, Brooks AG, et al. Conformational plasticity revealed by the cocrystal structure of NKG2D and its class I MHC-like ligand ULBP3. *Immunity*. 2001;15(6):1039-1049.

516. Matsumoto N, Mitsuki M, Tajima K, et al. The functional binding site for the C-type lectin-like natural killer cell receptor Ly49A spans three domains of its major histocompatibility complex class I ligand. *J Exp Med*. 2001;193(2):147-158.

517. Wang J, Whitman MC, Natarajan K, et al. Binding of the natural killer cell inhibitory receptor Ly49A to its major histocompatibility complex class I ligand. Crucial contacts include both H-2Dd AND beta 2-microglobulin. *J Biol Chem*. 2002;277(2):1433-1442.

518. Dimasi N, Sawicki MW, Reineck LA, et al. Crystal structure of the Ly49I natural killer cell receptor reveals variability in dimerization mode within the Ly49 family. *J Mol Biol*. 2002;320(3):573-585.

519. Dam J, Guan R, Natarajan K, et al. Variable MHC class I engagement by Ly49 natural killer cell receptors demonstrated by the crystal structure of Ly49C bound to H-2K(b). *Nat Immunol*. 2003;4(12):1213-1222.

520. Held W, Mariuzza RA. Cis-trans interactions of cell surface receptors: biological roles and structural basis. *Cell Mol Life Sci*. 2011;68(21): 3469-3478.

521. Herman A, Kappler JW, Marrack P, et al. Superantigens: mechanism of T-cell stimulation and role in immune responses. *Annu Rev Immunol*. 1991;9:745-772.

522. Rovira P, Buckle M, Abastado JP, et al. Major histocompatibility class I molecules present Urtica dioica agglutinin, a superantigen of vegetal origin, to T lymphocytes. *Eur J Immunol*. 1999;29(5):1571-1580.

523. Harata K, Muraki M. Crystal structures of Urtica dioica agglutinin and its complex with tri-N-acetylchitotriose. *J Mol Biol*. 2000;297(3): 673-681.

524. Saul FA, Rovira P, Boulot G, et al. Crystal structure of Urtica dioica agglutinin, a superantigen presented by MHC molecules of class I and class II. *Structure Fold Des*. 2000;8(6):593-603.

525. Papageorgiou AC, Acharya KR. Microbial superantigens: from structure to function. *Trends Microbiol*. 2000;8(8):369-375.

526. Jardetzky TS, Brown JH, Gorga JC, et al. Three-dimensional structure of a human class II histocompatibility molecule complexed with superantigen. *Nature*. 1994;368(6473):711-718.

527. Fields BA, Malchiodi EL, Li H, et al. Crystal structure of a T-cell receptor beta-chain complexed with a superantigen. *Nature*. 1996; 384(6605):188-192.

528. Petersson K, Hakansson M, Nilsson H, et al. Crystal structure of a superantigen bound to MHC class II displays zinc and peptide dependence. *EMBO J*. 2001;20(13):3306-3312.

529. Delon J, Germain RN. Information transfer at the immunological synapse. *Curr Biol* . 2000;10(24):R923-933.

530. Wang L, Zhao Y, Li Z, et al. Crystal structure of a complete ternary complex of TCR, superantigen and peptide-MHC. *Nat Struct Mol Biol* . 2007;14(2):169-171.

531. Saline M, Rodstrom KE, Fischer G, et al. The structure of superantigen complexed with TCR and MHC reveals novel insights into superantigenic T cell activation. *Nat Commun*. 2010;1:119.

532. Fremont DH, Rees WA, Kozono H. Biophysical studies of T-cell receptors and their ligands. *Curr Opin Immunol* . 1996;8(1):93-100.

533. Matsui K, Boniface JJ, Reay PA, et al. Low affinity interaction of peptide-MHC complexes with T cell receptors. *Science*. 1991;254(5039): 1788-1791.

534. Matsui K, Boniface JJ, Steffner P, et al. Kinetics of T-cell receptor binding to peptide/I-Ek complexes: correlation of the dissociation rate with T-cell responsiveness. *Proc Natl Acad Sci U S A* . 1994;91(26): 12862-12866.

535. Khilko SN, Jelonek MT, Corr M, et al. Measuring interactions of MHC class I molecules using surface plasmon resonance. *J Immunol Methods*. 1995;183(1):77-94.

536. Corr M, Slanetz AE, Boyd LF, et al. T cell receptor-MHC class I peptide interactions: affinity, kinetics, and specificity. *Science*. 1994;265(5174): 946-949.

537. Sykulev Y, Brunmark A, Jackson M, et al. Kinetics and affinity of reactions between an antigen-specific T cell receptor and peptide-MHC complexes. *Immunity*. 1994;1(1):15-22.

538. Alam SM, Travers PJ, Wung JL, et al. T-cell-receptor affinity and thymocyte positive selection. *Nature*. 1996;381(6583):616-620.

539. Alam SM, Davies GM, Lin CM, et al. Qualitative and quantitative differences in T cell receptor binding of agonist and antagonist ligands. *Immunity*. 1999;10(2):227-237.

540. Willcox BE, Gao GF, Wyer JR, et al. TCR binding to peptide-MHC stabilizes a flexible recognition interface. *Immunity*. 1999;10(3):357-365.

541. Natarajan K, Boyd LF, Schuck P, et al. Interaction of the NK cell inhibitory receptor Ly49A with H-2Dd: identification of a site distinct from the TCR site. *Immunity*. 1999;11(5):591-601.

542. Vales-Gomez M, Reyburn HT, Erskine RA, et al. Kinetics and peptide dependency of the binding of the inhibitory NK receptor CD94/NKG2-A and the activating receptor CD94/NKG2-C to HLA-E. *EMBO J*. 1999;18(15):4250-4260.

543. Fooksman DR, Dustin ML. Affinity measured by microcluster. *J Exp Med*. 2010;207(5):907-909.

544. Dustin ML. Making a little affinity go a long way: a topological view of LFA-1 regulation. *Cell Adhes Commun*. 1998;6(2-3):255-262.

545. Sabatino JJ Jr, Huang J, Zhu C, et al. High prevalence of low affinity peptide-MHC II tetramer-negative effectors during polyclonal CD4+ T cell responses. *J Exp Med*. 2011;208(1):81-90.

546. Huang J, Edwards LJ, Evavold BD, et al. Kinetics of MHC-CD8 interaction at the T cell membrane. *J Immunol*. 2007;179(11):7653-7662.

547. Altman JD, Moss PA, Goulder PJ, et al. Phenotypic analysis of antigenspecific T lymphocytes. *Science*. 1996;274(5284):94-96.

548. O'Herrin SM, Lebowitz MS, Bieler JG, et al. Analysis of the expression of peptide-major histocompatibility complexes using high affinity soluble divalent T cell receptors. *J Exp Med*. 1997;186(8):1333-1345.

549. Hanke T, Takizawa H, McMahon CW, et al. Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors. *Immunity*. 1999;11(1):67-77.

550. Mehta IK, Smith HR, Wang J, et al. A "chimeric" C57l-derived Ly49 inhibitory receptor resembling the Ly49D activation receptor. *Cell Immunol*. 2001;209(1):29-41.

551. Mehta IK, Wang J, Roland J, et al. Ly49A allelic variation and MHC class I specificity. *Immunogenetics*. 2001;53(7):572-583.

552. Braud VM, Allan DS, O'Callaghan CA, et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature*. 1998; 391(6669):795-799.

553. Cameron T, Norris P, Patel A, et al. Labeling antigen-specific CD4(+) T cells with class II MHC oligomers. *J Immunol Methods*. 2002;268(1):51.

554. Hugues S, Malherbe L, Filippi C, et al. Generation and use of alternative multimers of peptide/MHC complexes. *J Immunol Methods*. 2002; 268(1):83.

555. Malherbe L, Filippi C, Julia V, et al. Selective activation and expansion of high-affinity CD4+ T cells in resistant mice upon infection with Leishmania major. *Immunity*. 2000;13(6):771-782.

556. Lebowitz MS, O'Herrin SM, Hamad AR, et al. Soluble, high-affinity dimers of T-cell receptors and class II major histocompatibility complexes: biochemical probes for analysis and modulation of immune responses. *Cell Immunol*. 1999;192(2):175-184.

557. Crawford F, Kozono H, White J, et al. Detection of antigen-specific T cells with multivalent soluble class II MHC covalent peptide complexes. *Immunity*. 1998;8(6):675-682.

558. McCluskey J. The human leucocyte antigens and clinical medicine. In: Bradley J, McCluskey J, eds. *Clinical Immunology*. Oxford, NY; Oxford University Press; 1997;415-427.

559. Berman HM, Westbrook J, Feng Z, et al. The protein data bank. *Nucleic Acids Res*. 2000;28(1);235-242.

560. DeLano WL. *The PyMol Molecular Graphics System*. 2002. www.pymol.org.

561. Kruisbeek AM. Commonly used mouse strains. Current protocols in immunology. 2001;17:A.1C.1-A.1C.2.

562. Biddison, WE, Martin R. Peptide binding motifs for MHC class I and II molecules. *Current Protocols in Immunology*. 2001;36:A.1I.1-A.1I.7.

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Chapter 22

Antigen Processing and Presentation

Ted H. Hansen

Paul A. Roche

ANTIGEN PRESENTATION PATHWAYS

As part of the adaptive immune response, T cells mount immune responses to diseased cells and abnormal cells. Remarkably, the mechanism by which T cells discriminate self from non-self is based on pathogen- or tumor-derived proteins displayed on the cell surface by self-major histocompatibility complex (MHC) molecules. More specifically, when T cells detect diseased cells displaying MHC molecules loaded with peptides derived from foreign peptides, effector mechanisms are initiated to eliminate the diseased cell. By contrast, T cells that detect cells displaying MHC molecules loaded with peptides derived from self-proteins are either eliminated during ontogeny by negative selection or suppressed by peripheral tolerance mechanisms. Thus, at the molecular level, the T-cell receptors on T cells discriminates MHC loaded with self- versus non-self-peptides displayed at the cell surface of normal and diseased cells. The mechanisms by which peptide ligands for MHC molecules are generated is referred to as *antigen processing*, whereas the mechanism by which peptide/MHC complexes are displayed at the cell surface is called *antigen presentation*.

To assure the appropriate effector mechanism is generated to detect cells infected with pathogens in the cytosol or endocytic compartments, the antigen presentation pathways for classical cluster of differentiation (CD)8+ versus CD4+ T cells are different. More specifically, antigen derived from intracellular pathogens is typically bound to MHC-I proteins that are uniquely detected by CD8 T cells, whereas antigen derived from extracellular pathogens is typically bound to MHC-II proteins that are uniquely detected by CD4 T cells. To preferentially bind peptides derived from proteins synthesized inside the cell, the processing of peptides that bind MHC-I proteins occurs in the cytosol and the loading of these peptides occurs in the endoplasmic reticulum (ER). This pathway is commonly referred to as the classical MHC-I antigen-binding pathway. By contrast, to preferentially bind peptides from proteins synthesized outside the cell, the processing and loading of antigenic peptide ligands for MHC-II proteins occurs in an endocytic pathway terminating in the lysosomes. This pathway is typically referred to the classical MHC-II antigen-binding pathway. The differences between the MHC-I and MHC-II antigen presentation pathways are determined by their differential interaction with molecular chaperones. These same molecular chaperones enforce the quality control of antigen presentation to assure appropriate T cells are activated for detection and elimination of pathogen-infected cells or tumors.

ANTIGEN PROCESSING AND PRESENTATION BY MAJOR HISTOCOMPATIBILITY CLASS I (MHC-I) PROTEINS

Origin of MHC-I binding peptides

As detailed in Chapter 21, MHC-I proteins preferentially bind peptides of 8 to 10 amino acid lengths, and which peptides bind is determined by MHC-I allele-specific polymorphic residues that form the architecture of its peptide binding groove. Each MHC-I allele typically binds peptides with a consensus binding motif requiring a relatively specific amino acid central anchor and a C-terminal hydrophobic amino acid. Given the lack of restraints in most peptide positions and the large number of proteins synthesized by each cell relative to the number of MHC molecules, the selective processes controlling which peptides get presented is of considerable importance for the detection of pathogens and malignancies.

The source of the peptides that get presented during infection has been a question of considerable investigation. It has been known for some time that MHC-I-binding peptides are derived from membrane-bound, secreted, cytosolic, and nuclear proteins that are almost exclusively generated by the proteasome in the cytosol. However, the uncertainty of peptide source is based on two apparently incongruent observations. Most proteins from which MHC-I-binding peptides are derived have slow rates of turnover in cells ranging from hours to days; viral-derived peptides are presented by infected cells to CD8 T cells within a few minutes. This disparity between the degradation versus presentation kinetics is potentially explained by cumulative observations that a fraction of newly synthesized polypeptides are rapidly degraded, providing a peptide reservoir sufficient to support a robust CD8 T-cell response. Supporting evidence for this conclusion comes from studies that block protein synthesis with cycloheximide and detected a rapid decrease in the peptide supply for MHC-I binding.[1,2] One of the more elegant ways to monitor peptide transport into the ER was by transporter associated with antigen presentation (TAP) mobility in the ER membrane by photobleaching; establishing TAP mobility was proportional to the peptides in the cytosol. This assay was used to show a 90% reduction in peptide supply after 30 m treatment with cycloheximide, thus kinetically linking presentation closely to translation.[3] Indeed, there are now several reports that within 30 minutes after synthesis, antigenic peptides are presented at

the cell surface at levels that activate T cells.[4,5] What remained unclear from these studies is the biochemical and mechanistic basis for the generation of these rapidly transcribed proteins from which MHC-I-binding peptides are derived.

In a model that has gained wide acceptance, Yewdell and colleagues proposed that a rapid supply of MHC-I ligands is derived from aberrant protein production they termed defective ribosomal products or DriPs.[6,7] In their model, it was speculated that DriPs could be derived from unfolded or misfolded proteins of the proper sequence and length, proteins with errors in sequence or posttranslational modifications, prematurely terminated proteins, and proteins translated from the wrong start codon.[8] Regarding aberrant translations, there is evidence that MHC-presented peptides can arise from alternative reading frames, generating what has been called cryptic epitopes.[9] For example, Schwab et al. showed that inhibition of the eukaryotic translational initiation factor eIF2α resulted in the synthesis of cryptic peptides and intriguingly, viruses inhibit host translation by inactivating eIF2α.[10,11] However, most MHC-I-

presented peptides have native amino acid sequence and are derived from standard messenger ribonucleic acid (mRNA) translation products, suggesting cryptic epitopes represent only a minor component of MHC-presented peptides. In addition, the contribution of peptides derived from misfolded proteins in the secretory pathway is also likely modest. Although antigenic peptides from misfolded tyrosinase were found to be preferentially presented,[12] other studies show that several mutations causing misfolding do not lead to enhanced antigen presentation. Furthermore, ER quality control is typically too slow to account for the rapid supply of MHC-I-binding peptides.[13] The reason for this kinetic delay is that substrates for ER-associated degradation (ERAD) must be 1) translocated into the ER, 2) detected presumably by ER chaperones as being misfolded, 3) ubiquitinated and extracted from the ER, and 4) degraded by the proteasome in the cytosol. Thus the predominance of MHC-I-presented peptides likely does not come from cryptic epitopes or misfolded ER proteins identified by ER quality control.

To obviate trafficking into the ER, misfolded secretory proteins could be degraded rapidly based on degron detection such as unshielded hydrophobicity.[14] Alternatively, early in translation a lack of signal recognition particle engagement could lead to the mistargeting of secretory proteins to the cytosol. In support of this model, Schlosser et al.[15] showed that an epitope encompassing the signal peptidase cleavage site was efficiently presented by MHC-I proteins. To rapidly generate peptides, it has been speculated that cells may have select ribosomes called “immunoribosomes.”[16] Such immunoribosomes might be more adept at generating peptides with TAP access perhaps by compartmentalization and/or targeting proteins to the 20S proteasome for immediate destruction. In any case, there is considerable evidence that antigenic peptides can be rapidly presented by MHC-I within minutes after translation of the protein source. The physiologic significance of this rapid kinetics of antigen presentation is that it allows CD8 T cells to kill virus-infected cells before viral replication is completed and progeny are released. However, whether the production of peptides bound to MHC class I molecules is a deliberate process for degrading defective translation products and/or a stochastic event of normal protein translation remains to be determined.[17]

Peptide Trimming in the Cytosol

Degradation of cellular and antigenic peptides bound to MHC-I is largely mediated in the cytosol by the proteasome. The proteasome is responsible for the degradation of the majority of cytosolic and nuclear proteins, and in most cases proteasome targeted proteins are ubiquitinated. Ubiquitin (Ub) is typically coupled to internal lysine residues of proteins substrates, but coupling can also occur at the N terminus or on internal cysteine, serine, or threonine residues.[18,19,20,21] In this orchestrated process, activated Ub is transferred from one predominant Ub-activating enzyme (E1) to one of 30 to 40 mammalian Ub conjugating enzymes (E2s) and then to the substrate that is bound by one of hundreds of different ubiquitin protein ligases (E3s). The E3s are the major determinant of substrate specificity. This process can be repeated to form polyUb chains whereupon the next Ub moiety is added to one of the seven internal lysine residues of Ub. Proteins coupled with polyUb chains of four or more Ub moieties linked through Lys48 Ub residues are the prototypic signal for proteasome mediated degradation.

The cylindrical 20S catalytic particle (CP) of the proteasome is formed by four stacked rings

of seven subunits each. The inner two rings of the 20S proteasome are assembled from the beta subunits, three of which have catalytic activity with chymotryptic, tryptic, or caspase activity. Thus each proteasome has six active sites with the ability to cleave after most types of peptide bonds, although to differing efficiencies based on flanking residues. The catalytic sites are exposed to the interior of the central chamber of the 20S cylinder. The two outer rings of the 20S proteasome are assembled from alpha subunits forming a pore of 13 Angstrom, mandating protein substrates be partially denatured prior to entering the interior of the chamber. The gate of the 20S CP is normally closed, and access to the interior of the 20S proteasome is controlled by the complex of proteins termed the 19S regulatory particle that binds to each end of the 20S cylinder. The full assemblage of 19S-20S-19S complex constitutes the 26S proteasome. The lid of the 19S regulator particle binds and deubiquitinates protein substrates before they gain entry in the 20S CP; the base of the 19S regulatory particle contains ATPases that promote substrate unfolding and open the gate of the 20S CP to provide access of denatured protein substrates to the catalytic activity of the inner chamber.

The evolutionarily conserved function of the proteasome is for recycling amino acids and ubiquitin moieties. However, in mammals, modifications to the proteasome have been made to promote antigen presentation. In response to interferon (IFN)γ stimulation that occurs during inflammation, three catalytic subunits of the constitutively expressed proteasome are replaced by IFNγ-induced homologs forming what is called the immunoproteasome. More specifically, subunits β1, β2, and β5 of the constitutive proteasome are replaced by subunits β1i (LMP2), β2i (MECL-1), and β5i (LMP7) of the immunoproteasome. Suggesting an immunologic function,

region of the mouse and human MHCs juxtaposed to genes encoding the TAP heterodimer. Consistent with their immunologic relevance, the immunoproteasomes display enhanced cleavage of protein substrates after hydrophobic and basic amino acids, thus generating peptides with C-terminal residues preferred for binding to many MHC-I alleles. IFNγ stimulation also induces the expression of PA28α and PA28β that form the 11S regulatory particle, which can also bind to either end of the 20S CP. Interestingly, the PA28 regulatory particle does not contain ATPases, but it does induce conformational changes to open the 20S CP gate. Thus, PA28 may function to prevent the overdigestion of short peptides. This is an important issue, as it has been estimated that a nonamer peptide in the cytosol has a half-life of only 7 seconds.[22] However, studies testing PA28α/β-deficient mice demonstrated that PA28α/β is not required for overall antigen presentation during virus infection, but may affect degradation of select substrates.[23] In any case, the inclusion of the IFNγ-inducible β subunits into the 20S CP of immunoproteasome enhances antigen presentation by generating peptides with preferred C-terminal anchor residues, and in some same cases inclusion of the PA28 regulatory particle may prevent overdegradation of immunologically relevant proteins.

The proteasome generates the final C-terminal cleavage of most MHC-I-binding peptides. A recently reported exception to this is the generation of the tumor antigen MAGE-A3 by insulin-degrading enzyme.[23] Whereas insulin degrading enzyme is solely responsible for the generation of the human leukocyte antigen (HLA)-A1-restricted MAGE-A3 epitope, the HLA-B40-restricted MAGE-A3 epitope was proteasome dependent. Although there are several N-

terminal proteases in the cytosol, they have only a limited role in generation of the MHC-I-binding peptides. More specifically, tripeptidyl peptidase II, leucine amino peptidase, bleomycin hydrolase, and puromycin-sensitive aminopeptidase have all been found to affect generation of select MHC-I-binding epitopes, but these proteases have an overall negative effect on antigen presentation by MHC-I. Interestingly, the metalloprotease nardilysin that cleaves substrates on the N-terminus of arginine residues in dibasic pairs was implicated in three cytotoxic T lymphocyte epitopes and may be more generalizable given the preference for basic N-terminal resides for MHC-I binding.[24] However, as noted in the following, significant N terminal trimming of MHC-I-binding peptide occurs after transport from the cytosol into the ER.

Peptide Transport into the Endoplasmic Reticulum

Landmark studies of the mutagenized and immunoselected mouse cell line RMA-S resulted in the discovery of how peptides are transported from the cytosol into the ER lumen.[25,26,27] The low level of surface expression of MHC-I molecules by RMA-S that could be rescued by either low temperatures or culturing with known MHC-I-binding peptides was attributed to lack of peptide transport by TAP. TAP is a member of the adenosine triphosphate (ATP) binding cassette family. Mechanistically, hydrolysis of ATP is required both for peptide binding to the cytosolic face of TAP as well as peptide transport into the ER. Structurally, TAP is a heterodimeric complex of TAP1 and TAP2 subunits both encoded within the central region of the MHC of mouse and human. The translocation pore of TAP is formed by six transmembrane domains of each subunit, whereas the remaining transmembrane domains (four for TAP1 and three for TAP2) are involved in interaction with peptide loading complex (PLC).[28] Awaiting transport, both the N- and C-termini of peptides are bound to TAP[29]; peptides with higher affinity for binding TAP have a greater likelihood of transport, MHC binding, and presentation to T cells during infection.[30,31] TAP typically transports peptides of 8 to 16 residues, although longer peptides are less efficiently transported. As discussed in the next section, peptides with N-terminal extensions are trimmed in the ER.

Peptide Trimming in the Endoplasmic Reticulum

As noted previously, TAP transports many peptides with C-terminal hydrophobic residues required for MHC binding, but with N-terminal extensions, which need to be cleaved to conform to the MHC-binding motif of the expressed alleles. It is now clear that N-terminal trimming within the ER is carried out by the ER-associated aminopeptidase (ERAAP; ERAP1 in mice and ERAP1 in humans). Interestingly, unlike other aminopeptidases, ERAP1 preferentially cleaved peptides of 9 to 16 amino acids thus matching the same peptide length preference as transported by TAP.[32] More specifically, ERAP1 was found to degrade a model 13-mer to a 9-mer and then stop, an apparent adaptation to maximize optimal MHC-I binding. The length preference of ERAP1 could be explained by extended peptides binding to MHC-I before N-terminal trimming. However, this model is not supported by structural analyses of ERAP1 and the location of its active site.[33] Alternatively, recent analyses support a “molecular ruler” model. In this model, a hydrophobic pocket of ERAP1 binds the C-terminal peptide residue thereby positioning the peptide so that N-terminal cleavage occurs about nine amino acids away.[32,33] Thus, ERAAP/ERAP1 has unique structural features explaining its ability to trim extended peptide precursors while sparing ones of optimal length for MHC-I

binding. As further evidence of their specialized functions in antigen presentation by MHC-I proteins, ERAP1 preferentially binds and processes peptides with hydrophobic C-terminal residues consistent with the majority of the TAP-transported MHC-I-binding peptides. Interestingly, humans have a second ER-associated peptidase with homology to ERAP1 designated ERAP2.[34] ERAP2 preferentially binds peptides with C-terminal basic residues and thus may function in human cells to provide peptides for HLA-A3, -Aw68, and -B27 alleles that bind such peptides. Additionally, in humans the combined activity of both ERAP1 and ERAP2 (most likely as heterdimers) is required for the presentation of certain epitopes.[35] By contrast, in mice ERAAP/ERAP1 is sufficient to service their MHC-I alleles, all of which bind peptides with hydrophobic C-termini. As additional evidence for their tailoring for MHC-I-antigen presentation, the expression and trimming activity of ERAP1 and ERAP2 are upregulated upon IFNγ stimulation.

The impact of ERAP on specific epitopes and the overall MHC-binding peptide repertoire has been analyzed using

cell lines[36,37] and ERAAP-knockout mice.[38,39] These studies have shown that cytotoxic T lymphocyte detection of some but not all MHC-I/peptide complexes are ERAP1-dependent. For example, in vitro ribonucleic acid interference experiments suggested that ERAP1 is involved in formation of onethird of peptide/MHC-I complexes.[36,37] ERAAP-deficient mice had a 20% lower expression of K^b, D^b, K^d, and D^d alleles at the cell surface but a 70% lower expression of L^d. Of these mouse alleles, only L^d preferentially binds peptides with the motif of Pro in the second position, although about 20% of human HLA alleles also prefer peptides with a Pro in the second position. Notably, peptides with Pro in the second position are only transported by TAP with N-terminal extensions and are thus dependent upon ERAP1 for their generation. In vivo relevance of this conclusion was demonstrated by the observation that ERAAP-deficient BALB/c mice were found to be susceptible to *Toxoplasma gondii* infection resulting from the fact that the immunodominant epitope presented to CD8 T cells is L^d restricted.[40] Interestingly the ERAAP-deficient mice were also found to present several unique peptides that elicited potent CD8 T-cell responses demonstrating ERAAP functions, as a peptide editor altering the repertoire of peptides presented.[38]

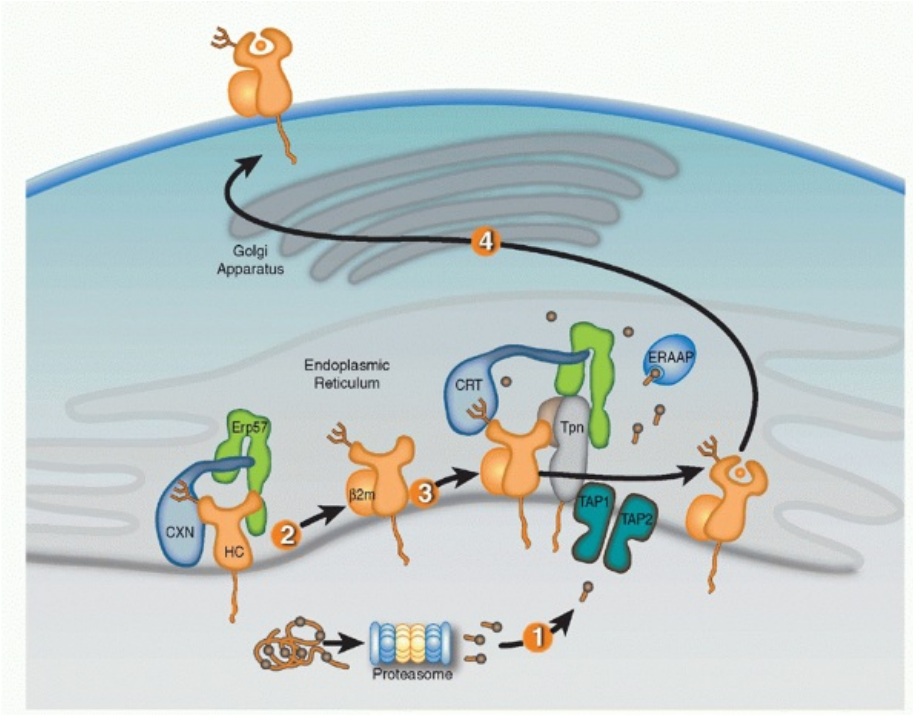

FIG. 22.1. Schematic Representation of the Sequential Events of Antigen Processing and Presentation by Major Histocompatibility Complex (MHC)-I Proteins. 1: Peptides are processed by the proteasome and translocated into the ER by TAP1/TAP2 heterodimers. **2:** MHC-I heavy chains (HCs) awaiting assembly with β2m are bound to calnexin (CXN)/ERp57 complexes. **3:** After assembly with β2m, HC/β2m heterodimers enter the peptide loading complex wherein tapasin (Tpn) bridges HC with transporter associated with antigen presentation (TAP)/TAP2, and calreticulin (CRT)/ERp57 complexes bridge HC with Tpn. **4:** Following the binding of a suitable peptide, which may require final N-terminal trimming by ERAAP, fully assembled MHC-I proteins (HC/β2m/peptide) traffic to the plasma membrane via the secretory pathway.

Chaperone-assisted Peptide Loading in the Endoplasmic Reticulum

Full assembly of the MHC-I heavy chains (HCs) with β2m and peptide within the ER is orchestrated by molecular chaperones that keep folding intermediates in a conformation capable of attaining full assembly (Fig. 22.1). Nascent HCs are transiently associated with the membrane-anchored

lectin calnexin (CXN) that, like its soluble paralog calreticulin (CRT), functions as a general chaperone for assembly of oligomeric glycoproteins. Quality control of glycoprotein assembly by CXN and CRT is carried out by monitoring terminal glucose residues on their N-linked glycans. More specifically, the folding sensor UDP-glucose glycoprotein glucotransferase allows incompletely assembled substrates to reassociate with CXN or CRT, and cycles of glucose addition and removal continue until substrates are either correctly folded and assembled or targeted for ERAD. After the MHC-I HC assembles with β2m, the HC/βm heterodimer exchanges CXN for CRT and enters the PLC consisting of CRT, ERp57, tapasin (Tpn), and TAP.[41,42,43,44] Optimal antigen presentation by MHC-I is dependent upon all

four components of the PLC (Tpn, TAP, CRT, and ERp57) that function to improve peptide loading in MHC-I proteins and their surface display.[45,46]

Knockout cell lines, genetically deficient mouse strains, and mutagenesis studies have provided key insights in the selective roles of PLC components in MHC-I assembly. CRT association with MHC-I proteins is dependent upon the N-linked glycan at residue Asn86 of the HC. Interestingly, the location of the glycan is important for CRT but not CXN association with HCs, likely reflecting geometric constraints imposed by PLC assembly. CRT-deficient cells have suboptimal peptide loading resulting in reduced surface MHC-I expression.[47] ERp57 is a thiol oxidoreductase that mediates disulfide bond formation of different substrates and is commonly associated with CXN and CRT. Interestingly, however, the function of ERp57 as a component of the PLC appears to be independent of the MHC-I redox state. ERp57 forms a disulfide bond with Tpn that is required for PLC construction.[48,49] It was originally proposed that the ERp57-Tpn complex prevents reduction of the α2 disulfide HC bond, thereby keeping HC in a peptide-receptive state.[50] However, other studies have detected little evidence the ERp57 controls redox state of MHC-I and more recently, the crystal structure of the ERp57-Tpn complex suggests that the role of the ERp57 in the PLC is structural, facilitating recruitment of peptide-accessible MHC-I.[49,51,52] As a likely reflection of its fundamental role in PLC construction, ERp57-deficient cells were found to have impaired peptide loading, surface expression, and antigen presentation by MHC-I proteins.

Tpn, an MHC-I-dedicated molecular chaperone, is required to bridge β2m-assembled HCs with TAP.[53] Playing a redundant role with other members of the PLC, Tpn also functions in ER retention of MHC with suboptimal peptide cargo.[54,55] Perhaps most importantly, Tpn functions as a peptide editor by stabilizing peptide-accessible MHC-I proteins and optimizing peptide cargo before release from the PLC. The mechanism of peptide editing by Tpn of MHC-I proteins was revealed using recombinant Tpn tethered to HLA,[56] and using recombinant Tpn-ERp57 conjugates added to Tpn-deficient cells.[57] These and other reports support the model that Tpn promotes the peptide exchange of MHC molecules in an affinity-dependent manner. The consensus model is that Tpn stabilizes the peptide binding groove of MHC-I in an “open” peptide-accessible conformation. The association of Tpn with peptide-accessible MHC-I then promotes peptide exchange until a peptide of suitable affinity binds to complete the folding of the ligand binding groove. This peptide-induced folding then induces the release of fully assembled MHC-I from the PLC. Logistically, how peptide editing by Tpn might occur was provided by mutagenesis studies of the MHC-I/Tpn interaction site. Two sites on the HC are critical for Tpn interaction: one site is in the α3 domain (residues 227 and 229) and the other in the α2 domain (residues 128-136)[43,58,59] (Fig. 22.2A). Based on the location of the latter site and molecular dynamic modeling, it has been theorized that the α2 interaction site might function as a folding sensor for C-terminal peptide anchoring in the MHC-I F pocket.[60,61] Consistent with this model, polymorphisms within the F pocket can have a profound effect of Tpn dependencies of different MHC-I alleles.[62] Based on mutagenesis and structural analyses, a credible model was constructed by Dong et al. of the MHC-I/PLC interactions[52] (see Fig. 22.2).

Although Tpn within the PLC is thought to enforce most of the quality control of antigen

presentation by MHC-I, there are also adjunct pathways. For example, the sensor UGT1 that adds a terminal glucose to unfolded ER proteins for rebinding CRT has been recently implicated in the quality control of antigen presentation by MHC-I. In support of this conclusion, using in vitro assays with recombinant components, UTG1 was reported to preferentially reglucosylate MHC-I proteins loaded with suboptimal peptides.[63] Also, as a post-ER quality control pathway, CRT was reported to use a KDEL-dependent mechanism to recycle suboptimally loaded MHC-I proteins from the early Golgi back the ER for improved peptide binding.[64]

Viral Immune Evasion of Antigen Presentation

As noted previously, several molecular components of antigen presentation were coopted from physiologic pathways of protein degradation and quality control. Using elegant mechanisms, viruses express diverse proteins that coopt these same pathways of protein degradation and quality control to evade immune detection by CD8 T cells.[65] Indeed, the specificity and potency of immune evasion proteins impairing antigen presentation makes them efficacious probes for physiologic pathways of relevance for MHC expression. Most of the well-characterized immune evasion proteins are expressed by deoxyribonucleic acid viruses with large genomes, particular viruses capable of latency or host coexistence. Strikingly, immune evasion proteins block several different steps of the antigen presentation pathway by MHC-I proteins (Fig. 22.3). For example, Epstein-Barr virus and Kaposi sarcoma-associated herpesvirus express proteins during latency that escape cytotoxic T lymphocyte detection by containing sequences that inhibit proteasome processing.[66,67,68,69,70] Alternatively, blocking peptide transport by inhibiting TAP function is a commonly used immune evasion strategy. For example, ICP47 of herpes simplex virus binds the cytosolic side of TAP and blocks peptide and not ATP binding,[71,72,73,74,75,76] whereas US6 of human cytomegalovirus (HCMV) binds the luminal side of TAP and induces a conformational change resulting in inhibition of ATP hydrolysis

and peptide translocation.[77,78,79,80,81] As a third mechanism of TAP blocking, UL49.5 of bovine herpesvirus induces TAP degradation.[82] There are also published examples of immune evasion proteins inhibiting Tpn function. For example, US3 of HCMV binds to Tpn and impairs optimization of peptide loading,[83,84] whereas E3-19K of AdV inhibits the ability of Tpn to bridge MHC-I with TAP.[85]

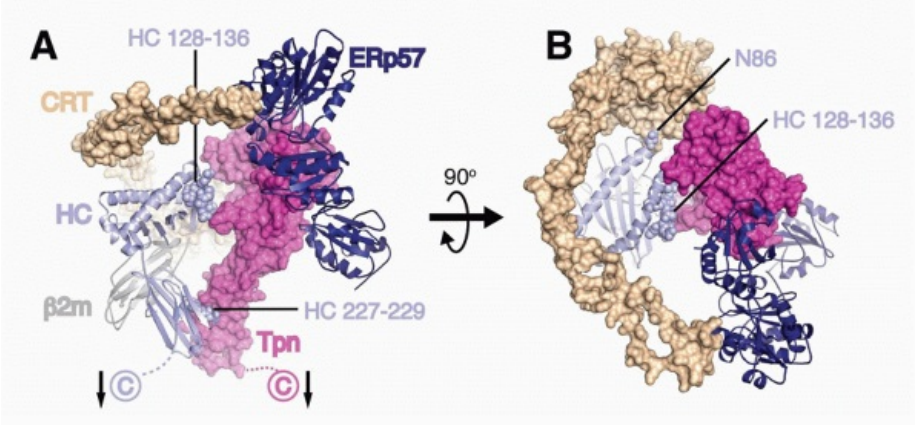

FIG. 22.2. A Model of the Peptide Loading Complex Based on Known Structures and Mutagenesis Data of Interaction Sites.[52] Heavy chains (HCs), β2m, and ERp57 are shown as ribbons, while calreticulin (CRT) and tapasin (Tpn) are shown as Connolly surfaces (1.4 Å probe). HCs and Tpn are membrane anchored as indicated with *arrows*, whereas transmembrane interactions between Tpn and TAP (not shown) attach the phospholipase C (PLC) components to TAP. HC sites predicted by mutagenesis studies to interact with Tpn (residues 227 to 229 and 128 to 136) and CRT (N86) are indicated as *spheres*. The long flexible P domain of CRT extends above the major histocompatibility complex (MHC)-I peptide binding platform before it noncovalently binds to ERp57, which is covalently attached to Tpn. **A:** PLC components are shown from the side to highlight Tpn contacts to the HC α2 and α3 domains. **B:** PLC components shown from above reveal coordinated chaperone binding of both MHCI α-helices near the antigenic peptide's C-terminus. The figure was rendered by Drs. William McCoy IV and Daved Fremont using Protein Data Bank (PDB) files kindly provided by Drs. Karin Reinisch and Peter Cresswell. The structures of the Tpn/ERp57 complex was published in Dong et al.,[52] the structure of CRT was modeled using PHYRE2[235] on its homolog calnexin,[236] and human leukocyte antigen-A2[237] is shown as a representative HC. It should be noted that the HC conformation shown is that attained after peptide occupancy, as the peptide-accessible HC conformation that binds the PLC has not been resolved.

Immune evasion proteins also employ strategies to misdirect trafficking of MHC-I proteins. For example, the aforementioned E3-19K protein of AdV binds MHC-I proteins and blocks their transport out of the ER.[86,87,88,89,90] Interestingly, cowpox virus expresses two different immune evasion proteins targeting MHC-I proteins that function in tandem. Cowpox virus 12 is a TAP function blocker that curtails peptide supply, whereas cowpox virus 203 returns fully assembled MHC-I proteins from the Golgi back to the ER.[91,92,93,94] To also misdirect assembled MHC-I proteins, the Nef protein of human immunodeficiency virus and gp48 protein of murine cytomegalovirus shuttle assembled MHC-I proteins from the Golgi to the lysosome.[95,96,97]

Interestingly, the aforementioned immune evasion proteins for the most part do not have cellular homologs, making it difficult to track their evolution. Striking exceptions to this

generality are the viral ER ubiquitin ligases called the viral MARCH (*m*embrane-*a*ssociated *R*ING-*CH*) proteins. The extensive family of MARCH proteins includes viral and cellular homologs that share a transmembrane orientation and a highly conserved atypical RING domain that confers E3 ubiquitin ligase activity.[98,99,100] Viral proteins mK3 of MHV68, and proteins kK3 and kK5 of Kaposi sarcoma-associated herpesvirus that function as immune evasion proteins were the founding members of the MARCH protein family.[101,102,103] Mechanistically, mK3 binds to TAP and awaits the entry of MHC-I into the PLC after which mK3 ubiquitinates the cytosolic tail of MHC-I HCs and thus induces their dislocation to the cytosol and degradation in the cytosol (ie, ERAD).[104,105,106,107] Of note, the extensively studied US2 and US11 proteins of HCMV also target ERAD of MHC-I proteins by recruiting cellular E3 ligases.[108,109,110,111,112,113] Indeed, studies of US2, US11 and mK3 continue to provide molecular insights into various mechanisms by which ERAD substrates are detected in the ER and dislocated to the cytosol.[114,115] In contrast to ERAD, the kK3 and kK5 MARCH ligases of Kaposi sarcoma-associated herpesvirus induce endocytosis and lysosomal degradation of MHC-I proteins. Interesting, kK3 like mK3 appears to ubiquitinate only MHC-I proteins, whereas kK5 targets other surface receptors including the T cell costimulation molecule CD86 (B7.2) as well as natural killer cell ligands, MHC class I-related chain A or B (MICA, MICB), and newly defined ligand for NKp80, activationinduced C-type lectin (AICL).[116] Inhibition of natural killer responses is of importance to the virus because downregulation of MHC-I proteins renders cells susceptible to natural killer cell lysis. Mechanistic studies of viral MARCH

proteins have provided several molecular insights into ubiquitin-dependent degradation pathways of particular relevance to antigen processing. In addition, there are about 10 cellular MARCH protein homologs in humans and mice, many of which detect immune receptors.[98,99,100] For example, as mentioned in the MHC-II section, MARCHI regulates MHC-II expression in B cells and dendritic cell (DC) subsets.[117,118,119] Thus, viral MARCH proteins were stolen from the host and adapted by the virus to function as immune evasion proteins using similar mechanisms.

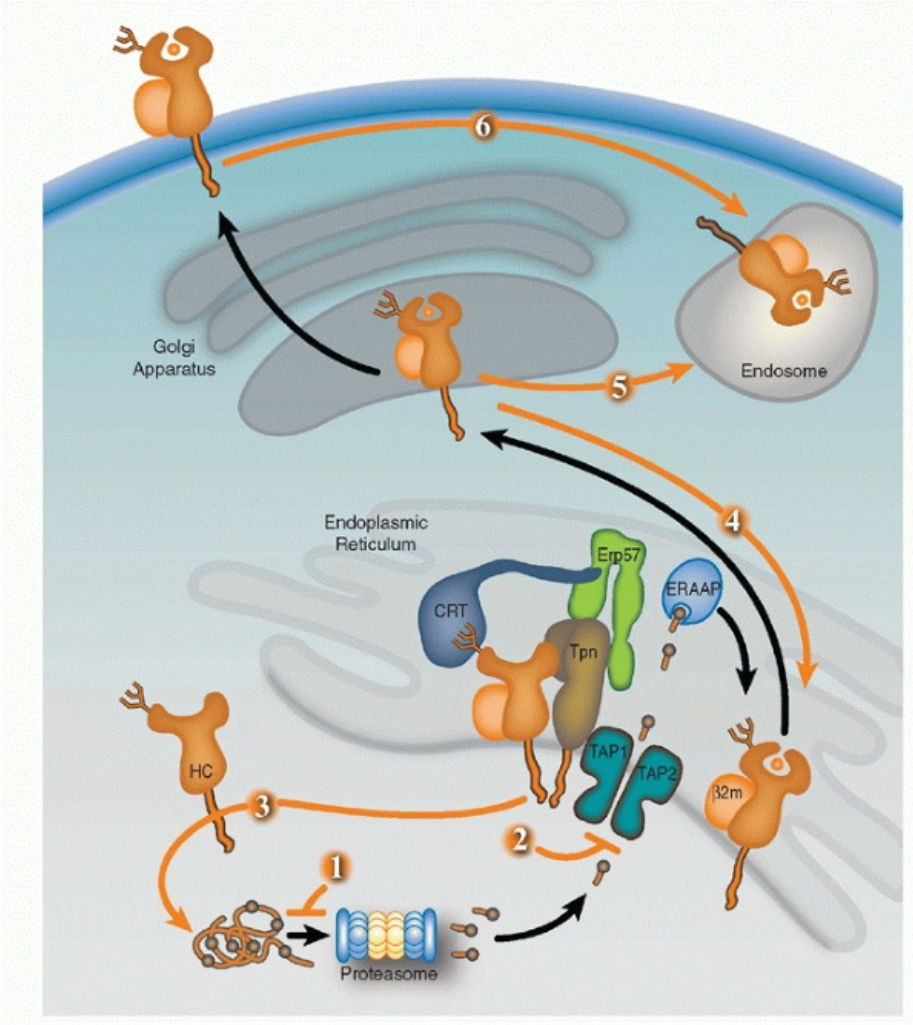

FIG. 22.3. Immune Evasion Strategies Used by Viral Proteins to Block Antigen Presentation by Major Histocompatibility Complex (MHC)-I Proteins. *Black arrows* demarcate physiologic pathways for antigen presentation, whereas the *numbered red arrows* demarcate reported immune evasion strategies by virus proteins. **1:** Blocking of antigen processing by the proteasome (examples are the EDNA1 protein of Epstein-Barr virus or the LANA1 protein of Kaposi sarcoma-associated herpesvirus). **2:** Blocking peptide transport by transporter associated with antigen presentation (examples include the ICP47 protein of herpes simplex virus, US6 protein of human cytomegalovirus, cpxv12 protein of cowpox virus, UL49.5 protein of bovine herpesvirus, and BNFL2a protein of Epstein-Barr virus). **3:** Dislocation of MHC-I proteins from the endoplasmic reticulum (ER) to the cytosol for proteasome-mediated degradation (examples are US2 and US11 proteins of human cytomegalovirusand mK3 protein of MHV68). **4:** ER retention and retrieval of MHC-I proteins (examples are E3-19K protein of AdV and the cpxv203 protein of cowpox virus). **5:** Mistrafficking of MHC-I proteins from the Golgi to an endosomal compartment (examples include gp48 protein of murine cytomegalovirus and Nef protein of human immunodeficiency virus-1). **6:** Rapid internalization of MHC-I proteins from the cell surface to an endosomal/lysosomal compartment (examples are kK3 and kK5 proteins of Kaposi sarcoma-associated herpesvirus).

ANTIGEN PROCESSING AND PRESENTATION BY MAJOR HISTOCOMPATIBILITY CLASS II (MHC-II) PROTEINS

Source of Antigen for MHC-II Presentation

As a general rule, the antigen-processing machinery that is utilized by MHC-I is specialized for the degradation, importation, and binding of peptides that are derived from antigens present in the cytosol of host cells. Although many viruses and bacteria thrive in the cytosol of infected cells, there are pathogens (such as *Toxoplasma gondii*) that reside in membrane-encapsulated intracellular compartments and do not access the cytosol.[120] Furthermore, almost any foreign material (including live or dead pathogens, fragments of apoptotic cells, or soluble proteins) can enter the cell by endocytosis and are therefore sequestered from the cytosol. It is for recognition of antigens such as this that MHC-II molecules exist; MHC-II binds peptides derived from antigens that gain access to intracellular compartments in cells by a variety of endocytic pathways.[121] Fluid-phase macropinocytosis is a process whereby extracellular fluid is taken up by plasma membrane protrusions that bring extracellular fluids and soluble proteins into the cell by endocytosis. Once internalized, macropinosomes fuse with early endosomes, thereby releasing their contents to the endocytic pathway. Immature (resting) DCs are particularly efficient in their capacity for macropinocytosis that enhances their significance as “sentinals” of the immune system.[122] Upon activation by encounter with foreign organisms, DCs greatly suppress macropinocytosis,[122,123,124] thereby limiting the ability of the activated DCs to sample their microenvironment and restricting the repertoire of antigenic peptides presented to T-lymphocytes.

As one can imagine, macropinocytosis is not a particularly efficient mechanism of antigen uptake. B cells possess antigen-specific receptors (immunoglobulins) that permit specific antigen internalization and presentation to T cells that can be 1000 times more efficient than fluid-phase endocytosis.[125] Curiously, the function and fate of B cells that generate specific MHC-II peptide complexes following immunoglobulin-mediated antigen uptake versus fluid-phase uptake of the same antigen differ significantly,[126] highlighting the importance of the receptor-mediated antigen uptake pathway in B cells. Similarly, macrophages and DCs express Fc receptors and complement receptors that allow efficient phagocytosis of large extracellular particles such as antibody-coated organisms or even apoptotic cells. Following endocytosis, the phagosome fuses with the membrane of a lysosome, forming a degradative phagolysosome that neutralizes and degrades internalized antigens into peptides.[127] Other surface receptors, such as mannose receptors[122,128,129] and DEC-205[130] on DCs, also allow for efficient antigen binding and uptake onto the endocytic pathway, and these receptors have been exploited as a vehicle to efficiently deliver bring foreign vaccine antigens into the MHC-II processing pathway in antigen-presenting cells (APCs).[131]

While antigens entering the endocytic pathway by phagocytosis, macropinocytosis, and receptor-mediated endocytosis give rise to the majority of peptides that bind to MHC-II, there is increasing evidence that cytosolic antigens are capable of entering endosomal MHC-II processing compartments and are degraded there into MHC-II-binding peptides. The most likely cellular mechanism responsible for this phenomena is process termed autophagy.[132,133] Macroautophagy is constitutively active in DCs and is a process that

leads to the “engulfment” of aggregated cytosolic protein complexes to form an autophagosome. Like phagosomes from extracellular antigens, the autophagosome fuses with a lysosome and results in the degradation of autophagosome contents into antigenic peptides.[134,135] A related process, termed chaperone-mediated autophagy, results from the direct import of cytosolic proteins into the lumen of endo/lysosomes and is facilitated by the chaperone protein hsc-70 and the lysosomal integral membrane protein Lamp2a.[136] Once inside this organelle, the imported protein is degraded into antigenic peptides just like “conventionally” internalized antigens. While this pathway clearly functions in vitro, the extent to which autophagy participates in MHC-II-restricted immune responses remains to be established.

MHC-II Assembly and Transport to the Endosomal Pathway

Because MHC-II is specialized to bind peptides generated in the endocytic pathway, there must be mechanisms in APCs to prevent the premature binding of peptides to MHC-II as the molecules assemble in the ER and traffic through the Golgi apparatus before their delivery to endosomal peptide loading compartments. In addition to the inability of MHC-II to bind to TAP/tapasin and other members of the MHC-I-peptide loading complex, MHC-II is unique in that it binds to a transmembrane chaperone protein termed the invariant chain (Ii). It is the association of Ii with the MHC-II-peptide binding site that prevents MHC-II molecules in the early secretory pathway from binding peptides.[137,138] Indeed, failure of newly synthesized MHC-II to bind Ii allows for the presentation of ER-imported peptides to CD4 T cells by MHC-II.[139] A specific region of Ii, termed the class II-associated Ii peptide (CLIP), serves to block the peptide-binding groove of nascent MHC-II molecules until it is ready for removal in endo-/lysosomes. The MHC-II-CLIP complex has been crystallized, and as one might expect, the structure of this complex is nearly identical to that of a MHC-II molecule containing an “antigenic” peptide.[140] As the name implies, unlike the highly polymorphic MHC-II molecule itself, Ii is nonpolymorphic. However, the human Ii mRNA can initiate protein synthesis using one of

two translation initiation sites (Fig. 22.4A), leading to generation of a major 33 kDa form of Ii (Ii-p33) as well as a minor population of larger Ii molecules whose translation is initiated using an upstream methionine (Ii-p35[141]). The use of alternative translation initiation sites appears to be unique to humans, as it is not seen in rodents.[142] Furthermore, alternative mRNA splicing in all species examined can give rise to an additional 8 kDa domain in each form of Ii, leading to the production of Ii-p41 and (in humans) Ii-p43.[143,144] This additional exon encodes a domain (see subsequent discussion) that regulates the activity of proteases in endo-/lysosomes.

The Ii molecule itself forms a trimer even in the absence of MHC-II, and each constituent subunit of this trimer contains either Ii-p33, -p35, -p41, or -p43 isoforms (see Fig. 22.4B). Ii homotrimers and mixed heterotrimers are generated, and each trimer contains an Ii molecule whose presence in the trimer is proportional to its level of expression.[145,146,147] Onto this “scaffold” of an Ii trimer, pairs of MHC-II αβ heterodimers assemble, ultimately forming a mature, nine-chain MHC-II-Ii complex.[148] The partially formed MHC-II-Ii complex containing an Ii trimer and only one or two αβ heterodimers is retained in the ER by interacting with the

chaperone calnexin,[149] presumably allowing the MHC-II molecule additional time to form a fully functional MHC-II-Ii nonamer. In addition, the longer p35 and p43 forms of human Ii contain an ER retention motif, and free Ii trimers containing one of these longer Ii forms are retained in the ER.[150,151] Curiously, completely folded MHC-II-Ii nonamers containing one of

these longer Ii isoforms are capable of exiting the ER and moving to antigen-processing compartments,[147,152] demonstrating that assembly with MHC-II masks the ER-retention motif on the Ii trimer.

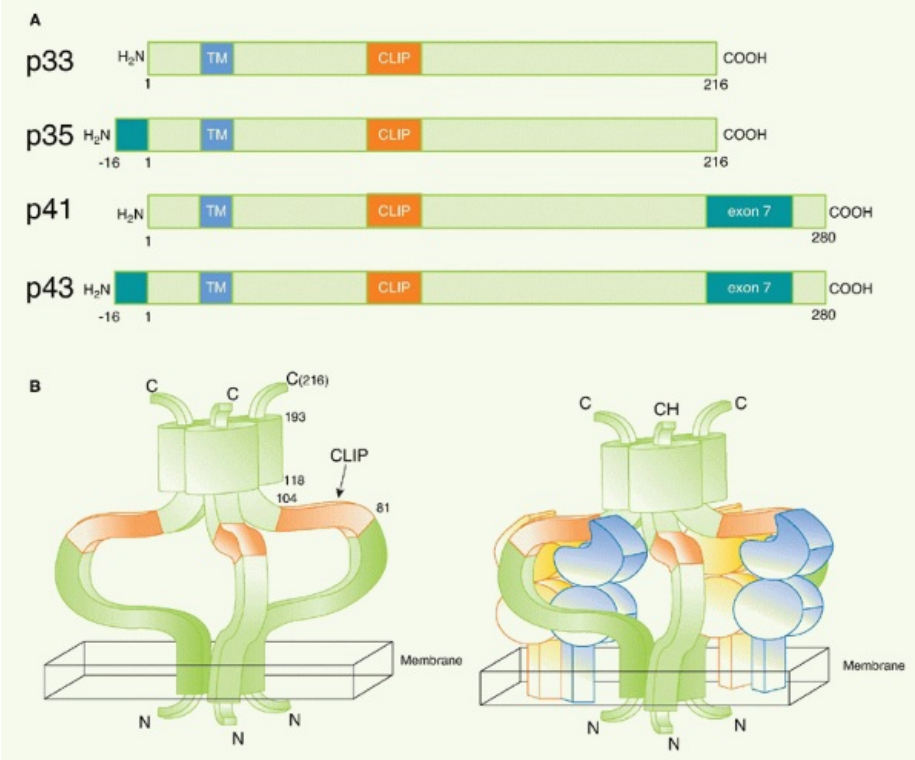

FIG. 22.4. Structure of Invariant Chain (Ii) and Major Histocompatibility Complex (MHC)-II-Ii Complexes. A: The major 216 amino acid form of human Ii is the 33 kDa Ii-p33 isoform. The use of an upstream initiation methionine results in the generation of Ii-p35 (which contains an endoplasmic reticulum-retention motif), and alternative splicing of each of these isoforms leads to the generation of Ii-p41 and Ii-p43 forms of Ii that possess proteinase inhibitor activity. The relative positions of the transmembrane domain (TM) and class II-associated Ii peptide region of each isoform of Ii are indicated. **B:** Models of Ii trimer (*left panel*) and the MHC-II-Ii complex (*right panel*). Only two of the three pairs of MHC-II αβ dimers are shown bound to the Ii trimer. The model shows the peptide-binding groove of MHC-II αβ dimers occupied by the CLIP region of Ii. (From Cresswell P. The biochemistry and cell biology of antigen processing. In: Paul WE. *Fundamental Immunology.* 5th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2003:613-629, reprinted with permission).

Ii association does more for MHC-II than simply prevent premature peptide binding. Assembly with Ii is critical for some alleles of MHC-II to fold property and achieve competence to leave

the ER, thus revealing a role for Ii as an assembly/folding molecular chaperone.[153] After leaving the ER, the MHC-II-Ii complex moves through the Golgi apparatus and is targeted to the endosomal pathway[150,154] (Fig. 22.5A). It is likely that MHC-II-Ii complexes arrive in the endocytic pathway by two distinct mechanisms and the relative contribution of each pathway may be cell-type specific. Some MHC-II-Ii complexes target directly from the trans-Golgi network to endosomes,[155,156] while other complexes move from the trans-Golgi network directly to the plasma membrane and enter endosomes only after endocytosis.[157] The movement of MHC-II-Ii complexes to the endosomal pathway is facilitated by recognition of two dileucine sorting motifs present in the cytosolic domain of every isoform of Ii.[158,159] The machinery that recognizes these motifs in the trans-Golgi network remains to be defined; however, it is clear that these motifs are recognized at the plasma membrane by the AP-2 adaptor protein complex.[160,161] This protein complex serves as a linker between Ii and the clathrin-mediated endocytosis pathway and serves to deliver plasma membrane-localized MHC-II-Ii to the early endosomes. Recognition and internalization of MHC-II-Ii from the plasma membrane is efficient and rapid,[157] and as a consequence of this, very little MHC-II-Ii is present on the plasma membrane of APCs at any given moment.

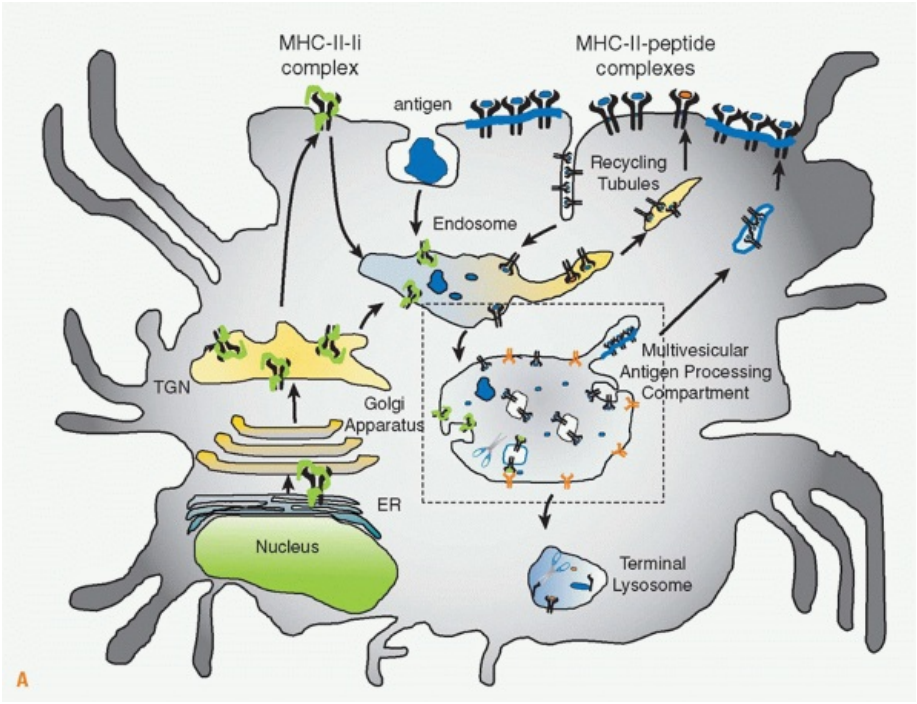

FIG. 22.5. Transport of Major Histocompatibility Complex (MHC)-II in Antigen-Presenting Cells. A: MHC-II associates with Ii in the endoplasmic reticulum, moves through the Golgi apparatus to the plasma membrane, and enters the endocytic pathway by clathrin-mediated endocytosis. Some fraction of MHC-II can also access these endocytic compartments directly from the Golgi apparatus/trans-Golgi network. Antigens access these same endocytic compartments by a number of different endocytosis pathways. The MHC-II-invariant chain (Ii) complex moves to late endosomal/prelysosomal antigen-processing compartments in which Ii is degraded, the MHC-II-associated class II-associated Ii peptide (CLIP) fragment of Ii is removed by DM,

and peptides derived from foreign antigens then bind to MHC-II (this compartment is highlighted in *panel B*). MHC-II-peptide complexes leave these intracellular antigen-processing compartments via vesicles or tubules that fuse with the plasma membrane, thereby delivering MHC-II-peptide complexes on the antigen-presenting cell surface. Once at the surface, MHC-II-peptide complexes can recycle between the plasma membrane and the endosomal system, potentially exchanging antigenic peptides. Some fraction of internalized MHC-II is also targeted for degradation in terminal lysosomes. *(continued)*

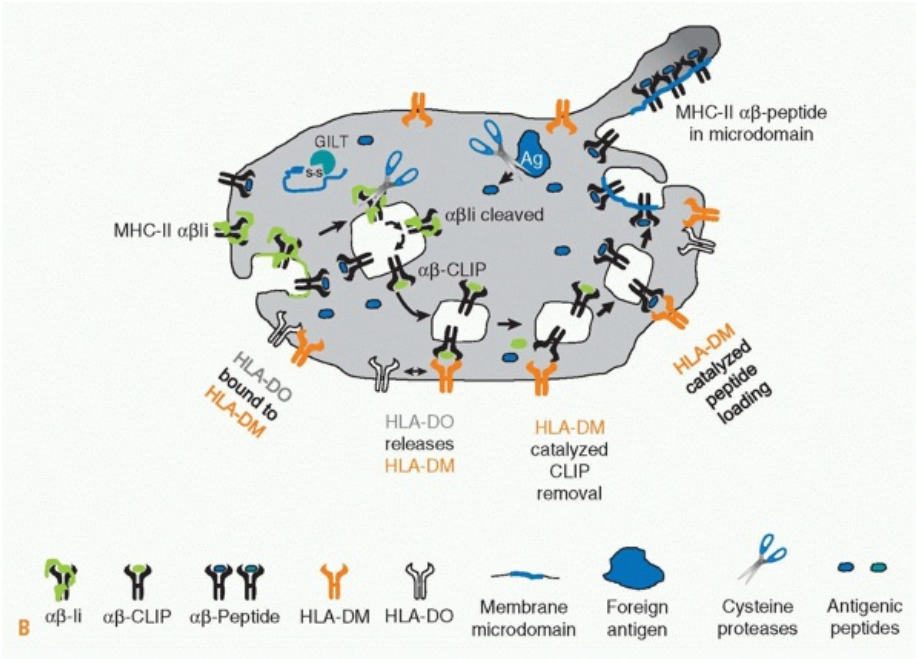

FIG. 22.5. *(continued)* **B:** Binding of peptides to MHC-II in antigen-processing compartments. The multivesicular antigen-processing compartment highlighted in *panel A* is shown here. MHC-II-Ii complexes are targeted to intraluminal vesicles of late endosomal multivesicular antigen-processing compartments where Ii proteolysis is initiated. This process results in the generation of MHC-II-CLIP complexes that are subject to editing by DM. DO binds to DM during intracellular transport and dissociates at the low pH of these compartments, thereby liberating free DM that catalyzes the removal of CLIP from the MHC-II-CLIP complex. DM also stabilizes the structure of peptide-free MHC-II until highaffinity peptide binding. Disulfide bonds present in protein antigens can be reduced by IFNγ-induced thiol reductase, thereby enhancing the formation of antigenic peptides generated by proteolysis. In this cartoon MHC-II, DM, and DO have particular membrane distributions; however, whether these distributions are maintained in all antigen-presenting cells and whether the reactions highlighted here occur on the indicated membranes remains to be determined. MHC-II molecules present in antigenprocessing compartments are present in distinct membrane microdomains, and after peptide binding, microdomain-associated MHC-II is transported to the plasma membrane in vesicles or tubules that bud from the limiting membrane of the antigen processing compartments (DM, HLA-DM; DO, HLA-DO).

Removal of Invariant Chain in Antigen-Processing Compartments

Regardless of the mechanism responsible for delivering MHC-II-Ii to endosomes, Ii must be removed in order to allow antigenic peptides to bind to nascent MHC-II. Ii is

degraded in discrete steps by proteolysis in acidic endosomes (see Fig. 22.5B), with proteolysis initiated at the luminal C-terminus of Ii in earlier endosomes and Ii degradation completed in more acidic late endosomal/prelysosomal structures. In vitro studies have shown that many different proteinases are capable of degrading Ii; however, in vivo, the initial cleavage of Ii is initiated by a proteinase whose activity in insensitive to the protease inhibitor leupeptin while additional cleavage is strictly leupeptin-dependent.[162] Leupeptin treatment of living APCs results in the accumulation of a 21 kDa Ii degradation intermediate that retains the nonameric MHC-II-Ii stoichiometry and is unable to bind antigenic peptides.[152] After the initial cleavage of Ii, a variety of proteinases can cleave the remaining MHC-II-associated Ii molecule; however, the terminal stages of Ii degradation in B cells and DCs are dependent on the cysteine proteinase cathepsin S.[163] Inhibition of cathepsin S activity or analysis of DCs isolated from cathepsin S-deficient mice reveal the accumulation of a 10 kDa Ii degradation product, highlighting the importance of this proteinase in Ii degradation. Curiously, MHC-II-expressing epithelial cells in the thymus do not rely on cathepsin S for Ii degradation but require cathepsin L for terminal Ii degradation.[164]

Proteolysis of Ii from both the luminal C-terminus and from residues adjacent to the luminal Ii transmembrane domain ultimately leaves a small fragment of Ii bound to MHC-II. This class II-associated Ii polypeptide, termed CLIP, is bound to the peptide-binding groove of MHC-II and serves as a "placeholder" to occupy the peptide-binding site until antigenic peptides are available to bind (see Fig. 22.4). While some MHC-II alleles have a very low affinity for CLIP and CLIP spontaneously dissociates from MHC-II,[165] in most cases CLIP must be actively removed from the peptide-binding site of MHC-II prior to antigenic peptide binding. A seminal finding in antigen processing and presentation came in 1994 with the discovery of HLA-DM (DM).[166,167] HLA-DM (known as H-2M in mice) is a nonpolymorphic transmembrane heterodimer whose α- and β-subunits are encoded in the MHC gene locus.

Like MHC-II, DM is expressed exclusively in APCs. An important function of DM is to facilitate the removal of CLIP from the MHC-II-CLIP complex.[168,169] DM functions enzymatically, and while the precise mechanism by which DM promotes CLIP dissociation from MHC-II remains to be completely elucidated, it is thought that DM disrupts the hydrogen bonding network in the peptide binding groove of MHC-II and thereby enhances the kinetics of CLIP dissociation from MHC-II.[170] Not only does DM catalyze CLIP removal from MHC-II, but DM also facilitates the dissociation of weakly bound antigenic peptides from MHC-II[168,170,171]; it is for this reason that DM has been termed a "peptide editor." Unlike the MHC-II molecule itself, DM contains a tyrosine-based endocytic sorting motif in its cytosolic domain that directly interacts with clathrin-associated adaptor proteins.[172] The presence of this signal allows for the entry of DM into the endocytic pathway after arrival at the cell surface. Most DM is localized in late endocytic compartments[173] that contain MHC-II-CLIP complexes,[174] and the

activity of DM is enhanced by the low pH of these later endocytic compartments.[168,169,175] DM editing activity in APCs can be regulated by its association with another MHC-encoded transmembrane protein termed HLA-DO (DO; H-2O in mice[176]). While the precise function of DO in antigen processing and presentation remains to be defined, in vitro studies have shown that DO binds to DM and inhibits the ability of DM to catalyze CLIP removal.[177,178] Like DM, DO expression is relatively restricted to APCs. Unlike MHC-II-Ii or DM, DO does not contain any intrinsic endo-/lysosomal sorting motifs. Instead, DO associated with DM in the ER and DM serves to "escort" DO to antigen-processing compartments.[179] It is in the low pH of these compartments that DO dissociates from DM, thereby enhancing DM activity. In this respect, DO acts as an inhibitor of DM function in much the same way that the CLIP region of Ii acts as an inhibitor of MHC-II peptide binding.

Antigen Processing and Peptide Loading onto MHC-II

While MHC-II in lysosome-like antigen-processing compartments must be "prepared" for peptide binding (by degrading MHC-II-associated Ii and removing CLIP by DM), internalized foreign antigens must be similarly prepared for binding to the nascent MHC-II molecule. This task is initiated by proteolysis by any number of endocytic proteinases. Antigen-processing compartments have a pH between 4.5 and 6.5, and most proteinases in these compartments have enzymatic activity optima at this pH. There is remarkable diversity in the proteinase pool in these compartments and great redundancy in the ability of distinct proteinases to degrade particular proteins. For this reason, it has been difficult to identify specific proteinases whose activity is required to generate specific antigenic peptides. There are exceptions to this, however, as asparaginyl cysteine endoprotease is required for the generation of an immunodominant epitope to tetanus toxoid[180] and both cathepsin L and cathepsin S affect quantitative and qualitative differences in the repertoire of peptides bound to MHC-II molecules.[181] It is also a known fact that many internalized foreign (and self-) antigens contain intrachain disulfide bonds, and reduction of these bonds would alter protein conformation and render a protein more (or less) sensitive to proteolysis in antigen-processing compartments. As its name implies, the expression of IFNγ-induced thiol reductase, GILT, can be induced by exposure to IFNγ.[182] This thiol reductase is expressed in antigen-processing compartments in APCs, and the activity of GILT is important for the presentation of antigenic epitopes that are generated from proteins that contain many intrachain disulfide bonds.[183]

Just as important as generation of antigenic peptides is the ability of the antigen-processing compartment to limit excessive proteolytic degradation of antigenic peptides. While asparaginyl cysteine endoprotease positively regulates expression tetanus toxoid epitopes, this same proteinase destroys expression of the immunodominant epitope of myelin basic protein,[184] a protein thought to be involved in the demyelinating autoimmune disease multiple sclerosis. Clearly, the proteinase activity of antigen-processing compartments must be sufficient to generate antigenic peptides capable of binding to MHC-II but not so great as to completely destroy these same epitopes. Measurement of proteinase activity in various APC subtypes has revealed that macrophages have high proteinase activity and efficiently degrade proteins, but are relatively pool generators of antigenic peptides.[185] By contrast,

DCs and B cells possess less proteinase activity, degrade proteins less efficiently, and are superb generators of antigen peptides.[185,186] This specialization of limiting proteinase activity helps DCs function as initiators of naïve T-cell activation and B cell capacity for immunoglobulin class switching after encounter with antigen-specific T cells.

It has been proposed that binding to the MHC-II molecules can serve as a mechanism to preserve the integrity of antigenic peptides. The peptide-binding groove of MHC-II is open, and in vitro binding experiments have shown that MHC-II is capable of binding to unfolded (but otherwise intact) protein antigens.[187] The association of distinct regions within a full-length (unfolded) protein with the peptidebinding site of MHC-II could therefore protect these regions from proteolytic degradation by endo-/lysosomal proteinases. This model of "bind first trim later" has been championed as a major mechanism of peptide loading onto MHC-II[188] and has been supported by experimental studies in B cells[189]; however, the extent to which "bind first trim later" versus "trim first bind later" predominates in distinct APC subsets and for different antigens remains to be determined.

Curiously, the Ii molecule itself also can serve to limit proteinase activity of antigen-processing compartments. The additional exon that is present in Ii isoforms that are products of alternative splicing (Ii-p41 and Ii-p43) encodes a domain that functions as an inhibitor of cathepsin L.[190,191] In vitro studies showed that coexpression with Ii-p41 inhibits the kinetics of Ii-p33 degradation,[192] a finding that is consistent with the role of Ii-p41 as a proteinase inhibitor. However, no defects in function have been identified using APCs isolated from mice expression only the Ii-p41,[193] leaving the importance of this domain in APC function an open issue.

DCs are particularly interesting APCs in that they can regulate their proteinase activity upon cell activation. Immature (resting) DCs are highly phagocytic and have a remarkable capacity for macropinocytosis. Nevertheless, these cells have little proteinase activity and are relatively inefficient generators of antigenic MHC-II-peptide complexes, potentially allowing them to serve as a "depot" for antigen internalized while the cell is in the quiescent state. It has even been shown that internalized proteins can remain intact in immature DCs for days.[194,195] Activation of the immature DC by any of a number of mechanisms (either by toll-like receptor signaling or stimulation by activated T cells) leads to a number of changes in the cell, with perhaps the most important being the activation of the vacuolar proton ATPase.[196] Activation of this ATPase leads to the acidification of antigen-processing compartments, enhances the activity of endo-/lysosomal proteinases that have low pH optima, and leads to the degradation of internalized antigens to form antigenic peptides capable of binding to MHC-II. Other changes also take place following activation of immature DCs, including downregulating expression of the endogenous cysteine proteinase inhibitor cystatin C[197] and reorganizing the internal membrane structure of the antigen processing compartments[198]; however, it is likely that acidification of these compartments is the major mechanism leading to enhanced antigen processing, peptide generation, and formation of MHC-II-peptide complexes upon DC activation.

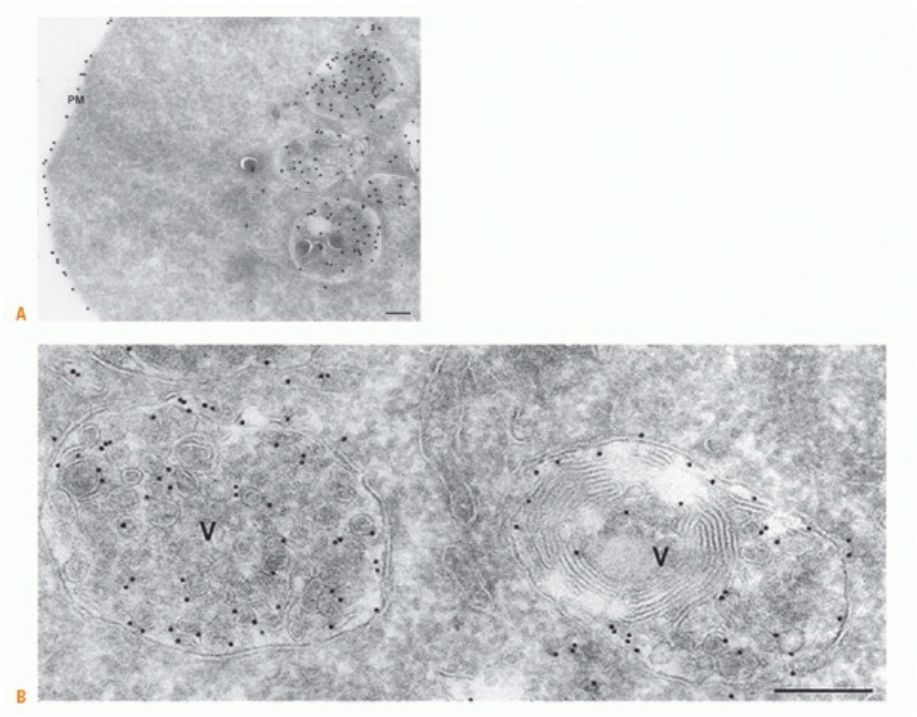

FIG. 22.6. Structure of Multivesicular Antigen-Processing Compartments. A: Ultrathin cryosections of a human B-cell line were stained with antibodies recognizing DM (10 nm gold) and total major histocompatibility complex (MHC)-II (15 nm gold). The plasma membrane stains almost exclusively for MHC-II while various forms of multivesicular antigen-processing compartments contain both MHC-II and DM (reprinted with permission Kleijmeer et al., *Methods*, 1996). **B:** Ultrathin cryosections of a mouse immature DC line were stained with antibodies recognizing MHC-II (10 nm gold). MHC-II staining is observed on the limiting membrane as well as in the intraluminal vesicles of a multivesicular body (*left*) and the numerous intraluminal membrane sheets in a multilamellar endosome (*right*) (reprinted with permission Kleijmeer et al.[198]).

Delivery of MHC-II to the Cell Surface

The sites of antigenic protein degradation and subsequent binding of peptides onto nascent MHC-II are commonly referred to as antigen-processing compartments. At one time, it was thought that APCs possessed a “special” compartment that served as the major antigen-processing compartment[199]; however, it is now clear that “antigen-processing compartments” refer to a heterogenous collection of intracellular (generally acidic) organelles that permit peptide binding onto MHC-II.[200] These organelles generally have characteristics of typical late endosomes/prelysosomes found in all cell types (Fig. 22.6); however, in APCs these organelles contain the specialized protein machinery required for antigen processing and presentation such as MHC-II, DM, DO, GILT, and Ii-processing enzymes. A “typical” antigenprocessing compartment is a late endosomal multivesicular body (MVB), an organelle that possesses intraluminal vesicles encapsulated in a limiting membrane.[201] These compartments can also have a multilamellar structure, containing numerous membrane sheets contained within an even

more acidic lysosome-like organelles. Curiously, the in DCs majority of MHC-II is present on the intraluminal vesicles of these MVBs while the CLIP editor DM and MHC-II-peptide complexes are enriched on the MVB-limiting membrane.[198] Although most MHC-II and DM are present on distinct MVB membranes, it is unclear on which membranes the process of Ii proteolysis, CLIP release, and peptide binding to MHC-II occurs. For example, while there is in vitro evidence that DM primarily interacts with MHC-II when these proteins are on the same membrane,[202,203] whether this is the intraluminal vesicle or the MVB-limiting membrane remains to be determined.

The presence of MHC-II-peptide complexes on the intraluminal vesicles of MVB presents a topological problem for the APC, as direct fusion of the MVB with the plasma membrane would release the intraluminal vesicles from these compartments to the extracellular space. Such a process would effectively result in the secretion of MHC-II from the APC. This process does occur in DCs with some frequency, and these secreted intraluminal vesicles are termed exosomes that are capable of stimulating naïve T cells in vivo.[204] While APC-derived exosomes are being utilized as cell-free vaccines,[205] their physiologic role remains to be unambiguously determined. The majority of MHC-II in antigen-processing compartments is not released as exosomes, but is instead either degraded following fusion of the MVB with lysosomes or inserted into the plasma membrane by “vesicle”-mediated protein transport. While vesicles containing intracellular MHC-II have been observed to fuse with the plasma membrane,[206] in APCs such as DCs it is likely that these “vesicles” are actually tubules that bud from the MVB membrane and subsequently fuse with the plasma membrane. The tubulation of these compartments is stimulated in DCs by APC activation,[198,207,208] and the encounter of DCs with T cells leads to the fusion of these tubules directly to the plasma membrane region at the point of DC:T-cell contact,[208] thereby delivering MHC-II-peptides generated in DCs directly to the site of T-cell receptor concentration at the immunologic synapse.

The cellular processes that govern the movement of MHC-II to the surface of APCs are similar to those that govern the movement of any late endosomal protein the surface of any cell. In some cases, the machinery regulating discrete transport steps remains a mystery while other steps are more well characterized. For example, the mechanism leading to the delivery of intraluminal vesicle-bound MHC-II back to the MVB0limiting membrane (from which tubules are formed) is termed back-fusion,[209] and the proteins and lipids that regulate this curious process remain to be identified. By contrast, it is known that the ability of MHC-II to insert into the plasma membrane is regulated by the small GTPase ARL14/ARF7 that couples the tubulovesicle membrane with the actin cytoskeleton.[210] Like other vesicle-mediated protein transport steps, microtubules and dynein/kinesin motors[211] as well as actin and myosin motors[212] are important for the locomotion of MHC-II into and out of antigen-processing compartments.

Once at the plasma membrane, MHC-II-peptide complexes are poised to interact with antigen-specific T-cell receptors. MHC-II-peptide complexes are clustered on the plasma membrane[195,213] and associate with distinct lipid raft-[214] and tetraspanin-membrane[215]

microdomains, thereby locally concentrating MHC-II on the cell surface. Biochemical studies have revealed that MHC-II associates with these membrane microdomains while still present in antigen-processing compartments even before antigenic peptide binding.[216] This finding is consistent with the observation that MVBs contain large amounts of cholesterol, a major constituent of lipid raft membrane microdomains. [217] While lipid raft- and tetraspanin-microdomain associations are important for the T-cell stimulatory function of APCs,[214,218] the molecular signals leading to membrane microdomain association of MHC-II remain to be determined.

MHC-II-peptide complexes are generated and reside in a relatively hostile (acidic, proteinase-rich) environment, and therefore it is not surprising that cell surface MHC-II has a relatively long half-life.[219,220] While this is true on activated B cells and mature DCs, MHC-II on the surface of immature DCs has a significantly reduced half-life. This is likely due to rapid endocytosis and lysosomal degradation of surface MHC-II in resting (immature) DCs.[221,222] The dichotomy between the endocytosis and survival of MHC-II in immature and mature DCs can be explained in part by the fact that internalized MHC-II is ubiquitinated in immature DCs and targeted for lysosomal degradation while internalized MHC-II is not ubiquitined in mature DCs and efficiently recycles back to the plasma membrane.[223,224] The E3 ubiquitin ligase March-I, whose expression appears to be limited to APCs, ubiquitinates the cytosolic domain of the MHC-II β-chain in APCs,[118] thereby preventing the recycling of internalized MHC-II and targeting ubiquitinated MHC-II for lysosomal degradation.[225] This mechanism ensures that the kinetics of MHC-II synthesis and degradation in immature DCs are similar and provides a means to halt the degradation of relevant MHC-II-peptide complexes upon DC activation.

Simply because MHC-II has a long half-life in B cells and DCs does not mean that these molecules do not internalize. In these APC subtypes, internalized MHC-II efficiently recycles from early (recycling) endosomes back to the plasma membrane,[221] thereby limiting their delivery to lysosomes and prolonging MHC-II half-life. The ability of internalized MHC-II to exchange one peptide for another in endosomes has been demonstrated in vitro[226,227]; however, it remains to be determined whether peptide exchange onto recycling MHC-II in vivo is a significant mechanism of peptide loading onto MHC-II. There is convincing evidence, however, that certain protein epitopes are primarily presented by recycling MHC-II.[228] Unlike presentation of epitopes from “classically processed” antigens, presentation of these epitopes does not require neosynthesis of MHC-II and is independent of Ii expression or DM activity. A particularly intriguing example of this pathway can be found in the analysis of an immunogenic epitope presentation of the influenza virus hemagglutinin,[228] in which presentation of one epitope is via the classical (late endosomal) processing and presentation pathway while another is presented via a nonclassical (early endosomal) recycling pathway.

Alternate Pathways of Antigen Presentation

In this chapter, we have reviewed what is typically called the classical MHC-I and MHC-II antigen-processing and -presentation pathways. In addition to these pathways, there are also interesting alternative antigen-presentation pathways. Although not the focus of this chapter,

these nonclassical pathways provide clear evidence that antigen presentation is more complex than a simple dichotomy of the two classical pathways. The most extensively studied of these alternative pathways is cross-presentation, a process by which CD8 T cells present exogenous antigen taken up by professional APCs such as DCs. Cross-presentation is critical for initiating CD8+ immune responses to pathogens that do not infect professional APCs or tumors that do not arise in professional APCs. Although several details remain unsolved, cell biologic approaches suggest that the loading of crosspresented antigens occurs by several different pathways each with hybrid features of both of the classical pathways.[229,230,231]

The presentation of lipid antigens by MHC-I like CD1 molecules also occurs by a nonclassical pathway.[232,233] Human CD1a, CD1b, and CD1c isoforms present bacterial lipids to conventional αβ T cells, whereas CD1d presents endogenous and microbial lipids to invariant natural killer T cells. Interestingly, disparate targeting motifs in their cytoplasmic tails facilitate the binding of lipid antigens in the early endosomes by CD1a, in the late endosome/lysosome by CD1b and CD1d, and at both sites by CD1c. This diversity of location of lipid antigen binding by different human CD1 isoforms allows surveying of different endosomal compartments for microbes. The assembly of CD1 isoforms in the ER uses molecular chaperones CXN, CRT, and ERp57 similar to the classical MHC-I pathways. As expected, the processing and loading of lipid antigens presented by CD1 isoforms requires unique players.[234] However, the trafficking of CD1 isoforms between the plasma membrane and endosomal compartments to acquire lipid antigens has parallels with the classical MHC-II pathway. In sum, with the underlying goal of providing maximal immunity for detection and protection against diverse pathogens, mammals have two classical and other nonclassical pathways of antigen presentation.

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REFERENCES

1. Reits EA, Vos JC, Gromme M, Neefjes J. The major substrates for TAP in vivo are derived from newly synthesized proteins. *Nature*. 2000;404:774.

2. Schubert U, Anton LC, Gibbs J, Norbury CC, Yewdell JW, Bennink JR. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature*. 2000;404:770.

3. Qian SB, Reits E, Neefjes J, Deslich JM, Bennink JR, Yewdell JW. Tight linkage between translation and MHC class I peptide ligand generation implies specialized antigen processing for defective ribosomal products. *J Immunol* . 2006;177:227.

4. Princiotta MF, Finzi D, Qian SB, et al. Quantitating protein synthesis, degradation, and endogenous antigen processing. *Immunity*. 2003; 18:343.

5. Dolan BP, Li L, Takeda K, Bennink JR, Yewdell JW. Defective ribosomal products are the major source of antigenic peptides endogenously generated from influenza A virus neuraminidase. *J Immunol*. 2010;184:1419.

6. Yewdell J. To DRiP or not to DRiP: generating peptide ligands for MHC class I molecules from biosynthesized proteins. *Mol Immunol*. 2002;39:139.

7. Yewdell JW, Anton LC, Bennink JR. Defective ribosomal products (DRiPs): a major source of antigenic peptides for MHC class I molecules? *J Immunol*. 1996;157:1823.

8. Dolan BP, Bennink JR, Yewdell JW. Translating DRiPs: progress in understanding viral and cellular sources of MHC class I peptide ligands. *Cell Mol Life Sci*. 2011;68:1481.

9. Starck SR, Shastri N. Non-conventional sources of peptides presented by MHC class I. *Cell Mol Life Sci*. 2011;68:1471.

10. Schwab SR, Li KC, Kang C, Shastri N. Constitutive display of cryptic translation products by MHC class I molecules. *Science*. 2003;301:1367.

11. Schwab SR, Shugart JA, Horng T, Malarkannan S, Shastri N. Unanticipated antigens: translation initiation at CUG with leucine. *PLoS Biol*. 2004;2:e366.

12. Ostankovitch M, Robila V, Engelhard VH. Regulated folding of tyrosinase in the endoplasmic reticulum demonstrates that misfolded full-length proteins are efficient substrates for class I processing and presentation. *J Immunol*. 2005;174:2544.

13. Golovina TN, Morrison SE, Eisenlohr LC. The impact of misfolding versus targeted degradation on the efficiency of the MHC class I-restricted antigen processing. *J Immunol*. 2005;174:2763.

14. Huang L, Kuhls MC, Eisenlohr LC. Hydrophobicity as a driver of MHC class I antigen processing. *EMBO J*. 2011;30:1634.

15. Schlosser E, Otero C, Wuensch C, et al. A novel cytosolic class I antigen-processing pathway for endoplasmic-reticulum-targeted proteins. *EMBO Rep*. 2007;8:945.

16. Yewdell JW, Nicchitta CV. The DRiP hypothesis decennial: support, controversy, refinement and extension. *Trends Immunol*. 2006;27:368.

17. Eisenlohr LC, Huang L, Golovina TN. Rethinking peptide supply to MHC class I molecules. *Nat Rev Immunol*. 2007;7:403.

18. Ciechanover A, Ben-Saadon R. N-terminal ubiquitination: more protein substrates join in. *Trends Cell Biol*. 2004;14:103.

19. Cadwell K, Coscoy L. Ubiquitination on nonlysine residues by a viral E3 ubiquitin ligase. *Science*. 2005;309:127.

20. Wang X, Herr RA, Chua WJ, Lybarger L, Wiertz EJ, Hansen TH. Ubiquitination of serine, threonine, or lysine residues on the cytoplasmic tail can induce ERAD of MHC-I by viral E3 ligase mK3. *J Cell Biol* . 2007;177:613.

21. Wang X, Herr RA, Rabelink M, Hoeben RC, Wiertz EJ, Hansen TH. Ube2j2 ubiquitinates hydroxylated amino acids on ER-associated degradation substrates. *J Cell Biol*. 2009;187:655.

22. Reits E, Griekspoor A, Neijssen J, et al. Peptide diffusion, protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I. *Immunity*. 2003;18:97.

23. Murata S, Udono H, Tanahashi N, et al. Immunoproteasome assembly and antigen presentation in mice lacking both PA28alpha and PA28beta. *EMBO J*. 2001;20:5898.

24. Kessler JH, Khan S, Seifert U, et al. Antigen processing by nardilysin and thimet oligopeptidase generates cytotoxic T cell epitopes. *Nat Immunol* . 2011;12:45.

25. Townsend A, Ohlen C, Bastin J, Ljunggren HG, Foster L, Karre K. Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature*. 1989;340:443.

26. Townsend A, Elliott T, Cerundolo V, Foster L, Barber B, Tse A. Assembly of MHC class I molecules analyzed in vitro. *Cell*. 1990;62:285.

27. Kelly A, Powis SH, Kerr LA, et al. Assembly and function of the two ABC transporter proteins encoded in the human major histocompatibility complex. *Nature*. 1992;355:641.

28. Koch J, Guntrum R, Heintke S, Kyritsis C, Tampe R. Functional dissection of the transmembrane domains of the transporter associated with antigen processing (TAP). *J Biol Chem*. 2004;279:10142.

29. Herget M, Baldauf C, Scholz C, et al. Conformation of peptides bound to the transporter associated with antigen processing (TAP). *Proc Natl Acad Sci U S A* . 2011;108:1349.

30. Burgevin A, Saveanu L, Kim Y, et al. A detailed analysis of the murine TAP transporter substrate specificity. *PLoS One*. 2008;3:e2402.

31. Peters B, Bulik S, Tampe R, van Endert PM, Holzhutter HG. Identifying MHC class I epitopes by predicting the TAP transport efficiency of epitope precursors. *J Immunol* .

2003;171:1741.

32. Chang SC, Momburg F, Bhutani N, Goldberg AL. The ER aminopeptidase, ERAP1, trims precursors to lengths of MHC class I peptides by a “molecular ruler” mechanism. *Proc Natl Acad Sci U S A* . 2005;102:17107.

33. Nguyen TT, Chang SC, Evnouchidou I, et al. Structural basis for antigenic peptide precursor processing by the endoplasmic reticulum aminopeptidase ERAP1. *Nat Struct Mol Biol*. 2011;18:604.

34. Tanioka T, Hattori A, Masuda S, et al. Human leukocyte-derived arginine aminopeptidase. The third member of the oxytocinase subfamily of aminopeptidases. *J Biol Chem*. 2003;278:32275.

35. Saveanu L, Carroll O, Lindo V, et al. Concerted peptide trimming by human ERAP1 and ERAP2 aminopeptidase complexes in the endoplasmic reticulum. *Nat Immunol* . 2005;6:689.

36. Serwold T, Gonzalez F, Kim J, Jacob R, Shastri N. ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. *Nature*. 2002;419:480.

37. York IA, Chang SC, Saric T, et al. The ER aminopeptidase ERAP1 enhances or limits antigen presentation by trimming epitopes to 8-9 residues. *Nat Immunol* . 2002;3:1177.

38. Hammer GE, Gonzalez F, Champsaur M, Cado D, Shastri N. The aminopeptidase ERAAP shapes the peptide repertoire displayed by major histocompatibility complex class I molecules. *Nat Immunol* . 2006;7:103.

39. Yan J, Parekh VV, Mendez-Fernandez Y, et al. In vivo role of ER-associated peptidase activity in tailoring peptides for presentation by MHC class Ia and class Ib molecules. *J Exp Med*. 2006;203:647.

40. Blanchard N, Shastri N. Coping with loss of perfection in the MHC class I peptide repertoire. *Curr Opin Immunol* . 2008;20:82.

41. Suh WK, Cohen-Doyle MF, Fruh K, Wang K, Peterson PA, Williams DB. Interaction of MHC class I molecules with the transporter associated with antigen processing. *Science*. 1994;264:1322.

42. Ortmann B, Androlewicz MJ, Cresswell P. MHC class I/beta 2-microglobulin complexes associate with TAP transporters before peptide binding. *Nature*. 1994;368:864.

43. Carreno BM, Solheim JC, Harris M, Stroynowski I, Connolly JM, Hansen TH. TAP associates with a unique class I conformation, whereas calnexin associates with multiple class I forms in mouse and man. *J Immunol* . 1995;155:4726.

44. Solheim JC, Harris MR, Kindle CS, Hansen TH. Prominence of beta 2-microglobulin, class I heavy chain conformation, and tapasin in the interactions of class I heavy chain with calreticulin and the transporter associated with antigen processing. *J Immunol* . 1997;158:2236.

45. Purcell AW, Elliott T. Molecular machinations of the MHC-I peptide loading complex. *Curr Opin Immunol* . 2008;20:75.

46. Peaper DR, Cresswell P. Regulation of MHC class I assembly and peptide binding. *Annu Rev Cell Dev Biol* . 2008;24:343.

47. Gao B, Adhikari R, Howarth M, et al. Assembly and antigen-presenting function of MHC class I molecules in cells lacking the ER chaperone calreticulin. *Immunity*. 2002;16:99.

48. Dick TP, Bangia N, Peaper DR, Cresswell P. Disulfide bond isomerization and the assembly of MHC class I-peptide complexes. *Immunity*. 2002;16:87.

49. Peaper DR, Wearsch PA, Cresswell P. Tapasin and ERp57 form a stable disulfide-linked dimer within the MHC class I peptide-loading complex. *EMBO J*. 2005;24:3613.

50. Kienast A, Preuss M, Winkler M, Dick TP. Redox regulation of peptide receptivity of major histocompatibility complex class I molecules by ERp57 and tapasin. *Nat Immunol* . 2007;8:864.

51. Garbi N, Tanaka S, Momburg F, Hammerling GJ. Impaired assembly of the major histocompatibility complex class I peptide-loading complex in mice deficient in the oxidoreductase ERp57. *Nat Immunol* . 2006;7:93.

52. Dong G, Wearsch PA, Peaper DR, Cresswell P, Reinisch KM. Insights into MHC class I peptide loading from the structure of the tapasin-ERp57 thiol oxidoreductase heterodimer. *Immunity*. 2009;30:21.

53. Ortmann B, Copeman J, Lehner PJ, et al. A critical role for tapasin in the assembly and function of multimeric MHC class I-TAP complexes. *Science*. 1997;277:1306.

54. Schoenhals GJ, Krishna RM, Grandea AG III, et al. Retention of empty MHC class I molecules by tapasin is essential to reconstitute antigen presentation in invertebrate cells. *EMBO J*. 1999;18:743.

55. Lybarger L, Yu YY, Chun T, et al. Tapasin enhances peptide-induced expression of H2-M3 molecules, but is not required for the retention of open conformers. *J Immunol* . 2001;167:2097.

56. Chen M, Bouvier M. Analysis of interactions in a tapasin/class I complex provides a mechanism for peptide selection. *EMBO J*. 2007;26:1681.

57. Wearsch PA, Cresswell P. Selective loading of high-affinity peptides onto major histocompatibility complex class I molecules by the tapasin-ERp57 heterodimer. *Nat Immunol*. 2007;8:873.

58. Lewis JW, Neisig A, Neefjes J, Elliott T. Point mutations in the alpha 2 domain of HLA-A2.1 define a functionally relevant interaction with TAP. *Curr Biol* .1996; 6:873.

59. Yu YY, Turnquist HR, Myers NB, Balendiran GK, Hansen TH, Solheim JC. An extensive region of an MHC class I alpha 2 domain loop influences interaction with the assembly complex. *J Immunol*. 1999;163: 4427.

60. Hansen TH, Lybarger L, Yu L, Mitaksov V, Fremont DH. Recognition of open conformers of classical MHC by chaperones and monoclonal antibodies. *Immunol Rev*. 2005;207:100.

61. Sieker F, Straatsma TP, Springer S, Zacharias M. Differential tapasin dependence of MHC class I molecules correlates with conformational changes upon peptide dissociation: a molecular dynamics simulation study. *Mol Immunol*. 2008;45:3714.

62. Williams AP, Peh CA, Purcell AW, McCluskey J, Elliott T. Optimization of the MHC class I peptide cargo is dependent on tapasin. *Immunity*. 2002;16:509.

63. Zhang W, Wearsch PA, Zhu Y, Leonhardt RM, Cresswell P. A role for UDP-glucose glycoprotein glucosyltransferase in expression and quality control of MHC class I molecules. *Proc Natl Acad Sci U S A* . 2011;108:4956.

64. Howe C, Garstka M, Al-Balushi M, et al. Calreticulin-dependent recycling in the early secretory pathway mediates optimal peptide loading of MHC class I molecules. *EMBO J*. 2009;28:3730.

65. Hansen TH, Bouvier M. MHC class I antigen presentation: learning from viral evasion strategies. *Nat Rev Immunol* . 2009;9:503.

66. Levitskaya J, Coram M, Levitsky V, et al. Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. *Nature*. 1995;375:685.

67. Dantuma NP, Heessen S, Lindsten K, Jellne M, Masucci MG. Inhibition of proteasomal degradation by the gly-Ala repeat of Epstein-Barr virus is influenced by the length of the repeat and the strength of the degradation signal. *Proc Natl Acad Sci U S A*. 2000;97:8381.

68. Bennett NJ, May JS, Stevenson PG. Gamma-herpesvirus latency requires T cell

evasion during episome maintenance. *PLoS Biol*. 2005;3:e120.

69. Zaldumbide A, Ossevoort M, Wiertz EJ, Hoeben RC. In cis inhibition of antigen processing by the latency-associated nuclear antigen I of Kaposi sarcoma herpes virus. *Mol Immunol*. 2007;44:1352.

70. Kwun HJ, da Silva SR, Shah IM, Blake N, Moore PS, Chang Y. Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen 1 mimics Epstein-Barr virus EBNA1 immune evasion through central repeat domain effects on protein processing. *J Virol*. 2007;81:8225.

71. Fruh K, Ahn K, Djaballah H, et al. A viral inhibitor of peptide transporters for antigen presentation. *Nature*. 1995;375:415.

72. Hill A, Jugovic P, York I, et al. Herpes simplex virus turns off the TAP to evade host immunity. *Nature*. 1995;375:411.

73. Ahn K, Meyer TH, Uebel S, et al. Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus ICP47. *EMBO J*. 1996;15:3247.

74. Tomazin R, Hill AB, Jugovic P, et al. Stable binding of the herpes simplex virus ICP47 protein to the peptide binding site of TAP. *EMBO J*. 1996;15:3256.

75. Lacaille VG, Androlewicz MJ. Herpes simplex virus inhibitor ICP47 destabilizes the transporter associated with antigen processing (TAP) heterodimer. *J Biol Chem*. 1998;273:17386.

76. Gorbulev S, Abele R, Tampe R. Allosteric crosstalk between peptidebinding, transport, and ATP hydrolysis of the ABC transporter TAP. *Proc Natl Acad Sci U S A* . 2001;98:3732.

77. Ahn K, Gruhler A, Galocha B, et al. The ER-luminal domain of the HCMV glycoprotein US6 inhibits peptide translocation by TAP. *Immunity*. 1997;6:613.

78. Hengel H, Koopmann JO, Flohr T, et al. A viral ER-resident glycoprotein inactivates the MHC-encoded peptide transporter. *Immunity*. 1997;6:623.

79. Lehner PJ, Karttunen JT, Wilkinson GW, Cresswell P. The human cytomegalovirus US6 glycoprotein inhibits transporter associated with antigen processing-dependent peptide translocation. *Proc Natl Acad Sci U S A* . 1997;94:6904.

80. Hewitt EW, Gupta SS, Lehner PJ. The human cytomegalovirus gene product US6 inhibits ATP binding by TAP. *EMBO J*. 2001;20:387.

81. Kyritsis C, Gorbulev S, Hutschenreiter S, Pawlitschko K, Abele R, Tampe R. Molecular mechanism and structural aspects of transporter associated with antigen

processing inhibition by the cytomegalovirus protein US6. *J Biol Chem*. 2001;276:48031.

82. Loch S, Klauschies F, Scholz C, et al. Signaling of a varicelloviral factor across the endoplasmic reticulum membrane induces destruction of the peptide-loading complex and immune evasion. *J Biol Chem*. 2008;283:13428.

83. Lee S, Yoon J, Park B, et al. Structural and functional dissection of human cytomegalovirus US3 in binding major histocompatibility complex class I molecules. *J Virol*. 2000;74:11262.

84. Park B, Kim Y, Shin J, et al. Human cytomegalovirus inhibits tapasindependent peptide loading and optimization of the MHC class I peptide cargo for immune evasion. *Immunity*. 2004;20:71.

85. Bennett EM, Bennink JR, Yewdell JW, Brodsky FM. Cutting edge: adenovirus E19 has two mechanisms for affecting class I MHC expression. *J Immunol*. 1999;162:5049.

86. Andersson M, Paabo S, Nilsson T, Peterson PA. Impaired intracellular transport of class I MHC antigens as a possible means for adenoviruses to evade immune surveillance. *Cell*. 1985;43:215.

87. Burgert HG, Kvist S. An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class I antigens. *Cell*. 1985;41:987.

88. Paabo S, Bhat BM, Wold WS, Peterson PA. A short sequence in the COOH-terminus makes an adenovirus membrane glycoprotein a resident of the endoplasmic reticulum. *Cell*. 1987;50:311.

89. Gabathuler R, Levy F, Kvist S. Requirements for the association of adenovirus type 2 E3/19K wild-type and mutant proteins with HLA antigens. *J Virol*. 1990;64:3679.

90. Liu H, Stafford WF, Bouvier M. The endoplasmic reticulum lumenal domain of the adenovirus type 2 E3-19K protein binds to peptide-filled and peptide-deficient HLA-A*1101 molecules. *J Virol*. 2005;79:13317.

91. Dasgupta A, Hammarlund E, Slifka MK, Fruh K. Cowpox virus evades CTL recognition and inhibits the intracellular transport of MHC class I molecules. *J Immunol*. 2007;178:1654.

92. Byun M, Wang X, Pak M, Hansen TH, Yokoyama WM. Cowpox virus exploits the endoplasmic reticulum retention pathway to inhibit MHC class I transport to the cell surface. *Cell Host Microbe*. 2007;2:306.

93. Byun M, Verweij MC, Pickup DJ, Wiertz EJ, Hansen TH, Yokoyama WM. Two mechanistically distinct immune evasion proteins of cowpox virus combine to avoid

antiviral CD8 T cells. *Cell Host Microbe*. 2009;6:422.

94. Alzhanova D, Edwards DM, Hammarlund E, et al. Cowpox virus inhibits the transporter associated with antigen processing to evade T cell recognition. *Cell Host Microbe*. 2009;6:433.

95. Schwartz O, Marechal V, Le GS, Lemonnier F, Heard JM. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. *Nat Med*. 1996;2:338.

96. Collins KL, Chen BK, Kalams SA, Walker BD, Baltimore D. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature*. 1998;391:397.

97. Schaefer MR, Wonderlich ER, Roeth JF, Leonard JA, Collins KL. HIV-1 Nef targets MHC-I and CD4 for degradation via a final common beta-COP-dependent pathway in T cells. *PLoS Pathog*. 2008;4:e1000131.

98. Wang X, Herr RA, Hansen T. Viral and cellular MARCH ubiquitin ligases and cancer. *Semin Cancer Biol* . 2008;18:441.

99. Nathan JA, Lehner PJ. The trafficking and regulation of membrane receptors by the RING-CH ubiquitin E3 ligases. *Exp Cell Res*. 2009;315:1593.

100. Ohmura-Hoshino M, Goto E, Matsuki Y, et al. A novel family of membrane-bound E3 ubiquitin ligases. *J Biochem*. 2006;140:147.

101. Stevenson PG, Efstathiou S, Doherty PC, Lehner PJ. Inhibition of MHC class I-restricted antigen presentation by gamma 2-herpesviruses. *Proc Natl Acad Sci U S A* . 2000;97:8455.

102. Coscoy L, Ganem D. Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. *Proc Natl Acad Sci U S A* . 2000;97:8051.

103. Ishido S, Wang C, Lee BS, Cohen GB, Jung JU. Downregulation of major histocompatibility complex class I molecules by Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins. *J Virol* . 2000;74:5300.

104. Lybarger L, Wang X, Harris MR, Virgin HW, Hansen TH. Virus subversion of the MHC class I peptide-loading complex. *Immunity*. 2003; 18:121.

105. Wang X, Lybarger L, Connors R, Harris MR, Hansen TH. Model for the interaction of gammaherpesvirus 68 RING-CH finger protein mK3 with major histocompatibility complex class I and the peptide-loading complex. *J Virol* . 2004;78:8673.

106. Wang X, Connors R, Harris MR, Hansen TH, Lybarger L. Requirements for the selective degradation of endoplasmic reticulum-resident major histocompatibility complex class I proteins by the viral immune evasion molecule mK3. *J Virol* . 2005;79:4099.

107. Boname JM, de Lima BD, Lehner PJ, Stevenson PG. Viral degradation of the MHC class I peptide loading complex. *Immunity*. 2004;20:305.

108. Wiertz EJ, Jones TR, Sun L, Bogyo M, Geuze HJ, Ploegh HL. The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell* . 1996;84:769.

109. Wiertz EJ, Tortorella D, Bogyo M, et al. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature*. 2001;384:432.

110. Loureiro J, Lilley BN, Spooner E, Noriega V, Tortorella D, Ploegh HL. Signal peptide peptidase is required for dislocation from the endoplasmic reticulum. *Nature*. 2006;441:894.

111. Lilley BN, Ploegh HL. A membrane protein required for dislocation of misfolded proteins from the ER. *Nature*. 2004;429:834.

112. Mueller B, Lilley BN, Ploegh HL. SEL1L, the homologue of yeast Hrd3p, is involved in protein dislocation from the mammalian ER. *J Cell Biol* . 2006;175:261.

113. Mueller B, Klemm EJ, Spooner E, Claessen JH, Ploegh HL. SEL1L nucleates a protein complex required for dislocation of misfolded glycoproteins. *Proc Natl Acad Sci U S A*. 2008;105:12325.

114. Lilley BN, Ploegh HL. Viral modulation of antigen presentation: manipulation of cellular targets in the ER and beyond. *Immunol Rev*. 2005;207:126.

115. Lybarger L, Wang X, Harris M, Hansen TH. Viral immune evasion molecules attack the ER peptide-loading complex and exploit ER-associated degradation pathways. *Curr Opin Immunol*. 2005;17:71.

116. Thomas M, Boname JM, Field S, et al. Down-regulation of NKG2D and NKp80 ligands by Kaposi's sarcoma-associated herpesvirus K5 protects against NK cell cytotoxicity. *Proc Natl Acad Sci U S A* . 2008;105:1656.

117. De GA, Camosseto V, Thibodeau J, et al. MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation. *Proc Natl Acad Sci U S A* . 2008;105:3491.

118. Matsuki Y, Ohmura-Hoshino M, Goto E, et al. Novel regulation of MHC class II

function in B cells. *EMBO J*. 2007;26:846.

119. Young LJ, Wilson NS, Schnorrer P, et al. Differential MHC class II synthesis and ubiquitination confers distinct antigen-presenting properties on conventional and plasmacytoid dendritic cells. *Nat Immunol*. 2008;9:1244.

120. Sinai AP, Joiner KA. Safe haven: the cell biology of nonfusogenic pathogen vacuoles. *Annu Rev Microbiol*. 1997;51:415.

121. Doherty GJ, McMahon HT. Mechanisms of endocytosis. *Annu Rev Biochem*. 2009;78:857.

122. Sallusto F, Cella M, Danieli C, Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med*. 1995;182:389.

123. Garrett WS, Chen LM, Kroschewski R, et al. Developmental control of endocytosis in dendritic cells by Cdc42. *Cell*. 2000;102:325.

124. West MA, Prescott AR, Eskelinen EL, Ridley AJ, Watts C. Rac is required for constitutive macropinocytosis by dendritic cells but does not control its downregulation. *Curr Biol*. 2000;10:839.

125. Lanzavecchia A. Antigen-specific interaction between T and B cells. *Nature*. 1985;314:537.

126. Nashar TO, Drake JR. The pathway of antigen uptake and processing dictates MHC class II-mediated B cell survival and activation. *J Immunol*. 2005;174:1306.

127. Trombetta ES, Mellman I. Cell biology of antigen processing in vitro and in vivo. *Annu Rev Immunol*. 2005;23:975.

128. Tan MC, Mommaas AM, Drijfhout JW, et al. Mannose receptor mediated uptake of antigens strongly enhances HLA-class II restricted antigen presentation by cultured dendritic cells. *Adv Exp Med Biol*. 1997;417:171.

129. Engering AJ, Cella M, Fluitsma D, et al. The mannose receptor functions as a high capacity and broad specificity antigen receptor in human dendritic cells. *Eur J Immunol*. 1997;27:2417.

130. Jiang W, Swiggard WJ, Heufler C, et al. The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature*. 1995;375:151.

131. Steinman RM, Banchereau J. Taking dendritic cells into medicine. *Nature*. 2007;449:419.

132. Munz C. Autophagy and antigen presentation. *Cell Microbiol*. 2006;8:891.

133. Crotzer VL, Blum JS. Cytosol to lysosome transport of intracellular antigens during immune surveillance. *Traffic*. 2008;9:10.

134. Paludan C, Schmid D, Landthaler M, et al. Endogenous MHC class II processing of a viral nuclear antigen after autophagy. *Science*. 2005;307:593.

135. Schmid D, Pypaert M, Munz C. Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. *Immunity*. 2007;26:79.

136. Zhou D, Li P, Lin Y, et al. Lamp-2a facilitates MHC class II presentation of cytoplasmic antigens. *Immunity*. 2005;22:571.

137. Roche PA, Cresswell P. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature*. 1990;345:615.

138. Teyton L, O'Sullivan D, Dickson PW, et al. Invariant chain distinguishes between the exogenous and endogenous antigen presentation pathways. *Nature*. 1990;348:39.

139. Long EO, LaVaute T, Pinet V, Jaraquemada D. Invariant chain prevents the HLA-DR-restricted presentation of a cytosolic peptide. *J Immunol*. 1994;153:1487.

140. Ghosh P, Amaya M, Mellins E, Wiley DC. The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. *Nature*. 1995;378:457.

141. Strubin M, Long EO, Mach B. Two forms of the Ia antigen-associated invariant chain result from alternative initiations at two in-phase AUGs. *Cell*. 1986;47:619.

142. Koch N, Lauer W, Habicht J, Dobberstein B. Primary structure of the gene for the murine Ia antigen-associated invariant chains (Ii). An alternatively spliced exon encodes a cysteine-rich domain highly homologous to a repetitive sequence of thyroglobulin. *EMBO J*. 1987;6:1677.

143. Strubin M, Berte C, Mach B. Alternative splicing and alternative initiation of translation explain the four forms of the Ia antigen-associated invariant chain. *EMBO J*. 1986;5:3483.

144. O'Sullivan DM, Noonan D, Quaranta V. Four Ia invariant chain forms derive from a single gene by alternate splicing and alternate initiation of transcription/translation. *J Exp Med*. 1987;166:444.

145. Marks MS, Blum JS, Cresswell P. Invariant chain trimers are sequestered in the rough endoplasmic reticulum in the absence of association with HLA class II antigens. *J Cell Biol*. 1990;111:839.

146. Lamb CA, Cresswell P. Assembly and transport properties of invariant chain trimers and HLA-DR- invariant chain complexes. *J Immunol*. 1992;148:3478.

147. Arunachalam B, Lamb CA, Cresswell P. Transport properties of free and MHC class II-associated oligomers containing different isoforms of human invariant chain. *Int Immunol*. 1994;6:439.

148. Roche PA, Marks MS, Cresswell P. Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature*. 1991;354:392.

149. Anderson KS, Cresswell P. A role for calnexin (IP90) in the assembly of class II MHC molecules. *EMBO J*. 1994;13:675.

150. Lotteau V, Teyton L, Peleraux A, et al. Intracellular transport of class II MHC molecules directed by invariant chain. *Nature*. 1990;348:600.

151. Schutze MP, Peterson PA, Jackson MR. An N-terminal double-arginine motif maintains type II membrane proteins in the endoplasmic reticulum. *EMBO J*. 1994;13:1696.

152. Newcomb JR, Cresswell P. Structural analysis of proteolytic products of MHC class II-invariant chain complexes generated in vivo. *J Immunol*. 1993;151:4153.

153. Bikoff EK, Germain RN, Robertson EJ. Allelic differences affecting invariant chain dependency of MHC class II subunit assembly. *Immunity*. 1995;2:301.

154. Romagnoli P, Layet C, Yewdell J, Bakke O, Germain RN. Relationship between invariant chain expression and major histocompatibility complex class II transport into early and late endocytic compartments. *J Exp Med*. 1993;177:583.

155. Neefjes JJ, Stollorz V, Peters PJ, Geuze HJ, Ploegh HL. The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. *Cell*. 1990;61:171.

156. Benaroch P, Yilla M, Raposo G, et al. How MHC class II molecules reach the endocytic pathway. *EMBO J*. 1995;14:37.

157. Roche PA, Teletski CL, Stang E, Bakke O, Long EO. Cell surface HLA-DR-invariant chain complexes are targeted to endosomes by rapid internalization. *Proc Natl Acad Sci U S A*. 1993;90:8581.

158. Bakke O, Dobberstein B. MHC class II-associated invariant chain contains a sorting signal for endosomal compartments. *Cell*. 1990;63:707.

159. Pieters J, Bakke O, Dobberstein B. The MHC class II-associated invariant chain contains two endosomal targeting signals within its cytoplasmic tail. *J Cell Sci*. 1993;106:831.

160. McCormick PJ, Martina JA, Bonifacino JS. Involvement of clathrin and AP-2 in the trafficking of MHC class II molecules to antigen-processing compartments. *Proc Natl Acad Sci U S A*. 2005;102:7910.

161. Walseng E, Bakke O, Roche PA. Major histocompatibility complex class II-peptide complexes internalize using a clathrin- and dynaminindependent endocytosis pathway. *J Biol Chem*. 2008;283:14717.

162. Blum JS, Cresswell P. Role for intracellular proteases in the processing and transport of class II HLA antigens. *Proc Natl Acad Sci U S A* . 1998;85:3975.

163. Riese RJ, Wolf PR, Bromme D, et al. Essential role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading. *Immunity*. 1996;4:357.

164. Shi GP, Villadangos JA, Dranoff G, et al. Cathepsin S required for normal MHC class II peptide loading and germinal center development. *Immunity*. 1999;10:197.

165. Sette A, Southwood S, Miller J, Appella E. Binding of major histocompatibility complex class II to the invariant chain-derived peptide, CLIP, is regulated by allelic polymorphism in class II. *J Exp Med*. 1995;181:677.

166. Morris P, Shaman J, Attaya M, et al. An essential role for HLA-DM in antigen presentation by class II major histocompatibility molecules. *Nature*. 1994;368:551.

167. Fling SP, Arp B, Pious D. HLA-DMA and -DMB genes are both required for MHC class II/peptide complex formation in antigen-presenting cells. *Nature*. 1994;368:554.

168. Sloan VS, Cameron P, Porter G, et al. Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nature*. 1995;375:802.

169. Denzin LK, Cresswell P. HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. *Cell*. 1995;82:155.

170. Weber DA, Evavold BD, Jensen PE. Enhanced dissociation of HLA-DR-bound peptides in the presence of HLA-DM. *Science*. 1996;274:618.

171. Kropshofer H, Vogt AB, Moldenhauer G, Hammer J, Blum JS, Hammerling GJ.

Editing of the HLA-DR-peptide repertoire by HLA-DM. *EMBO J*.1996;15:6144.

172. Marks MS, Roche PA, van Donselaar E, Woodruff L, Peters PJ, Bonifacino JS. A lysosomal targeting signal in the cytoplasmic tail of the beta chain directs HLA-DM to MHC class II compartments. *J Cell Biol* . 1995;131:351.

173. Sanderson F, Kleijmeer MJ, Kelly A, et al. Accumulation of HLA-DM, a regulator of antigen presentation, in MHC class II compartments. *Science*. 1994;266:1566.

174. Stang E, Guerra CB, Amaya M, Paterson Y, Bakke O, Mellins ED. DR/CLIP (class II-associated invariant chain peptides) and DR/peptide complexes colocalize in prelysosomes in human B lymphoblastoid cells. *J Immunol* . 1998;160:4696.

175. Sherman MA, Weber DA, Jensen PE. DM enhances peptide binding to class II MHC by release of invariant chain-derived peptide. *Immunity*. 1995;3:197.

176. Denzin LK, Fallas JL, Prendes M, Yi W. Right place, right time, right peptide: DO keeps DM focused. *Immunol Rev*. 2005;207:279.

177. Denzin LK, Sant'Angelo DB, Hammond C, Surman MJ, Cresswell P. Negative regulation by HLA-DO of MHC class II-restricted antigen processing. *Science*. 1997;278:106.

178. van Ham SM, Tjin EP, Lillemeier BF, et al. HLA-DO is a negative modulator of HLA-DM-mediated MHC class II peptide loading. *Curr Biol* . 1997;7:950.

179. Liljedahl M, Kuwana T, Fung-Leung WP, Jackson MR, Peterson PA, Karlsson L. HLA-DO is a lysosomal resident which requires association with HLA-DM for efficient intracellular transport. *EMBO J*. 1996;15:4817.

180. Manoury B, Hewitt EW, Morrice N, Dando PM, Barrett AJ, Watts C. An asparaginyl endopeptidase processes a microbial antigen for class II MHC presentation. *Nature*. 1998;396:695.

181. Hsieh CS, deRoos P, Honey K, Beers C, Rudensky AY. A role for cathepsin L and cathepsin S in peptide generation for MHC class II presentation. *J Immunol* . 2002;168:2618.

182. Arunachalam B, Phan UT, Geuze HJ, Cresswell P. Enzymatic reduction of disulfide bonds in lysosomes: characterization of a gamma-interferon-inducible lysosomal thiol reductase (GILT). *Proc Natl Acad Sci U S A* . 2000;97:745.

183. Maric M, Arunachalam B, Phan UT, et al. Defective antigen processing in GILT-free mice. *Science*. 2001;294:1361.

184. Manoury B, Mazzeo D, Fugger L, et al. Destructive processing by asparagine endopeptidase limits presentation of a dominant T cell epitope in MBP. *Nat Immunol*. 2002;3:169.

185. Delamarre L, Pack M, Chang H, Mellman I, Trombetta ES. Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science*. 2005;307:1630.

186. Delamarre L, Couture R, Mellman I, Trombetta ES. Enhancing immunogenicity by limiting susceptibility to lysosomal proteolysis. *J Exp Med*. 2006;203:2049.

187. Sette A, Adorini L, Colon SM, Buus S, Grey HM. Capacity of intact proteins to bind to MHC class II molecules. *J Immunol*. 1989;143:1265.

188. Sercarz EE, Maverakis E. Mhc-guided processing: binding of large antigen fragments. *Nat Rev Immunol*. 2003;3:621.

189. Moss CX, Tree TI, Watts C. Reconstruction of a pathway of antigen processing and class II MHC peptide capture. *EMBO J*. 2007;26:2137.

190. Bevec T, Stoka V, Pungercic G, Dolenc I, Turk V. Major histocompatibility complex class II-associated p41 invariant chain fragment is a strong inhibitor of lysosomal cathepsin L. *J Exp Med*. 1996;183:1331.

191. Guncar G, Pungercic G, Klemencic I, Turk V, Turk D. Crystal structure of MHC class II-associated p41 Ii fragment bound to cathepsin L reveals the structural basis for differentiation between cathepsins L and S. *EMBO J*. 1999;18:793.

192. Fineschi B, Arneson LS, Naujokas MF, Miller J. Proteolysis of major histocompatibility complex class II-associated invariant chain is regulated by the alternatively spliced gene product, p41. *Proc Natl Acad Sci U S A*. 1995;92:10257.

193. Takaesu NT, Lower JA, Yelon D, Robertson EJ, Bikoff EK. In vivo functions mediated by the p41 isoform of the MHC class II-associated invariant chain. *J Immunol*. 1997;158:187.

194. Inaba K, Turley S, Iyoda T, et al. The formation of immunogenic major histocompatibility complex class II-peptide ligands in lysosomal compartments of dendritic cells is regulated by inflammatory stimuli. *J Exp Med*. 2000;191:927.

195. Turley SJ, Inaba K, Garrett WS, et al. Transport of peptide-MHC class II complexes in developing dendritic cells. *Science*. 2000;288:522.

196. Trombetta ES, Ebersold M, Garrett W, Pypaert M, Mellman I. Activation of lysosomal function during dendritic cell maturation. *Science*. 2003;299:1400.

197. Pierre P, Mellman I. Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. *Cell*. 1998;93:1135.

198. Kleijmeer M, Ramm G, Schuurhuis D, et al. Reorganization of multivesicular bodies regulates MHC class II antigen presentation by dendritic cells. *J Cell Biol* . 2001;155:53.

199. Peters PJ, Neefjes JJ, Oorschot V, Ploegh HL, Geuze HJ. Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments [see comments]. *Nature*. 1991;349:669.

200. Kleijmeer MJ, Morkowski S, Griffith JM, Rudensky AY, Geuze HJ. Major histocompatibility complex class II compartments in human and mouse B lymphoblasts represent conventional endocytic compartments. *J Cell Biol* . 1997;139:639.

201. Geuze HJ. The role of endosomes and lysosomes in MHC class II functioning. *Immunol Today*. 1998;19:282.

202. Weber DA, Dao CT, Jun J, Wigal JL, Jensen PE. Transmembrane domain-mediated colocalization of HLA-DM and HLA-DR is required for optimal HLA-DM catalytic activity. *J Immunol*. 2001;167:5167.

203. Zwart W, Griekspoor A, Kuijl C, et al. Spatial separation of HLA-DM/HLA-DR interactions within MIIC and phagosome-induced immune escape. *Immunity*. 2005;22:221.

204. Thery C, Duban L, Segura E, Veron P, Lantz O, Amigorena S. Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes. *Nat Immunol* . 2002;3:1156.

205. Zitvogel L, Regnault A, Lozier A, et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med*. 1998;4:594.

206. Wubbolts R, Fernandez-Borja M, Oomen L, et al. Direct vesicular transport of MHC class II molecules from lysosomal structures to the cell surface. *J Cell Biol* . 1996;135:611.

207. Boes M, Cerny J, Massol R, et al. T-cell engagement of dendritic cells rapidly rearranges MHC class II transport. *Nature*. 2002;418:983.

208. Chow A, Toomre D, Garrett W, Mellman I. Dendritic cell maturation triggers retrograde MHC class II transport from lysosomes to the plasma membrane. *Nature*. 2002;418:988.

209. Murk JL, Stoorvogel W, Kleijmeer MJ, Geuze HJ. The plasticity of multivesicular bodies and the regulation of antigen presentation. *Semin Cell Dev Biol* . 2002;13:303.

210. Paul P, van den Hoorn T, Jongsma ML, et al. A genome-wide multidimensional RNAi screen reveals pathways controlling MHC class II antigen presentation. *Cell*. 2011;145:268.

211. Wubbolts R, Fernandez-Borja M, Jordens I, et al. Opposing motor activities of dynein and kinesin determine retention and transport of MHC class II-containing compartments. *J Cell Sci*. 1999;112(pt 6): 785.

212. Vascotto F, Lankar D, Faure-Andre G, et al. The actin-based motor protein myosin II regulates MHC class II trafficking and BCR-driven antigen presentation. *J Cell Biol*. 2007;176:1007.

213. Jenei A, Varga S, Bene L, et al. HLA class I and II antigens are partially co-clustered in the plasma membrane of human lymphoblastoid cells. *Proc Natl Acad Sci U S A*. 1997;94:7269.

214. Anderson HA, Hiltbold EM, Roche PA. Concentration of MHC class II molecules in lipid rafts facilitates antigen presentation. *Nat Immunol*. 2000;1:156.

215. Kropshofer H, Spindeldreher S, Rohn TA, et al. Tetraspan microdomains distinct from lipid rafts enrich select peptide-MHC class II complexes. *Nat Immunol*. 2002;3:61.

216. Poloso NJ, Muntasell A, Roche PA. MHC class II molecules traffic into lipid rafts during intracellular transport. *J Immunol*. 2004;173:4539.

217. Mobius W, van Donselaar E, Ohno-Iwashita Y, et al. Recycling compartments and the internal vesicles of multivesicular bodies harbor most of the cholesterol found in the endocytic pathway. *Traffic*. 2003;4:222.

218. Eren E, Yates J, Cwynarski K, et al. Location of major histocompatibility complex class II molecules in rafts on dendritic cells enhances the efficiency of T-cell activation and proliferation. *Scand J Immunol*. 2006;63:7.

219. Davis JE, Cresswell P. Lack of detectable endocytosis of B lymphocyte MHC class II antigens using an antibody-independent technique. *J Immunol*. 1990;144:990.

220. Lanzavecchia A, Reid PA, Watts C. Irreversible association of peptides with class II MHC molecules in living cells. *Nature*. 1992;357:249.

221. Cella M, Engering A, Pinet V, Pieters J, Lanzavecchia A. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature*. 1997;388:782.

222. Pierre P, Turley SJ, Gatti E, et al. Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature*. 1997;388:787.

223. Shin JS, Ebersold M, Pypaert M, Delamarre L, Hartley A, Mellman I. Surface expression of MHC class II in dendritic cells is controlled by regulated ubiquitination. *Nature*. 2006;444:115.

224. van Niel G, Wubbolts R, ten Broeke T, et al. Dendritic cells regulate exposure of MHC class II at their plasma membrane by oligoubiquitination. *Immunity*. 2006;25:885.

225. Walseng E, Furuta K, Bosch B, et al. Ubiquitination regulates MHC class II-peptide complex retention and degradation in dendritic cells. *Proc Natl Acad Sci U S A* . 2010;107:20465.

226. Pinet V, Vergelli M, Martin R, Bakke O, Long EO. Antigen presentation mediated by recycling of surface HLA-DR molecules. *Nature*. 1995;375:603.

227. Pathak SS, Blum JS. Endocytic recycling is required for the presentation of an exogenous peptide via MHC class II molecules. *Traffic*. 2000;1:561.

228. Sinnathamby G, Eisenlohr LC. Presentation by recycling MHC class II molecules of an influenza hemagglutinin-derived epitope that is revealed in the early endosome by acidification. *J Immunol* . 2003;170:3504.

229. Blanchard N, Shastri N. Cross-presentation of peptides from intracellular pathogens by MHC class I molecules. *Ann N Y Acad Sci* . 2010;1183:237.

230. Rock KL, Farfan-Arribas DJ, Shen L. Proteases in MHC class I presentation and cross-presentation. *J Immunol* . 2010;184:9.

231. van Endert P. Post-proteasomal and proteasome-independent generation of MHC class I ligands. *Cell Mol Life Sci* . 2001;68:1553.

232. Barral DC, Brenner MB. CD1 antigen presentation: how it works. *Nat Rev Immunol* . 2007;7:929.

233. Kasmar A, Van Rhijn I, Moody DB. The evolved functions of CD1 during infection. *Curr Opin Immunol* . 2009;21:397.

234. Darmoise A, Maschmeyer P, Winau F. The immunological functions of saposins. *Adv Immunol* . 2010;105:25.

235. Kelley LA, Sternberg MJ. Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc*. 2009;4:363.

236. Schrag JD, Bergeron JJ, Li Y, et al. The structure of calnexin, an ER chaperone involved in quality control of protein folding. *Mol Cell* . 2001;8:633.

237. Khan AR, Baker BM, Ghosh P, Biddison WE, Wiley DC. The structure and stability of an HLA-A*0201/octameric tax peptide complex with an empty conserved peptide-N-terminal binding site. *J Immunol*. 2000;164:6398.

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Chapter 23

Immunogenicity and Antigen Structure

Jay A. Berzofsky

Ira J. Berkower

THE NATURE OF ANTIGENIC DETERMINANTS RECOGNIZED BY ANTIBODIES

Haptens

In the antigen-antibody binding reaction, the antibodybinding site is often unable to accommodate the entire antigen. The part of the antigen that is the target of antibody binding is called an antigenic determinant, and there may be one or more antigenic determinants per molecule. To study antibody specificity, we need to have antibodies against single antigenic determinants. Small functional groups that correspond to a single antigenic determinant are called haptens. For example, these may be organic compounds, such as trinitrophenyl or benzene arsonate, a monosaccharide or oligosaccharide such as glucose or lactose, or an oligopeptide such as pentalysine. Although these haptens can bind to antibody, immunization with them usually will not provoke an antibody response (for exceptions, see Goodman[1]). Immunogenicity often can be achieved by covalently attaching haptens to a larger molecule, called the carrier. The carrier is immunogenic in its own right, and immunization with the hapten-carrier conjugate elicits an antibody response to both hapten and carrier. However, the antibodies specific for hapten can be studied by equilibrium dialysis using pure hapten (without carrier) or by immunoprecipitation using hapten coupled to a different (and non-cross-reacting) carrier or by inhibition of precipitation with free hapten.

This technique was pioneered by Landsteiner[2] and helped to elucidate the exquisite specificity of antibodies for antigenic determinants. For instance, the relative binding affinity of antibodies prepared against succinic acid-serum protein conjugates shows marked specificity for the maleic acid analog, which is in the cis configuration, as compared to the fumaric acid (trans) form.[3] Presumably, the immunogenic form of succinic acid corresponds to the cis form.[3] This ability of antibodies to distinguish cis from trans configurations was reemphasized in later studies measuring relative affinities of antibodies to maleic and fumaric acid conjugates[4] (Table 23.1A). Table 23.1B shows the specificity of antibodies prepared against p-azobenzenearsonate coupled to bovine gamma globulin.[5] As the hapten is coupled through the p-azo group to aromatic amino acids of the carrier, haptens containing bulky substitutions in the para position would most resemble the immunizing antigen. In fact, p-methyl-substituted benzene arsonate has a higher binding affinity than unsubstituted benzene arsonate. However, methyl substitution elsewhere in the benzene ring reduces affinity, presumably due to interference with the way hapten fits into the antibody-binding site. Thus, methyl substitutions can have positive or negative effects on binding energy, depending on where the substitution occurs. Table 23.1C shows the specificity of antilactose antibodies for lactose versus cellobiose.[6] These disaccharides differ only by the orientation of the hydroxyl

attached to C4 of the first sugar either above or below the hexose ring. The three examples in this table, as well as many others,[1] show the marked specificity of antibodies for cis-trans, ortho-meta-para, and stereoisomeric forms of the antigenic determinant.

Comparative binding studies of haptens have been able to demonstrate antibody specificity despite the marked heterogeneity of antibodies. Unlike the antibodies against a multideterminant antigen, the population of antibodies specific for a single hapten determinant is a relatively restricted population due to the shared structural constraints necessary for hapten to fit within the antibody-combining site. However, the specificity of an antiserum depends on the collective specificities of the entire population of antibodies, which are determined by the structures of the various antibody-binding sites. When studying the cross-reactions of hapten analogs, some haptens bind all antibodies but with reduced K_A. Other hapten analogs reach a plateau of binding because they fit some antibody-combining sites quite

well but not others (see discussion of cross-reactivity in Chapter 7). Antibodies raised in different animals may show different cross-reactivities with related haptens. Even within a single animal, antibody affinity and specificity are known to increase over time following immunization under certain conditions.[7] Thus any statements about the cross-reactivity of two haptens reflect both structural differences between the haptens that affect antigen-antibody fit and the diversity of antibody-binding sites present in a given antiserum.

TABLE 23.1 Exquisite Specificity of Antihapten Antibodies

Hapten	Structure	K_{rel} of Antibody Specific for	
A.		Maleic (cis)	Fumaric (trans)
	Maleanilate	1.0	<0.01
	Fumaranilate	<0.01	1.0
B.		Parasubstituted benzene arsonate	
	Benzene arsonate	1.0	
	o-Methyl benzene arsonate	0.2	
	m-Methyl benzene arsonate	0.8	
	p-Methyl benzene arsonate	1.9	
C.		Lactose	
	Lactose β Gal (1 → 4) Glu	1.00	
	Cellobiose β Glu (1 → 4) Glu	0.0025	

Part A from Pressman and Grossberg,[4] part B from Pressman et al.,[5] and part C from Karush,[6] with permission.

Carbohydrate Antigens

The antigenic determinants of a number of biologically important substances consist of carbohydrates. These often occur as glycolipids or glycoproteins. Examples of the former include bacterial cell wall antigens and the major blood group antigens, whereas the latter group includes "minor" blood group antigens such as Rh. In addition, the capsular polysaccharides of bacteria are important for virulence and are often targeted by protective antibodies. A number of spontaneously arising myeloma proteins have been found to show carbohydrate specificity, possibly reflecting the fact that carbohydrates are common environmental antigens. In the days prior to hybridoma technology, these carbohydratespecific myeloma proteins provided an important model for studying the reaction of antigen with a monoclonal antibody.

Empirically, the predominant antigenic determinants of polysaccharides often consist of short oligosaccharides (one to five sugars long) at the nonreducing end of the polymer chain.[8] This situation is analogous to a hapten consisting of several sugar residues linked to a large nonantigenic polysaccharide backbone. The remainder of the polysaccharide is important for immunogenicity, just as the carrier molecule was important for haptens. In addition, branch points in the polysaccharide structure allow for multiple antigenic determinants to be attached to the same macromolecule. This is important for immunoprecipitation by lattice formation, as discussed in Chapter 5. Several examples illustrating structural studies of oligosaccharide antigens are given later.

The technique used most widely to analyze the antigenic determinants of polysaccharides is called hapten inhibition.[8] In this method, the precipitation reaction between antigen and antibody is inhibited by adding short oligosaccharides. These oligosaccharides are large enough to bind with the same affinity and specificity as the polysaccharide, but because they are monomeric, no precipitate forms. As more inhibitor is added, fewer antibody-combining sites remain available for precipitation. Using antiserum specific for a single antigenic determinant, it is often possible to block precipitation completely with a short oligosaccharide corresponding to the nonreducing end of the polysaccharide chain. Besides showing the "immunodominance" of the nonreducing end of the chain, this result also shows that the structure of the antigenic determinant of polysaccharides depends on the sequence of carbohydrates and their linkage, rather than their conformation. For inhibition by hapten to be complete, the antigen-antibody system studied must be made specific for a single antigenic determinant. For optimal sensitivity, the equivalence point of antigen and antibody should be used.

We illustrate the types of carbohydrate antigens encountered by examining three classic examples in more detail: the salmonella O antigens, the blood group antigens, and dextrans that bind to myeloma proteins.

Immunochemistry of Salmonella O Antigens

The antigenic diversity among numerous salmonella species resides in the structural differences of the lipopolysaccharide (LPS) component of the outer membrane.[9] These molecules are the main target for antisalmonella antibodies. The polysaccharide moiety contains the antigenic determinant, whereas the lipid moiety is responsible for endotoxin

effects. The chemical structure of LPS can be divided into three regions (Fig. 23.1). Region I contains the antigenic O-specific polysaccharide, usually made up of repeated oligosaccharide units, which vary widely among different strains. Region II contains an oligosaccharide "common core" shared among many different strains. Failure to synthesize region II oligosaccharide or to couple completed region I polysaccharide to the growing region II core results in R (rough) mutants, which have "rough" colony morphology and lack the O antigen. Region III is the lipid part, called lipid A, which is shared among all salmonellae and serves to anchor LPS on the outer membrane. Early immunologic attempts to classify the O antigens of different salmonellae revealed a large number of cross-reactions between different strains. These were detected by preparing antiserum to one strain of salmonella and using it to agglutinate bacteria of a second strain. Each cross-reacting determinant was assigned a number, and each strain was characterized by a series of O antigen determinants (in aggregate, the "serotype" of the strain) based on its pattern of cross-reactivity. Each strain was classified within a group, based on sharing a strong O determinant. For example, group A strains share determinant 2, whereas

group B strains share determinant 4 (Table 23.2). However, within a group, each strain possesses additional O determinants, which serve to differentiate it from other members of that group. Thus, determinant 2 coexists with determinants 1 and 12 on *Salmonella paratyphi* A. This problem of cross-reactivity based on sharing of a subset of antigenic determinants is commonly encountered in complex antigen-antibody systems. The problem may be simplified by making antibodies monospecific for individual antigenic determinants. To do this, antibodies are absorbed to remove irrelevant specificities, or cross-reactive strains are chosen that share only a single determinant with the immunizing strain. The reaction of each determinant with its specific antibody can be thought of as an antigen-antibody system. Thus, for the strains shown in Table 23.2, antiserum to *Salmonella typhi* (containing anti-9 and anti-12 antibodies) may be absorbed with *S. paratyphi* A to remove anti-12, leaving a reagent specific for antigen 9 (see Table 23.2). Alternatively, the unabsorbed antiserum may be used to study the system antigen 12-anti-12 by allowing it to agglutinate *S. paratyphi* B, which shares only antigen 12 with the immunogen. Because the other determinants on *S. paratyphi* B were absent from the immunizing strain, the antiserum contains no antibodies to them.

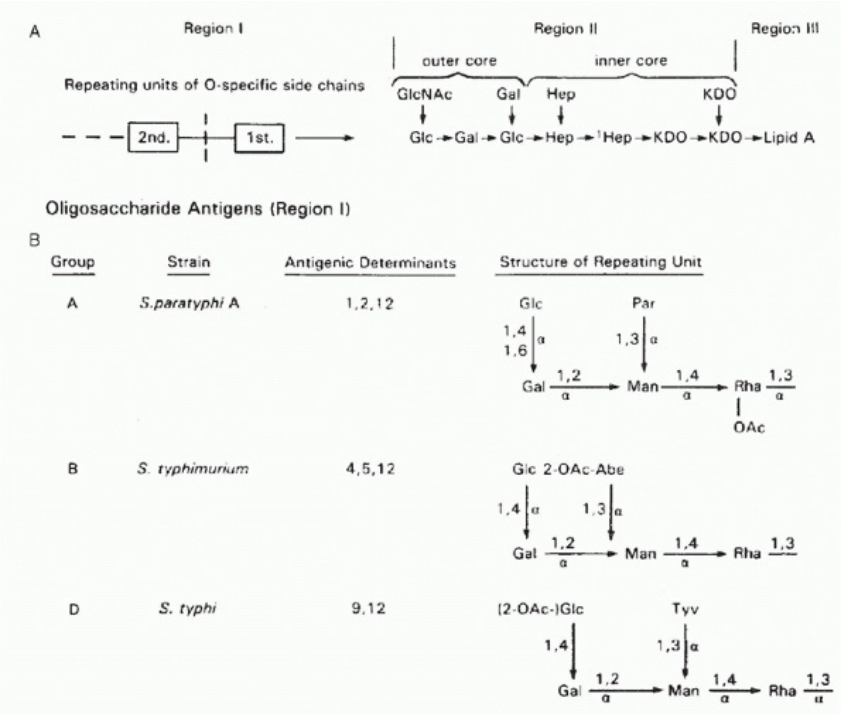

FIG. 23.1. Structure of Salmonella Lipopolysaccharide. Region I contains the unique O-antigen determinants, which consist of repeating units of oligosaccharides. These are attached to lipid moiety through the core polysaccharide. Three examples of oligosaccharide units are shown.[9] (Part A adapted from Kabat,[8] with permission; part B based on Jann and Westphal.[9])

TABLE 23.2 Salmonella Q Antigen Serotyping

Salmonella Strain	Serogroup	O Antigenic Determinants
S. paratyphi	A	A 1, 2, 12
S. paratyphi	B	B 1, 4, 5, 12
S. typhi	D	9, 12

Antiserum	Absorbed	Tested on	Single Determinant Measured
Anti-*S. typhi*		*S. paratyphi* B	12
Anti-*S. typhi*	*S. paratyphi* A	*S. typhi*	9

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Once the antigen-antibody reaction is made specific for a single determinant, a variety of oligosaccharides can be added

to test for hapten inhibition. Because the O antigens contain repeating oligosaccharide units, it is often possible to obtain model oligosaccharides by mild chemical or enzymatic degradation of the LPS itself. Once the most inhibitory oligosaccharide is found, its chemical structure is determined. Alternatively, a variety of synthetic monosaccharide, disaccharide, trisaccharide and oligosaccharides are tested for hapten inhibition of precipitation. For example, as shown in Table 23.3, antigen 1-anti-1 antibody precipitation is inhibited by methyl-α D-glucoside. Therefore, various disaccharides incorporating this structure were tested, of which α-D-Glu-(1 → 6)-D-Gal was the most inhibitory. Then various trisaccharides incorporating this sequence were tested. The results indicate the sequence and size of the determinant recognized by anti-1 antibodies to be a disaccharide with the previously discussed structure. The test sequences can be guessed by analyzing the oligosaccharide breakdown products of the LPS, which include tetramers of D-Glu-D-Gal-D-Man-L-Rham. The results in Table 23.3 also suggest that the difference between determinants 1 and 19 is the length of oligosaccharide recognized by antibodies specific for each determinant. This hypothesis is supported by the observation that determinant 1 is found in some strains with, and in other strains without, determinant 19; whereas determinant 19 is always found with determinant 1. As shown in Table 23.3, determinant 19 requires the full tetrasaccharide for maximal hapten inhibition, including the sequence coding for determinant 1. Besides identifying the antigenic structures, these results indicate that there is variation in the size of different antigenic determinants of polysaccharides.

TABLE 23.3 Analysis of Salmonella O-Antigen Structure by Hapten Inhibition

	Antigen System	
Maximum Inhibition by Hapten (%)	**1: anti-1**	**19: anti-19**
d-Glu	—	0
Me-α-D-Glu	35	10
α-D-Glu(1 → 6)-D-Gal	80	25
Glu.Gal.Man	80	70
Gtu.Gal.Man.L-Rham	>70	>70
Deduced structure	α-D-Glu(1 → 6)-D-Gal	D-Glu-D-Gal-D-Man-L-Rham

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Blood Group Antigens

The major blood group antigens A and B were originally detected by the ability of serum from individuals lacking either determinant to agglutinate red blood cells bearing them (for reviews, see Kabat,[8] Springer,[10] Marcus,[11] and Watkins[12]). In addition, group O individuals have an H antigenic determinant that is distinct from A or B types, and individuals in all three groups may have additional determinants such as the Lewis (Le) antigens. Although the ABH and Le antigenic determinants are found on a carbohydrate moiety, the carbohydrate may occur in a variety of biochemical forms. On cell surfaces, they are either glycolipids that are synthesized within the cell (AB and H antigens) or glycoproteins taken up from serum (Le antigens). In mucinous secretions, such as saliva, they occur as glycoproteins. Milk, ovarian cyst fluid, and gastric mucosa contain soluble oligosaccharides containing blood group reactivity. In addition, these antigens occur frequently in other species, including about half of the bacteria in the normal flora of the gut.[10] This widespread occurrence may account for the ubiquitous anti-AB reactivity of human sera, even in people never previously exposed to human blood group substances through transfusion or pregnancy.

The immunochemistry of these antigens was simplified greatly by the use of oligosaccharides in hapten inhibition studies. Group A oligosaccharides, for example, would inhibit the agglutination of group A red blood cells by anti-A antibodies. They could also inhibit the immunoprecipitation of group A-bearing glycoproteins by anti-A antibodies. Because the oligosaccharides are monomeric, their reaction with antibody does not form a precipitate but does block an antibody-combining site.

The inhibitory oligosaccharides from cyst fluid were purified and found to contain D-galactose, L-fucose, N-acetylgalactosamine, and N-acetylglucosamine. The most inhibitory oligosaccharides for each antigen are indicated in Figure 23.2. As can be seen in Figure 23.2, the ABH and Le antigens all share a common oligosaccharide core sequence, and the antigens appear to differ from each other by the sequential addition of individual sugars at the end or at branch points. Besides hapten inhibition, other biochemical data support this relationship among the different determinants. Enzymatic digestion of A, B, or H antigens yields a common core oligosaccharide from each. This product cross-reacts with antiserum specific for pneumococcal polysaccharide type XIV, which contains structural elements shared with blood group determinants, as shown at the bottom of Figure 23.2. In addition, this structure, known as precursor substance, has been isolated from ovarian cyst fluid.

Starting from precursor substance, the H determinant results from the addition of L-fucose to galactose, whereas Le^a determinant results from the addition of L-fucose to N-acetylglucosamine and Le^b from the addition of L-fucose to both sugars. Addition of N-acetylgalactosamine to H substance produces the A determinant, whereas addition of galactose produces the B determinant, in each case blocking reactivity of the H determinant.

The genetics of ABH and Le antigens is explained by this sequential addition of sugars via glycosyltransferases. The allelic nature of the AB antigens is explained by the addition of N-acetylgalactosamine, galactose, or nothing to the H antigen. The rare inherited trait of inability to synthesize the H determinants from precursor substance (Bombay phenotype) also blocks the expression of A and B antigens because the A and B transferases lack an acceptor substrate. However, the appearance of the Le^a antigen on red cells is independent of H antigen synthesis. Its structure, shown in Figure 23.2, can be derived directly from precursor substance without going through an H antigen intermediate. Comparing different individuals, the appearance of Le^a antigen on red blood cells correlates with its presence in

saliva, as the Le^a

antigen is not an intrinsic membrane component but must be absorbed from serum glycoproteins, which, in turn, depend on secretion. In addition to the independent synthetic pathway, the secretion of Le^a antigen is also independent of the secretory process for ABH antigens. Therefore, salivary nonsecretors of ABH antigens (which occur in 20% of individuals) may still secrete Le^a antigen if they have the fucosyl transferase encoded by the Le gene. In contrast, salivary secretion of ABH is required for red blood cells to express Le^b.

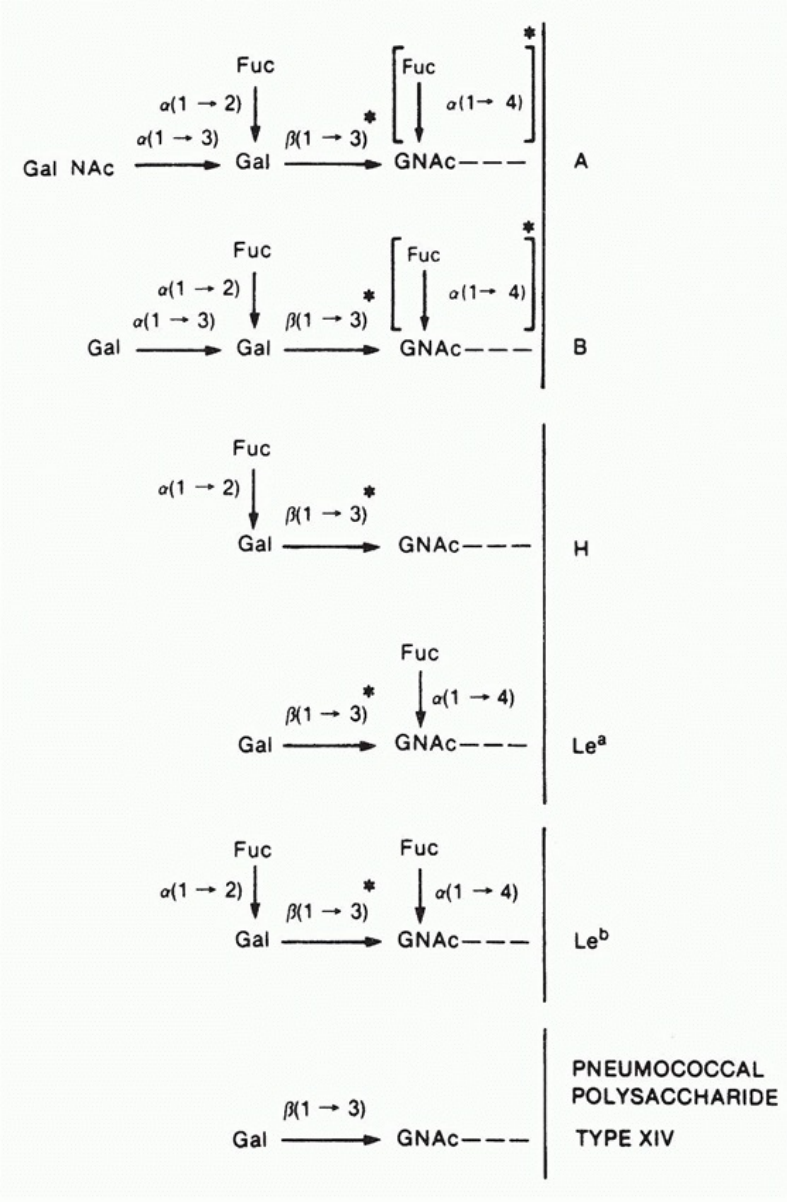


FIG. 23.2. Oligosaccharide Chain Specificity. Structure of the ABH and Le blood group antigens as determined by hapten inhibition studies.[8,11] There are two variants of each of these determinants. In type 1, the Gal-GNAc linkage is β(1 → 3), whereas in type 2, the Gal-GNAc linkage is β(1 → 4). In addition, there is heterogeneity in the A and B antigens with respect to the presence of the Le fucose attached to the GNAc. In the molecules that contain the extra fucose, when the Gal-GNAc linkage is β(1 → 3) (type 1), the fucose must be linked α(1 → 4), whereas the type 2 molecules, with the β(1 → 4)

Gal-GNAc linkage, contain α(1 → 3)-linked fucose. The *asterisks* indicate the sites of this variability in linkage.

Dextran-Binding Myeloma Proteins

Because polysaccharides are common environmental antigens, it is not surprising that randomly induced myeloma proteins were frequently found to have carbohydrate specificities. Careful studies of these monoclonal antibodies support the clonal expansion model of antibody diversity: heterogeneous antisera behave as the sum of many individual clones of antibody with respect to affinity and specificity. In the case of the Ig Aκ myeloma proteins W3129 and W3434, both antibodies were found to be specific for dextrans containing α-glu (1 → 6)glu bonds.[12] Hapten inhibition with a series of monosaccharide or oligosaccharides of increasing chain length indicated that the percentage of binding energy derived from the reaction with one glucose was 75%, two glucoses 95%, three glucoses 95% to 98%, and four glucoses 100%. This suggests that most binding energy between antidextran antibodies and dextran derives from the terminal monosaccharide, and that oligosaccharides of chain length four to six commonly fill the antibody-combining site. Human antidextran antisera behaved similarly, with tetrasaccharides contributing 95% of the binding energy. These experiments provided the first measure of the size of an antigenic determinant, four to six residues.[13,14] In addition, as was observed for antisera, binding affinity of myeloma proteins was highly sensitive to modifications of the terminal sugar and highly specific for α(1 → 6) versus α(1 → 3) glycosidic bonds. However, modification of the third or fourth sugar of an oligosaccharide had relatively less effect on hapten inhibition of either myeloma protein or of antisera reacting with dextran.

Studies with additional dextran-binding myeloma proteins[15] revealed that not all antipolysaccharide monoclonal antibodies are specific for the nonreducing end, as exemplified by QUPC 52. Competitive inhibition with monosaccharide and oligosaccharides revealed that <5% of binding energy derived from monosaccharides or disaccharides, 72% from trisaccharides, 88% from tetrasaccharides, and 100% from hexasaccharides, in marked contrast to other myeloma proteins. A second distinctive property of myeloma protein QUPC 52 was its ability to precipitate unbranched dextran of chain length 200. As the unbranched dextran has only one nonreducing end, and as the myeloma protein has only one specificity, lattice formation due to cross-linking between the nonreducing ends is impossible, and precipitation must be explained by binding some other determinant. Therefore, QUPC 52 appears to be specific for internal oligosaccharide units of three to seven chain length. The W3129 is specific for end determinants and will not precipitate unbranched dextran chains. Antibodies precipitating linear dextran were also detected in six antidextran human sera, comprising 48% to 90% of the total antibodies to branched chain dextran. Thus, antidextrans can be divided into those specific for terminal oligosaccharides and those specific for internal oligosaccharides; monoclonal examples of both types are available, and both types are present in human immune serum. Cisar et al.[15] speculated as to the different topology of the binding sites of W3129 or QUPC 52 necessary for terminal or internal oligosaccharide specificity. Both terminal and internal oligosaccharides have nearly identical chemical structures, differing at a single C-OH or glycoside bond. Perhaps the terminal oligosaccharide specificity of W3129 is due to the shape of the antibody-combining site—a cavity into which only the end can fit—whereas the

internal oligosaccharide-binding site of QUPC 52 could be a surface groove in the antibody, which would allow the rest of the polymer to protrude out at both ends. A more definitive

answer depends on x-ray crystallographic studies of the combining sites of monoclonal antibodies with precisely defined specificity, performed with antigen occupying the binding site.

With the advent of hybridoma technology, it became possible to produce monoclonal antibodies of any desired specificity. Immunizing mice with nearly linear dextran (the preferred antigen of QUPC 52), followed by fusion and screening (with linear dextran) for dextran-binding antibodies, yielded 12 hybridomas,[16] all with specificity similar to QUPC 52. First, oligosaccharide inhibition of all 12 monoclonals showed considerable increments in affinity up to hexasaccharides, with little affinity for disaccharides and only 49% to 77% of binding energy derived from trisaccharides.[17] Second, all 12 monoclonals had internal α(1 → 6) dextran specificity, as they could all precipitate linear dextran. Third,[9] out of 11 BALB/c monoclonals shared a cross-reactive idiotype with QUPC 52, whereas none shared idiotype with W3129.[18] These data support the hypothesis that different antibodies with similar specificity and similar groove-type sites may be derived from the same family of germline V_H genes bearing the QUPC 52 idiotype.[18]

The large number of environmental carbohydrate antigens and the high degree of specificity of antibodies elicited in response to each carbohydrate antigen suggest that a tremendous diversity of antibody molecules must be available, from which some antibodies can be selected for every possible antigenic structure. Studies of a series of 17 monoclonal anti-α(1 → 6) dextran hybridomas[19,20] have investigated whether the binding sites of closely related antibodies were derived from a small number of variable region genes, for both heavy and light chains, or whether antibodies of the same specificity could derive from variable region genes with highly divergent sequences. Each monoclonal had a groove-type site that could hold six or seven sugar residues (with one exception), based on inhibition of immunoprecipitation by different length oligosaccharides. Thus, unlike monoclonals to haptenated proteins, the precise epitope could be well characterized and was generally quite similar among the entire series.

Studies of the V_K sequences revealed that only three V_K groups were used in these hybridomas. Use of each V_K group correlated with the particular antigen used to immunize the animals, whether linear dextran or short oligosaccharides, so that 10 of the monoclonals from mice immunized the same way all used the same V_K.

In contrast, the 17 V_H chains were derived from at least five different germline genes from three different V_H gene families.[21] The two most frequently used germline V_H genes were found in seven and five monoclonals, respectively, with minor variations explainable by somatic mutations. The remarkable finding is that very different V_H chains (about 50% homologous) can combine with the same V_K to produce antibody-binding sites with nearly the same size, shape, antigen specificity, and affinity. Even when different V_H sequences combine with different V_K sequences, they can produce antibodies with very similar properties. Dextran binding depends on the antigen fitting into the groove and interacting favorably with the residues forming the sides and bottom of the groove. The results indicate that divergent variable region sequences, both in and out of the complementarity-determining regions, can be folded to form similar binding site contours, which result in similar immunochemical characteristics. Similar results have been reported in other antigen-antibody systems, such as phenyloxazolone.[22]

Additional studies of carbohydrate binding monoclonal antibodies have revealed significant

information about how the antibody variable regions can bind a carbohydrate structure with high affinity and specificity. Several examples are now available of crystal structures of carbohydrates bound to antibodies.

For example, monoclonal antibody Se155-4 is specific for the group B determinant of the salmonella O antigen, which consists of the sugars Gal-Abequose-Man.[23,24] The crystal structure of antibody bound to the polysaccharide shows that one hexose, abequose, fits into the binding pocket, while the rest of the interactions occur along the surface of the antibody, similar to the groove-type sites described previously. Binding energy depends on hydrogen bonds formed between the protein residues and the hydroxyl groups of the carbohydrate. The protein residues include aromatic amines, such as His 32, Trp 91, and Trp 96 of the light chain, as well as His 97 and His 35 of the heavy chain. In addition, one of the sugars is hydrogen bonded via a water molecule bridge to the amide bonds of the protein backbone. About three quarters of all sugar hydroxyl groups are involved in hydrogen bonds with the protein. Although each H-bond is relatively weak by itself, the combined effect of eight hydrogen bonds results in high-affinity binding. Antibody specificity derives from the fact that the carbohydrate fits into a binding pocket, where H-bond formation depends on precise interactions with amino acid residues that are oriented about the pocket. Surprisingly, most of these bonds are formed between sugar hydroxyls and aromatic amino acids that are neither charged nor very polar at neutral pH.

Similarly, monoclonal antibody BR96 and the humanized monoclonal hu3S193 are specific for the Le Y antigen, which resembles the Le B antigen described in Figure 23.2, except that the fucose-N-acetylglucosamine bond is β(1 → 3) instead of β(1 → 4). The Le Y antigen is commonly expressed on tumor cells of epithelial origin. The crystal structures have revealed the sources of the binding energy that results in affinity and specificity for this carbohydrate antigen.[25,26] These two monoclonals bind Le Y antigen in a large, deep pocket, which accommodates all four hexoses and correspond to the cavity type binding site predicted by Kabat.15 The terminal fucose goes in first while the other three sugars are hydrogen bonded to amino acid side chains lining the pocket, including Tyr 33, Tyr 35, and Gln 52 of the heavy chain and His 27 of the light chain. Once again, hydrogen bonds between hydroxyl groups of the sugars and aromatic amines (Trp and Tyr) of the protein play a dominant role in determining affinity and specificity of binding. A smaller number of H-bonds depend on amide groups of the protein backbone.

A third example is provided by human monoclonal 2G12, which has neutralizing activity against a broad spectrum of human immunodeficiency virus (HIV) isolates. This antibody binds the mannose-rich oligosaccharide side chains that form a protective surface, called a glycoshield, on the envelope glycoprotein gp120. The crystal structure shows that the two terminal mannose sugars of each oligosaccharide bind end on into a deep pocket of the antibody, in a cavity-type site.[27,28] Twelve hydrogen bonds form between the two terminal mannose residues and the protein, depending mainly on the amide groups of the protein backbone. Additional hydrogen bonds form between the third mannose residue and the side chain of Asp 100 of the heavy chain and between the fourth mannose residue and Tyr 94 of the light chain and Tyr 56 of the heavy chain. A unique feature of this antibody is the crossover of variable regions between heavy and light chains so that each binding site is made up of the V_H from one HL pair combining with the V_L chain of the opposite pair. This arrangement allows the antibody to bind one branch of an oligosaccharide and the opposite branch of a nearby oligosaccharide and makes it ideally suited for cross-linking the densely clustered oligosaccharides of gp120.

Immunogenicity of Polysaccharide Conjugates

Capsular polysaccharides are the main target of protective antibodies against bacterial infection, and, as such, are important vaccine antigens. In adults, the chain length of the polysaccharide is an important determinant of immunogenicity, and the polysaccharides induce a T-independent response that cannot be boosted on repeat exposure. In young children, whose maternal antibodies wane by 6 months of age and who most need immunity to pathogens such as *Haemophilus influenzae* type b and *Streptococcus pneumoniae* of multiple serotypes, the T-independent response to these polysaccharides is weak, regardless of chain length. To immunize children, the polysaccharides were coupled to a protein carrier to create a new T-dependent antigen that gained immunogenicity from T-cell help and boosted antibody titers with each successive dose. This strategy has produced highly successful conjugate vaccines against *H. influenzae* type b,[29] resulting in a markedly reduced incidence of meningitis caused by this agent in immunized children[30,31] and evidence of herd immunity even among unimmunized children. The same strategy has produced an effective vaccine against invasive disease[32] and otitis media[33] caused by the most prevalent serotypes of *S. pneumoniae*.

Protein and Polypeptide Antigenic Determinants

Like the proteins themselves, the antigen determinants of proteins consist of amino acid residues in a particular threedimensional array. The residues that make contact with complementary residues in the antibody-combining site are called contact residues. To make contact, of course, these residues must be exposed on the surface of the protein, not buried in the hydrophobic core. As the complementarity-determining residues in the hypervariable regions of antibodies have been found to span as much as 30 to 40 Å × 15 to 20 Å × 10 Å (D. R. Davies, personal communication), these contact residues comprising the antigenic determinant may cover a significant area of protein surface, as measured by x-ray crystallography of antibody-protein antigen complexes.[34,35,36,37] The size of the combining sites has also been estimated using simple synthetic oligopeptides of increasing length, such as oligolysine. In this case, a series of elegant studies[38,39,40] suggested that the maximum chain length a combining site could accommodate was six to eight residues, corresponding closely to that found earlier for oligosaccharides,[13,14] as discussed previously.

Several types of interactions contribute to the binding energy. Many of the amino acid residues exposed to solvent on the surface of a protein antigen will be hydrophilic. These are likely to interact with antibody contact residues via polar interactions. For instance, an anionic glutamic acid carboxyl group may bind to a complementary cationic lysine amino group on the antibody, or vice versa, or a glutamine amide side chain may form a hydrogen bond with the antibody. However, hydrophobic interactions can also play a major role. Proteins cannot exist in aqueous solution as stable monomers with too many hydrophobic residues on their surface. Those hydrophobic residues that are on the surface can contribute to binding to antibody for exactly the same reason. When a hydrophobic residue in a protein antigenic determinant or, similarly, in a carbohydrate determinant[8] interacts with a corresponding hydrophobic residue in the antibody-combining site, the water molecules previously in contact with each of them are excluded. The result is a significant stabilization of the interaction. A thorough review of these aspects of the chemistry of antigen-antibody binding is in Getzoff et al.[41]

Mapping Epitopes: Conformation versus Sequence

The other component that defines a protein antigenic determinant, besides the amino acid

residues involved, is the way these residues are arrayed in three dimensions. As the residues are on the surface of a protein, we can also think of this component as the topography of the antigenic determinant. Sela[42] divided protein antigenic determinants into two categories, sequential and conformational, depending on whether the primary sequence or the three-dimensional conformation appeared to contribute the most to binding. On the other hand, as the antibody-combining site has a preferred topography in the native antibody, it would seem a priori that some conformations of a particular polypeptide sequence would produce a better fit than others and therefore would be energetically favored in binding. Thus, conformation or topography must always play some role in the structure of an antigenic determinant.

Moreover, when one looks at the surface of a protein in a space-filling model, one cannot ascertain the direction of the backbone or the positions of the helices (contrast Figs. 23.3A and 23.3B).[43,44,45,46,47] It is hard to recognize whether two residues that are side by side on the surface are adjacent on the polypeptide backbone or whether they come from different parts of the sequence and are brought together by the folding of the molecule. If a protein maintains its native conformation when an antibody binds, then it must similarly

P.546

P.547

be hard for the antibody to discriminate between residues that are covalently connected directly and those connected only through a great deal of intervening polypeptide. Thus, the probability that an antigenic determinant on a native globular protein consists of only a consecutive sequence of amino acids in the primary structure is likely to be rather small. Even if most of the determinant were a continuous sequence, other nearby residues would probably play a role as well. Only if the protein were cleaved into fragments before the antibodies were made would there be any reason to favor connected sequences.

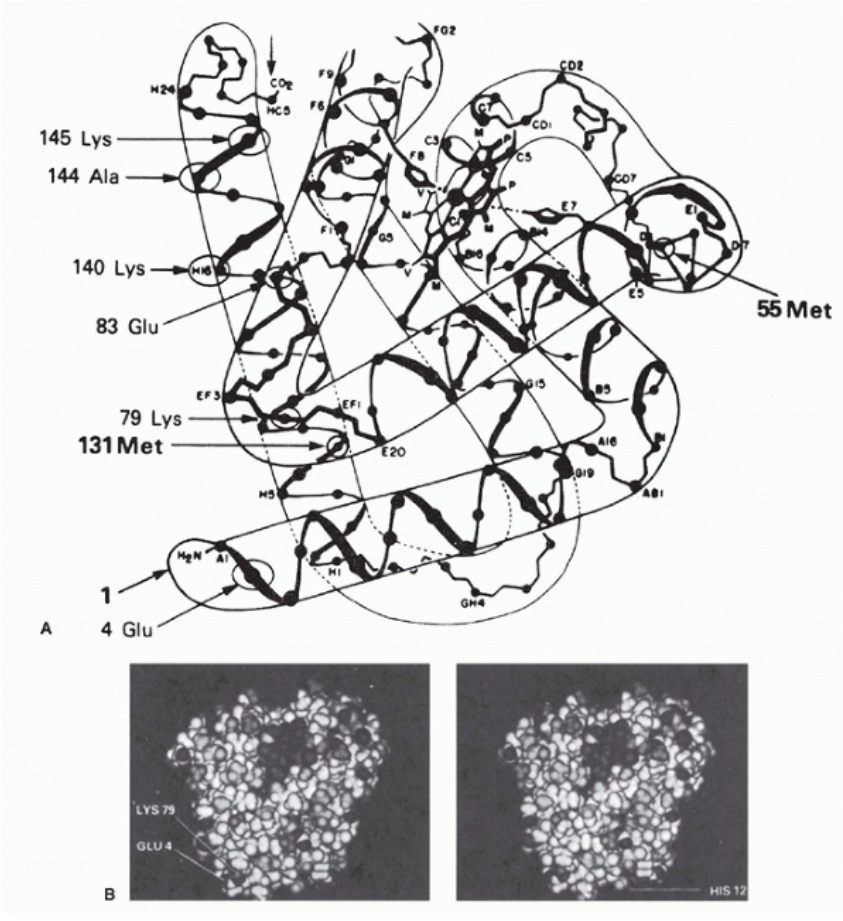


FIG. 23.3. A: Artist's representation of the polypeptide backbone of sperm whale myoglobin in its native three-dimensional conformation. The α helices are labeled A through H from the amino terminal to the carboxyl terminal. Side chains are omitted, except for the two histidine rings (F8 and E7) involved with the heme iron. Methionines at positions 55 and 131 are the sites of cleavage by cyanogen bromide (CNBr), allowing myoglobin to be cleaved into three fragments. Most of the helicity and other features of the native conformation are lost when the molecule is cleaved. A less drastic change in conformation is produced by removal of the heme to form apomyoglobin, as the heme interacts with several helices and stabilizes their positions relative to one another. The other labeled residues (Glu 4, Lys 49, Glu 83, Lys 140, Ala 144, and Lys 145) are residues that have been found to be involved in antigenic determinants recognized by monoclonal antibodies.[43] Note that cleavage by CNBr separates Lys 79 from Glu 4 and separates Glu 83 from Ala 144 and Lys 145. The "sequential" determinant of Koketsu and Atassi[44] (residues 15 to 22) is located at the elbow, lower right, from the end of the A helix to the beginning of the B helix. (Adapted from Dickerson.[45]) **B:** Stereoscopic views of a computer-generated space-filling molecular model of sperm whale myoglobin, based on the Takano[46] x-ray diffraction coordinates. This orientation, which corresponds to that in Panel A, is arbitrarily designated the "front view." The computer method was described by Feldmann et al.[47] The heme and aromatic carbons are *shaded darkest*, followed by carboxyl oxygens, then other oxygens, then primary amino groups, then other nitrogens, and finally side chains of aliphatic residues. The backbone and the side chains of nonaliphatic residues, except for the functional groups, are shown in *white*. Note that the direction of the helices is not apparent on the surface, in contrast to the backbone drawing in Panel A. The residues Glu 4, Lys 79, and His 12 are believed to be part of a topographic antigenic determinant recognized by a monoclonal antibody to myoglobin.[43] This stereo pair can be viewed in three dimensions using an inexpensive stereoviewer such as the "stereoscopes" sold by Abrams Instrument Corp. (Lansing, MI) or Hubbard Scientific Co. (Northbrook, IL). (Adapted from Berzofsky et al.[43])

This concept was analyzed and confirmed quantitatively by Barlow et al.,[48] who examined the atoms lying within spheres of different radii from a given surface atom on a protein. As the radius increases, the probability that all the atoms within the sphere will be from the same continuous segment of protein sequence decreases rapidly. Correspondingly, the fraction of surface atoms that would be located at the center of a sphere containing only residues from the same continuous segment falls dramatically as the radius of the sphere increases. For instance, for lysozyme, with a radius of 8 Å, fewer than 10% of the surface residues would lie in such a "continuous patch" of surface. These are primarily in regions that protrude from the surface. With a radius of 10 Å, almost none of the surface residues fall in the center of a continuous patch. Thus, for a contact area of about 20 Å × 25 Å, as found for a lysozyme-antibody complex studied by x-ray crystallography, none of the antigenic sites could be completely continuous segmental sites (see following discussion and Fig. 23.4). On the other hand, other analyses did not find a correlation of epitope residues with surface accessibility, suggesting that the situation is more complex.[49]

Antigenic sites consisting of amino acid residues that are widely separated in the primary protein sequence but brought together on the surface of the protein by the way it folds in its

native conformation have been called “assembled topographic” sites[50,51] because they are assembled from different parts of the sequence and exist only in the surface topography of the native molecule. By contrast, the sites that consist of only a single continuous segment of protein sequence have been called “segmental” antigenic sites.[50,51]

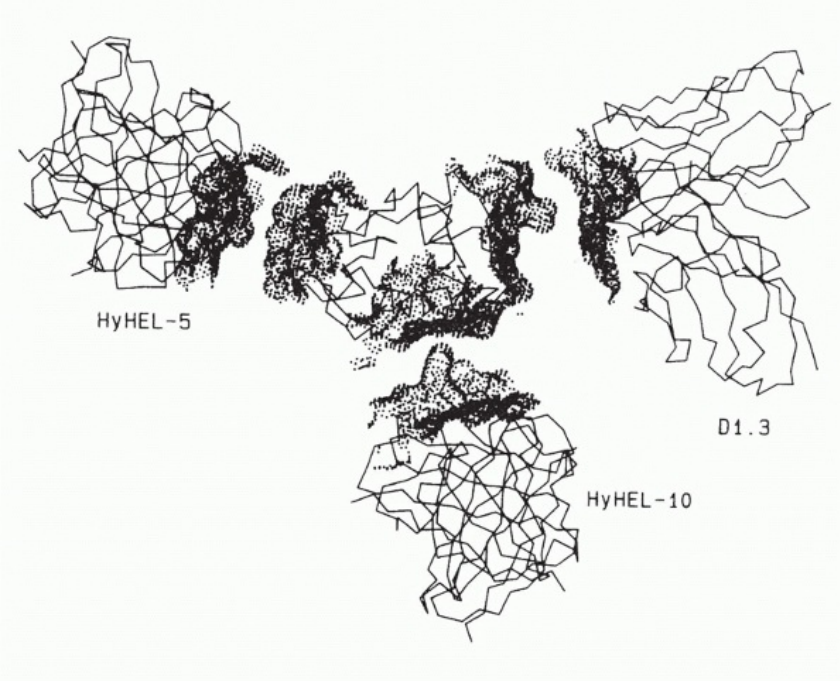


FIG. 23.4. Assembled Topographic Sites of Lysozyme Illustrated by the Footprints of Three Nonoverlapping Monoclonal Antibodies. Shown are the α-carbon backbones of lysozyme in the center and the Fv portions of three antilysozyme monoclonal antibodies D1.3, HyHEL-5, and HyHEL-10. The footprints of the antibodies on lysozyme and lysozyme on the antibodies (ie, their interacting surfaces) are shown by a *dotted representation*. Note that the three antibodies each contact more than one continuous loop of lysozyme and so define assembled topographic sites. Reproduced from Davies and Padlan[37] with permission.

In contrast to T-cell recognition of “processed” fragments retaining only primary and secondary structures, the evidence is overwhelming that most antibodies are made against the native conformation when the native protein is used as immunogen. For instance, antibodies to native staphylococcal nuclease were found to have about a 5000-fold higher affinity for the native protein than for the corresponding polypeptide on which they were isolated (by binding to the peptide attached to Sepharose).[52] An even more dramatic example is that demonstrated by Crumpton[53] for antibodies to native myoglobin or to apomyoglobin. Antibodies to native ferric myoglobin produced a brown precipitate with myoglobin but did not bind well to apomyoglobin, which, without the heme, has a slightly altered conformation. On the other hand, antibodies to the apomyoglobin, when mixed with native (brown) myoglobin, produced a white precipitate. These antibodies so strongly favored the conformation of apomyoglobin, from which the heme was excluded, that they trapped those molecules that vibrated toward that conformation and pulled the equilibrium state over to the apo form. One could almost say, figuratively, that the antibodies squeezed the heme out of the myoglobin. Looked at it thermodynamically, it is clear that the conformational

preference of the antibody for the apo versus native forms, in terms of free energy, had to be greater than the free energy of binding of the heme to myoglobin. Thus, in general, antibodies are made that are very specific for the conformation of the protein used as immunogen. Other more recent examples also show that antibodies can

enforce structures on disordered or denatured structures in proteins such as HIV-1 Tat[54] or influenza hemagglutinin.[55]

Synthetic peptides corresponding to segments of the protein antigen sequence can be used to identify the structures bound by antibodies specific for segmental antigenic sites. To identify assembled topographic sites, more complex approaches have been necessary. The earliest was the use of natural variants of the protein antigen with known amino acid substitutions, where such evolutionary variants exist.[50] Thus, substitution of different amino acids in proteins in the native conformation can be examined. The use of this method, which is illustrated later, is limited to studying the function of amino acids that vary among homologous proteins, that is, those that are polymorphic. It may now be extended to other residues by use of site-directed mutagenesis. A second method is to use the antibody that binds to the native protein to protect the antigenic site from modification[56] or proteolytic degradation.[57] A related but less sensitive approach makes use of competition with other antibodies.[58,59,60] A third approach, taking advantage of the capability of producing thousands of peptides on a solid-phase surface for direct binding assays,[61] is to study binding of a monoclonal antibody to every possible combination of six amino acids.[61] If the assembled topographic site can be mimicked by a combination of six amino acids not corresponding to any continuous segment of the protein sequence but structurally resembling a part of the surface, then one can produce a “mimotope” defining the specificity of that antibody.[61] Mimotopes have become widely used and can be combined with mutational analysis to map assembled topographic epitopes.[62] Mimetics have even been made for quaternary structural epitopes.[63] Many mimotope approaches use phase display peptide libraries to map epitopes of monoclonal antibodies.[64,65,66] However, other studies have been less optimistic about the ability to predict assembled topographic or discontinuous epitopes from mimotope binding[67] or random peptide libraries.[68]

Myoglobin also serves as a good model protein antigen for studying the range of variation of antigenic determinants from those that are more sequential in nature to those that do not even exist without the native conformation of the protein (see Fig. 23.3). A good example of the first more segmental type of determinant is that consisting of residues 15 to 22 in the amino terminal portion of the molecule. Crumpton and Wilkinson[69] first discovered that the chymotrypsin cleavage fragment consisting of residues 15 to 29 had antigenic activity for antibodies raised to either native or apomyoglobin. Two other groups[44,70] then found that synthetic peptides corresponding to residues 15 to 22 bind antibodies made to native sperm whale myoglobin, even though the synthetic peptides were only seven to eight residues long. Peptides of this length do not spend much time (in solution) in a conformation corresponding to that of the native protein. On the other hand, these synthetic peptides had a several hundred-fold lower affinity for the antibodies than did the native protein. Thus, even if most of the determinant was included in the consecutive sequence 15 to 22, the antibodies were still much more specific for the native conformation of this sequence than for the random conformation peptide. Moreover, there was no evidence to exclude the participation of other residues, nearby on the surface of myoglobin but not in this sequence, in the antigenic

determinant.[71,72,73,74*]

A good example of the importance of secondary structure is the case of the loop peptide (residues 64 to 80) of hen egg white lysozyme.[75] This loop in the protein sequence is created by the disulfide linkage between cysteine residues 64 and 80 and has been shown to be a major antigenic determinant for antibodies to lysozyme.[75] The isolated peptide 60 to 83, containing the loop, binds antibodies with high affinity, but opening of the loop by cleavage of the disulfide bond destroys most of the antigenic activity for antilysozyme antibodies.[75]

At the other end of the range of conformational requirements are those determinants involving residues far apart in the primary sequences that are brought close together on the surface of the native molecule by its folding in three dimensions, called assembled topographic determinants.[50,51] Of six monoclonal antibodies to sperm whale myoglobin studied by Berzofsky et al.,[43,76] none bound to any of the three cyanogen bromide (CNBr) cleavage fragments of myoglobin that together span the whole sequence of the molecule. Therefore, these monoclonal antibodies (all with affinities between 2×10^8 and 2×10^9 M^{-1}) were all highly specific for the native conformation. These were studied by comparing the relative affinities for a series of native myoglobins from different species with known amino acid sequences. This approach allowed the definition of some of the residues involved in binding to three of these antibodies. Two of these three monoclonal antibodies were found to recognize topographic determinants, as defined previously. One recognized a determinant including Glu 4 and Lys 79, which come within about 2 Å of each other to form a salt bridge in the native molecule (see Fig. 23.3A, B). The other antibody recognized a determinant involving Glu 83, Ala 144, and Lys 145 (see Fig. 23.3A). Again, these are far apart in the primary sequence but are brought within 12 Å of each other by the folding of the molecule in its native conformation. Similar examples have been reported for monoclonal antibodies to human myoglobin[77] and to lysozyme[37,58] as well as the HIV-1 envelope protein (neutralizing epitopes)[78,79] and the prion protein.[80] Other examples of such conformation-dependent antigenic determinants have been suggested using conventional antisera to such proteins as insulin,[81] hemoglobin,[82] tobacco mosaic virus,[83] and cytochrome c.[84] Moreover, the crystallographic structures of lysozyme-antibody[34,36,37] and neuraminidase-antibody[35] complexes, as well as HIV-1 envelope antibody complexes,[78,79] show clearly that, in both cases, the epitope bound is an assembled topographic site.

In the case of the three monoclonal antibodies binding to nonoverlapping sites of lysozyme (Fig. 23.4), it is clear that the footprints of all three antibody-combining sites cover more than one loop of polypeptide chain, and thus, each encompasses an assembled topographic site.[37] This result illustrates the concept that most antibody-combining sites must interact with more than a continuous loop of polypeptide chain and thus must define assembled topographic sites.[48] Another important example is represented by neutralizing antibodies to the HIV envelope protein that similarly bind assembled topographic sites[85,86] (see the end of this section).

How frequent are antibodies specific for topographic determinants compared to those that bind consecutive sequences when conventional antisera are examined? This question was studied by Lando et al.,[87] who passed goat, sheep, and rabbit antisera to sperm whale myoglobin over columns of myoglobin fragments, together spanning the whole sequence. After removal of all antibodies binding to the fragments, 30% to 40% of the antibodies

remained that still bound to the native myoglobin molecule with high affinity but did not bind to any of the fragments in solution by radioimmunoassay. Thus, in four of four antimyoglobin sera tested, 60% to 70% of the antibodies could bind peptides, and 30% to 40% could bind only native-conformation intact protein.

On the basis of studies such as these, it has been suggested that much of the surface of a protein molecule may be antigenic,[50,88] but that the surface can be divided up into antigenic domains.[43,73,74,77] Each of these domains consists of many overlapping determinants recognized by different antibodies.

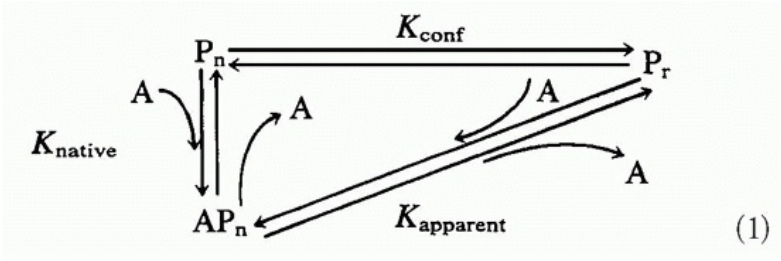
An additional interesting point is that in three published crystal structures of protein antigen-antibody complexes, the contact surfaces were broad, with local complementary pairs of concave and convex regions in both directions.[34,35,36,37] Thus, the concept of an antigen binding in the groove or pocket of an antibody may be oversimplified, and antibodies may sometimes bind by extending into pockets on an antigen.

Further information on the subjects discussed in this section is available in the reviews by Sela,[42] Crumpton,[53] Reichlin,[89] Kabat,[90] Benjamin et al.,[50] Berzofsky,[51] Getzoff et al.,[41] and Davis and Padlan.[37]

Conformational Equilibria of Protein and Peptide Antigenic Determinants

There are several possible mechanisms to explain why an antibody specific for a native protein will bind a peptide fragment in random conformation with lower affinity. Of course, the peptide may not contain all the contact residues of the antigenic determinant so that the binding energy would be lower. However, for cases in which all the residues in the determinant are present in the peptide, several mechanisms still remain. First, the affinity may be lower because the topography of the residues in the peptide may not produce as complementary a fit in the antibodycombining site as the native conformation would. Second, the apparent affinity may be reduced because only a small fraction of the peptide molecules are in a native-like conformation at any time, assuming that the antibody binds only to the native conformation. Because the concentration of peptide molecules in native conformation is lower than the total peptide concentration by a factor that corresponds to the conformational equilibrium constant of the peptide, the apparent affinity is also lower by this factor. This model is analogous to an allosteric model. A third, intermediate hypothesis would suggest that initial binding of the peptide in a nonnative conformation occurs with submaximal complementarity and is followed by an intramolecular conformational change in the peptide to achieve energy minimization by assuming a native-like conformation. This third hypothesis corresponds to an induced fit model. The loss of affinity is due to the energy required to change the conformation of the peptide, which in turn corresponds to the conformational equilibrium constant in the second hypothesis. To some extent, these models could be distinguished kinetically, as the first hypothesis predicts a faster “on” rate and a faster “off” rate than does the second hypothesis.[91] Such kinetic approaches have likewise been used to support an “encounter-docking” model related to this concept.[92]

Although not the only way to explain the data, the second hypothesis is useful because it provides a method to estimate the conformational equilibria of proteins and peptides.[52,93] The method assumes the second hypothesis, which can be expressed as follows:



where A = antibody, P_n = native peptide, and P_r = random conformation peptide so that

$$K_{apparent} = K_{conf}K_{native} \quad (2)$$

Thus, the ratio of the apparent association constant for peptide to the measured association constant for the native molecule should give the conformational equilibrium constant of the peptide. Note the implicit assumption that the total peptide concentration can be approximated by $[P_r]$. This will generally be true, as most peptide fragments of proteins demonstrate little native conformation, that is, $K_{conf} = [Pn] / [Pr]$ is much less than one. Also note that if the first hypothesis (or third) occurs to some extent, this method will overestimate K_{conf}. On the other hand, if the affinity for the peptide is lower because it lacks some of the contact residues of the determinant, this method will underestimate K_{conf} (by assuming that all the affinity difference is due to conformation). To some extent, the two errors may partially cancel out. When this method was used to determine the K_{conf} for a peptide from staphylococcal nuclease, a value of 2×10^{-4} (unitless because it is a ratio of two concentrations) was obtained.[52] Similarly, when antibodies raised to a peptide fragment were used, it was possible to estimate the fraction of time the native nuclease spends in nonnative

conformations.[93] In this case, the K_{conf} was found to be about 3000-fold in favor of the native conformation.

Antipeptide Antibodies that Bind to Native Proteins at a Specific Site

In light of the conformational differences between native proteins and peptides and the observed K_{conf} effects shown by antibodies to native proteins when tested on the corresponding peptides, it was somewhat surprising to find that antibodies to synthetic peptides show extensive cross-reactions with native proteins.[94,95] These two types of cross-reactions can be thought of as working in opposite directions: The binding of antiprotein antibodies to the peptide is inefficient, whereas the binding of antipeptide antibodies to the protein is quite efficient and commonly observed. This finding is quite useful, as automated solid-phase peptide synthesis has become readily available. This has been particularly useful in three areas: exploitation of protein sequences deduced by recombinant deoxyribonucleic acid (DNA) methods, preparation of site specific antibodies, and the attempt to focus the immune response on a single protein site that is biologically important but may not be particularly immunogenic. This section focuses on the explanation of the cross-reaction, uses of the cross-reaction, and the potential limitations regarding immunogenicity.

The basic assumption is that antibodies raised against peptides in an unfolded structure will bind the corresponding site on proteins folded into the native structure.[95] This is not immediately obvious, as antibody binding to antigen is the direct result of the antigen fitting into the binding site. Affinity is the direct consequence of “goodness of fit” between antibody and antigen, whereas antibody specificity is due to the inability of other antigens to occupy

the same site. How then can the antipeptide antibodies overcome the effect of K_{conf} and still bind native proteins with good affinity and specificity? The whole process depends on the antibodybinding site forming a three-dimensional space and the antigen filling it in an energetically favorable way.

Because the peptides are randomly folded, they rarely occupy the native conformation, so they are not likely to elicit antibodies against a conformation they do not maintain. If the antibodies are specific for a denatured structure, then, like the myoglobin molecules that were denatured to apomyoglobin by antibody binding,[53] the cross-reaction may depend on the native protein's ability to assume different conformational states. If the native protein is quite rigid, then the possibility of it assuming a random conformation is quite small; if it is a flexible three-dimensional spring, then local unfolding and refolding may occur all the time. Local unfolding of protein segments may permit the immunologic cross-reaction with antipeptide antibodies, as a flexible segment could assume many of the same conformations as the randomly folded peptide.[95] On the other hand, peptides with more stable conformations may be more likely to elicit antibodies that bind both the peptide and the native protein.[96] To this end, scaffolding has been used to maintain the conformation of peptides or protein fragments to be used as immunogens/vaccines, such as for respiratory syncytial virus[97] or HIV epitopes.[98,99]

In contrast, the ability of proteins to crystallize (a feature that allows the study of their structure by x-ray crystallography) has long been taken as evidence of protein rigidity.[100] In addition, the existence of discrete functional states of allosteric enzymes[101] provides additional evidence of stable structural states of a protein. Finally, the fact that antibodies can distinguish native from denatured forms of intact proteins is well known for proteins such as myoglobin.[53]

However, protein crystals are a somewhat artificial situation, as the formation of the crystal lattice imposes order on the components, each of which occupies a local energy minimum at the expense of considerable loss of randomness (entropy). Thus, the crystal structure may have artificial rigidity that exceeds the actual rigidity of protein molecules in solution. On the contrary, we may attribute some of the considerable difficulty in crystallizing proteins to disorder within the native conformation. Second, allosterism may be explained by two distinct conformations that are discrete without being particularly rigid. Finally, the ability to generate antiprotein antibodies that are conformation specific does not rule out the existence of antipeptide antibodies that are not. All antibodies are probably specific for some conformation of the antigen, but this need not be the crystallographic native conformation in order to achieve a significant affinity for those proteins or protein segments that have a “loose” native conformation.

Antipeptide antibodies have proved to be very powerful reagents when combined with recombinant DNA methods of gene sequencing.[95,102] From the DNA sequence, the protein sequence is predicted. A synthetic peptide is constructed, coupled to a suitable carrier molecule, and used to immunize animals. The resulting polyclonal antibodies can be detected with a peptide-coated enzyme-linked immunosorbent assay plate (see Chapter 7). They are used to immunoprecipitate the native protein from a ^{35}S-labeled cell lysate and thus confirm expression of the gene product in these cells. The antipeptide antibodies can also be used to isolate the previously unidentified gene product of a new gene. The site-specific antibodies are also useful in detecting posttranslational processing, as they bind all precursors and products that contain the site. In addition, because the antibodies bind only to the site

corresponding to the peptide, they are useful in probing structure-function relationships. They can be used to block the binding of a substrate to an enzyme or the binding of a virus to its cellular receptor.

Immunogenicity of Proteins and Peptides

Up to this point, we have considered the ability of antibodies to react with proteins or peptides as antigens. However, immunogenicity refers to the ability of these compounds to elicit antibodies following immunization. Several factors limit the immunogenicity of different regions of proteins, and these have been divided into those that are intrinsic to protein structure itself versus those extrinsic to the antigen that are related to the responder and vary from one animal or species to another.[51] In addition, we consider the special case of peptide immunogenicity, as it applies to vaccine development. The features of protein structure that have been suggested to explain the results include surface accessibility

of the site, hydrophilicity, flexibility, and proximity to a site recognized by helper T cells.

When the x-ray crystallographic structure and antigenic structure are known for the same protein, it is not surprising to find that a series of monoclonal antibodies binding to a molecule such as influenza neuraminidase choose an overlapping pattern of sites at the exposed head of the protein.[103]

The stalk of neuraminidase was not immunogenic apparently because it was almost entirely covered by carbohydrate. Beyond such things as carbohydrate, which may sterically interfere with antibody binding to protein, accessibility on the surface is clearly a sine qua non for an antigenic determinant to be bound by an antibody specific for the native conformation, without any requirement for unfolding of the structure.[51] Several measures of such accessibility have been suggested. All these require knowledge of the x-ray crystallographic three-dimensional structure. Some have measured accessibility to solvent by rolling a sphere with the radius of a water molecule over the surface of a protein.[104,105] Others have suggested that accessibility to water is not the best measure of accessibility to antibody and have demonstrated a better correlation by rolling a sphere with the radius of an antibody-combining domain.[106] Another approach to predicting antigenic sites on the basis of accessibility is to examine the degree of protrusion from the surface of the protein.[107] This was done by modeling the body of the protein as an ellipsoid and examining which amino acid residues remain outside ellipsoids of increasing dimensions. The most protruding residues were found to be part of antigenic sites bound by antibodies, but usually, these sites had been identified by using short synthetic peptides and so were segmental in nature. As noted previously, for an antigenic site to be contained completely within a single continuous segment of protein sequence, the site is likely to have to protrude from the surface, as otherwise residues from other parts of the sequence would fall within the area contacting the antibody.[48] However, inability of such surface or protrusion information to predict antigenic sites has also been encountered in some studies.[49]

Because the three-dimensional structure of most proteins is not known, other ways of predicting surface exposure have been proposed for the vast majority of antigens. For example, hydrophilic sites tend to be found on the water-exposed surface of proteins. Thus, hydrophilicity has been proposed as a second indication of immunogenicity.[108,109,110] This model has been used to analyze 12 proteins with known antigenic sites: The most hydrophilic site of each protein was indeed one of the antigenic sites. However, among the limitations are the facts that a significant fraction of surface residues can be nonpolar,[104,105] and that

several important examples of hydrophobic and aromatic amino acids involved in the antigenic sites are known.[42,83,111,112] Specificity of antibody binding likely depends on the complementarity of surfaces for hydrogen bonding and polar bonding as well as van der Waals contacts, whereas hydrophobic interactions and the exclusion of water from the interacting surfaces of proteins may contribute a large but nonspecific component to the energy of binding.[113] Another study suggested that amino acid pairs were better predictors of epitopes.[114]

A third factor suggested to play a role in immunogenicity of protein epitopes is mobility. Measurement of mobility in the native protein is largely dependent on the availability of a high-resolution crystal structure, so its applicability is limited to only a small subset of proteins. Furthermore, it has been studied only for antibodies specific for segmental antigenic sites; therefore, it may not apply to the large fraction of antibodies to assembled topographic sites. Studies of mobility have taken two directions. The case of antipeptide antibodies has already been discussed, in which antibodies made to peptides corresponding to more mobile segments of the native protein were more likely to bind to the native protein.[95,115] This is not considered just a consequence of the fact that more mobile segments are likely to be those on the surface and therefore more exposed because in the case of myohemerythrin (which was used as a model), two regions of the native protein that were equally exposed but less mobile did not bind nearly as well to the corresponding antipeptide antibodies.[116] However, as is clear from the previous discussion, this result applies to antibodies made against short peptides and therefore is not directly relevant to immunogenicity of parts of the native protein. Rather, it concerns the cross-reactivity of antipeptide antibodies with the native protein and therefore is of considerable practical importance for the purposes outlined in the section on antipeptide antibodies.

Studies in the other direction—that is, of antibodies raised against native proteins—would be by definition more relevant to the question of immunogenicity of parts of the native protein. Westhof et al.[117] used a series of hexapeptides to determine the specificity of antibodies raised against native tobacco mosaic virus protein and found that six of the seven peptides that bound antibodies to native protein corresponded to peaks of high mobility in the native protein. The correlation was better than could be accounted for just by accessibility because three peptides that corresponded to exposed regions of only average mobility did not bind antibodies to the native protein. However, when longer peptides—on the order of 20 amino acid residues—were used as probes, it was found that antibodies were present in the same antisera that bound to less mobile regions of the protein.[118] They simply had not been detected with the short hexapeptides with less conformational stability. Thus, it was not that the more mobile regions were necessarily more immunogenic but rather that antibodies to these were more easily detected with short peptides as probes. A similar good correlation of antigenic sites with mobile regions of the native protein in the case of myoglobin[117] may also be attributed to the fact that seven of the nine sites were defined with short peptides of six to eight residues.[71] Again, this result becomes a statement about cross-reactivity between peptides and native protein rather than about the immunogenicity of the native protein. For reviews, see Van Regenmortel[119] and Getzoff et al.[41]

To address the role of mobility in immunogenicity, an attempt was made to quantitate the relative fraction of antibodies specific for different sites on the antigen myohemerythrin.[120] The premise was that, although the entire surface of the protein may be immunogenic, certain regions may elicit significantly more antibodies than others and therefore may

be considered immunodominant or at least more immunogenic. Because this study was done with short synthetic peptides from 6 to 14 residues long based on the protein sequence, it was limited to the subset of antibodies specific for segmental antigenic sites. Among these, it was clear that the most immunogenic sites were in regions of the surface that were most mobile, convex in shape, and often of negative electrostatic potential. Other more recent studies corroborate the greater immunogenicity of more flexible segments of protein structures.[121] The role of these parameters has been reviewed.[41]

These results have important practical and theoretical implications. First, to use peptides to fractionate antiprotein antisera by affinity chromatography, peptides corresponding to more mobile segments of the native protein should be chosen when possible. If the crystal structure is not known, it may be possible to use peptides from amino or carboxyl termini or from exon-intron boundaries, as these are more likely to be mobile.[115] Second, these results may explain how a large but finite repertoire of antibody-producing B cells can respond to any antigen in nature or even artificial antigens never encountered in nature. Protein segments that are more flexible may be able to bind by induced fit in an antibody-combining site that is not perfectly complementary to the average native structure.[41,51] Indeed, evidence from the crystal structure of antigen-antibody complexes[122,123,124] suggests that mobility in the antibody-combining site as well as in the antigen may allow both reactants to adopt more complementary conformations on binding to each other, that is, a two-way induced fit. A nice example comes from the study of antibodies to myohemerythrin,[123] in which the data suggested that initial binding of exposed side chains of the antigen to the antibody promoted local displacements that allowed exposure and binding of other, previously buried residues that served as contact residues. The only way this could occur would be for such residues to become exposed during the course of an induced fit conformational change in the antigen.[41,123] In a second very clear example of induced fit, the contribution of antibody mobility to peptide binding was demonstrated for a monoclonal antibody to peptide 75 to 110 of influenza hemagglutinin, which was crystallized with or without peptide in the binding site and analyzed by x-ray crystallography for evidence of an induced fit.[124] Despite flexibility of the peptide, the antibody-binding site probably could not accommodate the peptide without a conformational change in the third complementarity determining region of the heavy chain, in which an asparagine residue of the antibody was rotated out of the way to allow a tyrosine residue of the peptide to fit in the binding pocket of the antibody.[124]

Regarding host-limited factors, immunogenicity is certainly limited by self-tolerance. Thus, the repertoire of potential antigenic sites on mammalian protein antigens such as myoglobin or cytochrome c can be thought of as greatly simplified by the sharing of numerous amino acids with the endogenous host protein. For mouse, guanaco, or horse cytochrome c injected into rabbits, each of the differences between the immunogen and rabbit cytochrome c is seen as an immunogenic site on a background of immunologically silent residues.[50,84,125] In another example, rabbit and dog antibodies to beef myoglobin bound almost equally well to beef or sheep myoglobin.[126] However, sheep antibodies bound beef but not sheep myoglobin, even though these two myoglobins differ by just six amino acids. Thus, the sheep immune system was able to screen out those clones that would be autoreactive with sheep myoglobin.

Ir genes of the host also play an important role in regulating the ability of an individual to make antibodies to a specific antigen.[127] These antigen-specific immune response genes are among the major histocompatibility complex (MHC) genes that code for transplantation

antigens. Structural mutations, gene transfer experiments, and biochemical studies[127] all indicate that Ir genes are actually the structural genes for MHC antigens. The mechanism of action of the MHC antigens works through their effect on helper T cells (described later in this chapter). There appear to be constraints on which B and T cells of a given specificity can help,[128,129] a process called T-B reciprocity.[130] Thus, if Ir genes control helper T-cell specificity, they will in turn limit which B cells are activated and which antibodies are made.

The immunogenicity of peptide antigens is also limited by intrinsic and extrinsic factors. With less structure to go on, each small peptide must presumably contain some non-self-structural feature in order to overcome self-tolerance. In addition, the same peptide must contain antigenic sites that can be recognized by helper T cells as well as by B cells. When no T-cell site is present, three approaches may be helpful: graft on a T-cell site, couple the peptide to a carrier protein, or overcome T-cell nonresponsiveness to the available structure with various immunologic agents, such as interleukin 2.

An example of a biologically relevant but poorly immunogenic peptide is the asparagine-alanine-asparagine-proline (NANP) repeat unit of the circumsporozoite (CS) protein of malaria sporozoites. A monoclonal antibody to the repeat unit of the CS protein can protect against murine malaria.[131] Thus, it would be desirable to make a malaria vaccine of the repeat unit of *Plasmodium falciparum* $(NANP)_n$. However, only mice of one MHC type ($H\text{-}2^b$) of all mouse strains tested were able to respond to $(NANP)_n$.[132,133] One approach to overcome this limitation is to couple $(NANP)_n$ to a site recognizable by T cells, perhaps a carrier protein such as tetanus toxoid.[134] In human trials, this conjugate was weakly immunogenic and only partially protective. Moreover, as helper T cells produced by this approach are specific for the unrelated carrier, a secondary or memory response would not be expected to be elicited by the pathogen itself.

Another choice might be to identify a T-cell site on the CS protein itself and couple the two synthetic peptides together to make one complete immunogen. The result with one such site, called Th2R, was to increase the range of responding mouse MHC types by one, to include $H\text{-}2^k$ as well as $H\text{-}2^b$.[135] This approach has the potential advantage of inducing a state of immunity that could be boosted by natural exposure to the sporozoite antigen. As CS-specific T and B cells are both elicited by the vaccine, natural exposure to the antigen could help maintain the level of immunity during the entire period of exposure.

Another strategy to improve the immunogenicity of peptide vaccines is to stimulate the T- and B-cell responses artificially by adding interleukin 2 to the vaccine. Results with myoglobin indicate that genetic nonresponsiveness can be overcome by appropriate doses of interleukin 2.[136] The same effect was found for peptides derived from malaria proteins.[137,137a]

One of the most important possible uses of peptide antigens is as synthetic vaccines. However, even though it is possible to elicit with synthetic peptides anti-influenza antibodies to nearly every part of the influenza hemagglutinin,[94] antibodies that neutralize viral infectivity have not been elicited by immunization with synthetic peptides. This may reflect the fact that antibody binding by itself often does not result in virus inactivation. Viral inactivation occurs only when antibody interferes with one of the steps in the life cycle of the virus, including binding to its cell surface receptor, internalization, and virus uncoating within the cell. Apparently, antibodies can bind to most of the exposed surface of the virus without affecting these functions. Only those antibodies that bind to certain “neutralizing” sites can

inactivate the virus. In addition, as in the case of the VP1 coat protein of poliovirus, certain neutralizing sites are found only on the native protein and not on the heat-denatured protein.[138] Thus, not only the site but also the conformation that is bound by the antibodies may be important for the antibody to inactivate the virus. These sites may often be assembled topographic sites not mimicked by peptide segments of the sequence. Perhaps binding of an antibody to such an assembled site can alter the relative positions of the component subsites so as to induce an allosteric neutralizing effect. Alternatively, antibodies to such an assembled site may prevent a conformational change necessary for activity of the viral protein.

One method of mapping neutralizing sites is based on the use of neutralizing monoclonal antibodies. The virus is grown in the presence of neutralizing concentrations of the monoclonal antibody, and virus mutants are selected for the ability to overcome antibody inhibition. These are sequenced, revealing the mutation that permits “escape” by altering the antigenic site for that antibody. This method has been used to map the neutralizing sites of influenza hemagglutinin[139] as well as poliovirus capsid protein VP1.[140] The influenza escaping mutations are clustered to form an assembled topographic site, with mutations distant from each other in the primary sequence of hemagglutinin but brought together by the three-dimensional folding of the native protein. At first, it was thought that neutralization was the result of steric hindrance of the hemagglutinin-binding site for the cell surface receptor of the virus.[141] However, similar work with poliovirus reveals that neutralizing antibodies that bind to assembled topographic sites may inactivate the virus at less than stoichiometric amounts, when at least half of the sites are unbound by antibody.[142] The neutralizing antibodies all cause a conformational change in the virus, which is reflected in a change in the isoelectric point of the particles from pH 7 to pH 4.[140,143] Antibodies that bind without neutralizing do not cause this shift. Thus, an alternative explanation for the mechanism of antibody-mediated neutralization is the triggering of the virus to self-destruct. Perhaps the reason that neutralizing sites are clustered near receptor-binding sites is that occupation of such sites by antibody mimics events normally caused by binding to the cellular receptor, causing the virus to prematurely trigger its cell entry mechanisms. However, in order to transmit a physiologic signal, the antibody may need to bind viral capsid proteins in the native conformation (especially assembled topographic sites), which antipeptide antibodies may fail to do. Antibodies of this specificity are similar to the viral receptors on the cell surface, some of which have been cloned and expressed without their transmembrane sequences as soluble proteins. The soluble recombinant receptors for poliovirus[144] and HIV-1[145,146,147] exhibit high-affinity binding to the virus and potent neutralizing activity in vitro. The HIV-1 receptor, cluster of differentiation (CD)4, has been combined with the human Ig heavy chain in a hybrid protein CD4-Ig,[148] which spontaneously assembles into dimers and resembles a monoclonal antibody, in which the binding site is the same as the receptor-binding site for HIV-1. In these recombinant constructs, high-affinity binding depends on the native conformation of the viral envelope glycoprotein gp120. Binding of CD4 to gp120 elicits a conformational change exposing a CD4-induced epitope, and fusions of CD4 domains to gp120 can be used as vaccines to elicit such antibodies.[149]

For HIV-1, two types of neutralizing antibodies have been identified. The first type binds a continuous or segmental determinant, such as the “V3 loop” sequence between amino acids 296 and 331 of gp120.[150,151,152] Antipeptide antibodies against this site can neutralize the virus.[150] However, because this site is located in a highly variable region of the envelope, these antibodies tend to neutralize a narrow range of viral variants with nearly the same

sequence as the immunogen. Even for this highly variable site, more broadly neutralizing antibodies can be obtained that recognize conserved conformations.[153,154,155] The second type of neutralizing antibody binds conserved sites on the native structure of gp120, allowing them to neutralize a broad spectrum of HIV-1 isolates. These antibodies are commonly found in the sera of infected patients,[156] and a panel of neutralizing monoclonals derived from these subjects has been analyzed.

These monoclonals can be divided into three types. One group, possibly the most common ones in human polyclonal sera, bind at or near the CD4 receptor-binding site of gp120.[79,157,158,159,160,161] A second type of monoclonal, called 2G12, binds a conformational site on gp120 that also depends on glycosylation, but has no direct effect on CD4 binding.[162] A third type, quite rare in human sera, is represented by monoclonal antibody 2F5[163] and binds a conserved site on the transmembrane protein gp41. Although this site is contained on a linear peptide ELDKWA, antibodies such as 2F5 cannot be elicited by immunizing with the peptide, again suggesting the conformational aspect of this site.[164,165] Indeed, the binding of antibodies to this membrane-proximal site has even been found to involve interaction of the antibody with the lipid membrane.[166] One might view this intriguing case as an example of a discontinuous or assembled topographic site created by the proximity of residues of the protein with structures in the lipid membrane, thus, spanning more than just different parts of the antigenic protein.

These monoclonals neutralize fresh isolates, as well as laboratory-adapted strains, and they neutralize viruses tropic for T cells or macrophages,[167] regardless of the use of CXCR4 or CCR5 as second receptor. These monoclonals, which target different sites, act synergistically. A cocktail combining all three types of monoclonals can protect monkeys against iv challenge or vaginal challenge with a simian immunodeficiency virus/HIV hybrid virus, indicating the potential for antibodies alone to prevent HIV infection.[168,169] Because each of the three conserved neutralizing determinants depends on the native conformation of the protein,[170] a prospective gp120 vaccine (or gp160 vaccine) would need to be in the native conformation to be able to elicit these antibodies.

ANTIGENIC DETERMINANTS RECOGNIZED BY T CELLS

Studies of T-cell specificity for antigen were motivated by the fact that the immune response to protein antigens is regulated at the T-cell level. A hapten, not immunogenic by itself, will elicit antibodies only when coupled to a protein that elicits a T-cell response in that animal. This ability of the protein component of the conjugate to confer immunogenicity on the hapten has been termed the “carrier effect.” Recognition of the carrier by specific helper T cells induces the B cells to make antibodies. Thus, the factors contributing to a good T-cell response appear to control the B-cell response as well.

“Nonresponder” animals display an antigen-specific failure to respond to a protein antigen, both for T cells and antibody responses. The “high responder” phenotype for each antigen is a genetically inheritable, usually dominant trait. Using inbred strains of mice, the genes controlling the immune response were found to be tightly linked to the MHC genes.[127,171] MHC-linked immune responsiveness has been shown to depend on the T-cell recognition of antigen bound within a groove of MHC antigens of the antigen-presenting cell (APC) (discussed herein below and see Chapters 21 and 22). The recognition of antigen in association with MHC molecules of the B cell is necessary for carrier-specific T cells to expand and provide helper signals to B cells.

In contrast to the range of antigens recognized by antibodies, the repertoire recognized by helper and cytotoxic T cells appears to be limited largely to protein and peptide antigens, although exceptions such as the small molecule tyrosine-azobenzene arsonate[172] exist. Once the antigenic determinants on proteins recognized by T cells are identified, it may be possible to better understand immunogenicity and perhaps even to manipulate the antibody response to biologically relevant antigens by altering the helper T-cell response to the antigen.

Defining Antigenic Structures

Polyclonal T-Cell Response

Significant progress in understanding T-cell specificity was made possible by focusing on T-cell proliferation in vitro, mimicking the clonal expansion of antigen specific clones in vivo. The proliferative response depends on only two cells: the antigen-specific T cell and an APC, usually a macrophage, dendritic cell, or B cell. The growth of T cells in culture is measured as the incorporation of [^{3}H]thymidine into newly formed DNA. Under appropriate conditions, thymidine incorporation increases with antigen concentration. This assay permits the substitution of different APCs and is highly useful in defining the MHC and antigen-processing requirements of the APCs.

Using primarily this assay, several different approaches have been taken to mapping T-cell epitopes. First, T cells immunized to one protein have been tested for a proliferative response in vitro to the identical protein or to a series of naturally occurring variants. By comparing the sequences of stimulatory and nonstimulatory variants, it was possible to identify potential epitopes recognized by T cells. For example, the T-cell response to myoglobin was analyzed by immunizing mice with sperm whale or horse myoglobin and testing the resulting T cells for proliferation in response to a series of myoglobins from different species with known amino acid substitutions.[173] Reciprocal patterns were observed in T cells from mice immunized with sperm whale or horse myoglobin. The response to the cross-stimulatory myoglobins was as strong as to the myoglobin used to immunize the mice. This suggested that a few shared amino acid residues formed an immunodominant epitope, and that most substitutions had no effect on the dominant epitope. A comparison of the sequences revealed that substitutions at a single residue could explain the pattern observed. All myoglobins that cross-stimulated sperm whale-immune T cells had Glu at position 109, whereas all that cross-stimulated horse-immune T cells had Asp at 109. No member of one group could stimulate T cells from donors immunized with a myoglobin of the other group. This suggested that an immunodominant epitope recognized by T cells was centered on position 109, regardless of which amino acid was substituted. Usually, this approach has led to correct localization of the antigenic site in the protein,[173,174,175] but the possibility of long-range effects on antigen processing must be kept in mind (see the section on “Antigen Processing”). Also, this approach using natural variants is limited in that it can focus on the correct region of the molecule but cannot define the boundaries of the site. Site-directed mutagenesis may therefore expand the capabilities of this approach.

A second approach is to use short peptide segments of the protein sequence, taking advantage of the fact that T cells specific for soluble protein antigens appear to see only segmental antigenic sites not assembled topographic ones.[127,176,177,178,179,180] These may be produced by chemical or enzymatic cleavage of the natural protein,[178,179,180,181,182,183,184,185,186] solid-phase peptide synthesis,[185,187,188,189,190] or recombinant DNA expression of cloned genes or gene

fragments.[191] In the case of class I MHC molecule-restricted cytotoxic T cells, viral gene deletion mutants expressing only part of the gene product have also been used.[192,193,194]

In the case of myoglobin-specific T cells, mapping of an epitope to residue Glu 109 was confirmed by use of a synthetic peptide 102 to 118, which stimulated the T cells.[189,195] The T cells elicited by a myoglobin with either Glu or Asp 109 could readily distinguish between synthetic peptides containing Glu or Asp at this position. Similar results were obtained with cytochrome c, where the predominant site recognized by T cells was localized with sequence variants to the region

around residue 100 at the carboxyl end of cytochrome.[174] Furthermore, the response to cytochrome c peptide 81 to 104 was as great as the response to the whole molecule. This indicated that a 24 amino acid peptide contained an entire antigenic site recognized by T cells. The T cells could distinguish between synthetic peptides with Lys or Gln at position 99, although both were immunogenic with the same MHC molecule.[196,197,198] This residue determined T-cell memory and specificity, and so presumably was interacting with the T-cell receptor (TCR). A similar conclusion could be drawn for residue 109 of myoglobin. However, this type of analysis must be used with caution. When multiple substitutions at position 109 were examined for T-cell recognition and MHC binding, residue 109 was found to affect both functions.[199] The ultimate use of synthetic peptides to analyze the segmental sites of a protein that are recognized by T cells was to synthesize a complete set of peptides, each staggered by just one amino acid from the previous peptide, corresponding to the entire sequence of hen egg lysozyme.[200] Around each immunodominant site, a cluster of several stimulatory peptides was found. The minimum "core" sequence consisted of just those residues shared by all antigenic peptides within a cluster, whereas the full extent of sequences spanning all stimulatory peptides within the same cluster defined the "determinant envelope." These two ways of defining an antigenic site differ, and one interpretation is that each core sequence corresponds to an MHC-binding site, whereas the determinant envelope includes the many ways for T cells to recognize the same peptide bound to the MHC.

In each case, the polyclonal T-cell response could be mapped to a single predominant antigenic site. These results are consistent with the idea that each protein antigen has a limited number of immunodominant sites (possibly one) recognized by T cells in association with MHC molecules of the high-responder type. If none of the antigenic sites could associate with MHC molecules on the APCs, then the strain would be a low responder, and the antigen would have little or no immunogenicity.

Monoclonal T Cells

Further progress in mapping T-cell sites depended on the analysis of cloned T-cell lines. These were either antigenspecific T-cell lines made by the method of Komoto and Fathman[201] or T-cell hybridomas made by the method of Kappler et al.[202] In the former method, T cells are allowed to proliferate in response to antigen and APCs, rested, and then restimulated again. After stimulation, the blasts can be cloned by limiting dilution and grown from a single cell in the presence of interleukin 2. In the second method, enriched populations of antigen-specific T cells are fused with a drug-sensitive T-cell tumor, and the fused cells are selected for their ability to grow in the presence of the drug. Then the antigen specificity of each fused cell line must be determined. The key to determining this in a tumor line is that antigen-specific stimulation of a T-cell hybridoma results in release of interleukin 2, even though proliferation is constitutive. T cells produced by either method are useful in defining epitopes, measuring their MHC associations and studying antigen-processing requirements.

Monoclonal T cells may be useful in identifying which of the many proteins from a pathogen are important for T-cell responses. For instance, Young and Lamb[203] have developed a way to screen proteins separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose for stimulation of T-cell clones and have used this to identify antigens of *Mycobacterium tuberculosis*.[204] Mustafa et al.[205] have even used T-cell clones to screen recombinant DNA expression libraries to identify relevant antigens of *Mycobacterium leprae*. Use of T cells to map epitopes has also been important in defining tumor antigens.[206,207,208,209,210,211]

Precise mapping of antigenic sites recognized by T cells was made possible by the fact that T cells would respond to peptide fragments of the antigen when they contain a complete antigenic determinant. A series of overlapping peptides can be used to walk along the protein sequence and find the antigenic site. Then, by truncating the peptide at either end, the minimum antigenic peptide can be determined. For example, in the case of myoglobin, a critical amino acid residue, such as Glu 109 or Lys 140, was found by comparing the sequences of stimulatory and nonstimulatory myoglobin variants and large CNBr cleavage fragments[212] as previously discussed, and then a series of truncated peptides containing the critical residue was synthesized with different overlapping lengths at either end.[185,189] Because solid-phase peptide synthesis starts from a fixed carboxyl end and proceeds toward the amino end, it can be stopped at various positions to produce a nested series of peptides that vary in length at the amino end. In this way, it was found that two of the Glu 109-specific T-cell clones responded to synthetic peptides 102 to 118 and 106 to 118 but not to peptide 109 to 118.[189] One clone responded to peptide 108 to 118, whereas the other did not. Thus, the amino end of the peptide recognized by one clone was Ser 108, whereas the other clone required Phe 106 and/or Ile 107. Similar fine specificity differences have been observed with T-cell clones specific for the peptides 52 to 61 and 74 to 96 of hen egg lysozyme,[182,213,214] the peptide 323 to 339 of chicken ovalbumin,[183] and the peptide 81 to 104 of pigeon cytochrome c[188]: The epitopes recognized by several T-cell clones overlap but are distinct. In addition, nine T-cell clones recognized a second T-cell determinant in myoglobin located around Lys 140, and each one responded to the CNBr cleavage fragment 132 to 153.[215] Further studies with a nested series of synthetic peptides showed that the stimulatory sequence is contained in peptide 136 to 145.[185]

These findings can be generalized to characterize a large number of epitopes recognized by T cells from a number of protein antigens (Table 23.4).[212,213,214,215,216,217,218,219,220,221,222,223,224,225,226,227] What these studies and others demonstrated about epitopes recognized by T cells is that in each case, the entire site is contained on a short peptide. MHC class I-restricted antigens also follow this rule,[228] even when the protein antigen is normally expressed on the surface of infected cells. This applies to viral glycoproteins, such as influenza hemagglutinin, which are recognized by cytolytic T cells after antigen processing[229] (see section on “Antigen Processing”). These peptides consist of no more than about 12 to 17 amino acid residues for class II MHC or 8 to 10 residues for class I. Within this size, they must contain all the information necessary to survive processing

within the APC, associate with the MHC antigen, and bind to the TCR, as discussed in the following sections.

TABLE 23.4 Examples of Immunodominant T-Cell Epitopes Recognized in Association with Class II MHC Molecules

Protein	T-Cell Antigenic Sites and Reference	Amphipathic Segments
Sperm whale myoglobin	69-78148	64-78
	102-118159	99-117
	132-145155	128-145
Pigeon cytochrome c	93-104158	92-103
Beef cytochrome c	11-25192	9-29
	66-80193	58-78
Influenza	109-119186	97-120
Hemagglutinin	130-140187	—
A/PR/8/34	302-313187,188	291-314
Pork insulin	B 5-16157	4-16
	A 4-14189	1-21
Chicken lysozyme	46-61185	—
	74-86184	72-86
	81-96175	86-102
	109-119145	—
Chicken ovalbumin	323-339153	329-346
Foot and mouth virus VP1	141-160191	148-165
Hepatitis B virus		
Pre-S	120-132190	121-135
Major surface antigen	38-52194	36-49

	95-109[194]	—
	140-154[194]	—
λ Repressor protein CI	12-26[195]	8-25
Rabies virus spike glycoprotein precursor	32-44[196]	29-46

Adapted with permission from Schwartz et al.[188]

Sequential Steps that Focus the T-Cell Response on Immunodominant Determinants

In contrast to antibodies that bind all over the surface of a native protein[50] (see "Protein and Polypeptide Antigenic Determinants" section), it has been observed that T cells elicited by immunization with the native protein tend to be focused on one or a few immunodominant sites.[230,231,232] This is true whether one deals with model mammalian or avian proteins such as cytochrome c,[179] myoglobin,[178,180] lysozyme,[182,214,233,234] insulin,[187,219] and ovalbumin,[183] or with bacterial, viral, and parasitic proteins from pathogens, such as influenza hemagglutinin[217] or nucleoprotein (NP),[228] staphylococcal nuclease,[235] or malarial CS protein.[135,236] Because the latter category of proteins shares no obvious homology to mammalian proteins, the immunodominance of a few sites cannot be attributed simply to tolerance for the rest of the protein because of homologous host proteins. Moreover, immunodominance is not simply the preemption of the response by a single clone of predominant T cells because it has been observed that immunodominant sites tend to be the focus for a polyclonal response of a number of distinct T-cell clones recognizing overlapping subsites within the antigenic site or having different sensitivities to substitutions of amino acids within the site.[182,183,188,189,200,213,214,237]

Immunodominant antigenic sites appear to be qualitatively different from other sites. For example, in the case of myoglobin, when the number of clones responding to different epitopes after immunization with native protein was quantitated by limiting dilution, it was observed that the bulk of the response to the whole protein in association with the high-responder class II MHC molecules was focused on a single site within residues 102 to 118[195] (Fig. 23.5). When T cells in the (high × low responder) F1 hybrid restricted to each MHC haplotype were compared, there was little difference in the responses to nondominant epitopes, and all the overall difference in magnitude of response restricted by the high versus low responder MHC could be attributed to the high response to the immunodominant determinant in the former and the complete absence of this response in the latter (see Fig. 23.5). Similar results were found for two different high-responder and two different low-responder MHC haplotypes.[195] Why did the response to the other sites not compensate for the lack of response to the immunodominant site in the low responders?

The greater frequency of T cells specific for the immunodominant site may in part be attributed to the large number of ways this site can be recognized by different T-cell clones, as mentioned previously, but this only pushes the problem back one level. Why is an immunodominant site the focus for so many different T-cell clones? Because the answer

cannot depend on any particular T cell, it must depend on other factors primarily involved in the steps in antigen processing and presentation by MHC molecules.

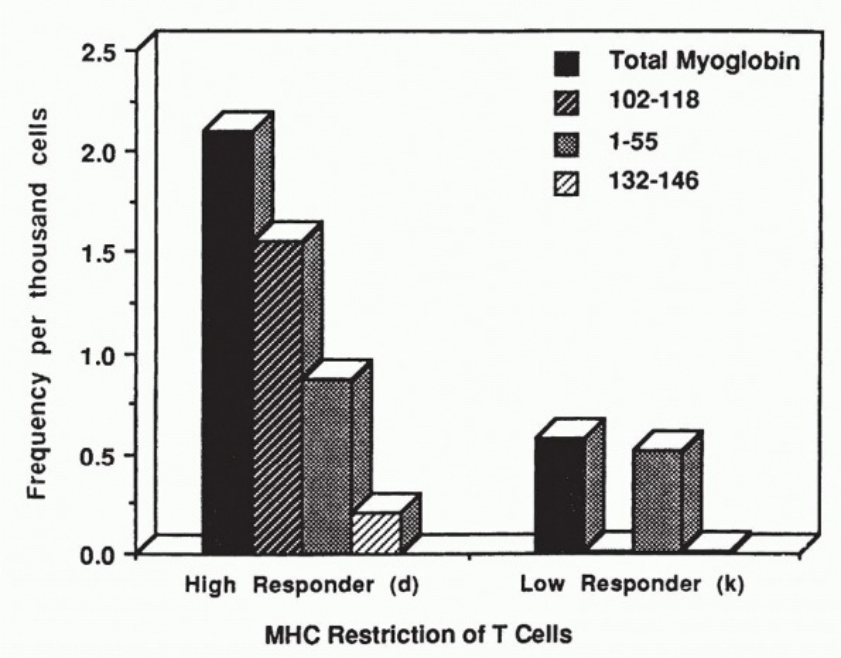


FIG. 23.5. Frequency of High- and Low-Responder Major Histocompatibility Complex (MHC)-Restricted T Cells in F1 Hybrid. High responsiveness may be accounted for by the response to a single immunodominant epitope. Lymph node T cells from (low-responder [H-2^k] × high-responder [H-2^d]) F1 hybrid mice immunized with whole myoglobin were plated at different limiting dilutions in microtiter wells with either high- or low-responder presenting cells and myoglobin as antigen. The cells growing in each well were tested for responsiveness to whole myoglobin and to various peptide epitopes of myoglobin. The frequency of T cells of each specificity and MHC restriction was calculated from Poisson statistics and is plotted on the ordinate. Most of the difference in T-cell frequency between high- and low-responder restriction types *(solid bars)* can be accounted for by the presence of T cells responding to the immunodominant site at residues 102 to 118, accounting for more than twothirds of the high-responder myoglobin-specific T cells, in contrast to the absence of such T cells restricted to the low-responder MHC type. (Based on the data in Kojima et al.[195].)

It has also been observed that some peptides may be immunogenic themselves, but the T-cell response they elicit is specific only for the peptide and does not cross-react with the native protein nor do T cells specific for the native protein recognize this site.[238,239,240] These are called cryptic determinants.[240] The reasons for these differences may involve the way the native protein is processed to produce fragments distinct from, but including or overlapping, the synthetic peptides used in experiments and also the competition among sites within the protein for binding to the same MHC molecules, as discussed further in the next section. To understand these factors that determine dominance or crypticity, one must understand the steps through which an antigen must go before it can stimulate a T-cell response.

Unlike B cells, T-cell recognition of antigen depends on the function of another cell, the

APC.[241] Antigen must pass through a number of intracellular compartments and survive processing and transport steps before it can be effectively presented to T cells. Following antigen synthesis in the cell (as in a virally infected cell) or antigen uptake via phagocytosis, pinocytosis, or, in some cases, receptor-mediated endocytosis, the subsequent steps include 1) partial degradation ("processing") into discrete antigenic fragments that can be recognized by T cells, 2) transport of these fragments into a cellular compartment where MHC binding can occur, 3) MHC binding and assembly of a stable peptide-MHC complex, and 4) recognition of that peptide-MHC complex by the expressed T-cell repertoire. At each step, a potential antigenic determinant runs the risk of being lost from the process, for example, by excessive degradation or failure to meet the binding requirements needed for transport to the next step. Only those peptides that surmount the four selective hurdles will prove to be antigenic for T cells. We will now consider each step in detail, for its contribution to the strength and specificity of the T-cell response to protein antigens.

Antigen Processing

Influence of Antigen Processing on the Expressed T-Cell Repertoire. Several lines of evidence indicate that antigen processing plays a critical role in determining which potential antigenic sites are recognized and, therefore, what part of the potential T-cell repertoire is expressed upon immunization with a protein antigen. Because the T cell does not see the native antigen but only the products of antigen processing, it is not unreasonable that the nature of these products would at least partly determine which potential epitopes could be recognized by T cells.

One line of evidence that processing plays a major role in T-cell repertoire expression came from comparisons that were made of the immunogenicity of peptide versus native molecule in the cases of myoglobin[238] or lysozyme.[239] In the case of myoglobin, a site of equine myoglobin (residues 102 to 118) that did not elicit a response when H-2^k mice were immunized with native myoglobin nevertheless was found to be immunogenic when such mice were immunized with the peptide.[238] Thus, the low responsiveness to this site in mice immunized with the native myoglobin was not due to either of the classical mechanisms of Ir gene defects—namely, a hole in the T-cell repertoire or a failure of the site to interact with MHC molecules of that strain. However, the peptide-immune T cells responded only poorly to native equine myoglobin in vitro. Thus, the peptide and the native molecule did not cross-react well in either direction. The problem was not simply a failure to process the native molecule to produce this epitope because (H-2^k × H-2^s) F1-presenting cells could present this epitope to H-2^s T cells when given native myoglobin but could not present it to H-2^k T cells. Also, because the same results applied to individual T-cell clones, the failure to respond to the native molecule was apparently not due to suppressor cells induced by the native molecule. Similar observations were made for the response to the peptide 74 to 96 of hen lysozyme in B10.A mice.[239] The peptide, not the native molecule, induced T cells specific for this site, and these T cells did not cross-react with the native molecule. With these alternative mechanisms excluded, we are left with the conclusion that an appropriate peptide was produced, but it differed from the synthetic peptide in such a way that a hindering site outside the minimal antigenic site interfered with presentation by presenting cells of certain MHC types. Further evidence consistent with this mechanism came from the work of Shastri et al.,[242] who found that different epitopes within the 74 to 96 region of lysozyme were immunodominant in H-2^b mice when different forms of the immunogen were used.

Another line of evidence came from fine specificity studies of individual T-cell clones. Shastri

et al.[243] observed that H-2^b T-cell clones specific for hen lysozymes were about 100-fold more sensitive to ring-necked pheasant lysozyme than to hen lysozyme. Nevertheless, they were equally sensitive to the CNBr cleavage fragments containing the antigenic sites from both lysozymes. Thus, regions outside the minimal antigenic site removable by CNBr cleavage presumably interfered with processing, presentation, or recognition of the corresponding site in hen lysozyme. Similarly, it was observed that a T-cell clone specific for sperm whale myoglobin, not equine myoglobin, responded equally well to the minimal epitope synthetic peptides from the two species.[238] Also, residues outside the actual site must be distinguishing equine from sperm whale myoglobin. Experiments using F1-presenting cells that can clearly produce this epitope for presentation to other T cells proved that the problem was not a failure to produce the appropriate fragment from hen lysozyme[239] or equine myoglobin.[238] Thus, these cases provide evidence that a structure outside the minimal site can hinder presentation in association with a particular MHC molecule.

Such a hindering structure was elegantly identified in a study by Grewal et al.[244] comparing hen egg lysozyme peptides presented by strains C57BL/6 and C3H.SW that share H-2^b but differ in non-MHC genes. After immunization with whole lysozyme, a strong T-cell response was seen to peptide 46 to 61 in C3H.SW mice but not at all in C57BL/6 mice. Because the F1 hybrids of these two strains responded, the lack of response in one strain was not due to a hole in

the T-cell repertoire produced by self-tolerance. It was found that peptide 46 to 60 bound directly to the I-A^b class II MHC molecule, whereas peptide 46 to 61 did not, indicating that the C-terminal Arg at position 61 hindered binding. Evidently, a non-MHC-linked difference in antigen processing allowed this Arg to be cleaved off the 46 to 61 peptide in C3H.SW mice, in which the peptide was dominant, but not in C57Bl/6 mice, in which the peptide was cryptic.

Even a small peptide that does not need processing may nevertheless be processed, and that processing may affect its interaction with MHC molecules. Fox et al.[245] found that substitution of a tyrosine for isoleucine at position 95 of cytochrome c peptide 93 to 103 enhanced presentation with Eβ^b but diminished presentation with Eβ^k when live APCs were used but not when the APCs were fixed and could not process antigen. Therefore, the tyrosine residue was not directly interacting with the different MHC molecule but was affecting the way the peptide was processed, which in turn affected MHC interaction.

Besides the mechanisms suggested previously, Gammon et al.[239] and Sercarz et al.[246] have proposed the possibility of competition between different MHC-binding structures (“agretopes”) within the same processed fragment. If a partially unfolded fragment first binds to MHC by one such site already exposed, further processing may stop, and other potential binding sites for MHC may never become accessible for binding. Such competition could also occur between different MHC molecules on the same presenting cell.[239] For instance, BALB/c mice, expressing both A^d and E^d, produce a response to hen lysozyme specific for 108 to 120, not for 13 to 35,[239] and this response is restricted to E^d. However, B10.GD mice that express only A^d respond well to 13 to 35 when immunized with lysozyme. The BALB/c mice clearly express an A^d molecule, so the failure to present this 13 to 35 epitope may be due to competition from E^d, which may preempt by binding the 108 to 120 site with higher affinity and preventing the 13 to 35 site from binding to A^d. Competition between different peptides binding to the same MHC molecule could also occur.

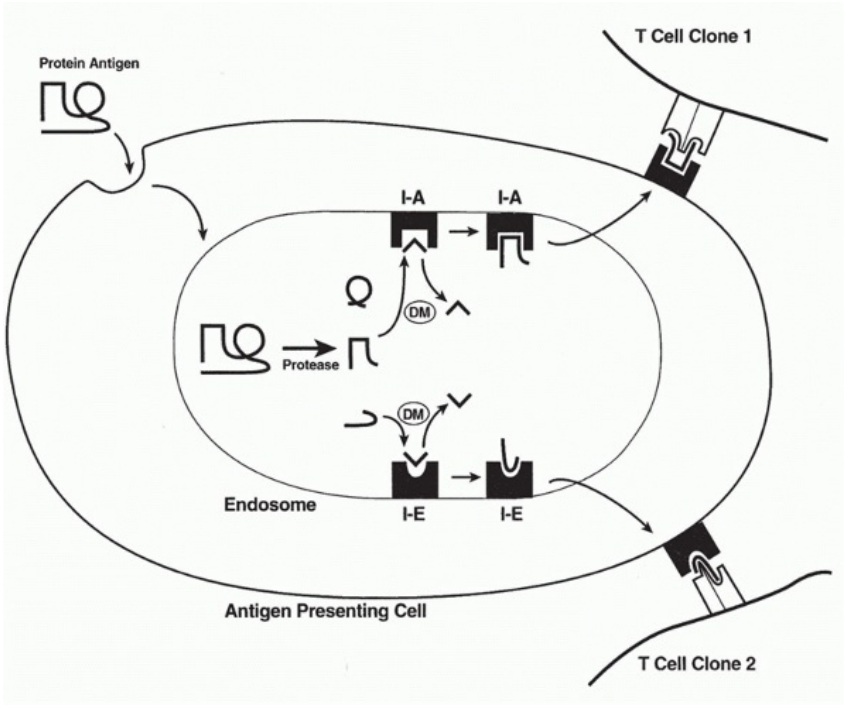


FIG. 23.6. Steps in Antigen Presentation by Class II Major Histocompatibility Complex (MHC) Molecules. Soluble antigen enters the presenting cell by phagocytosis, pinocytosis, or receptor-mediated endocytosis. It is partially degraded to peptide fragments by acid-dependent proteases in endosomes. Antigenic peptides associate with MHC class II molecules (I-A or I-E in the mouse) to form an antigenic complex that is transported to the cell surface. Before an MHC class II molecule can bind the peptide, it must release the class II-associated invariant chain peptide fragment of invariant chain from the binding groove, which is catalyzed by human leukocyte antigen (HLA)-DM. Binding of T-cell receptors to the peptide-MHC complex triggers T-cell proliferation, resulting in clonal expansion of antigen-specific T cells.

All these results, taken together, indicate that antigen processing not only facilitates interaction of the antigenic site with the MHC molecule and/or the TCR but also influences the specificity of these interactions and, in turn, the specificity of the elicited T-cell repertoire. The molecular mechanisms behind such effects are just now being elucidated, as described in the following sections.

Processing of Antigen for T Cells Restricted to Class II Major Histocompatibility Complex Molecules. It has long been known that T-cell responses such as delayed hypersensitivity in vivo or T-cell proliferation in vitro to exogenous proteins can be stimulated not only by the native protein but also by denatured protein[176] and fragments of native protein.[219] Indeed, this feature, along with the requirement for recognition in association with class II MHC molecules, distinguishes T- from B-cell responses. In a number of cases, the site recognized by cloned T cells has been located to a discrete synthetic peptide corresponding to a segment of the primary sequence of the protein. Examples include insulin,[187,219] cytochrome c,[188] lysozyme,[182,213] and myoglobin.[178,185,189] In each case, the stimulatory peptide must contain all the information required for antigen presentation and T-cell stimulation. The lack of conformational specificity does not indicate a lack of TCR specificity. Rather, it results from antigen processing into peptide fragments that

destroys conformational differences prior to binding the TCR. One way to accomplish this is via antigen processing, which involves the partial degradation of a protein antigen into peptide fragments (Fig. 23.6).

Evidence of processing came from the fact that a single protein antigen could stimulate T cells to different epitopes, each specific for a different MHC antigen. For example, when a series of myoglobin-specific T-cell clones were tested for both antigen specificity and MHC restriction, six clones were specific for a site centering on amino acid Glu 109, and all six recognized the antigen in association with I-A^d. Nine additional T-cell clones were specific for a second epitope centered on Lys 140 and were restricted to a different MHC antigen, I-E^d. Thus, the antigen behaved as if it was split up into distinct epitopes, each with its own ability to bind MHC.[215]

That T cells recognize processed antigen was demonstrated by the fact that inhibitors of processing can block antigen presentation. Early experiments by Ziegler and Unanue[247] showed that processing depends on intracellular degradative endosomes, as drugs such as chloroquine and ammonium chloride (NH4 Cl), which raise endosomal pH and inhibit acid-dependent proteases, could block the process. However, prior degradation of proteins into peptide fragments allows them to trigger T cells even in the presence of these inhibitors of processing.[248] For example, T-cell clone 14.5 recognizes the Lys 140 site of myoglobin equally well on the antigenic peptide (residues 132 to 153) as on the native protein (Fig. 23.7). The difference between these two forms of antigen is brought out by the presence of processing inhibitors. Leupeptin, for example, inhibits lysosomal proteases and blocks the T-cell responses to native myoglobin but not to peptide 132 to 153. Thus, native myoglobin cannot stimulate T cells without further processing, whereas the peptide requires little or no additional processing.[249]

Why is antigen processing necessary? For class II MHC molecules, experiments suggest that antigen processing may uncover functional sites that are buried in the native protein structure. For example, a form of intact myoglobin that has been partially unfolded through chemical modification can behave like a myoglobin peptide and can be presented by APC even in the presence of enough protease inhibitor or chloroquine to completely block the presentation of native myoglobin.[249] Denatured lysozyme could also be presented without processing to one T-cell clone.[184] This result suggests that the requirement for processing may simply be a steric requirement, that is, to uncover the two sites needed to form the trimolecular complex between antigen and MHC and between antigen and TCR. Thus, unfolding may be sufficient without proteolysis, and proteolysis may simply accomplish an unfolding analogous to Alexander's approach to the Gordian knot.

The importance of antigen unfolding for T-cell recognition and the ability of unfolding to bypass the need for antigen processing apply to a range of polypeptide sizes from small peptides to extremely large proteins. At one extreme, Lee et al.[250] found that even fibrinogen, of Mr 340,000, does not need to be processed if the epitope recognized is on the carboxy-terminal portion of the α chain, which is naturally unfolded in the native molecule. At the other extreme, even a small peptide of only 18 amino acid residues, apamin, requires processing unless the two disulfide bonds that hold it in the native conformation are cleaved artificially to allow unfolding.[251] Therefore, large size does not mandate processing, and small size does not necessarily obviate the need for processing, at least for class II presentation. The common feature throughout the size range seems to be the need for unfolding. This evidence, taken together with the earlier data on unfolding of myoglobin and

lysozyme, strongly supports the conclusion that unfolding, rather than size reduction, is the primary goal of antigen processing, and that either antigen presentation by MHC molecules or TCR recognition frequently requires exposure of residues not normally exposed on the surface of the native protein. This conclusion is supported by recent studies of peptides eluted from class II MHC molecules, and the crystal structures of class II MHC-peptide complexes, which show that longer peptides can bind with both ends extending beyond the two ends of the MHC groove[252,253,254] (see “Antigen Interaction with MHC Molecules” section).

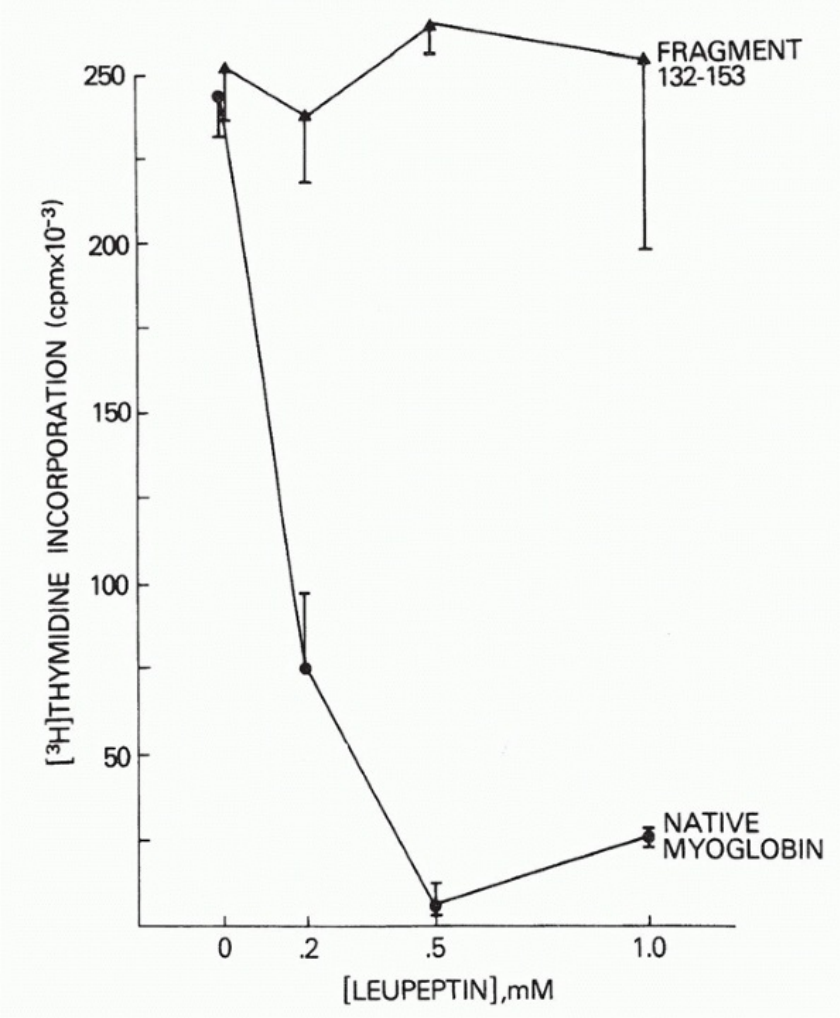


FIG. 23.7. Inhibition of Antigen Presentation by the Protease Inhibitor Leupeptin: Differential Effect on Presentation of the Same Epitope of Native Myoglobin or Peptide 132 to 153 to the Same Monoclonal T-Cell Population. Splenic cells from nonimmunized B10.D2 mice, as a source of antigen-presenting cells, were incubated with leupeptin at the concentration indicated for 15 minutes prior to and during exposure to 2 μM native myoglobin or 1 μM peptide fragment, washed, irradiated, and cultured at 400,000 cells per well with 10,000 T cells of clone 14.5; thymidine incorporation was measured after 4 days of culture.[249]

Besides proteolysis, unfolding may require the reduction of disulfide bonds between or within protein antigens. A gamma interferon-inducible lysosomal thiol reductase (GILT) is expressed in APCs and localizes to the late endosomal and lysosomal compartments where MHC class

occurs.[255] Unlike thioredoxin, this enzyme works at the acid pH of endosomes and uses Cys but not glutathione as a reducing agent. APCs from GILT knockout mice were tested for the ability to present hen egg lysozyme to hen egg lysozyme-specific T-cell lines.[256] For two epitopes, the T-cell response was insensitive to the GILT defect, even though they involved a disulfide bond in the native protein. But for one epitope, located between disulfide bonds, the T-cell response was completely inhibited when the APCs lacked GILT reductase. In this case, reduction of disulfide bonds was an essential step for antigen presentation, presumably needed to generate free peptides for MHC class II binding.

Processing of Antigen for T Cells Restricted to Class I Major Histocompatibility Complex Molecules. Early studies on class I-restricted T cells, such as cytolytic T cells (CTLs) specific for virus-infected cells, assumed that they responded mainly to unprocessed viral glycoproteins expressed on the surface of infected cells. However, since the mid-1980s, it has been clear that CTLs, like other T cells, recognize processed antigens. For example, influenza NP was a major target antigen for influenza specific CTLs, even though NP remains in the nucleus of infected cells and none is detectable on the cell surface.[257] Further support came from the finding that target cells that take up synthetic NP peptide 366 to 379 were lysed by NP-specific CTLs.[228] This constitutes evidence that antigen presented in association with class I molecules requires processing into antigenic fragments. Also, the demonstration that synthetic peptides could sensitize targets for CTLs introduced a powerful tool for mapping and studying CTL epitopes.

Even for influenza hemagglutinin, which is expressed on the surface of infected cells, surface expression was not required for antigenicity, implying that it is the processed antigen that stimulates a T-cell response. Target cells expressing leader-negative hemagglutinin, which is not transported to the cell surface but remains in the cytosol, were lysed equally well as those with surface hemagglutinin.[229] Similar conclusions were drawn from anchor-negative mutants.[258] Indeed, studies of HIV-1 gp160 genes with or without a leader sequence suggest that removal of the leader sequence can increase the amount of protein that is retained in the cytosol and is available for processing and presentation through the class I MHC processing pathway.[259] The explanation may be that the signal peptide results in cotranslational translocation of the growing peptide chain into the endoplasmic reticulum (ER), whereas proteins without a signal peptide remain in the cytosol, where they are accessible to the processing machinery of the class I pathway (see subsequent discussion). This cytosolic protein processing machinery consists primarily of the 26S proteasomes.[260,261] The specificity of such proteasomes to cleave at certain positions in a protein sequence thus provides the first hurdle that a potential epitope must surmount to be presented by class I MHC molecules to be cut out correctly but not destroyed by the proteasome.

In the standard proteasome, 14 distinct subunits assemble to form a high-molecular weight complex of about 580 kD with three distinct protease activities located on different subunits. The proteasome is a barrel-shaped structure, with the protease activities arrayed on the inner surface, and unfolded proteins are believed to enter the barrel at one end, leaving as peptides at the other end. The different proteases cut preferentially after aromatic or branched chain amino acids (chymotryptic-like activity of the β5 subunit), basic amino acids (trypsin-like activity of the β2 subunit), or acidic residues (glutamate preferring of the β1 subunit).[262,263] Protease activity is increased against misfolded proteins, such as

senescent proteins, which are tagged with ubiquitin and directed to the proteasome. In addition, viral proteins produced during infection and proteins synthesized with artificial amino acids are particularly susceptible to degradation by proteasomes. The products of protease digestion are peptides ranging from to 3 to 14 amino acids in length, including 9-mers, of just the right size for MHC binding. The chymotrypsin- and trypsin-like activities may be particularly important for antigenic peptides, as many peptides that naturally bind MHC end in hydrophobic or basic residues.[264]

The proteasome is the major processing machinery of the nonendosomal processing pathway. This is shown by the effect of proteasome inhibitors on MHC class I assembly and antigen presentation and by the effect of Large Multifunctional Peptidase (LMP-2) and LMP-7 mutations on antigen processing. A family of proteasome inhibitors have been described[262,263,265] that consist of short peptides, three to four amino acids in length, ending in an aldehyde, such as Ac-Leu Leu norLeu-al, carbobenzoxy-Leu Leu nor-Val-al,[263] or nonpeptides such as lactacystin.[266] Although the peptides appear to be directed primarily at the chymotrypsin-like protease activity, as false substrates, in fact, they inhibit all three types of protease activity.

By inhibiting antigen processing, these inhibitors induce a phenotype of reduced expression of MHC class I and inability to present antigen to class I-restricted CTL.[263] The MHC class I heavy chains remain in the ER, as shown by failure to become resistant to endoglycosidase H,[267] which occurs in the Golgi. They are also unable to form stable complexes with β2-microglobulin due to a lack of peptides. These effects are specific for the protease function because the inhibitors do not block presentation of synthetic peptides, which also rescue MHC class I expression, and because inhibition is reversible when inhibitor is removed. These results suggest that proteasomes are the primary supplier of antigenic peptides for class I, as other pathways are unable to compensate. However, it is also possible that the inhibitors could block other potential processing enzymes as well. An alternative processing pathway that bypasses the proteasome is provided by signal peptidase. As signal peptides are cleaved from proteins entering the ER, these hydrophobic peptides can bind MHC class I.[268] Particularly for MHC molecules such human leukocyte antigen (HLA)-A2, which prefer hydrophobic sequences, this peptidase can be an alternative source of antigenic peptides that are independent of proteasomes and transporter associated with antigen presentation (TAP)-1/2 transport (see section on “Transport”)because they are formed inside the ER.

The proteins destined for proteasomal processing include some normally short-lived proteins with a half-life of about 10 minutes, which constitute about 25% of the proteins in the cell. The rest arise from long-lived proteins, with a half-life

of about 1 day, which may be synthesized incorrectly. These defective ribosomal products are ubiquitinated and marked for rapid degradation in proteasomes.[269,270] In a normal cell, these can arise from errors in ribonucleic acid transcription, protein translation, assembly, folding, or targeting. But in a virally infected cell, misfolded viral proteins provide a ready supply of antigenic peptides for antigen presentation and T-cell recognition almost as soon as the virus starts to produce new viral proteins. Similarly, incorporation of amino acid analogues, such as canavavanine in place of arginine, creates misfolded proteins that are rapidly processed and more efficiently presented via the proteasomal pathway.[271]

Interestingly, the MHC itself encodes, near the class II region, three proteins, known as LMP-2 and LMP-7 for “lowmolecular-weight protein,” and MECL-1, for “multicatalytic endopeptidase complex-like 1,” which contribute to the proteasome structure. The LMP-2,

MECL-1, and LMP-7 subunits are upregulated by interferon-γ, and substitute for the subunits β1, β2, and β5, respectively, forming what has been dubbed an "immunoproteasome," present in professional APCs. All complexes with LMPs contain proteasome proteins, but only 5% to 10% of proteasomes contain LMP-2 and LMP-7. The ones without LMPs are called constitutive proteasomes.

These MHC-encoded subunits of the immunoproteasome shift the preference of proteasomes for cleaving after certain sequences, resulting in the production of different peptide fragments.[272,273,274] Proteasomes lacking LMP-2 through mutation or gene knockout have the same affinity, but decreased cleavage rate, for sequences ending in hydrophobic or basic amino acids. The effect is specific for these proteolytic sites, as the activity against sequences containing acidic amino acids actually increased.[273] Despite the shift in specific peptides released, the overall level of MHC class I expression was reduced only slightly in LMP-7 knockouts[274] and not at all in the LMP-2 knockouts. However, presentation of specific epitopes of the male H-Y antigen or of influenza NP was reduced by three-fold to five-fold in these knockouts. Toes et al.[275] quantitatively compared the cleavage fragments produced by standard proteasomes and immunoproteasomes and defined the prevalence of different amino acids on each side of the cleavage site. Consistent with earlier studies, there is a strong preference for both to cleave after leucine and also to a lesser extent after other hydrophobic residues, both aliphatic and aromatic. However, the immunoproteasomes have a stronger tendency to cleave after such hydrophobic residues and a much reduced cleavage frequency after acidic residues, Asp and Glu, than standard or constitutive proteasomes. This shift in specificity is concordant with the observation that class I MHC molecules tend to bind peptides with C-terminal hydrophobic or basic residues, not acidic ones. Thus, the immunoproteasomes in professional APCs may be more effective at generating antigenic peptides that can be presented by MHC molecules.[272,275] Protein degradation by proteasomes is processive, so the peptides released after 5% digestion are the same as the fragments released after 90% digestion. This suggests that intact proteins may enter the barrel, but they are not released at the other end until processing is complete.

Immunoproteasomes were shown to be essential for production of a hepatitis B virus core antigenic epitope[276] and to increase production of epitopes from adenovirus[277] and lymphocytic choriomeningitis virus.[278] Similarly, certain epitopes of latent membrane protein 2 of Epstein-Barr virus depended on immunoproteasomes.[279] In that case, the requirement for immunoproteasomes depended on the context of the peptide within the native protein. Incomplete protein synthesis due to puromycin or expression of the epitope surrounded by protein fragments with fewer membrane spanning domains allowed epitope generation by constitutive proteasomes.

On the other hand, some epitopes are generated more effectively by the constitutive proteasome than the immunoproteasome.[275,280] When the repertoire of seven defined class I MHC-restricted epitopes was compared in an elegant quantitative study in LMP-2-deficient or wild-type C57BL/6 mice, it was found that responses to the two epitopes that are immunodominant in wild-type mice were greatly reduced in the LMP-2-deficient mice, which lack immunoproteasomes, and two normally subdominant epitopes became dominant.[281] However, from adoptive transfer experiments in both directions, it was found that the reduced response to one normally dominant epitope was due to decreased production without immunoproteasomes, but that to the other was due to an altered T-cell repertoire in the LMP-2-deficient mice, presumably due to alterations in the peptides presented in the thymus. Further, the increased response to one of the subdominant determinants was related to

increased production of this peptide by the constitutive proteasomes compared to the immunoproteasomes. Thus, the immunoproteasome specificity plays a significant role in determining the repertoire of epitopes presented, and in selecting those that are immunodominant, as well as regulating the $CD8^+$ T-cell repertoire generated in the thymus.

Another protein associated with proteasomes is the proteasome activator PA28, which assembles into 11s structures.[282] Like LMP-2 and LMP-7,[272] PA28 is inducible by interferon-γ, and its induction causes a shift in proteasome function that may lead to the production of greater amounts of and different repertoires of antigenic peptides. For example, synthetic substrates were designed to test the ability of proteasomes to generate authentic MHC-binding peptides. These substrates contained the MHC-binding ligand flanked by the natural sequence as found in the original protein.

To generate the MHC-binding ligand, the proteasome would have to cleave the substrate twice.[283] By itself, the 20s proteasome was able to produce singly cleaved fragments, but with added PA28, doubly cut peptides were generated preferentially. Thus, PA28 favored the production of antigenic peptides, possibly by keeping the peptide in the proteasome until processing was complete. Alternatively, PA28 may coordinate the proteolytic activity of two adjacent sites to generate doubly cut peptides of just the right length (8- to 9-mers) to fit in the MHC groove. The distance between these nearby sites would determine the size of the peptides produced. The PA28 has been shown to increase generation of a dominant lymphocytic choriomeningitis virus epitope independently of the presence of the other interferon-γ-inducible components LMP-2, LMP-7, and MECL-1.[284]

The specificity of this proteasomal processing system determines the first step in winnowing the number of

protein segments that can become CTL epitopes, by selectively producing some peptide fragments in abundance and destroying others. Thus, it is probably not just coincidental that the C-terminal residues produced by proteasomal cleavage often serve as anchor residues for binding class I MHC molecules, or that the lengths of peptides produced are optimal for class I MHC binding.[272,285] Better understanding of the specificity of proteasomes will contribute to the new methods to predict dominant CD8+ T-cell epitopes.[232]

An analysis of proteasomal target sites was based on the proteasomal degradation pattern of three proteins: beta-casein, enolase, and prions.[286] The resulting peptides were analyzed by mass spectrometry, and the cleavage sites identified, including four amino acids on the amino end of each cleavage site and two amino acids on the carboxy end. The results are summarized in Figure 23.8. The three proteolytic activities of the proteasome were represented by favorable amino acids: Arg (trypsin-like), Tyr and Phe (chymotrypsin-like), and peptidylglutamylpeptide (Asp and Glu) at position P1. At this site, Pro was unfavored, as were Asn, Lys, and Ser. Additional negative effects were observed for Asp, Pro, and Ile at position P2. On the carboxy side of the cleavage site, at position P'1, the positive effect of Tyr was noted as well as negative effects of Ile, Phe, and Val.

Additional downstream processing steps, after the proteasome, are known to be important for generation of antigenic peptides. One of these occurs in the cytoplasm, prior to TAP transport, and others occur after transport into the ER. The proteasome creates a first draft of the peptides, which are then selected and trimmed to produce the final pool of antigenic peptides of optimal size and sequence for MHC binding while protecting the nascent peptides from degradation before they can bind MHC.

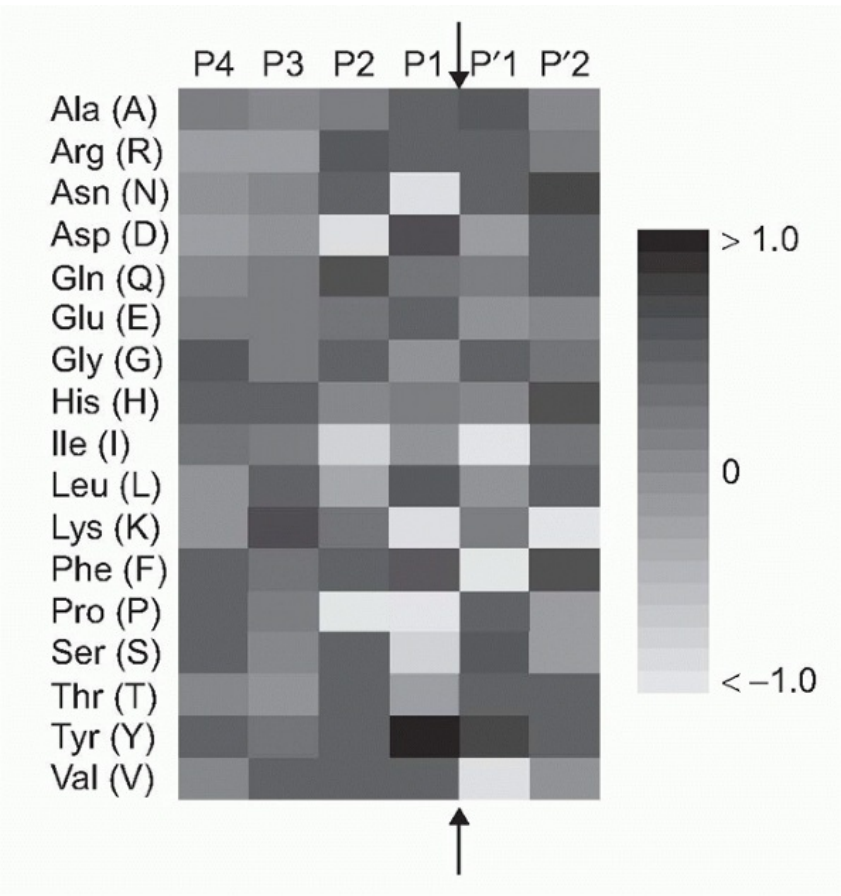


FIG. 23.8. The Effect of Specific Amino Acids on Proteasomal Cleavage between Positions P1 and P'1. Amino acids that favor cleavage are shown in *black*, whereas those that inhibit cleavage are shown in *white*. Reproduced from Donnes and Kohlbacher[286] with permission.

Recent studies have revealed that most peptides are released from the proteasome when they still need further processing to become antigenic peptides.[287,288] In the cytoplasm, the major proteolytic activity comes from an enzyme called tripeptidyl amino peptidase II (TPPII), which is located on a large particle. It has amino peptidase activity, which can remove 1 to 3 amino acids at a time and is useful for peptides of 15 amino acids or less. It also has endopeptidase activity that can cut in the middle of peptides larger than 15 amino acids, with the release of fragments of at least 9 amino acids. These are a significant source of 9-mers with new carboxyl ends, and this may be the only way to generate carboxyl ends other than the proteasome itself.

The importance of TPPII activity for antigen processing is shown by the fact that a specific inhibitor, butabindide, can prevent peptide loading of MHC, resulting in reduced surface expression. The most likely path for most protein antigens is to enter the proteasome and emerge as peptides of 15 amino acids or larger. These are then trimmed by TPPII, resulting in peptides ready for TAP transport. Longer peptides are trimmed internally by TPPII endoprotease, which may generate carboxyl ends needed for TAP binding. The resulting peptides are then transported by TAP into the ER, where they may be trimmed further to prepare them for MHC binding. An additional role of TPPII is shown by the generation of unique epitopes that are not produced by proteasomes. For example, an important T-cell epitope of the Nef protein of HIV requires TPPII processing. Proteasomal inhibitors have no

effect on it, but butabindide prevents its processing and presentation to HLA-A3- or A11-restricted T cells.[289] This pathway seems particularly important for generating epitopes ending in lysine groups, which bind these two MHC types but are rarely generated by proteasomes alone. TPPII can act in parallel with proteasomes or in tandem with them to release this epitope from intact protein or its partially degraded fragments.

Transport into a Cellular Compartment Where Major Histocompatibility Complex Binding Can Occur

The second hurdle a potential epitope must surmount is to be transported into the cellular compartment for loading onto MHC molecules. These compartments are different for class I and II molecules, as noted previously.

Transport Pathways Leading to Major Histocompatibility Complex Class I Presentation. The second hurdle for peptide presentation by class I MHC molecules is to get from the cytosol, where the peptides are produced, to the ER, where the newly synthesized class I MHC molecules are assembled and loaded with peptide. The discovery of a specific active transporter suggested that specificity of transport could further restrict the repertoire of peptides available to load onto class I MHC molecules. Genetic analysis of mutant cell lines that failed to load endogenous peptides onto class I MHC molecules revealed homozygous deletions of part of the MHC class II region near the DR locus. Molecular cloning of DNA from this region revealed at least 12 new genes, of which 2, called TAP-1 and TAP-2 showed a typical sequence for ABC transporter proteins.[290,291,292] Their function is

to transport processed peptides from the cytosol to the ER. Once in this compartment, peptides are handed off by TAP to newly formed MHC class I molecules and stabilize a trimolecular complex with β2-microglobulin. This complex is then transported to the cell surface, where antigen presentation occurs. Without the peptide transporters, empty dimers of MHC class I with β2-microglobulin form, but these are unstable. Excess free peptide would rescue MHC class I by stabilizing the few short-lived empty complexes that reach the surface, as shown by Townsend et al.[293] and Schumacher et al.[294] Thus, MHC-linked genes coding for proteolysis, peptide transport, and presentation at the cell surface have been identified. In effect, the MHC now appears to encode a complex system of multiple elements devoted to the rapid display of foreign protein determinants on the surface of an infected cell. By continuously sampling the output of the protein synthesizing machinery, this system permits rapid identification and destruction of infected cells by CTL before infectious virus can be released.

In an infected cell, as soon as viral proteins are made, peptide fragments generated by the proteasome become available to the TAP-1 and TAP-2 transporter proteins (Fig. 23.9). These transport the peptide fragments into the ER for association with newly formed MHC class I molecules, which would carry them to the cell surface for antigen presentation, all within 30 minutes. Indeed, the finding of a physical association between TAP and the nascent class I heavy chain/β2-microglobulin complex suggests that the peptide may be directly handed off from TAP to the new MHC molecule without being free in solution.[295,296] If TAP transport is highly selective, then some cytosolic peptides may fail to enter the ER for presentation with class I, but if it is promiscuous, then some peptides may be transported that were better off not presented, such as those leading to autoimmunity.

The idea that other proteins may control accessibility of MHC class I-binding sites for peptides originally came from the observation that two rat strains with the same MHC type ($RT1.A^a$) were nevertheless not histocompatible, and CTL could recognize the difference

between them.[297] The difference, called a cim effect, for class I modification, occurred because different peptides were binding the same MHC in the two strains.[298,299] The rat has two alleles for a peptide transporter supplying peptides to MHC. The one called TAP2A has peptide specificity matching that of RT1.A^a and delivers a broad set of peptides for MHC binding. The other transporter allele, called TAP-2B, supplies a different set of peptides that are discordant with RT1.A^a, so fewer types of peptides are bound. Although RT1.A^a would prefer to bind peptides with Arg at position 9, it has to settle for peptides with hydrophobic termini as provided by TAP-2B,[300] thereby accounting for the apparent histocompatibility difference. Thus, the specificity of TAP transport was shown to provide a selective step in narrowing the potential repertoire of CTL epitopes.

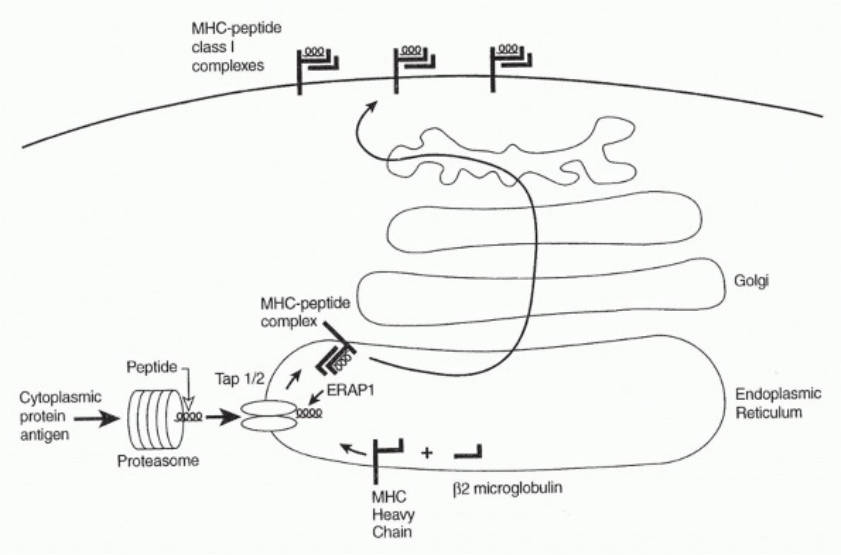


FIG. 23.9. Cytoplasmic Antigen-Processing Pathway leading to Major Histocompatibility Complex (MHC) Class I Presentation. Cytoplasmic antigen is degraded in the proteasome to fragments, which are transported into the endoplasmic reticulum by the transporter associated with antigen presentation (TAP) transporter. Peptide supplied by TAP forms a stable complex with MHC class I heavy chains and β2-microglobulin, and the complex is transported through the Golgi, where it achieves mature glycosylation, and out to the cell surface for presentation to class I-restricted T cells. Additional processing options by tripeptidyl amino peptidase II in the cytoplasm and endoplasmic reticulum aminopeptidase I in the endoplasmic reticulum, before and after TAP transport, allow greater flexibility in meeting the requirements of binding both TAP and MHC molecules.

To measure TAP specificity in other species, a transportable peptide bearing an N-linked glycosylation site was added to cells permeabilized by treatment with streptolysin. If the peptide was transported by TAP, it would enter the ER and cis Golgi, where it would be glycosylated.[301,302,303] The extent of glycosylation served as a measure of TAP function. When competitor peptides were added as well, TAP-mediated transport of the reporter peptide decreased, indicating saturation of peptide-binding sites. In this way, a series of related peptides could be tested for the ability to compete for TAP binding and transport in order to identify the requirements for TAP binding and transport.

TAP binding and transport depended strongly on peptide length.[303,304] Mouse TAP was shown to have a strong preference for peptides of nine residues or longer.[304] For human TAP, peptides shorter than seven amino acids long were not transported, regardless of sequence.[303] Peptides 8 to 11 amino acids long were almost all transported, with some variation in binding affinities depending on sequence.

Peptides 14 to 21 amino acids in length were transported selectively, whereas those longer than 24 amino acids were almost never transported intact. Thus, human TAP transport selected against peptides <7 or >24 amino acids in length, regardless of sequence. Unlike the rat, human and mouse TAP do not show allelic differences in peptide transport.

Although TAP can and must transport a wide variety of peptides, it may still have preferences for which peptides are transported most efficiently and which MHC types are provided with the peptides they need. For example, a self-peptide that naturally binds HLA-B27 was modified slightly to produce an N-linked glycosylation site, resulting in the sequence RRYQNSTEL.[303] Using the glycosylation of this peptide to measure transport, saturation of TAP by homologous peptides occurred with a 50% inhibitory concentration of < 1 μM. Other peptides with unrelated sequences also inhibited, often with equally high affinity. Not only did natural HLA-binding peptides compete but also did peptide variants lacking the MHC-binding motif at positions 2 and 9 (see subsequent discussion). Clearly, peptides binding different MHC types were transported by the same TAP protein, and even peptides that bound mouse MHC were transported by human TAP. In another example, using rat TAP proteins, peptides with Pro at position 2, 6, or 9 were found to be poor competitors for transport of a reference peptide.[302]

In a different approach, using a baculovirus system overexpressing TAP proteins in microsomes, the affinity of TAP binding was determined for a wide variety of synthetic peptides, allowing mapping of the important residues.[305,306] Binding, rather than transport, appears to be the major step determining TAP peptide selectivity.[307] Indeed, artificial neural networks have been developed to predict peptide binding to human TAP.[308] Using this scheme, it was found that peptides eluted from three different human class I molecules had higher predicted affinities for TAP than a control set of peptides with equal binding to those class I MHC molecules, supporting the hypothesis that TAP specificity contributes to the selection of the subset of peptides able to bind a class I molecule that actually bind in vivo.[308] Unlike MHC class I, there were no anchor positions at which a specific amino acid was required. However, there were several positions where substituting the wrong amino acid caused a marked reduction in TAP binding. In a typical MHC class I binding 9-mer, the strongest substitution effects were observed at position 9 (P9), followed by substitutions at P2 and P3, followed by P1. At the carboxy-terminal P9, the preferred residues were Tyr and Phe (as well as Arg and Lys), whereas Glu was worst, causing a 3 log reduction in binding. Similarly, substituting Pro at P2 caused a 1.5 to 2 log reduction in binding, as compared to preferred residues Arg, Val, and Ile.

These binding studies were extended to 231 peptides, which were evaluated for binding to both mouse and human TAP proteins.[309] Both TAP proteins showed strong positive selection favoring certain amino acids (Tyr and Phe) at position P9 and strong negative selection against others (Glu, Asp, and Ser) at P9. However, mouse TAP showed greater negative effects from basic amino acids (Arg or Lys) at P9. Both TAPs were sensitive to Pro at P1, P2, and P3, but human TAP was also sensitive to negative effects of amino acids Asp, Glu, and Gly at these positions. In both cases, TAP binding was insensitive to amino acid substitutions

at intermediate P4 through P8.

TAP preferences such as these would selectively transport some peptides more than others from cytoplasm to ER. Use of combinatorial peptide libraries independently confirmed that the critical residues influencing TAP transport were the first three N-terminal residues and the last C-terminal residue.[310]

Interestingly, these preferred residues are many of the same ones forming the MHC class I-binding motifs (P2 and P9). However, as the MHC-binding motifs differ from each other, it is not possible for TAP preferences to match them all. For example, the TAP preference for Arg at P2 and Phe, Tyr, Leu, Arg, or Lys at P9 overlaps with the binding motif of HLA-B27 and may favor the transport of peptide ligands for this MHC type. Remarkably, the variant B*2709, which does not prefer Tyr or Phe at P9, is not associated with autoimmune disease as in the more common form of HLA-B27. In contrast, HLA-B7 requires a Pro at P2, which greatly decreases TAP binding. Similarly, some peptides binding HLA-A2 have hydrophobic residues unfavorable for TAP binding, suggesting suboptimal compatibility between TAP and the most common HLA class I allele. Measurements with a series of naturally presented peptides from HLA-A2 and HLA-B27 indicated a mean 300-fold higher affinity of TAP for the HLA-B27 peptides than for those from HLA-A2, and some of the HLA-A2 peptides did not bind TAP at all.[308] How are these low-affinity peptides delivered to MHC? One suggestion is that peptide ligands for HLA-A2 and HLA-B7 may be transported as a larger precursor peptide containing the correct amino acids, which are then trimmed off to fit the MHC groove.

A series of studies support this mechanism, showing that longer peptide precursors are trimmed at the N-terminus by aminopeptidases in the ER to form HLA-binding peptides.[311] The ability to transport larger peptides, followed by trimming, could increase the range of permissible antigenic peptides, by facilitating transport of nonantigenic peptides, followed by trimming to the size and specificity needed for MHC binding. For example, following hepatitis B virus infection, certain peptide epitopes that are frequently recognized by CTL are nevertheless not bound or transported by TAP. By extending these peptides by one or two amino acids of the natural sequence at the amino end, their TAP binding was greatly enhanced, but at the expense of reduced MHC binding. When tested on permeabilized cells, the overall effect of the extended peptides was to increase MHC binding. Apparently, by improving TAP transport, these peptides were able to increase peptide delivery into the ER, where they were trimmed to produce peptides compatible with MHC binding.[312]

Similarly, it is known that peptides with a Pro at P2, needed to bind to certain MHC molecules but poorly transported, are produced from longer precursors that are transported by N-terminal trimming in the ER by aminopeptidases.[313] In fact, the inability of the aminopeptidase to cleave beyond a residue preceding a Pro naturally leads to trimming of peptides to produce ones with a Pro at P2. Alternatively, some of these peptides may derive from signal peptides and enter

the ER in a TAP-independent manner. As HLA-A2 tends to bind hydrophobic peptides, signal peptides, which are usually hydrophobic, may account for some HLA-A2-binding epitopes. The ER trimming enzyme has been identified as endoplasmic reticulum aminopeptidase I (ERAP I).[314,315,316] This enzyme is a 106 kD zinc metalloproteinase, and it is the major ER protease with broad specificity. It is inducible by interferon-γ and inhibited by the aminopeptidase inhibitor leucinethiol. Downregulation of ERAP I with small interfering ribonucleic acid in decreased expression of MHC class I, indicating that ERAP I contributes to the supply of peptides. ERAP I prefers peptides of 9 or 10 amino acids or longer and ignores peptides of 8 amino acids or fewer, which may allow it to generate antigenic peptides for

MHC binding without degrading them beyond recognition.

The aminopeptidase provides a way to relax the requirement for proteasomes to generate ends that can simultaneously bind TAP and MHC. In contrast, the fact that a comparable carboxypeptidase has not been identified suggests that carboxyl ends generated by proteasomal processing must be suitable for TAP transport and compatible with MHC binding. This requirement is quite stringent, as the carboxyl-terminal residue is the most important for TAP binding, and it is frequently an anchor residue for MHC as well.

The significance of selective peptide transport may be to limit immunity to self-peptides. If the match between HLA-B27 and TAP specificity is too good, it may contribute to the increased incidence of autoimmune disease associated with HLA-B27.[305] An effect of human TAP specificity in loading of peptides in viral infection has confirmed the biologic significance of TAP specificity.[312] TAP-binding specificity also limited the repertoire of alloantigenic peptides presented by HLA-B27.[317] Tapasin, which is involved in the TAP-binding process, may also contribute to selective MHC loading contributing to immunodominance by favoring binding of peptides with slow dissociation rates from MHC molecules.[318]

By combining the selectivity of proteasomal processing, TAP transport and MHC binding, it has been possible to generate models that correlate with known antigenic peptides.[319,320] These can be used to analyze the sequence of any given protein and to predict epitopes that may be recognized by T cells restricted to MHC class I. These may be important for analyzing the T-cell response to viral or neoplastic antigens and for generating synthetic vaccines capable of eliciting a T-cell response to these antigens. The importance of TAP proteins to antiviral immunity is shown by the fact that herpesviruses have targeted TAP-1 function as a way to interfere with antigen presentation to $CD8^+$ CTL. A herpes simplex virus immediate early viral protein called ICP47 binds to TAP and inhibits its function, causing reduced expression of new MHC class I molecules on the cell surface and inability to present viral or other antigens with MHC class I.[321,322,323] As a way to evade immune surveillance, this strategy could contribute to viral persistence in chronic infection and viral activation in recurrent disease as frequently occurs with herpes simplex virus-1 and herpes simplex virus-2. These findings also raise the possibility of making a live-attenuated ICP47-defective herpes simplex virus vaccine that would be more immunogenic than natural infection.

Transport Pathways Leading to Major Histocompatibility Complex Class II Presentation. Unlike the class I pathway, which delivers peptides to MHC, the MHC class II pathway transports MHC molecules to the endosomal compartment, where antigenic peptides are produced. During transport, the peptide-binding groove must be kept free of endogenous peptides. The cell uses one protein, called invariant chain (and its processed fragment CLIP), to block the binding site until needed, and another protein, HLA-DM, to facilitate release of CLIP peptides and their exchange for antigenic peptides as they become available.

MHC class II molecules assemble in the ER, where α and β chains form a complex with invariant chain.[324,325,326] Invariant chain binds MHC and blocks the peptide-binding groove, so endogenous peptides transported into the ER, for example by TAP, cannot bind.[325,327,328,329,330,331,332] The complex of α, β, and invariant chains, consisting of nine polypeptide chains in all,[333] is transported via the Golgi and directed by signals on invariant chain into endosome-/lysosome-like vesicles called MHC class II compartments. The compartments contain acid-activated proteases capable of digesting foreign proteins into antigenic peptides. In addition, they degrade invariant chain to a fragment called CLIP, corresponding to amino acids 80 to 103. As long as CLIP remains in the binding groove,

antigenic peptides cannot bind, so the rate of CLIP release limits the capacity of MHC to take up antigenic peptides.

Peptide loading can be measured by its effect on MHC structure. When an MHC class II molecule binds a peptide, it changes conformation, and certain monoclonal antibodies are specific for the peptide-bound conformation.[334] Also, the α-β complex becomes more stable after peptide binding, which can be detected by running the MHC on an SDS gel without boiling. The peptide bound form runs on gels as a large α-β dimer while MHC without peptides (but still bound to CLIP) is unstable under these conditions and falls apart to give α and β chain monomers on SDS gels.[335]

Mutant cell lines have been generated with a deletion between HLA-DP and HLA-DQ genes on chromosome 6.[334,336,337,338] These cells express normal levels of MHC class II structural proteins, HLA-DQ and HLA-DR, but fail to present protein antigens.[338] Some of their class II MHC proteins appear on the cell surface but more are retained in the MHC class II compartments. Biochemically, they still contain CLIP peptides,[339] rather than peptide antigens, and they have not achieved the conformation[334] or SDS stability of peptide-binding MHC class II complexes.[340] The defect was discovered to be due to loss of either of the two chains of a class II molecule, HLA-DM, and the phenotype can be corrected by adding back the missing gene.[341] In the presence of normal HLA-DM, MHC releases CLIP and binds antigenic peptides for presentation to T cells.

The importance of HLA-DM function for T-cell help in vivo was studied in H2-DM knockout mice.[342] These mice have reduced numbers of T cells, their class II MHC molecules reach the cell surface bearing high levels of CLIP peptide, and their B cells are unable to present certain antigens, such as ovalbumin, to T cells. When H2-DM knockout

mice were immunized with 4-hydroxy-5-nitrophenyl acetyl ovalbumin, specific IgG antibodies were reduced 20-fold, as compared to wild type. Germinal center formation and class switching were greatly reduced, and affinity maturation was not observed. The phenotype was more pronounced for some MHC types, such as I-A^b, than for others, such as I-A^k. Due to tighter binding of CLIP peptides, these MHC types may be more dependent on H2-DM to maintain empty class II molecules in a peptide receptive state.

In vitro studies with purified MHC class II molecules and biotin-labeled peptides have shown that HLA-DM can accelerate loading of exogenous peptides into HLA-DR-binding sites.[343,344] For example, loading of myelin basic protein fragment 90 to 102 was accomplished in 9 minutes with HLA-DM versus 60 minutes without it (Table 23.5). Other peptides were also loaded at the same rate, suggesting that the rate limiting step was the same for each: removal of CLIP peptides to expose the peptide binding sites on HLA-DR. The kinetic effect was optimal between pH 4.5 and 5.8, which is typical of the endosomal/lysosomal compartment where HLA-DM operates. HLA-DM did not affect the affinity, as measured by half-maximal binding, but it had a marked effect on the kinetics of binding.

Conversely, when biotinylated peptides were allowed to saturate HLA-DR-binding sites overnight, and then free peptides were removed, the off rate could be measured over time.[343,344] As shown in Table 23.5 (adapted from Sloan et al.[343]), the off rate for different peptides could be compared in the absence or presence of HLA-DM. The half-life for CLIP peptides was reduced from 11 hours to 20 minutes by adding HLA-DM. This could explain the enhanced loading of all other peptides, as they must wait for CLIP to come off. In the case of

antigenic peptides, myelin basic protein 90 to 112 was released 80-fold faster in the presence of DM than in its absence. However, another peptide, influenza hemagglutinin 307 to 319, was not affected at all. The differential effect on these antigenic peptides suggests that HLA-DM can serve a potential role in editing which peptides stay on MHC long enough to be presented and which are removed.[343] By releasing myelin basic protein preferentially and not the hemagglutinin peptide, HLA-DM would favor the stable MHC binding and presentation of hemagglutinin peptides over myelin basic protein peptides. The affinity of each peptide is determined by the fit between peptide and MHC groove, not by HLA-DM. However, DM can amplify the impact of the difference in affinity (ie, signal to noise ratio) by facilitating release of low-affinity peptides and allowing the high-affinity ones to remain. This editing function could have an important effect on which peptides get presented and elicit a T-cell response. HLA-DM could contribute to immunodominance of a peptide binding MHC with high affinity by releasing its lower affinity competitors. Alternatively, HLA-DM could contribute to self-tolerance by releasing self-peptides of low affinity before they could stimulate self-reactive T cells either at the time of positive selection (so they would fail to be positively selected) or in the periphery (so they would fail to be activated). When HLA-DM was engineered to be expressed on the cell surface, it also facilitated loading of exogenous peptides onto class II MHC molecules and affected the activation of T cells of different fine specificity for the same epitope.[345]

TABLE 23.5 Effect of Human Leukocyte Antigen-DM on Peptide on Rates and Off Rates for Binding to Human Leukocyte Antigen-DR1

Peptide	HLA-DM	Half-Time for Binding	Half-life for Release
CLIP (80-103)	-	60 min	11 hr
	+	9 min	0.3 hr
MBP (90-102)	-	62 min	86 hr
	+	9 min	1 hr
HA (307-319)	-	67 min	144 hr
	+	10 min	144 hr

CLIP, class II-associated invariant chain peptide; HA, hemagglutinin; HLA, human leukocyte antigen; MBP, myelin basic protein.

The on (association) and off (dissociation) rates of biotinylated peptide from purified soluble HLA-DR1 were measured by fluorescence assay, in the presence or absence of HLA-DM. The on rates of all three peptides are increased similarly in the presence of HLA-DM and probably reflect the rate-limiting dissociation of the bound CLIP fragment of the invariant chain. In contrast, once the peptides are bound, the off rates differ as a result of differences in affinity. Thus, HLA-DM catalyzes release of more weakly binding peptides and allows stable binding of higher affinity peptides.

In effect, this is an editing function of HLA-DM.

Adapted from the data of Sloan et al.[298]

Major Histocompatibility Complex Binding and Assembly of a Stable Peptide-Major Histocompatibility Complex

Antigen Interaction with Major Histocompatibility Complex Molecules.

Perhaps the most selective step a potential antigenic site must pass is to bind with sufficiently high affinity to an appropriate MHC molecule.

The response specificity of T cells to antigens on APCs or target cells provides a number of hints that antigen interacts directly with MHC molecules of the APCs to form an antigenic complex recognized by T cells. First, genes coding for immune responsiveness (Ir genes) to a specific antigen are tightly linked to genes for MHC-encoded cell surface molecules.[127,171] Second, it became apparent that T-cell recognition of antigen is the step at which MHC restriction occurs.[127,179,219,346] For example, in vitro T-cell responses to small protein and polypeptide antigens were found to parallel in vivo responses controlled by Ir genes, and T cells were exquisitely sensitive to differences in MHC antigens of the APCs in all their antigen recognition functions. This observation in vitro made it possible to separate the MHC of the T cell from that of the APC. The T-cell response to antigenic determinants on each chain of insulin depended on the MHC antigens of the APC. This was particularly apparent when T cells from an (A × B)F1 animal responded to antigen presented by APCs of either the A or B parental MHC type.[219,347] Neither parental APC stimulated an allogeneic response from (A × B)F1 T cells, and the response to antigen was now limited by the MHC of the APCs. This ability of the APCs to limit what could be presented to the T cells was termed "determinant selection."[219,347] It became obvious that even in a single (A × B)F1 animal, distinct sets of antigen-specific T cells exist that respond to each antigenic determinant only in association with MHC type A or B.[348]

Experiments on the fine specificity of antigen-specific T-cell clones suggested that the MHC of the APCs could influence the T-cell response in more subtle ways than just allowing or inhibiting it. Determinant selection implied that a given processed peptide should contain both a site for MHC interaction and a distinct functional site for TCR binding. Thus, a protein with multiple determinants could be processed into different peptides, each with a different MHC restriction, consistent with the independent Ir gene control of the response to each antigenic determinant on the same protein.[181] For example, T-cell clones specific for myoglobin responded to different antigenic determinants on different peptide fragments of myoglobin[215]: Those specific for one of the epitopes were always restricted to I-A, whereas those specific for the other were always restricted to I-E. The simplest interpretation was that each antigenic peptide contained an MHC association site for interacting with I-A or I-E. At the level of Ir genes, mouse strains lacking a functional I-E molecule could respond to one of the sites only, and those with neither I-A nor I-E molecules capable of binding to any myoglobin peptide would be low responders to myoglobin.

Evidence for a discrete MHC association site on peptide antigens came from studies with pigeon cytochrome c. The murine T-cell response to pigeon cytochrome c and its carboxy-terminal peptide (81 to 104) depends on the I-E molecules of the APCs.[179] However, distinct

structural sites on the synthetic peptide antigen appear to constitute two functional sites: an epitope site for binding to the TCR and an “agretope” (for “antigen restriction tope”) site for interacting with the MHC molecule of the APC.[179,196,197,198] Amino acid substitutions for Lys at position 99 on the peptide destroyed the ability to stimulate T-cell clones specific for the peptide, whereas the difference between Ala and a deletion at position 103 determined T-cell stimulation in association with some MHC antigens but not others, independent of the T-cell fine specificity. In addition, immunizing with the peptides substituted at position 99 elicited new T-cell clones that responded to the substituted peptide but not the original and showed the same pattern of genetic restriction, correlated with the residue at position 103, as the clones specific for the original peptide. These results implied that the substitutions at position 99 had not affected the MHC association site but independently altered the epitope site that interacts directly with the TCR. In contrast, position 103 was a likely subsite for MHC interaction, without altering the TCR-binding site.

It remained to be shown that MHC molecules without any other cell surface protein were sufficient for presentation of processed peptide antigens. This was demonstrated by Watts et al.,[349] who showed that glass slides coated with lipid containing purified I-A molecules could present an ovalbumin peptide to an ovalbumin-specific T-cell hybridoma. This result meant that no other special steps were required other than antigen processing and MHC association. Likewise, Walden et al.[350] specifically stimulated T-cell hybridomas with liposomes containing nothing but antigen and MHC molecules. Also, Norcross et al.[351] transformed mouse L cells with the genes for the I-A α and β chains and converted the fibroblasts (which do not express their own class II molecules) into I-A-expressing cells. These cells were able to present several antigens to I-A-restricted T-cell clones and hybridomas[351] and similar I-E transfectants presented to I-E-restricted T cells.[215] Thus, whatever processing enzymes are required are already present in fibroblasts, and the only additional requirement for antigen-presenting function is the expression of I-A or I-E antigens.

The planar membrane technique has been applied to determine the minimum number of MHC-antigen complexes per APC necessary to induce T-cell activation.[352] After pulsing the presenting cells with antigen, the cells were studied for antigen-presenting activity, and some of the cells were lysed to produce a purified fraction containing MHC charged with antigenic peptides. These MHC-peptide complexes were used to reconstitute planar membranes, and their potency was compared to a reference MHC preparation pulsed with a high peptide concentration in vitro and presumed to be fully loaded. In this way, the relative peptide occupancy of MHC-binding sites corresponding to any level of antigen presentation could be determined. For B cells and macrophages, the threshold of antigen loading necessary for triggering T cells was 0.2% of I-E^d molecules occupied by peptide, corresponding to about 200 MHC-peptide complexes per presenting cell. For artificial presenting cells, such as L cells transfected with I-E^d, the threshold was 23 times greater or 4.6% of MHC occupied by peptide. Similarly, when MHC-peptide binding was measured directly, using radiolabeled peptide to determine the minimum level of MHC-peptide complexes required for T-cell triggering, B cells were capable of presenting antigen with as few as 200 to 300 MHC-peptide complexes per cell.[353] A similar number of peptide-MHC class I molecule complexes was reported to be required on a cell for recognition by $CD8^+$ cytotoxic T cells.[354] These results explain how newly generated peptide antigens can bind enough MHC molecules to stimulate a T-cell response, even in the presence of competing cellular antigens, as a low level of MHC occupancy is sufficient. In addition, this threshold of presentation may explain how multivalent protein antigens, such as viral particles, with 100 to 200 protein copies each,

can be over 10^3-fold more immunogenic than the same weight of protein monomers.[355,356,357] Studies on the number of TCRs needed for triggering, based on titrating peptide and recombinant soluble class I MHC molecules on plastic, suggested that interaction of three to five TCRs with MHC-peptide complexes was sufficient, consistent with several T cells interacting with one APC.[358,359] Biochemical evidence for the direct association between processed peptide and MHC molecules was demonstrated by competition between peptides for antigen presentation[225,360,361,362,363] and then more directly by equilibrium dialysis,[364] molecular sieve chromatography,[365] or affinity labeling.[366] Equilibrium dialysis (see Chapter 7) was performed by incubating detergent-solubilized class II molecules with fluoresceinated or radioactive antigenic peptides, followed by dialysis against a large volume of buffer. Peptide can pass in or out of the dialysis bag, but the class II molecules are trapped inside. In the absence of binding by class II molecules, the labeled peptide would distribute itself equally between the inside and outside of the dialysis

chamber. However, when the appropriate class II molecules were added to the chamber, extra peptide molecules were retained inside it due to formation of a complex with MHC class II. In this way, direct binding of antigen and MHC was shown, and an affinity constant was determined.[364,365]

TABLE 23.6 Correlation between Major Histocompatibility Complex Restriction and Binding to Major Histocompatibility Complex Molecules

Competitor Peptide		Ova + A^d	Myo + E^d	HEL + A^k	Cyto + E^k
Ova	323-339	++++	−	++	+
Myo	106-118	++++	−	++	+/-
Myo	132-153	−	++++	−	++
HEL	46-61	+	+	++++	+
Cytochrome c	88-104	++	+/-	++	++++

HEL, hen egg lysozyme.

Data from Früh et al.322

A second approach was to form the antigen-MHC complex over 48 hours, followed by rapid passage over a Sephadex G50 sizing column (Sigma-Aldrich). The bound peptide came off the column early because it is the size of class II molecules (about 58 kD), while free peptide was usually included in the column and eluted later, as it is only approximately 2 kD.[365] Peptide bound to specific and saturable sites on MHC. Competitive binding showed that different peptide antigens with the same MHC restriction bind to the same site on the MHC class II molecule.[367,368] For example, Table 23.6 shows the results with peptide antigens

that are known to be presented with I-A or I-E antigens of the D or K haplotype. We observe that Ova peptide 323 to 329, which is presented with I-A^d, also binds well to purified I-A^d, whereas nonradioactive peptide competes for the peptide-binding sites of the I-A^d molecule. Similarly, the other I-A^d-restricted peptide, myoglobin 106 to 118, competes with Ova 323 to 339 for the same site. However, myoglobin 132 to 153, which is not restricted to I-A^d, does not compete for it but does compete for its own restriction element, I-E^d. Similarly, pigeon cytochrome c competes best for its restriction element I-E^k rather than I-A^k or I-E^d, which do not present cytochrome. Conversely, recombinant Eβ genes have been used to map separate sites on a class II MHC molecule for binding to peptide antigen and to the TCR.[369]

Using these two biochemical methods, it has been possible to explain major losses of peptide antigenicity resulting from amino acid substitutions in terms of their adverse effect on epitope or agretope function. For example, the response of each of two ovalbumin-specific T-cell clones was mapped to peptide 325 to 335 by using a nested set of synthetic peptides. Five substitutions were made for each amino acid in the peptide, and the resulting 55 different peptides were each tested for the ability to stimulate the clone.[370] Presumably, those peptides that failed to stimulate could be defective at an epitope or an agretope functional site. In fact, only two amino acids (Val 327 and Ala 332) were essential for MHC interaction, and changes at either of these resulted in a loss of antigenicity for the clone. Seven other amino acids were critical for T-cell stimulation but did not affect MHC binding. Thus, these must be part of the functional epitope. Interestingly, certain substitutions for His 328, Ala 330, and Glu 333 had effects on MHC binding, whereas others had effects on T-cell stimulation without affecting MHC binding. These amino acids might participate in both agretope and epitope functional sites, or, alternatively, the substitutions may affect the conformation of the peptide as it binds, thus indirectly affecting T-cell recognition[371] (see following discussion). The fact that substitutions at 9 of 11 amino acids could be tolerated without affecting MHC binding is consistent with the determinant selection hypothesis in that multiple antigenic peptides are capable of interacting with the same antigen-binding site on the MHC molecule.

Similarly, by using a T-cell clone specific for peptide 52 to 61 of hen egg lysozyme, substitutions at each amino acid were analyzed for the ability to bind to I-A^k and stimulate the clone.[372] Four of eleven amino acid residues were silent, whereas substitutions at three positions resulted in reduced binding to I-A^k. Substitutions at the remaining three positions resulted in decreased T-cell stimulation without affecting MHC association. The epitope was very sensitive to substitutions, even conservative ones such as changing Leu 56 to Ile, norLeu, or Val. The results in both of these studies confirmed by competitive binding that the MHC molecule contains a single saturable site for peptide binding. This site must be capable of binding a broad range of antigenic peptides. In binding the MHC groove, antigenic peptides assume the extended conformation that exposes the epitope for recognition by the TCR.

Although a full set of general principles explaining the specificity of antigen presentation and T-cell recognition has not yet emerged, it is studies such as these, combined with complementary structural studies characterizing the antigen-interacting portions of MHC molecules[214,253,254,369,373,374,375,376,377,378,379,380] (see Chapters 21 and 22) and of TCRs[381,382,383,384,385,386,387] (see Chapter 11) that will ultimately lead to an understanding of these principles.

One observation that came out of this type of structurefunction study was that a single peptide can bind to a class II MHC molecule in more than one way, and thus be seen by

different T cells in different orientations or conformations.[371,388] The same conclusion can be reached from an entirely different type of study, in which mutations are introduced into the MHC molecule. Mutations in the floor of the peptide-binding groove, which cannot directly interact with the TCR, can differentially affect recognition of a peptide by one clone and not another.[389,390,391] In a particularly thoroughly studied case, it was clear that the quantitative level of peptide binding was not affected by the mutation, but rather the change in the floor of the groove imposed an altered conformation on the peptide that differentially affected recognition by different T cells.[391] If indeed the TCR cannot detect the mutation in the MHC molecule except indirectly by its effect on the peptide conformation, then

one is forced to conclude that different T cells have preferences for different conformations of the same peptide bound to (what appears to the T cell as) the same MHC molecule.

Another general observation to come from this type of study is that substitution of amino acids often affects presentation by MHC and recognition by T cells through introduction of dominant negative interactions or interfering groups, whereas only a few residues are actually essential for peptide binding.[392] Both for class II binding[392,393,394,395] and for class I MHC binding,[396] most residues can be replaced with Ala or sometimes Pro without losing MHC binding, as long as a few critical residues are retained. Of course, T-cell recognition may require retention of other residues. If many of the amino acid side chains are not necessary for binding to the MHC molecule, then one might expect side chains of noncritical amino acids to occasionally interfere with binding, either directly or through an effect on conformation. That is exactly what was observed for a helper epitope from the HIV-1 envelope protein when a heteroclitic peptide— that is, one that stimulated the T cells at much lower concentrations than did the wild-type peptide—was obtained by replacing a negatively charged Glu with Ala or with Gln, which has the same size but no charge.[392] An Asp, negatively charged but smaller, behaved like the Glu. Thus, this residue was not necessary for binding to the class II MHC molecule, but a negatively charged side chain interfered with binding to the MHC molecule as measured by competition studies. Information about residues that interfere with binding has allowed the refinement of sequence motifs for peptides binding to MHC molecules to permit more reliable prediction of binding[397] (see following discussion).

This observation also provides a novel approach to make more potent vaccines by “epitope enhancement,” the process of modifying the internal sequence of epitopes to make them more potent, for example, by increasing affinity for an MHC molecule or TCR, or able to induce more broadly cross-reactive T cells specific for multiple strains of a virus.[398,399,400,401] Proof of principle that this approach can make more potent peptide vaccines has recently been obtained.[401] The modified “enhanced” helper T-cell epitope from the HIV-1 envelope protein described previously,[392] with Ala substituted for Glu, was shown to be immunogenic at 10-to 100-fold lower doses for in vivo immunization than the wild-type HIV-1 peptide to induce a T-cell proliferative response specific for the wild-type peptide. Further, when a peptide vaccine construct using this helper epitope coupled to a CTL epitope[402,403] was modified with the same Glu-to-Ala substitution, it was more potent at inducing $CD8^+$ CTL specific for the CTL epitope than was the original vaccine construct, even though the CTL epitope was unchanged (Fig. 23.10).[401] The increased potency of the vaccine construct was shown to be due to improved class II MHC-restricted help by genetic mapping using congenic strains of mice expressing the same class I MHC molecule to present the CTL epitope and the same background genes but differing in class II MHC

molecules.[401] Thus, class II-restricted help makes an enormous difference in induction of class I-restricted CTL, and epitope enhancement can allow construction of more potent vaccines, providing greater protection against viral infection.[404] Further, the improved help was found to be qualitatively, not just quantitatively, different, skewed more toward Th1 cytokines.[404] The mechanism was found to involve greater induction of CD40 ligand on the helper T cells, resulting in greater interleukin 12 production by the antigen-presenting dendritic cells, which in turn polarized the helper cells toward Th1 phenotype.[404] The dendritic cells conditioned with the helper T cells and the higher affinity peptide and then purified were also more effective at activating CD8+ CTL precursors in the absence of helper cells, supporting a mechanism of help mediated

through activation of dendritic cells.[405,406,407] This study showed also that such help was mediated primarily through upregulation of interleukin 12 production and CD80 and CD86 expression on the dendritic cell.[404] A similar or overlapping HIV envelope epitope recognized by human CD4+ T cells when presented by HLA-DR13 has been mapped and has undergone epitope enhancement as well.[408] Understanding this mechanism of epitope enhancement may contribute to the design of improved vaccines.

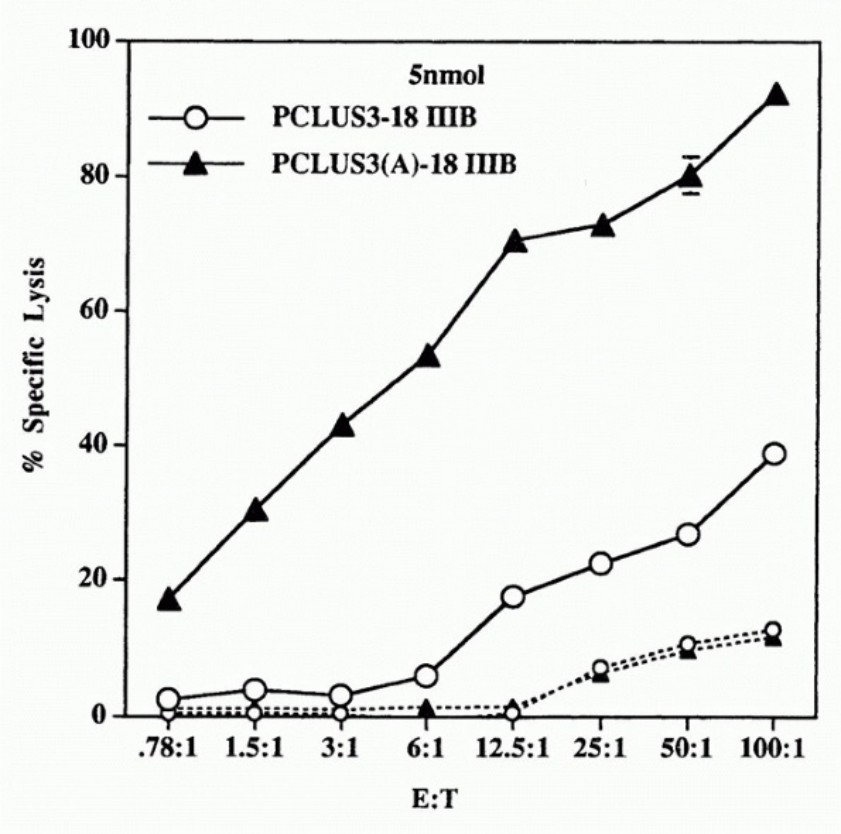


FIG. 23.10. Enhancement of Immunogenicity of a Peptide Vaccine for Induction of Class I Major Histocompatibility Complex (MHC)-Restricted Cytotoxic T-Lymphocytes by Modification of the Class II MHC-Binding Portion to Increase Cluster of Differentiation (CD)4+ T-Cell Help. Peptide vaccine PCLUS3-18 IIIB contains a class II MHC binding helper region, consisting of a cluster of overlapping determinants from the human immunodeficiency virsu-1 envelope protein gp160, and a class I MHC-binding cytolytic T cell (CTL) epitope, P18 IIIB. Modification of the helper

epitope to remove an adverse negative charge by replacement of a Glu with an Ala residue was shown to increase binding to the class II MHC molecule.[392] Here, introduction of the same modification of the helper epitope, to produce PCLUS3(A)-18IIIB, is shown to greatly increase immunogenicity in vivo for induction of CTL to the class I-binding P18IIIB portion. Immunization of A.AL mice with 5 nmol of either vaccine construct subcutaneously in montanide ISA 51 adjuvant, and stimulation of resulting spleen cells with P18IIIB for 1 week in culture, resulted in 33-fold more lytic units for lysing targets coated with P18IIIB when mice were immunized with the modified second-generation vaccine than when they were immunized with the original construct with the natural sequence. Thus, class II-restricted $CD4^+$ T-cell help has a major impact on induction of class I-restricted CTL, and this process of "epitope enhancement" can be used to make vaccines more potent than the natural viral antigens. Targets: BALB/c 3T3 fibroblasts with P18IIIB (*solid lines*) or no peptide (*dashed lines*). (Modified from Ahlers et al.[401] with permission.)

Similar epitope enhancement has been carried out for class I MHC-binding viral or tumor peptides as well,[409,410,411,412,413,414] and in one case has been found to result in greater clinical efficacy of a melanoma vaccine used for human immunotherapy.[415] Cautions have also been raised about the use of enhanced epitopes (sometimes also called agonistic or heteroclitic epitopes), in that the amino acid sequence modifications that affect MHC binding can also alter the conformation of the MHC-bound peptide and affect the specificity of T cells elicited.[416,417,418,419] The altered or enhanced peptides that elicit the most T cells specific for the wild-type epitope are the most effective at clearing tumors.[420] Thus, it is important in using epitope-enhanced peptides that the peptides still activate a large number of T cells that recognize the wild-type sequence. These results emphasize the importance of affinity for MHC molecules vaccine efficacy[421] and suggest that rational design of vaccines with higher affinity epitopes may produce more effective second-generation vaccines.[422,423] To that end, the discovery of sequence motifs predicting MHC molecule binding and the development of bioinformatics strategies to predict peptide affinity have proven a great impetus to the field.[424]

TABLE 23.7 Examples of Motifs for Peptides Binding to Class I and II Major Histocompatibility Complex Molecules

	Residue Number								
MHC Molecule	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Class I									
$H\text{-}2K^d$		Y							I, L, V
$H\text{-}2D^b$					N				M, I
								L,	

H-2K^b				F, Y			M
H-2L^d		P		(hydrophilic K, R)			M, L, F
H-2D^d		G	P	K, R			L
H-2K^k		E					I
HLA-A2.1		L, M					V
HLA-A3		L	(F)				Y, K
HLA-B27	K, R G	R	I, Y, F, W				K, R
Class II							
DRB1*0101	Y, V, L, F, I, A		L, A		A, G		L, A
DRB1*0301	L, I F, M V		D		K, R E, Q		Y, L F
DRB1*0401 (DR4Dw4)	F, Y W		no R, K		N, S T, Q Aliphatic	Polar Charged	Polar Aliphatic K

DRB1*0402 (DR4Dw10)	V, I, L, M	no	D, E	N, Q, S, T K	R, K H, N Q, P	Polar Aliphatic H
DRB1*1501 (DR2b)	L, V, I		F, Y I		I, L V, M F	
DQA1*0501	F, Y,		V, L		Y, F	
DQB1*0301	I, M L, V		I, M Y		M, L, V, I	

HLA, human leukocyte antigen; MHC, major histocompatibility complex.

Data from Goodman and Sercarz,234 Walden et al.,350 Sette et al.,370 Kurata and Berzofsky,371 Allen et al.,372 Brown et al.,373 and Bjorkman et al.374

In the case of class I MHC molecules, results defining sequence binding motifs generalize the conclusion that only a few critical “anchor” residues determine the specificity of binding to the MHC molecule (Table 23.7).[264,396,425,426,427,428,429] These motifs were defined by a detailed study of one peptide-MHC system[427] by sequencing the mixture of natural peptides eluted from a class I MHC molecule and finding that at

certain positions in the sequence, a single residue predominated within the pool of peptides,[425] and by separating and sequencing individual natural peptides eluted from a class I molecule and finding a conserved residue at certain positions.[426] The latter two studies also made the important observation that the natural peptides eluted from class I MHC molecules were all about the same length, eight or nine residues, and this was confirmed for a much larger collection of peptides eluted from HLA-A2 and analyzed by tandem mass spectrometry.[428] This finding was consistent with other studies demonstrating that a minimal nonapeptide was many orders of magnitude more potent than longer peptides in presentation by class I molecules to T cells.[430,431] This conservation of length was critical to the success of the approach of sequencing mixtures of peptides eluted from a class I molecule[425] because such a method requires that the conserved anchor residues all be at

the same distance from the N-terminus. The fact that Falk et al.[425] could find a single amino acid at certain positions, such as a Tyr at position 2 in peptides eluted from K^d, implies not only that most or all of the peptides bound to K^d had a Tyr that could be aligned but also that the peptides were already aligned as bound to the MHC molecule, with each one having just one residue N-terminal to the Tyr. This result implies that the position of the N-terminal residue is fixed in the MHC molecule. It is this fact that has made the identification of motifs for binding to class I molecules much more straightforward than finding motifs for binding class II molecules.

This conclusion has not only been confirmed but also explained by x-ray crystallographic data on class I peptide-MHC complexes.[376,377,378] It appears that both the N-terminal α-amino group and the C-terminal carboxyl group are fixed in pockets at either end of the MHC groove, independent of what amino acids are occupying those positions, and that the rest of the peptide spans these fixed points in a more or less extended conformation. The minimum length that can span the distance between these pockets is 8 residues, but 9 or 10 residues can be accommodated with a slight bulge or β turn in the middle of the peptide, explaining the narrow restriction on length. Between these ends, one or two pockets in the groove can accommodate the side chain of an amino acid, usually either at P2 binding in the B pocket or at P5 binding in the C pocket, depending on the particular MHC molecule. Additionally, the side chain of the C-terminal residue serves as an anchor in the F pocket at the end of the groove. These residues that fit into pockets correspond exactly to the “anchor” residues, at P2 or P5, and P8, P9, or P10, defined by the sequence motifs, and appear to be the primary determinants of specificity for peptide binding because the rest of the interactions are largely with peptide backbone atoms, including the α-amino and carboxyl groups, and therefore do not contribute to sequence specificity. This finding can explain both the breadth of peptides that can bind to a single MHC molecule because most of the binding involves only backbone atoms common to all peptides and also the exquisite specificity of binding is determined by the anchor residues that account for the Ir gene control of responsiveness.

In contrast, when natural self-peptides were eluted from class II MHC molecules,[252,432] the lengths were much more variable, ranging from 13 to 18 residues, and several variants of the same peptide were found with different lengths of extra sequence at one end or the other (“ragged ends”). This finding suggested that both ends of the peptide-binding groove of class II MHC molecules are open, in contrast to class I, so that additional lengths of peptide can hang out either side, and trimming does not have to be precise. However, a corollary is that the peptides eluted from class II molecules would not be aligned in a motif starting from the exact amino terminus and that was indeed what was found. Although a moderately conserved motif was found in some of the peptides eluted from the murine class II molecule I-A^d, consistent with the motif defined based on known antigenic peptides binding to I-A^d,[433] the motif was neither so clearly defined nor so highly conserved as in the class I case and required aligning of sequences to identify a core motif of about nine amino acid residues.[432] Subsequently, a number of motifs for peptides binding to human class II MHC molecules have been defined.[264,434,435,436,437,438,439] Unlike peptides eluted from MHC class I grooves, these class II-binding peptides may locate the core-binding motif at various distances from the amino or carboxyl end of the peptide.

The crystal structure of a peptide bound to a human class II MHC molecule, DR1, revealed that indeed the ends of the groove are open, and the peptide can extend beyond the groove in either direction.[254,440] In addition, the more broadly defined class II motifs in Table 23.7 can be explained by less stringent requirements for amino acid side chains to interact with

binding pockets in class II. In general, the MHC class II binding pockets are shallower than for class I, and a selected peptide derives less binding energy from each pocket. In fact, they form fewer H-bonds with the peptide side chains, and more H-bonds are directed at the peptide backbone, allowing a variety of different peptides to bind. Rather than requiring a specific amino acid at each position, the shallow binding pockets of MHC class II tend to exclude peptides based on unfavorable interactions, such as side chains too large to fit the binding pocket. Even one amino acid side chain that binds strongly to an MHC pocket is sufficient to anchor the peptide to MHC class II and set the frame for the interaction of the rest of the peptide with the MHC groove.

For example, binding of three peptides to the class II molecules I-A in mice or HLA-DQ in humans are shown in Figure 23.11. The first residue of the peptide motif is designated P1, the next is P2, and so on. The α-helical walls and β-sheet floor of the MHC class II groove (see Chapter 21) are peeled away to reveal the peptide backbone and side chains in relation to MHC-binding pockets. For the ovalbumin peptide $Ova^{323,324,325,326,327,328,329}$ binding to $I\text{-}A^{d}$, residues P1, P4, and P9 all point down into the binding pockets.[441] The best fit is between Val 327 and the P4 pocket, which creates mainly hydrophobic interactions with MHC and serves as the anchor residue. Residues P5 (His 328) and P8 (His 331) project upward for binding to the TCR. The shallow P4 pocket can tolerate only small hydrophobic side chains, such as Val, so it dictates which peptides can bind here. The other MHC

pockets, P1 and P9, accommodate many different residues, so they have little effect on which peptides can be presented by $I\text{-}A^{d}$.

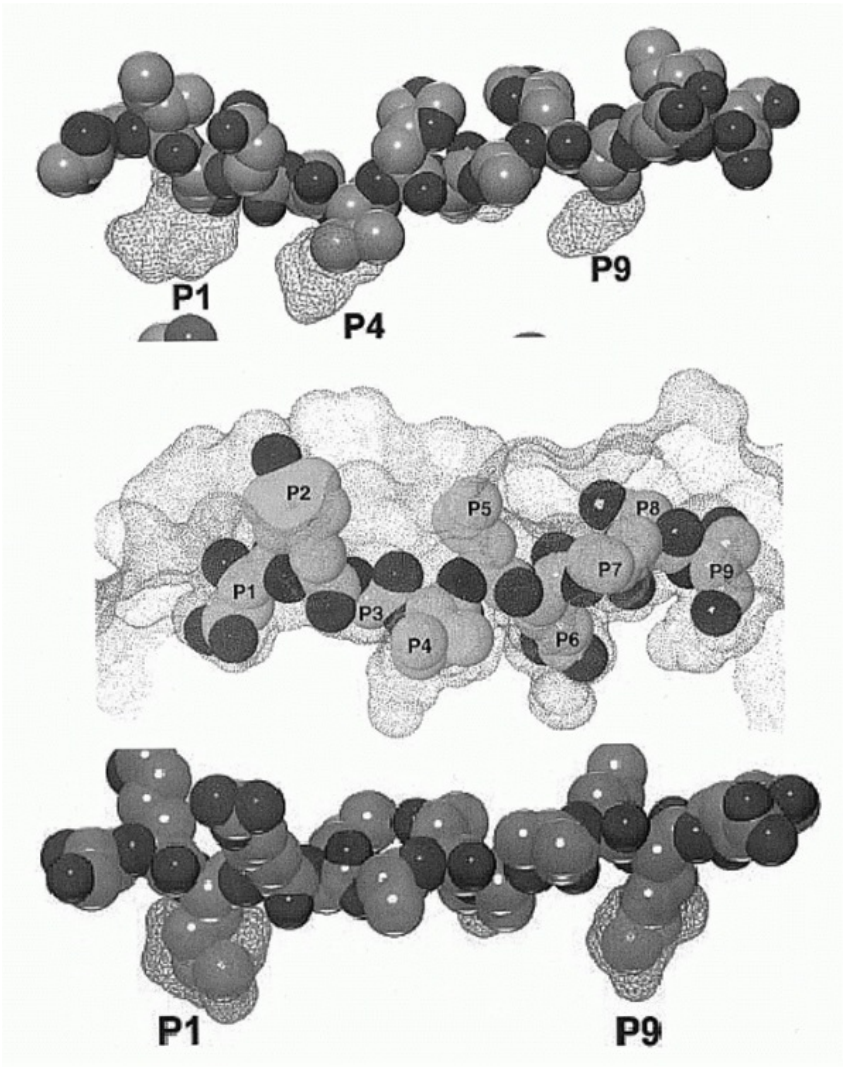


FIG. 23.11. Interaction of Peptides with the Binding Groove of Major Histocompatibility Complex Class II, as Determined by X-ray Crystallography. Anchor residue side chains fill binding pockets to a greater or lesser extent, supplemented by H-bonds to the peptide backbone. Examples include I-A^d with ovalbumin peptide 323 to 334 *(Top)*,[441] I-A^k with hen egg lysozyme 52 to 60 *(Middle)*,[442] and HLA-DR3 with invariant chain class II-associated invariant chain peptide *(Bottom)*.[443]

For hen egg lysozyme peptide[50,51,52,53,54,55,56,57,58,59,60,61,62] binding to I-A^k, interactions with MHC are observed for P1, P4, P6, and P9.[442] The P1 interaction is very different from I-A^d, as the P1 pocket is a perfect fit for Asp and has an arginine at the end of the tunnel to neutralize charge. This structure explains why nearly all peptides presented by I-A^k must have Asp at this position. In contrast, the P4 and P9 pockets are partially filled and will tolerate a number of different side chains at these positions. The P6 pocket requires a Glu or Gln here, even though the MHC residues deep in the pocket are acidic. It is presumed that one of the Glu residues must be protonated to allow Glu binding at this position. This arrangement of the peptide leaves P2, P5, and P8 exposed to solvent in the crystal structure and to the TCR during antigen presentation.

For the CLIP peptide binding to HLA-DR3, deep pockets at P1 and P9 are more fully occupied by the peptide side chains.[443] The pH-dependent binding is important because CLIP must be stable at neutral pH and unstable at acid pH in the presence of HLA-DM in order to perform its function.

Based on affinity for MHC, these interactions explain the peptide-binding motifs for MHC class II that select which peptides can be presented to T cells. In addition, these interactions orient the peptide in the MHC groove and determine which residues are accessible for recognition by the TCR.

T-Cell Receptor Recognition

The last hurdle that a potential antigenic determinant must surmount is recognition by a TCR within the repertoire of the individual responding. This repertoire may be limited by the availability of combinations of V, D, and J genes (see Chapter 11) in the genome that can combine to form an appropriate receptor, given the lack of somatic hypermutation in TCRs in contrast to antibodies,[383,444] and then by self-tolerance, as mediated by thymic or peripheral negative selection, or by limits on the repertoire that is positively selected in the thymus on existing self-peptide-MHC complexes. The available repertoire may also be influenced by prior exposure to cross-reactive antigens. In general, however, it has been hard to find holes in the repertoire.[445] Indeed, studies examining large panels of peptides binding to particular class I molecules for correlations between peptide affinity for MHC and T-cell responses have failed to find high-affinity peptides for which no T-cell response can be raised, whereas not all lower affinity peptides elicit a response.[446,447] These results suggest that if the peptide can bind well enough to the MHC molecule, there is virtually always a T cell that can see the complex. However, the array of other MHC molecules present can influence the breadth of the T-cell repertoire.[448] Furthermore, when TCR repertoires of mice and humans were compared for peptides presented by HLA-A2.1, they seemed to be capable of seeing the same spectrum of peptides.[449] Eleven peptides from hepatitis C virus proteins, each of which had a motif for binding to HLA-A2.1, were tested for recognition by CTL from HLA-A2.1 transgenic mice and human HLA-A2.1 positive patients infected with hepatitis C virus. The same four peptides that were recognized by the T cells from the mice were the ones recognized by the human T cells, whereas the others were not recognized well by either murine or human T cells. The selection of which peptides were recognized seemed to be determined by binding to the HLA-A2.1 molecule rather than by the availability of T cells. Thus, despite the differences in TCR genes in mice and humans, the repertoires are plastic enough that if a peptide passes the other three hurdles of processing, transport, and binding to MHC molecules, T cells can be elicited to respond to it in either species.[449] Likewise, no hole in the helper T-cell repertoire could be found to explain low responders to the recombinant hepatitis B vaccine.[450]

On the other hand, evidence exists that MHC binding is not the whole story. Schaeffer et al.[451] examined 14 overlapping peptides covering the sequence of staphylococcal nuclease with different class II MHC molecules, constituting 54 different peptide-MHC combinations. Clearly, MHC binding plays a major role because 12 out of 13 immunogenic peptides were high or intermediate binders to MHC molecules, whereas only 1 of 37 poor binders were immunogenic. Of high-affinity binders, five out of five peptides

were immunogenic. However, for intermediate affinity MHC-binding peptides, only 7 out of 12 were immunogenic. Similar results were found for the class I MHC molecule HLA-B*0702, in which it was found that 7 of 7 high-affinity binders but only 9 out of 12 intermediate affinity

binders, were immunogenic.[447] Thus, MHC binding alone is not sufficient to ensure immunogenicity. The T-cell repertoire was one factor suggested that might limit the spectrum of immunogenic peptides.

Indeed, examples for selection at the level of the TCR repertoire exist. A particularly elegant example described by Moudgil et al.[452] is one in which a peptide (46 to 61) of mouse lysozyme presented by I-A^k is recognized T cells from CBA/J and B10.A mice, expressing I-A^k and I-E^k, but not by T cells from B10.A(4R) mice expressing only I-A^k, even though the APC from B10.A(4R) mice can present the peptide to T cells from the other strains. T cells from the B10.A(4R) mice can respond to variant 46 to 61 peptides in which the C-terminal Arg is replaced by Ala, Leu, Phe, Asn, or Lys, indicating that the C-terminal Arg is hindering recognition, but not binding by I-A^k, and in this case, not processing as the B10.A(4R) APC can present the peptide. It appears that the hindrance interferes with recognition by TCRs available in B10.A(4R) mice, but not TCRs available in B10.A or CBA/J mice, or in (B10.A(4R) × CBA/J)F1 mice. Because the B10.A mice are congenic with the B10.A(4R) mice, the difference is not one of non-MHC-linked genes such as TCR structural genes or non-MHC self-antigens producing self-tolerance. Further, because the F1 mice respond, the difference is not due to a hole in the repertoire produced by a self-antigen of the B10.A(4R) mice. It was concluded that the CBA/J and B10.A mice contain an additional repertoire, positively selected on I-E^k or possibly an H-2D/L class I molecule, in which these strains differ, which can recognize the 46 to 61 peptide despite the hindering Arg at the C-terminus. An alternative related explanation is that strains that express I-E^k or D^k or D^d/L^d have an additional repertoire of TCRs positively selected on I-A^k-presenting self-peptides from processing of these other MHC molecules in the thymus. This example illustrates a case in point that subtle differences in TCRs, presumably caused in this case by positive selection, can lead to responsiveness or nonresponsiveness to a determinant that has already passed all of the three earlier hurdles, processing, transport, and MHC binding. Of course, there are some holes related to selftolerance, primarily related to the loss of response to dominant determinants[453] and especially loss of high-avidity T cells.[454,455,456,457] Interestingly, not only is there no loss of response to cryptic determinants but also T cells recognizing cryptic determinants of mouse lysozyme can be positively selected on other nonmouse lysozyme self-ligands in a lysozyme knockout mouse.[453] The selective loss of high-avidity T cells to immunodominant determinants has suggested a strategy to apply epitope enhancement to modify subdominant lower affinity tumor antigen peptides to increase their affinity for MHC molecules, and thereby make them more immunogenic in order by to take advantage of a repertoire not already crippled by loss of the high-avidity clones.[458]

Another elegant example of T-cell repertoire limitations on immunodominance comes from a study of mice deficient in LMP-2, and so unable to make immunoproteasomes. Reduced response to one normally immunodominant influenza epitope was found by T-cell adoptive transfer studies to be due to an alteration in the T-cell repertoire, presumably because of altered processing of self-peptides in the thymus.[281]

A recent study found another mechanism by which the T-cell repertoire contributes to immunodominance. It was found that the relative immunodominance of a dominant determinant of the HIV envelope protein among different strains of H-2D mice all presenting the same peptide-H-2D^d complex correlated with the avidity of the T cells responding in those strains.[459] The mechanism found was that highavidity T cells proliferate faster than low-avidity ones when exposed to antigen and therefore dominate the response. This

mechanism differs from those at earlier steps that progressively constrain the response, and thereby narrow the repertoire to dominant epitopes, whereas this mechanism selectively expands the dominant repertoire. This mechanism may also explain other recent findings such as the higher affinity of dominant compared to subdominant TCRs specific for human cytomegalovirus.[460] Because highavidity T cells are better able to clear virus infection,[461,462] a recent study even suggests that the driving force for MHC polymorphism is to create a large enough repertoire to select for high-avidity T cells.[463]

Several critical parameters have been reported to be involved in more effective T-cell activation during TCR-peptide-MHC interaction. In some situations, TCR avidity for the peptide-MHC complex appears to play a more critical role than dissociation rate[464] and a threshold for maximal activity may even be defined,[465] whereas in other situations, the half-life of the TCR-peptide-MHC interaction seems most critical.[466,467,468] Moreover, in situ in two-dimensional surface interactions, a rapid on-rate may compensate for a faster off-rate (shorter half-life) and allow effective TCR-peptide-MHC engagement, suggesting the total cumulative duration time (including multiple repeat engagements) may be most critical.[469,470,471,472]

As more is understood about the molecular basis of TCR recognition, with crystallographic data now available,[386,387,473] it becomes possible to apply epitope enhancement in a rational way to the affinity of the peptide-MHC complex to the TCR, as was described for the peptide affinity for MHC molecules. Sequence modifications in the peptide that increase the affinity for the TCR were shown to be more effective at expanding in vivo the T cells specific for tumor antigens.[474,475,476] Most of these modifications were found empirically, but a systematic study of substitutions throughout a number of peptides revealed a pattern in which peptides with conservative substitutions at P3, P5, or P7 were most likely to yield increased TCR affinity, narrowing the candidate list of peptides that require empirical screening.[477] This strategy provides a second type of epitope enhancement, derived from basic immunologic principles, to produce more effective vaccines.

The advent of DNA vaccines has made it possible to link a series T-cell epitopes in tandem and express them in the form of a multiepitope vaccine. These chimeric proteins have the

potential advantages of focusing the immune response on biologically important epitopes, combining T helper epitopes with CTL epitopes, using epitopes with high affinity for MHC, and linking the epitopes in any desired order to optimize immunogenicity. Ideally, antigen processing throughout the construct would produce multiple epitopes that could be presented independently. In the case of HIV, where antigenic variation is so common, these vaccines could be designed to overcome variation by including epitopes that are conserved within each geographical region (clades).

For example, Létourneau et al.[478] expressed a string of HIV sequence fragments from the gag, pol, vif, and env genes that were highly conserved within their own clades. As they progressed from one fragment to the next, they alternated sequences from different HIV clades: Immunity to multiple epitopes would produce broad based immunity across clades. Fourteen fragments were linked in tandem. Each fragment contained multiple T-cell epitopes, for a total of 270 known epitopes recognized with human MHC. In addition, T cells from most patients infected with HIV responded to peptide pools from the vaccine, indicating that the epitopes were processed and presented to T cells. But when the multiepitope vaccine was given as a DNA vaccine followed by two viral vector boosts, it elicited relatively weak T-cell

responses to two out of six peptide pools in HLA-A2 transgenic mice.

For these constructs to function as multiepitope vaccines, antigen processing between epitopes is required. In any given string, all of the epitopes are expressed equally, but different locations might be processed differently. In order to elicit T-cell immunity to multiple epitopes at the same time, there must be efficient processing and presenting of epitopes in all parts of the string. For example, Depla et al.[479] created a vaccine with 30 epitopes from hepatitis B virus that were restricted to five different MHC class I types. The immune response to the combined antigen was compared to individual peptides as an indication of the effect of antigen processing on presentation of internal epitopes. For two MHC types, the same 5 out of 12 epitopes were immunogenic, regardless of whether they were presented as a string of epitopes or as free peptides. However, for three other MHC types, whereas 14 out of 18 epitopes were immunogenic as peptides, only 3 out of 18 were immunogenic in the multiepitope vaccine. This difference suggests inefficient antigen processing at multiple sites, either because of failure to process the epitopes correctly or due to excessive degradation.

One way to control processing was suggested by Livingston et al.[480,481] These authors found that the first amino acid following each epitope is an important marker for antigen processing. They demonstrated that poor presentation of an internal epitope can be corrected by adding a spacer of as little as one amino acid between epitopes to improve the yield of processed peptides. The most favorable amino acid spacers were Lys, Arg, Asn, and Gln, followed by Cys, Gly, Ala, Thr, or Phe. The least active spacer residues were Asp, Val, Met, or Leu.[480,481] For example, an epitope from hepatitis B virus core antigen was poorly immunogenic when it was located next to a Phe residue, but its immunogenicity was enhanced by four logs when Lys was substituted for Phe at this position. These amino acid preferences may reflect the major protease activities of proteasomes: The most targeted amino acids could improve the yield of intact epitopes.

This strategy was used to construct a multiepitope vaccine from HIV antigens.[482] Twenty-one epitopes were identified, based on the T-cell response of infected humans, and these were linked together in a multiepitope DNA vaccine. Spacers of one to four amino acids were inserted between epitopes to optimize antigen processing. The DNA vaccine was used to immunize HLA transgenic mice, and the response to each epitope was compared to peptide vaccinated mice. For two HLA types, A2 and A3, the peptide and multiepitope vaccines were nearly equal, suggesting efficient processing. In addition, for HLA-A2, the potency of the T-cell response to different epitopes was largely independent of their position in the vaccine sequence, suggesting efficient antigen processing at various points throughout the chimeric protein. But for a third HLA type, B7, despite a normal peptide response, the multiepitope vaccine failed to elicit immunity to four out of seven epitopes tested, indicating that processing was limiting the response to these residues.

Design of an effective multiepitope vaccine depends on finding the optimal balance between antigen processing and epitope survival. Antigen processing between epitopes generates antigenic peptides, but processing within the epitope would destroy its antigenicity. Delamarre et al.[483] have compared pairs of proteins with the same sequence but different conformations, resulting in different susceptibilities to lysosomal processing. Ribonuclease (RNAse) A was relatively stable to lysosomal extracts of dendritic cells, whereas RNAse S was degraded quickly. Of these two forms of the same protein, RNAse A was a far more potent immunogen, as measured by T-cell proliferation or Delayed Type Hypersensitivity (DTH). Similarly, horseradish peroxidase is stable to lysosomal proteases in the native form, but is quickly degraded in the apo form. Native horse radish peroxidase (HRP) was a much more potent immunogen than the unstable apo form. These results indicate the harmful effect

of excessive processing on T-cell stimulation and immunogenicity.

Multiepitope immunogens have been designed to elicit CTLs by stimulating T helper cells and CTLs simultaneously. These immunogens have combined T helper and CTL epitopes in a variety of ways. Some vaccines contain at least one T helper epitope and one CTL epitope on each polypeptide chain.[484] Others have expressed them separately: A polypeptide consisting of multiple T helper epitopes was coadministered with a DNA plasmid coding for multiple CTL epitopes.[485] A third way would be to express alternating T helper and CTL epitopes in tandem in the same multiepitope vaccine, either as DNA or polypeptide. The T-cell response to paired T helper epitopes was generally weak,[484] whereas the response to a string of multiple T helper epitopes was reportedly stronger, although it did not confer any improvement on the immune response to CTL epitopes expressed separately.[485]

As with T-cell help for B cells, a multiepitope vaccine for CTLs should combine T helper and CTL epitopes in the same construct.[402] It would deliver both epitopes into

the same APC, to facilitate helper T cell effects on CTL induction. But the epitopes should target different cellular compartments, leading to the main processing pathways: T helper epitopes should travel to endosomes for processing and presentation with MHC class II. CTL epitopes should traffic to the cytoplasm for proteasomal processing and presentation with MHC class I.

Defining the Role of Individual Amino Acids and Effects of Altered Peptide Ligands

Once an antigenic peptide is identified, the next step is to map key amino acid residues by making a series of variant peptides, each of which differs from the native sequence by a single amino acid substitution, as previously described in the section on MHC binding. One approach, called an alanine scan, substitutes Ala for the natural amino acid at each position in the peptide or uses Ser or Gly to replace naturally occurring Ala. Ala is used because the side chain is only a methyl group, so it replaces whatever functional side chain is present with the smallest one other than that of Gly, which is not used because of its effects on conformation. Thus, one can ask whether loss of the naturally occurring side chain affects function, without the introduction of a new side chain that might itself affect function. Generally, each peptide will have several amino acids where Ala substitution destroys antigenicity. Some of these correspond to contact residues for the TCR, whereas others are contact residues for MHC. In many cases, the MHC-binding residues can be determined by testing the substituted peptides in a competitive MHC-binding assay (discussed previously). The amino acid substitutions that knockout T-cell proliferation but not MHC binding are presumed to be in the epitope recognized by the TCR directly, and these can be studied with additional substitutions. For example, this technique was used to compare the residues interacting with the MHC molecule or TCR when the same HIV-1 V3 loop peptide P18 (residues 308 to 322) was presented by three different MHC molecules, a human class I molecule, a mouse class I molecule, and a mouse class II molecule (Fig. 23.12).[486,487] Interestingly, there was a striking concordance of function of several of the residues as presented by all three MHC molecules (see Fig. 23.12). For example, Pro and Phe interacted with the MHC in all three cases, and the same Val interacted with the TCR in all three cases. Also, the same Arg in the middle of the peptide interacted with both the mouse class I and II molecules, and the C-terminal Ile was an anchor residue for both human and murine class I molecules.[486,487]

In the case of autoimmune T cells, these techniques have been used to study the number

and variety of epitopes recognized by self-reactive T cells. In the nonobese diabetic mouse, the β chain of insulin is a major target of T cells recovered from pancreatic islets.[488] Alanine scanning of β chain peptide 9 to 23 revealed two patterns of T-cell recognition for the same peptide. Some T cells recognize peptide 9 to 16, whereas others respond to peptide 13 to 23. Each epitope appears to have distinct sites for MHC and TCR binding, even though they come from the same peptide chain. Similarly, in systemic lupus erythematosus, human T cells specific for the Sm antigen are narrowly restricted to a few epitopes that are found on a small group of proteins.[489] On the Sm-B antigen, three epitopes were recognized. On Sm-D antigen, there were two. In each case, alanine scans showed that the same epitopes were recognized by distinct T-cell clones. These results are consistent with the hypotheses that the autoimmune response to insulin or to Sm antigen may be induced by abnormal exposure of a very few cryptic epitopes, or they may depend on selective loss of tolerance for a limited number of epitopes shared by a small subset of self-proteins.

TCRs may distinguish different chemical classes of amino acid side chains. An example of structural differences between amino acid side chains recognized by the TCR comes from an analysis of non-cross-reactive CTL that distinguishes homologous peptides from the V3 loop of different strains of HIV-1 envelope protein. The residue at P8 in the minimal determinant was identified as a key

“epitopic” TCR contact residue in both strain IIIB, which has a Val at this position, and strain MN, which has a Tyr at this position.[486,490,491] CTLs specific for strain IIIB do not recognize the MN sequence but will recognize peptides identical to MN except for the substitution of any aliphatic amino acid at that position, such as Val, Leu, or Ile.[400] In contrast, CTL specific for the MN strain do not recognize the IIIB sequence but will recognize the IIIB peptide if the Val at this position is replaced by a Tyr.[490] Moreover, they will see any MN variant in which the Tyr is replaced by another aromatic amino acid, such as Phe, Trp, or His.[400] Thus, the two non-cross-reactive TCRs see similar peptides but discriminate strongly between peptides with amino acids with aliphatic versus aromatic side chains. On the other hand, they do not distinguish strongly among different aliphatic residues or among different aromatic residues. Interestingly, however, in each category, the least active is the bulkiest member of the category, Ile and Trp, respectively, suggesting that these residues must fit into a pocket of limited size in the TCR.

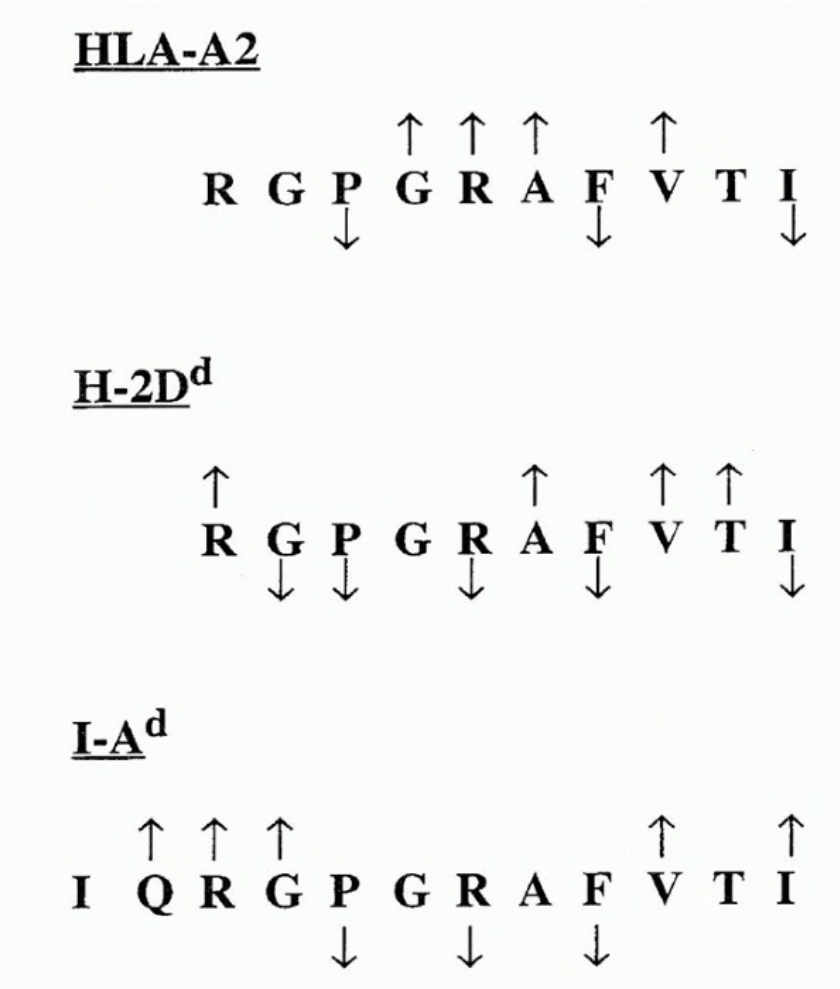


FIG. 23.12. Comparison of the Major Histocompatibility Complex (MHC)-Interacting ("Agretopic") and T-Cell Receptor (TCR)-Interacting ("Epitopic") Residues of the Same Human Immunodeficiency Virus (HIV)-1 Envelope V3 Loop Peptide as It Is Presented by Human Class I, Murine Class I, and Murine Class II MHC Molecules to Cluster of Differentiation (CD)8$^+$ Cytolytic T Cell and CD4+ Helper T Cells. The sequence of the optimal binding portion of peptide P18 IIIB from the HIV-1 envelope protein V3 loop for each MHC molecule, in single letter amino acid code, is shown. *Arrows pointing up* indicate residues determined to interact with the TCR, and *arrows pointing down* indicate residues determined to interact with the MHC molecule. Mapping of residue function for binding to the human class I MHC molecule HLA-A2.1 was described in Alexander-Miller et al.,[487] and binding to the murine class I molecule H-2D^d and the murine class II molecule I-A^d was described in Takeshita et al.[486] Note the common use of the Pro and Phe for binding all three MHC molecules and the use of the key Val residue for binding all the TCRs. Also, the murine MHC molecules both use the central Arg residue as a contact residue, whereas both class I molecules use the C-terminal Ile residue as an anchor residue. Thus, there is a surprising degree of concordance.

The interaction of peptide ligand with TCR can be studied by introducing single substitutions of conservative amino acids at these contact residues, such as Glu for Asp, Ser for Thr, or Gln for Asn. The TCR readily distinguishes among peptides with these minor differences at a

single residue, and the results have been revealing. Depending on affinity for the TCR, closely related (altered) peptides can elicit very different responses in T cells. Thus, although a substituted peptide may be very weak or nonstimulatory by itself, it may still act as a partial agonist or even a strong antagonist of an ongoing T-cell response. Antagonistic peptides can be demonstrated by pulsing APCs with native peptide antigen first so that one is not measuring competition for binding to MHC molecules, followed by pulsing with a 10-fold or greater excess of the antagonist before adding T cells. In the case of influenza hemagglutinin peptide 307 to 319 presented with HLA-DR1, peptide analogues such as Gln substituted for Asn 313 inhibited the proliferation of a human T-cell clone, even though they did not stimulate the clone. Anergy was not induced, and the antagonist peptide had to be present throughout the culture to inhibit the response.[492,493] Thus, lack of antagonist activity is another feature of the interaction between peptide (in complex with MHC molecule) and TCR that is required for the peptide to be a stimulatory antigenic determinant.

Partial agonists were first demonstrated using T-cell clones specific for an allelic form of mouse hemoglobin. These T cells were from CE/J mice, which express the Hb^s allele of mouse hemoglobin, after immunization with the Hb^d allele. The minimum antigenic peptide corresponds to amino acids 67 to 76 of the Hb^d sequence and differs from Hb^s at P72, P73, and P76.[494] Peptides substituted at each residue from amino acid 69 to 76 were tested for T-cell proliferation and cytokine release. Some substitutions, such as Gln for Asn at P72, blocked T-cell stimulation completely in both assays. Other substituted peptides, such as Asp for Glu at P73, lost T-cell proliferation but still stimulated interleukin 4 release, and these are considered partial agonists (Fig. 23.13).[495] Lack of stimulation was not due to failure to bind MHC, as both substituted peptides gave reasonable binding in a competitive-binding assay.[496] Similar alteration of cytokine profile by altering the peptide ligand can be seen in other systems.[493,497,498]

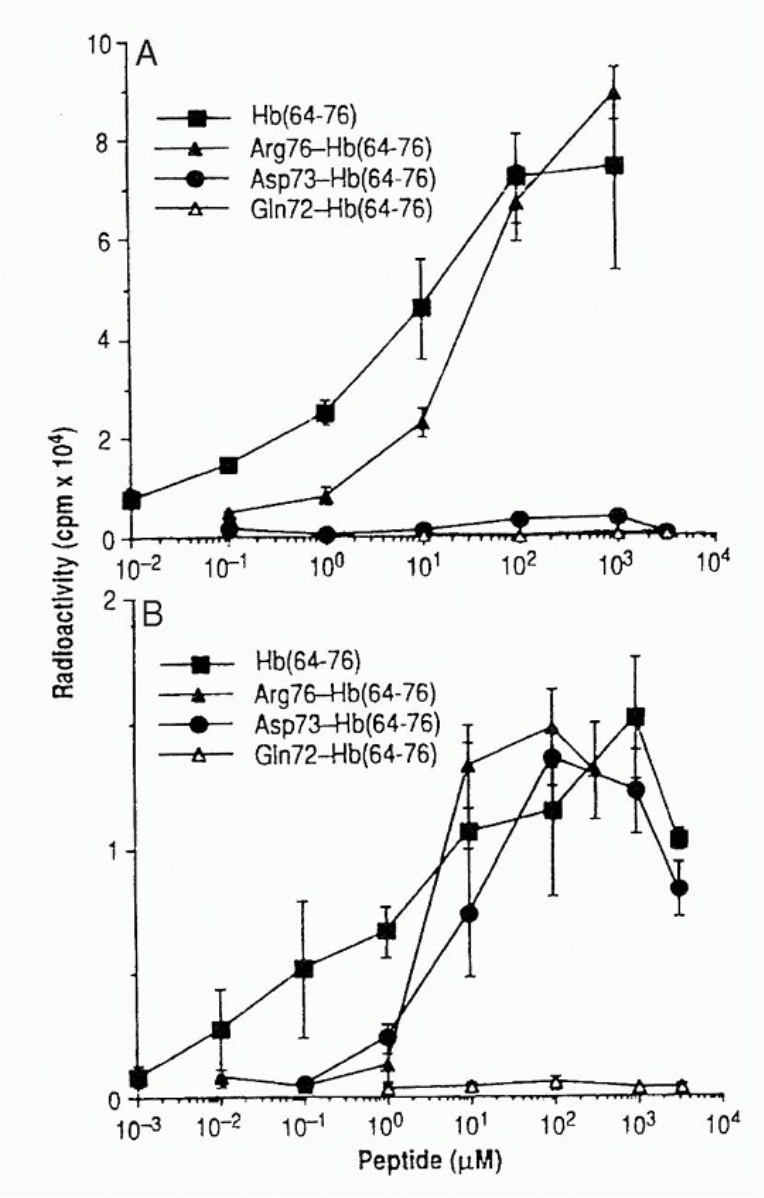


FIG. 23.13. Differential Effect of Altered Peptide Ligands on the Response to Peptide 64 to 76 from Hemoglobin. A: Proliferative response of a T-cell line incubated with antigen-presenting cells and the natural Hb (64-76) peptide or with peptides substituted at positions 72, 73, or 76. **B:** Interleukin 4 release by the T-cell line under the same conditions. The peptide substituted with Asp for Glu at position 73 is unable to induce T-cell proliferation, but it can still induce production of interleukin 4, so it is a partial agonist. In contrast, substitution of Gln for Asn at position 72 knocks out both responses equally. Modified from Evavold and Allen,[495] with permission.

For one of the Hb 64 to 76 specific T-cell clones, PL.17, substitutions at amino acids 70, 72, 73, or 76 reduced antigenic potency by 1000-fold or more, even though conservative amino acids were substituted. Although substitution of Ser for Ala 70 prevented T-cell stimulation in both assays, there was clearly some response to this peptide, as it induced expression of the interleukin 2 receptor.[499] In addition, once T cells were exposed to the Ser 70 peptide, they became unresponsive to subsequent exposure to the natural Hb^d peptide. This phenomenon closely resembled T-cell anergy and persisted for a week or more. The Ser 70 substitution

alters a contact residue of the peptide for the TCR of clone PL.17 and affects its affinity. Other T-cell clones, however, can respond to this peptide presented on the same MHC molecule (I-E^k). Other Hb^d peptides substituted at this position, such as Met and Gly 70, also induced anergy but not proliferation, whereas nonconservative substitutions such as Phe, Asn, Asp, and His 70 induced neither.[500]

Another well-studied example is influenza hemagglutinin peptide 306 to 318 as presented on human HLA-DR1. Based on the known crystal structure of the peptide-MHC complex,[253] amino acid substitutions could be targeted to contact residues for the TCR at P307, P309, P310, P312, P315, and P318.[501] At each position, nonconservative substitutions often rendered the peptide inactive, whereas conservative substitutions at several sites either gave full antigenicity or gave progressively lower stimulatory activity, down to 1000-fold less than native peptide while retaining the ability to induce anergy. For example, substituting His or Gly for Lys 307 gave 1000-fold reduced stimulation of T-cell proliferation but full ability to induce tolerance. Similarly, substituting His for Lys 315 gave complete loss of stimulation but nearly full anergy inducing activity. As before, induction of the interleukin 2 receptor (CD25) was a sign of T-cell activation by these altered peptide ligands, even when they did not induce proliferation. Unlike these peptides, the antagonists do not induce interleukin receptors or secretion, and they do not cause long-lasting tolerance. Overall, a number of altered peptide ligands have now been identified that, in appropriate complexes with MHC molecules, induce anergy or act as antagonists of the TCR and block activation by agonist ligands by delivering an abortive signal.[493]

Several methods have been found to anergize T cells to a specific antigen for up to a week, and all have the common theme of delivering a partial signal via the TCR, resulting in tolerance rather than stimulation. The first method was to expose the T cells to peptide plus APCs treated with the carbodiimide cross-linker ECDI.[502] This treatment may prevent accessory molecules on the presenting cell from interacting with the TCR complex or costimulatory signals from contributing to T-cell activation. The second method was to present peptide on presenting cells with mutated I-E molecules.[503,504] The third method was to use altered peptide ligands that act as TCR antagonists as described previously.[493,500,501] The final method was to block CD4 function with a monoclonal antibody, which would delay the recruitment of CD4 to the engaged TCR.[505] Because generation of a complete stimulatory signal requires the interaction of TCR and accessory molecules, modifications that affect either component can block signaling. An altered peptide ligand, with decreased affinity for the TCR, may form an unstable complex, which cannot stay together long enough to recruit accessory molecules and generate a complete signal.[505,506] Altered peptide ligands with low affinity for the TCR can also act as partial agonists that can compete with optimal agonists and reduce T-cell stimulation based on a similar mechanism (short dwell time of peptide-MHC complex on the TCR).[199]

Abnormal TCR signaling can be demonstrated by following the activity of protein kinases. Normal signaling produces phosphorylation of TCR subunits, such as ζ chain, as well as phosphorylation and activation of receptor-associated tyrosine kinases, such as ZAP-70. These kinases generate the downstream signal needed for T-cell activation. However, in each case studied, partial antigen signaling resulted in ζ chain phosphorylation without phosphorylation or activation of ZAP-70,[500,504,505] so downstream activation did not occur. This abnormal pattern occurred regardless of the method of anergy induction.

Partial signaling may be important for T-cell survival during negative selection in the thymus or in maintaining peripheral tolerance. By responding to self-antigens as if they were altered ligands presented in the thymus, T cells could use anergy induction as a successful strategy for avoiding clonal deletion. Similarly, peripheral tolerance may be an important mechanism for preventing autoimmune disease. Immunotherapy with altered peptide ligands could be envisioned as a way to block an ongoing response or induce tolerance to a specific antigen, such as the synovium in arthritis, or foreign MHC antigens in allograft rejection. However, a potential pitfall is that different T cells recognize the same peptide differently, so a peptide that is seen as an altered peptide ligand by some T-cell clones may be seen as a complete antigen by others. In addition, the choice of peptide would vary with MHC type. To be effective, an altered peptide ligand should antagonize or anergize polyclonal T cells and should work with each patient's MHC type.

A similar mechanism may be invoked to explain the generally weak immunogenicity of tumor antigens. According to this hypothesis, the only T cells capable of responding to self-antigens on tumors may have low-affinity receptors for them. In effect, the natural sequence is the altered ligand that induces tolerance. In some cases, this anergy can be overcome with modified peptides that have greater affinity for the TCR and induce a full stimulatory signal, resulting in an effective immune response to the tumor antigens,[474] as previously described under epitope enhancement.

Prediction of T-Cell Epitopes

The fact that T cells recognize processed fragments of antigens presented by MHC molecules leads to the ironic situation that T-cell recognition of antigen, which is more complex than antibody recognition due to the ternary complex needed between TCR, antigen, and MHC molecule, may actually be focused on simpler structures of the antigen than those seen by most antibodies specific for native protein antigens. In contrast to the assembled topographic antigenic sites seen by many antibodies,[50,51] T cells specific for processed antigens are limited to seeing short segments of continuous sequence.[177,233] Therefore, the tertiary structure of the protein plays little if any role in the structure of the epitope recognized by T cells, except as it may influence processing. However, the structure of the T-cell antigenic site itself must be limited to primary (sequence) and secondary structure, the latter depending only on local rather than long-range interactions. This limitation greatly simplifies the problem of identifying structural properties important to T-cell recognition because one can deal with sequence information, which can now be obtained from DNA without having a purified protein, and with the secondary structure implicit therein without having to obtain an x-ray crystallographic three-dimensional structure of the native protein—a much more difficult task.

Because the key feature necessary for a peptide to be recognized by T cells is its ability to bind to an MHC molecule, most approaches for predicting T-cell epitopes are based on

predicting binding to MHC molecules. These approaches, which have been reviewed,[424,507,508,509,510] can be divided into those that focus on specific individual MHC molecules one at a time, such as motif-based methods, and those that look for general structural properties of peptide sequences. We shall discuss first the methods based on general properties and then those directed to individual MHC molecules.

The first structural feature of amino acid sequences found associated with T-cell epitopes that remains in use today is helical amphipathicity,[227,511,512,513,514] which is statistically significant independent of the tendency to form a helix per se.[512] Because the x-ray

crystallographic structures of both MHC class I[376,377,378,515] and II molecules[253,443] have consistently shown peptides to be bound in extended, not α-helical, conformation, helicity per se has been abandoned as an associated structural feature of T-cell epitopes. However, as discussed in the following text, other explanations of amphipathic structures have been discovered that do not require the peptide to be bound to the MHC molecule as an α helix. Amphipathicity is the property of having hydrophobic and hydrophilic regions separated in space. It was observed that the immunodominant T-cell epitopes of myoglobin and cytochrome c corresponded to amphipathic helices.[185,189,516] DeLisi and Berzofsky[511] developed an algorithm to search for segments of protein sequence that could fold as amphipathic helices based on the idea that the hydrophobicity of the amino acids in the sequence must oscillate as one goes around an amphipathic helix. For the hydrophobic residues to line up on one side and the hydrophilic residues on the other, the periodicity of this oscillation must be approximately the same as the structural periodicity of the helix, about 100 degrees per residue (360 degrees per 3.6 residues per turn) (Fig. 23.14).

A microcomputer program implementing this analysis was published.[514] Margalit et al.[227] optimized the original approach,[511] correctly identifying 18 of the 23 immunodominant helper T cell antigenic sites from the 12 proteins in an expanded database ($p < .001$)[227] (see Table 23.4). Indeed, when the database was expanded to twice and then four times its original size, the correlation remained highly significant, and the fraction of sites predicted remained relatively stable (34/48 sites = 71%, $p < .003$; 61/92 sites = 66%, $p < .001$).[517,518] A similar correlation was found for 65% of peptides presented by class I MHC molecules.[518] A primary sequence pattern found in a substantial number of T-cell epitopes by Rothbard and Taylor[519] was consistent with one turn of an amphipathic helix. Also, another approach, called the “strip-of-the-helix” algorithm, which searches for helices with a hydrophobic strip down one face, found a correlation between amphipathic helices and determinants presented by both class II and I MHC molecules.[513,520]

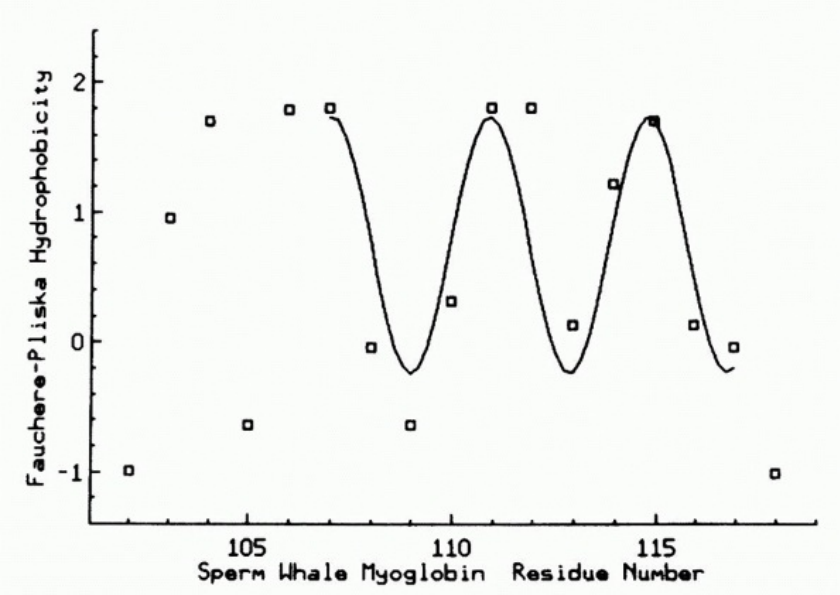


FIG 23.14. Plot of hydrophobicity of each amino acid in sperm whale myoglobin 102 to 118, according to the scale of Fauchère and Pliska,[604] as a function of amino acid sequence, showing least-squares fit of a sinusoidal function to the sequence of

hydrophobicities from 107 to 117. (From Berzofsky et al.,[180] with permission.)

Newer data suggest at least two explanations, not mutually exclusive, for this correlation in the absence of helical structure found in the peptides bound to MHC molecules.[521] First, crystal structures of peptides bound to class II MHC molecules have found that the peptides are bound in an extended conformation, but with a -130-degree twist like that of a type II polyproline helix,[253,443] and can be quite amphipathic because of this twist.[253] Although the -130-degree twist is distinct from that of an α helix, it gives a periodicity similar enough to be detected. Second, it was observed that spacing of the anchor residues in the motifs for peptides binding to class I and II MHC molecules was consistent with the spacing of turns of an α helix, for example, at P2 and P9 (seven residues apart like two turns of a helix) or at P5 and P9 (spaced like one turn of a helix).[521] Because the anchor residues are most often hydrophobic, this pattern resulted in an amphipathic periodicity pattern like that of an amphipathic α helix for just the anchor residues alone, seen in most motifs.[521] Thus, if the other residues have a random pattern, the anchor residue spacing alone, which is enforced by the spacing of the pockets in the MHC molecules that bind these anchor residues, will produce the amphipathic helical signal, even though the peptide is bound in an extended conformation. This amphipathic helical periodicity has held up as a correlate for peptides defined as T-cell epitopes[521] and has continued to be a useful predictive tool for identifying potential epitopes, successful in a number of studies, when one does not want to focus on individual MHC alleles or wants to find regions of high epitope density. Other structural properties such as coil content and exposure have been used as general predictors of MHC class II binding.[522]

Other approaches to predicting T-cell epitopes are generally based on sequences found to bind to specific MHC molecules.[424,507,508,509,510,523,524,525] The simplest approach is to apply standard sequence search algorithms to known protein sequences to locate motifs for peptides binding to particular MHC molecules, using collections of motifs identified in the literature.[264] This approach showed early success for epitopes in proteins from *Listeria monocytogenes*[526] and malaria,[527] but it also became apparent that only about 30% of sequences bearing motifs actually bound to the corresponding MHC molecules.[526,528,529] This discrepancy may relate to adverse interactions created by nonanchor residues[392,397,530] and

could be overcome to some extent by generating extended motifs taking into account the role of each residue in the sequence.[397,530]

To determine whether one could locate regions of proteins with high densities of motifs for binding multiple MHC molecules, Meister et al.[531] developed the algorithm Epimer, which determined the density of motifs per length of sequence. A surprising result was that the motifs were not uniformly distributed but clustered. This clustering may reflect the fact that many motifs are related, and that the same anchor residues are shared by several motifs, perhaps because MHC molecules are also related and their variable segments that define some of the binding pockets are sometimes exchanged by gene conversion events.[532] This hypothesis has now been confirmed and extended by studies showing that each anchor pocket can be grouped into families of MHC molecules sharing similar pockets and therefore anchor residues, but the families for the B, C, and F pockets do not coincide, so there is a

reassortment between pockets.[533,534] These observations allow prediction of motifs for additional MHC molecules. In the case of HIV, the densities of motifs for class I MHC binding were anomalous at both the low and high ends of the spectrum.[535] Clustering at the high end may be due to anchor sharing and showed no correlation with conserved or variable regions of the sequence. However, at the low end, long stretches with low motif density occurred preferentially in variable regions, suggesting that the virus was mutating to escape the CTL immune system.[535] This clustering may be useful in vaccine development because identification of sequences containing overlapping motifs for multiple MHC molecules may define promiscuously presented peptides that would elicit responses in a broad segment of the population.[531]

Another type of MHC allele-specific approach is the use of matrices defining the positive or negative contribution of each amino acid possible at each position in the sequence toward binding to an MHC molecule. A positive or negative value is assigned to each of the 20 possible amino acids that can occur at each position in a peptide sequence, and these are summed to give the estimated potential of that peptide for binding. The values in the matrix are derived from either experimental binding studies using peptide panels with single positions substituted with each possible amino acid,[439,536,537,538,539] or from comparisons of peptides known to bind in a compilation of the literature, if the number known is sufficiently large.[507,540] One improvement described is an amino acid similarity matrix that disfavors substitutions with opposite charge.[541] Davenport et al.[542,543] also developed a motif method based on Edman degradation sequencing of pooled peptides eluted from MHC molecules. All of these methods have had some success in predicting peptides binding to particular MHC molecules,[507] but they all require the assumption that each position in a peptide must be acting independently of its neighbors, which is a reasonable first approximation, but exceptions are known.[544,545] The more experimental data that goes into generating the matrix, the more reliable the predictions. Therefore, the predictive success may be greater for some of the more common HLA molecules for which more data exists. This matrix approach has been used for both class I and II MHC molecules. Current predictive matrix algorithms, compared to experimental screening, have been found to detect the vast majority of $CD8^+$ T-cell epitopes detected experimentally, for example, in vaccinia virus, in which the top-ranked 300 peptides using four different algorithms predicted 40 per 49 epitopes found.[546]

A potentially very useful observation is the finding that HLA class I molecules can be grouped into families (HLA supertypes) that share similar binding motifs.[438,536,547,548] The broader motifs that encompass several MHC molecules have been called supermotifs. For example, HLA-A*0301, A*1101, A*3101, A*3401, A*6601, A*6801, and A*7401 all fall into the HLA-A3 superfamily.[547] A peptide that carried this supermotif should be active in a broader range of individuals than one which was presented by a single HLA molecule. Moreover, as several HLA supertypes have been defined, it should be possible to design a vaccine effective in a large fraction of the population with only a limited number of well-selected antigenic determinants.[549]

Another approach for predicting peptides that bind to MHC molecules is based on free energy calculations of peptides docked into the groove of a known MHC structure, for which the crystallographic coordinates are known, or on structural modeling of the MHC molecule by homologous extension from another MHC molecule, when the crystal structure is not known, followed by peptide docking calculations.[550,551,552] It is important to use free energy rather

than energy, as the latter alone cannot find the most stable orientation of a side chain and cannot correctly rank order different side chains at the same position. This approach correctly predicts the structure of several known peptide-MHC complexes when starting with the crystal structure of a different complex, in each case to within 1.2 to 1.6 Å all-atom root-mean square deviation.[551] Using this structural modeling can allow one to extend motifs to nonanchor positions for cases where only anchor residue motifs are known and can allow one to predict new motifs for MHC molecules whose motifs have not yet been determined.

Another approach to predicting MHC-binding sequences uses a technique called threading that has been developed for predicting peptide secondary structure, based on threading a sequence through a series of known secondary structures, and calculating the energies of each structure. Altuvia et al.[553,554] showed that threading could be applied to peptides in the groove of MHC molecules, because when several peptides that bind to the same MHC molecule are compared crystallographically, the conformation of the peptides is fairly similar, as for example in several peptides cyrstallized bound to HLA-A2.1.[515] In testing the threading approach, Altuvia et al.[553,554] showed that known antigenic peptides are highly ranked among all peptides in a given protein sequence, and the rank order of peptides in competitive binding studies could be correctly predicted. The advantage of this approach is that it is independent of known binding motifs and can identify peptides that bind despite lack of the common motif for the MHC molecule in question. It can

also be used to rank a set of peptides all containing a known motif. For both class I and II MHC-binding peptides, a number of structure-based predictive approaches have been developed as well.[522,555,556,557,558]

Finally, artificial neural networks can be trained on a set of peptides that bind to a given MHC molecule to recognize patterns present in binding peptides.[559,560] When the predictions of the artificial neural networks are tested, the results can be used to further train the artificial neural networks to improve the predictive capability in an iterative fashion.

Recently, predictive algorithms for proteasomal cleavage sites[561,562] and TAP-transported peptides[319,563] have been developed. Combining these into approaches to predict epitopes based on all the steps a peptide must pass through, cleavage, transport, and MHC binding (except for TCR binding), has led to the most recent comprehensive algorithms for epitope prediction that can achieve up to 72% sensitivity.[286,320,508,564,565,566,567,568]

As all these methods are further developed and refined, they promise to allow accurate prediction of peptides that will bind to different MHC molecules and thus allow the design of vaccines without empirical binding studies until the end of the process. In addition, empirical high throughput methods for epitope mapping have been developed to speed the latter.[569] Further, localization of clusters of adjacent or overlapping binding sequences in a short segment of protein sequence can also be useful for selecting sequences that will be broadly recognized. Predictive algorithms for locating T-cell epitopes also have the potential use to reduce immunogenicity of therapeutic proteins (such as hormones, cytokines, and monoclonal antibodies) against which one would not want an immune response to develop.[570,571] For this purpose and others, it is useful to be able to predict all the epitopes likely to be recognized not just with a single MHC allele but with all of a person's MHC molecules or even those of a larger population; such methods have also been developed.[572]

RELATIONSHIP BETWEEN HELPER T-CELL EPITOPES AND B-CELL

EPITOPES ON A COMPLEX PROTEIN ANTIGEN

As we have seen, the factors that determine the location of antigenic sites for T and B cells, with the possible exception of self-tolerance, are largely different. Indeed, if B cells (with their surface antibody) bind sites that tend to be especially exposed or protruding—sites that are also more accessible and susceptible to proteolytic enzymes—then there is reason to think that T cells may have a lower probability of being able to recognize these same sites, which may be more likely to be destroyed during processing. Certainly, assembled topographic sites will be destroyed during processing. On the other hand, there are examples in which T cells and antibodies seem to see the same, or very closely overlapping, sites on a protein,[43,185,215,573,574,575] although fine specificity analysis usually indicates that the antibody and T cell fine specificities are not identical. The question dealt with here is whether there are any functional or regulatory factors in T cell-B cell cooperation that would produce a relationship between helper T cell specificity and B cell specificity for the same protein antigen.

Early evidence that helper T cells might influence the specificity of the antibodies produced came from a number of studies showing that Ir genes, which appeared to act through effects of T-cell help, could influence the specificity of antibodies produced to a given antigen.[181,576,577,578,579,580,581,582,583] It was hard to imagine how MHC-encoded Ir genes could determine which epitopes of a protein elicit antibodies, when such antibodies are generally not MHC restricted. One explanation suggested was that the Ir genes first select which helper T cells are activated, and these in turn influence which B cells, specific for particular epitopes, could be activated.[130] Because, for cognate help, the B cell has to present the antigen in association with an MHC molecule to the helper T cell, the Ir gene control of antibody specificity must operate at least partly at this step by selecting which helper T cell can be activated by and help a given B cell. Conversely, if the helper T cell selects a subset of B cells to be activated on the basis of their antibody specificity, then there is a reciprocal interaction between T and B cells influencing each other's specificity. Therefore this hypothesis was called “T-B reciprocity.”[130] Steric constraints on the epitopes that could be used by helper T cells to help a B cell specific for another particular epitope of the same protein were also proposed by Sercarz et al.[584]

The concept was first tested by limiting the fine specificity of helper T cells to one or a few epitopes and then determining the effect on the specificities of antibodies produced in response to the whole molecule. This was accomplished by inducing T-cell tolerance to certain epitopes[585] or using T cells from animals immune to peptide fragments of the protein.[128,586,587] In each case, the limitation on the helper T-cell specificity repertoire influenced the repertoire of antibodies produced.

One purpose of the B-cell surface Ig is to take up the specific antigen with high affinity, which is then internalized by receptor-mediated endocytosis and processed like any other antigen.[588,589,590,591,592,593,594,595] Therefore, the explanation was proposed that the surface Ig, which acts as the receptor to mediate endocytosis, sterically influences the rate at which different parts of the antigen are processed because what the B cell is processing is not free antigen but a monoclonal antibody-antigen immune complex.[130] This concept presupposes that many antibody-antigen complexes are stable near pH 6 in the endosome, and that what matters is the kinetics of production of large fragments, rather than the products of complete digestion, when both the antigen and the antibody may be degraded to single amino acids. Such protection from proteolysis of antigen epitopes by bound antibody can be demonstrated at least in vitro.[57] More recently, the effect of antigen-specific B-cell

surface Ig on the fragments produced by proteolytic processing of antigen was elegantly demonstrated by Davidson and Watts.[596] They demonstrated that the pattern of fragmentation of tetanus toxoid, as measured by SDS-polyacrylamide gel electrophoresis, produced during processing by B-lymphoblastoid clones specific for tetanus toxoid varied among B-cell clones depending on their specificity for different epitopes within the antigen.

Binding to the antibody may also influence which fragments are shuttled to the surface and which are shunted into true lysosomes for total degradation. Thus, different B cells bearing different surface Ig would preferentially process the antigen differently to put more of some potential fragments than others on their surface in contrast to nonspecific presenting cells that would process the antigen indifferently. By this mechanism, it is proposed that B-cell specificity leads to selective antigen presentation to helper T cells and, therefore, to selective help from T cells specific for some epitopes more than from T cells specific for others.[130]

To test this hypothesis, Ozaki and Berzofsky[129] made populations of B cells effectively monoclonal for purposes of antigen presentation by coating polyclonal B cells with a conjugate of monoclonal antimyoglobin coupled to anti-IgM antibodies. B cells coated with one such conjugate presented myoglobin less well to one myoglobin-specific T-cell clone than to others. B cells coated with other conjugates presented myoglobin to this clone equally well as to other clones. Therefore, the limitation on myoglobin presentation by this B cell to this T-cell clone depended on the specificity of both the monoclonal antibody coating the B cell and the receptor of the T-cell clone. It happened in this case that both the monoclonal antibody and the T-cell clone were specific for the same or closely overlapping epitopes. Therefore, it appears that the site bound by the B-cell surface Ig is less well presented to T cells. This finding is also consistent with a recent study of chimeric proteins in which one or more copies of an ovalbumin helper T-cell determinant were inserted in different positions.[597] Although the position of the ovalbumin determinants did not affect the antibody response to one epitope, the position did matter for antibody production to an epitope of the chimeric protein derived from insulin-like growth factor I. An ovalbumin determinant inserted distal to this epitope was much more effective in providing help than one inserted adjacent to the same epitope, when both constructs were used as immunogens, even though both constructs elicited similar levels of ovalbumin-specific T-cell proliferation in the presence of nonspecific presenting cells in vitro as a control for nonspecific effects of flanking residues on processing and presentation of the helper T-cell determinants. However, circumstantial evidence from the Ir gene studies mentioned previously suggests that T cells may preferentially help B cells that bind with some degree of proximity to the T-cell epitope, as there was a correlation between T cell and antibody specificity for large fragments of protein antigens under Ir gene control.[127,130,181,579,580,583] Therefore, antibodies may have both positive and negative selective effects on processing. Further studies on presentation of β-galactosidase-monoclonal antibody complexes by nonspecific APCs suggest similar conclusions.[598,599] Presumably, the conjugates are taken up via Ig fragment c (Fc) receptors on the presenting cells and processed differentially according to the site bound by the antibody so that they are presented differentially to different T-cell clones. Thus, non-B-presenting cells can be made to mimic specific B-presenting cells. This also suggests that circulating antibody may have a role in the selection of which T cells are activated in a subsequent exposure to antigen.

The issue of whether bound antibody enhanced or suppressed presentation of specific determinants to T cells was explored further by Watts et al.[600] and Simitsek et al.[601] They first found that a particular tetanus toxoid-specific Epstein-Barr virus-transformed human B-

cell clone 11.3 failed to present the tetanus toxoid epitope 1174 to 1189 to specific T cells, whereas it presented another epitope as well as did other B cells, and another B-cell clone presented the 1174 to 1189 epitope well. Moreover, the free 11.3 antibody also inhibited presentation of this epitope to T cells at the same time that it enhanced presentation of other epitopes by Fc receptor facilitated uptake.[600] They subsequently found that the same 11.3 B cell and antibody actually enhanced presentation of another epitope of tetanus toxoid, 1273 to 1284, by about 10-fold, even though both epitopes were within the footprint of the antibody as determined by protection from proteolytic digestion.[601] The enhancement could be mediated also by free antibody as well as Fab fragments thereof, indicating that the mechanism did not involve Fc receptor facilitated uptake. Furthermore, the 11.3 antibody had no effect on presentation of another determinant in the same tetanus toxoid C fragment, 947 to 967, which was not within the footprint of the antibody, and another antibody to the C fragment did not enhance presentation of 1273 to 1284. The authors concluded that the same antibody or surface Ig can protect two determinants from proteolysis but sterically hinder the binding of one to class II MHC molecules while facilitating the binding of the other.[601] The facilitation may involve protection from degradation. This antibody-mediated enhancement of presentation of selected epitopes to helper T cells can greatly lower the threshold for induction of a T-cell response and may thereby elicit responses to otherwise subdominant epitopes. It can also contribute to epitope spreading, for example, in autoimmune disease, in which an initial response to one dominant determinant leads to a subsequent response to other subdominant determinants, perhaps by helping for antibody production, which in turn facilitates presentation of the other determinants.

Taken together, these results support the concept of T-B reciprocity in which helper T cells and B cells each influence the specificity of the other's expressed repertoire.[130] This mechanism may also provide an explanation for some of the cases in which Ir genes have been found to control antibody idiotype.[602,603] These relationships probably play a significant role in regulating the fine specificity of immune response of both arms of the immune system. Therefore, they will also be of importance in the design of synthetic or recombinant fragment vaccines that incorporate both T- and B-cell epitopes to elicit an antibody response.

CONCLUSION

Overall, antibodies and T cells recognize different structural features in different contexts or environments, and thus complement each other to detect the spectrum of foreign (or self-) antigens encountered. Antibodies recognize three-dimensional structures on the exposed surface of molecules either in solution or on a cell surface.

Therefore, they are dependent on the conformation of the antigen and can recognize structures that are assembled on the surface of the antigen molecule by the way it folds but that are not contiguous in the primary sequence. However, they do not generally recognize structures buried within protein molecules or inside cells. In contrast, T cells are designed to recognize short segments of primary amino acid sequence of protein antigens, and thus are not dependent on the conformation of the original protein unless it affects processing. Furthermore, T cells particularly provide internal surveillance of proteins inside cells, recognizing peptide fragments of these presented by MHC molecules that carry these fragments to the cell surface. This surveillance of intracellular proteins requires a number of hurdles including proteolytic processing of the antigen into fragments, transport of these into the compartments where they are loaded onto MHC molecules, binding with appropriate specificity to the combining site of specific MHC molecules, and then finally recognition by an

appropriate TCR. These hurdles limit the number of amino acid sequences that can be recognized and account in part for immunodominance. Furthermore, the two major subsets of T cells also complement each other by recognizing exogenous and endogenous proteins processed and transported through different pathways and presented by difference classes of MHC molecules. Thus, the two major classes of T cells complement the types of structures and the locales surveyed by antibodies. For example, in the case of viruses, antibodies can detect intact virions and shed viral proteins in solution as well as viral proteins expressed on the surface of infected cells; $CD4^+$ T cells can recognize viral proteins taken up by APCs and processed in an endosomal pathway and $CD8^+$ T cells can detect proteins synthesized within the infected cell, whether or not they are ever expressed intact on the cell surface or secreted. Together, they provide the immune system with a strategy to detect all forms of foreign invaders as well as to protect the host through different effector mechanisms.

REFERENCES

1. Goodman JW. Antigenic determinants and antibody combining sites. In: Sela M, ed. *The Antigens*. New York, NY: Academic; 1975:127-187.

2. Landsteiner K. *The Specificity of Serological Reactions* . Cambridge, MA: Harvard University Press; 1945.

3. Landsteiner K, Van der Scheer J. Serological studies on azoproteins antigens containing azo components with aliphatic side chains. *J Exp Med*. 1934;59:751-768.

4. Pressman D, Grossberg AL. *The Structural Basis of Antibody Specificity* . New York, NY: Benjamin; 1968.

5. Pressman D, Siegel M, Hall LAR. The closeness of fit of antibenzoate antibodies about haptens and the orientation of the haptens in combination. *J Am Chem Soc*. 1954;76:6336-6341.

6. Karush F. The interaction of purified anti-b-lactoside antibody with haptens. *J Am Chem Soc*. 1957;79:3380-3384.

7. Eisen HN, Siskind GW. Variations in affinities of antibodies during the immune response. *Biochem*. 1964;3:996-1008.

8. Kabat EA. *Structural Concepts in Immunology and Immunochemistry* . 2nd ed. New York, NY: Holt Rinehart & Winston; 1976.

9. Jann K, Westphal O. Microbial polysaccharides. In: Sela M, ed. *The Antigens*. New York, NY: Academic; 1975:1-125.

10. Springer GF. Blood group and Forssman antigenic determinants shared between microbes and mammalian cells. In: Kallos P, Waksman BH, eds. *Progress in Allergy*. Basel, Switzerland: Karger; 1971:9-77.

11. Marcus DM. The ABO and Lewis blood-group system. Immunochemistry, genetics,

and relation to human disease. *N Engl J Med*. 1969;280:994-1006.

12. Watkins WM. Biochemistry and genetics of the ABO, Lewis, and P blood group systems. *Adv Hum Genet*. 1980;10:1-136.

13. Kabat EA. The upper limit for the size of the human antidextran combining site. *J Immunol*. 1960;84:82-85.

14. Kabat EA. The nature of an antigenic determinant. *J Immunol*. 1966;97:1-11.

15. Cisar J, Kabat EA, Dorner MM, Liao J. Binding properties of immunoglobulin combining sites specific for terminal or nonterminal antigenic determinants in dextran. *J Exp Med*. 1975;142:435-459.

16. Sharon J, Kabat EA, Morrison SL. Studies on mouse hybridomas secreting IgM or IgA antibodies to a(1->6)-linked dextran. *Mol Immunol*. 1981;18:831-846.

17. Sharon J, Kabat EA, Liao J, Potter M. Immunochemical characterization of binding sites of hybridoma antibodies specific for alpha (1 leads to 6) linked dextran. *Mol Immunol*. 1982;19:375-388.

18. Sharon J, D'Hoostelaere L, Potter M, Kabat EA, Morrison SL. A crossreactive idiotype, QUPC 52 IdX, present on most but not all anti-alpha (1 replaced by 6) dextran-specific IgM and IgA hydriboma antibodies with combining sites of different sizes. *J Immunol*. 1982;128:498-500.

19. Sikder SK, Akolkar PN, Kaladas PM, Morrison SL, Kabat EA. Sequences of variable regions of hybridoma antibodies to alpha (1——6) dextran in BALB/c and C57BL/6 mice. *J Immunol*. 1985;135:4215-4221.

20. Akolkar PN, Sikder SK, Bhattacharya SB, et al. Different VL and VH germline genes are used to produce similar combining sites with specificity for alpha(1——6) dextrans. *J Immunol*. 1987;138:4472-4479.

21. Brodeur PM, Riblet R. The immunoglobulin heavy chain variable region (Igh-v) locus in the mouse I. One-hundred Igh-v genes comprise seven families of homologous genes. *Eur J Immunol* . 1984;14:922-930.

22. Griffiths GM, Berek C, Kaartinen M, Milstein C. Somatic mutation and the maturation of immune response to 2-phenxyloxazolone. *Nature*. 1984;14:271-275.

23. Cygler M, Rose DR, Bundle DR. Recognition of a cell-surface oligosaccharide of pathogenic Salmonella by an antibody Fab fragment. *Science*. 1991;253:442-445.

24. Bundle DR, Eichler E, Gidney MA, et al. Molecular recognition of a Salmonella trisaccharide epitope by monoclonal antibody Se155-4. *Biochemistry*. 1994;33:5172-5182.

25. Jeffrey PD, Bajorath J, Chang CY, et al. The x-ray structure of an antitumour antibody

in complex with antigen. *Nat Struct Biol* . 1995;2:466-471.

26. Ramsland PA, Farrugia W, Bradford TM, Mark Hogarth P, Scott AM. Structural convergence of antibody binding of carbohydrate determinants in Lewis Y tumor antigens. *J Mol Biol* . 2004;340:809-818.

27. Calarese DA, Scanlan CN, Zwick MB, et al. Antibody domain exchange is an immunological solution to carbohydrate cluster recognition. *Science*. 2003;300:2065-2071.

28. Calarese DA, Lee HK, Huang CY, et al. Dissection of the carbohydrate specificity of the broadly neutralizing anti-HIV-1 antibody 2G12. *Proc Natl Acad Sci U S A* . 2005;102:13372-13377.

29. Schneerson R, Barrera O, Sutton A, Robbins JB. Preparation, characterization, and immunogenicity of Haemophilus influenzae type b polysaccharide-protein conjugates. *J Exp Med*. 1980;152:361-376.

30. Robbins JB, Schneerson R, Anderson P, Smith DH. The 1996 Albert Lasker Medical Research Awards. Prevention of systemic infections, especially meningitis, caused by Haemophilus influenzae type b. Impact on public health and implications for other polysaccharide-based vaccines. *JAMA*. 1996;276:1181-1185.

31. Murphy TV, White KE, Pastor P, et al. Declining incidence of Haemophilus influenzae type b disease since introduction of vaccination. *JAMA*. 1993;269:246-248.

32. Black S, Shinefield H, Fireman B, et al. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. *Pediatr Infect Dis J* . 2000;19:187-195.

33. Eskola J, Kilpi T, Palmu A, et al. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. *N Engl J Med* . 2001;344: 403-409.

34. Amit AG, Mariuzza RA, Phillips SEV, Poljak RJ. Three-dimensional structure of an antigen-antibody complex at 2.8 Å resolution. *Science*. 1986;233:747-758.

35. Colman PM, Laver WG, Varghese JN, et al. Three-dimensional structure of a complex of antibody with influenza virus neuraminidase. *Nature*. 1987;326:358-363.

36. Sheriff S, Silverton EW, Padlan EA, et al. Three-dimensional structure of an antibody-antigen complex. *Proc Natl Acad Sci U S A* . 1987; 84:8075-8079.

37. Davies DR, Padlan EA. Antibody-antigen complexes. *Annu Rev Biochem*. 1990;59:439-473.

38. Schlossman SF, Yaron A, Ben-Efraim S, Sober HA. Immunogenicity of a series of alpha,N-DNP-L-lysines. *Biochem*. 1965;4:1638-1645.

39. Schlossman SF, Levine H. Desensitization to delayed hypersensitivity reactions. With

special reference to the requirement for an immunogenic molecule. *J Immunol*. 1967;99:111-114.

40. Van Vunakis H, Kaplan J, Lehrer H, Levine L. Immunogenicity of polylysine and polyornithine when complexed to phosphorylated bovine serum albumin. *Immunochemistry*. 1966;3:393-402.

41. Getzoff ED, Tainer JA, Lerner RA, Geysen HM. The chemistry and mechanisms of antibody binding to protein antigens. *Adv Immunol*. 1988;43:1-98.

42. Sela M. Antigenicity: some molecular aspects. *Science*. 1969;166: 1365-1374.

43. Berzofsky JA, Buckenmeyer GK, Hicks G, Gurd FRN, Feldmann RJ, Minna J. Topographic antigenic determinants recognized by monoclonal antibodies to sperm whale myoglobin. *J Biol Chem*. 1982;257:3189-3198.

44. Koketsu J, Atassi MZ. Immunochemistry of sperm-whale myoglobin-XVI: accurate delineation of the single region in sequence 1-55 by immunochemical studies of synthetic peptides. Some conclusions concerning antigenic structures of proteins. *Immunochemistry*. 1974;11:1-8.

45. Dickerson RE. X-ray analysis and protein structure. In: Neurath H, ed. *The Proteins*. New York, NY: Academic; 1964:603-778.

46. Takano T. Structure of myoglobin refined at 2.0 Å resolution I crystallographic refinement of metmyoglobin from sperm whale. *J Mol Biol*. 1977;110:537-568.

47. Feldmann RJ, Bing DH, Furie BC, Furie B. Interactive computer surface graphics approach to the study of the active site of bovine trypsin. *Proc Natl Acad Sci U S A*. 1978;75:5409-5412.

48. Barlow DJ, Edwards MS, Thornton JM. Continuous and discontinuous protein antigenic determinants. *Nature*. 1986;322:747-748.

49. Lollier V, Denery-Papini S, Larre C, Tessier D. A generic approach to evaluate how B-cell epitopes are surface-exposed on protein structures. *Mol Immunol*. 2011;48:577-585.

50. Benjamin DC, Berzofsky JA, East IJ, et al. The antigenic structure of proteins: a reappraisal. *Annu Rev Immunol*. 1984;2:67-101.

51. Berzofsky JA. Intrinsic and extrinsic factors in protein antigenic structure. *Science*. 1985;229:932-940.

52. Sachs DH, Schechter AN, Eastlake A, Anfinsen CB. An immunological approach to the conformational equilibria of polypeptides. *Proc Natl Acad Sci U S A*. 1972;69:3790-3794.

53. Crumpton MJ. Protein antigen: the molecular bases of antigenicity and immunogenicity. In: Sela M, ed. *The Antigens*. New York, NY: Academic; 1974:1-79.

54. Serriere J, Dugua JM, Bossus M, et al. Fab'-induced folding of antigenic N-terminal peptides from intrinsically disordered HIV-1 tat revealed by x-ray crystallography. *J Mol Biol*. 2011;405:33-42.

55. Yewdell JW. Monoclonal antibodies specific for discontinuous epitopes direct refolding of influenza A virus hemagglutinin. *Mol Immunol*. 2010;47:1132-1136.

56. Burnens A, Demotz S, Corradin G, Binz H, Bosshard HR. Epitope mapping by chemical modification of free and antibody-bound protein antigen. *Science*. 1987;235:780-783.

57. Jemmerson R, Paterson Y. Mapping epitopes on a protein antigen by the proteolysis of antigen-antibody complexes. *Science*. 1986;232: 1001-1004.

58. Smith-Gill SJ, Wilson AC, Potter M, Prager EM, Feldmann RJ, Mainhart CR. Mapping the antigenic epitope for a monoclonal antibody against lysozyme. *J Immunol*. 1982;128:314-322.

59. Kohno Y, Berkower I, Minna J, Berzofsky JA. Idiotypes of anti-myoglobin antibodies: shared idiotypes among monoclonal antibodies to distinct determinants of sperm whale myoglobin. *J Immunol*. 1982; 128:1742-1748.

60. Streicher HZ, Cuttitta F, Buckenmeyer GK, Kawamura H, Minna J, Berzofsky JA. Mapping the idiotopes of a monoclonal anti-idiotypic antibodies: Detection of a common idiotope. *J Immunol*. 1986;136: 1007-1014.

61. Geysen HM, Rodda SJ, Mason TJ, Tribbick G, Schoofs PG. Strategies for epitope analysis using peptide synthesis. *J Immunol Methods*. 1987;102:259-274.

62. Denisova GF, Denisov DA, Yeung J, Loeb MB, Diamond MS, Bramson JL. A novel computer algorithm improves antibody epitope prediction using affinity-selected mimotopes: a case study using monoclonal antibodies against the West Nile virus E protein. *Mol Immunol*. 2008; 46:125-134.

63. Sadler K, Zhang Y, Xu JX, Yu QT, Tam JP. Quaternary protein mimetics of gp41 elicit neutralizing antibodies against HIV fusionactive intermediate state. *Biopolymers*. 2008;90:320-329.

64. Bublil EM, Freund NT, Mayrose I, et al. Stepwise prediction of conformational discontinuous B-cell epitopes using the Mapitope algorithm. *Proteins*. 2007;68:294-304.

65. Mayrose I, Shlomi T, Rubinstein ND, et al. Epitope mapping using combinatorial phage-display libraries: a graph-based algorithm. *Nucleic Acids Res*. 2007;35:69-78.

66. Pacios LF, Tordesillas L, Palacin A, Sanchez-Monge R, Salcedo G, Diaz-Perales A. LocaPep: localization of epitopes on protein surfaces using peptides from phage display libraries. *J Chem Inform Model*. 2011;51:1465-1473.

67. Irving MB, Craig L, Menendez A, et al. Exploring peptide mimics for the production of antibodies against discontinuous protein epitopes. *Mol Immunol*. 2010;47:1137-1148.

68. Sun PP, Chen WH, Huang YX, Wang HY, Ma ZQ, Lv YH. Epitope prediction based on random peptide library screening: benchmark dataset and prediction tools evaluation. *Molecules*. 2011;16: 4971-4993.

69. Crumpton MJ, Wilkinson JM. The immunological activity of some of the chymotryptic peptides of sperm-whale myoglobin. *Biochem J*. 1965;94:545-556.

70. Smith JA, Hurrell JGR, Leach SJ. A novel method for delineating antigenic determinants: peptide synthesis and radioimmunoassay using the same solid support. *Immunochemistry*. 1977;14:565-568.

71. Atassi MZ. Antigenic structure of myoglobin: the complete immunochemical anatomy of a protein and conclusions relating to antigenic structures of proteins. *Immunochemistry*. 1975;12:423-438.

72. Berzofsky JA, Buckenmeyer GK, Hicks G, et al. Topographic antigenic determinants detected by monoclonal antibodies to myoglobin. In: Celada F, Sercarz E, Shumaker V, eds. *Protein Conformation as Immunological Signal*. New York, NY: Plenum Press; 1983:165-180.

73. Hurrell JGR, Smith JA, Todd PE, Leach SJ. Cross-reactivity between mammalian myoglobins: linear vs. spatial antigenic determinants. *Immunochemistry*. 1977;14:283-288.

74. East IJ, Todd PE, Leach SJ. On topographic antigenic determinants in myoglobins. *Mol Immunol*. 1980;17:519-525.

75. Maron E, Shiozawa C, Arnon R, Sela M. Chemical and immunological characterization of a unique antigenic region in lysozyme. *Biochem*. 1971;10:763-771.

76. Berzofsky JA, Hicks G, Fedorko J, Minna J. Properties of monoclonal antibodies specific for determinants of a protein antigen, myoglobin. *J Biol Chem*. 1980;255:11188-11191.

77. East IJ, Hurrell JGR, Todd PE, Leach SJ. Antigenic specificity of monoclonal antibodies to human myoglobin. *J Biol Chem*. 1982;257: 3199-3202.

78. Zhou T, Georgiev I, Wu X, et al. Structural basis for broad and potent neutralization of HIV-1 by antibody VRC01. *Science*. 2010;329: 811-817.

79. Zhou TQ, Xu L, Dey B, et al. Structural definition of a conserved neutralization epitope on HIV-1 gp120. *Nature*. 2007;445:732-737.

80. Sasamori E, Suzuki S, Kato M, Tagawa Y, Hanyu Y. Characterization of discontinuous epitope of prion protein recognized by the mono clonal antibody T2. *Arch Biochem Biophysics*. 2010;501:232-238.

81. Arquilla ER, Bromer WW, Mercola D. Immunology conformation and biological activity of insulin. *Diabetes*. 1969;18:193-205.

82. Lau HKF, Reichlin M, Noble RW. Preparation of antibodies that bind to HbF but not to the isolated a and g subunits. *Fed Proc*. 1975;34: 975-980.

83. Benjamini E, Shimizu M, Yound JD, Leung CY. Immunochemical studies on the tobacco mosaic virus protein. VII. The binding of octanoylated peptides of the tobacco mosaic virus protein with antibodies to the whole protein. *Biochem*. 1968;7:1261-1264.

84. Urbanski GJ, Margoliash E. Topographic determinants on cytochrome c: I. The complete antigenic structures of rabbit, mouse, and guanaco cytochromes c in rabbits and mice. *J Immunol* . 1977;118:1170-1180.

85. Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature*. 1998;393:648-659.

86. Wyatt R, Kwong PD, Desjardins E, et al. The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature*. 1998;393:705-711.

87. Lando G, Berzofsky JA, Reichlin M. Antigenic structure of sperm whale myoglobin: I. Partition of specificities between antibodies reactive with peptides and native protein. *J Immunol* . 1982;129:206-211.

88. White TJ, Ibrahimi IM, Wilson AC. Evolutionary substitutions and the antigenic structure of globular proteins. *Nature*. 1978;274:92-94.

89. Reichlin M. Amino acid substitution and the antigenicity of globular proteins. *Adv Immunol* . 1975;20:71-123.

90. Kabat EA. The structural basis of antibody complementarity. *Adv Protein Chem*. 1978;32:1-76.

91. Berzofsky JA, Schechter AN. The concepts of crossreactivity and specificity in immunology. *Molec Immunol* . 1981;18:751-763.

92. Kourentzi K, Srinivasan M, Smith-Gill SJ, Willson RC. Conformational flexibility and kinetic complexity in antibody-antigen interaction. *J Mol Recog* . 2008;21:114-121.

93. Furie B, Schechter AN, Sachs DH, Anfinsen CB. An immunological approach to the conformational equilibria of staphylococcal nuclease. *J Mol Biol* . 1975;92:497-506.

94. Green N, Alexander H, Wilson A, et al. Immunogenic structure of the influenza virus hemagglutinin. *Cell* . 1982;28:477-487.

95. Lerner R. Antibodies of predetermined specificity in biology and medicine. *Adv*

Immunol. 1984;36:1-44.

96. Camacho CJ, Katsumata Y, Ascherman DP. Structural and thermodynamic approach to peptide immunogenicity. *Plos Computat Biol*. 2008;4.

97. McLellan JS, Correia BE, Chen M, et al. Design and characterization of epitope-scaffold immunogens that present the motavizumab epitope from respiratory syncytial virus. *J Mol Biol*. 2011;409:853-866.

98. Totrov M, Jiang XQ, Kong XP, et al. Structure-guided design and immunological characterization of immunogens presenting the HIV-1 gp120 V3 loop on a CTB scaffold. *Virology*. 2010;405:513-523.

99. Correia BE, Ban YEA, Friend DJ, et al. Computational protein design using flexible backbone remodeling and resurfacing: case studies in structure-based antigen design. *J Mol Biol*. 2011;405:284-297.

100. Perutz MF. Hemoglobin structure and respiratory transport. *Sci Am*. 1978;239:92-125.

101. Monod J, Wyman J, Changeux JP. On the nature of allosteric transitions: a plausible model. *J Mol Biol*. 1965;12:88-118.

102. Papkoff J, Lai MH-T, Hunter T, Verma IM. Analysis of transforming gene products from Moloney murine sarcoma virus. *Cell*. 1981;27: 109-119.

103. Colman PM, Varghese JN, Laver WG. Structure of the catalytic and antigenic sites in influenza virus neuraminidase. *Nature*. 1983;303: 41-44.

104. Lee B, Richards FM. The interpretation of protein structures: estimation of static accessibility. *J Mol Biol*. 1971;55:379-400.

105. Connolly ML. Solvent-accessible surfaces of proteins and nucleic acids. *Science*. 1983;221:709-713.

106. Novotny J, Handschumacher M, Haber E, et al. Antigenic determinants in proteins coincide with surface regions accessible to large probes (antibody domains). *Proc Natl Acad Sci U S A*. 1986;83:226-230.

107. Thornton JM, Edwards MS, Taylor WR, Barlow DJ. Location of “continuous” antigenic determinants in protruding regions of proteins. *EMBO J*. 1986;5:409-413.

108. Hopp TP, Woods KR. Prediction of protein antigenic determinants from amino acid sequences. *Proc Natl Acad Sci U S A*. 1981;78:3824-3828.

109. Fraga S. Theoretical prediction of protein antigenic determinants from amino acid sequences. *Can J Chem*. 1982;60:2606-2610.

110. Hopp TP. Protein surface analysis: methods for identifying antigenic determinants

and other interaction sites. *J Immunol Methods*. 1986; 88:1-18.

111. Leach SJ. How antigenic are antigenic peptides? *Biopolymers*. 1983;22: 425-440.

112. Todd PEE, East IJ, Leach SJ. The immunogenicity and antigenicity of proteins. *Trends Biochem Sci*. 1982;7:212-216.

113. Chothia C, Janin J. Principles of protein-protein recognition. *Nature*. 1975;256:705-708.

114. Chen J, Liu H, Yang J, Chou KC. Prediction of linear B-cell epitopes using amino acid pair antigenicity scale. *Amino Acids*. 2007;33: 423-428.

115. Tainer JA, Getzoff ED, Paterson Y, Olson AJ, Lerner RA. The atomic mobility component of protein antigenicity. *Annu Rev Immunol*. 1985;3:501-535.

116. Tainer JA, Getzoff ED, Alexander H, et al. The reactivity of anti-peptide antibodies is a function of the atomic mobility of sites in a protein. *Nature*. 1984;312:127-133.

117. Westhof E, Altschuh D, Moras D, et al. Correlation between segmental mobility and the location of antigenic determinants in proteins. *Nature*. 1984;311:123-126.

118. Al Moudallal Z, Briand JP, Van Regenmortel MHV. A major part of the polypeptide chain of tobacco masaic virus protein is antigenic. *EMBO J*. 1985;4:1231-1235.

119. Van Regenmortel MHV. Antigenic cross-reactivity between proteins and peptides: new insights and applications. *Trends Biochem Sci*. 1987;12:237-240.

120. Geysen HM, Tainer JA, Rodda SJ, et al. Chemistry of antibody binding to a protein. *Science*. 1987;235:1184-1190.

121. Scarabelli G, Morra G, Colombo G. Predicting interaction sites from the energetics of isolated proteins: a new approach to epitope mapping. *Biophys J*. 2010;98:1966-1975.

122. Edmundson AB, Ely KR, Herron JN. A search for site-filling ligands in the Meg Bence-Jones dimer: crystal binding studies of fluorescent compounds. *Mol Immunol*. 1984;21:561-576.

123. Getzoff ED, Geysen HM, Rodda SJ, Alexander H, Tainer JA, Lerner RA. Mechanisms of antibody binding to a protein. *Science*. 1987;235: 1191-1196.

124. Rini JM, Schulze-Gahmen U, Wilson IA. Structural evidence for induced fit as a mechanism for antibody-antigen recognition. *Science*. 1992;255:959-965.

125. Jemmerson R, Margoliash E. Topographic antigenic determinants on cytochrome c. Immunoadsorbent separation of rabbit antibody populations directed against horse cytochrome c. *J Biol Chem*. 1979;254: 12706-12716.

126. Cooper HM, East IJ, Todd PEE, Leach SJ. Antibody response to myoglobins: effect of host species. *Mol Immunol* . 1984;21:479-487.

127. Berzofsky JA. Ir genes: antigen-specific genetic regulation of the immune response. In: Sela M, ed. *The Antigens*. New York: Academic Press; 1987:1-146.

128. Manca F, Kunkl A, Fenoglio D, Fowler A, Sercarz E, Celada F. Constraints in T-B cooperation related to epitope topology on E. coli b-galactosidase. I. The fine specificity of T cells dictates the fine specificity of antibodies directed to conformation-dependent determinants. *Eur J Immunol* . 1985;15:345-350.

129. Ozaki S, Berzofsky JA. Antibody conjugates mimic specific B cell presentation of antigen: relationship between T and B cell specificity. *J Immunol* . 1987;138:4133-4142.

130. Berzofsky JA. T-B reciprocity: an Ia-restricted epitope-specific circuit regulating T cell-B cell interaction and antibody specificity. *Survey of Immunol Res* . 1983;2:223-229.

131. Potocnjak P, Yoshida N, Nussenzweig RS, Nussenzweig V. Monovalent fragments (Fab) of monoclonal antibodies to a sporozoite surface antigen (Pb44) protect mice against malarial infection. *J Exp Med*. 1980;151:1504.

132. Good MF, Berzofsky JA, Maloy WL, et al. Genetic control of the immune response in mice to a Plasmodium facilparum sporozoite vaccine: widespread non-responsiveness to a single malaria T epitope in highly repetitive vaccine. *J Exp Med*. 1986;164:655-660.

133. Del Giudice G, Cooper JA, Merino J, et al. The antibody response in mice to carrier-free synthetic polymers of Plasmodium falciparum circumsporozoite repetitive epitope is I-Ab-restricted: possible implications for malaria vaccines. *J Immunol* . 1986;137:2952-2955.

134. Herrington DA, Clyde DF, Losonsky G, et al. Safety and immunogenicity in man of a synthetic peptide malaria vaccine against Plasmodium falciparum sporozoites. *Nature*. 1987;328:257-259.

135. Good MF, Maloy WL, Lunde MN, et al. Construction of a synthetic immunogen: use of a new T-helper epitope on malaria circumsporozoite protein. *Science*. 1987;235:1059-1062.

136. Kawamura H, Rosenberg SA, Berzofsky JA. Immunization with antigen and interleukin-2 in vivo overcomes Ir genetic low responsiveness. *J Exp Med*. 1985;162:381-386.

137. Good MF, Pombo D, Lunde MN, et al. Recombinant human interleukin-2 (IL-2) overcomes genetic nonresponsiveness to malaria sporozoite peptides. Correlation of effect with biological activity of IL-2. *J Immunol* . 1988;141:972-977.

137a. Akaji K, Liu DT, Berkower IJ. unpublished results

138. Le Bouvier GL. The D-> C change in poliovirus particles. *Br J Exp Pathol* .

1959;40:605-620.

139. Gerhard W, Yewdell JW, Frankel ME, Webster RG. Antigenic structure of influenza virus hemagglutinin defined by hybridoma antibodies. *Nature*. 1981;290:713-717.

140. Emini EA, Kao SY, Lewis AJ, Crainic R, Wimmer E. Functional basis of poliovirus neutralization determined with mono-specific neutralizing antibodies. *J Virol*. 1983;46:466-474.

141. Wiley DC, Wilson EA, Skehel JJ. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature*. 1981;289:373-378.

142. Icenogle J, Shiwen H, Duke G, Gilbert S, Rueckert R, Anderegg J. Neutralization of poliovirus by a monoclonal antibody: kinetics and stoichiometry. *Virology*. 1983;127:412-425.

143. Mandel B. Interaction of viruses with neutralizing antibodies. In: Fraenkel-Conrat H, Wagner RR, eds. *Comprehensive Virology 15: Viral-Host Interactions*. New York, NY: Plenum; 1979.

144. Kaplan G, Freistadt MS, Racaniello VR. Neutralization of poliovirus by cell receptors expressed in insect cells. *J Virol*. 1990;64:4697-4702.

145. Smith DH, Byrn RA, Marsters SA, Gregory T, Groopman JE, Capon DJ. Blocking of HIV-1 infectivity by a soluble, secreted form of the CD4 antigen. *Science*. 1987;238:1704-1707.

146. Fisher RA, Bertonis JM, Meier W, et al. HIV infection is blocked in vitro by recombinant soluble CD4. *Nature*. 1988;331:76-78.

147. Hussey RE, Richardson NE, Kowalski M, et al. A soluble CD4 protein selectively inhibits HIV replication and syncytium formation. *Nature*. 1988;331:78-81.

148. Capon DJ, Chamow SM, Mordenti J, et al. Designing CD4 immunoadhesins for AIDS therapy. *Nature*. 1989;337:525-531.

149. DeVico A, Fouts T, Lewis GK, et al. Antibodies to CD4-induced sites in HIV gp120 correlate with the control of SHIV challenge in macaques vaccinated with subunit immunogens. *Proc Natl Acad Sci U S A* . 2007; 104:17477-17482.

150. Palker TJ, Clark ME, Langlois AJ, et al. Type-specific neutralization of the human immunodeficiency virus with antibodies to env-encoded synthetic peptides. *Proc Natl Acad Sci U S A*. 1988;85:1932-1936.

151. Rusche JR, Javaherian K, McDanal C, et al. Antibodies that inhibit fusion of HIV infected cells bind a 24 amino acid sequence of the viral envelope, gp120. *Proc Natl Acad Sci U S A*. 1988;85:3198-3202.

152. Goudsmit J, Debouck C, Meloen RH, et al. Human immunodeficiency virus type 1 neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees. *Proc Natl Acad Sci U S A* . 1988;85:4478-4482.

153. Gorny MK, VanCott TC, Hioe C, et al. Human monoclonal antibodies to the V3 loop of HIV-1 with intra- and interclade cross-reactivity. *J Immunol* . 1997;159:5114-5122.

154. Gorny MK, Williams C, Volsky B, et al. Human monoclonal antibodies specific for conformation-sensitive epitopes of V3 neutralize human immunodeficiency virus type 1 primary isolates from various clades. *J Virol* . 2002;76:9035-9045.

155. Sharon M, Kessler N, Levy R, Zolla-Pazner S, Gorlach M, Anglister J. Alternative conformations of HIV-1 V3 loops mimic beta hairpins in chemokines, suggesting a mechanism for coreceptor selectivity. *Structure (Camb)*. 2003;11:225-236.

156. Berkower I, Smith GE, Giri C, Murphy D. Human immunodeficiency virus-1: predominance of a group-specific neutralizing epitope that persists despite genetic variation. *J Exp Med* . 1989;170:1681-1695.

157. Kang CY, Nara P, Chamat S, et al. Evidence for non-V3-specific neutralizing antibodies that interfere with gp120/CD4 binding in human immunodeficiency virus 1-infected humans. *Proc Natl Acad Sci U S A* . 1991;88:6171-6175.

158. Berkower I, Murphy D, Smith CC, Smith GE. A predominant groupspecific neutralizing epitope of human immunodeficiency virus type 1 maps to residues 342 to 511 of the envelope glycoprotein gp120. *J Virol* . 1991;65:5983-5990.

159. Thali M, Olshevsky U, Furman C, Gabuzda D, Posner M, Sodroski J. Characterization of a discontinuous human immunodeficiency virus type 1 gp120 epitope recognized by a broadly reactive neutralizing human monoclonal antibody. *J Virol* . 1991;65:6188-6193.

160. Tilley SA, Honnen WJ, Racho ME, Hilgartner M, Pinter A. A human monoclonal antibody against the CD4-binding site of HIV1 gp120 exhibits potent, broadly neutralizing activity. *Res Virol* . 1991;142: 247-259.

161. Kessler JA 2nd, McKenna PM, Emini EA, et al. Recombinant human monoclonal antibody IgG1b12 neutralizes diverse human immunodeficiency virus type 1 primary isolates. *AIDS Res Hum Retroviruses*. 1997;13:575-582.

162. Trkola A, Purtscher M, Muster T, et al. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. *J Virol* . 1996;70:1100-1108.

163. Muster T, Steindl F, Purtscher M, et al. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. *J Virol* . 1993;67:6642-6647.

164. Eckhart L, Raffelsberger W, Ferko B, et al. Immunogenic presentation of a conserved gp41 epitope of human immunodeficiency virus type 1 on recombinant surface

antigen of hepatitis B virus. *J Gen Virol*. 1996;77:2001-2008.

165. Burton DR. A vaccine for HIV type 1: the antibody perspective. *Proc Natl Acad Sci U S A*. 1997;94:10018-10023.

166. Dennison SM, Stewart SM, Stempel KC, Liao HX, Haynes BF, Alam SM. Stable docking of neutralizing human immunodeficiency virus type 1 gp41 membrane-proximal external region monoclonal antibodies 2F5 and 4E10 is dependent on the membrane immersion depth of their epitope regions. *J Virol*. 2009;83:10211-10223.

167. Trkola A, Ketas T, Kewalramani VN, et al. Neutralization sensitivity of human immunodeficiency virus type 1 primary isolates to antibodies and CD4-based reagents is independent of coreceptor usage. *J Virol*. 1998;72:1876-1885.

168. Baba TW, Liska V, Hofmann-Lehmann R, et al. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. *Nat Med*. 2000;6: 200-206.

169. Mascola JR, Stiegler G, VanCott TC, et al. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat Med*. 2000;6: 207-210.

170. Steimer KS, Scandella CJ, Skiles PV, Haigwood NL. Neutralization of divergent HIV-1 isolates by conformation-dependent human antibodies to Gp120. *Science*. 1991;254:105-108.

171. Benacerraf B, McDevitt HO. Histocompatibility-linked immune response genes. *Science*. 1972;175:273-279.

172. Godfrey WL, Lewis GK, Goodman JW. The anatomy of an antigen molecule: functional subregions of L-tyrosine-p-azobenzenearsonate. *Mol Immunol*. 1984;21:969-978.

173. Berkower I, Buckenmeyer GK, Gurd FRN, Berzofsky JA. A possible immunodominant epitope recognized by murine T lymphocytes immune to different myoglobins. *Proc Natl Acad Sci U S A*. 1982;79: 4723-4727.

174. Solinger AM, Ultee ME, Margoliash E, Schwartz RH. The T-lymphocyte response to cytochrome c. I. Demonstration of a T-cell heteroclitic proliferative response and identification of a topographic antigenic determinant on pigeon cytochrome c whose immune recognition requires two complementing major histocompatibility complex-linked immune response genes. *J Exp Med*. 1979;150:830-848.

175. Katz ME, Maizels RM, Wicker L, Miller A, Sercarz EE. Immunological focusing by the mouse major histocompatibility complex: mouse strains confronted with distantly related lysozymes confine their attention to very few epitopes. *Eur J Immunol* . 1982;12:535-540.

176. Gell PGH, Benacerraf B. Studies on hypersensitivity II Delayed hypersensitivity to denatured proteins in guinea pigs. *Immunology*. 1959; 2:64-70.

177. Berzofsky JA. The nature and role of antigen processing in T cell activation. In: Cruse JM, Lewis RE Jr, eds. *The Year in Immunology 1984-1985*. Basel, Switzerland: Karger; 1985:18-24.

178. Livingstone A, Fathman CG. The structure of T cell epitopes. *Annu Rev Immunol*. 1987;5:477-501.

179. Schwartz RH. T-lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. *Annu Rev Immunol*. 1985;3:237-261.

180. Berzofsky JA, Cease KB, Cornette JL, et al. Protein antigenic structures recognized by T cells: potential applications to vaccine design. *Immunol Rev*. 1987;98:9-52.

181. Berzofsky JA, Richman LK, Killion DJ. Distinct H-2-linked Ir genes control both antibody and T cell responses to different determinants on the same antigen, myoglobin. *Proc Natl Acad Sci U S A*. 1979;76: 4046-4050.

182. Manca F, Clarke JA, Miller A, Sercarz EE, Shastri N. A limited region within hen egg-white lysozyme serves as the focus for a diversity of T cell clones. *J Immunol*. 1984;133:2075-2078.

183. Shimonkevitz R, Colon S, Kappler JW, Marrack P, Grey H. Antigen recognition by H-2-restricted T cells. II. A tryptic ovalbumin peptide that substitutes for processed antigen. *J Immunol*. 1984;133: 2067-2074.

184. Allen PM, Unanue ER. Differential requirements for antigen processing by macrophages for lysozyme-specific T cell hybridomas. *J Immunol*. 1984;132:1077-1079.

185. Berkower I, Buckenmeyer GK, Berzofsky JA. Molecular mapping of a histocompatibility-restricted immunodominant T cell epitope with synthetic and natural peptides: implications for antigenic structure. *J Immunol*. 1986;136:2498-2503.

186. Kurokohchi K, Akatsuka T, Pendleton CD, et al. Use of recombinant protein to identify a motif-negative human CTL epitope presented by HLA-A2 in the hepatitis C virus NS3 region. *J Virol*. 1996;70:232-240.

187. Thomas JW, Danho W, Bullesbach E, Fohles J, Rosenthal AS. Immune response gene control of determinant selection. III. Polypeptide fragments of insulin are differentially recognized by T but not by B cells in insulin immune guinea pigs. *J Immunol*. 1981;126:1095-1100.

188. Schwartz RH, Fox BS, Fraga E, Chen C, Singh B. The T lymphocyte response to cytochrome c. V. Determination of the minimal peptide size required for stimulation of T cell clones and assessment of the contribution of each residue beyond this size to antigenic potency. *J Immunol*. 1985;135:2598-2608.

189. Cease KB, Berkower I, York-Jolley J, Berzofsky JA. T cell clones specific for an amphipathic alpha helical region of sperm whale myoglobin show differing fine

specificities for synthetic peptides: A multi-view/single structure interpretation of immunodominance. *J Exp Med*. 1986;164:1779-1784.

190. Kurata A, Palker TJ, Streilein RD, Scearce RM, Haynes BF, Berzofsky JA. Immunodominant sites of human T-cell lymphotropic virus type 1 envelope protein for murine helper T cells. *J Immunol*. 1989;143: 2024-2030.

191. Lamb JR, Ivanyi J, Rees ADM, et al. Mapping of T cell epitopes using recombinant antigens and synthetic peptides. *EMBO J*. 1987;6: 1245-1249.

192. Townsend ARM, Gotch FM, Davey J. Cytotoxic T cells recognize fragments of the influenza nucleoprotein. *Cell*. 1985;42:457-467.

193. Walker BD, Flexner C, Birch-Limberger K, et al. Long-term culture and fine specificity of human cytotoxic T lymphocyte clones reactive with human immunodeficiency virus type 1. *Proc Natl Acad Sci U S A* . 1989;86:9514-9518.

194. Hosmalin A, Clerici M, Houghten R, et al. An epitope in HIV-1 reverse transcriptase recognized by both mouse and human CTL. *Proc Natl Acad Sci U S A* . 1990;87:2344-2348.

195. Kojima M, Cease KB, Buckenmeyer GK, Berzofsky JA. Limiting dilution comparsion of the repertoires of high and low responder MHC-restricted T cells. *J Exp Med*. 1988;167:1100-1113.

196. Heber-Katz E, Hansburg D, Schwartz RH. The la molecule of the antigen-presenting cell plays a critical role in immune response gene regulation of T cell activation. *J Mol Cell Immunol*. 1983;1:3-14.

197. Matis LA, Longo DL, Hedrick SM, Hannum C, Margoliash E, Schwartz RH. Clonal analysis of the major histocompatibility complex restriction and the fine specificity of antigen recognition in the T cell proliferative response to cytochrome c. *J Immunol*. 1983;130:1527-1535.

198. Hansburg D, Heber-Katz E, Fairwell T, Appella E. Major histocompatibility complex-controlled antigen presenting cell-expressed specificity of T cell antigen recognition. *J Exp Med*. 1983;158:25-39.

199. England RE, Kullberg MC, Cornette JL, Berzofsky JA. Molecular analysis of a heteroclitic T-cell response to the immunodominant epitope of sperm whale myoglobin: implications for peptide partial agonists. *J Immunol*. 1995;155:4295-4306.

200. Gammon G, Geysen HM, Apple RJ, et al. T cell determinant structure: cores and determinant envelopes in three mouse major histocompatibility complex haplotypes. *J Exp Med*. 1991;173:609-617.

201. Kimoto M, Fathman CG. Antigen-reactive T cell clones. I. Transcomplementing hybrid I-A-region gene products function effectively in antigen presentation. *J Exp Med*. 1980;152:759-770.

202. Kappler JW, Skidmore B, White J, Marrack P. Antigen-inducible H-2-restricted interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *J Exp Med*. 1981;153:1198-1214.

203. Young DB, Lamb JR. T lymphocytes respond to solid-phase antigen: a novel approach to the molecular analysis of cellular immunity. *Immunology*. 1986;59:167-171.

204. Lamb JR, Young DB. A novel approach to the identification of T-cell epitopes in Mycobacterium tuberculosis using human T-lymphocyte clones. *Immunology*. 1987;60:1-5.

205. Mustafa AS, Gill HK, Nerland A, et al. Human T-cell clones recognize a major M. leprae protein antigen expressed in E. coli. *Nature*. 1986; 319:63-66.

206. De Plaen E, Lurquin C, Van Pel A, et al. Immunogenic (tum-) variants of mouse tumor P815: cloning of the gene of tum- antigen P91A and identification of the tum- mutation. *Proc Natl Acad Sci U S A* . 1988;85:2274-2278.

207. Van der Bruggen P, Traversari C, Chomez P, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science*. 1991;254:1643-1647.

208. Guilloux Y, Lucas S, Brichard VG, et al. A peptide recognized by human cytolytic T lymphocytes on HLA-A2 melanomas is encoded by an intron sequence of the N-Acetylglucosaminyltransferase B gene. *J Exp Med*. 1996;183:1173-1183.

209. Kawakami Y, Eliyahu S, Delgado CH, et al. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc Natl Acad Sci U S A*. 1994;91:3515-3519.

210. Robbins PF, El-Gamil M, Li YF, et al. A mutated b-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. *J Exp Med*. 1996;183:1185-1192.

211. Wang R-F, Parkhurst MR, Kawakami Y, Robbins PF, Rosenberg SA. Utilization of an alternative open reading frame of a normal gene in generating a novel human cancer antigen. *J Exp Med*. 1996;183: 1131-1140.

212. Berkower I, Matis LA, Buckenmeyer GK, Gurd FRN, Longo DL, Berzofsky JA. Identification of distinct predominant epitopes recognized by myoglobin-specific T cells under control of different Ir genes and characterization of representative T-cell clones. *J Immunol*. 1984;132:1370-1378.

213. Shastri N, Oki A, Miller A, Sercarz EE. Distinct recognition phenotypes exist for T cell clones specific for small peptide regions of proteins. Implications for the mechanisms underlying major histocompatibility complex-restricted antigen recognition and clonal deletion models of immune response gene defects. *J Exp Med*. 1985;162:332-345.

214. Allen PM, McKean DJ, Beck BN, Sheffield J, Glimcher LH. Direct evidence that a class II molecule and a simple globular protein generate multiple determinants. *J Exp Med*. 1985;162:1264-1274.

215. Berkower I, Kawamura H, Matis LA, Berzofsky JA. T cell clones to two major T cell epitopes of myoglobin: Effect of I-A/I-E restriction on epitope dominance. *J Immunol*. 1985;135:2628-2634.

216. Hackett CJ, Dietzschold B, Gerhard W, et al. Influenza virus site recognized by a murine helper T cell specific for H1 strains. *J Exp Med*. 1983;158:294-302.

217. Hurwitz JL, Heber-Katz E, Hackett CJ, Gerhard WJ. Characterization of the murine TH response to influenza virus hemagglutinin: evidence for three major specificities. *J Immunol*. 1984;133:3371-3377.

218. Lamb JR, Eckels DD, Lake P, Woody JN, Green N. Human T cell clones recognize chemically synthesized peptides of influenza hemagglutinin. *Nature.* 1982; 300:66-69.

219. Rosenthal AS. Determinant selection and macrophage function in genetic control of the immune response. *Immunol Rev*. 1978;40: 136-152.

220. Milich DR, McLachlan A, Chisari FV, Thornton GB. Nonoverlapping T and B cell determinants on an hepatitis B surface antigen pre-S(2) region synthetic peptide. *J Exp Med*. 1986;164:532-547.

221. Francis MJ, Fry CM, Rowlands DJ, et al. Immunological priming with synthetic peptides of foot and mouth disease virus. *J Gen Virol*. 1985;66:2347-2352.

222. Corradin GP, Juillerat MA, Vita C, Engers HD. Fine specificity of a BALB/c T cell clone directed against beef apo cytochrome c. *J Mol Immunol*. 1983;20:763-768.

223. Corradin GP, Wallace CJA, Proudfoot AEI, Baumhuter S. Murine T cell response specific for cytochrome c. In: Sercarz EE, Berzofsky JA, eds. *The Immunogenicity of Protein Antigens: Repertoire and Regulation*. Boca Raton, FL: CRC Press; 1987:43-48.

224. Milich DR, Peterson DL, Leroux-Roels GG, Lerner RA, Chisari FV. Genetic regulation of the immune response to hepatitis B surface antigen (HBsAg). VI. Fine specificity. *J Immunol*. 1985;134:4203-4211.

225. Guillet JG, Lai MZ, Briner TJ, Smith JA, Gefter ML. Interaction of peptide antigens and class II major histocompatibility complex antigens. *Nature*. 1986;324:260-262.

226. Macfarlan RI, Dietzschold B, Wiktor TJ, et al. T cell responses to cleaved rabies virus glycoprotein and to synthetic peptides. *J Immunol*. 1984;133:2748-2752.

227. Margalit H, Spouge JL, Cornette JL, Cease K, DeLisi C, Berzofsky JA. Prediction of immunodominant helper T-cell antigenic sites from the primary sequence. *J Immunol*. 1987;138:2213-2229.

228. Townsend ARM, Rothbard J, Gotch FM, Bahadur G, Wraith D, McMichael AJ. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell*. 1986;44:959-968.

229. Townsend AR, Bastin J, Gould K, Brownlee GG. Cytotoxic T lymphocytes recognize influenza haemagglutinin that lacks a signal sequence. *Nature*. 1986;324:575-577.

230. Berzofsky JA. Structural features of protein antigenic sites recognized by helper T cells: what makes a site immunodominant? In: Cruse JM, Lewis RE Jr, eds. *The Year in Immunology 1985-1986*. Basel, Switzerland: Karger; 1986:28-38.

231. Berzofsky JA. Immunodominance in T lymphocyte recognition. *Immunol Lett*. 1988;18:83-92.

232. Yewdell JW, Bennink JR. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu Rev Immunol*. 1999;17:51-88.

233. Allen PM. Antigen processing at the molecular level. *Immunol Today*. 1987;8:270-273.

234. Goodman JW, Sercarz EE. The complexity of structures involved in T-cell activation. *Annu Rev Immunol*. 1983;1:465-498.

235. Finnegan A, Smith MA, Smith JA, Berzofsky JA, Sachs DH, Hodes RJ. The T cell repertoire for recognition of a phylogenetically distant protein antigen: peptide specificity and MHC restriction of staphylococcal nuclease specific T cell clones. *J Exp Med*. 1986;164:897-910.

236. Good MF, Pombo D, Quakyi IA, et al. Human T cell recognition of the circumsporozoite protein of Plasmodium falciparum. Immunodominant T cell domains map to the polymorphic regions of the molecule. *Proc Natl Acad Sci U S A* . 1988;85:1199-1203.

237. Nanda NK, Arzoo KK, Geysen HM, Sette A, Sercarz EE. Recognition of mutiple peptide cores by a single T cell receptor. *J Exp Med*. 1995;182: 531-539.

238. Brett SJ, Cease KB, Berzofsky JA. Influences of antigen processing on the expression of the T cell repertoire: evidence for MHC-specific hindering structures on the products of processing. *J Exp Med*. 1988;168: 357-373.

239. Gammon G, Shastri N, Cogswell J, et al. The choice of T-cell epitopes utilized on a protein antigen depends on multiple factors distant from as well as at the determinant site. *Immunol Rev*. 1987;98:53-73.

240. Sercarz EE, Lehmann PV, Ametani A, Benichou G, Miller A, Moudgil K. Dominance and crypticity of T cell antigenic determinants. *Annu Rev Immunol*. 1993;11:729-766.

241. Unanue ER. Antigen-presenting function of the macrophage. *Annu Rev Immunol*. 1984;2:395-428.

242. Shastri N, Gammon G, Horvath S, Miller A, Sercarz EE. The choice between two distinct T cell determinants within a 23 amino acid region of lysozyme depends upon structure of the immunogen. *J Immunol*. 1986;137:911-915.

243. Shastri N, Miller A, Sercarz EE. Amino acid residues distinct from the determinant region can profoundly affect activation of T cell clones by related antigens. *J Immunol*. 1986;136:371-376.

244. Grewal IS, Moudgil KD, Sercarz EE. Hindrance of binding to class II major histocompatibility complex molecules by a single amino acid residue contiguous to a determinant leads to crypticity of the determinant as well as lack of response to the protein antigen. *Proc Natl Acad Sci U S A* . 1995;92:1779-1783.

245. Fox BS, Carbone FR, Germain RN, Paterson Y, Schwartz RH. Processing of a minimal antigenic peptide alters its interaction with MHC molecules. *Nature*. 1988;331:538-540.

246. Sercarz E, Wilbur S, Sadegh-Nasseri S, et al. The molecular context of a determinant influences its dominant expression in a T cell response hierarchy through “fine processing.” In: Cinader B, Miller RG, eds. *Progress in Immunology VI*. New York, NY: Academic; 1986:227-237.

247. Ziegler HK, Unanue ER. Decrease in macrophage antigen catabolism caused by ammonia and chloroquine is associated with inhibition of antigen presentation to T cells. *Proc Natl Acad Sci U S A* . 1982;79:175-178.

248. Shimonkevitz R, Kappler J, Marrack P, Grey H. Antigen recognition by H-2 restricted T cells. I. Cell free antigen processing. *J Exp Med*. 1983;158:303-316.

249. Streicher HZ, Berkower IJ, Busch M, Gurd FRN, Berzofsky JA. Antigen conformation determines processing requirements for T-cell activation. *Proc Natl Acad Sci U S A* . 1984;81:6831-6835.

250. Lee P, Matsueda GR, Allen PM. T cell recognition of fibrinogen. A determinant on the Aa-chain does not require processing. *J Immunol*. 1988;140:1063-1068.

251. Régnier-Vigouroux A, Ayeb ME, Defendini ML, Granier C, Pierres M. Processing by accessory cells for presentation to murine T cells of apamin, a disulfide-bonded 18 amino acid peptide. *J Immunol*. 1988;140:1069-1075.

252. Rudensky AY, Preston-Hurlburt P, Hong SC, Barlow A, Janeway CA Jr. Sequence analysis of peptides bound to MHC class II molecules. *Nature*. 1991;353:622-627.

253. Stern LJ, Brown JH, Jardetzky TS, et al. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature*. 1994;368:215-221.

254. Stern LJ, Wiley DC. Antigenic peptide binding by class I and class II histocompatibility proteins. *Structure*. 1994;2:245-251.

255. Arunachalam B, Phan UT, Geuze HJ, Cresswell P. Enzymatic reduction of disulfide bonds in lysosomes: characterization of a gamma-interferon-inducible lysosomal thiol reductase (GILT). *Proc Natl Acad Sci U S A* . 2000;97:745-750.

256. Maric M, Arunachalam B, Phan UT, et al. Defective antigen processing in GILT-free mice. *Science*. 2001;294:1361-1365.

257. Townsend ARM, Skehel JJ. The influenza A virus nucleoprotein gene controls the induction of both subtype specific and crossreactive cytotoxic T cells. *J Exp Med*. 1984;160:552-563.

258. Braciale TJ, Braciale VL, Winkler M, et al. On the role of the transmembrane anchor sequence of influenza hemagglutinin in target cell recognition by class I MHC-restricted hemagglutinin-specific cytolytic T lymphocytes. *J Exp Med*. 1987;166:678-692.

259. Tobery T, Siliciano RF. Targeting of HIV-1 antigens for rapid intracellular degradation enhances cytotoxic T lymphocytes (CTL) recognition and the induction of De Novo CTL responses in vivo after immunization. *J Exp Med*. 1997;185:909-920.

260. York IA, Goldberg AL, Mo XY, Rock KL. Proteolysis and class I major histocompatibility complex antigen presentation. *Immunol Rev*. 1999; 172:49-66.

261. Yewdell JW, Bennink JR. Cut and trim: generating MHC class I peptide ligands. *Curr Opin Immunol*. 2001;13:13-18.

262. Vinitsky A, Antón LC, Snyder HL, Orlowski M, Bennink JR, Yewdell JW. The generation of MHC class I-associated peptides is only partially inhibited by proteasome inhibitors involvement of nonproteasomal cytosolic proteases in antigen processing? *J Immunol*. 1997;159: 554-564.

263. Rock KL, Gramm C, Rothstein L, et al. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell*. 1994;78:761-771.

264. Rammensee HG, Friede T, Stevanovíc S. MHC ligands and peptide motifs: first listing. *Immunogenetics*. 1995;41:178-228.

265. Vinitsky A, Cardozo C, Sepp-Lorenzino L, Michaud C, Orlowski M. Inhibition of the proteolytic activity of the multicatalytic proteinase complex (proteasome) by substrate-related peptidyl aldehydes. *J Biol Chem*. 1994;269:29860-29866.

266. Fenteany G, Standaert RF, Lane WS, Choi S, Corey EJ, Schreiber SL. Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science*. 1995;268:726-731.

267. Hughes EA, Ortmann B, Surman M, Cresswell P. The protease inhibitor, N-Acetyl-L-Leucyl-L-Leucyl-L-Norleucinal, decreases the pool of major histocompatibility complex class I-binding peptides and inhibits peptide trimming in the endoplasmic reticulum. *J Exp*

Med. 1996;183:1569-1578.

268. Henderson RA, Michel H, Sakaguchi K, et al. HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation. *Science*. 1992;255:1264-1266.

269. Yewdell J. To DRiP or not to DRiP: generating peptide ligands for MHC class I molecules from biosynthesized proteins. *Mol Immunol*. 2002; 39:139-146.

270. Princiotta MF, Finzi D, Qian SB, et al. Quantitating protein synthesis, degradation, and endogenous antigen processing. *Immunity*. 2003;18: 343-354.

271. Qian SB, Reits E, Neefjes J, Deslich JM, Bennink JR, Yewdell JW. Tight linkage between translation and MHC class I peptide ligand generation implies specialized antigen processing for defective ribosomal products. *J Immunol*. 2006;177:227-233.

272. Gaczynska M, Rock KL, Goldberg AL. g-Interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. *Nature*. 1993; 365:264-267.

273. Van Kaer L, Ashton-Rickardt PG, Eichelberger M, et al. Altered peptidase and viral-specific T cell response in LMP2 mutant mice. *Immunity*. 1994;1:533-541.

274. Fehling HJ, Swat W, Laplace C, et al. MHC class I expression in mice lacking the proteasome subunit LMP-7. *Science*. 1994;265:1234-1237.

275. Toes RE, Nussbaum AK, Degermann S, et al. Discrete cleavage motifs of constitutive and immunoproteasomes revealed by quantitative analysis of cleavage products. *J Exp Med*. 2001;194:1-12.

276. Sijts AJ, Ruppert T, Rehermann B, Schmidt M, Koszinowski U, Kloetzel PM. Efficient generation of a hepatitis B virus cytotoxic T lymphocyte epitope requires the structural features of immunoproteasomes. *J Exp Med*. 2000;191:503-514.

277. Sijts AJ, Standera S, Toes RE, et al. MHC class I antigen processing of an adenovirus CTL epitope is linked to the levels of immunoproteasomes in infected cells. *J Immunol*. 2000;164:4500-4506.

278. Schwarz K, van Den Broek M, Kostka S, et al. Overexpression of the proteasome subunits LMP2, LMP7, and MECL-1, but not PA28 alpha/beta, enhances the presentation of an immunodominant lymphocytic choriomeningitis virus T cell epitope. *J Immunol*. 2000;165:768-778.

279. Ito Y, Kondo E, Demachi-Okamura A, et al. Three immunoproteasome-associated subunits cooperatively generate a cytotoxic T-lymphocyte epitope of Epstein-Barr virus LMP2A by overcoming specific structures resistant to epitope liberation. *J Virol*. 2006;80: 883-890.

280. Morel S, Levy F, Burlet-Schiltz O, et al. Processing of some antigens by the standard proteasome but not by the immunoproteasome results in poor presentation by dendritic

cells. *Immunity*. 2000;12:107-117.

281. Chen W, Norbury CC, Cho Y, Yewdell JW, Bennink JR. Immunoproteasomes shape immunodominance hierarchies of antiviral CD8(+) T cells at the levels of T cell repertoire and presentation of viral antigens. *J Exp Med*. 2001;193:1319-1326.

282. Dubiel W, Pratt G, Ferrell K, Rechsteiner M. Purification of an 11 S regulator of the multicatalytic protease. *J Biol Chem*. 1992;267: 22369-22377.

283. Dick TP, Ruppert T, Groettrup M, et al. Coordinated dual cleavages induced by the proteasome regulator PA28 lead to dominant MHC ligands. *Cell*. 1996;86:253-262.

284. van Hall T, Sijts A, Camps M, et al. Differential influence on cytotoxic T lymphocyte epitope presentation by controlled expression of either proteasome immunosubunits or PA28. *J Exp Med*. 2000;192:483-494.

285. Goldberg AL, Rock KL. Proteolysis, proteasomes and antigen presentation. *Nature*. 1992;357:375-379.

286. Donnes P, Kohlbacher O. Integrated modeling of the major events in the MHC class I antigen processing pathway. *Protein Sci*. 2005;14: 2132-2140.

287. Reits E, Neijssen J, Herberts C, et al. A major role for TPPII in trimming proteasomal degradation products for MHC class I antigen presentation. *Immunity*. 2004;20:495-506.

288. Kloetzel PM. Generation of major histocompatibility complex class I antigens: functional interplay between proteasomes and TPPII. *Nat Immunol*. 2004;5:661-669.

289. Seifert U, Maranon C, Shmueli A, et al. An essential role for tripeptidyl peptidase in the generation of an MHC class I epitope. *Nat Immunol*. 2003;4:375-379.

290. Monaco JJ, Cho S, Attaya M. Transport protein genes in the murine MHC: possible implications for antigen processing. *Science*. 1990;250: 1723-1726.

291. Deverson EV, Gow IR, Coadwell WJ, Monaco JJ, Butcher GW, Howard JC. MHC class II region encoding proteins related to the multidrug resistance family of transmembrane transporters. *Nature*. 1990;348:738-741.

292. Trowsdale J, Hanson I, Mockridge I, Beck S, Townsend A, Kelly A. Sequences encoded in the class II region of the MHC related to the “ABC” superfamily of transporters. *Nature*. 1990;348:741-743.

293. Townsend A, Öhlén C, Bastin J, Ljunggren H-G, Foster L, Kärre K. Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature*. 1989;340:443-448.

294. Schumacher TNM, Heemels MT, Neefjes JJ, Kast WM, Melief CJM, Ploegh HL. Direct binding of peptide to empty MHC class I molecules on intact cells and in vitro. *Cell*. 1990;62:563-567.

295. Ortmann B, Androlewicz MJ, Cresswell P. MHC class I/b2-microglobulin complexes associate with TAP transporters before peptide binding. *Nature*. 1994;368:864-867.

296. Suh WK, Cohen-Doyle MF, Fruh K, Wang K, Peterson PA, Williams DB. Interaction of MHC class I molecules with the transporter associated with antigen processing. *Science*. 1994;264:1322-1326.

297. Livingstone AM, Powis SJ, Diamond AG, Butcher GW, Howard JC. A trans-acting major histocompatibility complex-linked gene whose alleles determine gain and loss changes in the antigenic structure of a classical class I molecule. *J Exp Med*. 1989;170:777-795.

298. Livingstone AM, Powis SJ, Günther E, Cramer DV, Howard JC, Butcher GW. Cim: an MHC class II-linked allelism affecting the antigenicity of a classical class I molecule for T lymphocytes. *Immunogenetics*. 1991;34:157-163.

299. Powis SJ, Deverson EV, Coadwell WJ, et al. Effect of polymorphism of an MHC-linked transporter on the peptides assembled in a class I molecule. *Nature*. 1992;357:211-215.

300. Powis SJ, Young LL, Joly E, et al. The rat cim effect: TAP allele-dependent changes in a class I MHC anchor motif and evidence against C-terminal trimming of peptides in the ER. *Immunity*. 1996;4:159-165.

301. Neefjes JJ, Momburg F, Hämmerling GJ. Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. *Science*. 1993;261:769-771.

302. Neefjes J, Gottfried E, Roelse J, et al. Analysis of the fine specificity of rat, mouse and human TAP peptide transporters. *Eur J Immunol* . 1995; 25:1133-1136.

303. Androlewicz MJ, Cresswell P. Human transporters associated with antigen processing possess a promiscuous peptide-binding site. *Immunity*. 1994;1:7-14.

304. Schumacher TNM, Kantesaria DV, Heemels MT, et al. Peptide length and sequence specificity of the mouse TAP1/TAP2 translocator. *J Exp Med*. 1994;179:533-540.

305. van Endert PM, Riganelli D, Greco G, et al. The peptide-binding motif for the human transporter associated with antigen processing. *J Exp Med*. 1995;182:1883-1895.

306. van Endert PM. Peptide selection for presentation by HLA class I: a role for the human transporter associated with antigen processing? *Immunol Res*. 1996;15:265-279.

307. Gubler B, Daniel S, Armandola EA, Hammer J, Caillat-Zucman S, van Endert PM. Substrate selection by transporters associated with antigen processing occurs during peptide binding to TAP. *Mol Immunol* . 1998;35:427-433.

308. Daniel S, Brusic V, Caillat-Zucman S, et al. Relationship between peptide

selectivities of human transporters associated with antigen processing and HLA class I molecules. *J Immunol*. 1998;161:617-624.

309. Burgevin A, Saveanu L, Kim Y, et al. A detailed analysis of the murine TAP transporter substrate specificity. *PLoS One*. 2008;3:e2402.

310. Uebel S, Kraas W, Kienle S, Wiesmuller KH, Jung G, Tampe R. Recognition principle of the TAP transporter disclosed by combinatorial peptide libraries. *Proc Natl Acad Sci U S A*. 1997;94:8976-8981.

311. Fruci D, Niedermann G, Butler RH, van Endert PM. Efficient MHC class I-independent amino-terminal trimming of epitope precursor peptides in the endoplasmic reticulum. *Immunity*. 2001;15:467-476.

312. Lauvau G, Kakimi K, Niedermann G, et al. Human transporters associated with antigen processing (TAPs) select epitope precursor peptides for processing in the endoplasmic reticulum and presentation to T cells. *J Exp Med*. 1999;190:1227-1240.

313. Serwold T, Gaw S, Shastri N. ER aminopeptidases generate a unique pool of peptides for MHC class I molecules. *Nat Immunol*. 2001;2:644-651.

314. Serwold T, Gonzalez F, Kim J, Jacob R, Shastri N. ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. *Nature*. 2002;419:480-483.

315. York IA, Chang SC, Saric T, et al. The ER aminopeptidase ERAP1 enhances or limits antigen presentation by trimming epitopes to 8-9 residues. *Nat Immunol*. 2002;3:1177-1184.

316. Saric T, Chang SC, Hattori A, et al. An IFN-gamma-induced aminopeptidase in the ER, ERAP1, trims precursors to MHC class I-presented peptides. *Nat Immunol*. 2002;3:1169-1176.

317. Paradela A, Alvarez I, Garcia-Peydro M, et al. Limited diversity of peptides related to an alloreactive T cell epitope in the HLA-B27-bound peptide repertoire results from restrictions at multiple steps along the processing-loading pathway. *J Immunol*. 2000;164:329-337.

318. Thirdborough SM, Roddick JS, Radcliffe JN, Howarth M, Stevenson FK, Elliott T. Tapasin shapes immunodominance hierarchies according to the kinetic stability of peptide-MHC class I complexes. *Eur J Immunol* . 2008;38:364-369.

319. Peters B, Bulik S, Tampe R, Van Endert PM, Holzhutter HG. Identifying MHC class I epitopes by predicting the TAP transport efficiency of epitope precursors. *J Immunol*. 2003;171:1741-1749.

320. Tenzer S, Peters B, Bulik S, et al. Modeling the MHC class I pathway by combining predictions of proteasomal cleavage, TAP transport and MHC class I binding. *Cell Mol Life Sci*. 2005;62:1025-1037.

321. York IA, Roop C, Andrews DW, Riddell SR, Graham FL, Johnson DC. A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. *Cell*. 1994;77:525-535.

322. Früh K, Ahn K, Djaballah H, et al. A viral inhibitor of peptide transporters for antigen presentation. *Nature*. 1995;375:415-418.

323. Hill A, Jugovic P, York I, et al. Herpes simplex virus turns off the TAP to evade host immunity. *Nature*. 1995;375:411-415.

324. Stockinger B, Pessara U, Lin RH, Habicht J, Grez M, Koch N. A role of Ia-associated invariant chains in antigen processing and presentation. *Cell*. 1989;56:683-689.

325. Brodsky FM, Guagliardi LE. The cell biology of antigen processing and presentation. *Annu Rev Immunol*. 1991;9:707-744.

326. Germain RN, Margulies DH. The biochemistry and cell biology of antigen processing and presentation. *Annu Rev Immunol*. 1993;11:403-450.

327. Elliot WL, Stille CJ, Thomas LJ, Humphreys RE. An hypothesis on the binding of an amphipathic, a helical sequence in Ii to the desetope of class II antigens. *J Immunol*. 1987;138:2949-2952.

328. Roche PA, Cresswell P. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature*. 1990;345: 615-618.

329. Teyton L, O'Sullivan D, Dickson PW, et al. Invariant chain distinguishes between the exogenous and endogenous antigen presentation pathways. *Nature*. 1990;348:39-44.

330. Roche PA, Cresswell P. Proteolysis of the class II-associated invariant chain generates a peptide binding site in intracellular HLA-DR molecules. *Proc Natl Acad Sci U S A*. 1991;88:3150-3154.

331. Bodmer H, Viville S, Benoist C, Mathis D. Diversity of endogenous epitopes bound to MHC class II molecules limited by invariant chain. *Science*. 1994;263:1284-1286.

332. Long EO, LaVaute T, Pinet V, Jaraquemada D. Invariant chain prevents the HLA-DR-restricted presentation of a cytosolic peptide. *J Immunol*. 1994;153:1487-1494.

333. Roche PA, Marks MS, Cresswell P. Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature*. 1991; 354:392-394.

334. Fling SP, Arp B, Pious D. HLA-DMA and -DMB genes are both required for MHC class II/peptide complex formation in antigen-presenting cells. *Nature*. 1994;368:554-558.

335. Sadegh-Nasseri S, Stern LJ, Wiley DC, Germain RN. MHC class II function preserved by low-affinity peptide interactions preceding stable binding. *Nature*. 1994;370:647-650.

336. Mellins E, Kempin S, Smith L, Monji T, Pious D. A gene required for class II-restricted antigen presentation maps to the major histocompatibility complex. *J Exp Med*. 1991;174:1607-1615.

337. Riberdy JM, Newcomb JR, Surman MJ, Barbosa JA, Cresswell P. HLA-DR molecules from an antigen-processing mutant cell line are associated with invariant chain peptides. *Nature*. 1992;360:474-477.

338. Morris P, Shaman J, Attaya M, et al. An essential role for HLA-DM in antigen presentation by class II major histocompatibility molecules. *Nature*. 1994;368:551-554.

339. Sette A, Ceman S, Kubo RT, et al. Invariant chain of peptides in most HLA-DR molecules of an antigen-processing mutant. *Science*. 1992;258: 1801-1804.

340. Denzin LK, Cresswell P. HLA-DM induces CLIP dissociation from MHC class II ab dimers and facilitates peptide loading. *Cell*. 1995;82: 155-165.

341. Denzin LK, Robbins NF, Carboy-Newcome C, Cresswell P. Assembly and intracellular transport of HLA-DM and correction of the class II antigen-processing defect in T2 cells. *Immunity*. 1994;1:595-606.

342. Alfonso C, Han JO, Williams GS, Karlsson L. The impact of H2-DM on humoral immune responses. *J Immunol*. 2001;167:6348-6355.

343. Sloan VS, Cameron P, Porter G, et al. Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nature*. 1995;375:802-806.

344. Sherman MA, Weber DA, Jensen PE. DM enhances peptide binding to class II MHC by release of invariant chain-derived peptide. *Immunity*. 1995;3:197-205.

345. Pezeshki AM, Cote MH, Azar GA, Routy JP, Boulassel MR, Thibodeau J. Forced expression of HLA-DM at the surface of dendritic cells increases loading of synthetic peptides on MHC class II molecules and modulates T cell responses. *J Immunol*. 2011;187:74-81.

346. Benacerraf B. A hypothesis to relate the specificity of T lymphocytes and the activity of I region-specific Ir genes in macrophages and B lymphocytes. *J Immunol*. 1978;120:1809-1812.

347. Rosenthal AS, Barcinski MA, Blake JT. Determinant selection is a macrophage dependent immune response gene function. *Nature*. 1977; 267:156-158.

348. Paul WE, Shevach EM, Pickeral S, Thomas DW, Rosenthal AS. Independent populations of primed F1 guinea pig T-lymphocytes respond to antigen-pulsed parental peritoneal exudate cells. *J Exp Med*. 1977;145:618-630.

349. Watts TH, Brian AA, Kappler JW, Marrack P, McConnell HM. Antigen presentation by supported planar membranes containing affinity-purified I-Ad. *Proc Natl Acad Sci U S A*. 1984;81:7564-7568.

350. Walden P, Nagy ZA, Klein J. Induction of regulatory T-lymphocyte responses by liposomes carrying major histocompatibility complex molecules and foreign antigen. *Nature*. 1985;315:327-329.

351. Norcross MA, Bentley DM, Margulies DH, Germain RN. Membrane Ia expression and antigen-presenting accessory cell function of L cells transfected with class II major histocompatibility genes. *J Exp Med*. 1984;160:1316-1337.

352. Demotz S, Grey HM, Sette A. The minimal number of class II MHCantigen complexes needed for T cell activation. *Science*. 1990;249: 1028-1030.

353. Harding CV, Unanue ER. Quantitation of antigen-presenting cell MHC class II/peptide complexes necessary for T-cell stimulation. *Nature*. 1990;346:574-576.

354. Christinck ER, Luscher MA, Barber BH, Williams DB. Peptide binding to class I MHC on living cells and quantitation of complexes required for CTL lysis. *Nature*. 1991;352:67-70.

355. Cabral GA, Marciano-Cabral F, Funk GA, et al. Cellular and humoral immunity in guinea pigs to two major polypeptides derived from hepatitis B surface antigen. *J Gen Virol*. 1978;38:339-350.

356. Kirnbauer R, Booy F, Cheng N, Lowy DR, Schiller JT. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc Natl Acad Sci U S A*. 1992;89:12180-12184.

357. Stoute JA, Slaoui M, Heppner G, et al. A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. *New Engl J Med*. 1997;336:86-91.

358. Takeshita T, Kozlowski S, England RD, et al. Role of conserved regions of class I MHC molecules in the activation of CD8+ CTL by peptide and purified cell-free class I molecules. *Int Immunol*. 1993;5:1129-1138.

359. Brower RC, England R, Takeshita T, et al. Minimal requirements for peptide mediated activation of CD8+ CTL. *Molec Immunol*. 1994;31: 1285-1293.

360. Werdelin O. Chemically related antigens compete for presentation by accessory cells to T cells. *J Immunol*. 1982;129:1883-1891.

361. Rock KL, Benacerraf B. Inhibition of antigen-specific T lymphocyte activation by structurally related Ir gene-controlled polymers. Evidence of specific competition for accessory cell antigen presentation. *J Exp Med*. 1983;157:1618-1634.

362. Rock KL, Benacerraf B. Inhibition of antigen-specific T lymphocyte activation by structurally related Ir gene-controlled polymers. II. Competitive inhibition of I-E. *J Exp Med*. 1984;160:1864-1879.

363. Guillet JG, Lai MZ, Briner TJ, et al. Immunological self, nonself discrimination. *Science*. 1987;235:865-870.

364. Babbitt BP, Allen PM, Matsueda G, Haber E, Unanue ER. The binding of immunogenic peptides to Ia histocompatibility molecules. *Nature*. 1985;317:359-361.

365. Buus S, Sette A, Colon SM, Jenis DM, Grey HM. Isolation and characterization of antigen-Ia complexes involved in T cell recognition. *Cell*. 1986;47:1071-1077.

366. Phillips ML, Yip CC, Shevach EM, Delovitch TL. Photoaffinity labeling demonstrates binding between Ia molecules and nominal antigen on antigen-presenting cells. *Proc Natl Acad Sci U S A*. 1986;83: 5634-5638.

367. Babbitt BP, Matsueda G, Haber E, Unanue ER, Allen PM. Antigenic competition at the level of peptide-Ia binding. *Proc Natl Acad Sci U S A* . 1986;83:4509-4513.

368. Buus S, Sette A, Colon SM, Miles C, Grey HM. The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides. *Science*. 1987;235:1353-1358.

369. Ronchese F, Schwartz RH, Germain RN. Functionally distinct subsites on a class II major histocompatibility complex molecule. *Nature*. 1987;329:254-256.

370. Sette A, Buus S, Colon S, Smith JA, Miles C, Grey HM. Structural characteristics of an antigen required for its interaction with Ia and recognition by T cells. *Nature*. 1987;328:395-399.

371. Kurata A, Berzofsky JA. Analysis of peptide residues interacting with MHC molecule or T-cell receptor: can a peptide bind in more than one way to the same MHC molecule? *J Immunol* . 1990;144:4526-4535.

372. Allen PM, Matsueda GR, Evans RJ, Dunbar JB Jr, Marshall GR, Unanue ER. Identification of the T-cell and Ia contact residues of a T-cell antigenic epitope. *Nature*. 1987;327:713-717.

373. Brown JH, Jardetzky T, Saper MA, Samraoui B, Bjorkman PJ, Wiley DC. A hypothetical model of the foreign antigen binding site of Class II histocompatibility molecules. *Nature*. 1988;332:845-850.

374. Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. Structure of the human class I histocompatibility antigen HLA-A2. *Nature*. 1987;329:506-512.

375. Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature*. 1987;329: 512-518.

376. Madden DR, Gorga JC, Strominger JL, Wiley DC. The structure of HLA-B27 reveals

nonamer self-peptides bound in an extended conformation. *Nature*. 1991;353:321-325.

377. Matsumura M, Fremont DH, Peterson PA, Wilson IA. Emerging principles for the recognition of peptide antigens by MHC class I molecules. *Science*. 1992;257:927-934.

378. Fremont DH, Matsumura M, Stura EA, Peterson PA, Wilson IA. Crystal structures of two viral peptides in complex with murine MHC class I H-2Kb. *Science*. 1992;257:919-927.

379. Germain RN, Ashwell JD, Lechler RI, et al. "Exon-shuffling" maps control of antibody and T-cell-recognition sites to the NH2 terminal domain of the class II major histocompatibility polypeptide Abeta. *Proc Natl Acad Sci U S A* . 1985;82:2940-2944.

380. Glimcher LH. T cells recognize multiple determinants on a single class II molecule, some of which depend on tertiary conformation. In: Sercarz EE, Berzofsky JA, eds. *Immunogenicity of Protein Antigens: Repertoires and Regulation* . Boca Raton, FL: CRC Press; 1987: 131-138.

381. Haskins K, Kappler J, Marrack P. The major histocompatibility complex-restricted antigen receptor on T cells. *Annu Rev Immunol* . 1984;2:51-66.

382. Meuer SC, Acuto O, Hercend T, Schlossman SF, Reinherz EL. The human T-cell receptor. *Annu Rev Immunol* . 1984;2:23-50.

383. Davis MM. Molecular genetics of the T cell-receptor beta chain. *Annu Rev Immunol* . 1985;3:537-560.

384. Kronenberg M, Siu G, Hood LE, Shastri N. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. *Annu Rev Immunol* . 1986;4:529-591.

385. Vasmatzis G, Cornette J, Sezerman U, DeLisi C. TcR recognition of the MHC-peptide dimer: structural properties of a ternary complex. *J Mol Biol* . 1996;261:72-89.

386. Garcia KC, Degano M, Stanfield RL, et al. An ab T cell receptor structure at 2.5Å and its orientation in the TCR-MHC complex. *Science*. 1996;274:209-219.

387. Garboczi DN, Ghosh P, Utz U, Fan QR, Biddison WE, Wiley DC. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature*. 1996;384:134-141.

388. Bhayani H, Paterson Y. Analysis of peptide binding patterns in different major histocompatibility complex/T cell receptor complexes using pigeon cytochrome c-specific T cell hybridomas. Evidence that a single peptide binds major histocompatibility complex in different conformations. *J Exp Med*. 1989;170:1609-1625.

389. Brett SJ, McKean D, York-Jolley J, Berzofsky JA. Antigen presentation to specific T cells by Ia molecules selectively altered by site-directed mutagenesis. *Int Immunol* . 1989;1:130-140.

390. McMichael AJ, Gotch FM, Santos-Aguado J, Strominger JL. Effect of mutations and variations of HLA-A2 on recognition of a virus peptide epitope by cytotoxic T lymphocytes. *Proc Natl Acad Sci U S A* . 1988;85: 9194-9198.

391. Racioppi L, Ronchese F, Schwartz RH, Germain RN. The molecular basis of class II MHC allelic control of T cell responses. *J Immunol* . 1991; 147:3718-3727.

392. Boehncke WH, Takeshita T, Pendleton CD, et al. The importance of dominant negative effects of amino acids side chain substitution in peptide-MHC molecule interactions and T cell recognition. *J Immunol* . 1993;150:331-341.

393. Rothbard JB, Busch R, Howland K, et al. Structural analysis of a peptide-HLA class II complex: identification of critical interactions for its formation and recognition by T cell receptor. *Int Immunol* . 1989;1:479-486.

394. Jardetzky TS, Gorga JC, Busch R, Rothbard J, Strominger JL, Wiley DC. Peptide binding to HLA-DR1: a peptide with most residues substituted to alanine retains MHC binding. *EMBO J*. 1990;9:1797-1803.

395. Rothbard JB, Busch R, Bal V, Trowsdale J, Lechler RI, Lamb JR. Reversal of HLA restriction by a point mutation in an antigenic peptide. *Int Immunol* . 1989;1:487-495.

396. Maryanski JL, Verdini AS, Weber PC, Salemme FR, Corradin G. Competitor analogs for defined T cell antigens: peptides incorporating a putative binding motif and polyproline or polyglycine spacers. *Cell* . 1990;60:63-72.

397. Ruppert J, Sidney J, Celis E, Kubo RT, Grey HM, Sette A. Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules. *Cell* . 1993;74:929-937.

398. Berzofsky JA. Epitope selection and design of synthetic vaccines: molecular approaches to enhancing immunogenicity and crossreactivity of engineered vaccines. *Ann N Y Acad Sci* . 1993;690:256-264.

399. Berzofsky JA. Designing peptide vaccines to broaden recognition and enhance potency. *Ann N Y Acad Sci* . 1995;754:161-168.

400. Takahashi H, Nakagawa Y, Pendleton CD, et al. Induction of broadly cross-reactive cytotoxic T cells recognizing an HIV-1 envelope determinant. *Science*. 1992;255:333-336.

401. Ahlers JD, Takeshita T, Pendleton CD, Berzofsky JA. Enhanced immunogenicity of HIV-1 vaccine construct by modification of the native peptide sequence. *Proc Natl Acad Sci U S A*. 1997;94:10856-10861.

402. Shirai M, Pendleton CD, Ahlers J, Takeshita T, Newman M, Berzofsky JA. Helper-CTL determinant linkage required for priming of anti-HIV CD8+ CTL in vivo with peptide vaccine constructs. *J Immunol* . 1994;152:549-556.

403. Ahlers JD, Dunlop N, Alling DW, Nara PL, Berzofsky JA. Cytokinein-adjuvant

steering of the immune response phenotype to HIV-1 vaccine constructs: GM-CSF and TNFa synergize with IL-12 to enhance induction of CTL. *J Immunol*. 1997;158:3947-3958.

404. Ahlers JD, Belyakov IM, Thomas EK, Berzofsky JA. High affinity T-helper epitope induces complementary helper and APC polarization, increased CTL and protection against viral infection. *J Clin Invest*. 2001;108:1677-1685.

405. Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature*. 1998;393:474-478.

406. Bennett SRM, Carbone FR, Karamalis F, Flavell RA, Miller JFAP, Heath WR. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature*. 1998;393:478-480.

407. Schoenberger SP, Toes REM, van der Voort EIH, Offringa R, Melief CJM. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature*. 1998;393:480-483.

408. Okazaki T, Pendleton CD, Sarobe P, et al. Epitope-enhancement of a CD4 HIV epitope toward the development of the next generation HIV vaccine. *J Immunol*. 2006;176:3753-3759.

409. Pogue RR, Eron J, Frelinger JA, Matsui M. Amino-terminal alteration of the HLA-A*0201-restricted human immunodeficiency virus pol peptide increases complex stability and in vitro immunogenicity. *Proc Natl Acad Sci U S A* . 1995;92:8166-8170.

410. Parkhurst MR, Salgaller ML, Southwood S, et al. Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. *J Immunol*. 1996; 157:2539-2548.

411. Sarobe P, Pendleton CD, Akatsuka T, et al. Enhanced in vitro potency and in vivo immunogenicity of a CTL epitope from hepatitis C virus core protein following amino acid replacement at secondary HLA-A2.1 binding positions. *J Clin Invest*. 1998;102:1239-1248.

412. Okazaki T, Pendleton DC, Lemonnier F, Berzofsky JA. Epitopeenhanced conserved HIV-1 peptide protects HLA-A2-transgenic mice against virus expressing HIV-1 antigen. *J Immunol*. 2003;171: 2548-2555.

413. Oh S, Terabe M, Pendleton CD, et al. Human CTL to wild type and enhanced epitopes of a novel prostate and breast tumor-associated protein, TARP, lyse human breast cancer cells. *Cancer Res*. 2004;64: 2610-2618.

414. Okazaki T, Terabe M, Catanzarot AT, Pendleton CD, Yarchoan R, Berzofsky JA. Possible therapeutic vaccine strategy against HIV escape from RT inhibitors studied in HLA-A2 transgenic mice. *J Virol*. 2006;80:10645-10651.

415. Rosenberg SA, Yang JC, Schwartzentruber DJ, et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic

melanoma. *Nat Med*. 1998;4:321-327.

416. Borbulevych OY, Do P, Baker BM. Structures of native and affinity-enhanced WT1 epitopes bound to HLA-A*0201: implications for WT1-based cancer therapeutics. *Mol Immunol*. 2010;47:2519-2524.

417. Cole DK, Edwards ESJ, Wynn KK, et al. Modification of MHC anchor residues generates heteroclitic peptides that alter TCR binding and T cell recognition. *J Immunol*. 2010;185:2600-2610.

418. Miles KM, Miles JJ, Madura F, Sewell AK, Cole DK. Real time detection of peptide-MHC dissociation reveals that improvement of primary MHC-binding residues can have a minimal, or no, effect on stability. *Mol Immunol*. 2011;48:728-732.

419. Hou YF, Kavanagh B, Fong L. Distinct CD8(+) T cell repertoires primed with agonist and native peptides derived from a tumor-associated antigen. *J Immunol*. 2008;180:1526-1534.

420. Jordan KR, McMahan RH, Kemmler CB, Kappler JW, Slansky JE. Peptide vaccines prevent tumor growth by activating T cells that respond to native tumor antigens. *Proc Natl Acad Sci U S A*. 2010;107:4652-4657.

421. Sette A, Vitiello A, Reherman B, et al. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. *J Immunol*. 1994;153:5586-5592.

422. Berzofsky JA, Ahlers JD, Derby MA, Pendleton CD, Arichi T, Belyakov IM. Approaches to improve engineered vaccines for human immunodeficiency virus (HIV) and other viruses that cause chronic infections. *Immunol Rev*. 1999;170:151-172.

423. Berzofsky JA, Ahlers JD, Belyakov IM. Strategies for designing and optimizing new generation vaccines. *Nat Rev Immunol*. 2001;1:209-219.

424. DeGroot AS, Meister GE, Cornette JL, Margalit H, DeLisi C, Berzofsky JA. Computer prediction of T-cell epitopes. In: Levine MM, Woodrow GC, Kaper JB, Cobon GS, eds. *New Generation Vaccines*. New York, NY: Marcel Dekker, Inc.; 1997:127-138.

425. Falk K, Rötzschke O, Stevanovic S, Jung G, Rammensee HG. Allelespecific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature*. 1991;351:290-296.

426. Jardetzky TS, Lane WS, Robinson RA, Madden DR, Wiley DC. Identification of self peptides bound to purified HLA-B27. *Nature*. 1991; 353:326-329.

427. Romero P, Corradin G, Luescher IF, Maryanski JL. H-2Kd-restricted antigenic peptides share a simple binding motif. *J Exp Med*. 1991;174: 603-612.

428. Hunt DF, Henderson RA, Shabanowitz J, et al. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science*. 1992;255:1261-

1263.

429. Corr M, Boyd LF, Padlan EA, Margulies DH. H-2Dd exploits a four residue peptide binding motif. *J Exp Med*. 1993;178:1877-1892.

430. Schumacher TNM, De Bruijn MLH, Vernie LN, et al. Peptide selection by MHC class I molecules. *Nature*. 1991;350:703-706.

431. Tsomides TJ, Walker BD, Eisen HN. An optimal viral peptide recognized by CD8+ T cells binds very tightly to the restricting class I major histocompatibility complex protein on intact cells but not to the purified class I protein. *Proc Natl Acad Sci U S A* . 1991;88:11276-11280.

432. Hunt DF, Michel H, Dickinson TA, et al. Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-Ad. *Science*. 1992;256:1817-1820.

433. Sette A, Buus S, Appella E, et al. Prediction of major histocompatibility complex binding regions of protein antigens by sequence pattern analysis. *Proc Natl Acad Sci U S A*. 1989;86:3296-3300.

434. Chicz RM, Urban RG, Gorga JC, Vignali DAA, Lane WS, Strominger JL. Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J Exp Med*. 1993;178:27-47.

435. Chicz RM, Urban RG, Lane WS, et al. Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. *Nature*. 1992;358:764-768.

436. Hammer J, Takacs B, Sinigaglia F. Identification of a motif for HLA-DR1 binding peptides using M13 display libraries. *J Exp Med*. 1992;176:1007-1013.

437. Hammer J, Valsasnini P, Tolba K, et al. Promiscuous and allele-specific anchors in HLA-DR-binding peptides. *Cell*. 1993;74:197-203.

438. Sinigaglia F, Hammer J. Motifs and supermotifs for MHC class II binding peptides. *J Exp Med*. 1995;181:449-451.

439. Marshall KW, Wilson KJ, Liang J, Woods A, Zaller D, Rothbard JB. Prediction of peptide affinity to HLA DRB1*0401. *J Immunol* . 1995;154:5927-5933.

440. Jardetzky TS, Brown JH, Gorga JC, et al. Three-dimensional structure of a human class II histocompatibility molecule complexed with superantigen. *Nature*. 1994;368:711-718.

441. Scott CA, Peterson PA, Teyton L, Wilson IA. Crystal structures of two I-A(d)-peptide complexes reveal that high affinity can be achieved without large anchor residues. *Immunity*. 1998;8:319-329.

442. Fremont DH, Monnaie D, Nelson CA, Hendrickson WA, Unanue ER. Crystal structure of I-A(k) in complex with a dominant epitope of lysozyme. *Immunity*. 1998;8:305-317.

443. Ghosh P, Amaya M, Mellins E, Wiley DC. The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. *Nature*. 1995;378:457-462.

444. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. *Nature*. 1988;334:395-402.

445. Ogasawara K, Maloy WL, Schwartz RH. Failure to find holes in the T cell repertoire. *Nature*. 1987;325:450-452.

446. Alexander J, Snoke K, Ruppert J, et al. Functional consequences of engagement of the T cell receptor by low affinity ligands. *J Immunol*. 1993;150:1-7.

447. Alexander J, Oseroff C, Sidney J, Sette A. Derivation of HLA-B*0702 transgenic mice: functional CTL repertoire and recognition of human B*0702-restricted CTL epitopes. *Hum Immunol*. 2003;64:211-223.

448. Firat H, Cochet M, Rohrlich PS, et al. Comparative analysis of the CD8(+) T cell repertoires of H-2 class I wild-type/HLA-A2.1 and H-2 class I knockout/HLA-A2.1 transgenic mice. *Int Immunol*. 2002;14: 925-934.

449. Shirai M, Arichi T, Nishioka M, et al. CTL responses of HLA-A2.1-transgenic mice specific for hepatitis C viral peptides predict epitopes for CTL of humans carrying HLA-A2.1. *J Immunol*. 1995;154:2733-2742.

450. Soroosh P, Shokri F, Azizi M, Jeddi-Tehrani M. Analysis of T-cell receptor beta chain variable gene segment usage in healthy adult responders and nonresponders to recombinant hepatitis B vaccine. *Scand J Immunol*. 2003;57:423-31.

451. Schaeffer EB, Sette A, Johnson DL, et al. Relative contribution of “determinant selection” and “holes in the T-cell repertoire” to T-cell responses. *Proc Natl Acad Sci U S A*. 1989;86:4649-4653.

452. Moudgil KD, Grewal IS, Jensen PE, Sercarz EE. Unresponsiveness to a self-peptide of mouse lysozyme owing to hindrance of T cell receptormajor histocompatibility complex/peptide interaction caused by flanking epitopic residues. *J Exp Med*. 1996;183:535-546.

453. Sinha P, Chi HH, Kim HR, et al. Mouse lysozyme-M knockout mice reveal how the self-determinant hierarchy shapes the T cell repertoire against this circulating self antigen in wild-type mice. *J Immunol*. 2004; 173:1763-1771.

454. Hernandez J, Lee PP, Davis MM, Sherman LA. The use of HLA A2.1/p53 peptide tetramers to visualize the impact of self tolerance on the TCR repertoire. *J Immunol*. 2000;164:596-602.

455. Theobald M, Biggs J, Hernández J, Lustgarten J, Labadie C, Sherman LA. Tolerance to p53 by A2.1-restricted cytotoxic T lymphocytes. *J Exp Med*. 1997;185:833-841.

456. Sandberg JK, Franksson L, Sundback J, et al. T cell tolerance based on avidity thresholds rather than complete deletion allows maintenance of maximal repertoire diversity. *J Immunol*. 2000;165:25-33.

457. Slifka MK, Blattman JN, Sourdive DJ, et al. Preferential escape of subdominant CD8+ T cells during negative selection results in an altered antiviral T cell hierarchy. *J Immunol*. 2003;170:1231-1239.

458. Gross DA, Graff-Dubois S, Opolon P, et al. High vaccination efficiency of low-affinity epitopes in antitumor immunotherapy. *J Clin Invest*. 2004;113:425-433.

459. Dzutsev AH, Belyakov IM, Isakov DV, Margulies DH, Berzofsky JA. Avidity of CD8 T cells sharpens immunodominance. *Int Immunol*. 2007;19:497-507.

460. Trautmann L, Rimbert M, Echasserieau K, et al. Selection of T cell clones expressing high-affinity public TCRs within Human cytomegalovirus-specific CD8 T cell responses. *J Immunol*. 2005;175: 6123-6132.

461. Alexander-Miller MA, Leggatt GR, Berzofsky JA. Selective expansion of high or low avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. *Proc Natl Acad Sci U S A*. 1996;93:4102-4107.

462. Gallimore A, Dumrese T, Hengartner H, Zinkernagel RM, Rammensee HG. Protective immunity does not correlate with the hierarchy of virus-specific cytotoxic T cell responses to naturally processed peptides. *J Exp Med*. 1998;187:1647-1657.

463. Messaoudi I, Patino JA, Dyall R, LeMaoult J, Nikolich-Zugich J. Direct link between MHC polymorphism, T cell avidity, and diversity in immune defense. *Science*. 2002;298:1797-1800.

464. Tian S, Maile R, Collins EJ, Frelinger JA. CD8(+) T cell activation is governed by TCR-Peptide/MHC affinity, not dissociation rate. *J Immunol*. 2007;179:2952-2960.

465. Schmid DA, Irving MB, Posevitz V, et al. Evidence for a TCR affinity threshold delimiting maximal CD8 T cell function. *J Immunol*. 2010;184: 4936-4946.

466. Carreno LJ, Bueno SM, Bull P, Nathenson SG, Kalergis AM. The halflife of the T-cell receptor/peptide-major histocompatibility complex interaction can modulate T-cell activation in response to bacterial challenge. *Immunology*. 2007;121:227-237.

467. Riquelme E, Carreno LJ, Gonzalez PA, Kalergis AM. The duration of TCR/pMHC interactions regulates CTL effector function and tumorkilling capacity. *Eur J Immunol* . 2009;39:2259-2269.

468. Carreno LJ, Riquelme EM, Gonzalez PA, et al. T-cell antagonism by short half-life

pMHC ligands can be mediated by an efficient trapping of T-cell polarization toward the APC. *Proc Natl Acad Sci U S A* . 2010; 107:210-215.

469. Aleksic M, Dushek O, Zhang H, et al. Dependence of T cell antigen recognition on T cell receptor-peptide MHC confinement time. *Immunity*. 2010;32:163-174.

470. Govern CC, Paczosa MK, Chakraborty AK, Husebyb ES. Fast on-rates allow short dwell time ligands to activate T cells. *Proc Natl Acad Sci U S A* . 2010;107:8724-8729.

471. Huppa JB, Axmann M, Mortelmaier MA, et al. TCR-peptide-MHC interactions in situ show accelerated kinetics and increased affinity. *Nature*. 2010;463:963-U143.

472. Huang J, Zarnitsyna VI, Liu BY, et al. The kinetics of two-dimensional TCR and pMHC interactions determine T-cell responsiveness. *Nature*. 2010;464:932-U156.

473. Ding YH, Smith KJ, Garboczi DN, Utz U, Biddison WE, Wiley DC. Two human T cell receptors bind in a similar diagonal mode to the HLA-A2/Tax peptide complex using different TCR amino acids. *Immunity*. 1998;8:403-411.

474. Slansky JE, Rattis FM, Boyd LF, et al. Enhanced antigen-specific antitumor immunity with altered peptide ligands that stabilize the MHC-peptide-TCR complex. *Immunity*. 2000;13:529-538.

475. Zaremba S, Barzaga E, Zhu M, Soares N, Tsang KY, Schlom J. Identification of an enhancer agonist cytotoxic T lymphocyte peptide from human carcinoembryonic antigen. *Cancer Res*. 1997;57: 4570-4577.

476. Fong L, Hou Y, Rivas A, et al. Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy. *Proc Natl Acad Sci U S A* . 2001;98:8809-8814.

477. Tangri S, Ishioka GY, Huang X, et al. Structural features of peptide analogs of human histocompatibility leukocyte antigen class I epitopes that are more potent and immunogenic than wild-type peptide. *J Exp Med*. 2001;194:833-846.

478. Letourneau S, Im EJ, Mashishi T, et al. Design and pre-clinical evaluation of a universal HIV-1 vaccine. *PLoS One*. 2007;2:e984.

479. Depla E, Van der Aa A, Livingston BD, et al. Rational design of a multiepitope vaccine encoding T-lymphocyte epitopes for treatment of chronic hepatitis B virus infections. *J Virol* . 2008;82:435-450.

480. Livingston B, Crimi C, Newman M, et al. A rational strategy to design multiepitope immunogens based on multiple Th lymphocyte epitopes. *J Immunol* . 2002;168:5499-5506.

481. Livingston BD, Newman M, Crimi C, McKinney D, Chesnut R, Sette A. Optimization of epitope processing enhances immunogenicity of multiepitope DNA vaccines. *Vaccine*. 2001;19:4652-4660.

482. Wilson CC, McKinney D, Anders M, et al. Development of a DNA vaccine designed to induce cytotoxic T lymphocyte responses to multiple conserved epitopes in HIV-1. *J Immunol*. 2003;171:5611-5623.

483. Delamarre L, Couture R, Mellman I, Trombetta ES. Enhancing immunogenicity by limiting susceptibility to lysosomal proteolysis. *J Exp Med*. 2006;203:2049-2055.

484. Spearman P, Kalams S, Elizaga M, et al. Safety and immunogenicity of a CTL multiepitope peptide vaccine for HIV with or without GM-CSF in a phase I trial. *Vaccine*. 2009;27:243-249.

485. Jin X, Newman MJ, De-Rosa S, et al. A novel HIV T helper epitopebased vaccine elicits cytokine-secreting HIV-specific CD4+ T cells in a phase I clinical trial in HIV-uninfected adults. *Vaccine*. 2009;27: 7080-7086.

486. Takeshita T, Takahashi H, Kozlowski S, et al. Molecular analysis of the same HIV peptide functionally binding to both a class I and a class II MHC molecule. *J Immunol*. 1995;154:1973-1986.

487. Alexander-Miller MA, Parker KC, Tsukui T, Pendleton CD, Coligan JE, Berzofsky JA. Molecular analysis of presentation by HLA-A2.1 of a promiscuously binding V3 loop peptide from the HIV-1 envelope protein to human CTL. *Int Immunol*. 1996;8:641-649.

488. Abiru N, Wegmann D, Kawasaki E, Gottlieb P, Simone E, Eisenbarth GS. Dual overlapping peptides recognized by insulin peptide B:9-23 T cell receptor AV13S3 T cell clones of the NOD mouse. *J Autoimmun*. 2000;14:231-237.

489. Talken BL, Schafermeyer KR, Bailey CW, Lee DR, Hoffman RW. T cell epitope mapping of the Smith antigen reveals that highly conserved Smith antigen motifs are the dominant target of T cell immunity in systemic lupus erythematosus. *J Immunol*. 2001;167:562-568.

490. Takahashi H, Merli S, Putney SD, et al. A single amino acid interchange yields reciprocal CTL specificities for HIV gp160. *Science*. 1989;246: 118-121.

491. Takahashi H, Houghten R, Putney SD, et al. Structural requirements for class-I MHC molecule-mediated antigen presentation and cytotoxic T-cell recognition of an immunodominant determinant of the HIV envelope protein. *J Exp Med*. 1989;170:2023-2035.

492. De Magistris MT, Alexander J, Coggeshall M, et al. Antigen analogmajor histocompatibility complexes act as antagonists of the T cell receptor. *Cell*. 1992;68:625-634.

493. Sette A, Alexander J, Ruppert J, et al. Antigen analogs/MHC complexes as specific T cell receptor antagonists. *Annu Rev Immunol*. 1994;12: 413-431.

494. Lorenz RG, Allen PM. Direct evidence for functional self-protein/lamolecule

complexes in vivo. *Proc Natl Acad Sci U S A* . 1988;85:5220-5223.

495. Evavold BD, Allen PM. Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science*. 1991;252: 1308-1310.

496. Evavold BD, Williams SG, Hsu BL, Buus S, Allen PM. Complete dissection of the Hb(64-76) determinant using Th1, Th2 clones and T cell hybridomas. *J Immunol* . 1992;148:347-353.

497. Pfeiffer C, Stein J, Southwood S, Ketelaar H, Sette A, Bottomly K. Altered peptide ligands can control CD4 T lymphocyte differentiation in vivo. *J Exp Med*. 1995;181:1569-1574.

498. Chaturvedi P, Yu Q, Southwood S, Sette A, Singh B. Peptide analogs with different affinities for MHC alter the cytokine profile of T helper cells. *Int Immunol* . 1996;8:745-755.

499. Sloan-Lancaster J, Evavold BD, Allen PM. Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. *Nature*. 1993;363:156-159.

500. Sloan-Lancaster J, Shaw AS, Rothbard JB, Allen PM. Partial T cell signaling: altered phospho-z and lack of Zap70 recruitment in APLinduced T cell anergy. *Cell* . 1994;79:913-922.

501. Tsitoura DC, Holter W, Cerwenka A, Gelder CM, Lamb JR. Induction of anergy in human T helper 0 cells by stimulation with altered T cell antigen receptor ligands. *J Immunol* . 1996;156:2801-2808.

502. Mueller DL, Jenkins MK, Schwartz RH. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu Rev Immunol* . 1989;7:445-480.

503. Racioppi L, Ronchese F, Matis LA, Germain RN. Peptide-major histocompatibility complex class II complexes with mixed agonist/antagonist properties provide evidence for ligand-related differences in T cell receptor-dependent intracellular signaling. *J Exp Med*. 1993;177:1047-1060.

504. Madrenas J, Wange RL, Wang JL, Isakov N, Samelson LE, Germain RN. Zeta phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. *Science*. 1995;267:515-518.

505. Madrenas J, Chau LA, Smith J, Bluestone JA, Germain RN. The efficiency of CD4 recruitment to ligand-engaged TCR controls the agonist/partial agonist properties of peptide-MHC molecule ligands. *J Exp Med*. 1997;185:219-229.

506. McKeithan TW. Kinetic proofreading in T-cell receptor signal transduction. *Proc Natl Acad Sci U S A*. 1995;92:5042-5046.

507. DeGroot AS, Jesdale BM, Berzofsky JA. Prediction and determination of MHC

ligands and T cell epitopes. In: Kaufmann SHE, Kabelitz D, eds. *Immunology of Infection*. London, United Kingdom: Academic Press; 1998:79-108.

508. Kessler JH, Melief CJM. Identification of T-cell epitopes for cancer immunotherapy. *Leukemia*. 2007;21:1859-1874.

509. De Groot AS, Cohen T, Ardito M, Moise L, Martin B, Berzofsky JA. Use of bioinformatics to predict MHC ligands and T-cell epitopes: application to epitope-driven vaccine design. In: Elsevier, ed. *Methods in Microbiology*. Vol. 37. 2010:35-66.

510. Salimi N, Fleri W, Peters B, Sette A. Design and utilization of epitopebased databases and predictive tools. *Immunogenetics*. 2010;62:185-196.

511. DeLisi C, Berzofsky JA. T cell antigenic sites tend to be amphipathic structures. *Proc Natl Acad Sci U S A*. 1985;82:7048-7052.

512. Spouge JL, Guy HR, Cornette JL, et al. Strong conformational propensities enhance T-cell antigenicity. *J Immunol*. 1987;138:204-212.

513. Stille CJ, Thomas LJ, Reyes VE, Humphreys RE. Hydrophobic strip-of-helix algorithm for selection of T cell-presented peptides. *Molec Immunol*. 1987;24:1021-1027.

514. Sette A, Doria G, Adorini L. A microcomputer program for hydrophilicity and amphipathicity analysis of protein antigens. *Mol Immunol*. 1986;23:807-810.

515. Madden DR, Garboczi DN, Wiley DC. The antigenic identity of peptide-MHC complexes: a comparison of the conformations of five viral peptides presented by HLA-A2. *Cell*. 1993;75:693-708.

516. Carbone FR, Fox BS, Schwartz RH, Paterson Y. The use of hydrophobic a-helix-defined peptides in delineating the T cell determinant for pigeon cytochrome c. *J Immunol*. 1987;138:1838-1844.

517. Cornette JL, Margalit H, DeLisi C, Berzofsky JA. Concepts and methods in the identification of T cell epitopes and their use in the construction of synthetic vaccines. *Methods Enzymol*. 1989;178:611-634.

518. Cornette JL, Margalit H, DeLisi C, Berzofsky JA. The amphipathic Helix as a structural feature involved in T-cell recognition. In: Epand RM, ed. *The Amphipathic Helix*. Boca Raton, FL: CRC Press; 1993:333-346.

519. Rothbard JB, Taylor WR. A sequence pattern common to T cell epitopes. *EMBO J*. 1988;7:93-100.

520. Reyes VE, Chin LT, Humphreys RE. Selection of class I MHCrestricted peptides with the strip-of-helix hydrophobicity algorithm. *Molec Immunol*. 1988;25:867-871.

521. Cornette JL, Margalit H, Berzofsky JA, DeLisi C. Periodic variation in side-chain

polarities of T-cell antigenic peptides correlates with their structure and activity. *Proc Natl Acad Sci U S A*. 1995;92:8368-8372.

522. Jorgensen KW, Buus S, Nielsen M. Structural properties of MHC class II ligands, implications for the prediction of MHC class II epitopes. *Plos One*. 2010;5.

523. Dimitrov I, Garnev P, Flower DR, Doytchinova I. MHC class II binding prediction-a little help from a friend. *J Biomed Biotechnol* . 2010;2010:705821.

524. Lin HH, Ray S, Tongchusak S, Reinherz EL, Brusic V. Evaluation of MHC class I peptide binding prediction servers: applications for vaccine research. *Bmc Immunol*. 2008;9.

525. Gupta A, Chaukiker D, Singh TR. Comparative analysis of epitope predictions: proposed library of putative vaccine candidates for HIV. *Bioinformation*. 2011;5:386-389.

526. Pamer EG, Harty JT, Bevan MJ. Precise prediction of a dominant class I MHC-restricted epitope of Listeria monocytogenes. *Nature*. 1991; 353:852-855.

527. Hill AVS, Elvin J, Willis AC, et al. Molecular analysis of the association of HLA-B53 and resistance to severe malaria. *Nature*. 1992;360: 434-439.

528. Lipford GB, Hoffman M, Wagner H, Heeg K. Primary in vivo responses to ovalbumin: probing the predictive value of the Kb binding motif. *J Immunol* . 1993;150:1212-1222.

529. Nijman HW, Houbiers JGA, Vierboom MPM, et al. Identification of peptide sequences that potentially trigger HLA-A2.1-restricted cytotoxic T lymphocytes. *Eur J Immunol*. 1993;23:1215-1219.

530. Altuvia Y, Berzofsky JA, Rosenfeld R, Margalit H. Sequence features that correlate with MHC restriction. *Molec Immunol*. 1994;31:1-19.

531. Meister GE, Roberts CGP, Berzofsky JA, DeGroot AS. Two novel T cell epitope prediction algorithms based on MHC-binding motifs; comparison of predicted and published epitopes from Mycobacterium tuberculosis and HIV protein sequences. *Vaccine*. 1995;13:581-591.

532. Kaufman JF, Auffray C, Korman AJ, Shackelford DA, Strominger J. The class II molecules of the human and murine major histocompatibility complex. *Cell*. 1984;36:1-13.

533. Zhang C, Anderson A, DeLisi C. Structural principles that govern the peptide-binding motifs of class I MHC molecules. *J Mol Biol* . 1998;281:929-947.

534. Sturniolo T, Bono E, Ding J, et al. Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices. *Nat Biotechnol*. 1999;17:555-561.

535. Zhang C, Cornette JL, Berzofsky JA, DeLisi C. The organization of human leukocyte antigen class I epitopes in HIV genome products: implications for HIV evolution and

vaccine design. *Vaccine*. 1997;15:1291-1302.

536. Hammer J, Bono E, Gallazzi F, Belunis C, Nagy Z, Sinigaglia F. Precise prediction of major histocompatibility complex class II-peptide interaction based on peptide side chain scanning. *J Exp Med*. 1994;180: 2353-2358.

537. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J Immunol*. 1994;152:163-175.

538. Fleckenstein B, Kalbacher H, Muller CP, et al. New ligands binding to the human leukocyte antigen class II molecule DRB1*0101 based on the activity pattern of an undecapeptide library. *Eur J Biochem*. 1996;240:71-77.

539. Schuler MM, Nastke MD, Stevanovikc S. SYFPEITHI: database for searching and T-cell epitope prediction. *Method Mol Biol*. 2007;409: 75-93.

540. Jesdale BM, Mullen L, Meisell J, Marznello M, Deocampo G, De Groot AS. *Epimatrix and Epimer, Tools for HIV Research*. Vaccines: Cold Spring Harbor Laboratory Press; 1997.

541. Kim Y, Sidney J, Pinilla C, Sette A, Peters B. Derivation of an amino acid similarity matrix for peptide: MHC binding and its application as a Bayesian prior. *Bmc Bioinformatics*. 2009;10.

542. Davenport MP, Ho Shon IAP, Hill AVS. An empirical method for the prediction of T-cell epitopes. *Immunogenetics*. 1995;42:392-397.

543. Davenport MP, Godkin A, Friede T, et al. A distinctive peptide binding motif for HLA-DRB1*0407, an HLA-DR4 subtype not associated with rheumatoid arthritis. *Immunogenetics*. 1997;45:229-232.

544. Leggatt GR, Hosmalin A, Pendleton CD, Kumar A, Hoffman S, Berzofsky JA. The importance of pairwise interactions between peptide residues in the delineation of T cell receptor specificity. *J Immunol*. 1998;161:4728-4735.

545. James EA, Moustakas AK, Bui J, Nouv R, Papadopoulos GK, Kwok WW. The binding of antigenic peptides to HLA-DR is influenced by interactions between Pocket 6 and Pocket 9. *J Immunol*. 2009;183:3249-3258.

546. Moutaftsi M, Peters B, Pasquetto V, et al. A consensus epitope prediction approach identifies the breadth of murine T(CD8+)-cell responses to vaccinia virus. *Nat Biotechnol*. 2006;24:817-819.

547. Sidney J, Grey HM, Southwood S, et al. Definition of an HLA-A3-like supermotif demonstrates the overlapping peptide-binding repertoires of common HLA molecules. *Hum Immunol*. 1996;45:79-93.

548. Kropshofer H, Max H, Halder T, Kalbus M, Muller CA, Kalbacher H. Self-peptides

from four HLA-DR alleles share hydrophobic anchor residues near the NH2-terminal including proline as a stop signal for trimming. *J Immunol*. 1993;151:4732-4742.

549. Sette A, Sidney J. Nine major HLA class I supertypes account for the vast preponderance of HLA-A and -B polymorphism. *Immunogenetics*. 1999;50:201-212.

550. Vajda S, Weng Z, Rosenfeld R, DeLisi C. Effect of conformational flexibility and solvation on receptor-ligand binding free energies. *Biochem*. 1994;33:13977-13988.

551. Sezerman U, Vajda S, DeLisi C. Free energy mapping of class I MHC molecules and structural determination of bound peptides. *Protein Science*. 1996;5:1272-1281.

552. Atanasova M, Dimitrov I, Flower DR, Doytchinova I. MHC class II binding prediction by molecular docking. *Molecular Informatics*. 2011;30:368-375.

553. Altuvia Y, Schueler O, Margalit H. Ranking potential binding peptides to MHC molecules by a computational threading approach. *J Mol Biol*. 1995;249:244-250.

554. Altuvia Y, Margalit H. A structure-based approach for prediction of MHC-binding peptides. *Methods*. 2004;34:454-459.

555. Antunes DA, Vieira GF, Rigo MM, Cibulski SP, Sinigaglia M, Chies JAB. Structural allele-specific patterns adopted by epitopes in the MHC-I cleft and reconstruction of MHC:peptide complexes to crossreactivity assessment. *Plos One*. 2010;5.

556. Bordner AJ. Towards universal structure-based prediction of class II MHC epitopes for diverse allotypes. *Plos One*. 2010;5.

557. Mohanapriya A, Lulu S, Kayathri R, Kangueane P. Class II HLA-peptide binding prediction using structural principles. *Hum Immunol*. 2009; 70:159-169.

558. Schiewe AJ, Haworth IS. Structure-based prediction of MHC-peptide association: algorithm comparison and application to cancer vaccine design. *J Mol Graphics Model*. 2007;26:667-675.

559. Brusic V, Rudy G, Harrison LC. Prediction of MHC binding peptides using artificial neural networks. In: Stonier RJ, Yu XH, eds. *Complex Systems Mechanism of Adaptation*. Amsterdam, The Netherlands: IOS Press; 1994:253-260.

560. Nielsen M, Lund O. NN-align. An artificial neural network-based alignment algorithm for MHC class II peptide binding prediction. *Bmc Bioinformatics*. 2009;10.

561. Altuvia Y, Margalit H. Sequence signals for generation of antigenic peptides by the proteasome: implications for proteasomal cleavage mechanism. *J Mol Biol*. 2000;295:879-890.

562. Ginodi I, Vider-Shalit T, Tsaban L, Louzoun Y. Precise score for the prediction of peptides cleaved by the proteasome. *Bioinformatics*. 2008;24:477-483.

563. Diez-Rivero CM, Chenlo B, Zuluaga P, Reche PA. Quantitative modeling of peptide binding to TAP using support vector machine. *Proteins*. 2010;78:63-72.

564. Peters B, Sette A. Generating quantitative models describing the sequence specificity of biological processes with the stabilized matrix method. *BMC Bioinformatics*. 2005;6:132.

565. Larsen MV, Lundegaard C, Lamberth K, et al. An integrative approach to CTL epitope prediction: a combined algorithm integrating MHC class I binding, TAP transport efficiency, and proteasomal cleavage predictions. *Eur J Immunol* . 2005;35:2295-2303.

566. Doytchinova IA, Flower DR. Class I T-cell epitope prediction: improvements using a combination of proteasome cleavage, TAP affinity, and MHC binding. *Mol Immunol* . 2006;43:2037-2044.

567. Doytchinova IA, Guan P, Flower DR. EpiJen: a server for multistep T cell epitope prediction. *BMC Bioinformatics*. 2006;7:131.

568. Larsen MV, Lundegaard C, Lamberth K, Buus S, Lund O, Nielsen M. Large-scale validation of methods for cytotoxic T-lymphocyte epitope prediction. *Bmc Bioinformatics*. 2007;8.

569. Pira GL, Ivaldi F, Moretti P, Manca F. High throughput T epitope mapping and vaccine development. *J Biomed Biotechnol* . 2010;2010:325720.

570. Scott DW, De Groot AS. Can we prevent immunogenicity of human protein drugs? *Ann Rheum Dis*. 2010;69:72-76.

571. Parker AS, Zheng W, Griswold KE, Bailey-Kellogg C. Optimization algorithms for functional deimmunization of therapeutic proteins. *BMC Bioinformatics*. 2010;11:180.

572. Cohen T, Moise L, Ardito M, Martin W, De Groot AS. A method for individualizing the prediction of immunogenicity of protein vaccines and biologic therapeutics: individualized T cell epitope measure (iTEM). *J Biomed Biotechnol* . 2010;2010.

573. Takahashi H, Cohen J, Hosmalin A, et al. An immunodominant epitope of the HIV gp160 envelope glycoprotein recognized by class I MHC molecule-restricted murine cytotoxic T lymphocytes. *Proc Natl Acad Sci U S A* . 1988;85:3105-3109.

574. Langton BC, Mackewicz CE, Wan AM, Andria ML, Benjamini E. Structural features of an antigen required for cellular interactions and for T-cell activation in an MHC-restricted response. *J Immunol* . 1988;141:447-456.

575. Thomas DB, Skehel JJ, Mills KHG, Graham CM. A single amino acid substitution in influenza haemagglutinin abrogates recognition by a monoclonal antibody and a spectrum of subtype-specific L3T4+ T cell clones. *Eur J Immunol* . 1987;17:133-136.

576. Mozes E, McDevitt HO, Jaton JC, Sela M. The nature of the antigenic determinant in

genetic control of antibody response. *J Exp Med*. 1969;130:493-504.

577. Mozes E, McDevitt HO, Jaton JC, Sela M. The genetic control of antibody specificity. *J Exp Med*. 1969;130:1263-1278.

578. Bluestein HG, Green I, Maurer PH, Benacerraf B. Specific immune response genes of the guinea pig. V: influence of the GA and GT immune response genes on the specificity of cellular and humoral immune responses to a terpolymer of L-glutamic acid, L-alanine, and L-tyrosine. *J Exp Med*. 1972;135:98-109.

579. Berzofsky JA, Schechter AN, Shearer GM, Sachs DH. Genetic control of the immune response to staphyloccal nuclease. III. Time course and correlation between the response to native nuclease and the response to its polypeptide fragments. *J Exp Med*. 1977;145:111-112.

580. Berzofsky JA, Schechter AN, Shearer GM, Sachs DH. Genetic control of the immune response to staphylococcal nuclease. IV. H-2-linked control of the relative proportions of antibodies produced to different determinants of native nuclease. *J Exp Med*. 1977;145:123-145.

581. Campos-Neto A, Levine H, Schlossman SJ. T cell regulation of specific B cell responses. *J Immunol*. 1978;121:2235-2240.

582. Campos-Neto A, Levine H, Schlossman SJ. Immune response gene control of antibody specificity. *Cellular Immunol* . 1982;69:128-137.

583. Kohno Y, Berzofsky JA. Genetic control of the immune response to myoglobin. V. Antibody production in vitro is macrophage and T celldependent and is under control of two determinant-specific Ir genes. *J Immunol*. 1982;128:2458-2464.

584. Sercarz E, Cecka JM, Kipp D, Miller A. The steering function of T cells in expression of the antibody repertoire directed against multideterminant protein antigen. *Ann Immunol Inst Pasteur*. 1977;128:599.

585. Cecka JM, Stratton JA, Miller A, Sercarz EE. Structural aspects of immune recognition of lysozymes. III: T cell specificity restriction and its consequences for antibody specificity. *Eur J Immunol* . 1976;6: 639-646.

586. Ferguson TA, Peters T Jr, Reed R, Pesce A, Michael JG. Immunoregulatory properties of antigenic fragments from bovine serum albumin. *Cellular Immunol* . 1983;78:1-12.

587. Kawamura H, Berkower I, Glover C, Berzofsky J. Helper T cell epitope specificity regulates B cell (antibody) specificity. *J Cell Biochem*. 1984;8A:211-220.

588. Chesnut RW, Colon SM, Grey HM. Antigen presentation by normal B cells B cell tumors and macrophages: functional biochemical comparison. *J Immunol*. 1982;128:1764-1768.

589. Chesnut RW, Colon SM, Grey HM. Requirements for the processing of antigen by antigen-presenting cells. I: functional comparison of B cell tumors and macrophages. *J Immunol*. 1982;129:2382-2388.

590. Lanzavecchia A. Antigen-specific interaction between T cells and B cells. *Nature*. 1985;314:537-539.

591. Chesnut RW, Grey HM. Studies on the capacity of B cells to serve as antigen-presenting cells. *J Immunol*. 1981;126:1075-1079.

592. Malynn BA, Wortis HH. Role of antigen-specific B cells in the induction of SRBC-specific T cell proliferation. *J Immunol*. 1984;132: 2253-2258.

593. Rock KL, Benacerraf B, Abbas AK. Antigen presentation by haptenspecific B lymphocytes. I. Role of surface immunoglobulin receptors. *J Exp Med*. 1984;160:1102-1113.

594. Tony HP, Parker DC. Major histocompatibility complex-restricted polyclonal B cell responses resulting form helper T cell recognition of antiimmunoglobulin presented by small B lymphocytes. *J Exp Med*. 1985;161:223-241.

595. Kawamura H, Berzofsky JA. Enhancement of antigenic potency in vitro and immunogenicity in vivo by coupling the antigen to anti-immunoglobulin. *J Immunol*. 1986;136:58-65.

596. Davidson HW, Watts C. Epitope-directed processing of specific antigen by B lymphocytes. *J Cell Biol*. 1989;109:85-92.

597. Löwenadler B, Lycke N, Svanholm C, Svennerholm AM, Krook K, Gidlund M. T and B cell responses to chimeric proteins containing hete rologous T helper epitopes inserted at different positions. *Molec Immunol*.1992;29:1185-1190.

598. Manca F, Fenoglio D, Kunkl A, Cambiaggi C, Sasso M, Celada F. Differential activation of T cell clones stimulated by macrophages exposed to antigen complexed with monoclonal antibodies. A possible influence of paratope specificity on the mode of antigen processing. *J Immunol*. 1988;140:2893-2898.

599. Manca F, Fenoglio D, Li Pira G, Kunkl A, Celada F. Effect of antigen/antibody ratio on macrophage uptake, processing, and presentation to T cells of antigen complexed with polyclonal antibodies. *J Exp Med*. 1991;173:37-48.

600. Watts C, Lanzavecchia A. Suppressive effect of antibody on processing of T cell epitopes. *J Exp Med*. 1993;178:1459-1463.

601. Simitsek PD, Campbell DG, Lanzavecchia A, Fairweather N, Watts C. Modulation of antigen processing by bound antibodies can boost or suppress class II major histocompatibility complex presentation of different T cell determinants. *J Exp Med*. 1995;181:1957-1963.

602. Bekoff MC, Levine H, Schlossman SF. T cell and Ir gene regulation of expression of a cross-reactive idiotype. *J Immunol* . 1982;129:1173-1180.

603. Kawamura H, Kohno Y, Busch M, Gurd FRN, Berzofsky JA. A major anti-myoglobin idiotype: influence of H-2-linked Ir genes on idiotype expression. *J Exp Med*. 1984;160:659-678.

604. Fauchère JL, Pliska V. Hydrophobic parameters p of amino-acid side chains from the partitioning of N-acetyl-amino-acid amides. *Eur J Med Chem* . 1983;18:369-374.

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Chapter 24

Fc Receptors and Their Role in Immune Regulation and Inflammation

Jeffrey V. Ravetch

Falk Nimmerjahn

HISTORICAL BACKGROUND

Cellular receptors for immunoglobulins (Igs) were anticipated by the description of cytophilic antibodies of the IgG class, identified by Boyden and Sorkin in 1960.[1] These antibodies conferred upon normal cells, like macrophages, the capacity to specifically absorb antigen. Using sheep red blood cells (RBCs) as the antigen resulted in rosette formation between the cytophilic antisheep RBC antibodies and macrophages and provided a convenient means of visualization of the binding of cytophilic antibodies with normal cells. Subsequent studies by Berken and Benacerraf[2] suggested that the crystallized fragment (Fc) of the cytophilic antibody interacted with a cell surface receptor on macrophages. Similar studies on B-lymphocytes extended the generality of these receptors and led to the term *Fc receptor* (FcR) to denote the surface molecules on lymphoid and myeloid cells that are capable of interacting with the Fc of immunoglobulin molecules.[3] Studies on IgE, IgM, and IgA demonstrated the existence of distinct receptors for those isotypes as well on various immune cell types. Detailed biochemical characterization of Fc receptors was inaugurated by the studies of Kulczycki et al.[4] on the high-affinity IgE FcR of mast cells, revealing a hetero-oligomeric $\alpha\beta\gamma_2$ subunit structure. A distinction between FcRs for the IgE and IgG isotypes emerged with the observation of the very high (10^{10} M^{-1}) binding affinity of IgE for its receptor in comparison with the low binding (10^6 M^{-1}) of IgG1 to its receptor. This distinction led to the realization that the functional IgG1 ligand was exclusively in the form of an immune complex (IC), whereas IgE binding occurred through monomer interaction with its receptor. This difference in binding affinity had significant functional implications for the structures of these receptors and mechanisms by which each isotype activated its target cell. Determination of the structure of these receptors was facilitated by their molecular cloning, beginning with the IgG FcRs[5,6] and followed by the IgE FcR.[7] Two distinct types of IgG receptors, differing in their transmembrane and cytoplasmic sequences, were identified that offered a molecular explanation for the apparent contradictory activation and inhibitory activities attributed to IgG FcRs. The primary structure of the subunits of the high-affinity IgE FcR revealed homology in the ligand binding α subunit to its IgG counterparts. However, the extent of similarity between these receptors became apparent with the observation that the γ chain subunit was common to both IgG and IgE FcRs, providing both assembly and signaling functions to these activation receptors.[8,9] This common structure suggested a functional link between IC diseases and allergic reactions, a prediction that was confirmed through mouse knockout studies of IgG FcRs.[10,11] The FcRs, through their dependence on the immunoreceptor tyrosine-based activation motif (ITAM) pathway of cellular activation, belonged to the family of immunoreceptors that included the antigen receptors on B and T cells. Three-dimensional crystal structures have been solved for the low-affinity IgG FcRs[12,13] and the high-affinity IgE FcR,[14] alone and in complex with their Ig ligands,[15,16] further establishing the close structural link between these Ig receptors.

The functional roles of IgG FcRs were suggested by the distribution of these receptors on both lymphoid and myeloid cells.[17] On myeloid cells, they were presumed to mediate effector cell activation, resulting in phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), and release of inflammatory mediators. However, the well-known ability of the classical pathway of complement to generate activated fragments in response to ICs capable of inducing inflammatory responses by myeloid cells complicated the interpretation of the physiological role of IgG FcRs. Thus, the contribution of IgG FcRs to the mechanism of IC-mediated inflammation, as distinct from the role of complement, remained uncertain. Insight into this distinction was gained through the generation of mouse strains specifically deficient or blocked in either FcRs[10,18,19,20] or components of the classical complement pathway.[21] Studies on IC-mediated inflammatory responses in these animals, such as the Arthus reaction, led to the realization that IgG FcRs and not the classical pathway of complement activation were the functional mediators of inflammatory responses triggered by ICs.[20,22,23] The situation for IgE was less confounding, and the identification and characterization of a high-affinity receptor for this isotype on mast cells offered a plausible explanation for many of the inflammatory features of allergic reactions,[24] validated later by mouse knockouts of this receptor. IgG ICs had also been observed to mediate suppression of B-cell responses; thus, the presence of an IgG FcR activity on B cells provided a possible, but uncharacterized, mechanism for this inhibitory activity. Molecular characterization of this inhibitory activity for the B-cell FcR, FcRIIB, resulted in the first detailed description of an inhibitory motif, now termed the immunoreceptor tyrosine-based inhibitory motif (ITIM),[25,26] and the signaling pathway by which it abrogates ITAM-triggered activation. The ITIM mechanism is now

recognized as ubiquitous and has resulted in the recognition of a large family of inhibitory receptors on immune cells that function to maintain proper

thresholds for activation and abrogate activation responses to terminate an immune reaction.[27]

FcRs are now recognized as central mediators of antibody-triggered responses, coupling the innate and adaptive immune responses in effector cell activation.[28] In addition to these specialized roles, the IgG FcRs have served as an example of the emerging class of balanced immunoreceptors, in which activation and inhibition are tightly coupled in response to ligand binding. Perturbations in either arm of the response have been shown to lead to pathological consequences and have been taken as a paradigm of how these systems are likely to work for those paired immunoreceptors with unknown ligand-binding functions. The newly described roles for FcRs in maintaining peripheral tolerance, shaping the antibody repertoire, regulating antigen-presenting cell (APC) maturation, and promoting mast cell survival indicate the diversity of functions that these receptors possess and their central role in modulating both afferent and efferent responses in the immune response.

This chapter focuses primarily on the IgG and IgE FcRs, for which substantial data on their structure, function, regulation, and role in a variety of physiological and pathological conditions are now available. The similarity in structure and signaling between those receptors and other members of this family, such as the IgA FcR and the recently discovered Fc-receptor for mouse and human IgM (FcμR), is also discussed. These FcRs will be referred to as “canonical Fc receptors,” reflecting their shared structural and functional properties. In contrast, lectins such as SIGN-R1 and DC-SIGN, which bind IgG Fc when sialylated, represent a novel class of FcRs with distinct structures and functions and will be discussed in detail in the following. Other Ig receptors with specialized functions in the transport of Igs, such as the FcRn[29] and the poly-Ig FcR,[30] will not be discussed here.

STRUCTURE AND EXPRESSION

Molecular Genetics

Two general classes of canonical FcRs are now recognized: the activation receptors, characterized by the presence of a cytoplasmic ITAM sequence associated with the receptor, and the inhibitory receptor, characterized by the presence of an ITIM sequence. These two classes of receptors function in concert and are usually found coexpressed on the cell surface. Thus coengagement of both signaling pathways is the rule, setting thresholds for and ultimately determining the physiological outcome of effector cell responses. Among the factors that determine this threshold level are the actual affinities of the individual activating and inhibitory receptors for a specific IgG ligand and the expression level of the receptor pairs on immune effector cells. Importantly, the affinity of different antibody isotypes and subclasses for their respective activating and inhibitory FcRs varies significantly, thus explaining the differential activity of antibody isotypes in vivo.[20,31,32] In addition, alleles of specific FcRs have been described that alter their affinity for individual subclasses thus accounting for the variable responses seen in a population to an antibody (see subsequent discussion). Noncanonical FcRs such as the IgM receptors FcμR, Fca/μR, the poly-Ig receptor, and the sialylated IgG Fc receptors SIGN-R1 and DC-SIGN, contain neither ITAM nor ITIM motifs.

Subunit Composition

Canonical FcRs are typically type I integral membrane glycoproteins consisting of, at the least, a ligand recognition *α* subunit that confers isotype specificity for the receptor. *α* subunits for IgG, IgE, IgM, and IgA have been described.[17,33,34,35] These subunits typically consist of two extracellular domains of the IgV type superfamily, a single transmembrane domain and a relatively short intracytoplasmic domain. In activation FcRs, a signaling subunit of the *γ* family is often found, resulting in an $\alpha\gamma_2$ complex. The inhibitory FcγRIIB molecule, in contrast, is expressed as a single-chain receptor. The *α* subunits have apparent molecular weights of between 40 and 75 kDa, and share significant amino acid sequence homology in their extracellular domains. Alternatively, spliced forms of FcγRIIB modify the intracytoplasmic domain of this molecule. For example, the B2 form lacks sequences that inhibit internalization and thus demonstrates enhanced internalization of ICs, in comparison with FcγRIIB1. However, all the splice variants contain the ITIM motif, a necessary and sufficient domain for mediating inhibitory signaling. The conservation of this sequence in mice and humans, its presence in all splice variants, and the hyperresponsive phenotypes generated in mice deficient in this receptor all support inhibition as the central function of FcγRIIB. The specific structures of the *α* subunits of the canonical FcRs are shown in Figure 24.1. The notable exceptions to the general structure just outlined are seen for the high-affinity FcγRI*α* subunit, which has three extracellular domains; the activation FcγRIIA*α* subunit, which does not require additional subunits for assembly or signaling; and the glycosylphosphatidyl-inositol (GPI)-linked FcγRIIIB, which attaches to the cell surface through a GPI linkage, rather than through a transmembrane domain.

The *γ* subunit is found associated with activation IgG, IgE, and IgA FcRs, as well as with non-FcR molecules, such as paired Ig-like receptor A (PIR-A) and natural killer (NK) cell cytotoxicity receptors, but not with IgM receptors, nor the lectins SIGN-R1 and DC-SIGN.[36,37,38] It is required for assembly of the *α* subunits of these receptors by protecting these subunits from degradation in the endoplasmic reticulum. The *γ*chain is found as a disulfide-linked homodimer, with a short extracellular domain containing the cysteine involved in dimerization, a transmembrane domain, and an intracytoplasmic domain containing the ITAM. An aspartic acid residue found in the transmembrane domain of the *γ*chain is often associated with a basic amino acid residue in the transmembrane domain of the *α* subunit.

The γsubunit belongs to a gene family that includes the T-cell receptor-associated ζ chain and the NK receptor DAP-10- and DAP-12-associated molecules.[39] FcγRIIIA can associate with the ζ chain, resulting in the $\alpha\zeta_2$ complex found in human NK cells.

A third subunit is found associated with the activation FcRs FcεRI and FcγRIII, the β subunit. This 33-kDa subunit has four transmembrane-spanning domains and amino and carboxy intracytoplasmic domains, belonging to the cluster

P.585

of differentiation (CD)20 family of tetraspan molecules.[24] An ITAM sequence is found in the intracytoplasmic carboxy domain. In mast cells and basophils, the β chain assembles into an $\alpha\beta\gamma_2$ complex with the α chain belonging to either FcγRIII or FcεRI. Its presence is required for assembly of FcεRI in rodents. In humans, however, $\alpha\gamma_2$ complexes of FcεRI are found in monocytes, Langerhans cells, and dendritic cells (DCs), in addition to the $\alpha\beta\gamma_2$ complexes found in mast cells and basophils. The ITAM motif found in the β subunit is not an autonomous activation sequence but functions as a signaling amplifier of the ITAM found in the γsubunits.[40]

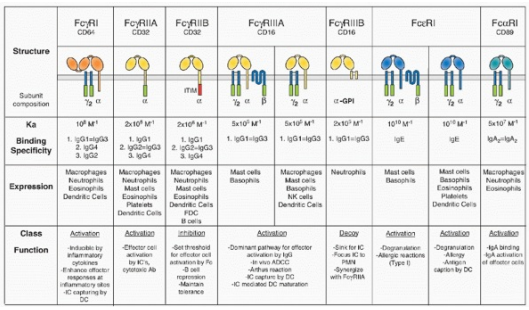

FIG. 24.1. Summary of Fc Receptor Structures, Expression Patterns, and in Vivo Functions. The immunoreceptor tyrosine-based activation motif signaling motif is indicated by the *green rectangle*; the immunoreceptor tyrosine-based inhibitor motif is indicated as a *red rectangle*. Alleles of FcγRIIA and FcγRIIIB and their binding properties are discussed in the text.

Gene Organization, Linkage, and Polymorphisms

All α subunits of canonical FcRs share a common gene organization, which indicates that the evolution of this family of receptors resulted from gene duplication from a common ancestor.[41] Sequence divergence then resulted in the acquisition of distinctive specificities for these related sequences. Most of the genes belonging to the expanded FcR family, including the recently identified Fcμ-receptor, the Fcα/μR, the poly-Ig receptor, the group of FcR-homologous or -like (FCRL) proteins, the α chains of FcγRI, FcγRII, FcγRIII, and FcεRI including the common FcR-γ chain (Fig. 24.2), are found on the long arm of chromosome 1.[34,35,42,43,44] This region is syntenic with a comparable region on mouse chromosome 1; however, FcγRIα and several FCRLs are found on mouse chromosome 3. In humans, the α subunit of the IgA receptor is found on chromosome 19 as is the lectin DC-SIGN (CD209) and the β subunit is on chromosome 11. The FcγRII-FcγRIII locus on chromosome 1 is further linked to a variety of lupus susceptibility genes found in that region, including the *Sle1* cluster.[45] A locus linked to atopy has been identified at 11q12-13 and further delineated polymorphisms of the β chain (I181V and V183L) that are associated with a heightened risk of atopy. However, a direct functional association of these polymorphisms with the known biological activities of the β chain has not been found.[46]

Polymorphisms in the α chains of the FcγRs have been described, most notably in FcγRIIA and FcγRIIIA; these polymorphisms result in differences in binding affinity to specific IgG subclasses.[47] For example, a histidine at position 131 in FcγRIIA results in higher affinity binding to IgG2 and IgG3 than does an arginine at that position. Similarly, FcγRIIIA with valine at position 158 of the α chain has a higher binding affinity for IgG1 and 3 than does the polymorphic form with phenylalanine at that position. These polymorphisms translate into a more robust ADCC response for the FcγRIIIA val/val and the FcγRIIA his/his haplotype in vitro and has been positively correlated with a better clinical response to antitumor antibodies inclunding the CD20-specific antibody rituximab, the Her2/neu-specific antibody trastuzumab, and the epidermal growth factor receptor-specific antibody cetuximab in human lymphoma, metastatic breast cancer, and colorectal cancer patient cohorts, respectively.[48,49,50,51,52,53] This data is consistent with previous results obtained with rituximab

P.586

and trastuzumab in mouse models, demonstrating an important role of cellular FcγRs for the activity of these therapeutic antibodies in vivo.[54,55] Four amino acids are polymorphic for FcγRIIIB at positions 18, 47, 64, and 88, which contribute to the neutrophil antigen polymorphisms for this receptor. Several studies have attempted to link specific FcR polymorphisms or copy number variations to autoimmune diseases, specifically to systemic lupus erythematosus (SLE).[56] Recent studies have reported associations in susceptibility to SLE in both murine and human populations with levels of FcγRIIB

expression or alleles of FcγRIIB.[57] Reduced expression of FcγRIIB on activated B cells, such as memory cells, has been seen in patients with SLE and chronic inflammatory demyelinating polyneuropathy (CIDP), and is associated with a promoter polymorphism.[58,59] In mouse strains that develop a spontaneous, lupus-like disease, restoring the level of FcγRIIB expression on their B cells to a wild-type level reverses disease.[60,61] A higher incidence of an allele of FcγRIIB has been reported in several populations with autoimmune disease. This allele, found in the transmembrane domain of the receptor, is suggested to result in a hypomorphic phenotype, similar to the reduced expression observed.[57] Of note, despite increasing the risk for development of SLE, this FcγRIIB allele seems to decrease the likelihood of malaria infections and the severity of disease and can be found at increased frequencies in areas of the world where malaria is endemic.[62,63]

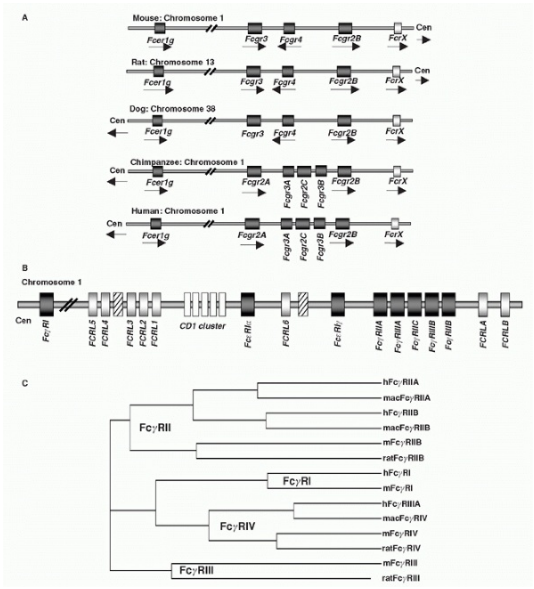

FIG. 24.2. Chromosomal Organization of Fc Receptor (FcR) and FcR-Like Genes. A: Localization of FcR genes in different species. **B:** The human FcR locus on chromosome 1. Classical FcR genes are shown in *dark grey*, FCRL genes as *light grey*, and the CD1 gene cluster on chromosome 1 as *white boxes*. Pseudogenes are indicated as hatched boxes. (adapted from Davis et al.[44]). **C:** The cladogramm shows the alignment of selective classical FcRs of humans (*h*), macaques (*mac*), mice (*m*), and rats.

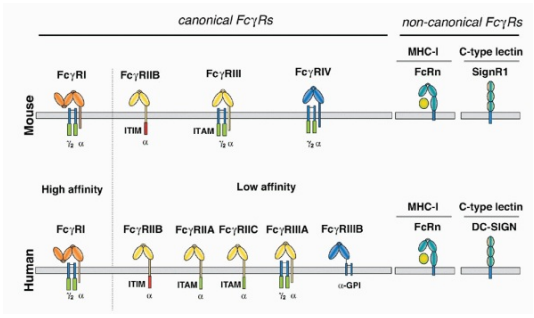

FIG. 24.3. Comparison of the Human and Mouse Canonical and Noncanonical Fcγ-Receptor Protein Family. In both species, canonical FcγRs can be distinguished by their affinity for the antibody Fc-portion (high or low affinity) and by the signaling pathways they trigger (activating versus inhibitory). The noncanonical neonatal FcR belongs to the family of major histocompatibility class I proteins and regulates immunoglobulin (Ig)G half life. Mouse SIGNR1 or human DC-SIGN are C-type lectin proteins that can only bind to IgG glycoforms rich in terminal sialic acid residues and are involved in the anti-inflammatory activity of IgG.

Species Comparisons

Detailed comparisons between the canonical FcRs in mice and humans have revealed several notable differences in both structure and expression of these molecules (Figs. 24.1, 24.2, 24.3). Whereas IgG, IgM, and IgE FcRs are conserved in these species, IgA FcRs are not. To date, a murine homolog for the IgA FcR has not been identified. In general, murine

and human IgG FcRs display comparable degrees of heterogeneity and complexity.[41,64] Specific differences, however, have been noted. For example, FcγRI is encoded by a single gene in the mouse, in comparison with three genes in the human.[43] Two genes for activation FcRs, FcγRIIA and C, are found in the human and not rodents, which is notable because of their unusual single-chain activation structure.[65] Both mice and human encode a gene referred to as FcγRIIIA, although recent studies have identified a novel mouse FcR with higher homology to the human FcγRIIIA called FcγRIV.[31,64,66] As mentioned previously, FcγRIIIB is unique among FcRs in being expressed as a GPI-anchored protein (see Fig. 24.3). Its expression is limited to human neutrophils, in comparison with FcγRIIIA, which is expressed widely on cells of the myeloid lineage, such as macrophages, NK cells, mast cells, and DCs. Finally, both mice and humans have only a single gene encoding the inhibitory FcγRIIB molecule. Among the noncanonical FcRs, the lectin DC-SIGN is the functional homologue of the murine SIGN-R1 molecule with respect to its ability to bind sialylated IgG Fc and mediate an antiinflammatory response.[67,68] While the overall structure of these proteins are similar (see Fig. 24.3), their patterns of expression are quite distinct. SIGN-R1 expression is restricted to marginal zone macrophages of the spleen and lymph node, whereas DC-SIGN expression is seen on myeloid DCs and macrophages in a variety of tissues, including spleen, bone marrow, and lymph node.[69]

The genes for the IgE FcR are conserved in mice and humans. The difference that is observed relates to the requirement for the β chain to achieve surface expression

in mice, precluding the expression of the $\alpha\gamma_2$ complex.[24] In humans, this form of the receptor is widely expressed on monocytes, Langerhans cells, and DCs, and is likely to be found on mast cells and basophils as well. This difference in FcεRI subunit composition is likely to result in functional differences as well. Although these specific interspecies differences are important, the fundamental organization of the canonical FcR system, with activation and inhibitory signaling through a shared ligand specificity coupled to opposing signaling pathways, is well conserved, as is the role of the noncanonical FcRs in mediating the immunomodulatory functions of sialylated IgG. Thus, conclusions regarding the function of this expanded FcR system in immunity by the analysis of murine models are relevant to an understanding of the role of these receptors to human immunity as well.

Expression

Canonical FcRs are expressed widely on cells of the myeloid lineage, including monocytes, macrophages, DCs, mast cells, basophils, neutrophils, eosinophils, and NK cells.[17,33] In addition, B cells and follicular DCs (FDCs) express the inhibitory FcγRIIB receptor, whereas T cells are generally negative for FcγR expression, but may express the Fc-receptor for IgM. Interestingly, the majority of FCRL proteins are expressed during varying stages of B-cell development. Despite their homology to the canonical FcRs, however, the FCRL proteins seem not to bind to Igs, rendering FcγRIIB the only IgG binding FcR on B cells. The specific expression pattern for each FcR varies, and these patterns are summarized in Figure 24.1. Because canonical FcRs represent a balanced system of activation and inhibition, the general rule of coexpression of FcRs of these classes is maintained. B cells use the B-cell antigen receptor as the activation coreceptor for FcγRIIB, whereas NK cells appear to utilize NK inhibitory receptors to modulate FcγRIIIA activation. The decoy FcγR, FcγRIIIB, is expressed exclusively on human neutrophils, on which it functions to concentrate and focus ICs without directly triggering cell activation, perhaps also playing role in neutrophil recruitment.[70] The FcγRIIA-FcγRIIB pair functions on neutrophils to modulate IC activation. FcεRI can be modulated by FcγRIIB, as demonstrated both in vitro and in vivo; mice deficient in FcγRIIB display enhanced IgE-triggered anaphylaxis[71] by virtue of the ability of IgE to bind with high affinity to FcεRI and with low affinity for FcγRIIB. Other mast cell inhibitory receptors, such as glycoprotein 49B1, modulate mast cell sensitivity to IgE: mice deficient in this molecule display enhanced anaphylactic responses to IgE stimulation.[72] Expression of the common *γ* chain is broad: it has been found on all myeloid and lymphoid cells examined to date. In contrast, the *β* chain appears to be quite restricted in its expression: it has been found only on mast cells and basophils.

Regulation of canonical FcR expression can occur at several levels. In general, cytokines involved in activation of inflammatory responses induce expression of activation FcγRs, whereas inhibitory cytokines downregulate these activation receptors. Transcriptional regulation of *α* chain levels has been documented for a variety of cytokines, including interferon-*γ*, interleukin (IL)-4, and transforming growth factor *β*.[73,74] In addition, complement component C5a binding to its receptor, C5aR, results in the induction of expression of activation FcγRs.[75] Induction of FcγRI, FcγRIIA, and FcγRIIIA *α* and *γ*chains in myeloid cells occurs upon interferon-*γ*treatment: IL-4 generally inhibits expression of these activation receptors but induces expression of the inhibitory FcγRIIB. Administration of intravenous gamma globulin, a widely used treatment for inflammatory diseases, has been shown to induce expression of the inhibitory FcγRIIB on effector macrophages and B cells in mice and humans.[59,76,77,78] This induction is not direct but mediated through other, noncanonical FcR pathways including molecules such as SIGNR1 and its human orthologe DC-SIGN (see Fig. 24.7). Engagement of these receptors by sialylated IgG results in an intrinsic Th2 pathway, ultimately leading to the expression of IL-4, which induces FcRIIB expression on inflammatory macrophages.[67,68,79,80] The situation in B cells is likely to be more complex, whereby regulation of FcγRIIB is critical for the maintenance of peripheral tolerance. Germinal center B cells downregulate FcγRIIB, perhaps in response to IL-4 production by T cells. Regulation of FcR expression has also been documented to occur upon binding of ligand. IgE regulates the expression of FcεRI by stabilizing the intracellular pool of receptor

upon receptor engagement.[81] Thus, high IgE levels result in the induction of surface expression of FcεRI. However, this same mechanism of regulation is not seen for FcγRs: mice deficient in IgG have FcγR levels comparable with those of wild-type animals. Competition for limiting subunits also contributes to regulation of receptor expression. In mast cells, it appears that the level of γ chain is limiting. Competition between α chains for the limiting concentration of γ chain has been documented in mouse knockouts, whereby levels of one receptor increase if the α chain of the other receptor is reduced.[82] This type of reciprocal regulation has recently also been observed for FcγRs in neutrophils and is likely to be significant in the cross-regulation of FcRs by different isotypes of Ig.[83]

Three-Dimensional Structure

The crystal structures of FcγRIIA, FcγRIIB, FcγRIIIA, and FcεRI have been solved, as have the cocrystals of FcγRIIIA-IgG1 Fc and FcεRI-IgE Fc[84] (Fig. 24.4). These studies demonstrate that the receptors have a common structure in which the two extracellular Ig domains fold in a strongly bent overall structure, arranged into a heart-shaped domain structure. A 1:1 stoichiometry between the receptor and ligand is observed, with the receptor inserted into the cleft formed by the two chains of the Fc fragment (Cγ2 or Cε3). The binding region of the FcR to Fc fragments consists mainly of rather flexible loops that rearrange upon complex formation. Only domain 2 and the linker region connecting domains 1 and 2 interact in the complex with different regions of both chains of the Fc. Conserved tryptophans located on the FcRs interact with proline to form a "proline sandwich." A solvent-exposed hydrophobic residue at position 155 is conserved among all FcRs and represents a binding site for the important IgG1 residue Leu 235 (not

found in IgE). Specificity is generated among the receptor-ligand pairs in a variable region connecting the two extracellular domains that is in contact with the lower hinge region of the Fc fragment (residues 234 to 238), a region not conserved among the IgGs and IgE. The binding region of FcRs to their Ig ligands does not overlap with other Fc binding molecules such as protein A, protein G, and FcRn.

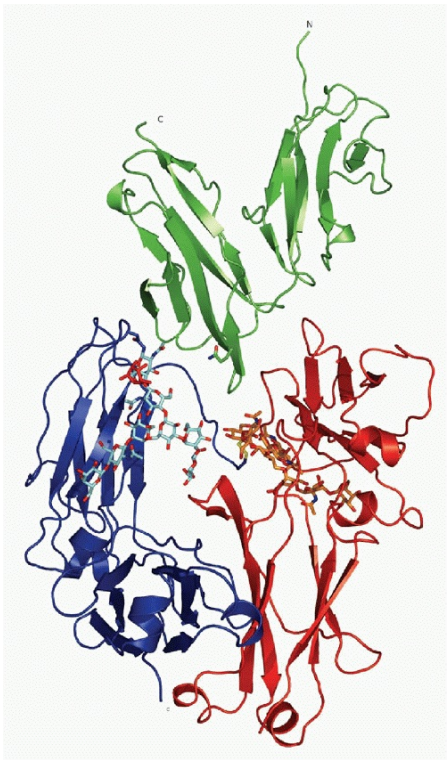

FIG. 24.4. Ribbon Diagram of Crystallizable Fragment (Fc) Receptor III-Immunoglobulin (IG)G1 Fc Structure. The carbohydrate moiety attached to both CH2 domains of the IgG Fc-fragment is shown as a stick and ball model. The extracellular domains of Fc receptor III are shown, together with the Fc fragment of IgG1. See text for details. Adapted from Sondermann et al.,[15] with permission.

The structure of the FcR bound to its ligand reveal that the antigen-binding fragment (Fab) arms are quite sharply bent and may adopt a perpendicular orientation toward the Fc. This arrangement would give the Fab arms maximal flexibility to bind antigen when the Fc fragment is oriented parallel to the membrane of the FcR-expressing cell. The asymmetrical interaction of the two Fc chains with a single FcR prevents a single antibody molecule from triggering dimerization of receptors and initiating signaling. Instead, dimerization is initiated by the interaction of antigen with the Fab arms, thus linking adaptive responses to effector cell triggering. Of note, the majority of the IgG-FcγR cocrystals have been generated with FcγRs produced in bacteria and therefore in an aglycosylated form. In general glycosylation at Asn

297 is critical for FcγR binding; modulation of FcγR binding by glycan modification has been observed for several classes of glycan modifications, including sialylation, fucosylation, and branching N-acetyl glucosamine.[85,86] The first IgG-FcγR cocrystal structure with differentially glycosylated IgG variants demonstrated that carbohydrate-carbohydrate interactions between IgG and FcγR are essential for high-affinity recognition of afucosylated IgG glycovariants by FcγRIIIA.[87] In contrast, low-affinity binding of FcγRIIIA to fucosylated IgG is independent of these carbohydrate interactions. These novel insights may offer new ways of optimizing IgG-FcγR interactions for enhanced therapeutic activity of IgG.

IN VITRO ACTIVITY

Binding Properties

As outlined in Figures 24.1 and 24.3, Ig binding to canonical FcRs falls into either high- or low-affinity binding classes. The high-affinity binding class is typified by FcεRI, with a binding affinity of 10^{10} M^{-1} for IgE, which ensures a monomeric interaction between IgE and its receptor. FcγRI binds with relatively high affinity for IgG1 and IgG3 (human) and IgG2a (mouse) with an affinity constant of 10^{8} M^{-1}. In contrast to these high-affinity FcRs, the low-affinity receptors, such as the human FcγRIIA, FcγRIIB, FcγRIIIA, FcγRIIIB, and FcαRI, and the mouse FcγRIIB, FcγRIII, and FcγRIV bind with affinities ranging from 5×10^{5} M^{-1} (FcγRIII) to 5×10^{7} M^{-1}. This low-affinity binding ensures that these receptors interact with ICs and not monomeric ligands. As described subsequently, this dependence on high-avidity and low-affinity interactions ensures that these receptors are activated only by physiologically relevant ICs and not by circulating monomeric Ig, thus avoiding inappropriate activation of effector responses. In general, low-affinity FcγRs bind IgG1 and IgG3 preferentially; their binding to IgG2 and IgG4 is of even lower affinity. As mentioned previously, polymorphisms in FcγRIIA and FcγRIIIA affect binding to IgG2 and IgG1, respectively, which may have significance in vivo in predicting responses to specific cytotoxic antibodies (Table 24.1). The isolated consideration of the affinity of antibody isotypes to their activating FcγRs is not sufficient, however, to explain the differences in in vivo activity. More importantly, the ratio of the affinities of an antibody isotype to the activating FcγRs compared to the inhibitory FcγRIIB (termed A/I-ratio) predicts antibody activity more consistently.[20,64] Thus, antibody isotypes with a high A/I-ratio, such as mouse IgG2a and IgG2b, will show a greater activity than counterparts with a lower ratio (see Table 24.1). The sugar moiety of the antibody Fc-portion is essential for FcR binding and the presence or absence of certain sugar residues can significantly impact on antibody-FcR binding.[20,80,88,89] The absence of fucose, for example, will selectively increase the affinity of human IgG1 or mouse IgG2b for human FcγRIIIa or mouse FcγRIV, respectively. In contrast, the presence of terminal sialic acid on the N-linked Fc glycan reduces affinities for FcRs by an order of magnitude with a concomitant reduction in in vivo activity.[80] Subunit interactions have also

been reported to influence affinity for ligand, as demonstrated for the common γchain associating with FcγRIIIA.[90] Its affinity for IgG1 is higher than the GPI-anchored form of this receptor, FcγRIIIB.

TABLE 24.1 Affinities of Mouse and Human Fc Receptors to Different Antibody Isotypes

	Mouse Soluble FcγR (K_A in M^{-1})					*Human Soluble FcγR (K_A in M^{-1})*						
	FcγRI	**FcγRIIB**	**FcγRIII**	**FcγRIV**	**A/I**	**FcγRI**	**FcγRIIA^{131R}**	**FcγRIIA^{131H}**	**FcγRIIB**	**FcγRIIIA^{158F}**	**FcγRIIIA^{158V}**	**A/I[a]**
mIgG1	n.b.	3.3×10^{6}	3.1×10^{5}	n.b.	0.1	n.d.	2.5×10^{5}	0.4×10^{5}	1×10^{5}	$< 10^{4}$[b]	n.d.	
mIgG2a	1.8×10^{8}	0.42×10^{6}	6.8×10^{5}	2.9×10^{7}	69	n.d.	3.2×10^{5}	1.7×10^{5}	1.6×10^{5}	1.0×10^{5}	n.d.	
mIgG2b	n.b.	2.2×10^{6}	6.4×10^{5}	1.7×10^{7}	7	n.d.	9.1×10^{4}	1.2×10^{5}	1.2×10^{5}	0.1×10^{5}	n.d.	
mIgG3	n.b.	n.b.	n.b.	n.b.	—	n.d.	$< 10^{4}$[b]	$< 10^{4}$[b]	$< 10^{4}$[b]	$< 10^{4}$[b]	n.d.	
hIgG1	3.8×10^{6}	2×10^{5}	3.5×10^{4}	2.2×10^{6}		9.1×10^{8}[d]	3.5×10^{5}[e]	5.2×10^{5}[e]	1-3.8 × 10^{5}[c,e]	0.4-1.1 × 10^{6}[e]	1.9-4.8 × 10^{6}[c,e]	1/13
hIgG2	n.b.	$< 10^{4}$[b]	$< 10^{4}$[b]	n.b		n.b.	1.0×10^{5}[e]	4.5×10^{5}[e]	0.2×10^{5}[e]	0.3×10^{5}[e]	0.7×10^{5}[e]	1.5/3.5
hIgG3	1.2×10^{6}	8.3×10^{4}	n.b.	$< 10^{4}$[b]		6.1×10^{7}[e]	9.1×10^{5}[e]	8.9×10^{5}[e]	1.7×10^{5}[e]	7.7×10^{6}[e]	9.8×10^{6}[e]	45/57
hIgG4	7.2×10^{4}	$< 10^{4}$[b]	$< 10^{4}$[b]	$< 10^{4}$[b]		3.4×10^{7}[e]	2.1×10^{5}[e]	1.7×10^{5}[e]	2×10^{5}[e]	2.0×10^{5}[e]	2.5×10^{5}[e]	1

Binding constants were obtained by surface plasmon resonance analysis with immobilized antibodies (FITC-isotype switch variants) and soluble Fc receptors (FcRs) produced by transient transfection in 293T cells. Shown are the association constants (K_A in M^{-1}) of soluble FcR binding to the indicated antibody isotypes as determined by surface plasmon resonance analysis.31,174,175,176 IG, immunoglobulin; n.b., no detectable binding or binding that is too low to be evaluated; n.d., that no surface plasmon resonance or other quantitative data are available.

[a] The two numbers indicate the A/I ratios for the low and high affinity allele of FcγRIII

[b] Indicates detectable but very low binding that did not allow to determine exact binding constants by surface plasmon resonance.

[c] *Maenaka et al.174; Okazaki et al.175

[d] #Paetz et al.176

[e] §Bruhns et al.177

The crystal structures of IgG1-FcγRIIIA and IgE-FcεRI reveal similarities in the binding properties of these two complexes. Of significance is the 1:1 stoichiometry of the complexes, which ensures that a single receptor binds to a single immunoglobulin molecule.[15,16] This property in turn ensures that activation occurs upon cross-linking of receptor complexes by multivalent ligands. Two binding sites on the receptor interact asymmetrically with two sites on the Fc molecule. The FcR inserts into the cleft formed by the two chains of the Fc molecule, burying a binding surface of 895 Å for each binding site. Alterations in the Fc structure that reduce the cleft, such as deglycosylation of IgG, inhibit FcR binding. Four distinct regions have been defined in the Fc domains involved in FcR interactions. For IgE, this includes residues 334 to 336, 362 to 365, 393 to 396, and 424. The homologous regions for IgG are residues 234 to 239, 265 to 269, 297 to 299, and 327 to 332. Interactions of these residues occur with the carboxy-terminal domain 2 of the respective FcRs. In view of the similarities of these complexes and the homologies among the receptors and their ligands, an obvious question that arises concerns the molecular basis for specificity. Attempts to resolve that question have relied on mutagenesis studies of the ligands and domain exchanges between receptors. For example, exchange of the FG loop in domain 2 of Fcε to Fcγ receptors confers detectable IgE binding; similarly, variation in this loop in FcγRs may provide interactions that determine IgG specificity for these receptors. Mutagenesis of IgG1 revealed that a common set of residues is involved in binding to all FcγRs, but FcγRII and FcγRIII also utilize distinct residues.[91] Several IgG1 residues not found at the IgG-FcR interface by crystallographic determination had a profound effect on binding, which indicates the greater complexity of these interactions in solution.

The implications of these structural studies are that the Fc domain of IgG may be selectively mutated to direct its binding to specific FcγRs. Fc mutants that selectively engage activation FcγRs (IIIA and IIA) while minimally interacting with inhibitory and decoy FcγRs (IIB and IIIB) would confer optimal cytotoxic potential for tumoricidal applications. Indications that such Fc engineering is possible are suggested by IgG mutants with selective binding to FcγRIII or FcγRII.[92,93,94]

Effector Cell Activation

The critical step in triggering effector cell response by canonical FcRs is mediated by the cross-linking of these receptors by Ig. This can occur either by interactions of low-affinity, high-avidity IgG ICs or of IgG opsonized cells with activation FcγRs or by the cross-linking of monomeric IgG or IgE bound to FcγRI or FcεR, respectively, by multivalent antigens binding to the Fab of the antibody. Crosslinking of ITAM-bearing FcRs results in common cellular responses, determined by the cell type, rather than the FcR. Thus, for example, FcεRI or FcγRIII cross-linking of mast cells results in degranulation of these cells, whereas crosslinking of macrophage expressed FcαRI or FcγRIII by opsonized cells triggers phagocytosis. These functions underlie the functional similarity of activation FcRs in which crosslinking mediates cellular responses by ITAM-mediated tyrosine kinase cascades. In addition to degranulation and phagocytosis, activation FcR cross-linking has been demonstrated to induce ADCC, the oxidative burst, and the release of cytokines and other inflammatory cell mediators. A sustained calcium influx is associated with these functions, as is transcription of genes associated with the activated state.

Cellular activation initiated by ITAM-bearing activation FcRs can be enhanced by coengagement with integrin and complement receptors. Although the ability of these receptors to mediate phagocytosis, for example, are

modest, synergistic interactions with FcRs result in sustained activation and enhancement. Synergistic interactions between activation FcRs and toll receptors, mannose receptors, and other pattern-recognition molecules have also been reported in vitro and suggest that interplay between the innate and adaptive effector mechanisms of an immune response are involved in mediating efficient protection from microbial pathogens.

In contrast to the activation of effector cell responses triggered by cross-linking of ITAM-bearing FcRs in vitro, cross-linking of an ITIM-bearing inhibitory receptor to an ITAM-bearing receptor results in the arrest of these effector responses. Homoaggregation of FcγRIIB by its crosslinking on effector cells by ICs does not result in cellular responses; rather, it is the coengagement of ITAM- and ITIM-bearing receptors that results in the functional generation of an inhibitory signal. In vitro, it is possible to ligate any ITAM-bearing receptor to any ITIM-bearing receptor with a resulting inhibitory response. This activity is used functionally to define putative ITIMs and has proved to be a useful device in dissecting the signaling

pathways induced by ITAM-ITIM coligation.

B-Lymphocyte Suppression

B-cell stimulation through the B-cell antigen receptor can be arrested by the coligation of FcγRIIB to the B-cell receptor (BCR). This occurs naturally when ICs, retained on FDCs in the germinal center, interact with both the BCR and FcγRIIB during the affinity maturation of an antibody response. In vitro suppression of B-cell activation has been demonstrated by coligation of BCR and FcγRIIB, resulting in arrest of calcium influx and proliferative responses triggered by the BCR,[25,95] the result of recruitment of the SH2-containing inositol 5′-phosphatase (SHIP)-1.[96] Calcium release from the endoplasmic reticulum is not affected, and there is thus an initial rise in intracellular calcium; however, this calcium flux is not sustained, because SHIP recruitment blocks calcium influx by uncoupling of the capacitance channel. Homoaggregation of FcγRIIB by ICs can trigger apoptosis in B cells, as demonstrated in the DT40 B-cell line and in murine splenocyte preparations.[97] This activity is retained in ITIM mutants and is dependent on the transmembrane sequence of FcγRIIB. Consistent with this, other studies showed that a SHIP-independent but BTK-, JNK1-, and cABL-dependent pathway is involved in induction of apoptosis upon FcγRIIB homoaggregation.[98] The potent inhibitory effect of FcγRIIB on B-cell activation has recently been used therapeutically to suppress autoantibody production by B cells. By engineering the Fcfragment of CD19-specific antibodies to have increased affinity to FcγRIIB, resulting in an enhanced coengagment of the inhibitory FcγRIIB with the BCR signaling complex, it was possible to suppress humoral immunity in mice and to shut down autoantibody production by B cells.[94] In contrast to mature B cells, which coexpress the BCR and FcγRIIB, plasma cells responsible for high-level antibody production downregulate BCR expression but maintain FcγRIIB expression. It was suggested that crossliniking of FcγRIIB by ICs on bone marrow plasma cells might be involved in inducing apoptosis in a fraction of plasma cells thereby creating niches for newly generated plasma cells during exposure to a new antigenic stimulus.[99]

SIGNALING

Immunoreceptor Tyrosine-Based Activation Motif Pathways

The general features of signal transduction through ITAM receptors are conserved among all members of this family, including T-cell receptors, BCRs, and various FcRs. The 19 amino acid-conserved ITAM is necessary and sufficient to generate an activation response, as demonstrated by the analysis of chimeric receptors. With a single exception, FcRs associate with accessory subunits that contain these signaling motifs. As described previously, the common *γ*chain contains an ITAM and is associated with FcεRI, FcγRI, FcγRIII, FcγRIV, and FcαRI. In addition, both FcεRI and FcγRIII may associate with the *β* subunit in mast cells. The ITAM found in the *β* chain does not function as an autonomous activation cassette, as has been found for most other ITAMs. Rather, it functions to amplify the activation response generated by the *γ* chain ITAM by increasing the local concentration of Lyn available for activation upon aggregation of the receptor.[40] FcγRIIA contains an ITAM in the cytoplasmic domain of its ligand recognition *α* subunit and is thus able to activate in the absence of any associated subunit.

Upon sustained receptor aggregation, Src family kinases that may be associated with the receptor in an inactive form become activated and rapidly tyrosine-phosphorylate the ITAM sequences, creating SH2 sites for the docking and subsequent activation of Syk kinases. Ligands that rapidly dissociate from their receptors result in nonproductive signaling complexes that fail to couple to downstream events and behave as antagonists.[100] The specific Src kinase involved for each FcR depends on the receptor and cell type in which it is studied. Thus, Lyn is associated with the FcεRI pathway in mast cells, Lck is associated with FcγRIIIA in NK cells, and both of these kinases as well as Hck are associated with FcγRI and FcγRIIA in macrophages. After activation of the Src kinase, tyrosine phosphorylation of the ITAM motif rapidly ensues, leading to the recruitment and activation of Syk kinases. This two-step process is absolutely necessary to transduce the aggregation signal to a sustainable intracellular response. Once activated, Syk kinases lead to the phosphorylation or recruitment of a variety of intracellular substrates, including PI3K, Btk and other Tec family kinases, phospholipase C-*γ*(PLC*γ*), and adaptor proteins such as SLP-76 and BLNK. The Ras pathway is also activated through Sos bound to Grb2 that is recruited upon phosphorylation of Shc. Ras phosphorylates Raf, which in turn leads to MEK kinase and MAP kinase activation. A summary of these intracellular pathways is shown in Figure 24.5A. A crucial step in this sequential activation cascade occurs with the activation of PI3K by Syk. By generating phosphatidyl inositol polyphosphates, such as PIP_3, PI3K leads to the recruitment of pleckstrin homology (PH) domain-expressing proteins such as Btk and PLC*γ*, which in turn leads to the

generation of inositol triphosphate and diacylglycerol, intermediates crucial to the mobilization of intracellular calcium and activation of protein kinase C, respectively.

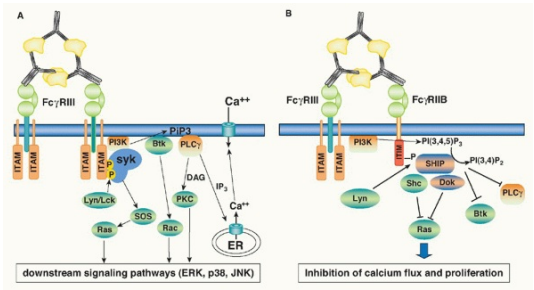

FIG. 24.5. Signaling by Activation and Inhibitory Fc Receptors (FcRs). A: FcγRIII signaling in natural killer cells is shown as an example of the activation class of FcRs. Cross-linking by an immune complex initiates the signaling cascade. The specific Src family kinase varies, depending on the cell type. **B:** FcR signaling in macrophages is shown as an example of simultaneous triggering of activation and inhibitory FcRs.

Immunoreceptor Tyrosine-Based Inhibitory Motif Pathways

The inhibitory motif, embedded in the cytoplasmic domain of the single-chain FcγRIIB molecule, was defined as a 13 amino acid sequence AENTITYSLLKHP, shown to be both necessary and sufficient to mediate the inhibition of BCR-generated calcium mobilization and cellular proliferation.[25,26] Significantly, phosphorylation of the tyrosine of this motif was shown to occur upon BCR coligation and was required for its inhibitory activity. This modification generated an SH2 recognition domain that is the binding site for the inhibitory signaling molecule SHIP.[96,101] In addition to its expression on B cells, where it is the only IgG FcR, FcγRIIB is widely expressed on macrophages, neutrophils, mast cells, DCs, and FDCs, absent only from T and NK cells. Studies on FcγRIIB provided the impetus to identify similar sequences in other surface molecules that mediated cellular inhibition and resulted in the description of the ITIM, a general feature of inhibitory receptors.

FcγRIIB displays multiple inhibitory activities. Coengagement of FcγRIIB to an ITAM-containing receptor leads to tyrosine phosphorylation of the ITIM by the Lyn kinase, recruitment of SHIP, and the inhibition of ITAM-triggered calcium mobilization and cellular proliferation (see Fig. 24.5B).[96,102,103] These two activities result from different signaling pathways; calcium inhibition requires the phosphatase activity of SHIP to hydrolyze PIP_3 and the ensuing dissociation of PH domain-containing proteins such as Btk and PLCγ.[104] The net effect is to block calcium influx and prevent sustained calcium signaling. Calciumdependent processes such as degranulation, phagocytosis, ADCC, cytokine release, and proinflammatory activation are all blocked. Arrest of proliferation in B cells is also dependent on the ITIM pathway, through the activation of the adaptor protein Dok and subsequent inactivation of MAP kinases.[105,106] The role of SHIP in this process has not been fully defined, inasmuch as it can affect proliferation in several ways. SHIP, through its catalytic phosphatase domain, can prevent activation of the PH domain survival factor Akt by hydrolysis of PIP_3.[107,108] SHIP also contains phosphotyrosine-binding domains that could act to recruit Dok to the membrane and provide access to the Lyn kinase that is involved in its activation. Dok-deficient B cells are unable to mediate FcγRIIB-triggered arrest of BCR-induced proliferation, while retaining their ability to inhibit a calcium influx, which demonstrates the dissociation of these two ITIM-dependent pathways.

Another inhibitory activity displayed by FcγRIIB is independent of the ITIM sequence and is displayed upon homoaggregation of the receptor. Under these conditions of FcγRIIB clustering, a proapoptotic signal is generated through the transmembrane sequence. This proapoptotic signal is blocked by recruitment of SHIP, which occurs upon coligation of FcγRIIB to the BCR, because of the Btk requirement for this apoptotic pathway.[97] This novel activity

has been reported only in B cells and has been proposed to act as a means of maintaining peripheral tolerance for B cells that have undergone somatic hypermutation. The in vivo relevance of this pathway remains to be proven.

IN VIVO FUNCTIONS

FcγReceptors in the Afferent Response

The ability of IgG ICs to influence the afferent response has been known since the 1950s and can be either enhancing or suppressive, depending on the precise combination of antibody and antigen and the mode of administration.[109] Investigators have attempted to define the molecular mechanisms behind these activities with the availability of defined mouse strains with mutations in activation or inhibitory FcRs and through the use of specific blocking antibodies to individual receptors. Direct effects on B cells stem from the ability of the inhibitory FcγRIIB molecule to influence the state of B-cell activation and survival. Because antigen is retained in the form of ICs on FDCs, it can interact with B cells by coengaging FcγRIIB with BCR, modulating the activation state of the cell. Mice deficient in this inhibitory receptor develop anti-deoxyribonucleic acid (DNA) and antichromatin antibodies and die of a fatal, autoimmune glomerulonephritis at 8 months of age. The phenotype is strain dependent and is not seen in BALB/c or 129 strains of mice.[110] Combining FcγRIIB deficiency with

defects in other inhibitory receptor pathways, such as PD-1, results in autoantibodies with different specifities and distinct pathological presentation. Thus, PD-1-deficient Balb/c mice develop cardiomyopathy resulting from anticardiac myosin antibodies. PD-1/FcγRIIB double deficient BALB/c mice develop antiuroepithelial antibodies and hydronephrosis.[111] FcγRIIB thus acts as a modifier of autoimmune disease, a conclusion further supported by studies that determined the contribution of the C57BL/6 background to the spontaneous lupus-like disease observed in those mice. The B6 background, by virtue of an incomplete light chain editing pathway, provides a source of autoreactive B cells in the periphery, which, when combined with defects in the inhibitory FcγRIIB pathway, leads to the accumulation of autoantibodies, pathogenic ICs, and disease.[112] Further support for this conclusion is provided by the observations that autoimmune disease-prone strains of mice, such as New Zealand black (NZB), BXSB, SB/Le, MRL, and nonobese diabetic, have reduced surface expression of FcγRIIB on activated B cells, attributed to DNA polymorphisms in the promoter region of the gene encoding this receptor.[113,114] This reduced expression of FcγRIIB is thus suggested to contribute to the increased susceptibility of these animals to the development of autoantibodies and autoimmune disease. Direct evidence that this is indeed the case comes from studies where FcγRIIB expression levels have been restored by retrovirus-mediated gene transfer in BXSB, NZM, and FcγRIIB knockout animals. These mice had dramatically reduced levels of autoreactive antibodies and were protected from the development of fatal autoimmune disease.[60] Consistent with these results obtained in mouse model systems, human patients with SLE and CIDP were demonstrated to have a reduced FcγRIIB expression level on B cells.[58,59]

Moreover, if FcγRIIB indeed functions in vivo to maintain peripheral tolerance, then its loss should allow for the emergence of autoantibodies when otherwise resistant animals are challenged with potentially crossreactive antigens. This hypothesis has been validated in models of collagen-induced arthritis and Goodpasture syndrome. FcγRIIB-deficient mice, with the nonpermissive H-2^b haplotype, develop arthritis when immunized with bovine type II collagen.[115] The loss of FcγRIIB thus bypasses the requirement for the specific H-2^q and H-2^r alleles previously demonstrated to be necessary in this model by allowing FcγRIIB-deficient autoreactive B-cell clones to expand and produce pathogenic autoantibodies. When the permissive DBA/1 strain (H-2^q) is made deficient in FcγRIIB, autoantibody development is augmented and disease is greatly enhanced. In a similar manner, immunization of H-2^b mice deficient in FcγRIIB with bovine type IV collagen results in crossreactive autoantibodies to murine type IV collagen, with dramatic pathogenic effects.[115,116] These mice develop hemorrhagic lung disease and glomerulonephritis with a “ribbon deposition” pattern of ICs in the glomeruli. These characteristics are indicative of Goodpasture syndrome, a human disease not previously modeled in an animal species.

Expression of the inhibitory FcγRIIB on B cells thus provides a mechanism for the suppressive effects of ICs on antibody production. Although FcγRIIB is expressed throughout peripheral B-cell development, recent data suggest that it represents a late checkpoint controlling the expansion of autoreactive IgG-positive plasma cells. In contrast, deficiency of the inhibitory receptor did not impact the generation of autoreactive IgM antibodies.[112] Taking the considerably higher pathogenic potential of IgG compared to IgM antibodies into account, this late stage of FcγRIIB-mediated regulation seems to be sufficient to prevent severe autoreactive processes.

The enhancing property of ICs on the afferent response is likely to arise from the expression of FcRs on APCs, such as DCs.[117,118,119] DCs express all three classes of IgG FcRs as well as FcεRI. Although in vitro studies have suggested that triggering of activation FcRs can induce DC maturation, the in vivo significance of this pathway has not been established.[120] The ability of FcRs, particularly FcγRI, to internalize ICs could provide a mechanism for enhanced presentation and augmented antibody responses, whereas the presence of the inhibitory FcγRIIB molecule appears to reduce the enhancing effect. Mice deficient in FcγRIIB display enhanced antibody responses to soluble antibody-antigen complexes, in some cases dramatically so, which is likely to result from enhanced presentation.[121,122] In addition, in vitro studies suggest that internalization through specific FcRs on APCs may influence the epitopes presented and T-cell response generated as a result. At present, a growing body of data suggests that FcRs are indeed involved in enhancement of the afferent response, by influencing antigen presentation and cognate T-cell interactions. FcγRIIB-deficient DCs pulsed ex vivo with antigen in the form of ICs induce a strong and protective cytotoxic immune response after transfer into naïve mice.[123] In contrast, wild-type DCs induce a much smaller and nonprotective response, indicating that the threshold set by co-crosslinking of the inhibitory and activating FcRs

prevents complete DC maturation. Moreover, blocking of IC binding to FcγRIIB on human DCs with an FcγRIIB-specific antibody resulted in spontaneous maturation of the cells by ICs present in low amounts in human serum.[124,125] Taken together, these data indicate that the inhibitory receptor is an important regulator of DC activation. As the DC maturation state will determine whether an activating or a tolerogenic signal will be delivered to T cells, FcRs might be important factors for the maintenance of peripheral tolerance in the cellular immune system. Transiently blocking FcγRIIB activity with monoclonal antibodies in vivo might thus be an interesting strategy to optimize immunotherapeutic and vaccination approaches. Further defining the precise role of each FcR expressed on APCs will require conditional knockouts of these molecules on specific DC populations to resolve the contribution of these systems to the generation of an appropriate antibody response.

FcγReceptors in the Efferent Response

The first canonical FcR knockout to be described was for the common activation subunit, the γchain, which resulted in the loss of surface assembly and signaling of FcγRI, FcγRIII and FcγRIV as well as FcεRI (10). Mice deficient in the common γ chain were systematically studied in diverse models of inflammation and found to be unable to mediate IgG-triggered inflammatory responses for cytotoxic or IC reaction; attributed to low-affinity activation receptors, the high-affinity FcγRI played a minimal role in the in vivo inflammatory response triggered by IgG.[22,126,127,128] The results were further confirmed by comparisons of mice deficient or blocked for either FcγRI, FcγRIII, or FcγRIV.[19,20,32,129] The loss of FcεRI ablated IgE-mediated anaphylaxis; this was demonstrated independently by gene disruption in the α subunit of that receptor.[11] Subsequent studies on mice deficient in the inhibitory FcγRIIB molecule established the opposing action of this receptor, in which mice deficient in that receptor displayed enhanced B-cell responses, autoimmunity, and augmented IgG-mediated inflammation in a subclass and effector cell-dependent manner.[18,20,32,55,128] The general finding, which is discussed in detail subsequently, illustrates that IgGs initiate their effector responses in vivo through coengagement of activating and inhibitory FcRs. The physiological response is thus the net of the opposing activation and inhibitory signaling pathways that each receptor triggers and is determined by the level of expression of each receptor and the selective avidity of the IgG ligand (see Table 24.1). This also explains the longstanding observation that different IgG isotypes have a differential activity in vivo (Fig. 24.6A). The absence of a murine homolog for FcαRI has precluded similar studies for that receptor. Studies on mice bearing a human transgene of FcαRI suggest that this receptor is involved in IgA nephropathy (Berger disease).[130]

Type I: Immediate Hypersensitivity

Both cutaneous and systemic models of passive anaphylaxis, induced by IgE, were studied in FcRγchain-deficient mice and were found to be absent, a finding fully consistent with the observations obtained in FcεRI-deficient mice and confirming the role of the high-affinity IgE receptor in mediating IgE-induced anaphylactic responses.[10,11,40,131] FcγRIIB-deficient mice, challenged in this model, displayed an unexpected enhancement of IgE-mediated anaphylaxis, which suggests a physiological interaction between this inhibitory receptor and FcεRI.[71] The molecular basis for this modulation of FcεRI signaling by FcγRIIB has not been

determined, although previous studies have indicated that IgE can bind with low affinity to FcγRII/FcγIII, which suggests that there exists a mechanism for coengagement of these receptors. Deletion of the mast cell inhibitory receptor glycoprotein 49B1 also results in enhanced IgE-induced anaphylaxis.[72] In addition to FcεRI, mast cells also express the IgG FcγRIIB and FcγRIII, but not FcγRIV. Passive systemic anaphylaxis induced by IgG was attenuated in FcRγ chain-deficient and FcγRIII-deficient mice, which indicates the capacity of IgG and FcγRIII to mediate mast cell activation in vivo. FcγRIIB-deficient mice displayed enhanced IgG-induced anaphylaxis. Active anaphylaxis, induced by immunization with antigen in alum, was enhanced in FcεRI-, FcγRIIB-, and glycoprotein 49B1-deficient mice and attenuated in FcRγ- and FcγRIII-deficient mice. All these animals displayed antigen-specific antibodies for IgE and IgGs, which indicates that the active anaphylaxis seen was attributed primarily to IgG antibodies. The reason for the enhancement of anaphylactic responses in FcεRI-deficient animals resulted from the increased expression of FcγRIII on mast cells in these mice, normally limited by competition of α chains for the available pool of the common γchain.[82] In the absence of FcεRI α chain, FcRγ chain is available to associate with FcγRIII α chain and assemble on the cell surface as a functional signaling receptor. A similar type of regulation was also described for expression of FcγRIII and FcγRIV on mouse neutrophils, where deletion of one receptor resulted in enhanced expression of the other γ-chain-dependent molecule.[83] These studies indicated the importance of the γchain in regulating the level of surface expression of FcεRI and FcγRIII. Because γ chain is also associated with other members of the activation/inhibition paired receptors expressed on mast cells, such as PIR-A/PIR-B, the intracellular competition between these diverse α subunits and the common γ chain determines the level of surface expression of individual receptors and thus their ability to respond to specific biological stimuli. The absolute level of surface expression of FcRs on mast cells is clearly of therapeutic significance in both IgE- and IgG-mediated inflammatory responses; modulation of γ chain expression could thus represent a new therapeutic avenue for intervention in diseases such as anaphylaxis and asthma.

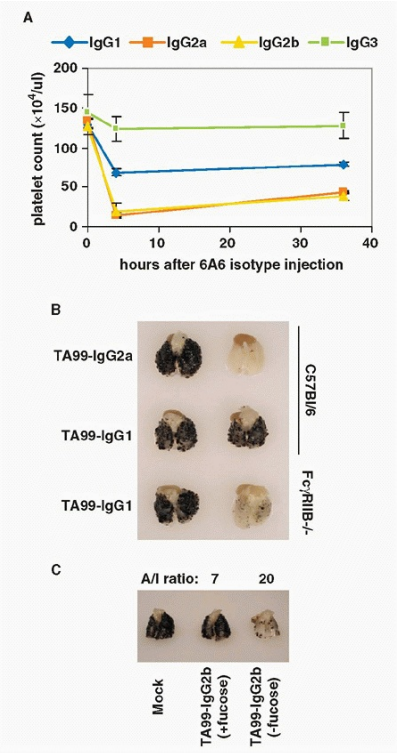

FIG. 24.6. Antibody Activity is Determined by Activating and Inhibitory Fc Receptors (FcRs). A: Mice were injected with the same amount of different 6A6 antibody isotypes, which recognize an integrin on mouse platelets and lead to FcR-dependent clearance of platelets from the blood as observed by the drop in platelet count after antibody injection. **B:** Wild-type or FcγRIIB knockout mice were injected intravenously with B16 melanoma cells on day 0 and with IgG1 and IgG2a isotype switch variants of the monoclonal antibody TA99 on alternate days. **C:** Mice were injected with antitumor antibodies containing or lacking branching fucose residues, thereby enhancing binding to activating FcRs and changing the A/I ratio (see text for details). Lungs were harvested on day 14. Adapted from Nimmerjahn and Ravetch,[20] with permission.

Type II Inflammation: Cytotoxic Immunoglobulin G

Cytotoxic IgGs are found in a variety of autoimmune disorders and have been developed for therapeutic indications in the treatment of infectious and neoplastic diseases. The mechanisms by which these antibodies trigger cytotoxicity in vivo have been investigated in FcR knockout mice. Anti-RBC antibodies trigger erythrophagocytosis of IgG-opsonized RBCs in an FcR-dependent manner; γ chain-deficient mice were protected from the pathogenic effect of these antibodies, whereas complement C3-deficient mice were indistinguishable from wild-type animals in their ability to clear the targeted RBCs.[132,133] FcγRIII plays the exclusive role in this process for the mouse IgG1 isotype. Murine IgG2a anti-RBC antibodies utilize primarily the FcγRIV receptor pathway despite the singular ability of murine IgG2a antibodies to bind as monomers to FcγRI. These and other studies suggest that the role of the high-affinity FcγRI in IgG-mediated inflammation is likely to be restricted to augmenting the effector response (determined by FcγRIII and IV) in situations that involve high concentrations of murine IgG2a antibodies which are found at localized inflammatory sites where FcγRI expression is induced on recruited macrophages.

Experimental models of immune thrombocytopenic purpura (ITP) in which murine IgG1 antiplatelet antibodies trigger thrombocytopenia and yielded results similar to those of the anti-RBC studies cited previously. The specific FcγR involved depended on the subclass of antibody used. IgG1 antibodies mediated their activity exclusively through FcγRIII, while IgG2a and 2b were FcγRIV dependent. In contrast, FcγRI- or C3-deficient mice were fully susceptible to antibody-induced thrombocytopenia.[20,31] FcγRIIB-deficient mice showed an isotype-specific enhancement of antibody-mediated platelet depletion, with the strongest impact on IgG1 and much smaller increases for IgG2a and IgG2b isotypes. This is consistent with the affinities of these isotypes for their specific activating and the inhibitory receptor, which will determine antibody activity in vivo (see Table 24.1). In a passive protection model of *Cryptococcus neoformans*-induced disease, passive immunization with mouse IgG1, IgG2a, and IgG2b antibodies resulted in protection in wild-type animals but not in FcRγchain-deficient animals.[134]

IgG antibodies raised to murine glomerular basement membrane preparations induce acute glomerulonephritis in a model of Goodpasture disease in wild-type but not FcRγ- or FcγRIV-deficient animals.[77,135,136] FcγRIIB-deficient animals displayed enhanced disease in this model, which indicates that the effector cells involved were constitutively expressing significant levels of FcγRIIB. Similar results were obtained when DBA/1 animals were

immunized with bovine type II collagen to induce arthritis. Deficiency of FcRγ chain protected these mice from the pathogenic effects of the anticollagen antibodies that were generated.[137] As mentioned previously, deficiency of FcγRIIB in the DBA/1 collagen-induced arthritis model resulted in enhanced disease, through increased autoantibody production and elevated effector responses.

A dramatic example of the importance of these pathways in determining the in vivo activity of cytotoxic antibodies was obtained in models of antitumor antibody response. In a syngenic murine model of metastatic melanoma, a murine IgG2a antimelanocyte antibody was able to reduce tumor metastasis in wild-type animals but was ineffective in FcRγ- or FcγRIV-deficient mice.[20,83,138] In the absence of FcγRIIB, the activity of an IgG1 antibody, matched in its antigen binding domain, was enhanced, which indicates that the in vivo cytotoxic activity of the antibody was the net of activation and inhibitory receptor engagement[20,55] (see Fig. 24.6B). These studies, together with similar studies performed with antiplatelet antibodies or defucosylated antibodies, demonstrated that the in vivo activity of a cytotoxic antibody could be predicted by a simple equilibrium binding model in which the ratio of the monomeric affinity constants for

the activation and inhibitory Fc receptors (A/I ratio) are the dominant parameters, as demonstrated for T-cell receptor-major histocompability complex interactions.[139] An example for the enhanced cytotoxic activity of an antitumor antibody due to increased affinity for activating FcRs is shown in Figure 24.6C. The generation of an afucosylated antitumor antibody resulted in an increase of the A/I ratio of 7 to 20 for this IgG subclass, resulting in enhanced antitumor activity in vivo. Xenograft models of human tumors transplated into nude mice demonstrated, for a variety of tumors and cytotoxic antibodies, the requirement for FcR effector activity. For example, human breast carcinoma or lymphoma lines transplanted into nude mice and treated with either the humanized IgG1 or chimerized IgG1 antibodies (trastuzumab and rituximab), respectively, revealed that the ability of these antibodies to modulate tumor growth was abrogated in FcRγ chain-deficient mice. A point mutation that eliminated FcR binding of the anti-Her2/neu murine IgG1 antibody 4D5 abolished the in vivo cytotoxic activity of the antibody against a human xenograft but did not affect the in vitro growth inhibitory activity; this again illustrates the difference between in vivo and in vitro mechanisms. Similar results were obtained for T-cell lymphoma xenograft models and anti-CD2 antibodies, among others.[140,141] The general conclusions that can be drawn from these studies support a dominant role for the low-affinity activating FcγRs in mediating cytotoxicity by IgG antibodies. FcγRIIB restricts the effector response for those antibodies with low A/I ratios and in situations in which the effector cell expresses this inhibitory molecule.

The relevance of these murine in vivo studies to the treatment of human populations with antitumor cytotoxic antibodies has been demonstrated in two studies that investigated the differential responses of patients treated with anti-CD20 (rituximab) for lymphoma.[48,52] Both studies demonstrated a highly significant correlation between patient response, as measured by the time to relapse, and alleles of FcγRIIIA. In patients with an allele of this low-affinity activation receptor (158V) that confers higher binding affinity for human IgG1 Fc, improved outcome was observed, as compared to those with a lower binding allele of this receptor (158F). In addition, lymphoma patients with the high affinity allele showed a significantly better clinical response after receiving antiidiotype vaccination.[51]

Type III Responses: Immune Complex-Mediated Inflammation

The classic example of this reaction, the Arthus reaction, has been studied in a variety of FcR- and complement-deficient animals. The initial studies were performed by using the cutaneous reverse passive Arthus reaction, in which antibody was injected intradermally and antigen was given intravenously. An inflammatory response, characterized by edema, hemorrhage, and neutrophil infiltration, developed within 2 hours. This reaction was elicited in a variety of complement- and FcR-deficient animals. The results from several independent studies confirmed the initial observations: that IgG ICs triggered cutaneous inflammatory reactions even in the absence of complement but displayed an absolute requirement for FcγR activation.[22] FcγRIIB modulated the magnitude of the response, with enhanced Arthus reactions observed in FcγRIIB-deficient strains.[18] The effector cell in the cutaneous reaction was determined to be the mast cell, as demonstrated by the use of mast cell-deficient strains and by mast cell reconstitution studies.[131] The generality of this result was demonstrated in similar reactions performed in the lung, illustrating the FcR dependence and relative complement independence of this response.[116] Thus, all studies have demonstrated an absolute dependence on FcR expression in the Arthus reaction. One model for IC-induced arthritis, the KRN/nonobese diabetic model, in which IgG1 anti-GPI antibodies are responsible for IC deposition in the synovium,[142] has been shown to depend on both FcγRIII and C3 but not on components of the classical complement pathway, such as C1q and C4; transfer of serum to animals deleted for FcγRIII or C3 prevented the development of disease.[143,144] Deficiency in the late components of complement, such as C5a or its receptor, have also been reported to result in a partial reduction in the magnitude of the response in IC-induced lung inflammation[145] and to result in a complete block in the KRN/nonobese diabetic arthritis model. The likely mechanism by which C5a exerts its effects is through upregulation of activating FcγRs, resulting in an amplification loop.[75] C5a is generated in this system as a result of FcR activation of effector cells and is independent of the classical, alternative, and mannan-binding protein pathways. Binding of C5a to the C5aR results in upregulation of activation receptors on these effector macrophages, thus augmenting the inflammatory response triggered by FcRs. These studies have led to a

revision of the hypotheses about the mechanism of IC-mediated inflammation, typified by the Arthus reaction, in which there is an absolute requirement for activating FcγRs in initiating mast cell activation by ICs. FcγR activation is, in turn, modulated by the inhibitory receptor FcγRIIB. The A/I value of a specific IgG antibody and the densities of these opposing signaling receptors determines the concentration threshold for IC activation and the magnitude of the effector response that can be obtained. The classical pathway of complement activation is not required; however, C5a activation, through the FcγR pathway, may enhance the response under some circumstances through an amplifying loop. The release of inflammatory mediators such as vasoactive amines, chemokines, and cytokines leads to the hallmarks of this reaction: edema, hemorrhage, and neutrophil infiltration at the site of IC deposition.

The significance of the FcR pathway in initiating IC inflammation in autoimmune disease was further established by investigating a spontaneous murine model of lupus, the B/W F1 mouse. The Arthus reaction results predicted the absolute requirement of activation FcγR in initiating inflammation and tissue damage in IC diseases such as lupus. The FcRγchain deletion was backcrossed onto the NZB and New Zealand white strains for eight generations, and the intercrossed progeny were segregated into B/W FcRγ-/- and FcRγ+/-. Anti-DNA antibodies and circulating ICs developed in all animals; IC and complement C3 deposition was similarly observed in all animals. However, mice deficient in the common γchain showed no evidence of glomerulonephritis

and had normal life expectancy, despite comparable levels of circulating ICs and glomerular deposition of these complexes along with complement C3. Mice heterozygous for the γchain mutation were indistinguishable from B/W F1 animals with wild-type γchains in developing glomerulonephritis and displaying reduced viability.[127] This spontaneous model supports the conclusions stated previously about the absolute requirement for FcγRIII in the activation of inflammatory disease by ICs: in the absence of this receptor, deposited ICs and C3 are not sufficient to trigger effector cell activation, which indicates that it is possible to uncouple pathogenic ICs from inflammatory disease by removing activating FcR engagement. Similar conclusions were reached in a murine model of Goodpasture disease where IC deposition, composed of mouse IgG2b antibodies, resulted in a fulminant glomerulonephritis.[77] Blocking the relevant activation FcR, FcγRIV, with a monoclonal antibody, protected the animals from fatal disease. These results further indicate that intervention in the effector stage of IC diseases, such as lupus and rheumatoid arthritis, would be accomplished by blocking activation FcγRs to prevent initiation of effector cell responses.

Immunoglobulin G-Dependent Neutralization and Induction of Agonistic Signaling

In contrast to these FcR-dependent activities, IgG molecules, by virtue of their exquisite specifity for discrete antigens, are expected to mediate FcγR-independent effects, such as neutralization of bacterial toxins or viruses. Here, IgG molecules would prevent the toxin or microorganism from binding to its cellular receptor, thereby protecting the host from the pathogenic effects of the infecting microbe. This picture changed, however, as it was demonstrated that both IgG-mediated toxin and virus neutralization can be dependent on cellular FcRs in vivo. Thus, neutralization of the anthrax toxin (called protective antigen component) by anti-protective antigen antibodies required FcR expression in vivo and anti-protective antigen antibodies that differed only by their Fc subclasses showed a hierarchy of protection with IgG2a and IgG2b antibodies affording a better protection than IgG1 antibodies, consistent with previous observation for cytotoxic IgG.[146,147] Similar results were obtained for the mechanism of activity of neutralizing antibodies protecting from influenza or human immunodeficiency virus infection.[148,149] These results suggest that current in vitro assays using only the pathogen-specific antibody, a target cell line might miss an important component required for antibody activity in vivo, and that novel assays including the FcR-dependent component might provide a better predictability of IgG activity.[150]

Beyond the central function of IgG in host defense, agonistic antibodies triggering signaling pathways in target cells are being evaluated for their therapeutic potential. For example, agonistic antibodies for tumor necrosis factor receptor family members such as anti-CD40 or anti-DR5 (drozitumab) are under evaluation for their ability to enhance tumor cell clearance by both direct and indirect mechanisms. Interestingly, the apoptosis inducing activity of anti-DR5 agonistic antibodies was found to be dependent on FcRs in vivo.[151] A similar dependence on FcRs for in vivo activity was demonstrated for agonistic anti-Fas antibodies.[152] A variety of CD40-specific agonistic antibodies have been used to deliver costimulatory signals to augment and sustain T-cell responses to result in tumor clearance.[151] Recent studies showed that the inhibitory FcγRIIB was essential for this agonistic activity and could not be replaced by activation FcγRs.[153,154] Although the exact mechanism of these FcR-dependent pathways remain to be established, these findings open the path toward generating improved agonistic anti-tumor necrosis factor receptor antibodies by Fc engineering. Consistent with this notion, a recent study showed that generating anti-CD40 antibodies with enhanced FcγRIIB binding considerably augmented their in vivo adjuvant activity.[154]

DISEASE ASSOCIATIONS

Autoimmunity and Tolerance

In view of their functional capacity to link autoantibodies to effector cells, FcRs have naturally been considered to have a pathogenic role in the development of autoimmune diseases. Several studies have attempted to correlate specific polymorphisms in FcγRIIA, FcγRIIIA, or

FcγRIIIB with incidence or severity of lupus or rheumatoid arthritis.[155] In view of the heterogeneity of these diseases, it is perhaps not surprising that inconsistent results have been obtained. Alleles that increase the ability of FcγRIIA to bind IgG2 or FcγRIIIA to bind IgG1 might be expected to correlate with disease severity in some populations. Indeed, these types of associations have been reported in some studies but not in others.[156] These variable results have often been explained as an indication that other genes may be in linkage disequilibrium with the FcR alleles under investigation. This is a plausible explanation when viewed in light of the autoimmunity susceptibility genes mapping in or near the region of the FcR genes, chromosome 1q21-24.[157] This region of chromosome 1 has been implicated in a variety of human and murine linkage studies. For example, the Sle1 alleles derived from NZB flank the FcγRIIB gene and form a linkage group with the ability to break tolerance to nuclear antigens, resulting in production of antichromatin antibodies. Epistatic interactions between FcγRIIB and lupus susceptibility genes have been demonstrated in the murine lupus model of B6.RIIB. Crossing the *yaa* gene to this strain accelerates the development of disease; 50% survival is decreased from 8 months to 4 months, with 100% fatality by 8 months. This increase in severity correlates with a change in the specificity of the autoantibodies, from diffuse antinuclear antibodies to antibodies that stain with a punctate, nucleolar pattern on antinuclear antibody staining. Recently, it has been shown that the yaa susceptibility locus contains a duplication of the toll-like receptor 7 gene in the pseudoautosomal region of the y-chromosome ([158]). Moreover, toll-like receptor 7 and toll-like receptor 9 with their ability to recognize potential self-antigens such as ribonucleic acid or DNA, respectively, were shown to be important components in the generation of pathogenic autoreactive antibodies.[159,160] Compared to other Yaa-independent SLE susceptibility loci, FcγRIIB may be a dominant factor for the development of autoantibodies

and the systemic activation of the immune system as demonstrated by a study using NZB/BXSB F1 animals congenic for the wild-type *fcgr2b* gene. In these mice, a dramatic reduction in the production of autoantibodies and immune cell activation was noted compared to mice with impaired FcγRIIB expression.[161] These data are consistent with studies that overexpressed FcγRIIB in autoimmune-prone mouse strains resulting in suppression of autoantibody production.[60,61]

Two types of polymorphisms in the FcγRIIB gene have been associated with SLE. Promoter polymorphisms have been described resulting in reduced expression of FcγRIIB on activated B cells in both mouse and human SLE and CIDP populations.[59,113,162] In addition, a polymorphism in the transmembrane domain of FcγRIIB has been identified that is associated with susceptibility to SLE in Japanese populations. This polymorphism results in an FcγRIIB protein with reduced ability to enter lipid rafts and thus behaves as a hypomorphic allele for inhibitory function.[163]

Together, these studies point to FcγRIIB as a susceptibility factor in the development of autoimmunity with the ability to interact with other susceptibility factors to modify both the afferent and efferent limbs of the autoimmune response.

Inflammation

Antibody-mediated inflammatory diseases have been clearly demonstrated to involve the coupling of pathogenic autoantibodies or ICs to cellular FcRs. Therapeutics targeted to disrupt these interactions are in development, beginning with a monoclonal antibody to human IgE that functions to reduce IgE binding to its high-affinity receptor and thereby prevent allergic and anaphylactic reactions.[164] Because IgE is required for the survival of mast cells as well as in the regulation of FcεRI expression, reduction in IgE has synergistic effects on the ligand, receptor, and effector cell. The success of this approach will undoubtedly lead to other approaches that target the receptor or its signaling pathway. Blocking FcγRIIIA or IIA is expected to mimic the phenotype of FcRgamma-deficient animals in models of IgG-induced disease. Early attempts to use this approach in ITP were promising but limited by the crossreactivity to receptors on neutrophils, which led to neutropenia and the development of immune response to the murine antibody.[165] Development of second-generation anti-FcγRIIIA antibodies with greater specificity and reduced toxicity now appears to be a viable approach for the treatment of autoimmune diseases. Moreover, using small molecules to inhibit signaling molecules such as Syk seems to have promising effects in preventing autoantibody dependent platelet depletion in mice and humans.[166]

An alternative approach to limiting the activation of FcRs is to utilize the endogenous inhibitory pathway to abrogate IgE or IgG activation of their cognate receptors through coligation to FcγRIIB. This mechanism has been proposed to explain the ability to induce desensitization for the treatment of allergic diseases.[33,167] Inducing production of IgG antibodies to an allergen may facilitate cross-linking of FcγRIIB to FcεRI. A similar approach involving anti-CD19 antibodies containing an engineered Fc-fragment with enhanced binding to FcγRIIB has been successfully used to shut down autoantibody production by human B cells in a xenograft mouse model system.[168] The ability to exploit the inhibitory pathway to reduce the activity of activation FcγRs has been demonstrated to account for some of the anti-inflammatory activity associated with high-dose intravenous gamma globulin (IVIG), which consists of the pooled IgG fraction of serum from thousands of donors.[76] The use of IVIG for the treatment of ITP and other autoimmune diseases is well established, although the mechanism of action has been elusive. In murine models of ITP, arthritis, and nephritis, it has been demonstrated that protection by IVIG is dependent on the presence of FcγRIIB; deletion of FcγRIIB or blocking FcγRIIB by a monoclonal antibody eliminates the ability of IVIG to protect the animal against an inflammatory response.[76,78,80] IVIG was demonstrated to lead

to the in vivo induction of FcγRIIB on splenic effector macrophages, which would raise the threshold required for platelet clearance by activating FcγRs on these cells.[20,31,78,83] A similar upregulation of the human inhibitory FcγRIIB was demonstrated in patients with CIDP receiving IVIG treatment as a first-line therapy.[59] Recently, it has become clear that the sialic acid-rich fraction of IgG antibodies in the IVIG preparation is responsible for the anti-inflammatory activity and FcγRIIB upregulation, enabling for the first time the generation of a recombinant IVIG replacement consisting of a sialic acid-rich IgG Fc fragment.[79,80] Human and mouse sialic acid-rich IgG has a reduced affinity for classical FcRs and acquires the capacity to bind to SIGNR1 strongly expressed on mouse splenic marginal zone macrophages.[68,80] By using SIGNR1-deficient mice expressing human DC-SIGN, it was demonstrated that human DC-SIGN expressed on macrophages or DCs can substitute for mouse SIGNR1 affording a candidate molecule essential for the anti-inflammatory activity of IVIG in humans.[67] Both molecules belong to the C-type lectin family and have been implicated in recognition of pathogenic microorganisms including human immunodeficiency virus and *Mycobacterium tuberculosis*.[169] Therefore, SIGNR1 and human DC-SIGN can be considered as noncanonical FcRs recognizing specific IgG glycovariants and, together with the family of toll-like receptors, provide a prime example for the dual usage of one molecule in the recognition of self- and non-self-ligands. Moreover, infusion of sialic acid-rich IgG resulted in an IL33-dependent expansion of basophils and IL4 production. IL4 is one of the interleukins known to upregulate FcγRIIB on innate immune effector cells, consistent with the upregulation of FcγRIIB noted in mouse model systems and humans. Together with previous data on the dependence of FcγRIIB upregulation on colony-stimulating factor-1-dependent macrophages, so called sensor macrophages, this suggests the following model for the anti-inflammatory activity of IVIG (Fig. 24.7). In this model, sialic acid-rich IVIG binds to mouse SIGNR1 or human DC-SIGN on sensor macrophages or DCs, which results in the production of IL33 that in turn leads to IL4 production by basophils, inducing the upregulation of FcγRIIB on effector macrophages, thereby raising the threshold for activation.[67] There is evidence that this

anti-inflammatory pathway triggered by IgG glycovariants rich in terminal sialic acid residues is of general importance for maintaining immune homeostasis. Thus, patients with rheumatoid arthritis and autoimmune-prone mouse strains have reduced levels of serum IgG-rich containing terminal sialic acid and galactose residues during active phases of the disease.[170,171] A similar reduction of this anti-inflammatory IgG variant is observed during aging, consistent with an increased probability to develop autoimmune disease.[172] This downmodulation of sialic acid-rich IgG was also observed during normal immunization conditions in mice, suggesting the general importance of this phenomenon during proinflammatory conditions.[80] The opposite type of regulation is seen in women with rheumatoid arthritis during pregnancy.[173] Parallelling the decreased frequency of arthritic flares an increased level of IgG glycoforms rich in terminal galactose and sialic acid residues was observed. In addition, inducing expression of FcγRIIB might be a clinically feasible approach and could be effective at modulating pathogenic autoantibodies from activation effector cell responses through activating FcRs.

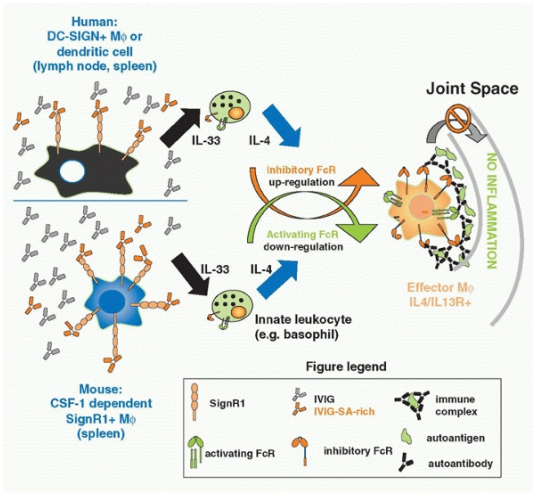

FIG. 24.7. Model for the Anti-inflammatory Activity of Intravenous Gamma Globulin (IVIG) in Mice and Humans. Sialic acid-rich antibodies in the IVIG preparation lose affinity for classical FcγRs and acquire the capacity to bind to molecules such as mouse SIGNR1 or human DC-SIGN on the surface of colonystimulating factor-1-dependent sensor macrophages or dendritic cells. This results in the production of IL33 and the expansion of innate leukocytes such as basophils followed by IL4 release resulting in the upregulation of the inhibitory FcγRIIB and downmodulation of activating FcγRs on effector macrophages inhibiting the release of inflammatory mediators and tissue destruction.

Studies on the Fc*α*RI receptor have demonstrated a role for this molecule in the

pathogenesis of IgA nephropathy, in which circulating macromolecular complexes are deposited in the mesangium, resulting in hematuria and eventually leading to renal failure. Soluble FcαRI is found in the circulating IgA complexes, which suggests a role for the receptor in the formation of these pathogenic complexes. A transgenic mouse expressing FcαRI spontaneously develops IgA nephropathy resulting from the interaction of polymeric mouse IgA and the human FcαRI receptor to release soluble receptor-IgA complexes, which leads to deposition in the mesangium and the sequelae of IgA neuropathy.

CONCLUSION

Receptors for the Fc of immunoglobulins provide an essential link between the humoral and adaptive response, translating the specificity of antibody diversity into cellular responses. The canonical FcRs mediate their biological responses through the coupling of Fc recognition to ITAM-/ITIM-based signaling motifs. A diverse array of biological responses depends on the FcR system, influencing both the afferent and efferent limbs of the immune response. Detailed biochemical, structural, and molecular biological data have provided a detailed understanding of how these receptors are regulated, are assembled, bind their ligand, and transduce specific cellular signals. FcRs play a significant role in vivo in maintaining peripheral tolerance by limiting the accumulation of autoreactive B cells that escape central tolerance checkpoints, such as light chain editing or potentially arise during somatic hypermutation in germinal centers, in modulating T-cell responses by regulating both antigen presentation and maturation by DCs, and in mediating the coupling of antigen recognition to effector-cell activation. They are the primary pathways by which pathogenic IgG and IgE antibodies trigger inflammatory responses in vivo. Allergic reactions, cytotoxic IgG responses, and IC-mediated inflammation are all critically dependent on FcR cross-linking and have resulted in a fundamental revision of the mechanisms

underlying such classic immunological responses as the Arthus reaction. Blocking of these receptors uncouples the pathogenic potential of autoantibodies and represents an important new therapeutic target for the development of anti-inflammatory therapeutic agents. Central to the correct functioning of these responses is the balance that is maintained through the pairing of activation and inhibitory receptors that coengage the IgG ligand; perturbations in either component can result in pathological responses. The study of FcRs defined the ubiquitous inhibitory motif, the ITIM, and has provided a paradigm for how these pathways modulate ITAM-based activation responses. Studies in mice deficient in individual FcRs have provided the necessary insights for defining comparable activities in human autoimmune diseases and suggest ways in which manipulation of the IgG-FcR interaction may lead to new classes of therapeutics for the treatment of these diseases. Modulation of the inhibitory response, a novel activity associated with IVIG to account for some of its anti-inflammatory activity in vivo, represents a novel approach to the regulation of Igmediated inflammation and suggests that therapeutic agents based on those pathways are likely to be effective. It seems clear that this anti-inflammatory pathway is of general importance for maintaining immune homeostasis, suggesting that IgG glycoforms rich in terminal sialic acid residues might be considered as a molecular switch either keeping the immune system in a resting state or, during infection or autoimmune responses, loosening the brakes thereby allowing full blown inflammation. In this pathway, noncanonical FcRs including SignR1 and human DC-SIGN recognizing specific IgG glycoforms are of central importance. Conversely, engineering of therapeutic antibodies targeted to eliminate infectious or neoplastic disease will probably benefit from optimization of their Fc domains for interaction with specific canonical FcRs.

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REFERENCES

1. Boyden SV, Sorkin E. The adsorption of antigen by spleen cells previously treated with antiserum in vitro. *Immunology*. 1960;3:272-283.

2. Berken A, Benacerraf B. Properties of antibodies cytophilic for macrophages. *J Exp Med*. 1966;123(1):119-144.

3. Paraskevas F, Lee ST, Orr KB, Israels LG. A receptor for Fc on mouse B-lymphocytes. *J Immunol*. 1972;108(5):1319-1327.

4. Kulczycki A Jr, Isersky C, Metzger H. The interaction of IgE with rat basophilic leukemia cells. I. Evidence for specific binding of IgE. *J Exp Med*. 1974;139(3):600-616.

5. Lewis VA, Koch T, Plutner H, Mellman I. A complementary DNA clone for a macrophage-lymphocyte Fc receptor. *Nature*. 1986;324(6095): 372-375.

6. Ravetch JV, Luster AD, Weinshank R, et al. Structural heterogeneity and functional domains of murine immunoglobulin G Fc receptors. *Science*. 1986;234(4777):718-725.

7. Kinet JP, Metzger H, Hakimi J, Kochan J. A cDNA presumptively coding for the alpha subunit of the receptor with high affinity for immunoglobulin E. *Biochemistry*. 1987;26(15):4605-4610.

8. Ra C, Jouvin MH, Blank U, Kinet JP. A macrophage Fc gamma receptor and the mast cell receptor for IgE share an identical subunit. *Nature*. 1989;341(6244):752-754.

9. Kurosaki T, Ravetch JV. A single amino acid in the glycosyl phosphatidylinositol attachment domain determines the membrane topology of Fc gamma RIII. *Nature*. 1989;342(6251):805-807.

10. Takai T, Li M, Sylvestre D, Clynes R, Ravetch JV. FcR gamma chain deletion results in pleiotrophic effector cell defects. *Cell*. 1994;76(3):519-529.

11. Dombrowicz D, Flamand V, Brigman KK, Koller BH, Kinet JP. Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor alpha chain gene. *Cell*. 1993;75(5):969-976.

12. Maxwell KF, Powell MS, Hulett MD, et al. Crystal structure of the human leukocyte Fc receptor, Fc gammaRIIa. *Nat Struct Biol*. 1999;6(5):437-442.

13. Sondermann P, Huber R, Jacob U. Crystal structure of the soluble form of the human fcgamma-receptor IIb: a new member of the immunoglobulin superfamily at 1.7 A resolution. *EMBO J*. 1999;18(5):1095-1103.

14. Garman SC, Kinet JP, Jardetzky TS. Crystal structure of the human high-affinity IgE receptor. *Cell*. 1998;95(7):951-961.

15. Garman SC, Wurzburg BA, Tarchevskaya SS, Kinet JP, Jardetzky TS. Structure of the Fc fragment of human IgE bound to its high-affinity receptor Fc epsilonRI alpha. *Nature*. 2000;406(6793):259-266.

16. Sondermann P, Huber R, Oosthuizen V, Jacob U. The 3.2-A crystal structure of the human IgG1 Fc fragment-Fc gammaRIII complex. *Nature*. 2000;406(6793):267-273.

17. Ravetch JV, Kinet JP. Fc receptors. *Annu Rev Immunol*. 1991;9:457-492.

18. Takai T, Ono M, Hikida M, Ohmori H, Ravetch JV. Augmented humoral and anaphylactic responses in Fc gamma RII-deficient mice. *Nature*. 1996;379(6563):346-349.

19. Hazenbos WL, Gessner JE, Hofhuis FM, et al. Impaired IgG-dependent anaphylaxis and Arthus reaction in Fc gamma RIII (CD16) deficient mice. *Immunity*. 1996;5(2):181-188.

20. Nimmerjahn F, Ravetch JV. Divergent immunoglobulin g subclass activity through selective Fc receptor binding. *Science*. 2005;310(5753):1510-1512.

21. Carroll MC. The role of complement and complement receptors in induction and regulation of immunity. *Annu Rev Immunol*. 1998;16:545-568.

22. Sylvestre DL, Ravetch JV. Fc receptors initiate the Arthus reaction: redefining the inflammatory cascade. *Science*. 1994;265(5175):1095-1098.

23. Ravetch JV, Clynes RA. Divergent roles for Fc receptors and complement in vivo. *Annu Rev Immunol*. 1998;16:421-432.

24. Kinet JP. The high-affinity IgE receptor (Fc epsilon RI): from physiology to pathology. *Annu Rev Immunol*. 1999;17:931-972.

25. Muta T, Kurosaki T, Misulovin Z, Sanchez M, Nussenzweig MC, Ravetch JV. A 13-amino-acid motif in the cytoplasmic domain of Fc gamma RIIB modulates B-cell receptor signalling. *Nature*. 1994;368(6466):70-73.

26. Amigorena S, Bonnerot C, Drake JR, et al. Cytoplasmic domain heterogeneity and functions of IgG Fc receptors in B lymphocytes. *Science*. 1992;256(5065):1808-1812.

27. Ravetch JV, Lanier LL. Immune inhibitory receptors. *Science*. 2000; 290(5489):84-89.

28. Ravetch JV, Bolland S. IgG Fc receptors. *Annu Rev Immunol*. 2001;19: 275-290.

29. Ghetie V, Ward ES. Multiple roles for the major histocompatibility complex class I-related receptor FcRn. *Annu Rev Immunol*. 2000;18: 739-766.

30. Mostov KE. Transepithelial transport of immunoglobulins. *Annu Rev Immunol*. 1994;12:63-84.

31. Nimmerjahn F, Bruhns P, Horiuchi K, Ravetch JV. FcgammaRIV: a novel FcR with distinct IgG subclass specificity. *Immunity*. 2005;23(1): 41-51.

32. Hamaguchi Y, Xiu Y, Komura K, Nimmerjahn F, Tedder TF. Antibody isotype-specific

engagement of Fcgamma receptors regulates B lymphocyte depletion during CD20 immunotherapy. *J Exp Med*. 2006;203(3): 743-753.

33. Daeron M. Fc receptor biology. *Annu Rev Immunol*. 1997;15:203-234.

34. Kubagawa H, Oka S, Kubagawa Y, et al. Identity of the elusive IgM Fc receptor (FcmuR) in humans. *J Exp Med*. 2009;206(12):2779-2793.

35. Shima H, Takatsu H, Fukuda S, et al. Identification of TOSO/FAIM3 as an Fc receptor for IgM. *Int Immunol*. 2010;22(3):149-156.

36. Kubagawa H, Chen CC, Ho LH, et al. Biochemical nature and cellular distribution of the paired immunoglobulin-like receptors, PIR-A and PIR-B. *J Exp Med*. 1999;189(2):309-318.

37. Maeda A, Kurosaki M, Kurosaki T. Paired immunoglobulin-like receptor (PIR)-A is involved in activating mast cells through its association with Fc receptor gamma chain. *J Exp Med*. 1998;188(5):991-995.

38. Moretta A, Bottino C, Vitale M, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu Rev Immunol*. 2001;19:197-223.

39. Lanier LL. Face off—the interplay between activating and inhibitory immune receptors. *Curr Opin Immunol*. 2001;13(3):326-331.

40. Dombrowicz D, Lin S, Flamand V, Brini AT, Koller BH, Kinet JP. Allergy-associated FcRbeta is a molecular amplifier of IgE- and IgGmediated in vivo responses. *Immunity*. 1998;8(4):517-529.

41. Qiu WQ, de Bruin D, Brownstein BH, Pearse R, Ravetch JV. Organization of the human and mouse low-affinity Fc gamma R genes: duplication and recombination. *Science*. 1990;248(4956):732-735.

42. Su Y, Brooks DG, Li L, et al. Myelin protein zero gene mutated in Charcot-Marie-tooth type 1B patients. *Proc Natl Acad Sci U S A*. 1993;90(22):10856-10860.

43. Maresco DL, Chang E, Theil KS, Francke U, Anderson CL. The three genes of the human FCGR1 gene family encoding Fc gamma RI flank the centromere of chromosome 1 at 1p12 and 1q21. *Cytogenet Cell Genet*. 1996;73(3):157-163.

44. Davis RS, Dennis G Jr, Odom MR, et al. Fc receptor homologs: newest members of a remarkably diverse Fc receptor gene family. *Immunol Rev*. 2002;190:123-136.

45. Morel L, Blenman KR, Croker BP, Wakeland EK. The major murine systemic lupus erythematosus susceptibility locus, Sle1, is a cluster of functionally related genes. *Proc Natl Acad Sci U S A*. 2001;98(4):1787-1792.

46. Sandford AJ, Moffatt MF, Daniels SE, et al. A genetic map of chromosome 11q, including the atopy locus. *Eur J Hum Genet*. 1995;3(3):188-194.

47. van der Pol W, van de Winkel JG. IgG receptor polymorphisms: risk factors for disease. *Immunogenetics*. 1998;48(3):222-232.

48. Cartron G, Dacheux L, Salles G, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. *Blood*. 2002;99(3):754-758.

49. Musolino A, Naldi N, Bortesi B, et al. Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. *J Clin Oncol*. 2008;26(11):1789-1796.

50. Paiva M, Marques H, Martins A, Ferreira P, Catarino R, Medeiros R. FcgammaRIIa polymorphism and clinical response to rituximab in non-Hodgkin lymphoma patients. *Cancer Genet Cytogenet*. 2008;183(1):35-40.

51. Weng WK, Czerwinski D, Timmerman J, Hsu FJ, Levy R. Clinical outcome of lymphoma patients after idiotype vaccination is correlated with humoral immune response and immunoglobulin G Fc receptor genotype. *J Clin Oncol*. 2004;22(23):4717-4724.

52. Weng WK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J Clin Oncol*. 2003;21(21):3940-3947.

53. Bibeau F, Lopez-Crapez E, Di Fiore F, et al. Impact of Fc{gamma}RIIa-Fc{gamma}RIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. *J Clin Oncol*. 2009;27(7):1122-1129.

54. Houghton AN, Scheinberg DA. Monoclonal antibody therapies-a “constant” threat to cancer. *Nat Med*. 2000;6(4):373-374.

55. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. *Nat Med*. 2000;6(4):443-446.

56. Bournazos S, Woof JM, Hart SP, Dransfield I. Functional and clinical consequences of Fc receptor polymorphic and copy number variants. *Clin Exp Immunol*. 2009;157(2):244-254.

57. Smith KG, Clatworthy MR. FcgammaRIIB in autoimmunity and infection: evolutionary and therapeutic implications. *Nat Rev Immunol* . 2010;10(5):328-343.

58. Mackay M, Stanevsky A, Wang T, et al. Selective dysregulation of the FcgammaIIB receptor on memory B cells in SLE. *J Exp Med*. 2006; 203(9):2157-2164.

59. Tackenberg B, Jelcic I, Baerenwaldt A, et al. Impaired inhibitory Fcgamma receptor IIB expression on B cells in chronic inflammatory demyelinating polyneuropathy. *Proc Natl Acad Sci U S A*. 2009;106(12):4788-4792.

60. McGaha TL, Sorrentino B, Ravetch JV. Restoration of tolerance in lupus by targeted inhibitory receptor expression. *Science*. 2005;307(5709): 590-593.

61. Brownlie RJ, Lawlor KE, Niederer HA, et al. Distinct cell-specific control of autoimmunity and infection by FcgammaRIIb. *J Exp Med*. 2008;205(4):883-895.

62. Clatworthy MR, Willcocks L, Urban B, et al. Systemic lupus erythematosus-associated defects in the inhibitory receptor FcgammaRIIb reduce susceptibility to malaria. *Proc Natl Acad Sci U S A*. 2007;104(17):7169-7174.

63. Waisberg M, Tarasenko T, Vickers BK, et al. Genetic susceptibility to systemic lupus erythematosus protects against cerebral malaria in mice. *Proc Natl Acad Sci U S A* . 2011;108(3):1122-1127.

64. Nimmerjahn F, Ravetch JV. Fcgamma receptors: old friends and new family members. *Immunity*. 2006;24(1):19-28.

65. Brooks DG, Qiu WQ, Luster AD, Ravetch JV. Structure and expression of human IgG FcRII(CD32). Functional heterogeneity is encoded by the alternatively spliced products of multiple genes. *J Exp Med*. 1989;170(4):1369-1385.

66. Ravetch JV, Perussia B. Alternative membrane forms of Fc gamma RIII(CD16) on human natural killer cells and neutrophils. Cell typespecific expression of two genes that differ in single nucleotide substitutions. *J Exp Med*. 1989;170(2):481-497.

67. Anthony RM, Kobayashi T, Wermeling F, Ravetch JV. Intravenous gammaglobulin suppresses inflammation through a novel T(H)2 pathway. *Nature*. 2011;475(7354):110-113.

68. Anthony RM, Wermeling F, Karlsson MC, Ravetch JV. Identification of a receptor required for the anti-inflammatory activity of IVIG. *Proc Natl Acad Sci U S A* . 2008;105(50):19571-19578.

69. Geijtenbeek TB, Gringhuis SI. Signalling through C-type lectin receptors: shaping immune responses. *Nat Rev Immunol* . 2009;9(7):465-479.

70. Coxon A, Cullere X, Knight S, et al. Fc gamma RIII mediates neutrophil recruitment to immune complexes. a mechanism for neutrophil accumulation in immune-mediated inflammation. *Immunity*. 2001;14(6): 693-704.

71. Ujike A, Ishikawa Y, Ono M, et al. Modulation of immunoglobulin (Ig) E-mediated systemic anaphylaxis by low-affinity Fc receptors for IgG. *J Exp Med*. 1999;189(10):1573-1579.

72. Daheshia M, Friend DS, Grusby MJ, Austen KF, Katz HR. Increased severity of local and systemic anaphylactic reactions in gp49B1-deficient mice. *J Exp Med*. 2001;194(2):227-234.

73. de Andres B, Mueller AL, Verbeek S, Sandor M, Lynch RG. A regulatory role for Fcgamma receptors CD16 and CD32 in the development of murine B cells. *Blood*. 1998;92(8):2823-2829.

74. Pricop L, Redecha P, Teillaud JL, et al. Differential modulation of stimulatory and inhibitory Fc gamma receptors on human monocytes by Th1 and Th2 cytokines. *J Immunol*. 2001;166(1):531-537.

75. Schmidt RE, Gessner JE. Fc receptors and their interaction with complement in

autoimmunity. *Immunol Lett*. 2005;100(1):56-67.

76. Samuelsson A, Towers TL, Ravetch JV. Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor. *Science*. 2001;291(5503):484-486.

77. Kaneko Y, Nimmerjahn F, Madaio MP, Ravetch JV. Pathology and protection in nephrotoxic nephritis is determined by selective engagement of specific Fc receptors. *J Exp Med*. 2006;203(3):789-797.

78. Bruhns P, Samuelsson A, Pollard JW, Ravetch JV. Colony-stimulating factor-1-dependent macrophages are responsible for IVIG protection in antibody-induced autoimmune disease. *Immunity*. 2003;18(4):573-581.

79. Anthony RM, Nimmerjahn F, Ashline DJ, Reinhold VN, Paulson JC, Ravetch JV. Recapitulation of IVIG anti-inflammatory activity with a recombinant IgG Fc. *Science*. 2008;320(5874):373-376.

80. Kaneko Y, Nimmerjahn F, Ravetch JV. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science*. 2006;313(5787):670-673.

81. Yamaguchi M, Lantz CS, Oettgen HC, et al. IgE enhances mouse mast cell Fc(epsilon)RI expression in vitro and in vivo: evidence for a novel amplification mechanism in IgE-dependent reactions. *J Exp Med*. 1997;185(4):663-672.

82. Dombrowicz D, Flamand V, Miyajima I, Ravetch JV, Galli SJ, Kinet JP. Absence of Fc epsilonRI alpha chain results in upregulation of Fc gammaRIII-dependent mast cell degranulation and anaphylaxis. Evidence of competition between Fc epsilonRI and Fc gammaRIII for limiting amounts of FcR beta and gamma chains. *J Clin Invest*. 1997;99(5): 915-925.

83. Nimmerjahn F, Lux A, Albert H, et al. FcgammaRIV deletion reveals its central role for IgG2a and IgG2b activity in vivo. *Proc Natl Acad Sci U S A* . 2010;107(45):19396-19401.

84. Sondermann P, Kaiser J, Jacob U. Molecular basis for immune complex recognition: a comparison of Fc-receptor structures. *J Mol Biol* . 2001;309(3):737-749.

85. Anthony RM, Nimmerjahn F. The role of differential IgG glycosylation in the interaction of antibodies with FcgammaRs in vivo. *Curr Opin Organ Transplant* . 2010 Dec 9. [Epub ahead of print.]

86. Ferrara C, Stuart F, Sondermann P, Brunker P, Umana P. The carbohydrate at FcgammaRIIIa Asn-162. An element required for high affinity binding to non-fucosylated IgG glycoforms. *J Biol Chem*. 2006;281(8): 5032-5036.

87. Ferrara C, Grau S, Jager C, et al. Unique carbohydrate-carbohydrate interactions are required for high affinity binding between FcγRIII and antibodies lacking core fucose. *Proc Natl Acad Sci U S A*. 2011;108(31):12669-12674.

88. Shinkawa T, Nakamura K, Yamane N, et al. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J Biol Chem*. 2003;278(5): 3466-3473.

89. Shields RL, Lai J, Keck R, et al. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibodydependent cellular toxicity. *J Biol Chem*. 2002;277(30):26733-26740.

90. Miller KL, Duchemin AM, Anderson CL. A novel role for the Fc receptor gamma subunit: enhancement of Fc gamma R ligand affinity. *J Exp Med*. 1996;183(5):2227-2233.

91. Shields RL, Namenuk AK, Hong K, et al. High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. *J Biol Chem*. 2001;276(9):6591-6604.

92. Zalevsky J, Leung IW, Karki S, et al. The impact of Fc engineering on an anti-CD19 antibody: increased Fcgamma receptor affinity enhances B-cell clearing in nonhuman primates. *Blood*. 2009;113(16):3735-3743.

93. Lazar GA, Dang W, Karki S, et al. Engineered antibody Fc variants with enhanced effector function. *Proc Natl Acad Sci U S A* . 2006;103(11):4005-4010.

94. Horton HM, Bernett MJ, Peipp M, et al. Fc-engineered anti-CD40 antibody enhances multiple effector functions and exhibits potent in vitro and in vivo antitumor activity against hematologic malignancies. *Blood*. 2010;116(16):3004-3012.

95. Choquet D, Partiseti M, Amigorena S, Bonnerot C, Fridman WH, Korn H. Cross-linking of IgG receptors inhibits membrane immunoglobulin-stimulated calcium influx in B

lymphocytes. *J Cell Biol*. 1993;121(2):355-363.

96. Ono M, Bolland S, Tempst P, Ravetch JV. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc(gamma)RIIB. *Nature*. 1996;383(6597):263-266.

97. Pearse RN, Kawabe T, Bolland S, Guinamard R, Kurosaki T, Ravetch JV. SHIP recruitment attenuates Fc gamma RIIB-induced B cell apoptosis. *Immunity*. 1999;10(6):753-760.

98. Tzeng SJ, Bolland S, Inabe K, Kurosaki T, Pierce SK. The B cell inhibitory Fc receptor triggers apoptosis by a novel c-Abl family kinase-dependent pathway. *J Biol Chem*. 2005;280(42):35247-35254.

99. Xiang Z, Cutler AJ, Brownlie RJ, et al. FcgammaRIIb controls bone marrow plasma cell persistence and apoptosis. *Nat Immunol*. 2007;8(4): 419-429.

100. Torigoe C, Inman JK, Metzger H. An unusual mechanism for ligand antagonism. *Science*. 1998;281(5376):568-572.

101. Tridandapani S, Pradhan M, LaDine JR, Garber S, Anderson CL, Coggeshall KM. Protein interactions of Src homology 2 (SH2) domain-containing inositol phosphatase (SHIP): association with Shc displaces SHIP from FcgammaRIIb in B cells. *J Immunol*. 1999;162(3): 1408-1414.

102. Daeron M, Latour S, Malbec O, et al. The same tyrosine-based inhibition motif, in the intracytoplasmic domain of Fc gamma RIIB, regulates negatively BCR-, TCR-, and FcR-dependent cell activation. *Immunity*. 1995;3(5):635-646.

103. Malbec O, Fong DC, Turner M, et al. Fc epsilon receptor I-associated lyn-dependent phosphorylation of Fc gamma receptor IIB during negative regulation of mast cell activation. *J Immunol*. 1998;160(4):1647-1658.

104. Bolland S, Pearse RN, Kurosaki T, Ravetch JV. SHIP modulates immune receptor responses by regulating membrane association of Btk. *Immunity*. 1998;8(4):509-516.

105. Tamir I, Stolpa JC, Helgason CD, et al. The RasGAP-binding protein p62dok is a mediator of inhibitory FcgammaRIIB signals in B cells. *Immunity*. 2000;12(3):347-358.

106. Yamanashi Y, Tamura T, Kanamori T, et al. Role of the rasGAP-associated docking protein p62(dok) in negative regulation of B cell receptor-mediated signaling. *Genes Dev*. 2000;14(1):11-16.

107. Aman MJ, Lamkin TD, Okada H, Kurosaki T, Ravichandran KS. The inositol phosphatase SHIP inhibits Akt/PKB activation in B cells. *J Biol Chem*. 1998;273(51):33922-33928.

108. Liu Q, Sasaki T, Kozieradzki I, et al. SHIP is a negative regulator of growth factor receptor-mediated PKB/Akt activation and myeloid cell survival. *Genes Dev*. 1999;13(7):786-791.

109. Heyman B. Regulation of antibody responses via antibodies, complement, and Fc receptors. *Annu Rev Immunol*. 2000;18:709-737.

110. Bolland S, Ravetch JV. Spontaneous autoimmune disease in Fc(gamma) RIIB-deficient mice results from strain-specific epistasis. *Immunity*. 2000;13(2):277-285.

111. Okazaki T, Otaka Y, Wang J, et al. Hydronephrosis associated with antiurothelial and antinuclear autoantibodies in BALB/c-Fcgr2b-/-Pdcd1-/- mice. *J Exp Med*. 2005;202(12):1643-1648.

112. Fukuyama H, Nimmerjahn F, Ravetch JV. The inhibitory Fcgamma receptor modulates autoimmunity by limiting the accumulation of immunoglobulin G+ anti-DNA plasma cells. *Nat Immunol*. 2005;6(1): 99-106.

113. Jiang Y, Hirose S, Abe M, et al. Polymorphisms in IgG Fc receptor IIB regulatory regions associated with autoimmune susceptibility. *Immunogenetics*. 2000;51(6):429-435.

114. Pritchard NR, Cutler AJ, Uribe S, Chadban SJ, Morley BJ, Smith KG. Autoimmune-prone mice share a promoter haplotype associated with reduced expression and function of the Fc receptor FcgammaRII. *Curr Biol*. 2000;10(4):227-230.

115. Yuasa T, Kubo S, Yoshino T, et al. Deletion of fcgamma receptor IIB renders H-2(b) mice susceptible to collagen-induced arthritis. *J Exp Med*. 1999;189(1):187-194.

116. Nakamura A, Yuasa T, Ujike A, et al. Fcgamma receptor IIB-deficient mice develop Goodpasture's syndrome upon immunization with type IV collagen: a novel murine model for autoimmune glomerular basement membrane disease. *J Exp Med*. 2000;191(5):899-

906.

117. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature*. 1998;392(6673):245-252.

118. Amigorena S, Bonnerot C. Fc receptors for IgG and antigen presentation on MHC class I and class II molecules. *Semin Immunol*. 1999;11(6):385-390.

119. Hamano Y, Arase H, Saisho H, Saito T. Immune complex and Fc receptor-mediated augmentation of antigen presentation for in vivo Th cell responses. *J Immunol*. 2000;164(12):6113-6119.

120. Regnault A, Lankar D, Lacabanne V, et al. Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J Exp Med*. 1999;189(2):371-380.

121. Wernersson S, Karlsson MC, Dahlstrom J, Mattsson R, Verbeek JS, Heyman B. IgG-mediated enhancement of antibody responses is low in Fc receptor gamma chain-deficient mice and increased in Fc gamma RII-deficient mice. *J Immunol*. 1999;163(2):618-622.

122. Baiu DC, Prechl J, Tchorbanov A, et al. Modulation of the humoral immune response by antibody-mediated antigen targeting to complement receptors and Fc receptors. *J Immunol*. 1999;162(6):3125-3130.

123. Kalergis AM, Ravetch JV. Inducing tumor immunity through the selective engagement of activating Fcgamma receptors on dendritic cells. *J Exp Med*. 2002;195(12):1653-1659.

124. Dhodapkar KM, Kaufman JL, Ehlers M, et al. Selective blockade of inhibitory Fcgamma receptor enables human dendritic cell maturation with IL-12p70 production and immunity to antibody-coated tumor cells. *Proc Natl Acad Sci U S A* . 2005;102(8):2910-2915.

125. Boruchov AM, Heller G, Veri MC, Bonvini E, Ravetch JV, Young JW. Activating and inhibitory IgG Fc receptors on human DCs mediate opposing functions. *J Clin Invest*. 2005;15:15.

126. Clynes R, Ravetch JV. Cytotoxic antibodies trigger inflammation through Fc receptors. *Immunity*. 1995;3(1):21-26.

127. Clynes R, Dumitru C, Ravetch JV. Uncoupling of immune complex formation and kidney damage in autoimmune glomerulonephritis. *Science*. 1998;279(5353):1052-1054.

128. Clynes R, Maizes JS, Guinamard R, Ono M, Takai T, Ravetch JV. Modulation of immune complex-induced inflammation in vivo by the coordinate expression of activation and inhibitory Fc receptors. *J Exp Med*. 1999;189(1):179-185.

129. Hazenbos WL, Heijnen IA, Meyer D, et al. Murine IgG1 complexes trigger immune effector functions predominantly via Fc gamma RIII (CD16). *J Immunol*. 1998;161(6):3026-3032.

130. Launay P, Grossetete B, Arcos-Fajardo M, et al. Fcalpha receptor (CD89) mediates the development of immunoglobulin A (IgA) nephropathy (Berger's disease). Evidence for pathogenic soluble receptor-Iga complexes in patients and CD89 transgenic mice. *J Exp Med*. 2000;191(11):1999-2009.

131. Miyajima I, Dombrowicz D, Martin TR, Ravetch JV, Kinet JP, Galli SJ. Systemic anaphylaxis in the mouse can be mediated largely through IgG1 and Fc gammaRIII. Assessment of the cardiopulmonary changes, mast cell degranulation, and death associated with active or IgE- or IgG1-dependent passive anaphylaxis. *J Clin Invest*. 1997;99(5):901-914.

132. Sylvestre D, Clynes R, Ma M, Warren H, Carroll MC, Ravetch JV. Immunoglobulin G-mediated inflammatory responses develop normally in complement-deficient mice. *J Exp Med*. 1996;184(6):2385-2392.

133. Fossati-Jimack L, Ioan-Facsinay A, Reininger L, et al. Markedly different pathogenicity of four immunoglobulin G isotype-switch variants of an antierythrocyte autoantibody is based on their capacity to interact in vivo with the low-affinity Fcgamma receptor III. *J Exp Med*. 2000;191(8):1293-1302.

134. Yuan R, Clynes R, Oh J, Ravetch JV, Scharff MD. Antibody-mediated modulation of Cryptococcus neoformans infection is dependent on distinct Fc receptor functions and IgG subclasses. *J Exp Med*. 1998;187(4): 641-648.

135. Suzuki Y, Shirato I, Okumura K, et al. Distinct contribution of Fc receptors and angiotensin II-dependent pathways in anti-GBM glomerulonephritis. *Kidney Int*. 1998;54(4):1166-1174.

136. Park SY, Ueda S, Ohno H, et al. Resistance of Fc receptor-deficient mice to fatal glomerulonephritis. *J Clin Invest*. 1998;102(6):1229-1238.

137. Kleinau S, Martinsson P, Heyman B. Induction and suppression of collagen-induced arthritis is dependent on distinct fcgamma receptors. *J Exp Med*. 2000;191(9):1611-1616.

138. Clynes R, Takechi Y, Moroi Y, Houghton A, Ravetch JV. Fc receptors are required in passive and active immunity to melanoma. *Proc Natl Acad Sci U S A* . 1998;95(2):652-656.

139. Stone JD, Cochran JR, Stern LJ. T-cell activation by soluble MHC oligomers can be described by a two-parameter binding model. *Biophys J*. 2001;81(5):2547-2557.

140. Zhang Z, Zhang M, Ravetch JV, Goldman C, Waldmann TA. Effective therapy for a murine model of adult T-cell leukemia with the humanized anti-CD2 monoclonal antibody, MEDI-507. *Blood*. 2003;102(1): 284-288.

141. Zhang M, Zhang Z, Garmestani K, et al. Activating Fc receptors are required for antitumor efficacy of the antibodies directed toward CD25 in a murine model of adult t-cell leukemia. *Cancer Res*. 2004;64(16):5825-5829.

142. Ji H, Ohmura K, Mahmood U, et al. Arthritis critically dependent on innate immune system players. *Immunity*. 2002;16(2):157-168.

143. Korganow AS, Ji H, Mangialaio S, et al. From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. *Immunity*. 1999;10(4):451-461.

144. Matsumoto I, Staub A, Benoist C, Mathis D. Arthritis provoked by linked T and B cell recognition of a glycolytic enzyme. *Science*. 1999;286(5445):1732-1735.

145. Baumann U, Kohl J, Tschernig T, et al. A codominant role of Fc gamma RI/III and C5aR in the reverse Arthus reaction. *J Immunol* . 2000;164(2):1065-1070.

146. Nimmerjahn F, Ravetch JV. Antibody-mediated modulation of immune responses. *Immunol Rev*. 2010;236:265-275.

147. Abboud N, Chow SK, Saylor C, et al. A requirement for FcgammaR in antibody-mediated bacterial toxin neutralization. *J Exp Med*. 2010; 207(11):2395-2405.

148. Huber VC, Lynch JM, Bucher DJ, Le J, Metzger DW. Fc receptor-mediated phagocytosis makes a significant contribution to clearance of influenza virus infections. *J Immunol*. 2001;166(12):7381-7388.

149. Hessell AJ, Hangartner L, Hunter M, et al. Fc receptor but not complement binding is important in antibody protection against HIV. *Nature*. 2007;449(7158):101-104.

150. Verma A, Ngundi MM, Meade BD, De Pascalis R, Elkins KL, Burns DL. Analysis of the Fc gamma receptor-dependent component of neutralization measured by anthrax toxin neutralization assays. *Clin Vaccine Immunol*. 2009;16(10):1405-1412.

151. Wilson NS, Yang B, Yang A, et al. An Fcgamma receptor-dependent mechanism drives antibody-mediated target-receptor signaling in cancer cells. *Cancer Cell* . 2011;19(1):101-113.

152. Xu Y, Szalai AJ, Zhou T, et al. Fc gamma Rs modulate cytotoxicity of anti-Fas antibodies: implications for agonistic antibody-based therapeutics. *J Immunol* . 2003;171(2):562-568.

153. White AL, Chan HT, Roghanian A, et al. Interaction with Fc{gamma} RIIB is critical for the agonistic activity of anti-CD40 monoclonal antibody. *J Immunol* . 2011;187(4):1754-1763.

154. Li F, Ravetch JV. Inhibitory Fcgamma receptor engagement drives adjuvant and anti-tumor activities of agonistic CD40 antibodies. *Science*. 2011;333(6045):1030-1034.

155. Dijstelbloem HM, van de Winkel JG, Kallenberg CG. Inflammation in autoimmunity: receptors for IgG revisited. *Trends Immunol*. 2001;22(9):510-516.

156. Nimmerjahn F, Ravetch JV. Fcgamma receptors as regulators of immune responses. *Nat Rev Immunol* . 2008;8(1):34-47.

157. Wakeland EK, Liu K, Graham RR, Behrens TW. Delineating the genetic basis of systemic lupus erythematosus. *Immunity*. 2001;15(3): 397-408.

158. Pisitkun P, Deane JA, Difilippantonio MJ, Tarasenko T, Satterthwaite AB, Bolland S. Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication.

Science. 2006;312(5780):1669-1672.

159. Pasare C, Medzhitov R. Control of B-cell responses by toll-like receptors. *Nature*. 2005;438(7066):364-368.

160. Ehlers M, Fukuyama H, McGaha TL, Aderem A, Ravetch JV. TLR9/MyD88 signaling is required for class switching to pathogenic IgG2a and 2b autoantibodies in SLE. *J Exp Med*. 2006;203(3):553-561.

161. Lin Q, Hou R, Sato A, et al. Inhibitory IgG Fc receptor promoter region polymorphism is a key genetic element for murine systemic lupus erythematosus. *J Autoimmun*. 2010;34(4):356-363.

162. Blank MC, Stefanescu RN, Masuda E, et al. Decreased transcription of the human FCGR2B gene mediated by the -343 G/C promoter polymorphism and association with systemic lupus erythematosus. *Hum Genet*. 2005;117(2-3):220-227.

163. Floto RA, Clatworthy MR, Heilbronn KR, et al. Loss of function of a lupus-associated FcgammaRIIb polymorphism through exclusion from lipid rafts. *Nat Med*. 2005;11(10):1056-1058.

164. Heusser C, Jardieu P. Therapeutic potential of anti-IgE antibodies. *Curr Opin Immunol*. 1997;9(6):805-813.

165. Bussel JB. Fc receptor blockade and immune thrombocytopenic purpura. *Semin Hematol*. 2000;37(3):261-266.

166. Podolanczuk A, Lazarus AH, Crow AR, Grossbard E, Bussel JB. Of mice and men: an open-label pilot study for treatment of immune thrombocytopenic purpura by an inhibitor of Syk. *Blood*. 2009;113(14):3154-3160.

167. Strait RT, Morris SC, Finkelman FD. IgG-blocking antibodies inhibit IgE-mediated anaphylaxis in vivo through both antigen interception and Fc gamma RIIb cross-linking. *J Clin Invest*. 2006;116(3):833-841.

168. Horton HM, Chu SY, Ortiz EC, et al. Antibody-mediated coengagement of FcgammaRIIb and B cell receptor complex suppresses humoral immunity in systemic lupus erythematosus. *J Immunol*. 2011;186(7):4223-4233.

169. Geijtenbeek TB, den Dunnen J, Gringhuis SI. Pathogen recognition by DC-SIGN shapes adaptive immunity. *Future Microbiol*. 2009;4(7): 879-890.

170. Parekh RB, Dwek RA, Sutton BJ, et al. Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature*. 1985;316(6027):452-457.

171. Bond A, Cooke A, Hay FC. Glycosylation of IgG, immune complexes and IgG subclasses in the MRL-lpr/lpr mouse model of rheumatoid arthritis. *Eur J Immunol* . 1990;20(10):2229-2233.

172. Parekh R, Roitt I, Isenberg D, Dwek R, Rademacher T. Age-related galactosylation of the N-linked oligosaccharides of human serum IgG. *J Exp Med*. 1988;167(5):1731-1736.

173. van de Geijn FE, Wuhrer M, Selman MH, et al. Immunoglobulin G galactosylation and sialylation are associated with pregnancy-induced improvement of rheumatoid arthritis and the postpartum flare: results from a large prospective cohort study. *Arthritis Res Ther*. 2009;11(6):R193.

174. Maenaka K, van der Merwe PA, Stuart DI, Jones EY, Sondermann P. The human low affinity Fcgamma receptors IIa, IIb, and III bind IgG with fast kinetics and distinct thermodynamic properties. *J Biol Chem*. 2001;276(48):44898-44904.

175. Okazaki A, Shoji-Hosaka E, Nakamura K, et al. Fucose depletion from human IgG1 oligosaccharide enhances binding enthalpy and association rate between IgG1 and FcgammaRIIIa. *J Mol Biol* . 2004;336(5):1239-1249.

176. Paetz A, Sack M, Thepen T, et al. Recombinant soluble human Fcgamma receptor I with picomolar affinity for immunoglobulin G. *Biochem Biophys Res Commun*. 2005;338(4):1811-1817.

177. Bruhns P, Iannascoli B, England P. et al. Specificity and affinity of human Fcgamma receptors and their polymorphic variants for human IgG subclasses. *Blood*. 2009;113(16):3716-3725.

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Chapter 25

Type I Cytokines and Interferons, and Their Receptors

Warren J. Leonard

OVERVIEW AND ISSUES OF NOMENCLATURE

Cytokines are proteins that are secreted by cells and transduce signals via specific cell surface receptors on either the cytokine-producing cell (autocrine actions) or on other target cells (paracrine actions). From this operational type of description, it is clear that the distinction between cytokines, growth factors, and hormones may be imprecise. In general, cytokines and growth factors are similar, except that the term cytokine most often refers to molecules involved in host defense that have actions on leukocytes, whereas the term growth factor more often refers to molecules acting on other somatic cell types. Cytokines most often act locally. For example, in the interaction between a T cell and an antigen-presenting cell, cytokines are produced and usually exert potent actions locally, with rather limited biologic halflives in the circulation. In contrast, hormones are released and then disseminated via the bloodstream throughout the body, with actions on distal target organs. Nevertheless, this distinction between cytokines and hormones is not absolute, with certain cytokines acting at longer distances as well.

In the immune system, terms such as *monokines* and *lymphokines* were originally devised to identify the cellular source for cytokines.[1] Specifically, monokines included molecules such as interleukin (IL)-1, which was first recognized to be produced by monocytes, and lymphokines included molecules such as IL-2, which was first described as a T-cell growth factor produced by T lymphocytes. The monokine/lymphokine nomenclature can be problematic; however, when a cytokine is synthesized by more than one type of cell. This resulted in the adoption of the term cytokine, as proposed by Stanley Cohen in 1974.[2,3] The term cytokine refers to a protein made by a cell (“cyto”) that acts (“kine”) on target cells. Cytokines can have very broad ranges of actions, including on cell development, differentiation, growth, cytolytic activity of effector cells, survival, apoptosis, and chemotaxis.

Many cytokines are referred to as *interleukins* to indicate molecules that are produced by one leukocyte and act on another.[4] However, this term is also problematic as some interleukins (eg, IL-1 and IL-6) are additionally produced by cells other than leukocytes and/or exert actions on cell types beyond the immune system. For example, IL-7 is produced by stromal and epithelial cells rather than by typical leukocytes. Furthermore, nomenclature can be inconsistent. For example, IL-7 is highly related to another cytokine that is denoted as thymic stromal lymphopoietin (TSLP) rather than as an interleukin (see section on IL-7 and Thymic Stromal Lymphopoietin), even though both can be produced by stromal and epithelial cells and share a receptor component as well as select actions on lymphocytes. The more descriptive name of TSLP correctly describes its production by thymic stroma but obscures its production by skin epithelial cells, and that major target cells include dendritic cells (DCs) and cluster of differentiation (CD)4+ T cells.

Among the many classes of cytokines, the “type I” cytokines are distinctive in their sharing a similar four α-helical bundle structure, as detailed in the section on Type I Cytokines

—Structural Considerations, and correspondingly, their receptors share characteristic features that have led to their description as the cytokine receptor superfamily, hematopoietin receptors, or type I cytokine receptors.[5,6,7,8] Although many interleukins are type I cytokines, some are not. For example, two of the major "proinflammatory cytokines," IL-1 and IL-6, are interleukins, but IL-6 is a type I cytokine, whereas IL-1 is not. One interleukin, IL-8, is a CXC family chemokine, an entirely different type of molecule involved in chemotaxis. Moreover, IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B, and IL-29 are more similar to interferons (IFNs) and are denoted as type II cytokines.

In summary, the term inter leuk in indicates a relationship to leukocytes, whereas the identification of a cytokine as a type I or type II cytokine indicates general properties of its three-dimensional structure. Knowing that a molecule is a type I cytokine is instructive as it indicates a likely general structure for the cytokine receptor as well as the mechanism of signal transduction. In contrast, the identification of a molecule as an interleukin provides little information other than that it often, but not always, is a type I or type II cytokine of immunologic interest.

In addition to molecules of primarily immunologic interest, other important proteins, such as growth hormone, prolactin, erythropoietin (Epo), thrombopoietin, and leptin, are type I cytokines and their receptors are type I cytokine receptors. Despite having their major actions outside the immune system, these cytokines nevertheless share important signal transduction pathways with type I cytokines of immunologic interest. By focusing on type I cytokines and IFNs and their receptors, this chapter necessarily focuses on cytokines that are evolutionarily related and share common signaling pathways, instead of focusing on common functions per se. For example, although IL-6 has overlapping actions with IL-1 and tumor necrosis factor (TNF)-α, these latter proinflammatory cytokines are not discussed in this chapter

because they are not type I cytokines, and the signaling pathways they use are distinct from those used by IL-6. This illustrates the important concept that similar end functions can be mediated via more than one type of signaling pathway. This is not to minimize the observation that many type I cytokines in fact do have similar/overlapping functions, as detailed in the section on "cytokine redundancy."

The field of IFN research is older than the cytokine field, but both fields more recently have developed in parallel. In fact, one can consider the IFNs to be the first cytokines that were identified. IFN was discovered as an antiviral activity in 1957. This turned out to be type I IFN (IFNα/β). Type II IFN (IFNγ) was discovered in 1965. Over time, it was recognized that type I cytokines and IFNs/type II cytokines share a number of common features, including signaling pathways.

An unfortunate nomenclature issue exists that should be noted. There are specialized popuplations of T cells that include T helper 1 (Th1) and T helper 2 (Th2) cells (see following discussion), which produced specialized sets of cytokines, such as IFNγ by Th1 and IL-4 by Th2 cells. These are sometimes called type 1 and type 2 cytokines (for Th1 and Th2 cytokines); unfortunately, this jargon can result in confusion as IL-4 is a type I (ie, four α-helical bundle) cytokine that is functionally a type 2 cytokine (in that it is produced by Th2 cells), where IFNγ is a type II structure cytokine produced by Th1 cells.

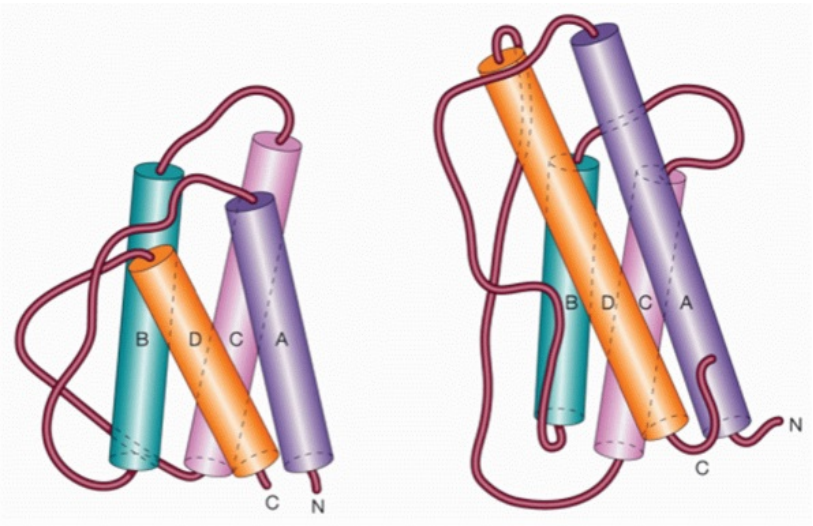

FIG. 25.1. Schematic of Four α-Helical Bundle Cytokines. Schematic drawing showing typical short-chain and long-chain four helical bundle cytokines. Although these both exhibit an "up-up-down-down" topology to their four α helices, note that in the short-chain cytokines, the AB loop is in behind the CD loop, whereas in the long-chain cytokines the situation is reversed. See text. The figure was provided by Dr. Alex Wlodawer, National Cancer Institute.

TYPE I CYTOKINES AND THEIR RECEPTORS

Type I Cytokines—Structural Considerations

Type I cytokines typically share only limited amino acid sequence identity, but strikingly, all type I cytokines whose structures have been solved by nuclear magnetic resonance and/or x-ray crystallographic methods achieve similar three-dimensional structures,[5,6,7,8,9] and those whose structures have not yet been solved are believed to share similar three-dimensional structures. Type I cytokines contain four α helices and thus are designated as four α-helical bundle cytokines (Fig. 25.1). Within their structures, the first two and last two α helices are each connected by long overhand loops, resulting in an "up-up-down-down" topologic structure, as the first two helices (A and B) can be oriented in an "up" orientation and the last two helices (C and D) can be oriented in a "down" orientation, as viewed from the N- to C-terminal direction. As shown in Figure 25.1, the N- and C-termini of the cytokines are positioned on the same part of the molecule.

Type I cytokines are either "short chain" or "long chain" four α-helical bundle cytokines based on the lengths of the α helices.[8] Some of the short-chain cytokines include IL-2,

IL-3, IL-4, IL-5, granulocyte macrophage-colony stimulating factor (GM-CSF), IL-7, IL-9, IL-13, IL-15, IL-21, macrophagecolony stimulating factor (M-CSF), stem cell factor (SCF), and TSLP, whereas long-chain cytokines include growth hormone, prolactin, Epo, thrombopoietin, leptin, IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), novel neurotrophin-1 (NNT-1)/B cell-stimulating factor-3 (BSF-3)/cardiotrophin-like factor (CLC), and granulocytecolony stimulating factor (G-CSF) (Table 25.1).[8,10,11] The α helices are approximately 15 amino acids long in short-chain helical cytokines and 25 amino acids long in long-chain cytokines. Additional differences include differences in the angles between the pairs of helices, and the AB loop is "under" the CD loop in the short cytokines but "over" the CD loop in the long cytokines (see Fig.

25.1).[7,8,12] Moreover, short-chain cytokines have β structures in the AB and CD loops, whereas long-chain cytokines do not.

TABLE 25.1 Four Helical Bundle Cytokines

Short-Chain Cytokines	Long-Chain Cytokines
IL-2	IL-6
IL-4	IL-11
IL-7	Oncostatin M
IL-9	Leukemia inhibitory factor
IL-13	CNTF
IL-15	Cardiotropin-1
IL-21	NNT-1/BSF-3
TSLP	
IL-3	Growth hormone
IL-5[a]	Prolactin
GM-CSF	Erythropoietin
	Thrombopoietin
M-CSF[a,b]	Leptin
SCF[b]	G-CSF

BSF-3, B cell-stimulating factor-3; CNTF, ciliary neurotrophic factor; G-CSF, granulocytecolony stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; M-CSF, macrophage-colony stimulating factor; NNT-1, novel neurotrophin-1; SCF, stem cell factor; TSLP, thymic stromal lymphopoietin.

[a] Dimers.

[b] Different from the other four helical bundle cytokines in that the M-CSF and SCF receptors (CSF-1R and c-kit, respectively) have intrinsic tyrosine kinase activity and are not type I cytokine receptors.

The division of type I cytokines into short-chain and longchain cytokines has evolutionary considerations and correlates with grouping of their receptor chains for these two subfamilies of type I cytokines. An analysis of short-chain cytokines has revealed that 61 residues comprise the family framework, including most of the 31 residues that contribute to the buried inner core. The similarities and differences in the structures of IL-2, IL-4, and GM-CSF have been analyzed. 5 Among these cytokines, there is considerable variation in the intrachain disulfide bonds that stabilize the structures. For example, IL-4 has three intrachain disulfide bonds, GM-CSF has two, and IL-2 has only one. In IL-4, the first disulfide bond (between Cys 24 and Cys 65) connects loop AB to BC, the second disulfide bond (between Cys 46 and Cys 99) connects helix B and loop CD, and the third disulfide bond (between Cys 3 and Cys 127) connects the residue preceding helix B with helix D. In GM-CSF, the N-terminus of helix B and the N-terminus of β strand CD are connected by one disulfide bond, whereas the other disulfide bond connects the C-terminus of helix C and a strand following helix D. In IL-2, a single essential disulfide bond between Cys 58 and Cys 105 connects helix B to strand CD. Thus, each cytokine has evolved distinctive disulfide bonds to stabilize its structure, although it is typical that helix B is connected to the loop between helices C and D. The structures formed by helices A and D are more conserved than those formed by helices B and C, primarily due to the interhelical angles; helix D and the connecting region are the most highly conserved elements among the three cytokines.[5] This is of particular interest, as the regions of type I cytokines that are most important for cytokine receptor interactions (based on analogy to the growth hormone receptor structure, see subsequent discussion) include helices A and D and residues in the AB and CD loops, whereas helices B and C do not form direct contacts.[5]

Variations on these typical four α-helical bundle structures can occur. For example, IL-5 is unusual in that it is a dimer, wherein the ends containing the N- and C-termini are juxtaposed, and helix D is “exchanged” between the two covalently attached monomers so that helix D of each molecule actually forms part of the four helix bundle of the other.[13] M-CSF is also a dimer, but no exchange of helix D occurs.[12]

The IFNs form related albeit distinctive structures from type I cytokines and are designated as type II cytokines.[8] IFNβ has an extra helix that is positioned in place of the CD strand.[14] IFNγ is a dimer, each of which consists of six α helices[15] (Fig. 25.2). Two of these helices are interchanged, including one from each four α-helical bundle.[12,15] IL-10, which is closely related to IFNγ, has a similar structure,[16] as

presumably do the more recently identified IL-10-like molecules, including IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B, and IL-29. Interestingly, although not universally the case, most four α-helical bundle cytokines have four exons, with helix A in exon 1, helices B and C in exon 3, and helix D in exon 4.[7] A related organization is found for IFNγ, as well as for the long-chain helical cytokines, growth hormone, and G-CSF. However, there are exceptions, for example, IL-15 is divided into nine exons, whereas the IFNαs and IFNβ are encoded by single exons.

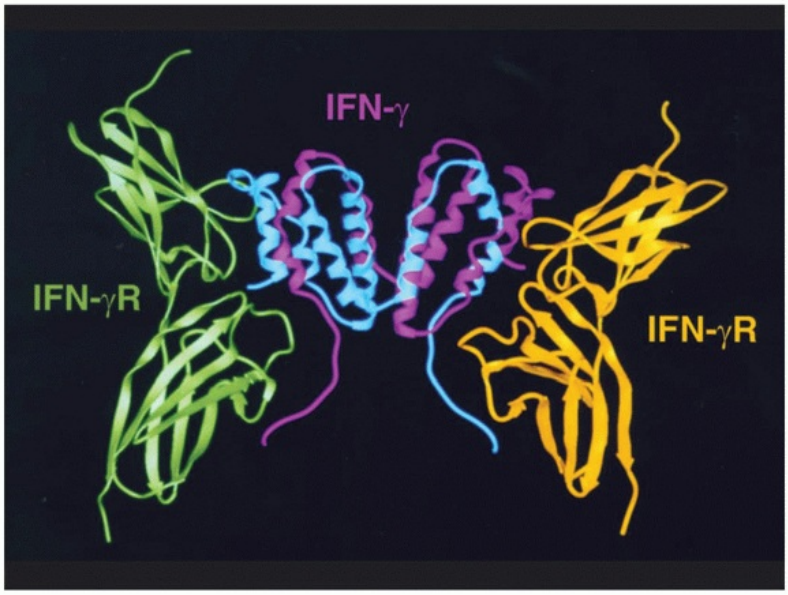

FIG. 25.2. Structure of the Interferon γReceptor (IFNγR). Shown are ribbon diagrams of the structures of the IFNγ/IFNγR complex as an example of a type II cytokine/cytokine receptor. In the IFNγR, only IFNGR-1 complexed to the IFNγ dimer is shown, as the full structure with IFNGR-2 is not available. See text for discussion of the structure. The IFNγ-IFNGR-1 structure is from Walter et al.[401] The figure was provided by Dr. Alex Wlodawer, National Cancer Institute.

Receptors for Type I Cytokines

The first report suggesting that type I cytokines interacted with receptors with similar features identified similarities in the sequences of the erythropoietin receptor and the IL-2 receptor β chain,[17] and subsequent analysis of a large number of type I cytokine receptors established these receptors as a superfamily.[18] Type I cytokine receptors are generally type I membrane-spanning glycoproteins (N-terminus extracellular, C-terminus intracellular). The only exceptions are proteins such as the CNTF receptor α chain (see the following discussion), which lacks a cytoplasmic domain and instead has a glycosylphosphatidylinositol (GPI) anchor; however, the orientation of this protein is otherwise similar to that of a type I membrane protein. In their extracellular domains, a number of conserved features have been noted (Table 25.2). These include four conserved cysteine residues that are involved in intrachain disulfide bonding, and a tryptophan residue, located two amino acids C-terminal to the second conserved cysteine. In addition, a membrane proximal WSXWS (Trp-Ser-X-Trp-Ser) motif is generally conserved, although again exceptions exist, for example, in the growth hormone receptor, the motif is a substantially different YGEFS (Tyr-Gly-Glu-Phe-Ser) sequence, and in the IL-23R, it is WQPWS (Trp-Gln-Pro-Trp-Ser). In some cases, such as the common cytokine receptor β chain, β_C, shared by the IL-3, IL-5, and GM-CSF receptors (see subsequent discussion), the extracellular domain is extended, with a duplication of the domains containing the four conserved cysteines and the WSXWS motif. Another shared feature of type I cytokine receptors is the presence of fibronectin type III domains.

The two pairs of conserved cysteine residues are typically encoded in two adjacent exons, and the exon containing the WSXWS motif is typically just 5′ to the exon encoding the transmembrane domain. Although serines can be encoded by six different codons (ie, sixfold degeneracy in codon usage), only two of these (AGC and AGT) dominate as the codons used for the serines in WSXWS. All of these features indicate a common ancestral type I

cytokine receptor.

TABLE 25.2 Features Common to Type I Cytokine Receptors

Extracellular domain

1. Four conserved cysteine residues, involved in intrachain disulfide bonds
2. WSXWS motif
3. Fibronectin type III modules

Cytoplasmic domain

1. Box 1/Box 2 regions—The Box 1 region is a proline-rich region that is involved in the interaction of Janus family tyrosine kinases.

Overall, analogous to limited sequence identity between type I cytokines, there is only limited sequence identity among type I receptor molecules. Nevertheless, they appear to form similar structures, based on the known structures for the receptors for growth hormone, prolactin, erythropoietin, IL-4, IL-13, IL-6, and IL-2[9,19,20,21,22,23,24,25] as well as the modeling of other cytokine receptor molecules based on the known structures. The cytokines and their receptors have presumably coevolved, with the differences in amino acid sequences between different cytokines allowing for their distinctive interactions with their cognate receptor chains. Despite amino acid differences, there are also several sets of cytokines that coevolved to interact with shared receptor chains, which form type I cytokine and cytokine receptor subfamilies.[8,11]

In addition to these noted similarities in the extracellular domains, there are sequence similarities that are conserved in the cytoplasmic domain of cytokine receptors. In particular, membrane proximal "Box 1/Box 2" regions are conserved (see Table 25.2), with the proline-rich Box 1 region being the most conserved.[26] This will be discussed in greater detail related to its role in the binding of JAK kinases.

Type I Cytokine Receptors Are Homodimers, Heterodimers, or Higher Order Receptor Oligomers

The first cytokine receptor structure solved was that for growth hormone (Fig. 25.3).[19] Prior to x-ray crystallographic analysis, it was believed that growth hormone bound to its

receptor with a stoichiometry of 1:1, but x-ray crystal solution structure revealed that a single growth hormone molecule interacts with a dimer of the growth hormone receptor, in which each receptor monomer contributes a total of seven β strands. Perhaps the most striking finding is that totally different parts of growth hormone interact with the same general region of each receptor monomer. The three-dimensional structure for the growth hormone/growth hormone receptor complex is shown in Figure 25.3. Solving the structure also clarified the basis for the assembly of the growth hormone receptor complex.[19] Growth hormone first interacts with one receptor monomer via a relatively large and high-affinity interaction surface (site I), spanning ~1230 $Å^2$. A second receptor monomer then interacts with the growth

hormone/growth hormone receptor complex via two contact points: one on growth hormone (spanning ~900 $Å^2$) (site II) and the other on the first receptor monomer (spanning ~500 $Å^2$) (site III), located much more proximal to the cell membrane. Thus, three extracellular interactions are responsible for the formation and stabilization of the growth hormone/growth hormone receptor complex. Mutations in critical residues in site I should prevent growth hormone binding to its receptor, whereas inactivating mutations in site II can be predicted to prevent dimerization and signal transduction, suggesting a basis by which different classes of antagonists might be identified.

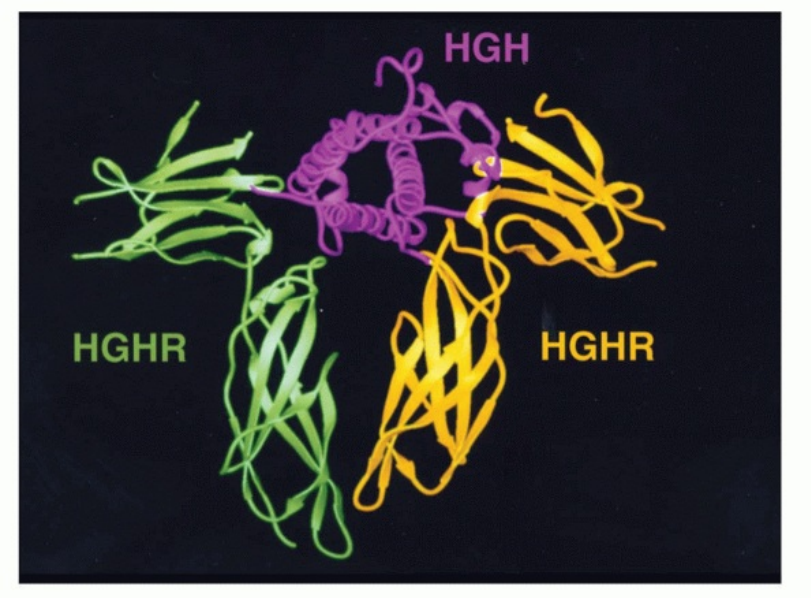

FIG. 25.3. Structure of the Growth Hormone Receptor. Shown are ribbon diagrams of the structure of the growth hormone receptor as an example of a type I cytokine receptor. For growth hormone, both growth hormone receptor monomers are shown. See text for discussion of the structures. The growth hormone/growth hormone receptor structure is from de Vos et al.[19] The figure was provided by Dr. Alex Wlodawer, National Cancer Institute.

The growth hormone/growth hormone receptor structure revealed that the growth hormone receptor extracellular domain is composed of two fibronectin type III modules, each of which is approximately 100 amino acids long and contains seven β strands, resulting in the formation of an immunoglobulin (Ig)-like structure. The contact surface between ligand and receptor occurs in the hinge region that separates these two fibronectin type III modules. Analysis of a growth hormone-prolactin receptor complex revealed a similar structure for the prolactin receptor.[20]

The growth hormone/growth hormone receptor structure served as a paradigm for the structures of other type I cytokine receptors. As a receptor homodimer, it immediately served as a model for other homodimers, such as the Epo receptor, whose structure was solved[21] using a small protein mimetic (20 amino acid long peptide) of Epo.[27] The Epo receptor structure is similar to that of the growth hormone receptor, although the “site III” stem region interaction surface in the Epo receptor is much smaller than that in the growth hormone receptor, comprising only 75 $Å^2$.[21]

In addition to the structural similarities for the growth hormone and Epo receptors and

perhaps other homodimeric type I cytokine receptors, a similar structure was achieved by the heterodimeric growth hormone/growth hormone receptor/prolactin receptor structure,[20] in which one of the growth hormone receptor monomers is replaced by a prolactin receptor molecule. Thus, the two surfaces of growth hormone interact either with two identical monomers of growth hormone receptor or with two nonidentical monomers in the case of the growth hormone receptor/prolactin receptor heterodimer.

It seems reasonable that cytokine-receptor systems with a homodimeric receptor are evolutionarily older than those with heterodimeric receptors, and that the coordination of two different receptor chains in heterodimeric receptors would have evolved in order to allow higher levels of specialization. In this regard, it is interesting that growth hormone and Epo, whose actions are vital for growth and erythropoiesis, bind to receptor homodimers, whereas the heterodimeric structures that typify the immune system are perhaps more “specialized” functions that arose later in evolution.

Interestingly, all type I cytokines known to interact with homodimers (growth hormone, prolactin, Epo, and G-CSF) are long-chain helical cytokines, although other long-chain helical cytokines (eg, cytokines whose receptors contain gp130; see following discussion) interact with heteromeric receptors. The short-chain cytokines that signal through homodimers are SCF and M-CSF, but in these cases, the receptors (c-kit and CSF-1R, respectively) are different from type I cytokine receptors in that they contain intrinsic tyrosine kinase domains. Thus, SCF and M-CSF are not typical type I cytokines and all other short-chain cytokines signal through heterodimers or more complex receptor structures (eg, IL-2 and IL-15 receptors have three components).

Heterodimeric receptors are involved when site II on a cytokine has evolved to a point where it interacts with a different receptor molecule than site I does. This latter situation is the case for many cytokines, including all short-chain type I cytokines except for SCF and M-CSF. Overall, several sets of type I cytokines fall into distinct groups, wherein each group shares at least one common receptor component. This phenomenon is observed for certain sets of both shortchain and long-chain four α-helical bundle cytokines, and depending on the set of cytokines, the shared chain interacts either with site I or site II.

The structures of the low- and high-affinity forms of the IL-2 receptor have now been solved[9,23,28]; these are the first complete structures for a short-chain cytokine/receptor complex.[29] Moreover, they are of added interest in that the low-affinity receptor involves the interaction of IL-2 with IL-2Rα, which is not a type I cytokine receptor protein but instead is a distinctive sushi-domain containing protein, whereas the high-affinity receptor includes not only IL-2Rα but also IL-2Rβ and γ_C, which are both type I cytokine receptor proteins that are shared either with IL-15 (IL-2Rβ) or with IL-4, IL-7, IL-9, IL-15, and IL-21 (γ_C),[30] as discussed in the following text. In the structure of IL-2 bound to its high-affinity receptor (Fig. 25.4), there is a long peptide connecting the IL-2Rα globular head and transmembrane segment, allowing the binding site on this protein to extend relatively far from the cell surface in order to bind the dorsal surface of IL-2. Both the IL-2/IL-2Rα and IL-2/IL-2Rβ contacts are independent, and IL-2Rα does not appear to contact either IL-2Rβ or γ_C; however, IL-2/IL-2Rβ forms a composite surface with γ_C, somewhat analogous to the composite surface formed by growth hormone and one growth hormone receptor monomer for binding to a second monomer. As anticipated, the surface interaction between IL-2 and γ_C is smaller than that between IL-2 and either of the other chains. In addition to the heterodimeric IL-2 receptor structure,[23] the IL-6[31,32] and LIF[33] receptor complexes,

the IL-4/IL-13 receptor[34] complexes, and GM-CSF receptor structure[35] have been solved.

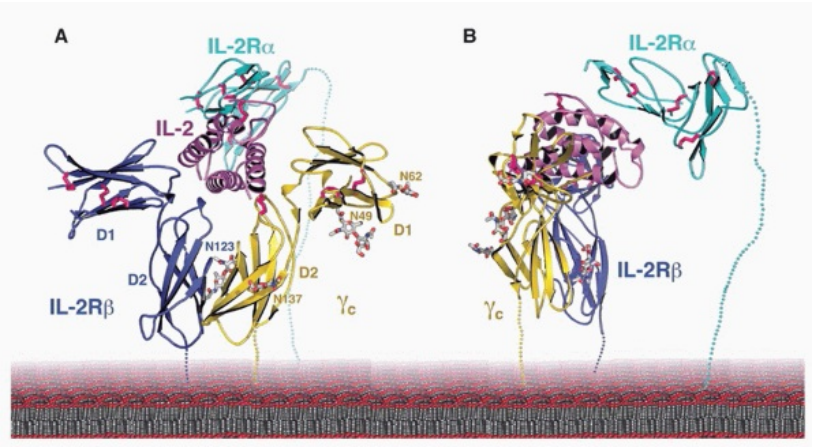

FIG. 25.4. Structure of the High-Affinity Interleukin (IL)-2 Receptor. This is the first structure for a short-chain type I cytokine complexed to its complete receptor. It is particularly interesting in that it includes the sushi-domain containing IL-2Rα chain as well. Reprinted from Wang et al.,[23] with permission of Dr. Garcia and *Science* magazine.

TYPE I CYTOKINE RECEPTOR FAMILIES AND THEIR RELATIONS

Cytokines that Share the Common Cytokine Receptor γChain (Interleukin-2, Interleukin-4, Interleukin-7, Interleukin-9, Interleukin-15, and Interleukin-21)

The receptors for six different immunologically important cytokines, IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, share the common cytokine receptor γ chain, γ_c (CD132).[11,30,36,37,38,39,40,41,42,43,44,45] These cytokines are all short-chain four α-helical bundle cytokines; basic features of these cytokines are summarized in Table 25.3. The properties of these cytokines and their distinctive receptor chains are summarized in the following text, followed by a discussion of the discovery that they share a common receptor component and the implications thereof.

IL-2 is important not only for its function but also historically, as it was the first type I cytokine that was cloned,[46] the first type I cytokine for which a receptor component was cloned,[47,48] and the first short-chain type I cytokine whose receptor structure was solved.[23] Many general principles have derived from studies of this cytokine, including its being the first cytokine that was demonstrated to act in a growth factor-like fashion through specific high-affinity receptors, analogous to the growth factors being studied by endocrinologists and biochemists.[49,50]

Mature IL-2 is a 133 amino acid long peptide that can act as a major T-cell growth factor, in keeping with its original discovery as a T-cell growth factor.[51] Although IL-2 is not produced by resting T cells, it is rapidly and potently induced following antigen encounter with resting CD4+ T cells, and transcription and synthesis of IL-2 are often used as indicators of T-cell receptor (TCR)-mediated cellular activation. Although the antigen determines the specificity of the T-cell immune response, the interaction of IL-2 with high-affinity IL-2 receptors regulates the magnitude and duration of the subsequent response, based on the amount of IL-2 produced, the levels of high-affinity receptors expressed, and the duration of IL-2 production and receptor expression. IL-2 can act in either an autocrine or paracrine fashion, depending on whether the producing cell is also the responding cell or whether the responding cell is a nonproducing cell. The gene encoding IL-2 is located on chromosome

4,[52] and like many other helical cytokines, its gene consists of four exons.[7] IL-2 binds to three different classes of receptors. These are formed by different combinations of three different chains, IL-2Rα,[47,48,53] IL-2Rβ,[54,55,56,57] and a protein initially called IL-2Rγ[36] but now known as the common cytokine receptor γ chain, γ_c.[11,30,37] These different classes of IL-2 receptors are discussed in the following text.

In addition to its being a T-cell growth factor, IL-2 has other important actions as well (see Table 25.3).[29] For example, it can increase Ig synthesis and J chain transcription in B cells,[50,58,59] potently augment the cytolytic activity of

natural killer (NK) cells,[60,61,62] induce the cytolytic activity of lymphokine activated killer cells, promote the elimination of autoreactive cells in a process known as antigen-induced (or activation-induced) cell death,[29,50,63] and promote the differentiation of regulatory T (T_{reg}) cells,[64,65] cells that suppress inappropriate responses and are important for immunologic tolerance. Low-dose IL-2 is sufficient to promote T_{reg}-cell survival, and thereby, for example, can protect mice from developing autoimmune diabetes.[66] Interestingly, IL-2 can also prime CD8+ T cells during a primary response to undergo enhanced proliferation in vivo during a secondary response,[67,68] and autocrine IL-2 is believed to be required for secondary expansion of CD8+ memory T cells.[69] There appears to be a complex interplay between IL-2 and inflammatory signals to regulate effector and memory cytolytic T-lymphocytes generation in lymphocytic choriomeningitis virus infection, with persistent IL-2 promoting effector rather than memory cytotoxic T-lymphocyte development.[70,71] In *Listeria monocytogenesis* infection, Th1 effector memory cells highly express the transcription factor T-Bet and IL-2Rα, and IL-2Rα appears to be critical for the development of these cells.[72] Presumably because of its critical role in T_{reg} development, the absence of IL-2, IL-2Rα, or IL-2Rβ leads to autoimmunity. Interestingly, the production of IL-2 by mast cells has been reported to contribute to the suppression of chronic allergic dermatitis by its increasing the relative number of T_{reg} cells at the site of inflammation.[73]

TABLE 25.3 Features of Cytokines Whose Receptors Share γ_c

Cytokine	Major Source	Size[a]	Actions	Chromosome Location (h/m)	Genomic Org
IL-2	Activated T cells (Th1 cells)	h153 aa/20aa m169aa/20aa 15.5 kDa	T-cell growth factor B-cell growth, Ig production, J chain expression Induces LAK activity Induces tumor infiltrating lymphocyte activity Augments NK activity Critical roles in antigen-induced cell	4q26-27/3	Four exons

			death Stimulates macrophage/monocyte Antitumor effects Promotes T_{reg} development Promotes Th1 and Th2 differentiation Inhibits Th17 differentiation		
IL-4	Activated T cells (Th2 cells) CD4+NK1.1+ natural T cells	h153/24 aa m140 aa/20 aa 18 kDa	B-cell proliferation Ig class switch: IgG1, IgE production Augment MHC II, Fcε receptors, IL-4Rα, and IL-2Rβ expression Th2 cell differentiation Antitumor effects	5q31.1/11	Four exons
IL-7	Stromal cells	h177 aa/25aa m154aa/25aa 17-25 kDa	Thymocyte growth T-cell growth Pre-B-cell growth in mice but not human Survival and growth of peripheral T cells CD4+ and CD8+ T-cell homeostasis	8q12-13/3	Six exons
IL-9	Activated Th cells	h144aa/18aa m144aa/18aa 14 kDa	Th helper clones Erythroid progenitors B cells Mast cells/allergic responses Fetal thymocytes	5q31-35/13	Five exons
IL-15	Monocytes and many cells outside the immune system[b]	h162aa/48aa m162aa/48aa 14-15 kDa	Mast cell growth NK cell development and activity T-cell proliferation CD8+ T-cell homeostasis	4q31/8	Nine exons
IL-21	Activated CD4+ T cells	h162aa/31 aa m146aa/24 aa	Comitogen for T-cell proliferation Inhibits B-cell proliferation to anti-IgM + IL-4 Augments B-cell proliferation to anti-CD40	4q67-27/3	Five exons

	Conflicting reports related to NK cells Cooperates with IL-7 and IL-15 to expand CD8 cells Antitumor effects Drives terminal B-cell differentiation to plasma cells Proapoptotic for B and NK cells Promotes Tfh differentiation Promotes Th17 differentiation

CD, cluster of differentiation; Ig, immunoglobulin; IL, interleukin; LAK, lymphokine activated killer; MHC, major histocompatibility complex; NK, natural killer; Tfh, T follicular helper; Th, T helper; T_{reg}, regulatory T.

[a] h and m refer to human and mouse, respectively. The number of amino acids refers to the length of the open reading frame/length of signal peptide. The number of amino acids in the mature protein is therefore the difference between these numbers. Note that for IL-15, residues 1 to 29 have been identified as a signal peptide and 30 to 48 as a propeptide.

[b] More IL-15 messenger ribonucleic acid is produced in skeletal muscle, kidney, placenta, and lung than in thymus or spleen. It is important to note, however, that IL-15 messenger ribonucleic acid is widely expressed without concomitant production of IL-15 protein so that the source of biologically meaningful IL-15 may be more limited.

IL-2 is also important in inducing or inhibiting Th differentiation. It is required for efficient Th1 74 and Th2 differentiation,[75,76,77,78] which are populations of T helper cells first described based on patterns of cytokine production by mouse T cells.[79] This theme was then extended to human cells as well,[80,81,82,83,84] although there are some variations in humans and mice in terms of the degree of how tightly restricted cytokine expression is. IFNγ is the cytokine most reliably produced by Th1 cells; IL-2 is also produced by these cells, although without as rigorous an association. In contrast, IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 are produced by Th2 cells. In both species, certain type I cytokines (eg, IL-3 and GM-CSF) are produced by both Th1 and Th2 cells. IL-12 and the transcription factors signal transducer and activator of transcription (STAT)4 and T-Bet are the major transcription factors promoting Th1 differentiation, whereas IL-4 and the transcription factor STAT6 drive Th2 differentiation. For Th1 cells, IL-2 acts by STAT5-dependent induction and expression of IL-12Rβ1/IL-12Rβ2 and T-Bet,[74] whereas for Th2 differentiation, IL-2 via STAT5 promotes IL-4[75,76] and IL-4Rα[77] expression. In addition, IL-2 also affects chromatin remodeling at the *Ifng* locus during Th1 differentiation.[85] IL-2 also inhibits Th17 differentiation,[86] in part via downregulation of IL-6Rα/gp130 expression[74] and by inducing T-bet,[74,87] which prevents Runx1-mediated activation of RORγt, a transcription factor that drives Th17 differentiation.[87]

Like IL-2, IL-4 is produced primarily by activated CD4+ T cells.[88,89] IL-4 is also produced by

CD4+NK1.1+ “natural” T cells, denoted as natural killer T (NKT) cells,[90] and by mast cells and basophils.[88] IL-4 is the major B-cell growth factor, and it promotes Ig class switch, enhancing the production and secretion of mouse IgG1 (human IgG4) and being essential for the production of IgE.[88] IL-4 is involved in the physiologic response to parasites, including helminths, and for allergen sensitization. IL-4 induces expression of class II major histocompatibility complex molecules and increases cell surface expression of the CD23 (the low-affinity IgE receptor) on B cells. In addition to its actions on B cells, IL-4 can also act as a T-cell growth factor, inducing proliferation in both human and mouse T cells, and is critical for normal differentiation of Th2 cells.[89] Moreover, when IL-4 and transforming growth factor (TGF)-β are combined, cells that produce IL-9 (Th9) cells are induced (see following discussion). When combined with phorbol 2-myristate 3-acetate, IL-4 is also a potent comitogen for thymocytes. Importantly, IL-4 can inhibit certain responses of cells to IL-2.[91] Moreover, IL-4 can exert actions on macrophages, hematopoietic precursor cells, stromal cells, and fibroblasts.[92] The gene encoding IL-4 is located on human chromosome 5 (5q23.3-31.2) and mouse chromosome 11,[92,93] in the same region as IL-3, IL-5, IL-13, and GM-CSF. The type I IL-4 receptor is expressed on T cells and other hematopoietic cells consists of the 140 kDa IL-4Rα protein[88,94,95,96] and γ_C.[38,39] Expression of IL-4Rα tends to be quite low, and cells that potently respond to IL-4 often express only a few hundred receptors per cell. In addition to the type I IL-4 receptor, an alternate form of the receptor (the type II IL-4 receptor), containing IL-4Rα and IL-13Rα1, although not expressed on mature T cells, is expressed on many other cell types and can transduce IL-4 signals into these cells. For example, IL-13Rα1 is expressed on neonatal Th1 cells and the type II IL-4 receptor has been implicated as mediating apoptosis of these cells.[97]

IL-7 is not produced by lymphocytes but instead is a 152 amino acid long cytokine that is produced by stromal cells and certain other cells.[98,99,100] Based on the analysis of patients with X-linked severe combined immunodeficiency (SCID), JAK3-deficient SCID, and IL-7Rα-deficient SCID, IL-7 receptor-dependent signaling is essential for T-cell development in humans (discussed subsequently). The major role of IL-7 is to enhance thymocyte survival, growth, and differentiation[101,102,103,104,105] as well as low-affinity peptide-induced proliferation, and thus it promotes homeostatic proliferation of naive and memory CD8+ T cells.[100,105,106,107] Additionally, IL-7 can regulate the homeostasis of CD4+ memory T cells[108,109] and also can stimulate the growth of mature T cells.[30,105,110,111] Although IL-7, as noted previously, is believed to primarily be a stromal factor, IL-7 has also been noted to be produced by the liver and kidney but not spleen or lymph nodes after toll-like receptor (TLR) signaling. Although basal liver production is low, after TLR signaling, hepatic IL-7 can promote CD4 and CD8 T-cell survival and promotes antigen-specific T-cell responses.[112] In addition, in the mouse, IL-7 is vital for the growth of mouse pre-B cells,[96,98,103,104,111] and transient IL-7 signaling can inhibit Ig heavy chain gene rearrangements.[113] IL-7 via STAT5 has also been shown to inhibit Ig-κ recombination in pro-B cells.[114] In contrast to its requirement for B-cell development in the mouse, there is normal B-cell development in patients with defective IL-7 signaling, as is found in patients with X-linked SCID, JAK3-deficient

SCID, and IL-7Rα-deficient SCID (see following discussion). Thus, human B cells can develop normally in the absence of IL-7 responsiveness, demonstrating that in humans, IL-7 is not vital for the growth of human pre-B cells,[30,115] and it remains unknown whether IL-7 plays important roles in human B-cell biology. Interestingly, IL-7 also acts on DCs, and this

appears to limit the homeostatic proliferation of T cells under lymphopenic conditions.[100,116] Clinically, IL-7 has been of interest in terms of its ability to induce T-cell proliferation and to thereby increase T-cell numbers, with a greater effect on CD8+ than on CD4+ T cells, as well as to augment the diversity of the TCR repertoire[117]; IL-7 also can increase antigen-specific responses following vaccination[100] and can promote antiviral immunity, apparently in part due to its induction of IL-22 and repression of SOCS3.[118] The gene encoding IL-7 is located on human chromosome 8q12 to 8q13[119] and mouse chromosome 3. The functional IL-7 receptor contains the 75 kDa IL-7Rα[120] and γ_c.[37,40] Interestingly, signaling via the TCR, IL-2 or IL-7 can downregulate IL-7Rα expression,[121,122] with PI 3-kinase/AKT and GFI1B being implicated in its downregulation in T cells.[122,123,124] The downregulation of IL-7Rα not only can both decrease responsiveness of cells but can also increase the availability of the cytokine for other cells that are poised to respond.[11] Induction of IL-7Rα requires PU.1 in B cells[125] and another Ets family protein, GABP, in T cells.[123]

IL-9 was originally described as a mouse T-cell growth factor[126] that is produced by activated T cells and can support the growth of T-helper clones but not of cytolytic clones.[127] In contrast to IL-2, its production is delayed, suggesting its involvement in later, perhaps secondary signals. In the mouse, IL-9 can exert proliferative effects on erythroid progenitors, B cells, B-1 cells, mast cells, and fetal thymocytes. IL-9 is identical to mast cell growth-enhancing activity, a factor present in conditioned medium from splenocytes,[128] and synergizes with IL-3 for maximal mast cell proliferation. IL-9 is highly expressed in the lung of patients with asthma,[129] and overexpression leads to airway inflammation and Th2 cytokine production.[130] Interestingly, IL-9 is substantially made in the lung by innate lymphoid cells, and in these cells neutralizing antibodies to IL-9 lowered expression of IL-13 and IL-5, supporting a link between IL-9 and the regulation of the Th2 response.[131] Consistent with the action of IL-9 on thymocytes in vitro, IL-9 transgenic mice develop thymic lymphomas, and IL-9 is a major antiapoptotic factor for such tumors.[132] Nevertheless, IL-9 knockout mice have normal T-cell development[133]; instead, they exhibit a defect in pulmonary goblet cell hyperplasia and mastocytosis following challenge with *Schistosoma mansoni* eggs, a synchronous pulmonary granuloma formation model. However, there was no defect in eosinophilia or granuloma formation.[133] Mice expressing an IL-9 transgene in the lung exhibit airway inflammation and bronchial hyperresponsiveness; nevertheless, IL-9-/- mice exhibit normal eosinophilia and airway hyperreactivity in an ovalbumin-induced inflammatory model.[134,135,136] Thus, although IL-9 can contribute to allergic/pulmonary responses, there are compensatory cytokines that substitute for IL-9 in at least certain settings. IL-9 can be produced by the Th9 populations of cells, which can be induced by IL-4 plus TGF-β in a fashion that is dependent on PU.1 and interferon regulatory factor (IRF)-4.[137] Interestingly, the addition of IL-25 further enhances the production of IL-9.[138] IL-9-producing cells are typically IL-9+IL-10+Foxp3-effector T cells and can be derived from Th2 cells.[139,140] Overall, IL-9 is believed to play a key role in allergic responses; additionally, given the ability of both Th17 and T_{reg} cells to produce IL-9 during autoimmunity and transplantation, it will be interesting to further clarify the role of IL-9 in autoimmunity.[141] Currently, there is some confusion, as one study showed that antibody blockade of IL-9 or IL-9Rα blocks experimental autoimmune encephalomyelitis (EAE) disease progression,[142] whereas another study showed that IL-9Rα knockout mice develop more severe EAE.[138] While mouse IL-9 is active on human cells, human IL-9 is not biologically active on mouse cells (the opposite situation

from that for IL-2). Human IL-9 is located on chromosome 5q31 to 5q35,[143] which is also the location for the genes encoding IL-3, IL-4, IL-5, IL-13, and GM-CSF. In contrast, mouse IL-9 is "isolated" on chromosome 13, while IL-3, IL-4, IL-5, IL-13, and GM-CSF are clustered on chromosome 11. IL-9 binds to the 64 kDa IL-9Rα binding protein, which is similar in size to γ_c,[144] and the functional IL-9 receptor consists of IL-9Rα plus γ_c.[30,41,42]

IL-15 was identified as a T-cell growth factor that also unexpectedly was expressed in the supernatant of a human T-lymphotropic virus type I (HTLV-I)-transformed T-cell line.[145,146] Although IL-15 messenger ribonucleic acid (RNA) is produced by a range of nonlymphocytic cell types, it is difficult to detect physiologic levels of IL-15 protein.[147] Its main site of synthesis appears to be DCs and monocytes, and unlike IL-2, IL-15 is not produced by activated T cells.[148] IL-15 receptors are widely expressed; although IL-15 is perhaps most important for the development of NK cells[149,150,151] and CD8+ memory T cells,[150,151] it also has both paracrine and autocrine actions on DCs, including promoting the survival of these cells.[11] Interestingly, it also regulates the TCR repertoire of γδ intraepithelial lymphocytes[152] and cross talk between different types of DCs.[153] The receptor for IL-15 on T cells contains IL-2Rβ,[43,147,154] γ_c,[43] and an IL-15-specific protein, IL-15Rα. IL-15Rα shares a number of structural similarities with IL-2Rα, including that it is a sushi domaincontaining protein (IL-2Rα has two sushi domains, whereas IL-15Rα has one),[155] and the *IL2RA* and *IL15RA* genes are closely positioned on human chromosome 10p14.[156] A distinctive feature of IL-15 signaling is that it signals substantially by a process called *transpresentation,* wherein IL-15Rα on the surface of dendritic cells or monocytes will transpresent IL-15 to responding cells such as CD8+ T cells or NK cells that express IL-2Rβ + γ_c.[148,157] IL-15Rα has a longer cytoplasmic domain than IL-2Rα and appears to be critical for IL-15Rα function, although potentially not for transpresentation.[158] Interestingly, transpresentation can be utilized by IL-2 as well.[159] The very high affinity of IL-15Rα for IL-15 is explained by a large number of ionic interactions mediated by the sushi domain.[160] Note that these responding cells can also express IL-15Rα. In contrast to IL-2, which is a growth factor as well as a mediator of antigen-induced (or activationinduced) cell death and promoter of T_{reg} differentiation, the

role of IL-15 appears to be more focused on growth of CD8+ T cells,[161] maintaining long-lasting, high-avidity T-cell responses to foreign pathogens (ie, CD8+ T-cell memory).[148,162] This ability has suggested it could have potential as a vaccine adjuvant.[163] Overall, the fact that IL-15 promotes the proliferation and differentiation of B, T, and NK cells; the cytolytic activity of CD8 T cells; and the maturation of dendritic cells, yet, unlike IL-2, fails to stimulate immunosuppressive T_{reg} cells indicates that it has an array of properties that make it potentially promising as an anticancer therapeutic agent; as a result, a number of ongoing clinical trials are now in progress.[164]

IL-21 is the most recently identified member of the IL-2 family of cytokines. IL-21 can bind to specific receptors and exert actions on T, B, NK cells, DCs, and macrophages.[165] It augments T-cell proliferation as a comitogen,[166] can cooperate with IL-7 or IL-15 to drive the expansion of freshly isolated mouse CD8+ T cells, and it can augment the antitumor activity of CD8+ T cells.[167] IL-21 promotes the differentiation of Th17 cells[168,169] as well as the generation of T follicular helper cells.[170] In the case of Th17 differentiation, IL-21 is part of an IL-6 to IL-21 to IL-23 signaling cascade[169,171,172] to drive the differentiation of these

cells in an RORγ-dependent manner.[173] Polarization of Th17 cells is dependent on TCR stimulation, TGF-β, and IL-6 but is independent of IL-23, which instead may be required for maintaining/expanding these cells.[174,175,176,177]

The actions of IL-21 on B cells are particularly complex. It augments B-cell proliferation when combined with anti-CD40 or lipopolysaccharide (LPS) but inhibits proliferation in response to anti-IgM + IL-4[166]; this inhibition is reversed if anti-CD40 is additionally provided. It induces apoptosis of incompletely activated B cells, perhaps serving a role analogous to that of IL-2 in activation-induced cell death of T cells to eliminate incompletely activated cells.[165] In contrast, IL-21 drives terminal differentiation to plasma cells of more fully activated cells. Strikingly, IL-21 can drive plasma cell differentiation of both peripheral memory B cells and cord blood B cells, at least in part explained by its ability to induce expression of BLIMP1.[178,179] Strikingly, IL-21 regulates not only BLIMP-1 but also a broad range of genes via a functional cooperation between STAT3 and IRF4.[180] In IL-21R knockout mice, following immunization, IgG1 is diminished related to a role for IL-21 in class switching to IgG1 and IgG3, whereas IgE is elevated, related to the ability of IL-21 to inhibit Cε transcription[181] and/or to its ability to augment the apoptosis of IgE-producing B cells. Analysis of IL-21R/IL-4 double knockout mice has revealed that IL-21 cooperates with IL-4 to globally regulate Ig production in that these mice exhibit a panhypogammaglobulinemia, mimicking the T-cell phenotype in humans with X-linked SCID.[182] IL-21 can also cooperate with IL-15 and Flt-3 ligand to increase development of NK cells[166] and augment antitumor activity[183]; however, it was also reported to oppose the actions of IL-15[184] and can also direct NK cell apoptosis. Interestingly, IL-21 exerts potent antitumor effects, including against large established solid tumors.[11,165] In the pMEL-1 CD8 TCR transgenic system, the effector cells recognize a melanoma antigen. When cells are adoptively transferred into tumor-bearing mice and animals are vaccinated with the cognate antigen, the addition of IL-21 or IL-21 plus IL-15 has potent antitumor effects.[167] Moreover, treatment of cells with IL-21 in vitro prior to adoptive transfer results in markedly enhanced antitumor effects in vivo, conferring a distinctive differentiation program from that conferred by IL-2.[185] IL-21 is now in phase II clinical trials for cancer. In addition to its anticancer activity, IL-21 can also promote autoimmunity. Elevated IL-21 levels have been reported in the BXSB-*Yaa* mouse model of systemic lupus erythematosus,[178] and elevated IL-21 levels have been found in a subset of humans with systemic lupus erythematosus (P. Lipsky, personal communication). Elevated IL-21 has also been associated with other autoimmune processes, including in the non-obese diabetic (NOD) mouse.[181] More importantly, in mouse models of type 1 diabetes, lupus, and experimental allergic uveitis, no autoimmune disease develops on an IL-21R knockout background.[186,187,188] IL-21 unexpectedly can also be immunosuppressive via its induction of IL-10.[189,190] Consistent with this, an IL-21/IL-10/STAT3 pathway is required for normal development of memory CD8+ T cells after lymphocytic choriomeningitis virus (LCMV) infection[191]; similarly, STAT3, presumably by an overlapping mechanism is important for human T-cell memory development, as evidenced by studies in patients with autosomal dominant hyper-IgE syndrome, which is caused by dominant negative mutations in STAT3.[192] IL-21 has complex roles in viral responses. For LCMV, IL-21 is required to control chronic infection,[193] whereas IL-21 mediates the pathogenic response after infection with pneumonia virus of mice.[194] Interestingly, for hepatitis B virus, IL-21 appears to be critical in determining the age-dependent effectiveness of immune responses, wherein decreased IL-21 production in younger individuals hinders the generation of critical lymphoid responses to the virus, whereas more robust IL-21 production in adults is associated with viral clearance that

occurs in 95% of patients.[195]

The receptor for IL-21 consists of IL-21R plus γ_C.[30,44] IL-21R is most related to IL-2Rβ, and like IL-2Rβ, its expression is induced following cellular stimulation with anti-CD3 or phytohemagglutinin, and in addition, its expression is augmented in T cells following transformation with HTLV-I.[196] Both human and mouse IL-21 can act on cells of the other species. IL-21 is on human chromosome 4q26 to 4q27, while its receptor is on chromosome 16p11, immediately downstream of the *IL4R* gene.

Thus, IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 collectively exhibit partially overlapping roles related to T cells, NK cells, B cells, and mast cells, and together would be expected to play vital roles for normal development and/or function of these cellular lineages. As discussed in the following, IL-2, IL-7, IL-9, and IL-15 utilize primarily STAT5A and STAT5B; IL-4 activates primarily STAT6; and IL-21 activates STAT1, STAT3, STAT5A, and STAT5B, with STAT3 being the dominant STAT for this cytokine.[11] The fact that these six cytokines share γ_C is of particular interest, especially as the gene encoding γ_C is mutated in patients with the most common form of severe combined immunodeficiency in humans.

X-Linked Severe Combined Immunodeficiency Disease Results from Mutations in the Gene Encoding γ_C

The γ chain was originally identified as a third component of the IL-2 receptor[36] after it became clear that IL-2 receptor α and β chains alone were not sufficient to transduce an IL-2 signal. The hypothesis that the γ chain was a shared component of receptors for cytokines in addition to IL-2 was motivated from a comparison of the clinical phenotypes in humans that result from defective expression of IL-2 versus. the γ chain. In 1993, it was discovered that mutations in the gene encoding the γ chain resulted in X-linked SCID (the disease is also designated as SCIDX1).[30,197] X-linked SCID is characterized by profoundly diminished numbers of T cells and NK cells[30,45,197,198,199,200,201] (Table 25.4). Although the B cells are normal in number, they are nonfunctional, apparently due to a lack of T-cell help as well as an intrinsic B-cell defect.[45,200,202] In contrast to the profoundly decreased number of T cells in patients with X-linked SCID, IL-2-deficient patients[203,204] and mice[205] have normal numbers of T cells (the phenotypes of mice deficient in type I and type II cytokines and their receptor, JAK kinases, and STAT proteins are summarized in Table 25.15). This observation indicated that defective IL-2 signaling was unlikely to be responsible for X-linked SCID, making the finding that the gene encoding the γ chain was mutated in X-linked SCID all the more unexpected. Thus, the conundrum was why a defect in a component of a receptor would cause a more severe than a defect in the corresponding cytokine. This led to the hypothesis that the γ chain was critical for other cytokine receptors as well.[197] In this model, defective IL-2 signaling either did not contribute to the defects in X-linked SCID or these defects were explained by the simultaneous inactivation of multiple signaling pathways.[45,197] Indeed, it was found that the γ chain was also an essential component of both the IL-4 and IL-7 receptors on T cells,[37,38,39,40] leading to it being renamed as the common cytokine receptor γ chain, γ_C.[37,38] IL-9, IL-15, and IL-21 were subsequently also shown to share γ_C[30] (Fig. 25.5).

The sharing of γ_C by six different cytokine receptors revealed that X-linked SCID is a disease of defective cytokine signaling. The major deficiencies in X-linked SCID can be attributed to defects related to different cytokines. Based on the dramatically diminished T-cell

development not only in IL-7-deficient[104] or IL-7Rα-deficient[103] mice but also in *IL-7R*-deficient humans with T-B+NK+ SCID,[206,207] yet normal T-cell development in mice deficient in IL-2,[205] IL-4,[208,209] both IL-2 and IL-4,[210] IL-9,[133] IL-15,[151] IL-15Rα,[150] IL-21R,[182,184] and most if not all of the defect in T-cell development in patients with X-linked SCID can be attributed to defective IL-7 signaling.[30] In addition to profoundly diminished numbers of T cells, humans with X-linked SCID lack NK cells. As discussed previously, NK-cell development is defective in IL-15- and IL-15Rα-deficient mice, indicating that it is defective IL-15 signaling that is responsible for the defective NK-cell development in X-linked SCID.[30]

TABLE 25.4 Features of X-Linked Severe Combined Immunodeficiency

1. Absent or profoundly diminished numbers of T cells and mitogen responses
2. Absence of NK cells
3. Normal numbers of B cells, but defective B-cell responses
4. IgM can be normal but greatly diminished Igs of other classes
5. XSCID carrier females exhibit nonrandom X-inactivation patterns in their T cells and NK cells; the X-inactivation pattern is random in surface IgM-positive B cells but nonrandom in more terminally differentiated B cells.

Ig, immunoglobulin; NK, natural killer; XSCID, X-linked severe combined immunodeficiency.

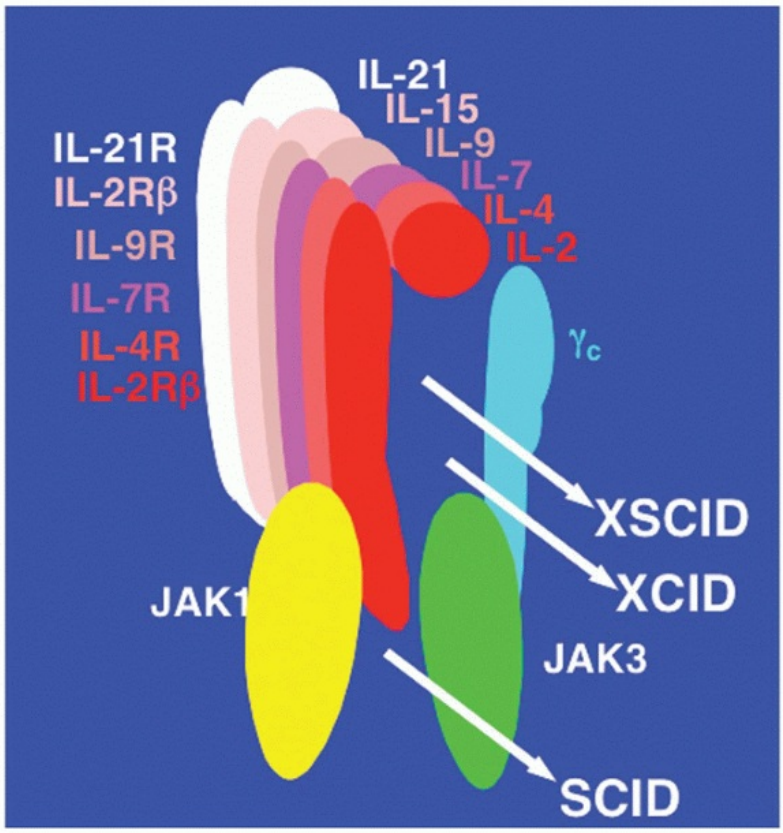

FIG. 25.5. Schematic of the Receptors for Interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15, and IL-21, Showing Interactions with JAK1 and JAK3. The figure shows that IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 all share γ_C. IL-2Rα and IL-15Rα are not shown. Whereas the distinctive chains associate with JAK1, γ_C associates with JAK3. Mutations in γ_C cause X-linked severe combined immunodeficiency or more moderate forms of X-linked immunodeficiency. Mutations in JAK3 cause an autosomal recessive form of severe combined immunodeficiency (see text).

In contrast to the greatly diminished number of T cells and absent NK cells in patients with X-linked SCID, B-cell numbers are normal. This is in contrast to the greatly diminished numbers of B cells in γ_C-deficient mice[211,212] as well as mice deficient in either IL-7 or IL-7Rα, indicating that IL-7 is not required for pre-B-cell development in humans and underscoring a major difference for IL-7 in human versus mouse biology. Indeed, patients with *IL-7R*-deficient SCID have a T-B+NK+ form of SCID.[206,207] Although B cells develop in patients with X-linked SCID, they are nonfunctional. This is due in part to a lack of T-cell help (given the near absence of T cells in X-linked SCID), but a variety of data indicate an intrinsic B-cell defect as well.[30] As discussed

previously, analysis of IL-4/IL-21R double knockout mice indicate that defective signaling by IL-4 and IL-21 appear to explain the intrinsic B-cell defect in X-linked SCID.[182]

Rationale for the Sharing of γ_C

Why should there have been evolutionary pressure to maintain the sharing of γ_C, given the obvious increased risk associated with sharing a receptor component when it is mutated? There are at least two different types of models.[45,213] First, the sharing of γ_C could be a

basis for shared actions. For example, given that IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 can each act as T-cell growth factors, at least in vitro, it is possible that γ_c might couple to signal transducing molecule(s) that promote T-cell growth. Second, the sharing of γ_c might represent a means by which one cytokine using γ_c can modulate the signals of the others. To understand this model, it is important to recognize that, in contrast to antigen receptor complexes, cytokine receptor components individually are targeted to the cell surface, and the formation/stability of receptor complexes is promoted or stabilized by ligand binding, as noted in the discussion of growth hormone, wherein the second receptor monomer recognizes the combined surface of growth hormone and the first growth hormone receptor monomer[19] or for the IL-2 receptor where γ_c "sees" a combined IL-2/IL-2Rβ surface.[23] This latter finding is consistent with γ_c originally having been coprecipitated with IL-2Rβ in the presence but not in the absence of IL-2,[214] and dimerization of IL-2Rβ and γ_c is known to be required for efficient signaling.[215,216] Thus, receptor heterodimerization at a minimum is stabilized by the cytokine and physiologically may be absolutely dependent on the presence of the cytokine. In the absence of stable preformed cytokine receptor complexes between γ_c and the other receptor chains, one can envision that γ_c might be differentially recruited to different receptors based on the relative amount of a cytokine or its binding efficiency. In a situation where γ_c is limiting, a cytokine might then not only induce its own action but could also simultaneously inhibit the action of another cytokine that was less efficient at recruiting γ_c to its cognate receptor complex.

An analysis of mice deficient in IL-2,[205] IL-2Rα,[217] IL-2Rβ,[218] and γ_c[211,212] provides the interesting observation that although the mice lacking γ_c have defective signaling in six different cytokine pathways, mice deficient in IL-2, IL-2Rα, and IL-2Rβ appear to be less healthy than the γ_c-knockout mice with more markedly activated T cells and autoimmunity that is not evident in γ_c-deficient mice. This is at least in part explained by the fact that IL-2-deficient mice lack T_{reg} cells and thus develop autoimmune disease,[65] whereas γ_c-deficient mice lack T cells due to defective IL-7 signaling and thus lack effector T cells. These and other data indicate that γ_c plays a major role in regulating lymphoid homeostasis, as originally indicated.[219]

TABLE 25.5 Features of Cytokines whose Receptors Share the Common β Chain, β_c

Cytokine	Major Source	Size	Cellular Targets	Chromosome Location (h/m)	Exons
IL-3	T cells	h152/19aa m166/26 aa 22-34 kDa	Multiple lineages	5q31.1/11	5
IL-5	T cells	h139/22aa m133/21 45 kDa dimer	Eosinophils B cells (?)	5 q31.1/11	4

GM-CSF	T cells	h144/17aa m141/17aa 23 kDa	Granulocytes Macrophages	5q31.1/11	4

GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin.

Cytokines whose Receptors Share the Common β Chain, β_c (Interleukin-3, Interleukin-5, and Granulocyte Macrophage-Colony Stimulating Factor)

The hematopoietic cytokines, IL-3, IL-5, and GM-CSF (Table 25.5) are all synthesized by T cells and exert effects on cells of hematopoietic lineage.[220,221,222] These cytokines are vital for proliferation as well as differentiation of myeloid precursor cells. Of these three cytokines, IL-3 is the most pluripotent[221] and historically was also called multi-CSF, reflecting the large number of lineages on which it can act. It can act to promote proliferation, survival, and development of multipotent hematopoietic progenitor cells and of cells that have become dedicated to a range of different lineages, including granulocyte, macrophage, eosinophil, mast cell, basophil, megakaryocyte, and erythroid lineages. It induces a range of effects, including, for example, inducing the production off IL-4 by basophils.[223] IL-3 also can exert end-function effects, such as enhancing phagocytosis and cytotoxicity. GM-CSF is mainly restricted to the granulocyte and monocyte/macrophage lineages, but its actions are nevertheless still quite broad.[220] It is both a growth and survival factor. In addition, it can expand the number of antigen-presenting cells, such as DCs, and thereby may greatly expand the ability of the host to respond to antigen. IL-23 and RORγt can augment GM-CSF production in Th cells, whereas IL-12, IFNγ, and IL-27 negatively regulate its expression, and GM-CSF is now known to be required for the initiation of autoimmune neuroinflammation in experimental autoimmune encephalitis. Collectively, these results suggest that GM-CSF is important for encephalitogenic actions of both Th1 and Th17 cells.[224,225] GM-CSF mediates autoimmune effects at least in part by enhancing IL-6-dependent

Th17 cell development and survival.[226] Whereas IL-3 and GM-CSF can act on eosinophils, they act at much earlier stages than IL-5, presumably expanding the number of eosinophil-committed precursors cells. IL-5 stimulates the eosinophilic lineage and eosinophil release from the bone marrow and is essential for expanding eosinophils after helminth infections,[227,228] and can mediate the killing of *S. mansoni*. IL-5 can also induce Ig production in B cells activated by contact with activated Th cells in mouse systems, and IL-5 and IL-5Rα knockout mice have diminished CD5+ B1 cells and decreased thymocytes until approximately 6 weeks of age.[227,228] Interestingly, the production of IL-5 by memory Th2 cells is driven by GATA3 but downregulated by expression of eomesodermin, which interacts with GATA3. Most memory Th2 cells produce the transcription factor Eomes, but the $CD62^{lo}CXCR3^{lo}$ population has decreased Eomes and is the population of cells that produces IL-5.[229]

On cells that express receptors for more than one of these cytokines, such as eosinophilic progenitors that express receptors for IL-3, IL-5, and GM-CSF, or on mouse pre-B cells, which express receptors for IL-3 and IL-5, the signals induced are indistinguishable.[221,222] Thus, the differential lineage specificities of these cytokines are determined by the cellular distribution of their receptors rather than by fundamental differences in the signals that are induced by each cytokine. These observations are explained by studies demonstrating that each of these three cytokines has its own unique 60 to 80 kDa α chain (ie, IL-3Rα, IL-5Rα,

and GM-CSFRα),[230,231,232,233,234,235,236] but that they share a common 120 to 130 kDa β chain, β_C.[222,237,238,239] The α chains are the principal binding proteins for the cytokines, whereas the shared β_C subunit augments binding affinity but does not exhibit binding activity in the absence of the proper α chain. The α chains have relatively short cytoplasmic domains (approximately 55 amino acids long for IL-3Rα, IL-5Rα, and GM-CSFRα) and are not believed to play major roles in signaling function, whereas β_C, with its cytoplasmic domain of 432 amino acids, is the primary determinant of the signal. As a result, there is a relative compartmentalization of binding and signaling function for these cytokines, although the cytoplasmic domains of the GM-CSFRα and IL-5Rα chains (and by analogy, perhaps the IL-3Rα chain), as well as β_C, appear to be capable of at least modulating the growth signals in transfected cells.[222,240,241,242] In any case, the sharing of β_C helps to explain why the signals induced by IL-3, IL-5, and GM-CSF are similar on cells that can respond to more than one of these cytokines. The situation for the β_C family of cytokines is therefore quite different from the receptors for γ_C family cytokines, wherein the chains with the largest cytoplasmic domains (IL-2Rβ, IL-4Rα, IL-7Rα, IL-9Rα, and IL-21R) not only contribute most to signaling specificity but also are the proteins principally involved in ligand binding (note that for IL-2 and IL-15, IL-2Rα and IL-15Rα, respectively, serve important roles as well). The shared chain, γ_C, serves a vital accessory function (hence the development of X-linked SCID when the *IL2RG* gene is mutated), but it contributes less to cytokine binding and does not provide an obvious basis for signaling specificity (see subsequent discussion).

An interesting feature of the β_C family of hematopoietic cytokines is that there appears to be considerable redundancy of function so that knockout mice that lack the ability to respond to all three cytokines (GM-CSF, IL-3, and IL-5) due to deletion of β_C as well as the mouse IL-3-specific β_C-like protein (discussed in the following text) nevertheless exhibit relatively normal hematopoiesis. These observations do not minimize the potency of these particular cytokines but instead underscore a substantial redundancy for a particularly important set of functions.[221,222,243] It is also noteworthy that β_C-deficient mice exhibit defective host responses to infectious challenge, suggesting that these hematopoietic cytokines play a vital role in promoting immune function.

Cytokines whose Receptors Share gp130 (Interleukin-6, Interleukin-11, Oncostatin M, Ciliary Neurotropic Factor, Leukemia Inhibitory Factor, Cardiotrophin-1, Novel Neurotrophin-1/B Cell-Stimulating Factor-3/Cardiotrophin-Like Factor, and Interleukin-27)

There are now eight cytokines that are known to utilize gp130 as a signal transducing molecule.[244,245,246,247,248,249,250,251,252,253,254,255] Some of the properties of these cytokines are summarized in Table 25.6. This family is often referred to as the IL-6 family of cytokines and includes IL-6, IL-11, OSM, LIF, CNTF, CT-1, NNT/BSF-3/CLC, and IL-27. This group of cytokines comprises molecules with a diverse range of actions, ranging beyond the hematopoietic and

immune systems to the central nervous and cardiovascular systems, making them even more "multifunctional" than the γ_C and β_C families of cytokines, whose actions are more restricted to the lymphoid and hematopoietic systems.

TABLE 25.6 Cytokines whose Receptors Share gp130[a]

Cytokine	Chromosome Location (h/m)
IL-6	7p21/5
IL-11	19q13.3-13.4/7
LIF	22q12.1-12.2/11
OSM	22q12.1-12.2/11
CNTF	11q12.2/19
CT-1	16p11.1-11.2/7
NNT-1/BSF-3/CLC	11q13/19
IL-27	

Overlapping actions of several gp130 cytokines

	IL-6	IL-11	LIF	OSM	CNTF	CT-1
Growth of myeloma cells	+	-	+	+	+	?
Maintenance of embryonic stem cell pluripotency	-	-	+	+	+	+
Induction of hepatic acute phase proteins	+	+	+	+	+	+
Induction of cardiac hypertrophy	-	+	+	+	+/-	+
Induction of osteoclast formation	-	+	+	+	?	?
Enhanced neuronal survival/differentiaion	+	+	+	+	+	+
Inhibit adipogenesis	?	+	+	?	?	?

BSF-3, B cell-stimulating factor-3; CLC, cardiotrophin-like factor; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; IL, interleukin; LIF, leukemia inhibitory factor; NNT-1, novel neurotrophin-1; OSM, oncostatin M.

[a]Most of the data in this table are derived from Yin et al.248

Note that NNT-1 can support survival of chicken embryonic and sympathetic neurons, can induce amyloid A, and analogous to IL-6 can induce B-cell hyperplasia.

IL-6 was originally identified and then cloned as a B-cell differentiation factor that stimulated terminal differentiation/maturation of B cells into antibody-producing plasma cells.[256,257,258] However, IL-6 also can exert effects for T-cell growth and differentiation (and thus is a thymocyte "comitogen"), induce myeloid differentiation into macrophages, induce acute-phase protein synthesis of hepatocytes, and exert actions on keratinocytes, mesangial cells, hematopoietic stem cells, the development of osteoclasts, and neural differentiation of PC12 cells.[250] IL-6 is also a mediator of amplified lymphocyte trafficking during febrile inflammatory responses, which is dependent on IL-6-mediated activation of L-selectin.[259] IL-6 binds to an 80 kDa IL-6 binding protein, denoted IL-6Rα, which has a comparatively short 82 amino acid long cytoplasmic domain.[257,260] This IL-6-IL-6Rα complex then interacts with and recruits the 130 kDa signal transducing molecule, gp130, which together with IL-6Rα can form a functional IL-6 receptor.[250] From a structural perspective, gp130 contains a total of six fibronectin type III modules, with the four conserved cysteine residues and the WSXWS motif being located in the second and third of these modules, starting from the N-terminus. As such, these regions are topologically positioned at greater distance external to the cell membrane than is the case for the other type I cytokine receptors discussed previously.

The IL-6 system illustrates a novel twist related to the properties of their principal binding proteins: The cytoplasmic domain of IL-6Rα is superfluous for signaling; a soluble form of the IL-6Rα extracellular domain is in fact sufficient for ligand binding and coordination with gp130, a process termed *IL-6 transsignaling*.[261] Thus, in the presence of soluble IL-6Rα and IL-6, many cell types that express gp130 but not IL-6Rα are capable of signaling in response to IL-6. It was observed that IL-6 signaling requires the dimerization of gp130 and that the overall complex containing two molecules each of IL-6, IL-6Rα, and gp130 (a dimer of a trimer or a hexamer),[32,262,263,264,265] providing a possible paradigm for the stoichiometry of subunits for other members of the IL-6 family of cytokines.[24]

IL-11 was identified as a factor produced by a stromal cell line in response to stimulation with IL-1.[266,267] It has a number of effects on hematopoiesis, particularly in combination with IL-3 and SCF. Because IL-11 exhibited "IL-6-like activities," a complementary deoxyribonucleic acid (DNA) was isolated based on the presence of IL-6-like activity in the presence of antibodies to IL-6.[268] Other actions of IL-11 include the ability to stimulate the proliferation of lymphoid and hematopoietic progenitor cells, stimulate megakaryocytic progenitors and megakaryocyte maturation, and stimulate erythroid progenitors (an action not shared by IL-6).[266,267] Overall, it acts on T cells, B cells, hematopoietic cells, epithelium, endothelial cells, and osteoclasts.[269] Like IL-6, IL-11 induces acute-phase proteins and augments antigen-specific B-cell responses, but it does not stimulate human myeloma cells.[266,267,268,270] Subsequently, adipogenesis inhibitory factor was cloned and found to be identical to IL-11,[271] revealing another action of IL-11. IL-11 is also produced by lung eosinophils and various structural cells in the lung and is expressed in patients with modest-to-severe asthma.[272] IL-11 signals via a receptor complex containing both IL-11Rα[273] and gp130.[244] Interestingly, IL-11Rα messenger RNA can be alternatively spliced to yield a form lacking the cytoplasmic domain, and like IL-6Rα, a soluble form of IL-11Rα can coordinate with IL-11 to transsignal in cells expressing gp130.[274] Studies on the stoichiometry of the IL-11 receptor complex failed to reveal dimerization of gp130 to itself or LIFRβ. Thus, assuming that it forms a hexameric receptor complex, only five of the members are known: two molecules of IL-11 and IL-11Rα, respectively, and one of gp130, suggesting that another

component may still be found.[275] IL-11 and STAT3 are expressed in gastrointestinal cancers associated with inflammation, suggesting a possible link to cancer.[269]

LIF is another multifunctional cytokine originally cloned based on the activity associated with its name.[276] LIF can suppress the differentiation of pluripotent embryonic stem cells, inhibit adipogenesis (like IL-11), and induce monocyte differentiation of the M1 murine leukemia cell line, thus mimicking a number of the actions of IL-6.[277] In addition, it exerts a number of actions in the central nervous system, and LIF was shown to be identical to cholinergic neural differentiation factor.[278] and can induce acetylcholine synthesis while simultaneously suppressing catecholamine production, thereby inducing cholinergic function while suppressing noradrenergic function.[278] LIF has been shown to be essential for embryo implantation.[279] LIF binds to a receptor (LIFRβ) that is structurally related to gp130,[280] but the functional LIF receptor (LIFR) receptor requires the heterodimerization of LIFRβ and gp130 as well.[246] Interestingly, whereas IL-6 promotes Th17 differentiation, this is inhibited by LIF, apparently based on LIF's ability to activate ERKs and to augment the expression of SOCS-3, which can inhibit STAT3 activation.[281] Interestingly, LIF can also promote the differentiation of T_{reg} cells and may exhibit therapeutic potential in multiple sclerosis.[282] LIF maintains mouse embryonic stem cells, but LIF is not capable of maintaining human embryonic stem cells or mouse epiblast stem cells in the pluripotent state.[283]

CNTF was discovered based on its ability to promote neuronal survival.[284,285] CNTF signals through a receptor comprising LIFRβ and gp130 but requires a specific binding protein,[286,287] now denoted CNTF receptor (CNTFR)α. Interestingly, CNTFRα lacks transmembrane and cytoplasmic domains and instead is a GPI-linked receptor molecule. CNTFRα appears to provide a receptor-cytokine surface with which gp130 and LIFRβ can interact. Thus, CNTF is like IL-6 in that each requires initial binding to a receptor component (CNTFRα or IL-6Rα) that does not require its own cytoplasmic domain for signaling. Whereas IL-6 signaling involves homodimerization of gp130, CNTF signaling involves the heterodimerization of LIFRβ and gp130. In fact, the functional CNTFR appears to be a hexameric structure containing two molecules of CNTF, two of CNTFRα, and one each of gp130 and LIFRβ.[288] The receptor is expressed largely within the nervous system and in skeletal muscle, accounting for largely restricted actions of CNTF.[286]

OSM is a growth regulator that was originally identified based on its ability to inhibit the growth of A375 human melanoma cells.[289,290] OSM is a potent growth factor for Kaposi sarcoma in patients with acquired immunodeficiency syndrome.[291,292] Not only OSM can bind directly to gp130 and signals through a receptor combination of gp130 and LIFRβ[246] but also has an alternative receptor comprising a specific OSM receptor subunit (OSMRβ) and gp130.[293] These are now known as the type I and type II OSM receptors (OSMRs), respectively. OSM can enhance the development of both endothelial cells and hematopoietic cells, possibly by increasing hamangioblasts, a common precursor for endothelial and hematopoietic cells.[251]

CT-1 was initially isolated based on its actions on cardiac muscle cells.[294] However, it is now clear that it is a multifunctional cytokine with hematopoietic, neuronal, and developmental effects, in addition to its effects on cardiac development and hypertrophy.[295,296] Like OSM and LIF, CT-1 can also signal through a heterodimer of LIFRβ and gp130.[249] Interestingly, the CT-1 receptor on motor neurons may involve a third receptor

component, possibly GPI linked.[297,298]

NNT-1/BSF-3, like CNTF, can also support the survival of chicken embryonic sympathetic and motor neurons.[253] Interestingly, in mice, NNT-1/BSF-3 can augment the effects of IL-1 and IL-6 and is a B-cell-stimulating factor (hence the term BSF-3). The NNT-1 receptor contains LIFRβ and gp130.[253] NNT-1 is also known as CLC, and this forms a complex with a soluble receptor protein known as cytokine-like factor-1. Together, this complex is a second ligand for CNTFR.[299] It appears that CLC can also interact directly with soluble CNTFR to form a related cytokine.[254]

IL-27 is an IL-6-related cytokine[300] that represents a dimer of the p28 protein (also known as IL-30) and EB13 (Epstein-Barr virus-induced gene 3) (also known as IL-27B), which can induce proliferation of naive CD4+ T cells. p28 can exist by itself independently of EB13 and is capable of antagonizing gp130-mediated signaling by IL-6, and indeed, overexpression of p28 in transgenic mice prevents normal development of germinal centers and antibody production.[301] Together with IL-12, IL-23, CLC/cytokine-like factor-1, and CLC/soluble CNTFR, this is one of five cytokines that represent dimers including a type I cytokine and a soluble receptor-like protein. IL-27 signals via gp130 and the WSX-1/TCCR receptor,[300] which is discussed in the section on diseases of cytokine receptors and related molecules. IL-27 has proinflammatory and anti-inflammatory effects. Although it was suggested to promote Th1 responses, it is clear that it can also antagonize such responses, and IL-27 can promote effector responses of both CD4+ and CD8+ T cells, of NK cells, and it additionally stimulates mast cells. The anti-inflammatory effects of IL-27 are indicated by the absence of WSX-1 resulting in increased mast cell and macrophage responses.[302,303]

Thus, eight cytokines (IL-6, IL-11, LIF, CNTF, OSM, CT-1, NNT-1/BSF-3, and IL-27) all have receptors that are dependent on gp130.[304] These can be divided into two sets of cytokines: those known to not require LIFRβ (IL-6, IL-11, and IL-27) and those that use both gp130 and LIFRβ (LIF, CT-1, OSM, CNTF, and NNT-1/BSF-3) (Table 25.7), with OSM having two forms of receptors, each of which contains gp130 but only one of which contains LIFRβ. When cytokines share essentially the same receptor, one can hypothesize that two cytokines might exert identical actions on cells that can respond to both cytokines. It is clear that the presence of IL-6Rα, IL-11Rα, and CNTFRα (either on the cell surface or as a soluble receptor form, discussed subsequently) determines whether a cell can respond to IL-6, IL-11, and CNTF. This raises the interesting question as to whether functional homologues of these proteins will also exist for LIF, OSM, CT-1, NNT-1/BSF-3, and IL-27.

TABLE 25.7 Composition of Receptors for the Interleukin-6 Family of Cytokines

Cytokines whose receptors do not contain LIFRβ	
IL-6	IL-6Rα + gp130
IL-11	IL-11Rα + gp130
OSM	OSMRβ + gp130
Cytokines whose receptors contain LIFRβ	
LIF	LIFRβ + gp130

OSM	LIFRβ + gp130
CNTF	CNTFRα + LIFRβ + gp130
CT-1	LIFRβ + gp130 + ?CT1Rα
NNT-1/BSF-3 /CLC + CLF-1	CNTFRα + LIFRβ + gp130
CLC + soluble CNTFRα	LIFRβ + gp130
Cytokines whose receptors contain OSMRβ	
OSM	OSMRβ + gp130
IL-31	IL-31R + OSMRβ

BSF-3, B cell-stimulating factor-3; CLC, cardiotrophin-like factor; CLF-1, cytokinelike factor-1; CNTF, ciliary neurotrophic factor; CNTFR, CNTF receptor; CT-1, cardiotrophin-1; IL, interleukin; LIF, leukemia inhibitory factor; NNT-1, novel neurotrophin-1; OSM, oncostatin M.

Note that the sharing of CNTFRα by CNTF and the dimeric NNT-1/BSF-3/CLC - CLF-1 ligand helps to explain why the phenotype in CNTF-/- mice is less severe than that found in CNTFRα-/- mice.

Significance of the Sharing of Receptor Chains

Interestingly, γ_C, β_C, and gp130 all contribute to signaling, but none of these shared cytokine receptor proteins has primary binding activity for any known cytokine. Instead, they each increase binding affinity in the context of the primary binding protein for each cytokine. Consequently, the capacity of a cell to respond to a given cytokine is determined by the unique binding chain(s), but signaling pathways can be shared.

Other Receptors with Similarities to gp130 (Granulocyte-Colony Stimulating Factor Receptor, OB-R, Interleukin-12Rβ1, Interleukin-12Rβ2, and Interleukin-31R)

As noted previously, LIFRβ and OSMRβ bear some similarities to gp130.[293] In addition, the G-CSF receptor, the leptin receptor (also denoted OB-R, for obesity receptor), IL-12/IL-23 receptor components, and IL-31R all resemble gp130. The amino acid identity among these different receptors, compared pairwise, ranges from 18% to 32%, with LIFRβ and OSMRβ being the most similar. Although G-CSF is granulocyte-colony stimulating factor, it may have

a broader role. For example, G-CSF has been shown to stimulate myoblast proliferation and thereby to affect skeletal muscle formation,[305] indicating broad pleiotropic actions by this cytokine.

Leptin

Leptin is the product of the obesity (ob) gene, an adipose tissue-derived cytokine that plays a

role in body weight homeostasis.[306,307,308] Additionally, leptin affects thymic homeostasis, increasing thymocyte number and having antiapoptotic effects in the thymus and periphery. It is proinflammatory, promoting Th1 differentiation, and augmenting the production of TNF-α, IL-1, and IL-6 by monocytes/macrophages.[308] The leptin receptor, OB-R, was cloned and found to be most closely related to the gp130 signal transducer, G-CSF receptor, and LIFRβ .[309] Interestingly, this receptor is encoded by the "diabetes gene," which is mutated in db/db mice.[310]

Interleukin-12, IL-23, and IL-35

As noted previously, IL-12 is the major inducer of Th1 cells. IL-12 is primarily produced by phagocytic cells in response to bacterial and intracellular parasites, such as *Toxoplasma gondii*, but it is also produced by other antigen-presenting cells, such as B cells.[311] IL-12 potently induces the production of IFNγ by NK cells and T cells and is also a growth factor for preactivated but not resting NK and T cells. IL-12 was originally discovered as NK cell stimulatory factor.[312] IL-12 can also induce the production of IL-2, IL-3, GM-CSF, IL-9, TNF-α, and M-CSF, although inducing IFNγ is perhaps its most important known action.[311,313,314] As is discussed in the section on immunodeficiency diseases, IL-12 is essential for the proper clearing of mycobacterial infections. It is interesting that Th2 cells do not respond to IL-12; the lack of responsiveness of these cells to IL-12 results from their loss of expression of the IL-12Rβ2 subunit of the IL-12 receptor.[315] Apparently, IL-4 inhibits IL-12Rβ2 expression, whereas IL-2[74] and IFNγ[315] induce its expression. The abilities of mice to survive infections is critically linked to the Th patterns of cytokines. For example, the ability to survive *T. gondii* infection is dependent on IFNγ/IL-12 production (a Th1 pattern).[83]

IL-12 can be thought of as having vital roles in both innate immunity and acquired immune responses. It is rapidly produced by NK cells and then T cells in response to antigens or foreign pathogens. This rapid response facilitates the activation of first-line defense against infections. In addition, however, IL-12 is also required for the subsequent differentiation of specialized T-cell populations, including its STAT4-dependent priming of Th1 cells for optimal production of IFNγ and IL-2. IL-12 can act synergistically with hematopoietic growth factors, such as IL-3 and SCF, to support the proliferation and survival of hematpoietic stem cells.[311] Structurally, IL-12 is a covalently linked dimer of 35 (IL-12A) and 40 (IL-12B) kDa peptides[311]; thus, successful production of IL-12 requires that a cell transcribe both the p35 and p40 genes.[316] Interestingly, whereas p35 bears sequence similarity to IL-6 and G-CSF, p40 is homologous to the extracellular domains of IL-6Rα, CNTFRα, and G-CSF receptor and bears some of the features typical of type I receptors, including four conserved cysteines, a conserved tryptophan, and a WSEWAS motif, which has similarity to the typical WSXWS motif.[311] Moreover, as both IL-12 receptor (IL-12Rβ1 and IL-12Rβ2) chains bear some similarity to gp130,[312,316,317] one can think of p40 as a functional homologue of the soluble p80 IL-6Rα chain. Thus, for this cytokine, part of the "receptor" has become part of the cytokine. Interestingly, all cells that produce IL-12 synthesize much more p40 than p35, suggesting that the careful control of signaling is at the level of the "primordial" p35 cytokine part of IL-12. p40 is on human chromosome 5q31 to 5q33 while p35 is on 3p12 tp 3p13.2.[311] As noted previously, it is interesting that IL-2 induces expression of IL-12Rβ1 and IL-12Rβ2.

IL-23 is similar to IL-12 in that it also contains p40.[318] However, rather than also containing p35, IL-23 is a dimer of a p40 and p19 (IL-23A). IL-23 signals via a receptor that contains IL-12Rβ1 but not IL-12Rβ2.[319] Instead, another receptor chain, denoted IL-23R, is the second component of the IL-23R[320]; both IL-12Rβ2 and IL-23R are located on chromosome 1 within

150 kb of each other.[320] Both IL-12 and IL-23 activate JAK2 and TYK2. IL-12 and IL-23 can activate STAT1, STAT3, STAT4, and STAT5. STAT4 is the dominant STAT protein activated by IL-12.[320] Interestingly, as compared to human IL-23R, mouse IL-23R contains a 20 amino acid duplicated region that spans the WQPWS motif. IL-23 appears to play a vital role in the maintenance, rather than in the initial differentiation of Th17 cells (IL-17-producing cells), and IL-23 is essential for resistance to certain diseases, such as EAE, which were originally believed to be Th1-mediated diseases, as elimination of IL-23 by eliminating either the p40 subunit shared by IL-12 and IL-23 or by eliminating p19 confers resistance to EAE.[175,321] Whereas IL-23 promotes Th17 cell numbers, IL-27 can negatively regulate their development.[321] In the CD40-mediated model of inflammatory bowel disease, IL-12 p35 secretion controlled wasting disease and serum cytokine production but did not affect mucosal pathology, which instead was associated with IL-23/p19.[322] Interestingly, IL-23R has been identified as an inflammatory bowel disease gene,[323] and IL-23 can drive intestinal inflammation via direct actions on T cells.[324] Separate from Th17 cells, a population of innate lymphoid cells that produce IL-17, IL-22, and IFNγ in response to IL-23 have been identified as the mediators of inflammatory bowel disease.[325] IL-23 and its receptor are nevertheless critical for the terminal differentiation of Th17 cells.[169,326] IL-27 is a related cytokine (discussed previously), but it can act as an inhibitor of Th1 responses associated with intracellular infections such as *T. gondii*. Whereas host defense to *T. gondii* is dependent on IL-12 and IFNγ, IL-27-deficient mice control the parasite replication but develop a lethal inflammatory disease that is dependent on CD4+ T cells. Additionally, IL-27 can inhibit Th2 actions.[303]

IL-35 is produced by T_{reg} cells and plays a role in immunosuppression. It is a heterodimer of IL-12A (p35) and IL-27B (EBI3).[327] IL-35 treatment of naive mouse or human T cells induces regulatory cells that have been called iTR35 cells[328] and promote tolerance and tumor progression.

Interleukin-31

IL-31 is a four α-helical bundle cytokine that fits into the greater IL-6 extended family in that its receptor consists of IL-31RA and the OSMRβ.[329] IL-31R is a gp130-like type 1 receptor, with four splice variants. IL-31 is produced primarily by activated T cells and when overexpressed results in pruritis, alopecia, and skin lesions, indicating a potential role in dermatitis.[329,330]

Other Examples of Shared Receptor Molecules

Interleukin-7 and Thymic Stromal Lymphopoietin Share Interleukin-7 Receptor α Chain

In addition to IL-7, a second stromal factor, TSLP has been identified that shares at least some actions with IL-7.[11,331,332,333] The TSLP receptor is a heterodimer of TSLPR and IL-7Rα.[334,335] Interestingly, TSLPR is 24% identical to γ_c, making it the cytokine receptor most like γ_c.[334] Human and mouse TSLP share only 43% amino acid identity, and human and mouse TSLP receptors share only 39% identity.[336] This is very low for human and mouse orthologues of cytokine receptors, for example, human and mouse γ_c are 70% identical.[337] In addition to their wide sequence divergence, mouse and human TSLP were originally suggested to substantially differ in their actions. Mouse TSLP was first reported to be a B-cell differentiation factor that is important for the development of IgM+ immature B cells from pre-

B cells and to also be a weak thymic comitogen. In contrast, human TSLP appeared not to exert effects on these lineages but was found to be an epithelial-derived cytokine that potently activated DCs related to Th2 allergic responses, an action not then known to be shared by mouse TSLP.[338,339,340] However, it is now clear that mouse TSLP can also activate DCs and that it plays a critical role in both atopic disease and asthma in mouse models, and that these effects of TSLP are mediated via actions on both DCs and CD4+ T cells.[341,342,343,344,345,346,347] TSLP can also promote immunity to helminth parasites,[333] and TSLP is also important for mucosal healing in the dextran sulfate sodium-induced colitis model.[348] Interestingly, human TSLP is expressed by Hassall corpuscles[349] and was suggested to promote selection of T_{reg} cells in human thymus, thus contributing to central tolerance.[350] It is striking, however, that mice lacking TSLP receptor have normal numbers of T_{reg} cells, indicating that TSLP is not absolutely essential for this function, and that other molecules can at least substitute for TSLP. Indeed, IL-7 is such a molecule.[350a] TSLP is now known to be produced by a range of cell types, including epithelial cells, fibroblasts, keratinocytes, stromal cells, basophils, mast cells, and DCs,[11] and to in turn act on multiple lineages, including DCs, CD4+ and CD8+ T cells, mast cells, NKT cells, eosinophils, and B cells.[11,333,351] Interestingly, TSLP promotes Th2 differentiation and has been shown to promote metastasis in breast cancer and pancreatic cancer.[352,353,354] From a signaling perspective, whereas IL-7 activates JAK1 and JAK3, TSLP activates JAK1 and JAK2, making it the only type 1 cytokine to use this combination of JAK kinases to activate STAT5.[347] Although IFNγ also uses JAK1 and JAK2, it instead activates STAT1.[355]

Two Types of Interleukin-4 Receptors, One of which also Responds to Interleukin-13

As detailed previously, on T cells, IL-4 acts through a receptor comprising IL-4Rα and γ_C (the type I IL-4 receptor)[38,39]; however, IL-4 can also signal through type II IL-4 receptors comprising IL-4Rα and IL-13Rα1 but not γ_C.[356] IL-13 is another cytokine that shares some actions with IL-4 and was originally described as a T-cell-derived cytokine capable of inhibiting inflammation,[357] although it is now clear that other cells such as NK cells and mast cells can also produce IL-13. IL-13 can induce identical signals to IL-4 on non-T cells that respond to IL-4 but lacks effects on mature T cells, as these cells do not bind IL-13.[358,359] The shared actions of IL-4 and IL-13 include the ability to 1) decrease expression of inflammatory cytokines, 2) induce major histocompatibility complex class II expression, 3) induce CD23 expression and IgE production by B cells in humans, 4) inhibit IL-2-induced proliferation of chronic lymphocytic leukemia cells of B-cell origin, and 5) costimulate with anti-CD40 antibodies. This is explained by the observation that the type II IL-4 receptor consists of IL-4Rα plus IL-13Rα1,[360,361] with both IL-4 and IL-13 inducing indistinguishable signals on cells expressing this receptor. Interestingly, IL-4 binds primarily to IL-4Rα and IL-13 binds primarily to IL-13Rα1. This situation may be analogous to the situation for LIF, CT-1, and OSM, which can all act through receptors containing LIFRβ and gp130 but differ in their abilities to directly interact with each of these receptor proteins. An additional IL-13 binding protein, IL-13Rα2, has much higher binding affinity for IL-13 than does IL-13Rα1.[362] IL-13Rα2 is nonfunctional in terms of signaling and instead acts as a “decoy” receptor.

Although IL-13 was originally believed to be substantially redundant with IL-4, it has important distinctive actions as well. IL-13 may be vital in asthma, as blocking IL-13 can inhibit pathophysiologic changes of asthma.[363,364] It also is clear that IL-13 regulates eosinophilic infiltration, airway hyperresponsiveness, and mucus secretion, and that it is a potent mediator

of fibrosis.[357] Moreover, IL-13 that is produced by innate natural helper cells in the lung contributes to asthma.[365] The phenotype of IL-13 knockout mice suggested a role for IL-13 the ability to expel helminths,[366] and it is clear that IL-13 specifically can modulate resistance to intracellular organisms, including *Leishmania major* and *Listeria monocytogenes*.[357] IL-13 has also been identified as a factor that is secreted by and can stimulate the growth of Hodgkin and Reed-Sternberg cells.[367]

Although IL-4 and IL-13 are both critical for host defense to helminths, the use of reporter mice revealed that Th2 cells produce both cytokines. Tfh cells in lymph nodes as well as basophils make IL-4, whereas innate helper type 2 cells secrete IL-13. Thus, although both are produced during helminth infection, there are distinctive sites of production, correlating with distinctive roles.[368]

An Example of Multiple Affinities of Binding for a Single Cytokine: Three Classes of Interleukin-2 Receptors

Although cytokines typically signal via a single class of highaffinity cell surface receptor, more complex situations exist.

The IL-2 system provides the very interesting illustration of a system with three classes of affinities of receptors (Table 25.8). In addition to the high-affinity receptor (IL-2Rα + IL-2Rβ + γ_c, $K_d \approx 10^{-11}$ M), there are both low-affinity (IL-2Rα alone, $K_d \approx 10^{-8}$ M) and intermediate-affinity (IL-2Rβ + γ_c, $K_d \approx 10^{-9}$ M) receptors.[29,50] Low- and high-affinity receptors are expressed on activated lymphocytes, whereas intermediate-affinity receptors are found on resting lymphocytes, particularly on NK cells. Both intermediate- and high-affinity receptors can signal, suggesting that IL-2Rβ and γ_c are necessary and sufficient for signaling, in keeping with the theme of dimerization indicated previously. Given that the intermediate-affinity form is functional, what then is the rationale for having a high-affinity IL-2 receptor that also contains IL-2Rα? This is a particularly important question in view of the fact IL-2Rα has an extremely short cytoplasmic domain that does not appear to play a role in signaling. The importance of IL-2Rα is clearly demonstrated by the severely abnormal phenotype of IL-2Rα-deficient mice, which exhibit autoimmunity, inflammatory bowel disease, and premature death,[217] and by the observation that IL-2Rα mutations can cause an autoimmune syndrome in humans as well.[369] A clue to the importance of IL-2Rα comes from the kinetics of association of IL-2 with each chain. Although the IL-2Rα appears to lack a direct signaling function, it has a very fast on-rate for IL-2 binding.[370] Thus, the combination of this rapid onrate with the slow off-rate from IL-2Rβ/γ_c dimers results in high-affinity binding that is vital for responding to the very low concentrations of IL-2 that are physiologically present in vivo. Moreover, as activated T cells express approximately 10 times as many low-affinity than high-affinity receptors, IL-2Rα may serve as an efficient means of recruitment and concentration of IL-2 on the cell surface, allowing more efficient formation of IL-2/IL-2Rβ/γ_c signaling complexes.

TABLE 25.8 Classes of Interleukin-2 Receptors

Affinity	K_d	Where Expressed	Composition	Functional
	10^{-8}			

Low	10^{-8} M	Activated cells	IL-2Rα	No
Intermediate	10^{-9} M	Resting cells	IL-2Rβ and γ_C	Yes
High	10^{-11} M	Activated cells	IL-2Rα, IL-2Rβ, and γ_C	Yes

IL, interleukin

As noted previously, IL-2Rα is not a type I cytokine receptor and in fact has homology to the recognition domain of complement factor B.[371] Subsequently, the IL-15 receptor α chain was shown to also have a similar structure to IL-2Rα,[155] with both IL-2Rα and IL-15Rα having what are called "sushi" domains, which contribute to ligand binding. The fact that IL-2 and IL-15 both have related α chains is consistent with the close relationship between IL-2 and IL-15, and that the receptors for both IL-2 and IL-15 contain both IL-2Rβ and γ_C.

As IL-2Rα cannot transduce a signal by itself, the detection of IL-2Rα on the cell surface does not necessarily reflect IL-2 responsiveness. Because IL-2Rα was discovered before IL-2Rβ and γ_C, many papers in the literature have evaluated IL-2 receptor expression based on IL-2Rα expression alone, and this may not reflect IL-2 responsiveness. IL-2Rα is also expressed on a subpopulation of double negative thymocytes, but it remains unclear if IL-2 physiologically acts on these cells. Each of the components of the IL-2 receptor is located on a different chromosome: Human IL-2Rα is located on chromosome 10p14 to 10p15,[372] IL-2Rβ is at 22q,[373,374] and γ_C is at Xq13.1,[197] while the mouse homologues are located at chromosomes 2, 15, and X.

Erythropoietin, Thrombopoietin, and Stem Cell Factor

Epo was the first cytokine that was biochemically purified and is vital for erythropoiesis, whereas thrombopoietin is critical for thrombopoiesis. These cytokines each bind to receptors that are homodimers.[21,375] Interestingly, Epo signaling may depend in part on the functional cooperation of the Epo receptor and c-kit, the receptor for SCF.[376] This latter receptor has intrinsic tyrosine kinase activity and is not a type I cytokine receptor. Epo was the first cytokine with demonstrated clinical efficacy, for example, in renal insufficiency. Although it is dominantly associated with erythrocyte production, Epo receptors have been found more broadly on immune cells. Recently, Epo has been shown to have effects on a number of cell types, blocking NF-κB p65 and resulting in inhibition of the induction of TNF-α and inducible nitric oxide synthase in activated macrophages, with diminished control of *Salmonella* infection, whereas in a model of chemical-induced colitis, such inhibition of NF-κB-dependent immune modulators limits tissue damage and disease severity.[377] It will therefore be important to determine the full scope of immunologic actions that are mediated by Epo.

CYTOKINE AND CYTOKINE RECEPTOR PLEIOTROPY AND REDUNDANCY

It is well recognized that many cytokines exhibit the phenomena of cytokine "pleiotropy" and "redundancy."[304] Cytokine pleiotropy refers to the ability of a cytokine to exert many different types of responses, often on different cell types, whereas cytokine redundancy refers to the fact that many different cytokines can induce similar actions. One set of cytokines that exhibit cytokine pleiotropy is the γ_C family of cytokines. For example, IL-2 can induce T-cell growth,

augment B-cell Ig synthesis, increase the cytolytic activity of lymphokineactivated killer and NK cells, and play an essential role in mediating activation-induced cell death; IL-4 can induce B-cell growth and Ig class switch; and IL-7 not only plays a major role in thymocyte development but also can stimulate mature T cells, and at least in the mouse, can act as a pre-B-cell growth factor. The gp130 family cytokines also exhibit broad actions. For example, IL-6 exerts effects ranging from that of

a comitogen for thymocyte activation to that of a mediator of the acute-phase response in liver. Regarding cytokine redundancy, it has already been highlighted that IL-2, IL-4, IL-7, and IL-15 receptors contain γ_C can act as T-cell growth factors, and that IL-3 has actions that overlap with IL-5 and GM-CSF.

The recognition that cytokines not only have overlapping actions but also share receptor components, led to the concepts of "cytokine receptor pleiotropy" and "cytokine receptor redundancy."[304] The first of these terms can be defined by the ability of a single cytokine receptor subunit to function in more than one receptor. Thus, examples include the sharing of γ_C, β_C, gp130, IL-4Rα, LIFRβ, and OSMRβ, as summarized previously, as well as the sharing of IL-2Rβ by IL-2 and IL-15 receptors, the sharing of IL-4Rα and IL-13Rα in type II IL-4 receptors and IL-13 receptors, and the sharing of IL-7Rα by the IL-7 and TSLP receptors. Another way of viewing receptor pleiotropy is that certain receptor chains are useful "modules" that function in more than one context.

The final term, cytokine receptor subunit redundancy, is the one with fewest examples. There is one well-documented example in mice but not in humans. IL-3 signals through IL-3Rα plus either β_C or an alternative unique IL-3Rβ that shares 91% amino acid identity with β_C and appears to be a completely functionally redundant protein for IL-3 signaling, but IL-3Rβ cannot substitute for β_C in the context of IL-5 or GM-CSF signaling.[239] Other potential examples exist. For example, in type I and type II IL-4 receptors, IL-4Rα coordinates with either γ_C or IL-13Rα1, respectively, and there are two types of OSM receptors, both of which contain gp130, but one of which contains a specific OSM receptor while the other contains LIFRβ. What remains unknown, however, is whether the signals mediated by these different types of receptors are truly identical so that there is redundancy, or whether there are distinctive features to the signals that IL-4 and OSM induce via the different receptors. Indeed, mice lacking IL-13Rα1 have exacerbated Th2 responses and is essential for allergen-induced airway hyperreactivity, suggesting that type 2 IL-4 receptor signaling (either via IL-4 or IL-13) is not identical to that mediated by the type 1 IL-4 receptor (via IL-4).[378]

In addition to these examples related to type I cytokines, the IL-10 subfamily of type II cytokines is interesting in that IL-10Rβ (IL-10R1), IL-20Rα (IL-10R1), IL-20Rβ (IL-20R2), and IL-22Rα (IL-22R) are each shared receptor components, collectively affecting signaling in response to IL-10, IL-19, IL-20, IL-22, and IL-24.[304,379] Specifically, IL-10 signals via a receptor containing IL-10R1 and IL-10R2, IL-19 signals via a receptor containing IL-20R1 and IL-20R2, IL-20 signals via receptors containing IL-20R2 and either IL-20R1 or IL-22R, IL-22 signals via receptors containing IL-22R and IL-10R2, and IL-24 signals through receptors containing IL-20R2 plus either IL-20R1 or IL-22R. Two other IL-10 family members, IL-28 and IL-29, both signal via IL-28R + IL-10R2.[380] Interestingly, IL-10 is believed to be a dimer; IL-26 may also be a dimer, but the other family members are monomers.[379]

SOLUBLE RECEPTORS

Soluble forms of many cytokine receptors have been identified, including those for IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-13Rα2, GM-CSF, type I and type II IFNs, IL-22, and TNF.[381,382,383] As is clear from this list of cytokines, soluble receptors are not restricted to receptors for type

I cytokines, and in the case of IL-2, the principal soluble receptor protein is IL-2Rα, which is not a type I cytokine receptor. Soluble receptors can be created by alternative splicing that truncates the protein N-terminal to the transmembrane domain, resulting in a secreted protein rather than a membrane-anchored membrane in the case of IL-4Rα, IL-5Rα, IL-6Rα, IL-7Rα, IFNAR-2, and GM-CSFRα. Alternatively, they can be created by proteolytic cleavage of the membrane receptor as is found for the receptors for IL-2Rα and TNFRI and TNFRII[382] (Table 25.9). Although it is conceivable that a distinct gene might encode the soluble forms of a receptor, no examples have been reported. In the cases where proteolytic cleavage occurs, the identity of the proteases has not been identified. The major questions related to these soluble receptors are as follows: 1) Do they have physiologic or pathophysiologic functions? 2) How do their affinities compare to the corresponding cell surface receptor? 3) Do they have diagnostic, prognostic, and therapeutic applications?

Unfortunately, there is little information available on the in vivo role of soluble receptors. In general, when analyzed using in vitro studies, soluble receptors can compete with their corresponding cell surface receptors, thereby serving negative regulatory roles. For example, this appears to be the case for the IL-22 soluble receptor. However, soluble IL-6Rα exerts an agonistic role because, as summarized previously, IL-6 signaling occurs equally well via gp130 when the soluble rather than transmembrane form of IL-6Rα interacts with IL-6. Nevertheless, a mutated form of IL-6Rα that cannot interact with gp130 but still binds IL-6 can effectively inhibit the actions of IL-6.[384] In the case of IL-2Rα, there is no reported physiologic function for soluble IL-2Rα, as the affinity of the released receptor is, as expected, similar to that of the low-affinity receptor ($K_d \approx 10^{-8}$ M), making it unlikely to effectively compete with the high-affinity cell surface receptor ($K_d \approx 10^{-11}$ M). However, this and other soluble receptors could serve as cytokine carrier proteins and potentially could increase stability of a cytokine by protecting it from proteolysis.[382] This theoretically could be similar to the role of IL-2/anti-IL-2 conjugates in exhibiting higher activity.[385] Moreover, there are potential diagnostic and prognostic uses for measuring the level of shed receptors (Table 25.10).

TABLE 25.9 Soluble Cytokine Receptors

Generated by Alternative Splicing	Generated by Proteolytic Cleavage of Mature Receptor
sIL-4Rα	sIL-2Rα
sIL-5Rα	sTNFR
sIL-6Rα	
sIL-7Rα	
sGM-CSFRα	
sIFNAR-2	

GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; TNFR, tumor necrosis factor receptor.

TABLE 25.10 Soluble Interleukin-2 Receptors in Human Disease

Malignancies
Hematologic
Adult T-cell leukemia
Hairy cell leukemia
Acute lymphocytic leukemia
Chronic lymphocytic leukemia (B cell)
Acute myelogenous leukemia
Chronic myelogenous leukemia (especially in blast crisis)
Malignant lymphomas
Hodgkin disease
Non-Hodgkin lymphomas
Nonhematologic
Adenocarcinoma of lung, breast, pancreas
Small cell bronchogenic carcinoma
Ovarian, cervical, and endometrial cancers
Nasopharyngeal carcinoma
Melanoma
Infections
Human immunodeficiency virus
Tuberculosis
Rubeola

Infectious mononucleosis
Other diseases
End-stage renal disease
Rheumatoid arthritis
Systemic lupus erythematosus
Scleroderma
Sarcoidosis
After transplantation
After interleukin-2 administration

In adults, the mean sIL-2Rα levels are 280 ± 161 units/mL (levels tend to be higher in pediatric populations). The situations where the levels exceed 5000 units/mL are adult T-cell leukemia, hairy cell leukemia, chronic myelogenous leukemia, and after interleukin-2 administration. The situations where levels are between 1000 and 5000 units/mL include acute myelogenous leukemia, chronic lymphocytic leukemia, non-Hodgkin lymphomas, acquired immunodeficiency syndrome associated with Kaposi sarcoma, tuberculosis, rubeola, and end-stage renal disease. Data are from Kurman et al.381 and Fernandex-Botran et al.382

INTERFERONS (TYPE II CYTOKINES) AND THEIR RECEPTORS

IFNs represent an evolutionarily conserved family (Table 25.11) of cytokines that are related to the IL-10 family of type II cytokines. IFNs were discovered in 1957 on the basis of their antiviral activity and were the first cytokines that were discovered.[386] IFNs are known as either type I or type II IFNs,[387,388,389,390,391,392,393,394,395,396] where type I interferons include IFNα (originally known as leukocyte IFN) and IFNβ (originally known as fibroblast IFN), and IFNε, IFNκ, IFNω, IFNδ, and IFNτ. IFNδ and IFNτ are absent in humans. IFNω is closely related to the IFNαs and was formerly designated as an IFNα. There are multiple (at least 12) IFNαs in mice and humans. In contrast, the other type I IFNs, IFNβ and IFNω, are each encoded by single human and mouse genes near the IFNα cluster, and in addition, there are multiple pseudogenes most closely related to IFNα and IFNω. The type I IFNs are clustered on human chromosome 9 and mouse chromosome 4. Type II interferon is IFNγ,[314] which is encoded by a single gene on human chromosome 12 and mouse chromosome 10. IFNα/β is produced after viral infection in many cell types, although plasmacytoid DCs appear to be the largest producers of IFNα/β. These type I IFNs inhibit viral replication and induce the apoptosis of virally infected cells. In addition, IFNα/β mediate the activation of macrophages and NK cells, and they additionally affect the proliferation and survival of CD8+ T cells.

TABLE 25.11 Type II Cytokines

		Chromosomal Location (h/m)
Type I IFNs		
IFNα	Many genes	9p22/4
IFNβ	Single gene	9p21/4
IFNω	Single gene	9p21/4
IFNτ	Many genes	
Type II IFN		
IFNγ	Single gene	12q14/10
IL-10 family cytokines		
IL-10	Single gene	1q31-32/1
IL-19	Single gene	1q32/1
IL-20	Single gene	1q32/1
IL-22	Single gene	12q14-15/10
IL-24	Single gene	1q32/1
IL-26	Single gene	12q14-15/
IL-28	Single gene	19q13/7
IL-29	Single gene	19q13

IFN, interferon; IL, interleukin.

The grouping of the IFNαs and IFNβ together as type I IFNs is logical not only because of the similar amino acid sequences and structures of these IFNs but also based on the fact that they share the same receptor and induce essentially the same signals.[397,398] Although DNA array analysis does show some differences in the genes induced by IFNα and IFNβ, the basis for these differences are unclear.[399] These signals include not only antiproliferative and antiviral activities but also the ability to stimulate cytolytic activity in lymphoyctes, NK cells, and macrophages. In contrast, IFNγ has a distinct receptor. Type I and type II IFN receptors share a sufficient degree of similarity to each other so as to form a family.[400] The structure of the IFNγ receptor[401] is shown in Figure 25.2. IFN receptors are referred to as type II cytokine receptors, and there are substantial differences between these receptors and the type I cytokine receptors.[8] Because both type I and type II IFNs bind to type II cytokine receptors,

IFNs are occasionally referred to as type II cytokines but more generally are referred to as IFNs. Based on the similarity of the

IL-10 receptor to the IFNγ receptors,[8] IL-10 was designated as a type II cytokine, and indeed when its x-ray crystal was determined, IL-10 was found to be topologically related to IFNγ.[16] As noted previously, more recently a series of IL-10-related cytokines has been identified, including IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B, and IL-29, and these are also designated as type II cytokines, with IL-28A, IL28B, and IL-29 also known as IFNλs. Among type II cytokines, IFNγ has helices similar to those of the type I short-chain helical cytokines, but its short helices that occupy the AB and CD loops positions exhibit the long-chain cytokine-like AB over CD topology. IL-10 and IFNαβ have long-chain structures.[8] Thus, the theme of short-chain and long-chain type I cytokines also extends to the IFNs and the IL-10 family of type II cytokines.

Type I IFNs signal through a receptor known as the type I IFN receptor.[388,392,394,396,397] The receptor consists of at least two chains.[402,403,404,405] In contrast to the α and β chain nomenclature typical for type I cytokine receptors, IFN receptor chains are officially denoted as IFNAR-1 (previously also denoted IFN-αR1, IFNAR1, and IFN-Rα) and IFNAR-2 (previously also known as IFNα/β receptor, IFNαR2, IFNAR2, and IFNRβ).[406] IFNAR-2 has both short and long forms as well as a soluble form.[407] The long form has a much larger cytoplasmic domain and serves a more important role in signal transduction. Whereas IFNAR-1 cannot bind IFNα, IFNAR-2 binds with low affinity, and the combination of both chains results in high-affinity binding[407] and function. As detailed in the following, IFNAR-1 binds the Janus family tyrosine kinase TYK2, whereas IFNAR-2 binds JAK1. In addition to these cellular receptors, it is interesting that vaccinia virus and other orthopoxviruses encode a soluble form of type I IFN receptor that is related to the IL-1 receptors and that is capable of binding IFNα, IFNβ, and IFNω.[408,409] This form of IFN receptor is therefore not a member of the type II cytokine family but instead is an Ig superfamily protein. IFNαs are typically species specific, although IFNα8 is a human type I IFN that can bind to the mouse receptor. IFNAR-1 confers species specificity of binding. As noted previously, type I IFNs are induced by viral infection. Infection with viruses results in the generation of nonself RNAs in the cell, which are recognized by retinoic acid inducible gene-I (RIG-I)-like receptors, which include RIG-I, MDA5, and LGP2, each of which contains a helicase domain. Retinoic acid inducible gene-I-like receptors are essential for the production of tpe I interferons as well as IL-6, and cells lacking both RIG-I and MDA5 are completely defective in the production of type I IFNs.[410]

IFNγ was first recognized in 1965 as immune IFN, named as IFNγ in 1980, cloned in 1982, and then confirmed to be the same as primary macrophage activating factor.[388,397,411,412,413] In addition to its antiviral activity, IFNγ also has a range of actions on proinflammatory and anti-inflammatory host responses.[393] For example, IFNγ can prime macrophages to manifest antimicrobial and antitumor effects. Moreover, following IL-12-mediated differentiation of Th1 cells, IFNγ is a major secreted product. This production is critical for host response to a range of infectious pathogens, including, for example, *T. gondii*. IFNγ inhibits the generation of Th2 cells as well as Th17 cells. IFNγ is produced by a range of cells, including T_{reg} cells and in this context appears to result in apoptosis of naive cells and Th2 effector T cells. It is produced by NK cells and can mediate NK-dependent lysis of tumor cells, augment IgG2a isotype switching of B cells, and activate DCs. Moreover, IFNγ can either contribute to disease protection or can be protective, depending on the context.[413] Finally, IFNγ- or anti-IFNγ-based therapy are being tried in a range of conditions.[314]

IFNγ is encoded by four exons on chromosome 12. IFNγ forms a functional homodimer with an apparent molecular weight of 34 kDa, whereas little of the monomeric form can be detected and it is not biologically active. Each IFNγ monomer has six α helices, four of which resemble the shortchain helical cytokines, and there is no β sheet structure. The subunits interact in an antiparallel fashion. In contrast to the ability of many different cells to produce IFNα, IFNγ is more restricted, with it being produced primarily by NK cells, CD8+ T cells, and the Th1 subclass of CD4+ T cells.[388] Many signals, including antigen stimulation, IL-12, and IL-18, can induce the production of IFNγ. IFNγ exerts its effects through specific receptors that are expressed on all types of cells except erythrocytes. Interestingly, even platelets express IFNγ receptors, raising the possibility that they can serve a function in transporting IFNγ in the circulation.[388] The functional human receptor consists of two chains[414]: IFNGR-1, formerly also denoted IFNγR1 or IFNγRα[406,415] a 90 kDa protein whose gene is located on human chromosome 6q16 to 6q22 and mouse chromosome 10,[412] and IFNGR-2, also denoted as IFNγRβ,[416,417] located on human chromosome 21q22.1 and mouse chromosome 16.[412] IFNGR-1 is required for ligand binding, whereas IFNGR-2 plays a role in signaling. JAK1 associates with the Leu-Pro-Lys-Ser sequence in the membrane proximal region of the cytoplasmic domain of IFNGR-1,[418] whereas JAK2 binds to IFNGR-2.[419] The fact that IFNγ is a homodimer explains how its binding induces the homodimerization of IFNGR-1, which then allows the recruitment of IFNGR-2. Thus, the functional IFNγ receptor is believed to contain two molecules each of IFNGR-1 and IFNGR-2. Normal IFNγ production is dependent on IL-12, and defective IFNγ signaling is associated with failure to appropriately clear mycobacterial and other infections (discussed subsequently).

Both type I and type II IFNs are important for cancer immunoediting, a process in which neoplastic growth is suppressed by the immune system and tumor immunogenicity is shaped. IFNγ can suppress tumor development, but type I IFNs are required to initiate the antitumor response, at least in part via actions on DCs.[420]

Interleukin-10, a Type II Cytokine, and the Related Cytokines Interleukin-19, Interleukin-20, Interleukin-22, Interleukin-24, Interleukin-26, Interleukin-28A, Interleukin-28B, and Interleukin-29

IL-10 is a type II cytokine that originally was identified as cytokine synthesis inhibitory factor,[380,421,422] produced by Th2 cells. However, it then was observed to be produced by T_{reg} cells as well, and it is now clear that IL-10 is much

more broadly expressed, including by Th1, Th17 cells, B cells, macrophages, NKT, NK, T_{reg}, monocytes, and myeloid DCs but not plasmacytoid DCs, keratinocytes, as well as mast cells and eosinophils.[423,424] IL-10 production by T_{reg} cells helps to explain their suppressive effects, and IL-10 production by T_{reg} cells is required for their suppression of Th17-mediated inflammation.[425] The IL-10 gene contains five exons and is located on chromosome 1 in both mice and humans.[422] IL-10 has an open reading frame of 178 amino acids, including the signal peptide, and the mature protein is 18 kDa. Human IL-10 receptor maps to 11q23.3. IL-10 can inhibit the production of a number of cytokines, including IL-2, IL-3, IFNγ, GM-CSF, and TNF-α. Production of IL-10 is substantially dependent on the transcription factor E4BP4 (also called NFIL-3).[426] IL-10 production is induced by pathogen-derived products, with TLR2 signaling being critical for its induction by antigen-presenting cells and macrophages, although TLR-independent induction of IL-10 has also been observed.[423] Interestingly, IL-21 has been shown to be a potent inducer of IL-10 and to mediate IL-27 and part of the IL-6-

mediated induction of IL-10 as well.[189,190] IL-10 inhibits monocyte/macrophage/DC-dependent T-cell proliferation, in part, by markedly decreasing synthesis of a variety of cytokines, expression of costimulatory molecules, and chemokines, to name just some of its inhibitory effects.[380] In addition to these indirect effects on T cells, IL-10 can exert direct stimulatory effects on thymocytes and T cells in vitro and promote the development of B1 cells and activity of NK cells. IL-10-deficient mice develop a form of inflammatory bowel disease that is similar to Crohn disease.[422,427] Interestingly, the BCRF1 protein that is encoded by Epstein-Barr virus is very similar to IL-10 and shares many of its biologic properties as a macrophage "deactivating" factor and as a costimulator of proliferation of B cells.[428] The Epstein-Barr virus IL-10 homologue is a selective agonist, although its binding to the IL-10 receptor is somewhat impaired.[428] IL-10 is a major inhibitor of Th1 functions.[422] Although it was originally suggested that IL-10 might also favor Th2 development, IL-4 is the major mediator of Th2 cell development, and IL-10 instead plays a major role in limiting and terminating inflammatory responses.[422]

The IL-10 receptor is most closely related to IFN receptors, making it a type II cytokine receptor[429,430] and corresponding to the close structural relationship of IL-10 to IFNγ. The receptor for IL-10 consists of an IL-10R1 chain and IL-10R2[422]; this latter protein is the same as CRF2-4, which was first identified as an "orphan" IFN receptor family member that is located on chromosome 21 within 35 kb of IFNGR-2.[431,432] The major STAT protein used by IL-10 is STAT3.[423]

A series of IL-10-related cytokines have been been identified, including IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B, and IL-29.[379,424,433,434,435,436,437] IL-19 was discovered as a gene that was induced in LPS-stimulated monocytes. IL-19 has been suggested to potentially be involved in the pathogenesis of chronic inflammatory diseases, such as psoriasis. Like IL-19, IL-20 is produced by myeloid cells; it was also found to be produced by epidermal cells, with overexpression resulting in aberrant epidermal differentiation. IL-20 is believed to have a role in psoriasis, and in psoriatic skin, expression of IL-20 is primarily detected in DCs.[379,438] IL-20 also appears to contribute to rheumatoid arthritis, atherosclerosis, and stroke; recently, IL-20 has been implicated in bone loss, with elevated IL-20 in patients with osteopenia and osteoporosis in in ovariectomized mice. Moreover, IL-20R1-deficient mice have increased bone density as did treatment with anti-IL-20.[439]

IL-22 was found as an IL-9-induced cytokine that can in turn induce acute-phase reactant production by hepatocytes and accordingly that it might be involved in inflammatory responses. It was first observed to be produced by activated Th1 cells but was subsequently noted to be more broadly produced by CD4+ and CD8+ T cells, NK cells, lymphoid tissueinducer cells and mucosal RORγt+NKp46+ cells in humans and mice,[440,441,442] and to be produced by Th17 cells. IL-22 is often coexpressed with IL-10, and STAT3, the Notch pathway, and the aryl hydrocarbon receptor promoter production of these cytokines; in contrast, TGF-β, ICOS, and IL-27 augment IL-10 production yet inhibit IL-22.[424] IL-22 mediates cross talk between leukocytes and tissue epithelia, with target cells primarily in the digestive and respiratory systems, but it also can act on keratinocytes, can promote innate immunity,[443] and can cooperate with IL-17A to effect epithelial and healing responses. In particular, IL-22 can contribute to epithelial resistance to injury after microbial infection of the gut or lungs, and thereby may play a role in host defense and tissue repair.[442] Interestingly, the transcription factor c-Maf mediates suppression of IL-22 production by TGF-β.[444]

IL-24 was originally discovered as melanoma differentiation-associated antigen 7. IL-24 is

induced in peripheral blood mononuclear cells by mitogen stimulation and is produced not only by Th2 cells but also by myeloid cells. Overexpression of IL-24 can induce apoptosis in a wide range of tumor cells, including, for example, melanoma and malignant gliomas, and infection with adenoviral-drive melanoma differentiation-associated antigen 7 can sensitize tumors to ionizing radiation.[380] Like IL-19, IL-20, and IL-22, IL-24 is expressed in psoriatic but not normal skin and like these other cytokines, functional receptors are highly expressed on keratinocytes. IL-10, IL-19, IL-20, IL-22, and IL-24 are all increased in synovial fluid cells in rheumatoid arthritis and thus are potential mediators of this autoimmune disease. IL-20 and IL-22 in particular may be associated with lupus.[379]

IL-26 was originally discovered as AK155. Relatively little is known regarding the function of IL-26.

IL-28A and IL-28B (IFNλ1 and IFNλ2) and IL-29 (IFNλ3) are also called type III IFNs and are induced in plasmacytoid DCs by viral infection and exhibit antiviral activity, analogous to type I IFNs; nevertheless, they bind to a receptor consisting of IL-10R2 and IL-28R[445] and differ in terms of potency and kinetics from IFNα.[379] IL-28A, IL-28B, and IL-29 induce relatively weak antiviral responses, and the IL-28R chain is relatively restricted in its expression, as compared to the IFNα receptor. IL-28A appears to enhance antiviral activity induced by TLR3 and TLR9 and to be important for host defense to influenza A.[379] Interestingly, the genes encoding IL-10, IL-19, IL-20, and IL-24 colocalize at human chromosome 1q32, whereas those encoding IL-22

and IL-26 are at 12q14 to 12q15 near the *IFNG* gene, and IL-28 and IL-29 are at 19q13.[380,446] Thus, these type II cytokines can be subdivided into three different groups based at least in part on chromosome localization.

Overall, IL-10 family cytokines are diverse and can be subdivided into the IL-19, IL-20, IL-22, IL-24, and IL-26 subfamily that acts on tissue epithelial cells and in the case of IL-20 in wound healing, whereas IL-28A, IL-28A, and IL-29 exhibit antiviral activity and are more IFN-like in their biology.[379]

SPECIES SPECIFICITY OF CYTOKINES

There are no general rules for the species specificity of human and mouse cytokines, and how the cytokines and their receptor chains have coevolved. To provide illustrative examples of each situation, human IL-2 can stimulate both human and mouse cells, whereas mouse IL-2 exhibits little action on human cells.[59] Conversely, human IL-12 does not work on mouse cells, whereas mouse IL-12 is biologically active on both mouse and human cells.[311] This selective property of IL-12 is dependent on the species origin of p35. Finally, IL-4 exhibits rather strict specificity so that human and mouse IL-4 only induce responses on human and mouse cells, respectively.[92,93] As noted previously, IFNαs are generally specifies specific, although IFNα8 is not. In addition to these examples, varying degrees of relative species specificity have been demonstrated, depending on the cytokine. Thus, a cytokine may or may not be restricted in its specificity to its own species, and if it does occur, cross-species activity may have attenuated potency.

SIGNALING THROUGH INTERFERON AND CYTOKINE RECEPTORS

Our understanding of signaling through IFN and cytokine receptors has tremendously increased in the past few years. Multiple signaling pathways/molecules have been observed for various cytokines. Collectively, these include the JAK-STAT pathway, IRF family proteins, Ras/mitogen-activated protein (MAP) kinase pathway, Src and Zap70 and related proteins, PI 3-kinase, IRS-1 and IRS-2, and phosphatases. Each of these pathways will be discussed in

turn. The JAK-STAT pathways are important for both type I and IFNs/type II cytokines, while the IRF proteins play central functions more so for the IFNs than type I cytokines.

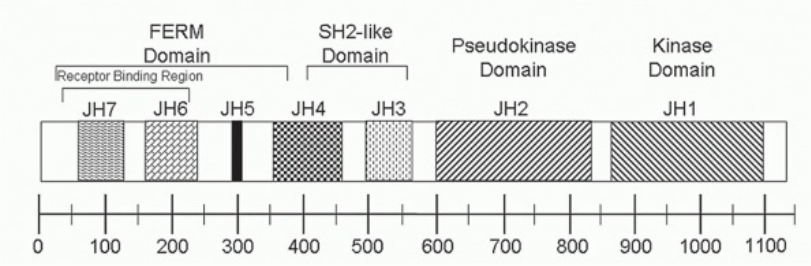

FIG. 25.6. Schematic of JAK Kinases. Shown are the locations of the seven JH domains. JH1 is the catalytic domain. JH2 is the pseudokinase domain, the presence of which prompted the naming of this family as Janus family tyrosine kinases. As noted in the text, the JH nomenclature has limitations and in fact masks the presence of an SH2 domain that spans parts of the JH4 and JH3 domains. Also shown is the conserved tyrosine (Y1007 in JAK2) whose phosphorylation is required for maximal catalytic activity.

OVERVIEW OF JAKS AND STATS

The JAK-STAT pathway[30,355,397,447,448,449] is particularly exciting in that it serves as a rapid mechanism by which signals can be transduced from the membrane to the nucleus. JAK kinases are known as Janus family tyrosine kinases, and the acronym "STAT" denotes signal transducer and activator of transcription. STAT proteins are substrates for JAK kinases. A tremendous amount of information is now available on JAKs and STATs that demonstrate their importance related to development, differentiation, proliferation, cellular transformation, and tumorigenesis.

JAKs

The JAK kinases are 116 to 140 kDa, and comprise approximately 1150 amino acids.[355,397] The seven regions of conserved sequences in JAK kinases, denoted JH1 to JH7, are depicted in Figure 25.6. One of the hallmark features of these kinases is that in addition to the presence of a catalytic tyrosine kinase domain (JH1), there is also a pseudokinase region (JH2). The name Janus kinase reflects the two faces of the mythological Roman god, with one face representing the true kinase and the other the pseudokinase. Although the JH nomenclature was used historically, it has obvious limitations in that except for the JH1 catalytic and JH2 pseudokinase domains, and it remains unclear whether or not the other JH regions correspond to discrete domains. Modeling has indicated five important regions, including an N-terminal domain, a FERM (4.1, ezrin, radixin, moesin) domain, and SH2, kinase-like (which spans two of the JH domains), and kinase domains.[450]

There are four mammalian JAK kinases, JAK1,[451] JAK2,[452] JAK3,[453,454] and TYK2,[455] each of which was identified as part

of a study intended to identify new kinases.[355] At least one JAK kinase is activated by every IFN and cytokine, and some cytokines activate two or three JAK kinases.[355,397,447,448] Table 25.12 lists a number of features of each JAK kinase, whereas Table 25.13 summarizes the JAK kinases that are activated by a variety of cytokines.

TABLE 25.12 Features of Janus Family Kinases

Kinase	Inducible versus Constitutive	Size	Chromosomal Location (H/M)
JAK1	Constitutive	135 kDa	1p31.3/4
JAK2	Constitutive	130 kDa	9p23-24/19
JAK3	Inducible	116 kDa	19p13/8
TYK2	Constitutive	140 kDa	19p13.2/9

Because JAK1, JAK2, and TYK2 are ubiquitously expressed, each cell type expresses either three or in some cases all four JAK kinases (eg, lymphoid cells that also express JAK3). The JAK kinases that are activated by a specific cytokine are those that can bind to its receptor's cytoplasmic domain. JAK kinases physically bind to the membrane proximal Box 1/Box 2 region of the cytoplasmic domains,[454,455,456,457,458,459] and the N-terminal region of the JAK is required for this function.[460,461,462] In cytokine receptors, the Box 1 region is proline rich,[26] suggesting that JAK kinases may have SH3-like domains in their N-terminal regions to mediate these interactions. As each cytokine or IFN receptor is a homodimer, heterodimer, or higher order oligomer, it is reasonable to assume that at least two JAK molecules (either two molecules of one JAK or one molecule each of two different JAKs) will be activated, as one JAK will be associated with each receptor chain. In accord with their ubiquitous expression, JAK1, JAK2, and TYK2 are activated by a variety of different sets of cytokines (see Tables 25.12 and 25.13). For example, JAK1 is activated not only by type I and II IFNs but also by the γ_C family of cytokines (eg, IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21), whereas JAK2 is activated not only by IFNγ but also by growth hormone, Epo, prolactin, and the hematopoietic cytokines, IL-3, IL-5, and GM-CSF.[355,447] TYK2 is somewhat more restricted in that it is activated by IFNα/β and IL-12/IL-23; the significance of its activation by gp130 family cytokines is less clear. Interestingly, JAK1, JAK2, and TYK2 are recruited by each of the cytokine receptors that share gp130 as a signal transducing molecule, raising the question as to whether these cytokines require all three JAK kinases for optimal function or whether any one or two is/are sufficient. At least for IL-6, JAK1 is vital,[463,464] whereas the importance of JAK2 and TYK2 is less clear.

TABLE 25.13 Cytokines and the JAKs They Activate

Cytokine	JAK Kinase(s) Activated
IFNα/β	JAK1, TYK2

IFNγ	JAK1, JAK2
Growth hormone	JAK2
Prolactin	JAK2
Erythopoietin	JAK2
Thrombopoietin	JAK2
IL-10	JAK1, TYK2
IL-12, IL-23	JAK2, TYK2
G-CSF	JAK1, JAK2
γ_C family	
IL-2, IL-4,[a] IL-7, IL-9, IL-15, IL-21	JAK1, JAK3
β_C family	
IL-3, IL-5, GM-CSF	JAK2,?JAK1
gp130 family	
IL-6, IL-11, CNTF, LIF, OSM, CT-1	JAK1, JAK2, TYK2
TSLP	JAK1, JAK2

CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN, interferon; IL, interleukin; LIF, leukemia inhibitory factor; OSM, oncostatin M; TSLP, thymic stromal lymphopoietin.

[a] Note that IL-4 activates JAK1 and JAK3 when it acts through the type I IL-4 receptor (IL-4Rα + γ_C, found for example on T cells). However, JAK3 is not activated when IL-4 signals through the type II IL-4 receptors (IL-4Rα + IL-13Rα1, a form of receptor that is expressed on a number of non-T cells, including fibroblasts).

JAK3 is different from the other JAK kinases in that it is much more inducible. Moreover, JAK3 is only activated by cytokines whose receptors contain γ_C.[30,45] Interestingly, each cytokine whose receptor contains γ_C activates not only JAK3 but also JAK1. The basis for the activation of both JAK1 and JAK3 by IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 is that JAK1 associates with each of the distinctive signaling chains (IL-2Rβ, IL-4Rα, IL-7Rα, IL-9Rα, and IL-21R), whereas JAK3 associates with γ_C.[30,45] Although this could be a coincidental result of coevolution of these cytokine systems, these observations raise the possibility that JAK1 is

the JAK kinase that most efficiently functionally cooperates with JAK3.

Given the wide range of cytokines that activate any particular JAK kinase and that in some cases multiple cytokines can activate the same set of JAKs, it is clear that the JAK kinases by themselves do not determine signaling specificity. For example, IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 all activate JAK1 and JAK3 but induce a range of actions. Moreover, JAK2 is the only JAK kinase that is activated by growth hormone and erythropoietin, cytokines with different target cells and biologic functions. Interestingly, JAK2 is the JAK that interacts with all cytokine receptors that form homodimers. Given that homodimeric receptors are likely the oldest cytokine receptors in evolution, this suggests that JAK2 might be the "primordial" JAK kinase from which others evolved.

Importance of JAK Kinases in Signaling

In addition to the activation of JAK kinases by multiples cytokines and IFNs, a variety of other data indicate their importance for signaling. One of the vital series of experiments that led to the establishment of the critical role of JAK kinases in IFN signaling involved a group of mutant cell lines that were defective for IFN signaling, but wherein signaling could be rescued by genetic complementation.[397] Defective signaling in response to IFNα and IFNβ was found in a mutant fibroblast cell line (U1 cells) lacking TYK2; defective signaling in response to IFNα,

IFNβ, and IFNγ was found in a mutant cell line lacking JAK1 (U4 cells), and defective IFNγ signaling was found in cells lacking JAK2.[397] A variety of other data have indicated the importance of JAK kinases in cytokine signaling pathways. First, dominant negative JAK2 inhibits signaling by erythropoietin and growth hormone,[465,466] while a dominant negative JAK3 inhibits signaling in response to IL-2[467]; as noted previoulsy, JAK1 is vital for IL-6 signaling. Second, humans[468,469] and mice[470,471,472] deficient in JAK3 exhibit developmental and signaling defects. Third, humans with an activating mutation in JAK2 develop polycythemia vera,[473] and a JAK2 inhibitor inhibited the growth of acute lymphoblastic leukemia cells in vitro.[474] Fourth, a patient with mutation in TYK2 developed a form of immunodeficiency.[475] Fifth, in Drosophila, the *hopscotch* gene encodes a JAK kinase, wherein loss-of-function alleles result in lethality and decreased proliferation, whereas a gain-offunction allele, *hopscotch Tumorous-lethal* results in melanotic tumors and hypertrophy of the hematopoietic organs.[476,477] Sixth, in zebrafish, JAK1 is vital for normal cell migration and anterior specification.[478] Seventh, as discussed in the following, JAK kinases are constitutively activated in many cell lines infected with a number of viruses, including HTLV-I, v-Abl, spleen focus-forming virus, and with v-Src.[479,480,481,482] These data together underscore the vital roles of JAKs in cytokine signaling. Depending on the function of the particular cytokine (eg, development, differentiation, or proliferation), the particular JAK kinases potentially may be involved in a variety of processes, and when dysregulated, in at least certain settings, they appear to contribute to cellular transformation.

JAK3 Mutations Result in an Autosomal Recessive Form of Severe Combined Immunodeficiency that is Indistinguishable from X-Linked Severe Combined Immunodeficiency

A very large number of different mutations in γ_c have been observed in X-linked SCID. As might be expected, in cases where it has been examined, amino acid substitutions in the extracellular domain result in defective cytokine binding. In contrast, mutations or truncations in the γ_c cytoplasmic domain result in defective signaling. Analysis of a family in which a number of males exhibit a moderate form of X-linked combined immunodeficiency revealed

that this disease also resulted from a mutation in γ_c[41] (Leu 271 → Gln) that resulted in a decrease, but not total loss of JAK3 association, in contrast to the loss of JAK3 interaction seen with mutations in the γ_c cytoplasmic domain that cause X-linked SCID. Thus, the severity of the immunodeficiency inversely correlated with the degree of JAK3 activation.[30,45] Moreover, it was predicted that JAK3 was required for T-cell and NK-cell development, and that mutations in JAK3 might result in a clinical phenotype indistinguishable from that in X-linked SCID.[41] Indeed, this is the case in humans.[468,469] As one would hypothesize, many mutations in JAK3 have been identified.[355] Presumably, any mutation that interferes with its ability to interact with γ_c, with its catalytic activity, or with recruitment of substrates could result in clinical disease, ranging from moderate-tosevere immunodeficiency. Analogous to the similarity of X-linked SCID and JAK3-deficient SCID, mice deficient in either γ_c or JAK3 also have indistinguishable phenotypes.[211,212,470,471,472] These in vivo data underscore the vital role of JAK3 in mediating γ_c-dependent functions and suggested that JAK3 is essential for most, if not all, γ_c functions. Some in vitro data indicate that γ_c may do more than to recruit JAK3,[467,483] and interestingly, γ_c can interact with calpain,[484] but the recruitment of JAK3 is clearly essential, and the defects in T-cell and NK-cell development associated with γ_c or JAK3 deficiency are indistinguishable. Because JAK3 deficiency is not clinically or phenotypically more severe than γ_c deficiency in humans and mice, it seems likely that γ_c is the major, if not only, protein with which JAK3 associates. Moreover, as had been predicted based on the association of γ_c with JAK3,[41] JAK3 inhibitors are immunosuppressive, and one JAK inhibitor has now been approved by the FDA.[485] Although JAK3 was suggested to associate with CD40,[486] a functional role for JAK3 in CD40 signaling has been questioned.[487] The phenotypes of mice lacking each of the four JAK kinases are summarized in Table 25.14.

JAK2 Mutations and Translocations

The JAK2V617F mutations has now been identified in most of patients with polycythemia vera,[473,488] some of whom have homozygous and some have heterozygous mutations. The homozygous patients had duplications of the mutated allele. This mutation results in a constitutively activated kinase and presumably confers factor-independent growth, something that has been demonstrated in transfected cell lines. Moreover, patients with polycythemia exhibit erythropoietin-independent BFU-E. Furthermore, the *JAK2* locus is involved in a chromosomal translocation to create the TEL-JAK2 protein that is causally related to a human leukemia,[489] further underscoring the relationship of JAK2 to the growth of hematopoietic cells.

TYK2 Mutations and Human Immunodeficiency

A mutation in TYK2 has now been identified in a patient with primary immunodeficiency.[475] The patient had clinical defects more severe than anticipated from TYK2-deficient mice, with increased susceptibility to viral infections and atypical mycobacterial infections and defective signaling in response to IL-23, IL-6, and IL-10. Additionally, the patient had hyper-IgE production, which perhaps corresponds to enhanced Th2 cell-mediated allergic inflammation in TYK2-/- mice.[490] Because this was a single patient, it remains to be determined if other individuals with TYK2 deficiency will have this full range of clinical problems.

ACTIVATION OF JAKS AND THE JAK-STAT PARADIGM

The paradigm of JAK-STAT activation is shown in Figure 25.7, which also shows activation of

other pathways, such as the Ras-MAP kinase and PI 3-kinase/AKT/p70 S6

P.626

P.627

P.628

kinase pathways. Following IFN or cytokine engagement, dimerization or higher order oligomerization of receptor complexes is induced. This in turn allows the juxtapositioning of JAK kinases, facilitating transphosphorylation and activation. In receptors with only two chains, the direct transphosphorylation of one JAK by the other seems likely to occur. In more complex receptors, such as the IFNγ system, because the receptor is a heterotetramer with two IFNGR-1 chains that each bind JAK1 and two IFNGR-2 chains that each bind JAK2, it is not clear if JAK1 and JAK2 transactivate each other or if one of the JAK kinases plays a dominant role. Indeed, one study suggests that JAK2 may phosphorylate both itself and JAK1, thereby increasing the catalytic activities of both kinases.[491] JAK1 in turn then phosphorylates IFNGR-1, allowing the recruitment of STAT1. In this model, it is additionally suggested that JAK2 phosphorylates STAT1.[491] Interestingly, a kinase-dead mutant of JAK1 was able to mediate the induction of certain IFNγ-induced genes, indicating a potential "structural" role for JAK1, but catalytically active JAK1 was essential for the establishment of the antiviral state,[491] emphasizing the essential role of both JAK1 and JAK2 for normal IFNγ function.

TABLE 25.14 Phenotypes of Mice Deficient in Type I and Type II Cytokines, Their Receptors, JAKs and STATs

Type I Cytokines and Their Receptors	
γ_c family	
IL-2[205,692,693]	Normal thymic and peripheral T-cell development. Decreased polyclonal T-cell responses in vitro, but more normal in vivo responses to pathogenic challenges. Autoimmunity with marked changes in levels of serum immunoglobulin isotypes. Ulcerative colitis-like inflammatory bowel disease.
IL-4[208,209]	Defective Th2 cytokine responses and class switch; defective IgG1 and IgE production.
IL-2/IL-4[210]	Some features of both IL-2 and IL-4 knockout mice. No gross abnormalities of T-cell development.
IL-7[104]	Greatly diminished thymic and peripheral T-cell development and B lymphopoiesis, resulting in profound lymphopenia.
IL-9[133,135]	No T-cell defect. Defect in pulmonary goblet cell hyperplasia and mastocytosis following

	challenge with *Schistoma mansoni* eggs. No defect in eosinophilia or granuloma formation.
IL-15151	Defective NK-cell development. Defect in CD8 memory T-cell homeostasis.
IL-2Rα217	Normal initial lymphoid development, but massive enlargement of peripheral lymphoid organs, polyclonal T- and B-cell expansions, and activated T cells, with impaired activation-induced cell death. Autoimmunity with increasing age, including hemolytic anemia and inflammatory bowel disease.
IL-2Rβ218	Severe autoimmunity including autoimmune hemolytic anemia. Death within approximately 3 months. Deregulated T-cell activation. Dysregulated B-cell differentiation and altered Ig profile.
γ_c211,212	Greatly diminished thymic development but double negative, double positives, and single positives all represented. Age-dependent accumulation of peripheral $CD4_+$ T cells with an activated memory phenotype. Greatly diminished numbers of conventional B cells, although B1 cells are present. No NK cells or γδ cells. Absent gut-associated lymphoid tissue, including Peyer patches.
IL-4Rα694	Like IL-4-/- mice, they have defective Th2 cytokine responses and class switch; defective IgG1 and IgE production. In addition, they cannot expel *Nippostrongylus brasiliensis*, presumably because of defective IL-13 signaling.
IL-7Rα103	Greatly diminished thymic and peripheral T-cell development and B lymphopoiesis, resulting in profound lymphopenia.
IL-15Rα150	Defective NK cell development. Defect in CD8 memory T-cell homeostasis.
IL-13366,695	Defective Th2 cell development and the ability to expel helminths. Impaired diarrhea to OVA.
IL-4/IL-13 DKO695	Produce almost no IgE, highly resistant to OVA-induced diarrhea.

IL-13Rα1378,695	Exacerbated Th2 responses, with diminished mortality after infection with *S. mansoni* and greater susceptibility to *N. brasiliensis*. However, partially impaired allergic diarrhea to OVA.
IL-21R182,184	Decreased IgG1, elevated IgE.
IL-21R/IL-4 DKO182	Panhypogammaglobulinemia (mimicks the B-cell phenotype in humans with XSCID).
β_c family	
IL-5227	Decreased basal level of eosinophils and defective induction of eosinophils following infectious challenge. Developmental defect in $CD5_+$ B1 cells. Normal antibody and cytotoxic T-cell responses.
GM-CSF696,697	Normal basal hematopoiesis. Unexpected abnormalities of the lung; abnormal pulmonary homeostasis.
IL-3Rβ698	No defects (due to redundant function of β_C).
β_C243,698	Defective responses to IL-5 and GM-CSF but normal responses to IL-3 (due to redundant function of IL-3Rβ). Diminished eosinophils: both basal levels and in responses to infectious challenge. Unexpected abnormalities of the lung characterized by pulmonary proteinosis and reduced phagocytosis by alveolar macrophages. In other words, the defects are a combination of those found in the IL-5- and GM-CSF-deficient mice.
IL-3Rβ + β_C DKO698	Same phenotype as β_C-deficient mice, except that they cannot respond to IL-3.
gp130 family	
IL-6699	Impaired acute-phase responses following infection or tissue damage. Decreased numbers of hematopoietic progenitor cells.
gp130700,701	Embryonic lethal. Extreme hypoplastic development of the myocardium; although

	the ventricular wall was very thin, trabeculation within the ventricle chamber was normal. Hematologic abnormalities characterized by greatly reduced CFU-S and somewhat reduced CFU-Gm and BFU-E. Markedly diminished size of thymus and numbers of thymocytes. Reduced primordial germ cells in embryonic gonads. Diminished size of placenta.
LIF702,703,704	Decreased hematopoietic progenitor cells. Normal sympathetic neurons but deficient neurotransmitter switch in vitro. Defective blastocyst implantation.
LIFRβ705,706	Postnatal lethality. Normal hematopoietic and germ cell compartments but multiple neurologic, skeletal, placental, and metabolic defects. The greater severity than found in LIF-deficient mice reflects that LIFRβ is shared by several cytokines, including CNTF, LIF, OSM, and CT-1.
CNTF707	Progressive atrophy and loss of motor neurons.
CNTFRα708	Severe motor neuron deficient resulting in perinatal mortality. The more severe phenotype than in CNTF-deficient mice was unexpected and suggests another cytokine may utilize CNTFRα.
IL-11Rα709	Blastocysts can implant but decidualization cannot occur, associated with failure of pregnancy. Fetal lethal phenotype.
IL-12 p40710	Impaired but not completely lacking in their ability to produce IFNγ and to mount a Th1 response in vivo. Elevated secretion of IL-4; normal production of IL-2 and IL-10. Substantially decreased CTL responses. Resistant to infection with intracellular pathogens.
IL-12Rβ1711	Defective IL-12 signaling. IL-2 but not IL-12 could augment NK activity. Defective IFNγ production in response to ConA or anti-CD3. Severe defect in Th1 differentiation.
IL-12 p35175,712	Selective loss of IL-12 function without loss of IL-23. Less resistant to infection than p40

	and IL-12Rβ1 KO.
IL-12Rβ2713	Selective loss of IL-12 function without loss of IL-23. Less resistant to infection than p40 and IL-12Rβ1 KO.
IL-23 p19175,714	Complete resistance to EAE, defective DTH response.
EBI3715	Increased pathologic features of colitis and shorter survival than in p28 KO mice, indicating that IL-35 rather IL-27 is protective.
IL-27 p28715	Decreased pathologic features of colitis and longer survival than in EBI3 KO mice, indicating that IL-35 rather than IL-27 is protective.
Epo, Tpo, G-CSF, and M-CSF	
Epo716	Embryonic lethal. Complete block of fetal liver erythropoiesis, resulting in severe anemia, yet normal development of BFU-E and CFU-E progenitor cells.
EpoR717	Same as Epo-deficient mice.
TpoR718	Decreased megakaryocytes and platelets, but other hematopoietic cells are present in normal numbers.
G-CSF719 Neutropenia and impaired neutrophil mobility. Diminished granulocytes and macrophage precursors.	
M-CSF720,721	Osteopetrosis, absence of teeth. Females are infertile, suggesting an unexpected role for M-CSF.
M-CSF + GM-CSF721	A combination of defects of both M-CSF and GM-CSF, with osteopetrosis and alveolar proteinosis. Early death of pneumonia.
Type II cytokines and their receptors	
IFNAR1722,723,724	Normal development. Defective immune defense against most viral infections tested,

	including lymphocytic choriomeningitis virus, Semliki Forest virus, Theiler virus, vesicular stomatitis virus. Normal resistance to *Listeria monocytogenes*, *Leishmania major*, *Mycobacterium bovis*, and *Mycobacterium avium*.
IFNγ[725,726,727]	Normal lymphoid development. Impaired resistance to *Listeria monocytogenes*, *Leishmania major*, *M. bovis,* and *M. avium*. Can mount curative responses to a number of viruses. $CD4_+$ effector cells default to the Th2 pathway after infection with *Leishmania*. Succumb to infection with *Toxoplasmosis gondii*.
IFNGR1[723,724,728]	Normal lymphoid development. Impaired resistance to *Listeria monocytogenes, Leishmania major, M. bovis,* and *M. avium* Can mount curative responses to a number of viruses.
IL-10[422,427]	Normal lymphocyte development and antibody responses. Chronic enterocolitis, anemia, and growth retardation. Augmented inflammatory responses.
IL-19[729]	Increased susceptibility to dextran sulfate sodium-induced colitis, increased accumulation of macrophages, and production of proinflammatory cytokines and IFNγ.
IL-20R1[39]	Increased bone density with defective osteoclast differentiation.
IL-20R2[730]	Anti-TCR stimulation results in elevated IL-2 and IFNγ production, with increased antigen-specific CD4 and CD8 cells that produce IFNγ, but diminished IL-10.
IL-22[731]	Increased susceptibility to infection with *Citrobacter rodentium*, with augmented bacterial burden, intestinal epithelial damage, and mortality.
IL-28A[732]	Normal clearance of a range of viruses but diminished antiviral activity in response to TLR3 and TLR9 agonists. Expression of IL-28α on nonhematopoietic cells appears to be the most critical.

STATs and JAKs

STAT1[557,558]	Defective responses to both type I and type II IFNs. Defective response to certain viruses and bacterial antigens.
STAT2[559]	Defective signaling in response to type I IFNs; interestingly, the defect in not as severe in STAT2-deficient macrophages as it is in STAT2-deficient fibroblasts.
STAT3[560,561,562,563,564]	Embryonic lethal. Embryos implant but cannot grow. The fact that this phenotype is even more severe than that seen with gp130 suggests a role for STAT3 via a gp130-independent cytokine. By Cre-lox methodology, *STAT3* was also selectively targeted in T cells, which exhibit defective IL-2-induced proliferation that correlates with a defect in IL-2-induced IL-2Rα expression; as expected, T cells also exhibit defective signaling in response to IL-6. *STAT3*-deficient neutrophils and macrophages show defective IL-10 signaling. STAT3 is also essential for normal involution of the mammary epithelium, for wound healing, and for normal hair cycle processes. *STAT3*-deficient DCs exhibit defective Flt3L-dependent expansion of DCs.
STAT4[566,567]	Defective Th1 development. Essentially, the same phenotype as in IL-12-deficient mice, including defective IL-12-mediated boosting of NK cell cytolytic activity.
STAT5A[511,571]	Defective lobuloalveolar development in the mammary gland, a syndrome resulting from defective prolactin signaling. Defective IL-2-induced IL-2Rα expression and associated defects in IL-2-induced T-cell proliferation. Defective superantigen-induced expansion of Vβ8 T cells. Defective antigeninduced recruitment of eosinophils into the lung as well as antigen-induced IgG1 production.
STAT5B[512,572]	Defective growth analogous to Laron dwarfism, a disease of defective growth hormone signaling. Defective IL-2-induced IL-2Rα expression. More severe defects in IL-2-induced T-cell proliferation and NK-cell

	proliferation. Defective antigen-induced recruitment of eosinophils into the lung as well as antigen-induced IgG1 production. Defective NK cytolytic activity.
Partial STAT5A/STAT5B513,514	Defective signaling in response to prolactin and growth hormone. Absent NK-cell development. Major defect in T-cell proliferation and TCR signaling. Anemia.
Complete STAT5A/STAT5B573	Fetal lethality; absent T-cell development.
STAT6568,569,570	Defective Th2 development. Essentially, the same phenotype as IL-4-deficient mice. Defective B-cell proliferation.
JAK1463	Perinatal lethality, with defective signaling by gp130-dependent cytokines (IL-6, IL-11, CNTF, OSM, LIF, CT-1, and NNT-1/BSF-3). Defective signaling by γ_C-dependent cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21; of these, IL-2 and IL-7 were formally evaluated).
JAK2733,734	Fetal lethality with profound anemia due to defective signaling in response to Epo.
JAK3470,471,472	Very similar and possibly identical to γ_C-deficient mice. Defective signaling in response to γ_C-dependent cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21). Greatly diminished T cells in thymus and spleen, but then age-dependent peripheral expansion of CD4+ T cells. Unlike humans with JAK3 mutations, Jak3-deficient mice have greatly diminished B-cell numbers as well.
TYK2735,736,737	Mice lacking TYK2 exhibit diminished signaling in response to IFNα/β and IL-12, but it is not abrogated. Primarily STAT3 activation is diminished, even though STAT1/STAT2 and STAT4, respectively, are the STAT proteins that are primarily activated by IFNα/β and IL-12. Additionally, there are diminished responses to IFNγ and IL-18.

BFU, burst forming unit; BSF-3, B cell-stimulating factor-3; CD, cluster of differentiation; CFU, colony forming unit; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DKO, double

knockout; DTH, delayed type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; Epo, erythropoietin; G-CSF, granulocytecolony stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN, interferon; Ig, immunoglobulin; IL, interleukin; KO, knockout; LIF, leukemia inhibitory factor; M-CSF, macrophage-colony stimulating factor; NK, natural killer; NNT-1, novel neurotrophin-1; OSM, oncostatin M; STAT, signal transducer and activator of transcription; TCR, T-cell receptor; Th, T helper; Tpo, thrombopoietin; XSCID, X-linked severe combined immunodeficiency.

The previous discussion assumes that transphosphorylation of JAK kinases is a mechanism for the amplification of catalytic activity. Unless other kinases are involved, however, implicit to this idea is that the JAK kinases themselves must exhibit some basal activity that is amplified to a higher level by autophosphorylation or transphosphorylation. It is reasonable to speculate that both models may be operative, depending on the system. Consistent with JAK kinases being activated by phosphorylation, mutagenesis of a critical tyrosine in TYK2[492] or JAK2[493] (eg, tyrosine 1007 in the case of JAK2) in the activation loop of the kinase domain inhibits activity. It is also conceivable that phosphorylation of other tyrosines on the JAKs may create appropriate motifs for the recruitment of additional signaling molecules.

The function of the pseudokinase domain remains unclear. No other metazoan protein tyrosine kinases contain such a domain. The JH2 lacks the third glycine in the critical Gly-X-Gly-X-X-Gly motif, is missing an aspartic acid that serves as the proton acceptor that is typically conserved in the catalytic loop of both tyrosine and serine kinases, and is missing the conserved phenylalanine in the Asp-Phe-Glu motif that binds adenosine triphosphate[355] together, pre

sumably explaining the lack of catalytic function of the JH2 pseudokinase domain.[452] Despite the lack of catalytic activity, there are data in support of vital functions for this region. While the kinase domain alone can act as an active kinase, it is interesting that a mutation in the JAK kinase JH2 domain can hyperactivate the Drosophila (*hop*$^{Tum\text{-}l}$/DSTAT) JAK-STAT pathway, and that the corresponding Glu695 to Lys mutation in mouse JAK2 also resulted in increased autophosphorylation of JAK2 and phosphorylation of STAT5 in transfected cells.[494] Moreover, the JH2 domain may play an important role in mediating the interaction of JAKs with STAT proteins.[495]

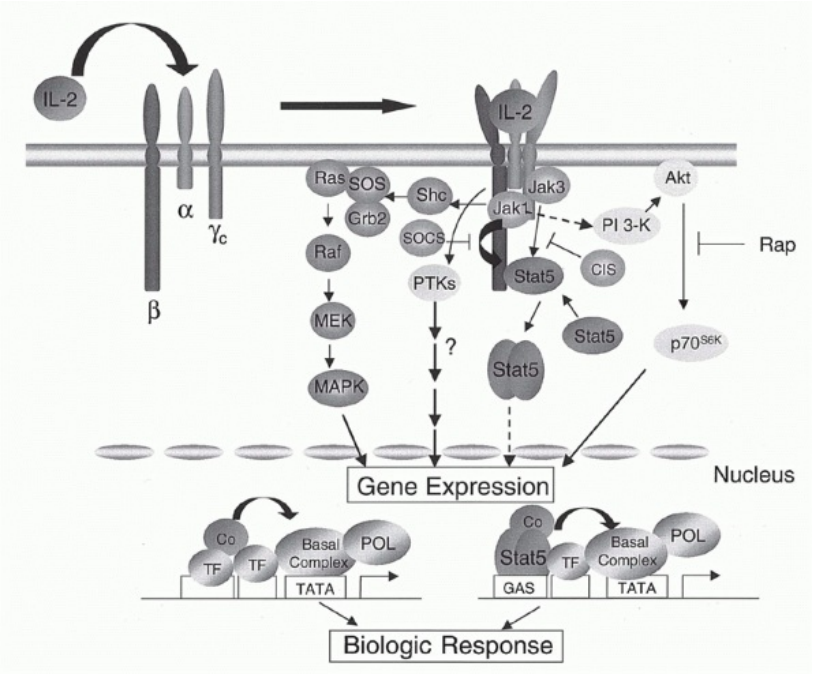

FIG. 25.7. Schematic of Cytokine Signaling Showing Multiple Signaling Pathways Activated by Interleukin (IL)-2. Shown is the association of JAK1 and JAK3 with different chains of the receptor. Activation of JAK kinases results in tyrosine phosphorylation of IL-2Rβ. This allows the docking of STAT5 proteins via their SH2 domain. The STATs themselves are tyrosine phosphorylated, dimerize, and translocate to the nucleus where they modulate expression of target genes. The schematic also indicates that another phosphotyrosine mediates recruitment of SHC, which then can couple to the Ras/Raf/MEK/mitogen-activated protein kinase pathway. Also shown is the important phosphatidyl inositol 3-kinase pathway. These and other pathways are activated by many type I cytokines.

Given that JAK kinases are associated with a wide range of IFN and cytokine receptors, it was predicted that JAKs would mediate signals involved in multiple processes. These include important roles in development (as demonstrated by the lack of T-cell and NK-cell development associated with JAK3 deficiency, a defect at least partially due to defective signaling in response to IL-7 and IL-15), signaling in response to cytokines that are mitogenic growth factors (eg, IL-2, IL-3, etc.), and in the antiviral response (IFNs). Studies in zebrafish revealed not only a role for JAK1 in early vertebrate development but also revealed that during early development JAK1 kinase was exclusively of maternal origin.[478] These developmental roles for JAK1 in zebrafish are consistent with the importance of a JAK kinase in early Drosophila development as well.[496]

SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION PROTEINS ARE SUBSTRATES FOR JAKS THAT AT LEAST IN PART HELP DETERMINE SPECIFICITY

Given that there are only four JAK kinases but scores of cytokines, it is clear that JAKs by themselves cannot fully determine specificity. Indeed, different cytokines with different actions activate the same JAKs. One level of specificity comes from the same JAKs having different substrates, depending

on the receptor. The best characterized substrates for JAKs are the STAT

proteins,[355,447,448] and these appear to provide some, but not all of the specificity, particularly given that there are only seven STAT proteins. Among the mutant cell lines with defects in IFN signaling, in addition to the ones with defects in JAKs noted previously, others were defective in STAT proteins, providing perhaps the earliest data proving a vital role for STAT proteins in signaling in response to IFNs.

STAT proteins are classically considered to be latent transcription factors that initially exist in the cytosol but then must be activated by phosphorylation, with subsequent translocation to the nucleus. STATs were first discovered as factors that bound to the promoters of interferon-inducible genes. The seven mammalian STAT proteins are STAT1,[497] STAT2,[498] STAT3,[499,500] STAT4,[501,502] STAT5A,[503,504,505,506,507] STAT5B,[505,507,508] and STAT6.[509] Table 25.15 summarizes the cytokines that activate each of the STATs. Additionally, some STATs (eg, STAT3) are known to have more than one isoform with distinct functions.[510] Although the STATs conserve a reasonable level of homology, STAT5A and STAT5B are unusually closely related, being 91% identical at the amino acid level.[505,506,507,508] It is interesting that mouse and human STAT5A are more related than STAT5A and STAT5B within a single species. The same is true for mouse and human STAT5B, suggesting that there has been evolutionary pressure to maintain the difference between STAT5A and STAT5B and that these two proteins might have certain distinctive functions. In this regard, STAT5A and STAT5B knockout mice exhibit both similarities as well as some differences in their phenotypes[511,512,513,514]; however, given different levels of each STAT5 protein in certain tissues, it remains to be determined if the different phenotypes result from different intrinsic actions of STAT5A and STAT5B versus differences in the total level of STAT5.

TABLE 25.15 Cytokines and the Signal Transducer and Activator of Transcriptions They Activate

Cytokine	STATs Activated
IFNα/β	STAT1, STAT2, STAT4
IFNγ	STAT1
Growth hormone	STAT5A, STAT5B
Prolactin	STAT5A, STAT5B
Erythopoietin	STAT5A, STAT5B
Thrombopoietin	STAT5A, STAT5B
IL-10	STAT3
IL-12	STAT4, STAT3
G-CSF	STAT3

γ_c family	
IL-2, IL-7, IL-9, IL-15, IL-21	STAT5A, STAT5B, STAT3, STAT1
IL-4	STAT6, STAT5A, STAT5B
IL-13	STAT6
TSLP	STAT5A, STAT5B
β_c family	
IL-3, IL-5, GM-CSF	STAT5A, STAT5B
gp130 family	
IL-6, IL-11, CNTF, LIF, OSM, CT-1	STAT3
Leptin	STAT3

CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN, interferon; IL, interleukin; LIF, leukemia inhibitory factor; STAT, signal transducer and activator of transcription; TSLP, thymic stromal lymphopoietin.

Note that for IL-2, IL-7, IL-9, and IL-15, STAT5A and STAT5B appear to be the major STATs that are activated, although STAT3 in particular and STAT1 to a lesser degree can also be activated. For IL-21, STAT3 is the dominant STAT protein activated, followed by STAT1 and STAT5A/STAT5B.44,585

As active STAT proteins exist as dimers, the ability of at least some STATs to form heterodimers (eg, STAT1 with STAT2 or STAT3[448]) increases the number of different complexes that can form. In addition, further complexity can be generated by the ability of at least some of the STATs to exist in alternatively spliced forms,[508,515] some of which are inactive.

A schematic of STAT proteins is shown in Figure 25.8. The STATs can be divided into two basic groups: those that are longer (STAT2 and STAT6, approximately 850 amino acids) and those that are shorter (STAT1, STAT3, STAT4, STAT5A, and STAT5B, between 750 and 800 amino acids). The STAT genes cluster in three locations. Mouse STAT2 and STAT6 are both located on chromosome 10; STAT1 and STAT4 are located on chromosome 1; and STAT3, STAT5A, and STAT5B are located on chromosome 11.[516] Correspondingly, human STAT5A and STAT5B are closely positioned on chromosome 17q.[508]

The classic model is that in order for STATs to be “activated” and to be able to function as transcriptional activators, a number of cellular events must occur. They first bind to phosphorylated tyrosines on cytokine receptors, are tyrosine phosphorylated, dissociate, dimerize, translocate from the cytosol to the nucleus, bind to target DNA sequences, and activate gene expression. A number of conserved structural features common to all STATs help to explain these functions. These include an SH2 domain, a conserved tyrosine residue,

a DNA-binding domain, and a C-terminal transactivation domain, and an N-terminal STAT tetramerization region (Fig. 25.8). In addition, there is now evidence for the biologic function of nuclear STATs that are not phosphorylated, as discussed subsequently.

Docking of Signal Transducer and Activator of Transcription on Receptors or Other Molecules, their Tyrosine Phosphorylation, and Dimerization

Each STAT protein has an SH2 domain that plays two important roles: 1) for receptor docking, as for example, has been shown for STAT1 docking on IFNGR-1,[517] STAT2 docking on IFNAR-1,[518] STAT3 docking on gp130,[519] STAT5A and STAT5B docking on IL-2Rβ and IL-7Rα,[356,520] and STAT6 docking on IL-4Rα[521]; and 2) for STAT dimerization that is mediated by the SH2 of one STAT interacting with the conserved phosphorylated tyrosine of another STAT protein. In the case of the IFNα receptor, no STAT1 docking site on IFNAR-1 or IFNAR-2 has been identified, and it is believed that STAT1 may interact with STAT2 after STAT2 is itself tyrosine phosphorylated.[355] It is also possible that STATs can dock on JAKs, given the ability to directly coprecipitate JAKs and STATs.[480,495] After

docking has occurred, a conserved tyrosine (tyrosine 701 in STAT1, tyrosine 694 in STAT5A, etc.) can be phosphorylated. This phosphorylation is required for SH2 domain-mediated STAT dimerization, and the phosphorylation likely occurs while the STAT is docked on the receptor in physical proximity to receptor-associated JAK kinases. Following STAT phosphorylation, the STAT protein dissociates from the receptor, and its dimerization with itself or another STAT is presumably then favored over its reassociation with the cytokine receptor chain given that STAT dimerization involves two reciprocal phosphotyrosine-SH2 interactions, whereas docking on a receptor involves only one interaction. Thus, efficient activation of STATs requires the presence in STATs of a conserved SH2 domain and a critical tyrosine.

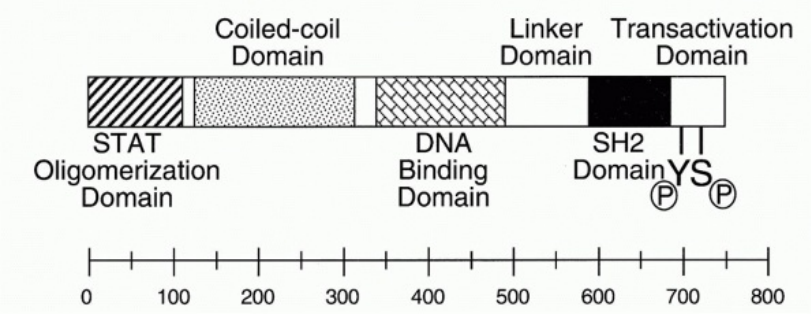

FIG. 25.8. Architecture of a Typical Signal Transducer and Activator of Transcription (STAT) Protein. Shown are the locations of the following important regions: 1) The N-terminal region has been shown to mediate the interaction of STAT dimers bound to adjacent gamma activated sites (known to be important for STAT1 but presumably true for all STATs), 2) the DNA-binding domain, 3) the SH2 domain that mediates STAT docking on receptors and STAT homodimerization/heterodimerization following tyrosine phosphorylation, and 4) the location of the conserved tyrosine whose phosphorylation allows the SH2-mediated dimerization. At least in STAT1 and STAT3, Serine 727, which is C-terminal to the conserved tyrosine, is an important site for phosphorylation. In the case of STAT1, p48 interacts downstream of the STAT dimerization domain. CBP/p300 interacts with two sites at both the N-terminus and C-terminus. Although it has been suggested that the region between the DNA-binding domains and the SH2 domain is an SH3 domain, this remains unproven, and no

interactions with proline-rich regions have been reported; as a result, the labeling of this region as an SH3 domain has been omitted. Note that this structure is typical of that for STAT1, STAT3, STAT4, STAT5A, and STAT5B. The main features are conserved in STAT2 and STAT6, but these are approximately 50 to 100 amino acids longer.

Whereas some receptor proteins such as IFNGR-1[517] and IL-7Rα[356] have a single STAT docking site (for STAT1 and STAT5, respectively), a number of receptor molecules, including IL-2Rβ,[356,520] IL-4Rα,[521] gp130,[519] Epo receptor (EpoR),[522] and IL-10R1,[523] have more than one docking site for their respective STATs. The presence of more than one site not only provides functional redundancy but also potentially could allow the simultaneous activation of two STAT molecules, providing a high local concentration of phosphorylated STATs to facilitate their dimerization.

Signal Transducer and Activator of Transcription Nuclear Translocation and Deoxyribonucleic Acid Binding

Following dimerization, the STATs translocate into the nucleus where they can bind to DNA as transcription factors. The mechanism for nuclear translocation was originally mysterious given the absence of an obvious nuclear localization signal.[524] However, it was shown that tyrosine-phosphorylated STAT1 dimers can directly interact with importin-α5, allowing internalization. This suggested that there indeed was a nuclear localization signal that is normally masked, and mutation of Leu407 does not interfere with tyrosine phosphorylation, dimerization, or DNA binding but prevents nuclear localization.[524] Following its dephosphorylation, nuclear STAT1 is exported to the cytosol by a process that is dependent on the chromosome region maintenance 1 (CRM1) export reporter.[525] Thus, both import and export of STAT1 appear to be regulated processes.[526]

Whereas most STAT dimers directly bind to DNA, in the case of IFN-α/β, STAT1-STAT2 heterodimers are formed, and these bind DNA in conjunction with a 48 kDa DNA-binding protein known as IRF9; the STAT1-STAT2-IRF9 complex is known as IFN-stimulated gene factor 3 (ISGF3).[448] In the case of other STAT dimers, accessory proteins are not required for DNA binding. The motif recognized by ISGF3 complexes is AGTTTNCNTTTCC (known as an ISRE, for IFN-stimulated response element), whereas the other STAT complexes bind more semipalindromic TTCNmGAA motifs that are generally denoted as gamma-activated site (GAS) motifs for IFNγ-activated sequences, reflecting their original discovery in the context of IFNγ.[355,397,447]

A series of chimeric STAT proteins were used to delineate a DNA-binding domain of approximately 180 amino acids, with two conserved subdomains. Although multiple STATs bind to the same motifs, their relative efficiencies vary, indicating fine specificities conferred by the different

DNA-binding domains. For example, whereas STAT1 homodimers favor a TTCN3GAA motif, STAT6 prefers a TTCN4GAA motif.[521] These differences partially explain why different STATs modulate the expression of nonidentical sets of target genes. The structures of STAT1 and STAT3β bound to DNA[527,528] (Fig. 25.9) almost resembles that of a vertebral column, wherein the DNA represents the spinal cord. The N-terminal and coiled-coil domains are spatially the furthest from the DNA, whereas the DNA-binding domain, linker, and SH2 domain surround the DNA, with the stability apparently being provided by the SH2-phosphotyrosine interaction between the STAT monomers and each STAT monomer-DNA interaction with the DNA, presumably via a “half GAS site,”[529] as discussed under STAT

tetramerization.

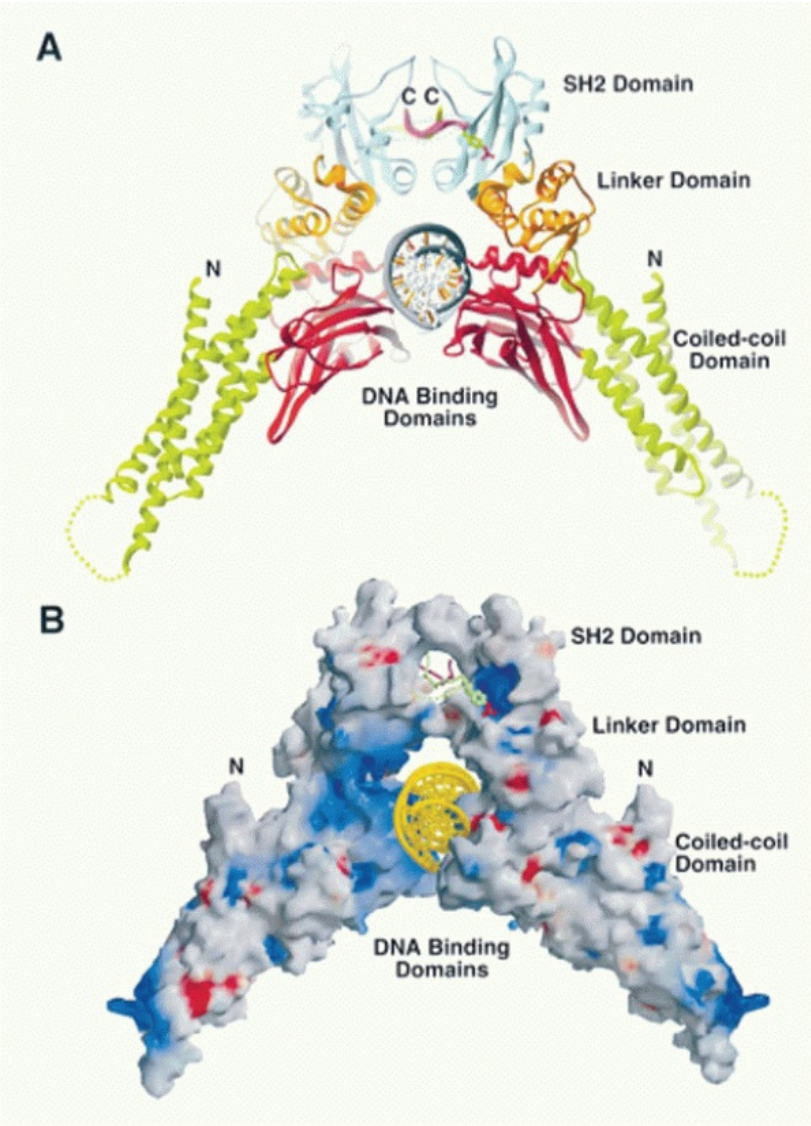

FIG. 25.9. Three-Dimensional Structure of a Signal Transducer and Activator of Transcription 1 Dimer Bound to Deoxyribonucleic Acid. Reproduced from Chen et al.[527] and Becker et al.,[528] with permission of Dr. Kuryian and Cell Press.

N-terminal regions mediate cooperative DNA binding of STAT proteins when multiple STAT-binding sites occur in close proximity,[530,531] for example, in the IFNγ gene.[530] In the IL-2Rα gene, IL-2 response elements have been described in both the 5′ regulatory region and the first intron, each of which has tandem GAS motifs that functionally cooperate to mediate IL-2-induced IL-2Rα transcription.[532,533,534,535]

STAT proteins were the first transcription factors that were identified as targets for tyrosine phosphorylation. Previously, tyrosine phosphorylation was primarily associated with membrane proximal events, but the tyrosine phosphorylation of STATs is associated with a membrane proximal event, as the STATs dock on cell surface receptors. This phosphorylation then allows the rapid dimerization that facilitates nuclear localization and DNA binding. STATs can interact directly with JAK kinases (first shown for STAT5 and JAK3),[480] suggesting that STATs may at times dock on JAKs rather than on receptors.

Nonphosphorylated Signal Transducer and Activator of Transcription Proteins Can Be Nuclear

In addition to the classical tyrosine phosphorylation-mediated dimerization and nuclear translocation, it is now clear that STAT proteins can exist in the nucleus even without being tyrosine phosphorylated and in that context can modulate gene expression.[536,537,538] Moreover, nonphosphorylaed STATs (eg, STAT4) can form N-domain-mediated dimers.[539]

Optimal Binding Sites for Signal Transducer and Activator of Transcription Proteins

The optimal binding motifs for several STAT proteins have been determined. For STAT1, STAT3, and STAT4, a TTCCSGGAA motif was defined,[448,530] while STAT5A and STAT5B optimally bind a TTCYNRGAA motif[529] and STAT6 binds a TTCNTNGGAA motif (where Y is C or T and R is G or A).[521,529] Whereas dimeric STAT protein binding strongly preferred canonical motifs, the range of sequences recognized by STAT5 tetramers is broader, often occurring to two imperfect motifs or with one canonical motif with an associated "TTC" or "GAA" half GAS motif. The optimal inter-GAS motif spacing is 11-12 bp, with relative secondary peaks of 6-7 bp and 16 bp as well.[529a] The presence of suboptimal GAS motifs spaced at appropriate distances to allow tetrameric binding may allow greater specificity via cooperative binding of STAT oligomers. A subset of genes bind STAT5 tetramers and STAT5 tetramers are critical for cytokine responses and normal immune function.

Transcriptional Activation by Signal Transducer and Activator of Transcription

In addition to tyrosine phosphorylation, some STATs can be phosphorylated on serine, and STAT1 and STAT3 phosphorylation at Serine 727 in the C-terminal transactivation domain is required for full activity.[540,541,542] The Serine 727 region resembles a MAP kinase recognition site, and one study indicated that MAP kinase activity is required for IFNα/β-induced gene expression.[543] Serine 727 mediates the interaction of STAT1 with MCM5, a member of the minichromosome maintenance family of proteins; this interaction presumably is important for maximal transcriptional activation.[544] In contrast, STAT2 is not serine phosphorylated.[448] STAT5A and STAT5B are serine phosphorylated,[545] and this phosphorylation may be important in cellular transformation.[546,547]

In addition to this regulated modification of the STAT proteins, STATs can also interact with other factors. For

example, as noted previously, STAT1-STAT2 heterodimers bind DNA only after interacting with IRF9 to form ISGF3.[448] STAT1 interacts with and synergizes with Sp1 for transcriptional activation in the ICAM-1 gene[548] and with TRADD to influence IFNγ signaling.[549] An alternatively spliced shorter form of STAT3, denoted STAT3β, associates with c-Jun to enhance transcriptional activity,[550] and full-length STAT3 can also associate with c-Jun.[551] Moreover, certain STATs can interact with the potent transcriptional coactivators CBP/p300.[552,553,554] In the case of STAT1, this is mediated by interactions involving both the N- and C-terminal regions of STAT1 and the CREB and E1A binding regions of CBP, respectively.[553] STAT5A has been shown to associate with the glucocorticoid receptor.[555] Additionally, the IL-2-response element in the IL-2Rα gene requires not only STAT5 binding but also the binding of Elf-1, an Ets family transcription factor, to a nearby site.[532] Thus, active STAT complexes appear to involve the coordination of STAT proteins with other

factors. The corepressor silencing mediator for retinoic acid receptor and thyroid hormone receptor was identified as a potential STAT5-binding partner that binds to both STAT5A and STAT5B and potentially plays a negative regulatory role.[556]

Specificity of Signal Transducer and Activator of Transcription Proteins

Analogous to the JAKs, the same STATs are activated by multiple cytokines. The phenotypes of mice lacking expression of each of the seven STAT proteins are known, and these knockout models have helped to clarify which cytokines critically depend on a given STAT protein. STAT1 knockout mice exhibit defects that are very selective for the actions of type I and type II IFNs,[557,558] suggesting that STAT1 is only vital for the actions of IFNs, even though a variety of other cytokines have been reported to activate STAT1. Although it is possible that STAT1 plays an important but redundant role for at least some of these other cytokines, the phenotype of STAT1-deficient mice indicates a need for caution in the interpretation of in vitro experiments that use high concentrations of cytokines or cell lines. STAT2-deficient mice exhibit defects consistent with selective inactivation of IFNα/β signaling.[559] STAT3-deficient mice exhibit fetal lethality; the embryos implant, but they exhibit defective development and growth.[560] Deletion of STAT3 within specific lineages has revealed that mice lacking STAT3 in T cells[561] have normal lymphoid development but exhibit a defect in IL-2-induced IL-2Rα expression, somewhat analogous to what is seen in STAT5A- and STAT5B-deficient mice (see the following discussion). They also exhibit defective Th17 differentiation due to defective signaling by IL-6 and IL-21.[169] Neutrophils and macrophages lacking STAT3 exhibit defective signaling to IL-10, and it is known that STAT3 is important for the normal involution of the mammary epithelium, for wound healing, and for normal hair cycle.[562,563] DCs lacking STAT3 exhibit a defect in Flt3L-dependent differentiation,[564] and antigen-presenting cells that are deficient in STAT3 exhibit disruption of priming of antigen-specific CD4+ T cells.[565] STAT4-deficient mice exhibit a phenotype similar to that of IL-12-deficient mice (ie, defective Th1 development), consistent with the observation that STAT4 is activated by IL-12.[566,567] Analogously, STAT6-deficient mice exhibit a phenotype similar to that of IL-4-deficient mice (ie, defective Th2 development),[568,569,570] in keeping with the observation that STAT6 is only activated by IL-4 and the closely related cytokine, IL-13. Interestingly, mice lacking STAT5A exhibit a defect in prolactin-mediated effects, including defective lobuloalveloar proliferation,[571] whereas mice lacking STAT5B have defective growth similar to that found in Laron dwarfism.[572] Thus, although STAT5A and STAT5B appear to always be coordinately induced, each of these STATs is important in vivo. In addition to these defects, both STAT5A- and STAT5B-deficient mice have defects in T-cell development and signaling.[511,512] STAT5A-deficient mice have diminished numbers of splenocytes and exhibit a defect in IL-2-induced IL-2Rα expression.[511] STAT5B-deficient mice have similar defects but also have diminished thymocytes.[512] Most dramatically, these mice have a major defect in the proliferation of freshly isolated splenocytes[512] and defective NK-cell development.[512] Mice lacking both STAT5A and STAT5B in lymphoid cells exhibit a dramatic defect in T-cell development,[573] and even hypomorphic expression of both STAT5A and STAT5B is sufficient to result in a lack of NK-cell development.[513] Presumably, the lack of T-cell development relates to defective IL-7 signaling, whereas the lack of NK-cell development relates to defective IL-15-dependent STAT5 activation. STAT5A/STAT5B double knockout mice exhibit lethality that is associated with severe anemia that develops in these mice.[574]

Signal Transducer and Activator of Transcription Proteins are Evolutionarily Old

Just as Drosophila has a JAK kinase, there is a Drosophila STAT, denoted as either DSTAT or STAT92E.[575,576] The existence of JAK kinases and STAT proteins in lower organisms suggest that the system is evolutionarily old. A STAT has been identified in *Dictyostelium* that recognizes the sequence TTGA,[577] has highest sequence similarity to STAT5B, and can bind mammalian IFN-stimulated response elements.[577] Interestingly, *Saccharomyces cerevisiae* do not appear to have STATs as no SH2 domains have been identified in the entire *S. cerevisiae* genome.

What Types of Functions Are Mediated by Signal Transducer and Activator of Transcriptions?

First and foremost, STAT proteins can translocate to the nucleus and bind to regulatory regions of target genes and influence transcription. Although STATs are generally activators of transcription, they can repress. For example, STAT1 can function as a transcriptional repressor of the c-myc gene.[578]

STAT proteins were discovered based on the study of IFN-inducible genes as part of studies intended to understand the cellular differentiation events that lead to development of the antiviral state. In addition to roles in differentiation, STAT proteins can contribute, directly or indirectly, to survival and/or mitogenic/proliferative responses that typify hematopoietic

and immunologic cytokines, such as IL-3, IL-5, GM-CSF, IL-2, and IL-4.[355] First, a number of in vitro systems have demonstrated that viruses or viral oncogenes are associated with activated JAK-STAT pathways, suggesting a role for STATs in cellular transformation. Indeed, STAT3 and STAT5 have been implicated as oncogenes, with compelling data for transformation capability of STAT3 in cell lines, persistently tyrosine-phosphorylated STATs in several types of leukemia and lymphoma, and the development of T-lymphoblastic lymphomas in STAT5 transgenic mice.[579] The role of STAT3 in the tumor microenvironment makes it an attractive target for cancer immunotherapy.[580] Second, there is diminished proliferation in a number of the STAT knockout mice that have been analyzed. For example, STAT4-deficient cells exhibit diminished cellular proliferation to IL-12566,567; STAT6-deficient cells exhibit diminished proliferation to IL-4[568,569]; and STAT5-deficient mice have diminished T-cell proliferation to IL-2.[511,512] Some of these effects are indirect, based on modulation of receptor expression. For example, STAT6-deficient mice exhibit decreased IL-4Rα expression. Similarly, the absence of STAT5 results in decreased IL-2-induced IL-2Rα expression as well as decreased proliferation.[511,512] Thus, in at least some cases, decreased proliferation results at least in part from decreased expression of receptor components. STAT5A and STAT5B regulate BCL-XL induction, indicating their ability to affect cell survival.[574] Finally, STAT1 has been linked to cell growth arrest and induction of the cdk inhibitor $p21^{WAF1/CIP1}$,[581] and activation of STAT1 occurs in thanatophoric dysplasia type II dwarfism as the result of a mutant fibroblast growth factor receptor.[582] In this chondrodysplasia, the mutant fibroblast growth factor receptor induces nuclear translocation of STAT1, expression of $p21^{WAF1/CIP1}$, and growth arrest, suggesting a possible relationship to the disease. Thus, different STATs may potentially mediate either growth expansion or growth arrest. Moreover, STATs may potentially play other types of roles, as well. For example, STAT3 has been reported to serve as an adapter to couple PI 3-kinase to the IFNAR-1 component of type I IFN receptors.[583]

A more complete understanding of the actions of the different STAT proteins may be

facilitated with a compilation of the genes that are regulated by each STAT. Progress in this area has been made for a number of cytokines by microarray or RNA-Seq analysis (eg, for the IFNs[399]). It is also important to recognize that not all cytokine signals are dependent on STATs, as illustrated, for example, by STAT1-independent IFN signals,[584] STAT5-independent IL-2 signals, 520 and STAT1/STAT3-independent IL-21 signals.[585]

Do Other Proteins Bind to GAS Motifs?

At least one non-STAT protein can bind to GAS motifs. The *BCL6* gene is often mutated or has undergone translocations in diffuse large cell B-cell lymphomas. Interestingly, BCL6 binds to GAS motifs capable of binding STAT6 and specifically can inhibit IL-4 action.[586] Mice lacking BCL6 expression exhibit defective germinal center formation, suggesting that formation of germinal centers may be at least partially dependent on BCL6 regulated (presumably negative) control of certain STAT-responsive genes.[586]

OTHER LATENT TRANSCRIPTION FACTORS AS EXAMPLES OF CYTOPLASMIC TO NUCLEAR SIGNALING (NF-κB, NFAT, AND SMADS)

An exciting feature of STAT proteins is that they exist in an inactive form in the cytosol and then are rapidly translocated to the nucleus. The rapid activation within minutes of signals from cell membrane to nuclear DNA binding makes the STAT acronym apt, analogous to the urgency associated with "STAT" emergency physician orders in clinical medicine. The rapid activation of STAT proteins is somewhat analogous to several other transcription factors.[587] NF-κB also undergoes rapid nuclear translocation but by a completely different mechanism from STATs. In contrast to STAT proteins where the tyrosine phosphorylation of the STATs is an initiator of nuclear translocation, for NF-κB, it is the serine phosphorylation and/or uquitination of IκB that results in its dissociation and/or destruction, allowing the release and translocation of NF-κB. There is the classical NF-κB pathway used by many cytokines, which involves the IKKα/IKKβ/IKKγ-dependent phosphorylation of IκB, resulting in the activation of NFκB1 (p50)/Rel-A (p65) heterodimers as well as the alternative pathway in which a homodimer of IKKα mediates the activation of NFκB2 (p52)/Rel-B heterodimers.[588,589,590,591,592,593,594] A third example of cytosolic to nuclear translocation occurs with nuclear factor of activated T-cell (NFAT) family proteins,[595,596,597] which are vital for regulating transcription of a number of cytokines, including for example IL-2, IL-4, and GM-CSF. NFAT is translocated to the nucleus where it associates with activator protein 1 (AP-1) family proteins to form a functional complex. It is the activation of calcineurin and dephosphorylation of NFAT that allows its nuclear translocation. A fourth example of cytosolic to nuclear translocation occurs with the SMAD proteins that mediate TGF-β signaling, which contributes to Th17 differentiation (discussed subsequently). For SMADs, the phosphorylation is on serine and the kinase is intrinsic to the receptor, but like STATs, activation of these latent transcription factors is rapid and initiated by the binding of a growth factor. Thus, several mechanisms, each involving phosphorylation or dephosphorylation, have evolved to allow cytoplasmic to nuclear translocation of latent transcription factors.

OTHER SUBSTRATES FOR JAKS

As JAKs are potent cytosolic tyrosine kinases, it is evident that the JAKs may do more than just phosphorylate tyrosine residues on receptors where STAT proteins dock as well as phosphorylating the STATs. In vitro data indicate that JAK kinases also can phosphorylate receptor tyrosines other than the docking sites for STATs. For example, in the case of IL-2Rβ, JAK1 can phosphorylate not only tyrosines 392 and 510, which are STAT docking sites, but also tyrosine 338, which is a docking site for SHC,[520] all of which are required for maximal

proliferation. JAK kinases are known to autophosphorylate themselves or transphosphorylate other JAKs. Other molecules are potentially phosphorylated by JAK kinases, including the STAM adapter molecule[598] and the p85 subunit of PI 3-kinase.[599]

Interferon Regulatory Factor Family Proteins

IRFs form a family of nine proteins that are regulated by type I IFNs.[395,600,601] They each have a well-conserved N-terminal DNA-binding domain that forms a helix-loop-helix structure as well as a C-terminal interaction domain. IRFs are critical for a number of actions, including type I IFN-dependent gene transcription.[395] Select IRFs play critical roles in TLR-mediated IFN induction. IRFs can have negative as well as positive effects. For example, IFNγ-mediated repression of IL-4 is mediated by IRF protein(s).[602]

OTHER SIGNALING MOLECULES IMPORTANT FOR CYTOKINES

Other Tyrosine Kinases besides JAKS

In addition to their activation of JAK kinases, a number of cytokines can activate Src family kinases. For example, IL-2 can activate p56lck[603,604] in T cells and p59fyn and p53/p56 lyn in B-cell lines.[605,606] The activation of some of these kinases has been reported to be mediated by associating with the "A" region of IL-2Rβ. Another tyrosine kinase, Syk, has been reported to associate with the S region of IL-2Rβ.[607] However, the significance of these interactions is less clear than that for JAK kinases. First, cells lacking Lck can vigorously proliferate in response to IL-2.[608,609] Second, when the A region is deleted, proliferation still occurs, albeit at a lower level than seen with wild-type IL-2Rβ.[610] However, Y338, which is required for the recruitment of SHC, is in the A region and is required for normal proliferation.[520] Thus, it is possible that the decrease in proliferation associated with deletion of the A region relates more to the loss of Y338 than it does to the loss of association of Lck. Moreover, in *Il2rb*-/- mice reconstituted with an IL-2Rβ A-region mutant, proliferation is increased rather than decreased.[611] Additional investigation is required to clarify the role of activation of Src family kinases by IL-2 and other cytokines as well as the significance of the Syk interaction with IL-2Rβ. As Syk and JAK1 both associate with the S region of IL-2Rβ, mutations that delete the S region simultaneous prevent both associations, making it hard to determine the specific role of Syk. Syk-deficient mice exhibit normal IL-2 proliferation,[612] further suggesting that Syk may not play an important role in IL-2-induced proliferation. The G-CSF receptor also can form a complex with Lyn and Syk,[613] but again Syk-deficient mice do not exhibit a defect in G-CSF signaling.[612] β_C has also been reported to interact with Src family kinases[614]; gp130 has been reported to associate with a number of other kinases, including Btk, Tec, and Fes[615,616,617]; and IL-4Rα has been shown to interact with Fes.[618] However, relatively little is known about the significance of tyrosine kinases besides JAKs in cytokine signaling. Additionally, PI 3-kinase, p38, ERK, and JNK can each become activated, leading to activation of many downstream transcription factors, including NF-κB, AP1, PU.1, IRF1, IRF4, and IRF8, to mediate a program of gene expression.

IRS Proteins

IRS-1 was discovered as a tyrosine phosphorylated substrate of the insulin receptor.[619] IRS proteins have a large number of phosphotyrosine docking sites, particularly for the p85 subunit of PI 3-kinase, and they presumably serve to recruit important accessory molecules.

Interestingly, both insulin and IL-4 could induce tyrosine phosphorylation of an IRS-1-like molecule in hematopoietic cells. The 32D myeloid progenitor cells lack IRS-1 and could only signal in response to insulin or IL-4 when they were transfected with IRS-1.[620] Both the insulin receptor and IL-4Rα proteins contain NPXY sequences that are important for IRS-1 or IRS-2 binding; in IL-4Rα, this is contained within a sequence denoted as the I4R motif.[621]

Other cytokines have subsequently been shown to activate IRS-1 and/or IRS-2. For example, growth hormone can induce phosphorylation of IRS-1[622] and IRS-2[623]; IFNγ and LIF induce phosphorylation of IRS-2[623]; and the γ_c-dependent cytokines IL-2, IL-7, and IL-15 can induce tyrosine phosphorylation of IRS-1 and IRS-2 in T cells.[624] The significance of these findings remain unclear, as 32D cells reconstituted with a complete IL-2 receptor can proliferative vigorously in response to IL-2,[608] whereas as noted previously, in these same cells, IL-4 responsiveness requires coexpression of both IL-4Rα and IRS-1, indicative of differing roles for IRS proteins for different cytokines.

Phosphatidylinositol 3-Kinase

PI 3-kinase is a lipid kinase that consists of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit.[625] PI 3-kinase phosphorylation and activation can be induced by a number of cytokines,[626,627,628,629] and the use of inhibitors, such a wortmannin or LY294002, have demonstrated its importance in signaling for at least certain cytokines. IRS-1 has multiple docking sites for PI 3-kinase (YXXM motifs) and thus for some cytokines, such as IL-4, the association of IRS-1 might be the mechanism by which PI 3-kinase can be recruited.

The Ras/Mitogen-Activated Protein Kinase Pathway

Another major signaling pathway for a number of cytokines is the Ras/MAP kinase pathway.[630] This pathway presumably is used by cytokines whose receptors recruit the SHC adaptor molecule, which in turn mediates the recruitment of Grb2 and Sos, eventually leading to the activation of Ras. In turn, Ras couples to the MAP kinase pathway through a welldefined signaling cascade. Certain cytokines, such as IL-2 and IL-3, appear to use this pathway, whereas others, such as IL-4, do not, indicating that this pathway is differentially important depending on the cytokine that is being used.

DOWNMODULATION OF CYTOKINE SIGNALS

Much of the previous discussion has centered on the mechanisms by which cytokines induce signals. However, the mechanisms by which cytokine signals can be terminated are also extremely important. There are multiple levels at which negative regulation can occur. These include 1) regulating a balance between the production (transcriptional and translational control) of the cytokine, its receptor, and/or downstream signaling molecules and the degradation

of these same molecules; and 2) regulation of the activation state of the receptor and downstream signaling molecules. For example, these can be mediated by transcriptional repressors or molecules that either inhibit cytokine signaling (eg, SOCS proteins) or reverse activated states (eg, phosphatases).

Transcriptional control of cytokine production is a widely used mechanism. Many T-cell-derived cytokines such as IL-2, IL-3, and IL-4 are only produced by activated T cells, and their production is lost with the loss of activation. IL-15 provides an example where translation of the protein is carefully regulated, in part by the existence of multiple upstream antithymocyte globulins.[147] Most cytokine receptor chains are constitutively expressed, but some like IL-21R and IL-2Rβ are regulated in part by signals that act through the TCR. The

most regulated receptor chain may be IL-2Rα, whose expression is absent on resting lymphocytes but strongly induced following stimulation with antigens, mitogens, and certain cytokines, but the transcriptional/translational control of most cytokine receptors is poorly studied.

Because phosphorylation events are vital for the creation of phosphotyrosine docking sites, dephosphorylation is an obvious mechanism of control. Indeed, two tyrosine phosphatases, Shp-1 (formerly also known as SHP, HCP, SH-PTP1, and PTP1C) and Shp-2 (formerly also known as Syp and PTP1D) have been shown to play roles related to cytokine signaling.[631,632] Shp-1 mutations cause the motheaten (*me*) and viable motheaten (*me*v) phenotypes in mice.[633,634] The viable motheaten mouse had a less severe phenotype that is associated with increased numbers of erythroid progenitor cells and hyperresponsiveness to Epo,[635] suggesting that Shp-1 might normally diminish responsiveness to Epo. Indeed, it was demonstrated that Shp-1 binds directly to the EpoR when Y429 is phosphorylated.[636] This tyrosine is located in a "negative" regulatory region of the EpoR, and when mutated, Epo-responsive cells can grow in lower concentrations of Epo. Following Shp-1 binding to Y429, dephosphorylation and inactivation of JAK2 is facilitated.[636] Thus, the negative regulation of Epo signaling appears to be at the level of a receptor-dependent inactivation of a JAK kinase. Shp-1 has also been shown to interact with β_C and to mediate diminished IL-3-induced signaling[637] and to be able to associate with both TYK2[638] and JAK2.[639]

Shp-2 has generally been considered primarily an "activating" phosphatase; it is therefore interesting that it can also interact with JAK1, JAK2, and TYK2.[640] In addition to the presumed dephosphorylation of JAK kinases by phosphatases, STAT proteins also appear to be regulated at the level of tyrosine dephosphorylation,[641] and interestingly, it has been shown that dephosphorylation of phosphotyrosine on STAT1 dimers requires extensive spatial reorientation of the two monomers within the dimer, something that is facilitated by the N-terminal domain that is involved in tetramerization.[642] Finally, another type of phosphatase, namely the lipid phosphatases, known as SHIP and SHIP2, can act as negative regulators of cytokine signals.[643] In addition to Shp-1 and Shp-2, CD45, PTP1B, and T-cell PTP (TCPTP) have been reported to regulate JAK kinases. CD45 appears to play roles related to Epo and IFN signaling. PTP1B has been reported to dephosphorylate JAK2 and TYK2, and TCPTP has been reported to dephosphorylate JAK1 and JAK3.[449] In the cytosol, Shp-2 and PTP1B have also been reported to dephosphorylate cytosolic STAT5 and nuclear STAT1 and STAT3, whereas TCPTP has been reported to dephosphorylate STAT1 and STAT3 in both cytosol and nucleus, but additional work is needed to determine whether these events are occurring physiologically in vivo.[449]

In addition to dephosphorylation, another mode of negative regulation is by degradation. In addition to the degradation of receptor molecules, STAT1 itself is a target of the ubiquitin-proteasome pathway.[644] Finally, it is possible that regulation also can occur at the level of alternative splicing. In this regard, alternatively spliced versions of some of the STATs[448,508,515] have been reported.

THE CIS/SOCS/JAB/SSI FAMILY OF INHIBITORY ADAPTER PROTEINS

In 1995, the protoype molecule for an interesting class of proteins was discovered. The prototype molecule was named CIS, for cytokine inducible, SH2-containing protein, and it was shown to negatively regulate the actions of a set of cytokines.[645,646] CIS is rapidly induced by a variety of cytokines, including IL-2, IL-3, GM-CSF, and Epo, to physically associate with both the β_C and Epo receptors.[645,646] Subsequently, a related protein,

variably denoted SOCS-1, JAB (JAK-binding protein), and SSI-1 (STAT-induced STAT inhibitor-1) was identified that could negatively regulate the activity of other cytokines, including IL-6[647,648,649] and IL-2, among others. Interestingly, this protein could associate with JAK family kinases, whereas this function has not been reported for CIS. A total of eight CIS/SOCS/JAB/SSI family members have been identified that collectively regulate signals in response to multiple cytokines.[650,651,652,653,654] These proteins are now generally known as SOCS proteins and share a central SH2 domain and a region known as a C-terminal region known as a SOCS box. Additional SOCS box-containing proteins lack SH2 domains and include proteins known as ASBs (ankyrin repeat-containing proteins with a SOCS box), SSBs (SPRY domain-containing proteins with a SOCS box), and WSBs (WD40 repeat-containing proteins with a SOCS box). SOCS proteins tend to be expressed at very low levels in nonactivated cells and to be induced by cytokines and pathogens. Following their induction, they negatively influence cytokine signaling, serving as negative feedback regulators. Their actions can extend beyond type I cytokines, for example, SOCS-1 can negatively regulate LPS responses.[655]

Knockout mice for a number of the SOCS proteins have been prepared, alone and in combination. CIS knockout mice are relatively normal, but CIS transgenic mice exhibit low body weight, failure of lactation, and diminished numbers of NK and NKT cells as well as altered TCR-mediated responses, presumably related to its ability to inhibit STAT5-dependent responses including those related to growth hormone, prolactin, IL-15, and IL-2, as well as others. SOCS-1 knockout exhibit multiorgan inflammation and neonatal lethality as the result of augmented responsiveness

to IFNγ, and this lethality can be prevented if the mice are crossed to IFNγ-deficient mice. Interestingly, SOCS-1-/- mice also indicate an essential role for SOCS-1 in thymocyte differentiation, and there are diminished numbers of maturing B cells. Deletion of SOCS-1 within thymocytes/T cells/NKT cells results in multiple lymphoid abnormalites with increased CD8+ T cells due to increased sensitivity to γ_C-dependent cytokines, including IL-7.[656] SOCS-2 knockout mice exhibit gigantism, ostensible due to dysregulated growth hormone signaling. SOCS-2 knockout T cells exhibit enhanced Th2 differentiation, and knockout mice exhibit elevated Th2 responses after helminth infection.[657] SOCS-3 knockout mice exhibit embryonic lethality due to placental insufficiency and dysregulated responses to LIF and IL-6 as well as hematopoietic defects. Conditional SOCS-3 knockout mice have revealed a physiologic role for SOCS-3 in regulating G-CSF signaling in myeloid cells and for "emergency" granulopoiesis[658] as well as for IL-6/gp130 signaling.[659,660] SOCS-6 knockout have mild growth retardation.[449,652,661] Additional SOCS knockout mice and various combinations continue to be generated, adding additional information to this important area of negative regulation of STAT-dependent signaling.

PROTEIN INHIBITORS OF ACTIVATED SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION PROTEINS

Protein inhibitors of activated STAT (PIAS) proteins are other negative regulators of cytokine actions,[449,662,663,664,665,666] with PIAS-1 being an inhibitor of STAT1-binding activity and PIAS-3 being a similar inhibitor of STAT3-binding activity. These PIAS proteins block the DNA-binding activity of STAT dimers. Two other members, PIASx (which has both α and β splice variants) and PIASy, have also been described, with PIASx inhibiting STAT4 and PIASY inhibiting STAT1. They appear to act at least in part as transcriptional corepressors by recruiting other proteins such as histone deacetylase. In addition, PIASx and PIASy have added actions not restricted to the context of STAT inhibition. For example, PIASy is a nuclear matrix-associated SUMO E3 ligase (a ubiquitin-related protein) that can repress the activitity

of the Wnt-responsive transcription factor, LEF1.[665] PIASy coexpression results in the covalent modification of LEF1 by SUMO. Thus, as a class, PIAS proteins have more than one type of action.[449,667]

DISEASES OF CYTOKINE RECEPTORS AND RELATED MOLECULES

A Range of Cytokine-Related Causes of Severe Combined Immunodeficiency

As detailed previously, mutations in the γ_C cause X-linked SCID, and mutations in JAK3 cause a similar T-B+NK-SCID. Given that γ_C-dependent cytokines activate primarily STAT5A and STAT5B as signaling molecules downstream of the JAKs, it remains an open question as to whether mutations in these STAT proteins also cause human disease. Although one could hypothesize that mutations in either STAT5A or STAT5B alone might not cause a phenotype due to potential redundancy, mice lacking STAT5A or STAT5B exhibit defects in T- and NK-cell numbers as well as T-cell proliferation and NK cytolytic activity,[511,512] and mice lacking both STAT5A and STAT5B have an even more profound defect, with absent T-cell[573] and NK-cell development.[513] Moreover, STATB mutations have been found and cause growth hormone insensitivity, IGF-1 deficiency, and immune dysfunction.[573a,573b]

It can be predicted that human immunodeficiencies might also result from mutations in some of the cytokines whose receptors contain γ_C or from mutations in other components of the receptors for these cytokines. Indeed, as noted previously, patients with IL-2 deficiency exhibit a SCID-like syndrome, due to inadequate function of their T cells, and recently, an unusual immunodeficiency has been found to result from a mutation in IL-2Rα.[369] One patient with defective IL-2Rβ expression also had an immunodeficiency syndrome characterized by autoimmunity,[668] somewhat analogous to IL-2Rβ-deficient mice. Given that mutations in IL-7Rα in humans cause T-B+NK+ SCID,[206] mutations in IL-7 might be predicted to cause a similar syndrome. The one major difference might be that IL-7-deficient humans might not be capable of receiving a successful bone marrow transplant if stromal IL-7 is required for the graft. Such patients have not yet been identified. Given the defective NK-cell development and CD8+ memory T-cell development in IL-15- and IL-15Rα-deficient mice, it is likely that these types of defects would also occur in humans lacking either of these proteins. However, again, such patients have not yet been identified. Although IL-9 transgenic mice develop lymphomas,[669] IL-9-deficient mice exhibit defects related to mast cells and mucous production rather than lymphoid defects. Thus, defects related to the IL-9 system seem unlikely as causes of SCID. At present, defective expression of IL-2, IL-2Rα, IL-2Rβ, IL-7Rα, γ_C, and JAK3 are the only cytokinerelated mutations that have been found to cause SCID, with TYK2 being implicated in another form of immunodeficiency. More time will be required to determine whether mutations in other cytokines, cytokine receptors, JAKs, or STATs can also cause SCID.

Defects in the Ability to Clear Mycobacterial Infections and Chronic Mucocutaneous Candidiasis

A number of immunodeficiencies have been characterized where affected individuals cannot properly clear mycobacterial infections. These have also turned out to be diseases of defective cytokine signaling. Mutations have been found in the components of either IL-12 itself or in the IFN or IL-12/IL-23 receptors, with mutations having been found in either the gene encoding the p40 subunit of IL-12 (which as noted previously is also a component of IL-23),[670] in IL-12Rβ1 (a component of both the IL-12 and IL-23 receptors),[671] or in either the IFNGR-1 or IFNGR-2 components of IFNγ receptors.[672,673] The critical role of IL-12 for Th1 cell-mediated differentiation and production of IFNγ provides the explanation for finding

similar clinical syndromes in humans lacking the p40, IL-12Rβ1, IFNGR1, or IFNGR-2. Moreover,

one patient with a mutation in the STAT1 gene was also identified with a similar clinical syndrome,[674] indicating that as anticipated, STAT1 is a critical mediator of IFNγ signaling. Interestingly, this patient had a mutation on only one STAT1 allele, but the mutation was a dominant negative mutations that selectively inhibits the formation of STAT1 dimers (hence abrogating IFNγ signaling) but yet had at most only a modest effect on the ability to form ISGF3, hence leaving signaling in response to IFNα/β relatively intact. Chronic mucocutaneous candidiasis can be caused by autosomal dominant IL-17F or autosomal recessive IL-17RA deficiency or by gain-of-function heterozygous STAT1 mutant alleles that impair IL-17 immunity.[675]

Somatic mutations that activate STAT3 occur in benign liver adenomas, suggesting a role for IL-6-STAT3 pathway in human hepatocellular tumorigenesis.[676]

Mutations in the WSX-1/TCCR Type I Receptor

Interestingly, there is a type I cytokine receptor denoted as TCCR or WSX-1 that is related to IL-12β2 and its mutations results in defective Th1-related responses and diminished IFNγ production, resulting in susceptible to *Leishmania major* and *Listeria monocytogenes*.[254,677,678] TCCR/WSX-1 is an essential component of the receptor for IL-27[300] and also has been found to be required for resistance to *Trypanosoma cruzi*.[679,680]

Other Diseases Associated with Cytokine Receptors

A number of other diseases have been reported that related to cytokine receptors. First, mutations in the growth hormone receptor have been found in a form of dwarfism (Laron dwarfism)[681] in which target cells cannot respond to growth hormone. Interestingly, some aspects of STAT5B deficiency are related to this syndrome. Second, a single patient with a form of congenital neutropenia (Kostmann syndrome) has been found to have a mutation in one of his G-CSF receptor alleles.[682] Third, a kindred of patients with familial erythrocytosis has truncation in the erythropoietin receptor, resulting in hypersensitivity to Epo.[683] Fourth, an altered virally encoded form (v-mpl) of the thrombopoietin receptor (c-mpl) was originally identified as the oncogene of the myeloproliferative leukemia virus.[684] Finally, gain-of-function mutations in *IL7R*[685] and genomic aberrations of *TSLPR* (also known as *CRLF2*)[686,687,688] have been found in childhood acute lymphoblastic leukemias, sometimes associated with mutations in JAK2 or JAK1 or TLSPR itself.

Modulation of Cytokines and the Clinic

Certain diseases are associated with increased levels of cytokines or other situations where treatment with an anticytokine receptor antibody is a rationale therapy. One major example is Castleman disease, which is associated with overproduction of IL-6 by lymph node cells, leading to the successful treatment of this disease with IL-6 receptor blockade.[257,689] Anti-IL-6R-based therapy also has utility for rheumatoid arthritis and possibly for Crohn disase.[257,690] Blocking the IL-2 receptor has been used in the treatment of patients with adult T-cell leukemia and other neoplasias, and the use of humanized and conjugated antibodies have produced responses in a number of individuals. Humanized anti-Tac monoclonal antibody, marketed under the name daclizumab, has shown very strong efficacy in the treatment of allograft rejection as well as in T-cell-mediated autoimmune disorders, including multiple sclerosis, uveitis, and tropical spastic paraparesis. Conjugated antibodies

to both IL-2Rα and IL-2Rβ are also being tested for use in a variety of malignant disorders, wherein the malignant cells express these proteins. Thus, there is the possibility of therapy, either based on blocking the cytokine or based on eliminating the responding cells.[691]

CONCLUSION

Type I cytokines and IFNs are involved in the regulation of an enormous number of immunologic and nonimmunologic processes. There has been a progressive transition from viewing these as discrete molecules with special actions to sets of molecules that can be grouped according to shared receptor components and common signaling pathways. Signaling is one area where our understanding has greatly expanded; the pathways that are activated are similar for many cytokines, even when the biologic functions they induce are dramatically different. Although some of the differences can be explained by "compartmentalization" according to which cells produce the cytokine and which cells express receptors that allow them to respond to the cytokine, a tremendous amount still needs to be learned about how distinctive signals are triggered as well as more regarding the sets of genes that are induced by each cytokine. These will provide vital information important to the quest to completely understand the mechanisms by which type I cytokines and IFNs can effect their actions. At the same time, the generation of knockout mice for most cytokines and their receptors, as well as many signaling molecules, has provided in vivo clues as to vital functions served by these cytokines. Caution is clearly needed, however, in generalizing from these findings to human biology, given some apparently major differences in roles served, such as the essential role played by IL-7 in both humans and mice for T-cell development, whereas IL-7 is also essential for B-cell development in mice but not in humans. The identification of so many humans disorders associated with cytokines and cytokine receptors has tremendously helped to teach us more about normal human biology as well.

REFERENCES

1. Dumonde DC, Wolstencroft RA, Panayi GS, Matthew M, Morley J, Howson WT. "Lymphokines": non-antibody mediators of cellular immunity generated by lymphocyte activation. *Nature*. 1969;224(214):38-42.

2. Cohen S, Bigazzi PE, Yoshida T. Commentary. Similarities of T cell function in cell-mediated immunity and antibody production. *Cell Immunol*. 1974;12(1):150-159.

3. Waksman BH. *Immunology, The Making of a Modern Science* . London, United Kingdom: Academic Press; 1995.

4. Oppenheim JJ, Gery I. From lymphodrek to interleukin 1 (IL-1). *Immunol Today*. 1993;14(5):232-234.

5. Wlodawer A, Pavlovsky A, Gustchina A. Hematopoietic cytokines: similarities and differences in the structures, with implications for receptor binding. *Protein Sci*. 1993;2(9):1373-1382.

6. Bazan JF. Neuropoietic cytokines in the hematopoietic fold. *Neuron*. 1991;7(2):197-208.

7. Rozwarski DA Gronenborn AM, Clore GM, et al. Structural comparisons among the short-chain helical cytokines. *Structure*. 1994;2(3):159-173.

8. Sprang SR, Bazan JF. Cytokine structural taxonomy and mechanisms of receptor engagement. *Curr Opin Struct Biol* . 1993;3:815.

9. Wang X, Lupardus P, Laporte SL, Garcia KC. Structural biology of shared cytokine receptors. *Ann Rev Immunol* . 2009;27:29-60.

10. Zhang F, Basinksi MB, Beals JM, et al. Crystal structure of the obese protein leptin-E100. *Nature*. 1997;387(6629):206-209.

11. Rochman Y, Spolski R, Leonard WJ. New insights into the regulation of T cells by gamma(c) family cytokines. *Nat Rev Immunol* . 2009;9(7):480-490.

12. Davies DR, Wlodawer A. Cytokines and their receptor complexes. *FASEB J*. 1995;9(1):50-56.

13. Milburn MV, Hassell AM, Lambert MH, et al. A novel dimer configuration revealed by the crystal structure at 2.4 A resolution of human interleukin-5. *Nature*. 1993;363(6425):172-176.

14. Senda T, Shimazu T, Matsuda S, et al. Three-dimensional crystal structure of recombinant murine interferon-beta. *EMBO J*. 1992;11(9):3193-3201.

15. Ealick SE, Cook WJ, Vijay-Kumar S, et al. Three-dimensional structure of recombinant human interferon-gamma. *Science*. 1991;252(5006): 698-702.

16. Zdanov A, Schalk-Hihi C, Gustchina A, Tsang M, Weatherbee J, Wlodawer A. Crystal structure of interleukin-10 reveals the functional dimer with an unexpected topological similarity to interferon gamma. *Structure*. 1995;3(6):591-601.

17. D'Andrea AD, Fasman GD, Lodish HF. Erythropoietin receptor and interleukin-2 receptor beta chain: a new receptor family. *Cell*. 1989;58(6): 1023-1024.

18. Bazan JF. Structural design and molecular evolution of a cytokine receptor superfamily. *Proc Natl Acad Sci U S A* . 1990;87(18):6934-6938.

19. de Vos AM, Ultsch M, Kossiakoff AA. Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science*. 1992;255(5042):306-312.

20. Somers W, Ultsch M, De Vos AM, Kossiakoff AA. The X-ray structure of a growth hormone-prolactin receptor complex. *Nature*. 1994;372(6505):478-481.

21. Livnah O, Stura EA, Johnson DL, et al. Functional mimicry of a protein hormone by a peptide agonist: the EPO receptor complex at 2.8 A. *Science*. 1996;273(5274):464-471.

22. Hage T, Sebald W, Reinemer P. Crystal structure of the interleukin-4/receptor alpha chain complex reveals a mosaic binding interface. *Cell*. 1999;97(2):271-281.

23. Wang X, Rickert M, Garcia KC. Structure of the quaternary complex of interleukin-2 with its alpha, beta, and gammac receptors. *Science*. 2005;310(5751):1159-1163.

24. Lupardus PJ, Skiniotis G, Rica AJ, et al. Structural snapshots of full-length Jak1, a transmembrane gp130/IL-6/IL-6Ralpha cytokine receptor complex, and the receptor-Jak1 holocomplex. *Structure*. 2011;19(1):45-55.

25. Lupardus PJ, Birnbaum ME, Garcia KC. Molecular basis for shared cytokine recognition revealed in the structure of an unusually high affinity complex between IL-13 and IL-13Ralpha2. *Structure*. 2010;18(3): 332-342.

26. Murakami M, Narazaki M, Hibi M, et al. Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family. *Proc Natl Acad Sci U S A*. 1991;88(24):11349-11353.

27. Wrighton NC, Farrell FX, Chang R, et al. Small peptides as potent mimetics of the protein hormone erythropoietin. *Science*. 1996;273(5274): 458-464.

28. Stauber DJ, Debler EW, Horton PA, Smith KA, Wilson IA.Crystal structure of the IL-2 signaling complex: paradigm for a heterotrimeric cytokine receptor. *Proc Natl Acad Sci U S A*. 2006;103(8): 2788-2793.

29. Kim HP, Imbert J, Leonard WJ. Both integrated and differential regulation of components of the IL-2/IL-2 receptor system. *Cytokine Growth Factor Rev*. 2006;17(5):349-366.

30. Leonard WJ. Cytokines and immunodeficiency diseases. *Nat Rev Immunol*. 2001;1(3):200-208.

31. Chow D, He X, Snow AL, Rose-John S, Garcia KC. Structure of an extracellular gp130 cytokine receptor signaling complex. *Science*. 2001;291(5511):2150-2155.

32. Boulanger MJ, Chow DC, Brevnova EE, Garcia KC. Hexameric structure and assembly of the interleukin-6/IL-6 alpha-receptor/gp130 complex. *Science*. 2003;300(5628):2101-2104.

33. Boulanger MJ, Bankovich AJ, Kortemme T, Baker D, Garcia KC. Convergent mechanisms for recognition of divergent cytokines by the shared signaling receptor gp130. *Mol Cell*. 2003;12(3):577-589.

34. LaPorte SL, Juo SZ, Vaclavikova J, et al. Molecular and structural basis of cytokine receptor pleiotropy in the interleukin-4/13 system. *Cell*. 2008;132(2):259-272.

35. Hansen G, Hercus TR, McClure BJ, et al. The structure of the GM-CSF receptor complex reveals a distinct mode of cytokine receptor activation. *Cell*. 2008;134(3):496-507.

36. Takeshita T, Asao H, Ohtani K, et al. Cloning of the gamma chain of the human IL-2 receptor. *Science*. 1992;257(5068):379-382.

37. Noguchi M, Nakamura Y, Russel SM, et al. Interleukin-2 receptor gamma chain: a functional component of the interleukin-7 receptor. *Science*. 1993;262(5141):1877-1880.

38. Russell SM, Keegan AD, Harada N, et al. Interleukin-2 receptor gamma chain: a functional component of the interleukin-4 receptor. *Science*. 1993;262(5141):1880-1883.

39. Kondo M, Takeshita T, Ishii N, et al. Sharing of the interleukin-2 (IL-2) receptor gamma chain between receptors for IL-2 and IL-4. *Science*. 1993;262(5141):1874-1877.

40. Kondo M, Takeshita T, Higuchi M, et al. Functional participation of the IL-2 receptor gamma chain in IL-7 receptor complexes. *Science*. 1994;263(5152):1453-1454.

41. Russell SM, Johnston JA, Noguchi M, et al. Interaction of IL-2R beta and gamma c chains with Jak1 and Jak3: implications for XSCID and XCID. *Science*. 1994;266(5187):1042-1045.

42. Kimura Y, Takeshita T, Kondo M, et al. Sharing of the IL-2 receptor gamma chain with the functional IL-9 receptor complex. *Int Immunol*. 1995;7(1):115-120.

43. Giri JG, Ahdieh M, Eisenman J, et al. Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO J*. 1994;13(12):2822-2830.

44. Asao H, Okuyama C, Kumaki S, et al. Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex. *J Immunol*. 2001;167(1):1-5.

45. Leonard WJ. The molecular basis of X-linked severe combined immunodeficiency: defective cytokine receptor signaling. *Annu Rev Med*. 1996;47:229-239.

46. Taniguchi T, Matsui H, Fujita T, et al. Structure and expression of a cloned cDNA for human interleukin-2. *Nature*. 1983;302(5906):305-310.

47. Leonard WJ, Depper JM, Crabtree GR, et al. Molecular cloning and expression of cDNAs for the human interleukin-2 receptor. *Nature*. 1984;311(5987):626-631.

48. Nikaido T, Shimizu A, Ishida N, et al. Molecular cloning of cDNA encoding human interleukin-2 receptor. *Nature*. 1984;311(5987): 631-635.

49. Lin JX, Leonard WJ. Signaling from the IL-2 receptor to the nucleus. *Cytokine Growth Factor Rev*. 1997;8(4):313-332.

50. Lin JX, Leonard WJ. *The Cytokine Handbook*. 4th ed. San Diego, CA: Academic Press; 2001.

51. Morgan DA, Ruscetti FW, Gallo R. Selective in vitro growth of T lymphocytes from normal human bone marrows. *Science*. 1976;193(4257):1007-1008.

52. Siegel LJ, Harper ME, Wong-Staal F, Gallo RC, Nash WG, O'Brien SJ. Gene for T-cell growth factor: location on human chromosome 4q and feline chromosome B1. *Science*. 1984;223:175-178.

53. Leonard WJ, Depper JM, Uchiyama T, Smith KA, Waldmann TA, Greene WC. A monoclonal antibody that appears to recognize the receptor for human T-cell growth factor; partial characterization of the receptor. *Nature*. 1982;300(5889):267-269.

54. Sharon M, Klausner RD, Cullen BR, Chizzonite R, Leonard WJ. Novel interleukin-2 receptor subunit detected by cross-linking under highaffinity conditions. *Science*. 1986;234(4778):859-863.

55. Tsudo M, Kozak RW, Goldman CK, Waldmann TA. Demonstration of a non-Tac peptide that binds interleukin 2: a potential participant in a multichain interleukin 2 receptor complex. *Proc Natl Acad Sci U S A* . 1986;83(24):9694-9698.

56. Teshigawara K, Wang HM, Kato K, Smith KA. Interleukin 2 high-affinity receptor expression requires two distinct binding proteins. *J Exp Med*. 1987;165(1):223-238.

57. Hatakeyama M, Tsudo M, Minamoto S, et al. Interleukin-2 receptor beta chain gene: generation of three receptor forms by cloned human alpha and beta chain cDNA's. *Science*. 1989;244(4904):551-556.

58. Blackman MA, Tigges MA, Minie ME, Koshland ME. A model system for peptide hormone action in differentiation: interleukin 2 induces a B lymphoma to transcribe the J chain gene. *Cell*. 1986;47(4):609-617.

59. Waldmann TA. The multi-subunit interleukin-2 receptor. *Annu Rev Biochem*. 1989;58:875-911.

60. Siegel JP, Sharon M, Smith PL, Leonard WJ. The IL-2 receptor beta chain (p70): role in mediating signals for LAK, NK, and proliferative activities. *Science*. 1987;238(4823):75-78.

61. Lanier LL, Phillips JH. Natural killer cells. *Curr Opin Immunol* . 1992;4(1):38-42.

62. Janssen RA, Mulder NH, The TH, de Leij L. The immunobiological effects of interleukin-2 in vivo. *Cancer Immunol Immunother* . 1994;39(4):207-216.

63. Lenardo M, Cham KM, Hornung F, et al. Mature T lymphocyte apoptosis —immune regulation in a dynamic and unpredictable antigenic environment. *Ann Rev Immunol* . 1999;17:221-253.

64. Malek TR, Yu A, Vincek V, Scibelli P, Kong L. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. Implications for the nonredundant function of IL-2. *Immunity*. 2002;17(2):167-178.

65. Malek TR, Castro I. Interleukin-2 receptor signaling: at the interface between tolerance and immunity. *Immunity*. 2010;33(2):153-165.

66. Tang Q, Adams JY, Penaranda C, et al. Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity*. 2008;28(5):687-697.

67. Williams MA, Tyznik AJ, Bevan MJ. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature*. 2006;441(7095):890-893.

68. Schoenberger SP, Janssen EM. IL-2 gets with the program. *Nat Immunol*. 2006;7(8):798-800.

69. Feau S, Arens R, Togher S, Schoenberger SP. Autocrine IL-2 is required for secondary population expansion of CD8(+) memory T cells. *Nat Immunol*. 2011;12(9):908-913.

70. Pipkin ME, Sacks JA, Cruz-Guilloty F, et al. Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity*. 2010;32(1):79-90.

71. Kalia V, Sarkar S, Subramaniam S, Haining WN, Smith KA, Ahmed R. Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo. *Immunity*. 2010; 32(1):91-103.

72. Pepper M, Pagan AJ, Igyarto BZ, Taylor JJ, Jenkins MK. Opposing signals from the Bcl6 transcription factor and the interleukin-2 receptor generate T helper 1 central and effector memory cells. *Immunity*. 2011;35(4):583-595.

73. Hershko AY, Suzuki R, Charles N, et al. Mast cell interleukin-2 production contributes to suppression of chronic allergic dermatitis. *Immunity*. 2011;35(4):562-571.

74. Liao W, Lin JX, Wang L, Li P, Leonard WJ. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. *Nat Immunol*. 2011;12(6):551-559.

75. Zhu J, Cote-Sierra J, Guo L, Paul WE. Stat5 activation plays a critical role in Th2 differentiation. *Immunity*. 2003;19(5):739-748.

76. Cote-Sierra J, Foucras G, Guo L, et al. Interleukin 2 plays a central role in Th2 differentiation. *Proc Natl Acad Sci U S A* . 2004;101(11): 3880-3885.

77. Liao W, Schones DE, Oh J, et al. Priming for T helper type 2 differentiation by interleukin 2-mediated induction of interleukin 4 receptor alpha-chain expression. *Nat Immunol*. 2008;9(11):1288-1296.

78. Liao W, Lin JX, Leonard WJ. IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation. *Curr Opin Immunol* . 2011;23(5):598-604.

79. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*. 1986;136(7):2348-2357.

80. Lucey DR, Clerici M, Shearer GM. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. *Clin Microbiol Rev*. 1996;9(4):532-562.

81. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature*. 1996;383(6603):787-793.

82. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today*. 1996;17(3):138-146.

83. Jankovic D, Sher A, Yap G. Th1/Th2 effector choice in parasitic infection: decision making by committee. *Curr Opin Immunol* . 2001;13(4):403-409.

84. Romagnani S. *Cytokine Reference*. Edited by JJ Oppenheim and M Feldman. San Diego, CA: Academic Press; 2001:99-112.

85. Shi M, Lin TH, Appell KC, Berg LJ. Janus-kinase-3-dependent signals induce chromatin remodeling at the Ifng locus during T helper 1 cell differentiation. *Immunity*. 2008;28(6):763-773.

86. Laurence A, Tato CM, Davidson TS, et al. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity*. 2007;26(3): 371-381.

87. Lazarevic V, Chen X, Shim JH, et al. T-bet represses T(H)17 differentiation by preventing Runx1-mediated activation of the gene encoding RORgammat. *Nat Immunol* . 2011;12(1):96-104.

88. Nelms K, Keegan AD, Zamorano J, Ryan JJ, Paul WE. The IL-4 receptor: signaling mechanisms and biologic functions. *Ann Rev Immunol* . 1999; 17:701-738.

89. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Ann Rev Immunol*. 2010;28:445-489.

90. Yoshimoto T, Bendelac A, Watson C, Hu-Li J, Paul WE. Role of NK1.1+ T cells in a TH2 response and in immunoglobulin E production. *Science*. 1995;270(5243):1845-1847.

91. Paul W. *IL-4: Structure and Function*. Boca Raton, FL: CRC Press; 1992.

92. Paul WE. Interleukin-4: a prototypic immunoregulatory lymphokine. *Blood*. 1991;77(9):1859-1870.

93. Boulay JL, Paul WE. Hematopoietin sub-family classification based on size, gene organization and sequence homology. *Curr Biol* . 1993; 3(9):573-581.

94. Mosley B, Beckmann MP, March CJ, et al. The murine interleukin-4 receptor: molecular cloning and characterization of secreted and membrane bound forms. *Cell*. 1989;59(2):335-348.

95. Idzerda RL, March CJ, Mosley B, et al. Human interleukin 4 receptor confers biological responsiveness and defines a novel receptor superfamily. *J Exp Med*. 1990;171(3):861-873.

96. Galizzi JP, Zuber CE, Harada N, et al. Molecular cloning of a cDNA encoding the human interleukin 4 receptor. *Int Immunol* . 1990;2(7): 669-675.

97. Li L, Lee HH, Bell JJ, et al. IL-4 utilizes an alternative receptor to drive apoptosis of Th1 cells and skews neonatal immunity toward Th2. *Immunity*. 2004;20(4):429-440.

98. Namen AE, Lupton S, Hjerrild K, et al. Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature*. 1988;333(6173):571-573.

99. Goodwin RG, Lupton S, Schmierer A, et al. Human interleukin 7: molecular cloning and growth factor activity on human and murine B-lineage cells. *Proc Natl Acad Sci U S A*. 1989;86(1):302-306.

100. Mackall CL, Fry TJ, Gress RE. Harnessing the biology of IL-7 for therapeutic application. *Nat Rev Immunol* . 2011;11(5):330-342.

101. Murray R, Suda T, Wrighton N, Lee F, Zlotnik A. IL-7 is a growth and maintenance factor for mature and immature thymocyte subsets. *Int Immunol* . 1989;1(5):526-531.

102. Watson JD, Morrissey PJ, Namen AE, Conlon PJ, Widmer MB. Effect of IL-7 on the growth of fetal thymocytes in culture. *J Immunol* . 1989;143(4):1215-1222.

103. Peschon JJ, Morrissey PJ, Grabstein KH, et al. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med*. 1994;180(5):1955-1960.

104. von Freeden-Jeffry U, Vieiea P, Lucian LA, McNiel T, Burdach SE, Murray R. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med*. 1995;181(4):1519-1526.

105. Fry TJ, Mackall CL. Interleukin-7: from bench to clinic. *Blood*. 2002;99(11):3892-3904.

106. Tan JT, Dudl E, LeRoy E, et al. IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc Natl Acad Sci U S A* . 2001;98(15):8732-8737.

107. Schluns KS, Kieper WC, Jameson SC, Lefrancois L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat Immunol* . 2000;1(5):426-432.

108. Schluns KS, Lefrancois L. Cytokine control of memory T-cell development and survival. *Nat Rev Immunol* . 2003;3(4):269-279.

109. Seddon B, Tomlinson P, Zamoyska R. Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells. *Nat Immunol* . 2003;4(7):680-686.

110. Chazen GD, Pereira GM, LeGros G, Gillis S, Shevach EM. Interleukin 7 is a T-cell growth factor. *Proc Natl Acad Sci U S A* . 1989;86(15): 5923-5927.

111. Morrissey PJ, Goodwin RD, Nordan RP, et al. Recombinant interleukin 7, pre-B cell growth factor, has costimulatory activity on purified mature T cells. *J Exp Med*. 1989;169(3):707-716.

112. Sawa Y, Arima Y, Ogura H, et al. Hepatic interleukin-7 expression regulates T cell responses. *Immunity*. 2009;30(3):447-457.

113. Chowdhury D, Sen R. Transient IL-7/IL-7R signaling provides a mechanism for feedback inhibition of immunoglobulin heavy chain gene rearrangements. *Immunity*. 2003;18(2):229-241.

114. Malin S, McManus S, Cobaleda C, et al. Role of STAT5 in controlling cell survival and immunoglobulin gene recombination during pro-B cell development *Nat Immunol*. 2010;11(2):171-179.

115. Leonard WJ. The defective gene in X-linked severe combined immunodeficiency encodes a shared interleukin receptor subunit: implications for cytokine pleiotropy and redundancy. *Curr Opin Immunol* . 1994;6(4):631-635.

116. Guimond M, Veenstra RG, Grindler DJ, et al. Interleukin 7 signaling in dendritic cells regulates the homeostatic proliferation and niche size of CD4+ T cells. *Nat Immunol*. 2009;10(2):149-157.

117. Sportes C, Hakim FT, Memon SA, et al. Administration of rhIL-7 in humans increases in vivo TCR repertoire diversity by preferential expansion of naive T cell subsets. *J Exp Med*. 2008;205(7): 1701-1714.

118. Pellegrini M, Calzascia T, Toe JG, et al. IL-7 engages multiple mechanisms to overcome chronic viral infection and limit organ pathology. *Cell*. 2011;144(4):601-613.

119. Brunton LL, Lupton SD. An STS in the human IL7 gene located at 8q12-13. *Nucleic Acids Res*. 1990;18(5):1315.

120. Goodwin RG, Friend D, Ziegler SF, et al. Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. *Cell*. 1990;60(6):941-951.

121. Xue HH, Kovanen PE, Pose-Masison CA, et al. IL-2 negatively regulates IL-7 receptor alpha chain expression in activated T lymphocytes. *Proc Natl Acad Sci U S A* . 2002;99(21):13759-13764.

122. Park JH, Yu Q, Erman B,et al. Suppression of IL7Ralpha transcription by IL-7 and other prosurvival cytokines: a novel mechanism for maximizing IL-7-dependent T cell survival. *Immunity*. 2004;21(2): 289-302.

123. Xue HH, Bollenbacher J, Rovella V, et al. GA binding protein regulates interleukin 7 receptor alpha-chain gene expression in T cells. *Nat Immunol*. 2004;5(10):1036-1044.

124. Chandele A, Joshi NS, Zhu J, Paul WE, Leonard WJ, Kaech SM. Formation of IL-7Ralphahigh and IL-7Ralphalow CD8 T cells during infection is regulated by the opposing functions of GABPalpha and Gfi-1. *J Immunol*. 2008;180(8):5309-5319.

125. DeKoter RP, Lee HJ, Singh H. PU.1 regulates expression of the interleukin-7 receptor in lymphoid progenitors. *Immunity*. 2002;16(2):297-309.

126. Van Snick J, Goethals A, Renauld JC, et al. Cloning and characterization of a cDNA for a new mouse T cell growth factor (P40). *J Exp Med*. 1989;169(1):363-368.

127. Demoulin JB, Renauld JC. Interleukin 9 and its receptor: an overview of structure and function. *Int Rev Immunol* . 1998;16(3-4):345-364.

128. Hultner L, Moeller J, Schmitt E, et al. Thiol-sensitive mast cell lines derived from mouse bone marrow respond to a mast cell growthenhancing activity different from both IL-3 and IL-4. *J Immunol* . 1989;142(10):3440-3446.

129. Erpenbeck VJ, Hohlfeld JM, Volkmann B, et al. Segmental allergen challenge in patients with atopic asthma leads to increased IL-9 expression in bronchoalveolar lavage fluid lymphocytes. *J Allergy Clin Immunol* . 2003;111(6):1319-1327.

130. Temann UA, Ray P, Flavell RA. Pulmonary overexpression of IL-9 induces Th2 cytokine expression, leading to immune pathology. *J Clin Invest*. 2002;109(1):29-39.

131. Wilhelm C, Hirota K, Stieglitz B, et al. An IL-9 fate reporter demonstrates the induction of an innate IL-9 response in lung inflammation. *Nat Immunol*. 2011;12(11):1071-1077.

132. Renauld JC, Vink A, Louahed J, Van Snick J. Interleukin-9 is a major antiapoptotic factor for thymic lymphomas. *Blood*. 1995;85(5):1300-1305.

133. Townsend JM, Fallon GP, Matthews JD, Smith P, Jolin EH, McKenzie NA. L-9-deficient mice establish fundamental roles for IL-9 in pulmonary mastocytosis and goblet cell hyperplasia but not T cell development. *Immunity*. 2000;13(4):573-583.

134. Temann UA, Geba GP, Rankin JA, Flavell RA. Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. *J Exp Med*. 1998;188(7):1307-1320.

135. McMillan SJ, Bishop B, Townsend MJ, McKenzie AN, Lloyd CM. The absence of interleukin 9 does not affect the development of allergeninduced pulmonary inflammation nor airway hyperreactivity. *J Exp Med*. 2002;195(1):51-57.

136. Renauld JC. New insights into the role of cytokines in asthma. *J Clin Pathol*. 2001;54(8):577-589.

137. Staudt V, Bothur E, Klein M, et al. Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity*. 2010;33(2):192-202.

138. Noelle RJ, Nowak EC. Cellular sources and immune functions of interleukin-9. *Nat Rev Immunol*. 2010;10(10):683-687.

139. Dardalhon V, Awasthi A, Kwon H, et al. IL-4 inhibits TGF-betainduced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat Immunol*. 2008;9(12):1347-1355.

140. Veldhoen M, Uyttekhove C, van Snick J, et al. Transforming growth factor-beta ‘reprograms’ the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol* . 2008;9(12): 1341-1346.

141. Elyaman W, Bradshaw EM, Uyttenhove C, et al. IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells. *Proc Natl Acad Sci U S A*. 2009;106(31):12885-12890.

142. Nowak EC, Weaver CT, Turner H, et al. IL-9 as a mediator of Th17-driven inflammatory disease. *J Exp Med*. 2009;206(8):1653-1660.

143. Modi WS, Pollock DD, Mock BA, et al. Regional localization of the human glutaminase (GLS) and interleukin-9 (IL9) genes by in situ hybridization. *Cytogenet Cell Genet*. 1991;57(2-3):114-116.

144. Renauld JC, Druez C, Kermouni A, et al. Expression cloning of the murine and human interleukin 9 receptor cDNAs. *Proc Natl Acad Sci U S A* . 1992;89(12):5690-5694.

145. Grabstein KH, Eisenman J, Shaneback K, et al. Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. *Science*. 1994;264(5161):965-968.

146. Burton JD, Bamford RN, Peters C, et al. A lymphokine, provisionally designated interleukin T and produced by a human adult T-cell leukemia line, stimulates T-cell proliferation and the induction of lymphokineactivated killer cells. *Proc Natl Acad Sci U S A*. 1994;91(11):4935-4939.

147. Waldmann TA, Tagaya Y. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Ann Rev Immunol* . 1999;17: 19-49.

148. Waldmann TA. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat Rev Immunol* . 2006;6(8):595-601.

149. Carson WE, Gigi JG, Lindemann MJ, et al. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J Exp Med*. 1994;180(4):1395-1403.

150. Lodolce JP, Boone DL, Chai S, et al. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity*. 1998;9(5):669-676.

151. Kennedy MK, Glaccum M, Brown SN, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J Exp Med*. 2000;191(5):771-780.

152. Zhao H, Nguyen H, Kang J. Interleukin 15 controls the generation of the restricted T cell receptor repertoire of gamma delta intestinal intraepithelial lymphocytes. *Nat Immunol*. 2005;6(12):1263-1271.

153. Kuwajima S, Sato T, Ishida K, Tada H, Tezuka H, Ohteki T. Interleukin 15-dependent crosstalk between conventional and plasmacytoid dendritic cells is essential for CpG-induced immune activation. *Nat Immunol* . 2006;7(7):740-746.

154. Bamford RN, Grant AJ, Burton JD, et al. The interleukin (IL) 2 receptor beta chain is shared by IL-2 and a cytokine, provisionally designated IL-T, that stimulates T-cell

proliferation and the induction of lymphokine-activated killer cells. *Proc Natl Acad Sci U S A*. 1994;91(11):4940-4944.

155. Giri JG, Kumaki S, Ahdieh M, et al. Identification and cloning of a novel IL-15 binding protein that is structurally related to the alpha chain of the IL-2 receptor. *EMBO J*. 1995;14(15):3654-3663.

156. Anderson DM, Kumaki S, Ahdieh M, et al. Functional characterization of the human interleukin-15 receptor alpha chain and close linkage of IL15RA and IL2RA genes. *J Biol Chem*. 1995;270(50):29862-29869.

157. Dubois S, Mariner J, Waldmann TA, Tagaya Y. IL-15Ralpha recycles and presents IL-15 In trans to neighboring cells. *Immunity*. 2002;17(5): 537-547.

158. Wu Z, Xue HH, Bernard J, et al. The IL-15 receptor {alpha} chain cytoplasmic domain is critical for normal IL-15Ralpha function but is not required for trans-presentation. *Blood*. 2008;112(12):4411-4419.

159. Wuest SC, Edwan JH, Martin JF, et al. A role for interleukin-2 transpresentation in dendritic cell-mediated T cell activation in humans, as revealed by daclizumab therapy. *Nat Med*. 2011;17(5):604-609.

160. Lorenzen I, Dingley AJ, Jacques Y, Grotzinger J. The structure of the interleukin-15 alpha receptor and its implications for ligand binding. *J Biol Chem*. 2006;281(10):6642-6647.

161. Waldmann TA, Dubois S, Tagaya Y. Contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for immunotherapy. *Immunity*. 2001;14(2):105-110.

162. Ma A, Koka R, Burkett P. Diverse functions of IL-2, IL-15, and IL-7 in lymphoid homeostasis. *Ann Rev Immunol.* 2006;24:657-679.

163. Oh S, Berzofsky JA, Burke DS, Waldmann TA, Perera LP. Coadministration of HIV vaccine vectors with vaccinia viruses expressing IL-15 but not IL-2 induces long-lasting cellular immunity. *Proc Natl Acad Sci U S A* . 2003;100(6):3392-3397.

164. Steel JC, Waldmann TA, Morris JC. Interleukin-15 biology and its therapeutic implications in cancer. *Trends Pharmacol Sci* . 2012;33(1): 35-41.

165. Spolski R, Leonard WJ. Interleukin-21: basic biology and implications for cancer and autoimmunity. *Ann Rev Immunol* . 2008;26:57-79.

166. Parrish-Novak J, Dillon SR, Nelson A, et al. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature*. 2000;408(6808):57-63.

167. Zeng R, Spolski R, Finkelstein SE, et al. Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function. *J Exp Med*. 2005; 201(1):139-148.

168. Korn T, Bettelli E, Gao W, et al. IL-21 initiates an alternative pathway to induce

proinflammatory T(H)17 cells. *Nature*. 2007;448(7152): 484-487.

169. Zhou L, Ivanov II, Spolski R, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol*. 2007;8(9):967-974.

170. Vogelzang A, McGuire HM, Yu D,Sprent J, Mackay CR, King C. A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity*. 2008;29(1):127-137.

171. Harrington LE, Hatton RD, Mangan PR, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol*. 2005;6(11):1123-1132.

172. Park H, Li Z, Yan XO, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol*. 2005;6(11):1133-1141.

173. Ivanov II, McKenzie BS, Zhou L, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell*. 2006;126(6):1121-1133.

174. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity*. 2006;24(6):677-688.

175. Cua DJ, Sherlock J, Chen Y, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature*. 2003;421(6924):744-748.

176. Veldhoen M, Hocking RJ, Flavell RA, Stockinger B. Signals mediated by transforming growth factor-beta initiate autoimmune encephalomyelitis, but chronic inflammation is needed to sustain disease. *Nat Immunol*. 2006;7(11):1151-1156.

177. Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*. 2006;441(7090):235-238.

178. Ozaki K, Spolski R, Ettinger R, et al. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J Immunol*. 2004;173(9):5361-5371.

179. Ettinger R, Sims GP, Fairhurst AM, et al. IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. *J Immunol*. 2005;175(12):7867-7879.

180. Kwon H, Thierry-Mieg D, Thierry-Mieg J, et al. Analysis of interleukin-21-induced Prdm1 gene regulation reveals functional cooperation of STAT3 and IRF4 transcription factors. *Immunity*. 2009;31(6):941-952.

181. Leonard WJ, Spolski R. Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. *Nat Rev Immunol*. 2005; 5(9):688-698.

182. Ozaki K, Spolski R, Feng CG, et al. A critical role for IL-21 in regulating immunoglobulin production. *Science*. 2002;298(5598):1630-1634.

183. Wang G, Tschoi M, Spolski R, et al. In vivo antitumor activity of interleukin 21 mediated by natural killer cells. *Cancer Res*. 2003;63(24): 9016-9022.

184. Kasaian MT, Whitters MJ, Carter LL, et al. IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity. *Immunity*. 2002; 16(4):559-569.

185. Hinrichs CS, Spolski R, Paulos CM, et al. IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy. *Blood*. 2008;111(11):5326-5333.

186. Spolski R, Kashyap M, Robinson C, Yu Z, Leonard WJ. IL-21 signaling is critical for the development of type I diabetes in the NOD mouse. *Proc Natl Acad Sci U S A* . 2008;105(37):14028-14033.

187. Bubier JA, Sproule TJ, Foreman O, et al. A critical role for IL-21 receptor signaling in the pathogenesis of systemic lupus erythematosus in BXSB-Yaa mice. *Proc Natl Acad Sci U S A*. 2009;106(5):1518-1523.

188. Wang L, Yu CR, Kim HP, et al. Key role for IL-21 in experimental autoimmune uveitis. *Proc Natl Acad Sci U S A* . 2011;108(23): 9542-9547.

189. Spolski R, Kim HP, Zhu W, Levy DE, Leonard WJ. IL-21 mediates suppressive effects via its induction of IL-10. *J Immunol* . 2009;182(5): 2859-2867.

190. Spolski R, Leonard WJ. IL-21 is an immune activator that also mediates suppression via IL-10. *Crit Rev Immunol* . 2009;30(6):559-570.

191. Cui W, Liu Y, Weinstein JS, Craft J, Kaech SM. An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells. *Immunity*. 2011;35(5):792-805.

192. Siegel AM, Heimall J, Freeman AF, et al. A critical role for STAT3 transcription factor signaling in the development and maintenance of human T cell memory. *Immunity*. 2011;35(5):806-818.

193. Elsaesser H, Sauer K, Brooks DG. IL-21 is required to control chronic viral infection. *Science*. 2009;324(5934):1569-1572.

194. Spolski R, Wang L, Wan CK, et al. IL-21 Promotes the pathologic immune response to pneumovirus infection. *J Immunol* . 2012;188(4): 1924-1932.

195. Publicover J, Goodsell A, Nishimura S, et al. IL-21 is pivotal in determining age-dependent effectiveness of immune responses in a mouse model of human hepatitis B. *J Clin Invest*. 2011;121(3):1154-1162.

196. Ozaki K, Kikly K, Michalovich D, Young PR, Leonard WJ. Cloning of a type I cytokine

receptor most related to the IL-2 receptor beta chain. *Proc Natl Acad Sci U S A* . 2000;97(21):11439-11444.

197. Noguchi M, Yi H, Rosenblatt HM, et al. Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell*. 1993;73(1):147-157.

198. Conley ME. Molecular approaches to analysis of X-linked immunodeficiencies. *Ann Rev Immunol*. 1992;10:215-238.

199. Fischer A, Cavazzana-Calvo M, De Saint Basile G, et al. Naturally occurring primary deficiencies of the immune system. *Ann Rev Immunol* . 1997;15:93-124.

200. Buckley RH. Primary immunodeficiency diseases due to defects in lymphocytes. *N Engl J Med*. 2000;343(18):1313-1324.

201. Cunningham-Rundles C, Ponda PP. Molecular defects in T- and B-cell primary immunodeficiency diseases. *Nat Rev Immunol* . 2005;5(11): 880-892.

202. Haddad E, Le Deist F, Aucourturier P, et al. Long-term chimerism and B-cell function after bone marrow transplantation in patients with severe combined immunodeficiency with B cells: a single-center study of 22 patients. *Blood*. 1999;94(8):2923-2930.

203. Weinberg K, Parkman R. Severe combined immunodeficiency due to a specific defect in the production of interleukin-2. *N Engl J Med* . 1990;322(24):1718-1723.

204. Chatila T, Castigli E, Pahwa R, et al. Primary combined immunodeficiency resulting from defective transcription of multiple T-cell lymphokine genes. *Proc Natl Acad Sci U S A*. 1990;87(24):10033-10037.

205. Schorle H, Holtschke T, Hunig T, Schimpl A, Horak I. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature*. 1991;352(6336):621-624.

206. Puel A, Ziegler SF, Buckley RH, Leonard WJ. Defective IL7R expression in T(-)B(+)NK(+) severe combined immunodeficiency. *Nat Genetics*. 1998;20(4):394-397.

207. Puel A, Leonard WJ. Mutations in the gene for the IL-7 receptor result in T(-)B(+)NK(+) severe combined immunodeficiency disease. *Curr Opin Immunol* . 2000;12(4):468-473.

208. Kuhn R, Rajewsky K, Muller W. Generation and analysis of interleukin-4 deficient mice. *Science*. 1991;254(5032):707-710.

209. Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Kohler G. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature*. 1993;362(6417):245-248.

210. Sadlack B, Kuhn R, Schorle H, et al. Development and proliferation of lymphocytes in mice deficient for both interleukins-2 and -4. *Eur J Immunol* . 1994;24(1):281-284.

211. DiSanto JP, Muller W, Guy-Grand D, Fischer A, Rajewsky K. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor gamma chain. *Proc Natl Acad Sci U S A*. 1995;92(2): 377-381.

212. Cao X, Shores EW, Hu-Li J, et al. Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. *Immunity*. 1995;2(3):223-238.

213. Leonard WJ. Dysfunctional cytokine receptor signaling in severe combined immunodeficiency. *J Investig Med* . 1996;44(6):304-311.

214. Takeshita T, Asao H, Suzuki J, Sugamura K. An associated molecule, p64, with high-affinity interleukin 2 receptor. *Int Immunol* . 1990;2(5): 477-480.

215. Nakamura Y, Russell SM, Mess SA, et al. Heterodimerization of the IL-2 receptor beta- and gamma-chain cytoplasmic domains is required for signalling. *Nature*. 1994;369(6478):330-333.

216. Nelson BH, Lord JD, Greenberg PD. Cytoplasmic domains of the interleukin-2 receptor beta and gamma chains mediate the signal for T-cell proliferation. *Nature*. 1994;369(6478):333-336.

217. Willerford DM, Chen J, Ferry JA, Davidson L, Ma A, Alt FW. Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. *Immunity*. 1995;3(4):521-530.

218. Suzuki H, Kindug TM, Surlonger C, et al. Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta. *Science*. 1995;268(5216):1472-1476.

219. Nakajima H, Shores EW, Noguchi M, Leonard WJ. The common cytokine receptor gamma chain plays an essential role in regulating lymphoid homeostasis. *J Exp Med*. 1997;185(2):189-195.

220. Metcalf D. *The Hematopoietic Colony Stimulating Factors* . Amsterdam, The Netherlands: Elsevier; 1984.

221. Hara T, Miyajima A. Function and signal transduction mediated by the interleukin 3 receptor system in hematopoiesis. *Stem Cells*. 1996;14(6):605-618.

222. Geijsen N, Koenderman L, Coffer PJ. Specificity in cytokine signal transduction: lessons learned from the IL-3/IL-5/GM-CSF receptor family. *Cytokine Growth Factor Rev* . 2001;12(1):19-25.

223. Hida S, Yamasaki S, Sakamoto Y, et al. Fc receptor gamma-chain, a constitutive component of the IL-3 receptor, is required for IL-3-induced IL-4 production in basophils. *Nat Immunol* . 2009;10(2):214-222.

224. Codarri L, Gyulveszi G, Tosevski V, et al. RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat Immunol* . 2011;12(6):560-567.

225. El-Behi M, Ciric B, Dai H, et al. The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat Immunol*. 2011;12(6):568-575.

226. Sonderegger I, Iezzi G, Maier R, Schmitz N, Kurrer M, Kopf M. GMCSF mediates autoimmunity by enhancing IL-6-dependent Th17 cell development and survival. *J Exp Med*. 2008;205(10):2281-2294.

227. Kopf M, Brombacker F, Hodgkin PD, et al. IL-5-deficient mice have a developmental defect in CD5+ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. *Immunity*. 1996;4(1):15-24.

228. Yoshida T, Ikuta K, Sugaya H, et al. Defective B-1 cell development and impaired immunity against Angiostrongylus cantonensis in IL-5R alpha-deficient mice. *Immunity*. 1996;4(5):483-494.

229. Endo Y, Iwamura C, Kuwahara M, et al. Eomesodermin controls interleukin-5 production in memory T helper 2 cells through inhibition of activity of the transcription factor GATA3. *Immunity*. 2011;35(5): 733-745.

230. Itoh N, Yonehara S, Schreurs J, et al. Cloning of an interleukin-3 receptor gene: a member of a distinct receptor gene family. *Science*. 1990;247(4940):324-327.

231. Hara T, Miyajima A. Two distinct functional high affinity receptors for mouse interleukin-3 (IL-3). *EMBO J*. 1992;11(5):1875-1884.

232. Kitamura T, Sato N, Arai K, Miyajima A. Expression cloning of the human IL-3 receptor cDNA reveals a shared beta subunit for the human IL-3 and GM-CSF receptors. *Cell*. 1991;66(6):1165-1174.

233. Takaki S, Tominaga A, Hitoshi Y, et al. Molecular cloning and expression of the murine interleukin-5 receptor. *EMBO J*. 1990;9(13): 4367-4374.

234. Tavernier J, Devos R, Cornelis S, et al. A human high affinity interleukin-5 receptor (IL5R) is composed of an IL5-specific alpha chain and a beta chain shared with the receptor for GM-CSF. *Cell*. 1991;66(6): 1175-1184.

235. Gearing DP, King JA, Gough NM, Nicola NA. Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor. *EMBO J*. 1989;8(12):3667-3676.

236. Park LS, Martin U, Sorenson R, et al. Cloning of the low-affinity murine granulocyte-macrophage colony-stimulating factor receptor and reconstitution of a high-affinity receptor complex. *Proc Natl Acad Sci U S A* . 1992;89(10):4295-4299.

237. Gorman DM, Itoh N, Kitamura T, et al. Cloning and expression of a gene encoding an interleukin 3 receptor-like protein: identification of another member of the cytokine receptor gene family. *Proc Natl Acad Sci U S A* . 1990;87(14):5459-5463.

238. Hayashida K, Kitamura T, Gorman DM, Arai K, Yokota T, Miyajima A. Molecular

cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF): reconstitution of a high-affinity GM-CSF receptor. *Proc Natl Acad Sci U S A*. 1190;87(24):9655-9659.

239. Miyajima A, Kitamura T, Harada N, Yokota T, Arai K. Cytokine receptors and signal transduction. *Ann Rev Immunol* . 1992;10:295-331.

240. Sakamaki K, Miyajima I, Kitamura T, Miyajima A. Critical cytoplasmic domains of the common beta subunit of the human GM-CSF, IL-3 and IL-5 receptors for growth signal transduction and tyrosine phosphorylation. *EMBO J*. 1992;11(10):3541-3549.

241. Cornelis S, Fache I, Van der Heyden J, et al. Characterization of critical residues in the cytoplasmic domain of the human interleukin-5 receptor alpha chain required for growth signal transduction. *Eur J Immunol* . 1995;25(7):1857-1864.

242. Kouro T, Kikuchi Y, Kanazawa H, et al. Critical proline residues of the cytoplasmic domain of the IL-5 receptor alpha chain and its function in IL-5-mediated activation of JAK kinase and STAT5. *Int Immunol* . 1996;8(2):237-245.

243. Nishinakamura R, Miyajima A, Mee PJ, Tybulewicz VL, Murray R. Hematopoiesis in mice lacking the entire granulocyte-macrophage colony-stimulating factor/interleukin-3/interleukin-5 functions. *Blood*. 1996;88(7):2458-2464.

244. Hilton DJ, Hilton AA, Raicevic A, et al. Cloning of a murine IL-11 receptor alpha-chain; requirement for gp130 for high affinity binding and signal transduction. *EMBO J*. 1994;13(20):4765-4775.

245. Hibi M, Murakami M, Saito M, Hirano T, Taga T, Kishimoto T. Molecular cloning and expression of an IL-6 signal transducer, gp130. *Cell*. 1990;63(6):1149-1157.

246. Gearing DP, Corneau MR, Friend DJ, et al. The IL-6 signal transducer, gp130: an oncostatin M receptor and affinity converter for the LIF receptor. *Science*. 1992;255(5050):1434-1437.

247. Taga T, et al. Functional inhibition of hematopoietic and neurotrophic cytokines by blocking the interleukin 6 signal transducer gp130. *Proc Natl Acad Sci U S A* . 1992;89(22):10998-11001.

248. Yin T, Taga T, Tsang ML, et al. Involvement of IL-6 signal transducer gp130 in IL-11-mediated signal transduction. *J Immunol* . 1993;151(5): 2555-2561.

249. Pennica D, Shaw KJ, Swanson TA, et al. Cardiotrophin-1. Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. *J Biol Chem*. 1995;270(18):10915-10922.

250. Taga T, Kishimoto T. Gp130 and the interleukin-6 family of cytokines. *Ann Rev Immunol*. 199715:797-819.

251. Miyajima A, Kinshita T, Tanaka M, Kamiya A, Mukouyama Y, Hara T. Role of Oncostatin M in hematopoiesis and liver development. *Cytokine Growth Factor Rev* . 2000;11(3):177-183.

252. Bravo J, Heath JK. Receptor recognition by gp130 cytokines. *EMBO J*. 2000;19(11):2399-2411.

253. Senaldi G, Varnum BC, Sarmiento U, et al. Novel neurotrophin-1/B cell-stimulating factor-3: a cytokine of the IL-6 family. *Proc Natl Acad Sci U S A* . 1999;96(20):11458-11463.

254. Trinchieri G, Pflanz S, Kastelein RA. The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. *Immunity*. 2003;19(5):641-644.

255. Pflanz S, Hibbert L, Mattson J, et al. WSX-1 and glycoprotein 130 constitute a signal-transducing receptor for IL-27. *J Immunol* . 2004; 172(4):2225-2231.

256. Hirano T, Yaukawa K, Harada H, et al. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature*. 1986;324(6092):73-76.

257. Kishimoto T. Interleukin-6: from basic science to medicine—40 years in immunology. *Ann Rev Immunol* . 2005;23:1-21.

258. Tanaka T, Narazaki M, Kishimoto T. Therapeutic targeting of the interleukin-6 receptor. *Ann Rev Pharmacol Toxicol* . 2012;52: 199-219.

259. Chen Q, Wang WC, Bruce R, et al. Central role of IL-6 receptor signaltransducing chain gp130 in activation of L-selectin adhesion by feverrange thermal stress. *Immunity*. 2004;20(1):59-70.

260. Yamasaki K, Taga T, Hirata Y, et al. Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor. *Science*. 1988;241(4867):825-828.

261. Rose-John S, Neurath MF. IL-6 trans-signaling: the heat is on. *Immunity*. 2004;20(1):2-4.

262. Murakami M, Hibi M, Nakagawa N, et al. IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. *Science*. 1993;260(5115):1808-1810.

263. Paonessa G, Graziani R, De Serio A, et al. Two distinct and independent sites on IL-6 trigger gp 130 dimer formation and signalling. *EMBO J*. 1995;14(9):1942-1951.

264. Ward LD, Howlett GJ, Discolo G, et al. High affinity interleukin-6 receptor is a hexameric complex consisting of two molecules each of interleukin-6, interleukin-6 receptor, and gp-130. *J Biol Chem*. 1994; 269(37):23286-23289.

265. Skiniotis G, Boulanger MJ, Garcia KC, Walz T. Signaling conformations of the tall cytokine receptor gp130 when in complex with IL-6 and IL-6 receptor. *Nat Struct Mol Biol*. 2005;12(6):545-551.

266. Du XX, Williams DA. Interleukin-11: a multifunctional growth factor derived from the hematopoietic microenvironment. *Blood*. 1994; 83(8):2023-2030.

267. Goldman SJ. Preclinical biology of interleukin 11: a multifunctional hematopoietic cytokine with potent thrombopoietic activity. *Stem Cells*. 1995;13(5):462-471.

268. Paul SR, Bennett D, Calvetti JA, et al. Molecular cloning of a cDNA encoding interleukin 11, a stromal cell-derived lymphopoietic and hematopoietic cytokine. *Proc Natl Acad Sci U S A*. 1990;87(19): 7512-7516.

269. Putoczki T, Ernst M. More than a sidekick: the IL-6 family cytokine IL-11 links inflammation to cancer. *J Leukocyte Biol* . 2010;88(6): 1109-1117.

270. Kobayashi S, Teramura M, Oshimi K, Mizoguchi H. Interleukin-11. *Leuk Lymphoma*. 1995;15(1-2):45-49.

271. Kawashima I, Ohsumi J, Mita-Honjo K, et al. Molecular cloning of cDNA encoding adipogenesis inhibitory factor and identity with interleukin-11. *FEBS Lett*. 1991;283(2):199-202.

272. Zheng T, Zhu Z, Wang J, Homer RJ, Elias JA. IL-11: insights in asthma from overexpression transgenic modeling. *J Allergy Clin Immunol* . 2001;108(4):489-496.

273. Cherel M, Sorel M, Lebeau B, et al. Molecular cloning of two isoforms of a receptor for the human hematopoietic cytokine interleukin-11. *Blood*. 1995;86(7):2534-2540.

274. Baumann H, Wang Y, Morella KK, et al. Complex of the soluble IL-11 receptor and IL-11 acts as IL-6-type cytokine in hepatic and nonhepatic cells. *J Immunol* . 1996;157(1):284-290.

275. Neddermann P, Graziani R, Ciliberto G, Paonessa G. Functional expression of soluble human interleukin-11 (IL-11) receptor alpha and stoichiometry of in vitro IL-11 receptor complexes with gp130. *J Biol Chem*. 1996;271(48):30986-30991.

276. Gearing DP, Gough NM, King JA, et al. Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF). *EMBO J*. 1987;6(13):3995-4002.

277. Hilton DJ, Gough NM. Leukemia inhibitory factor: a biological perspective. *J Cell Biochem*. 1991;46(1):21-26.

278. Yamamori T, Fukada K, Aebersold R, et al. The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. *Science*. 1989;246(4936):1412-1416.

279. Piccinni MP, Scaletti C, Vultaggio A, Maggi E, Romagnani S. Defective production of LIF, M-CSF and Th2-type cytokines by T cells at fetomaternal interface is associated with pregnancy loss. *J Reprod Immunol* . 2001;52(1-2):35-43.

280. Gearing DP, Thut CJ, VandeBos T, et al. Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp130. *EMBO J*. 1991;10(10):2839-2848.

281. Cao W, Yang Y, Wang Z, et al. Leukemia inhibitory factor inhibits T helper 17 cell differentiation and confers treatment effects of neural progenitor cell therapy in autoimmune disease. *Immunity*. 2011;35(2):273-284.

282. Metcalfe SM. LIF in the regulation of T-cell fate and as a potential therapeutic. *Genes Immunity*. 2011;12(3):157-168.

283. Pera MF, Tam PP. Extrinsic regulation of pluripotent stem cells. *Nature*. 2010;465(7299):713-720.

284. Lin LF, Mismer D, Lile JD, et al. Purification, cloning, and expression of ciliary neurotrophic factor (CNTF). *Science*. 1989;246(4933): 1023-1025.

285. Stockli KA, Lottspeich F, Sendtner M, et al. Molecular cloning, expression and regional distribution of rat ciliary neurotrophic factor. *Nature*. 1989;342(6252):920-923.

286. Davis S, Aldrich TH, Valensuela DM, et al. The receptor for ciliary neurotrophic factor. *Science*. 1991;253(5015):59-63.

287. Davis S, Aldrich TH, Stahl N, et al. LIFR beta and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor. *Science*. 1993;260(5115):1805-1808.

288. De Serio A, Graziani R, Laufer R, Ciliberto G, Paonessa G. In vitro binding of ciliary neurotrophic factor to its receptors: evidence for the formation of an IL-6-type hexameric complex. *J Mol Biol* . 1995;254(5):795-800.

289. Zarling JM, Shoyab M, Marguardt H, et al. Oncostatin M: a growth regulator produced by differentiated histiocytic lymphoma cells. *Proc Natl Acad Sci U S A* . 1986;83(24):9739-9743.

290. Malik N, Kallestad JC, Gunderson NL, et al. Molecular cloning, sequence analysis, and functional expression of a novel growth regulator, oncostatin M. *Mol Cell Biol* . 1989;9(7):2847-2853.

291. Nair BC, DeVico AL, Nakamura S, et al. Identification of a major growth factor for AIDS-Kaposi's sarcoma cells as oncostatin M. *Science*. 1992;255(5050):1430-1432.

292. Miles SA, Martinez-Maza O, Rezai A, et al. Oncostatin M as a potent mitogen for AIDS-Kaposi's sarcoma-derived cells. *Science*. 1992; 255(5050):1432-1434.

293. Mosley B, De Imus C, Friend D, et al. Dual oncostatin M (OSM) receptors. Cloning and characterization of an alternative signaling subunit conferring OSM-specific receptor activation. *J Biol Chem*. 1996;271(51):32635-32643.

294. Pennica D, King KL, Shaw KJ, et al. Expression cloning of cardiotrophin 1, a cytokine that induces cardiac myocyte hypertrophy. *Proc Natl Acad Sci U S A* . 1995;92(4):1142-1146.

295. Pennica D, Wood WI, Chien KR. Cardiotrophin-1: a multifunctional cytokine that signals via LIF receptor-gp 130 dependent pathways. *Cytokine Growth Factor Rev* .

1996;7(1):81-91.

296. Latchman DS. Cardiotrophin-1: a novel cytokine and its effects in the heart and other tissues. *Pharmacol Ther*. 2000;85(1):29-37.

297. Pennica D, Arce V, Swanson TA, et al. Cardiotrophin-1, a cytokine present in embryonic muscle, supports long-term survival of spinal motoneurons. *Neuron*. 1996;17(1):63-74.

298. Robledo O, Fourcin M, Chevalier S, et al. Signaling of the cardiotrophin-1 receptor. Evidence for a third receptor component. *J Biol Chem*. 1997;272(8):4855-4863.

299. Elson GC, Lelievre E, Guillet C, et al. CLF associates with CLC to form a functional heteromeric ligand for the CNTF receptor complex. *Nat Neurosci*. 2000;3(9):867-872.

300. Pflanz S, Timans JC, Cheung J, et al. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. *Immunity*. 2002;16(6):779-790.

301. Stumhofer JS, Tait ED, Quinn WJ 3rd, et al. A role for IL-27p28 as an antagonist of gp130-mediated signaling. *Nat Immunol*. 2010;11(12): 1119-1126.

302. Hunter CA. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat Rev Immunol*. 2005;5(7):521-531.

303. Kastelein RA, Hunter CA, Cua DJ. Discovery and biology of IL-23 and IL-27: related but functionally distinct regulators of inflammation. *Ann Rev Immunol*. 2007;25:221-242.

304. Ozaki K, Leonard WJ. Cytokine and cytokine receptor pleiotropy and redundancy. *J Biol Chem*. 2002;277(33):29355-29358.

305. Hara M, Yuasa S, Shimoji K, et al. G-CSF influences mouse skeletal muscle development and regeneration by stimulating myoblast proliferation. *J Exp Med*. 2011;208(4):715-727.

306. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friendman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature*. 1994;372(6505):425-432.

307. Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature*. 1998;395(6704):763-770.

308. La Cava A, Matarese G. The weight of leptin in immunity. *Nat Rev Immunol*. 2004;4(5):371-379.

309. Tartaglia LA, Dembski M, Weng X, et al. Identification and expression cloning of a leptin receptor, OB-R. *Cell*. 1995;83(7):1263-1271.

310. Chen H, Charlat O, Tartaglia LA, et al. Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell*.

1996;84(3):491-495.

311. Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigenspecific adaptive immunity. *Ann Rev Immunol*. 1995;13:251-276.

312. Kobayashi M, Fitz L, Ryan M, et al. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J Exp Med*. 1989;170(3): 827-845.

313. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* . 2003;3(2):133-146.

314. Trinchieri G. Type I interferon: friend or foe? *J Exp Med*. 2010; 207(10):2053-2063.

315. Szabo SJ, Dighe AS, Gubler U, Murphy KM. Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J Exp Med*. 1997;185(5):817-824.

316. Gubler U, Chua AO, Schoenhaut DS, et al. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc Natl Acad Sci U S A*. 1991;88(10):4143-4147.

317. Chua AO, Wilkinson VL, Presky DH, Gubler U. Cloning and characterization of a mouse IL-12 receptor-beta component. *J Immunol*. 1995;155(9):4286-4294.

318. Gubler U, Presky DH. Molecular biology of interleukin-12 receptors. *Ann N Y Acad Sci*. 1996;795:36-40.

319. Oppmann B, Lesley R, Blom B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity*. 2000;13(5):715-725.

320. Parham C, Chirica M, Timans J, et al. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rbeta1 and a novel cytokine receptor subunit, IL-23R. *J Immunol*. 2002;168(11):5699-5708.

321. Stumhofer JS, Laurence A, Wilson EH, et al. Interleukin 27 negatively regulates the development of interleukin 17-producing T helper cells during chronic inflammation of the central nervous system. *Nat Immunol*. 2006;7(9):937-945.

322. Uhlig HH, McKenzie BS, Hue S, et al. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. *Immunity*. 2006;25(2):309-318.

323. Duerr RH, Taylor KD, Brant SR, et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science*. 2006;314(5804):1461-1463.

324. Ahern PP, Schiering C, Buonocore S, et al. Interleukin-23 drives intestinal inflammation through direct activity on T cells. *Immunity*. 2010;33(2):279-288.

325. Geremia A, Arancibia-Carcamo CV, Fleming MP, et al. IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease. *J Exp Med*. 2011;208(6):1127-1133.

326. McGeachy MJ, Chen Y, Tato CM, et al. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nat Immunol*. 2009;10(3):314-324.

327. Collison LW, Workman CJ, Kuo TT, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature*. 2007;450(7169): 566-569.

328. Collison LW, Chaturvedi V, Henderson AL, et al. IL-35-mediated induction of a potent regulatory T cell population. *Nat Immunol*. 2010;11(12):1093-1101.

329. Dillon SR, Sprecher C, Hammond A, et al. Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. *Nat Immunol*. 2004;5(7):752-760.

330. Zhang Q, Putheti P, Zhou Q, Liu Q, Gao W. Structures and biological functions of IL-31 and IL-31 receptors. *Cytokine Growth Factor Rev*. 2008;19(5-6):347-356.

331. Friend SL, Hosier S, Nelson A, et al. A thymic stromal cell line supports in vitro development of surface IgM+ B cells and produces a novel growth factor affecting B and T lineage cells. *Exp Hematol*. 1994;22(3):321-328.

332. Sims JE, Williams DE, Morrissey PJ, et al. Molecular cloning and biological characterization of a novel murine lymphoid growth factor. *J Exp Med*. 2000;192(5):671-680.

333. Ziegler SF, Artis D. Sensing the outside world: TSLP regulates barrier immunity. *Nat Immunol*. 2010;11(4):289-293.

334. Pandey A, Ozaki K, Baumann H, et al. Cloning of a receptor subunit required for signaling by thymic stromal lymphopoietin. *Nat Immunol*. 2000;1(1):59-64.

335. Park LS, Martin U, Garka K, et al. Cloning of the murine thymic stromal lymphopoietin (TSLP) receptor: formation of a functional heteromeric complex requires interleukin 7 receptor. *J Exp Med*. 2000;192(5): 659-670.

336. Reche PA, Soumelis V, Gorman DM, et al. Human thymic stromal lymphopoietin preferentially stimulates myeloid cells. *J Immunol*. 2001;167(1):336-343.

337. Cao X, Kozak CA, Kiu JY, Noguchi M, O'Connell E, Leonard WJ. Characterization of cDNAs encoding the murine interleukin 2 receptor (IL-2R) gamma chain: chromosomal mapping and tissue specificity of IL-2R gamma chain expression. *Proc Natl Acad Sci U S A*. 1993;90(18):8464-8468.

338. Soumelis V, Reche PA, Kanzler H, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol*. 2002;3(7):673-680.

339. Leonard WJ. TSLP: finally in the limelight. *Nat Immunol*. 2002;3(7): 605-607.

340. Liu YJ, Soumelis V, Watanabe N, et al. TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. *Ann Rev Immunol*. 2007;25:195-219.

341. Al-Shami A, Spolski R, Kelly J, et al. A role for thymic stromal lymphopoietin in CD4(+) T cell development. *J Exp Med*. 2004;200(2):159-168.

342. Al-Shami A, Spolski R, Kelly J, Keane-Myers A, Leonard WJ. A role for TSLP in the development of inflammation in an asthma model. *J Exp Med*. 2005;202(6):829-839.

343. Yoo J, Omori M, Gyramati D, et al. Spontaneous atopic dermatitis in mice expressing an inducible thymic stromal lymphopoietin transgene specifically in the skin. *J Exp Med*. 2005;202(4):541-549.

344. Zhou B, Comeau MR, De Smedt T, et al. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nat Immunol*. 2005;6(10):1047-1053.

345. Rochman I, Watanabe N, Arima K, Liu YJ, Leonard WJ. Cutting edge: direct action of thymic stromal lymphopoietin on activated human CD4+ T cells. *J Immunol*. 2007;178(11):6720-6724.

346. Rochman Y, Leonard WJ. The role of thymic stromal lymphopoietin in CD8+ T cell homeostasis. *J Immunol*. 2008;181(11):7699-7705.

347. Rochman Y, Kashyap M, Robinson GW, et al. Thymic stromal lymphopoietin-mediated STAT5 phosphorylation via kinases JAK1 and JAK2 reveals a key difference from IL-7-induced signaling. *Proc Natl Acad Sci U S A* . 2010;107(45):19455-19460.

348. Reardon C, Lechmann M, Brustle A, et al. Thymic stromal lymphopoetin-induced expression of the endogenous inhibitory enzyme SLPI mediates recovery from colonic inflammation. *Immunity*. 2011;35(2): 223-235.

349. Watanabe N, Wang YH, Lee HK, et al. Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. *Nature*. 2005;436(7054):1181-1185.

350. Liu YJ. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Ann Rev Immunol*. 2005;23:275-306.

350a. Mazzucchelli R, Hixon JA, Spolski R, et al. Development of regulatory T cells requires IL-7Rα stimulation by IL-7 or TSLP. *Blood*. 2008;112(8):283-292.

351. Kashyap M, Rochman Y, Spolski R, Samsel L, Leonard WJ. Thymic stromal lymphopoietin is produced by dendritic cells. *J Immunol*. 2011;187(3):1207-1211.

352. Pedroza-Gonzalez A, Xu K, Wu TC, et al. Thymic stromal lymphopoietin fosters human breast tumor growth by promoting type 2 inflammation. *J Exp Med*. 2011;208(3):479-490.

353. De Monte L, Reni M, Tassi E, et al. Intratumor T helper type 2 cell infiltrate

correlates with cancer-associated fibroblast thymic stromal lymphopoietin production and reduced survival in pancreatic cancer. *J Exp Med*. 2011;208(3):469-478.

354. Olkhanud PB, Rochman Y, Bodogai M, et al. Thymic stromal lymphopoietin is a key mediator of breast cancer progression. *J Immunol*. 2011;186(10):5656-5662.

355. Leonard WJ, O'Shea JJ. Jaks and STATs: biological implications. *Ann Rev immunol*. 1998;16:293-322.

356. Lin JX, Migone TS, Tsang M, et al. The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. *Immunity*. 1995;2(4):331-339.

357. Wynn TA. IL-13 effector functions. *Ann Rev Immunol*. 2003;21: 425-456.

358. Zurawski G, de Vries JE. Interleukin 13, an interleukin 4-like cytokine that acts on monocytes and B cells, but not on T cells. *Immunol Today*. 1994;15(1):19-26.

359. Zurawski SM, Chomarat P, Djoussou O, et al. The primary binding subunit of the human interleukin-4 receptor is also a component of the interleukin-13 receptor. *J Biol Chem*. 1995;270(23):13869-13878.

360. Hilton DJ, Zhang JG, Metcalf D, Alexander WS, Nicola NA, Wilson TA. Cloning and characterization of a binding subunit of the interleukin 13 receptor that is also a component of the interleukin 4 receptor. *Proc Natl Acad Sci U S A* . 1996;93(1):497-501.

361. Aman MJ, Tayebi N, Obiri NI, Puri RK, Modi WS, Leonard WJ. cDNA cloning and characterization of the human interleukin 13 receptor alpha chain. *J Biol Chem*. 1996;271(46):29265-29270.

362. Caput D, Laurent P, Kaghad M, et al. Cloning and characterization of a specific interleukin (IL)-13 binding protein structurally related to the IL-5 receptor alpha chain. *J Biol Chem*. 1996;271(28):16921-16926.

363. Grunig G, Warnock M, Wakil AE, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science*. 1998;282(5397): 2261-2263.

364. Wills-Karp M, Luyimvazi J, Xu X, et al. Interleukin-13: central mediator of allergic asthma. *Science*. 1998;282(5397):2258-2261.

365. Cormier SA, Kolls JK. Innate IL-13 in virus-induced asthma? *Nat Immunol*. 2011;12(7):587-588.

366. McKenzie GJ, Emson CI, Bell SE, et al. Impaired development of Th2 cells in IL-13-deficient mice. *Immunity*. 1998;9(3):423-432.

367. Kapp U, Yah WC, Patterson B, et al. Interleukin 13 is secreted by and stimulates the growth of Hodgkin and Reed-Sternberg cells. *J Exp Med*. 1999;189(12):1939-1946.

368. Liang HE, Reinhardt RL, Bando JK, Sullivan BM, Ho IC, Lockslev RM. Divergent

expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. *Nat Immunol*. 2011;13(1):58-66.

369. Sharfe N, Dadi HK, Shahar M, Roifman CM. Human immune disorder arising from mutation of the alpha chain of the interleukin-2 receptor. *Proc Natl Acad Sci U S A* . 197;94(7):3168-171.

370. Smith KA. The interleukin 2 receptor. *Annu Rev Cell Biol* . 1989;5: 397-425.

371. Leonard WJ, Depper JM, Kanehisa M, et al. Structure of the human interleukin-2 receptor gene. *Science*. 1985;230(4726):633-639.

372. Leonard WJ, Donlon TA, Lebo RV, Greene WC. Localization of the gene encoding the human interleukin-2 receptor on chromosome 10. *Science*. 1985;228(4707):1547-1549.

373. Shibuya H, Yoneyama M, Nakamura Y, et al. The human interleukin-2 receptor beta-chain gene: genomic organization, promoter analysis and chromosomal assignment. *Nucleic Acids Res*. 1990;18(13):3697-3703.

374. Gnarra JR, Otani H, Wang MG, McBride OW, Sharon M, Leonard WJ. Human interleukin 2 receptor beta-chain gene: chromosomal localization and identification of 5' regulatory sequences. *Proc Natl Acad Sci U S A* . 1990;87(9):3440-3444.

375. Watowich SS, Wu H, Scolovsky M, Klingmuller U, Constantinescu SN, Lodish HF. Cytokine receptor signal transduction and the control of hematopoietic cell development. *Annu Rev Cell Dev Biol* . 1996;12:91-128.

376. Wu H, Klingmuller U, Besmer P, Lodish HF. Interaction of the erythropoietin and stem-cell-factor receptors. *Nature*. 1995;377(6546):242-246.

377. Nairz M, Schroll A, Moschen AR, et al. Erythropoietin contrastingly affects bacterial infection and experimental colitis by inhibiting nuclear factor-kappaB-inducible immune pathways. *Immunity*. 2011;34(1): 61-74.

378. Ramalingam TR, Pesce JT, Sheikh F, et al. Unique functions of the type II interleukin 4 receptor identified in mice lacking the interleukin 13 receptor alpha1 chain. *Nat Immunol*. 2008;9(1):25-33.

379. Ouyang W, Rutz S, Crellin NK, Valdez PA, Hymowitz SG. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Ann Rev Immunol* . 2011;29:71-109.

380. Pestka S, Krause CD, Sarkar D, Walkter MR, Shi Y, Fisher PB. Interleukin-10 and related cytokines and receptors. *Ann Rev Immunol* . 2004;22:929-979.

381. Kurman C, Rubin L, Nelson D. *Article: Soluble Products of Immune Activation: Soluble Interleukin-2 Receptor (sIL-2R, tac Protein)*. Washington, DC: American Society for Microbiology; 1992.

382. Fernandez-Botran R, Chilton PM, Ma Y. Soluble cytokine receptors: their roles in immunoregulation, disease, and therapy. *Adv immunol*. 1996;63:269-336.

383. Kotenko SV, Izotova LS, Mirochnitchenko OV, et al. Identification, cloning, and characterization of a novel soluble receptor that binds IL-22 and neutralizes its activity. *J Immunol*. 2001;166(12):7096-7103.

384. Salvati AL, Lahm A, Paonessa G, Ciliberto G, Toniatti C. Interleukin-6 (IL-6) antagonism by soluble IL-6 receptor alpha mutated in the predicted gp130-binding interface. *J Biol Chem*. 1995;270(20):12242-12249.

385. Webster KE, Walters S, Kohler RE, et al. In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and longterm acceptance of islet allografts without immunosuppression. *J Exp Med*. 2009;206(4):751-760.

386. Isaacs A, Lindenmann J. Virus interference. I. The interferon. *Proc R Soc Lond B Biol Sci*. 1957;147(927):258-267.

387. De Maeyer E, De Maeyer-Guignard J. Type I interferons. *Int Rev Immunol*. 1998;17(1-4):53-73.

388. Farrar MA, Schreiber RD. The molecular cell biology of interferongamma and its receptor. *Ann Rev Immunol*. 1993;11:571-611.

389. Roberts RM, Liu L, Alexenko A. New and atypical families of type I interferons in mammals: comparative functions, structures, and evolutionary relationships. *Prog Nucleic Acid Res Mol Biol*. 1997;56:287-325.

390. Roberts RM, Liu L, Guo Q, Leaman D, Bixby J. The evolution of the type I interferons. *J Interferon Cytokine Res*. 1998;18(10):805-816.

391. Hayes MP, Zoon KC. Production and action of interferons: new insights into molecular mechanisms of gene regulation and expression. *Prog Drug Res*. 1994;43:239-270.

392. Taniguchi T, Takaoka A. The interferon-alpha/beta system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. *Curr Opin Immunol*. 2002;14(1):111-116.

393. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol*. 2006;6(11):836-848.

394. van Boxel-Dezaire AH, Rani MR, Stark GR. Complex modulation of cell type-specific signaling in response to type I interferons. *Immunity*. 2006;25(3):361-372.

395. Honda K, Takaoka A, Taniguchi T. Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. *Immunity*. 2006;25(3):349-360.

396. Vilcek J. Fifty years of interferon research: aiming at a moving target. *Immunity*. 2006;25(3):343-348.

397. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. *Annu Rev Biochem*. 1998;67:227-264.

398. Langer J, Garotta G, Pestka S. Interferon receptors. *Biotherapy*. 1996;8(3-4):163-174.

399. de Veer MJ, Holko M, Frevel M, et al. Functional classification of interferon-stimulated genes identified using microarrays. *J Leukoc Biol*. 2001;69(6):912-920.

400. Bazan JF. Shared architecture of hormone binding domains in type I and II interferon receptors. *Cell*. 1990;61(5):753-754.

401. Walter MR, Windsor WT, Nagabhushan TL, et al. Crystal structure of a complex between interferon-gamma and its soluble high-affinity receptor. *Nature*. 1995;376(6537):230-235.

402. Uze G, Lutfalla G, Gresser I. Genetic transfer of a functional human interferon alpha receptor into mouse cells: cloning and expression of its cDNA. *Cell*. 1990;60(2):225-234.

403. Novick D, Cohen B, Rubinstein M. The human interferon alpha/beta receptor: characterization and molecular cloning. *Cell*. 1994;77(3):391-400.

404. Domanski P, Witte M, Kellum M, et al. Cloning and expression of a long form of the beta subunit of the interferon alpha beta receptor that is required for signaling. *J Biol Chem*. 1995;270(37):21606-21611.

405. Lutfalla G, Holland SJ, Cinato E, et al. Mutant U5A cells are complemented by an interferon-alpha beta receptor subunit generated by alternative processing of a new member of a cytokine receptor gene cluster. *EMBO J*. 1995;14(20):5100-5108.

406. ISICR-Newsletter. Nomenclature for interferon receptors and interferon d. *International Society for Interferon and Cytokine Research* . 1997;4:1.

407. Domanski P, Colamonici OR. The type-I interferon receptor. The long and short of it. *Cytokine Growth Factor Rev*. 1996;7(2):143-151.

408. Symons JA, Alcami A, Smith GL. Vaccinia virus encodes a soluble type I interferon receptor of novel structure and broad species specificity. *Cell*. 1995;81(4):551-560.

409. Spriggs MK. Poxvirus-encoded soluble cytokine receptors. *Virus Res*. 1994;33(1):1-10.

410. Kato H, Takahasi K, Fujita T. RIG-I-like receptors: cytoplasmic sensors for non-self RNA. *Immunol Rev*. 2011;243(1):91-98.

411. Gray PW, Leung DW, Pennica D, et al. Expression of human immune interferon cDNA in E. coli and monkey cells. *Nature*. 1982;295(5849): 503-508.

412. Bach EA, Aguet M, Schreiber RD. The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Ann Rev Immunol*. 1997;15:563-591.

413. Young HA, Romero-Weaver AL, Savan R, Maher SG, Weiss JM. *Class II Cytokines*. Kerala, India: Research Signpost, Fort Post Office; 2007.

414. Marsters SA, Pennica D, Bach E, Schreiber RD, Ashkenazi A. Interferon gamma signals via a high-affinity multisubunit receptor complex that contains two types of polypeptide chain. *Proc Natl Acad Sci U S A* . 1995;92(12):5401-5405.

415. Aguet M, Dembic Z, Merlin G. Molecular cloning and expression of the human interferon-gamma receptor. *Cell*. 1988;55(2):273-280.

416. Soh J, Donnelly RJ, Kotenko S, et al. Identification and sequence of an accessory factor required for activation of the human interferon gamma receptor. *Cell*. 1994;76(5):793-802.

417. Hemmi S, Bohni R, Stark G, Di Marco F, Aguet M. A novel member of the interferon receptor family complements functionality of the murine interferon gamma receptor in human cells. *Cell*. 1994;76(5): 803-810.

418. Kaplan DH, Greenlund AC, Tanner JW, Shaw AS, Schreiber RD. Identification of an interferon-gamma receptor alpha chain sequence required for JAK-1 binding. *J Biol Chem*. 1996;271(1):9-12.

419. Sakatsume M, Igarashi K, Winestock KD, Garotta G, Larner AC, Finbloom DS. The Jak kinases differentially associate with the alpha and beta (accessory factor) chains of the interferon gamma receptor to form a functional receptor unit capable of activating STAT transcription factors. *J Biol Chem*. 1995;270(29):17528-17534.

420. Diamond MS, Kinder M, Matsushita H, et al. Type I interferon is selectively required by dendritic cells for immune rejection of tumors. *J Exp Med*. 2011;208(10):1989-2003.

421. Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med*. 1989;170(6):2081-2095.

422. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Ann Rev Immunol*. 2001;19:683-765.

423. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. *Nat Rev Immunol*. 2010;10(3):170-181.

424. Rutz S, Ouyang W. Regulation of interleukin-10 and interleukin-22 expression in T helper cells. *Curr Opin Immunol* . 2011;23(5):605-612.

425. Chaudhry A, Samstein RM, Treuting P, et al. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity*. 2011;34(4):566-578.

426. Motomura Y, Kitamura H, Hijikata A, et al. The transcription factor E4BP4 regulates the production of IL-10 and IL-13 in CD4+ T cells. *Nat Immunol*. 2011;12(5):450-459.

427. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*. 1993;75(2):263-274.

428. Liu Y, de Waal Malefyt R, Briere F, et al. The EBV IL-10 homologue is a selective agonist with impaired binding to the IL-10 receptor. *J Immunol*. 1997;158(2):604-613.

429. Ho AS, Liu Y, Khan TA, Hsu DH, Bazan JF, Moore KW. A receptor for interleukin 10 is related to interferon receptors. *Proc Natl Acad Sci U S A* . 1993;90(23):11267-11271.

430. Liu Y, Wei SH, Ho AS, de Waal Malefyt R, Moore KW. Expression cloning and characterization of a human IL-10 receptor. *J Immunol*. 1994;152(4):1821-1829.

431. Lutfalla G, Gardiner K, Uze G. A new member of the cytokine receptor gene family maps on chromosome 21 at less than 35 kb from IFNAR. *Genomics*. 1993;16(2):366-373.

432. Gibbs VC, Pennica D. CRF2-4: isolation of cDNA clones encoding the human and mouse proteins. *Gene*. 1997;186(1):97-101.

433. Xie MH, Aggarwal S, Ho WH, et al. Interleukin (IL)-22, a novel human cytokine that signals through the interferon receptor-related proteins CRF2-4 and IL-22R. *J Biol Chem*. 2000;275(40):31335-31339.

434. Dumoutier L, Leemans C, Lejeune D, Kotenko SV, Renauld JC. Cutting edge: STAT activation by IL-19, IL-20 and mda-7 through IL-20 receptor complexes of two types. *J Immunol*. 2001;167(7):3545-3549.

435. Wang M, Tan Z, Zhang R, Kotenko SV, Liang P. Interleukin 24 (MDA-7/MOB-5) signals through two heterodimeric receptors, IL-22R1/IL-20R2 and IL-20R1/IL-20R2. *J Biol Chem*. 2002;277(9):7341-7347.

436. Gallagher G, Dickensheets H, Eskdale J, et al. Cloning, expression and initial characterization of interleukin-19 (IL-19), a novel homologue of human interleukin-10 (IL-10). *Genes Immun*. 2000;1(7):442-450.

437. Blumberg H, Conklin D, Xu WF, et al. Interleukin 20: discovery, receptor identification, and role in epidermal function. *Cell*. 2001;104(1): 9-19.

438. Wegenka UM. IL-20: biological functions mediated through two types of receptor complexes. *Cytokine Growth Factor Rev* . 2010;21(5):353-363.

439. Hsu YH, Chen WY, Chan CH, Wu CH, Sun ZJ, Chang MS. Anti-IL-20 monoclonal antibody inhibits the differentiation of osteoclasts and protects against osteoporotic bone loss. *J Exp Med*. 2011;208(9):1849-1861.

440. Colonna M. Interleukin-22-producing natural killer cells and lymphoid tissue inducer-like cells in mucosal immunity. *Immunity*. 2009;31(1):15-23.

441. Ouyang W. Distinct roles of IL-22 in human psoriasis and inflammatory bowel disease. *Cytokine Growth Factor Rev* . 2010;21(6):435-441.

442. Vivier E, Spits H, Cupedo T. Interleukin-22-producing innate immune cells: new players in mucosal immunity and tissue repair? *Nat Rev Immunol* . 2009;9(4):229-234.

443. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. IL-22 increases the innate immunity of tissues. *Immunity*. 2004;21(2):241-254.

444. Rutz S, Noubade R, Eidenschenk C, et al. Transcription factor c-Maf mediates the TGF-beta-dependent suppression of IL-22 production in T(H)17 cells. *Nat Immunol* . 2011;12(12):1238-1245.

445. Sheppard P, Kindsvogel W, Xu W, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat Immunol* . 2003;4(1):63-68.

446. Fickenscher H, Hor S, Kupers H, Knappe A, Wittmann S, Sticht H. The interleukin-10 family of cytokines. *Trends Immunol*. 2002;23(2):89-96.

447. Darnell JE Jr, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science*. 1994;264(5164):1415-1421.

448. Horvath CM, Darnell JE. The state of the STATs: recent developments in the study of signal transduction to the nucleus. *Curr Opin Cell Biol* . 1997;9(2):233-239.

449. Shuai K, Liu B. Regulation of JAK-STAT signalling in the immune system. *Nat Rev Immunol*. 2003;3(11):900-911.

450. Giordanetto F, Kroemer RT. Prediction of the structure of human Janus kinase 2 (JAK2) comprising JAK homology domains 1 through 7. *Protein Eng*. 2002;15(9):727-737.

451. Wilks AF, Harpur AG, Kurban RR, Ralph SJ, Zurcher G, Ziemiecki A. Two novel protein-tyrosine kinases, each with a second phosphotransferase-related catalytic domain, define a new class of protein kinase. *Mol Cell Biol* . 1991;11(4):2057-2065.

452. Harpur AG, Andres AC, Ziemiecki A, Aston RR, Wilks AF. JAK2, a third member of the JAK family of protein tyrosine kinases. *Oncogene*. 1992;7(7):1347-1353.

453. Johnston JA, Kawamura M, Kirken RA, et al. Phosphorylation and activation of the Jak-3 Janus kinase in response to interleukin-2. *Nature*. 1994;370(6485):151-153.

454. Witthuhn BA, Silvennoinen O, Miura O, et al. Involvement of the Jak-3 Janus kinase in signalling by interleukins 2 and 4 in lymphoid and myeloid cells. *Nature*. 1994;370(6485):153-157.

455. Krolewski JJ, Lee R, Eddy R, Shows TB, Dalla-Favera R. Identification and chromosomal mapping of new human tyrosine kinase genes. *Oncogene*. 1990;5(3):277-282.

456. Gurney AL, Wong SC, Henzel WJ, de Sauvage FJ. Distinct regions of c-Mpl cytoplasmic domain are coupled to the JAK-STAT signal transduction pathway and Shc phosphorylation. *Proc Natl Acad Sci U S A* . 1995;92(12):5292-5296.

457. DaSilva L, Howard OM, Rui H, Kirken RA, Farrar WL. Growth signaling and JAK2 association mediated by membrane-proximal cytoplasmic regions of prolactin receptors. *J Biol Chem*. 1994;269(28): 18267-18270.

458. Witthuhn BA, Quelle FW, Silvennoinen O, et al. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell*. 1993;74(2): 227-236.

459. Tanner JW, Chen W, Young RL, Longmore GD, Shaw AS. The conserved box 1 motif of cytokine receptors is required for association with JAK kinases. *J Biol Chem*. 1995;270(12):6523-6530.

460. Zhao Y, Wagner F, Frank SJ, Kraft AS. The amino-terminal portion of the JAK2 protein kinase is necessary for binding and phosphorylation of the granulocyte-macrophage colony-stimulating factor receptor beta c chain. *J Biol Chem*. 1995;270(23):13814-13818.

461. Kohlhuber F, Rogers NC, Watling D, et al. A JAK1/JAK2 chimera can sustain alpha and gamma interferon responses. *Mol Cell Biol* . 1997;17(2):695-706.

462. Chen M, Cheng A, Chen YQ, et al. The amino terminus of JAK3 is necessary and sufficient for binding to the common gamma chain and confers the ability to transmit interleukin 2-mediated signals. *Proc Natl Acad Sci U S A* . 1997;94(13):6910-6915.

463. Rodig SJ, Meraz MA, White JM, et al. Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokineinduced biologic responses. *Cell*. 1998;93(3):373-383.

464. Guschin D, Rogers N, Briscoe J, et al. A major role for the protein tyrosine kinase JAK1 in the JAK/STAT signal transduction pathway in response to interleukin-6. *EMBO J*. 1995;14(7):1421-1429.

465. Frank SJ, Yi W, Zhao Y, et al. Regions of the JAK2 tyrosine kinase required for coupling to the growth hormone receptor. *J Biol Chem*. 1995;270(24):14776-14785.

466. Zhuang H, Patel SV, He TC, et al. Inhibition of erythropoietin-induced mitogenesis by a kinase-deficient form of Jak2. *J Biol Chem*. 1994;269(34):21411-21414.

467. Kawahara A, Minami Y, Miyazaki T, Ihle JN, Taniguchi T. Critical role of the interleukin 2 (IL-2) receptor gamma-chain-associated Jak3 in the IL-2-induced c-fos and c-myc, but not bcl-2, gene induction. *Proc Natl Acad Sci U S A* . 1995;92(19):8724-8728.

468. Russell SM, Tavebi N, Nakajima H, et al. Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. *Science*. 1995;270(5237):797-800.

469. Macchi P, Villa A, Giliani S, et al. Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID). *Nature*. 1995;377(6544):65-68.

470. Nosaka T, van Deursen JM, Tripp RA, et al. Defective lymphoid development in mice lacking Jak3. *Science*. 1995;270(5237):800-802.

471. Thomis DC, Gurniak CB, Tivol E, Sharpe AH, Berg LJ. Defects in B lymphocyte maturation and T lymphocyte activation in mice lacking Jak3. *Science*. 1995;270(5237):794-797.

472. Park SY, Saijo K, Takahaski T, et al. Developmental defects of lymphoid cells in Jak3 kinase-deficient mice. *Immunity*. 1995;3(6):771-782.

473. Levine RL, Wadleigh M, Cools J, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell* . 2005;7(4): 387-397.

474. Meydan N, Grunberger T, Dadi H, et al. Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor. *Nature*. 1996;379(6566):645-648.

475. Minegishi Y, Saito M, Morio T, et al. Human tyrosine kinase 2 deficiency reveals its requisite roles in multiple cytokine signals involved in innate and acquired immunity. *Immunity*. 2006;25(5):745-755.

476. Harrison DA, Binari R, Nahreini TS, Gilman M, Perrimon N. Activation of a Drosophila Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects. *EMBO J*. 1995;14(12):2857-2865.

477. Luo H, Hanratty WP, Dearolf CR. An amino acid substitution in the Drosophila hopTum-l Jak kinase causes leukemia-like hematopoietic defects. *EMBO J*. 1995;14(7):1412-1420.

478. Conway G, Margoliath A, Wong-Madden S, Roberts RJ, Gilbert W. Jak1 kinase is required for cell migrations and anterior specification in zebrafish embryos. *Proc Natl Acad Sci U S A*. 1997;94(7):3082-3087.

479. Danial NN, Pernis A, Rothman PB. Jak-STAT signaling induced by the v-abl oncogene. *Science*. 1995;269(5232):1875-1877.

480. Migone TS, Lin JX, Cereseto A, et al. Constitutively activated Jak-STAT pathway in T cells transformed with HTLV-I. *Science*. 1995;269(5220):79-81.

481. Ohashi T, Masuda M, Ruscetti SK. Induction of sequence-specific DNA-binding factors by erythropoietin and the spleen focus-forming virus. *Blood*. 1995;85(6):1454-1462.

482. Yu CL, Meyer DJ, Campbell GS, et al. Enhanced DNA-binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein. *Science*. 1995;269(5220):81-83.

483. Nelson BH, McIntosh BC, Rosencrans LL, Greenberg PD. Requirement for an initial signal from the membrane-proximal region of the interleukin 2 receptor gamma(c) chain for Janus kinase activation leading to T cell proliferation. *Proc Natl Acad Sci U S A* . 1997;94(5):1878-1883.

484. Noguchi M, Sarin A, Aman MJ, et al. Functional cleavage of the common cytokine

receptor gamma chain (gammac) by calpain. *Proc Natl Acad Sci U S A* . 1997;94(21):11534-11539.

485. Changelian PS, Flanagan ME, Ball DJ, et al. Prevention of organ allograft rejection by a specific Janus kinase 3 inhibitor. *Science*. 2003; 302(5646):875-878.

486. Hanissian SH, Geha RS. Jak3 is associated with CD40 and is critical for CD40 induction of gene expression in B cells. *Immunity*. 1997;6(4): 379-387.

487. Jabara HH, Buckley RH, Roberts JL, et al. Role of JAK3 in CD40-mediated signaling. *Blood*. 1998;92(7):2435-2440.

488. Levine RL, Gilliland DG. JAK-2 mutations and their relevance to myeloproliferative disease. *Curr Opin Hematol* . 2007;14(1):43-47.

489. Lacronique V, Boureux A, Valle VD, et al. A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. *Science*. 1997; 278(5341):1309-1312.

490. Seto Y, Nakajima H, Suto A, et al. Enhanced Th2 cell-mediated allergic inflammation in Tyk2-deficient mice. *J Immunol* . 2003;170(2): 1077-1083.

491. Briscoe J, Rogers NC, Witthuhn BA, et al. Kinase-negative mutants of JAK1 can sustain interferon-gamma-inducible gene expression but not an antiviral state. *EMBO J*. 1996;15(4):799-809.

492. Gauzzi MC, Valezquez L, McKendry R, Mogensen KE, Fellous M, Pellegrini S. Interferon-alpha-dependent activation of Tyk2 requires phosphorylation of positive regulatory tyrosines by another kinase. *J Biol Chem*. 1996;271(34):20494-20500.

493. Feng J, Witthuhn BA, Matsuda T, Kohlhuber F, Kerr IM, Ihle JN. Activation of Jak2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop. *Mol Cell Biol*. 1997;17(5):2497-2501.

494. Luo H, Rose P, Barber D, et al. Mutation in the Jak kinase JH2 domain hyperactivates Drosophila and mammalian Jak-Stat pathways. *Mol Cell Biol* . 1997;17(3):1562-1571.

495. Fujitani Y, Hibi M, Fukada T, et al. An alternative pathway for STAT activation that is mediated by the direct interaction between JAK and STAT. *Oncogene*. 1997;14(7):751-761.

496. Binari R, Perrimon N. Stripe-specific regulation of pair-rule genes by hopscotch, a putative Jak family tyrosine kinase in Drosophila. *Genes Dev*. 1994;8(3):300-312.

497. Fu XY. A transcription factor with SH2 and SH3 domains is directly activated by an interferon alpha-induced cytoplasmic protein tyrosine kinase(s). *Cell*. 1992;70(2):323-335.

498. Fu XY, Schindler C, Improta T, Aebersold R, Darnell JE Jr. The proteins of ISGF-3, the interferon alpha-induced transcriptional activator, define a gene family involved in

signal transduction. *Proc Natl Acad Sci U S A* . 1992;89(16):7840-7843.

499. Zhong Z, Wen Z, Darnell JE Jr. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science*. 1994;264(5155):95-98.

500. Akira S, Nishio Y, Inoue M, et al. Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. *Cell*. 1994;77(1):63-71.

501. Yamamoto K, Quelle FW, Thierfelder WE, et al. Stat4, a novel gamma interferon activation site-binding protein expressed in early myeloid differentiation. *Mol Cell Biol* . 1994;14(7):4342-4349.

502. Zhong Z, Wen Z, Darnell JE Jr. Stat3 and Stat4: members of the family of signal transducers and activators of transcription. *Proc Natl Acad Sci U S A* . 1994;91(11):4806-4810.

503. Hou J, Schindler U, Henzel WJ, Wong SC, McKnight SL. Identification and purification of human Stat proteins activated in response to interleukin-2. *Immunity*. 1995;2(4):321-329.

504. Wakao H, Gouilleux F, Groner B. Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. *EMBO J*. 1994;13(9):2182-2191.

505. Mui AL, Wakao H, Harada N, O'Farrell AM, Miyajima A. Interleukin-3, granulocyte-macrophage colony-stimulating factor, and interleukin-5 transduce signals through two forms of STAT5. *J Leukoc Biol* . 1995; 57(5):799-803.

506. Azam M, Erdjument-Bromage H, Kreider BL, et al. Interleukin-3 signals through multiple isoforms of Stat5. *EMBO J*. 1995;14(7):1402-1411.

507. Liu X, Robinson GW, Gouilleux F, Groner B, Hennighausen L. Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue. *Proc Natl Acad Sci U S A* . 1995;92(19):8831-8835.

508. Lin JX, Mietz J, Modi WS, John S, Leonard WJ. Cloning of human Stat5B. Reconstitution of interleukin-2-induced Stat5A and Stat5B DNA binding activity in COS-7 cells. *J Biol Chem*. 1996;271(18):10738-10744.

509. Hou J, Schindler U, Henzel WJ, Ho TC, Breasseur M, McKnight SL. An interleukin-4-induced transcription factor: IL-4 Stat. *Science*. 1994;265(5179):1701-1706.

510. Maritano D, Sugrue ML, Tininini S, et al. The STAT3 isoforms alpha and beta have unique and specific functions. *Nat Immunol* . 2004;5(4):401-409.

511. Nakajima H, Liu XW, Wynshaw-Boris A, et al. An indirect effect of Stat5a in IL-2-induced proliferation: a critical role for Stat5a in IL-2-mediated IL-2 receptor alpha chain induction. *Immunity*. 1997;7(5):691-701.

512. Imada K, Bloom ET, Nakajima H, et al. Stat5b is essential for natural killer cell-mediated proliferation and cytolytic activity. *J Exp Med*. 1998;188(11):2067-2074.

513. Teglund S, McKay C, Schuetz E, et al. Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. *Cell*. 1998;93(5):841-850.

514. Moriggl R, Topham DJ, Teglund S, et al. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. *Immunity*. 1999;10(2):249-259.

515. Wang D, Stravopodis D, Teglund S, Kitazawa J, Ihle JN. Naturally occurring dominant negative variants of Stat5. *Mol Cell Biol* . 1996;16(11):6141-6148.

516. Copeland NG, Gilbert DJ, Schindler C, et al. Distribution of the mammalian Stat gene family in mouse chromosomes. *Genomics*. 1995;29(1):225-228.

517. Greenlund AC, Farrar MA, Viviano BL, Schreiber RD. Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). *EMBO J*. 1994;13(7):1591-1600.

518. Yan H, Krishnan K, Greenlund AC, et al. Phosphorylated interferonalpha receptor 1 subunit (IFNaR1) acts as a docking site for the latent form of the 113 kDa STAT2 protein. *EMBO J*. 1996;15(5):1064-1074.

519. Stahl N, Farruggella TK, Boulton TG, et al. Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. *Science*. 1995;267(5202):1349-1353.

520. Friedmann MC, Migone TS, Russell SM, Leonard WJ. Different interleukin 2 receptor beta-chain tyrosines couple to at least two signaling pathways and synergistically mediate interleukin 2-induced proliferation. *Proc Natl Acad Sci U S A* . 1996;93(5):2077-2082.

521. Schindler U, Wu P, Rothe M, Brasseur M, McKnight SL. Components of a Stat recognition code: evidence for two layers of molecular selectivity. *Immunity*. 1995;2(6):689-697.

522. Klingmuller U, Bergelson S, Hsiao JG, Lodish HF. Multiple tyrosine residues in the cytosolic domain of the erythropoietin receptor promote activation of STAT5. *Proc Natl Acad Sci U S A*. 1996;93(16): 832-8328.

523. Weber-Nordt RM, Riley JK, Greenlund AC, Moore KW, Darnell JE, Schreiber RD. Stat3 recruitment by two distinct ligand-induced, tyrosine-phosphorylated docking sites in the interleukin-10 receptor intracellular domain. *J Biol Chem*. 1996;271(44):27954-27961.

524. McBride KM, Banninger G, McDonald C, Reich NC. Regulated nuclear import of the STAT1 transcription factor by direct binding of importin-alpha. *EMBO J*. 2002;21(7):1754-1763.

525. McBride KM, McDonald C, Reich NC. Nuclear export signal located within theDNA-binding domain of the STAT1transcription factor. *EMBO J*. 2000;19(22):6196-6206.

526. Reich NC. STAT dynamics. *Cytokine Growth Factor Rev* . 2007;18 (5-6):511-518.

527. Chen X, Veinkemeier U, Zhao Y, Jeruzalmi D, Darnell JE Jr, Kuriyan J. Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA. *Cell*. 1998;93(5):827-839.

528. Becker S, Groner B, Muller CW. Three-dimensional structure of the Stat3beta homodimer bound to DNA. *Nature*. 1998;394(6689): 145-151.

529. Soldaini E, John S, Moro S, Bollenbacher J, Schindler U, Leonard WJ. DNA binding site selection of dimeric and tetrameric Stat5 proteins reveals a large repertoire of divergent tetrameric Stat5a binding sites. *Mol Cell Biol* . 2000;20(1):389-401.

529a. Lin JX, Peng L, Liu D, et al. Critical role of STAT5 transcription factor tetramerization for cytokine responses and normal immune function. *Immunity*. 2012;36:586-599.

530. Xu X, Sun YL, Hoey T. Cooperative DNA binding and sequence-selective recognition conferred by the STAT amino-terminal domain. *Science*. 1996;273(5276):794-797.

531. Vinkemeier U, Cohen SL, Moarefi I, Chait BT, Kuriyan J, Darnell JE Jr. DNA binding of in vitro activated Stat1 alpha, Stat1 beta and truncated Stat1: interaction between NH2-terminal domains stabilizes binding of two dimers to tandem DNA sites. *EMBO J*. 1996;15(20):5616-5626.

532. Sperisen P, Wang SM, Soldaini E, et al. Mouse interleukin-2 receptor alpha gene expression. Interleukin-1 and interleukin-2 control transcription via distinct cis-acting elements. *J Biol Chem*. 1995;270(18): 10743-10753.

533. John S, Robbins CM, Leonard WJ. An IL-2 response element in the human IL-2 receptor alpha chain promoter is a composite element that binds Stat5, Elf-1, HMG-I(Y) and a GATA family protein. *EMBO J*. 1996;15(20):5627-5635.

534. Lecine P, Algarte M, Rameil P, et al. Elf-1 and Stat5 bind to a critical element in a new enhancer of the human interleukin-2 receptor alpha gene. *Mol Cell Biol* . 1996;16(12):6829-6840.

535. Kim HP, Kelly J, Leonard WJ. The basis for IL-2-induced IL-2 receptor alpha chain gene regulation: importance of two widely separated IL-2 response elements. *Immunity*. 2001;15(1):159-172.

536. Chatterjee-Kishore M, Wright KL, Ting JP, Stark GR. How Stat1 mediates constitutive gene expression: a complex of unphosphorylated Stat1 and IRF1 supports transcription of the LMP2 gene. *EMBO J*. 2000;19(15):4111-4122.

537. Meyer T, Begitt A, Lodige I, van Rossum M, Vinkemeier U. Constitutive and IFN-gamma-induced nuclear import of STAT1 proceed through independent pathways. *EMBO J*. 2002;21(3):344-354.

538. Yang J, Chatterjee-Kishore M, Staugaitis SM, et al. Novel roles of unphosphorylated STAT3 in oncogenesis and transcriptional regulation. *Cancer Res*. 2005;65(3):939-947.

539. Ota N, Brett TJ, Murphy TL, Fremont DH, Murphy KM. N-domaindependent nonphosphorylated STAT4 dimers required for cytokine-driven activation. *Nat Immunol*. 2004;5(2):208-215.

540. Wen Z, Zhong Z, Darnell JE Jr. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell*. 1995;82(2):241-250.

541. Zhang X, Blenis J, Li HC, Schindler C, Chen-Kiang S. Requirement of serine phosphorylation for formation of STAT-promoter complexes. *Science*. 1995;267(5206):1990-1994.

542. Horvath CM, Darnell JE Jr. The antiviral state induced by alpha interferon and gamma interferon requires transcriptionally active Stat1 protein. *J Virol*. 1996;70(1):647-650.

543. David M, Petricoin E 3rd, Benjamin C, Pine R, Weber MJ, Larner AC. Requirement for MAP kinase (ERK2) activity in interferon alpha- and interferon beta-stimulated gene expression through STAT proteins. *Science*. 1995;269(5231):1721-1723.

544. Zhang JJ, Zhao Y, Chait BT, et al. Ser727-dependent recruitment of MCM5 by Stat1alpha in IFN-gamma-induced transcriptional activation. *EMBO J*. 1998;17(23):6963-6971.

545. Xue HH, Fink DW Jr, Zhang X, Qin J, Turck CW, Leonard WJ. Serine phosphorylation of Stat5 proteins in lymphocytes stimulated with IL-2. *Int Immunol*. 2002;14(11):1263-1271.

546. Friedbichler K, Hoelbl A, Li G, et al. Serine phosphorylation of the Stat5a C-terminus is a driving force for transformation. *Front Biosci*. 2012;17:3043-3056.

547. Friedbichler K, Kerenyi MA, Kovacic B, et al. Stat5a serine 725 and 779 phosphorylation is a prerequisite for hematopoietic transformation. *Blood*. 2010;116(9):1548-1558.

548. Look DC, Pelletier MR, Tidwell RM, Roswit WT, Holtzman MJ. Stat1 depends on transcriptional synergy with Sp1. *J Biol Chem*. 1995;270(51):30264-30267.

549. Wesemann DR, Qin H, Kokorina N, Benveniste EN. TRADD interacts with STAT1-alpha and influences interferon-gamma signaling. *Nat Immunol*. 2004;5(2):199-207.

550. Schaefer TS, Sanders LK, Nathans D. Cooperative transcriptional activity of Jun and Stat3 beta, a short form of Stat3. *Proc Natl Acad Sci U S A* . 1995;92(20):9097-9101.

551. Zhang X, Wrzeszczynska MH, Horvath CM, Darnell JE Jr. Interacting regions in Stat3 and c-Jun that participate in cooperative transcriptional activation. *Mol Cell Biol*. 1999;19(10):7138-7146.

552. Bhattacharya S, Eckner R, Grossman S, et al. Cooperation of Stat2 and p300/CBP in signalling induced by interferon-alpha. *Nature*. 1996;383(6598):344-347.

553. Zhang JJ, Vinkemeier U, Gu W, Chakravarti D, Hovarth Sm, Darnell JE Jr. Two contact regions between Stat1 and CBP/p300 in interferon gamma signaling. *Proc Natl Acad Sci U S A*. 1996;93(26):15092-15096.

554. Horvai AE, Xi L, Korvus E, et al. Nuclear integration of JAK/STAT and Ras/AP-1 signaling by CBP and p300. *Proc Natl Acad Sci U S A* . 1997;94(4):1074-1079.

555. Stocklin E, Wissler M, Gouilleux F, Groner B. Functional interactions between Stat5 and the glucocorticoid receptor. *Nature*. 1996; 383(6602):726-728.

556. Nakajima H, Brindle PK, Handa M, Ihle JN. Functional interaction of STAT5 and nuclear receptor co-repressor SMRT: implications in negative regulation of STAT5-dependent transcription. *EMBO J*. 2001;20(23):6836-6844.

557. Durbin JE, Hackenmiller R, Simon MC, Levy DE. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell*. 1996;84(3):443-450.

558. Meraz MA, White JM, Sheehan KC, et al. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell*. 1996;84(3):431-442.

559. Park C, Li S, Cha E, Schindler C. Immune response in Stat2 knockout mice. *Immunity*. 2000;13(6):795-804.

560. Takeda K, Noguchi K, Shi W, et al. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc Natl Acad Sci U S A* . 1997;94(8):3801-3804.

561. Akaishi H, Takeda K, Kaisho T, et al. Defective IL-2-mediated IL-2 receptor alpha chain expression in Stat3-deficient T lymphocytes. *Int Immunol* . 1998;10(11):1747-1751.

562. Akira S. Roles of STAT3 defined by tissue-specific gene targeting. *Oncogene*. 2000;19(21):2607-2611.

563. Chapman RS, Lourenco PC, Tonner E, et al. Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. *Genes Dev*. 1999;13(19):2604-2616.

564. Laouar Y, Welte T, Fu XY, Flavell RA. STAT3 is required for Flt3L-dependent dendritic cell differentiation. *Immunity*. 2003;19(6):903-912.

565. Cheng F, Wang HW, Cuenca A, et al. A critical role for Stat3 signaling in immune tolerance. *Immunity*. 2003;19(3):425-436.

566. Kaplan MH, Sun YL, Hoey T, Grusby MJ. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature*. 1996;382(6587):174-177.

567. Thierfelder WE, van Deursen JM, Yamamoto K, et al. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature*. 1996;382(6587):171-174.

568. Takeda K, Tanaka T, Shi W, et al. Essential role of Stat6 in IL-4 signalling. *Nature*. 1996;380(6575):627-630.

569. Kaplan MH, Schindler U, Smiley ST, Grusby MJ. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity*. 1996;4(3):313-319.

570. Shimoda K, van Deursen J, Sangster MY, et al. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature*. 1996;380(6575):630-633.

571. Liu X, Robinson GW, Wagner KU, et al. Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev*. 1997;11(2):179-186.

572. Udy GB, Towers RP, Snell RG, et al. Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proc Natl Acad Sci U S A* . 1997;94(14):7239-7244.

573. Yao Z, Cui Y, Watford WT, et al. Stat5a/b are essential for normal lymphoid development and differentiation. *Proc Natl Acad Sci U S A* . 2006;103(4):1000-1005.

573a. Kofoed EM, Hwa V, Little B, et al. Growth hormone insensitivity associated with a STAT5b mutation. *N Engl J Med* . 2003;349(12): 1139-1147.

573b. Scaglia PA, Martínez AS, Feigerlová E, et al. A novel missense mutation in the SH2 domain of the STAT5B gene results in a transcriptionally inactive STAT5b associated with severe IGF-I deficiency, immune dysfunction, and lack of pulmonary disease. *J Clin Endocrinol Metab*. 2012;97(5):E830-E839.

574. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF. Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. *Cell*. 1999;98(2):181-191.

575. Yan R, Small S, Desplan C, Dearolf CR, Darnell JE Jr. Identification of a Stat gene that functions in Drosophila development. *Cell*. 1996;84(3):421-430.

576. Hou XS, Melnick MB, Perrimon N. Marelle acts downstream of the Drosophila HOP/JAK kinase and encodes a protein similar to the mammalian STATs. *Cell*. 1996;84(3):411-419.

577. Kawata T, Shevchenko A, Fukuzawa M, et al. SH2 signaling in a lower eukaryote: a STAT protein that regulates stalk cell differentiation in dictyostelium. *Cell*. 1997;89(6):909-916.

578. Ramana CV, Grammatikakis N, Chernov M, et al. Regulation of c-myc expression by IFN-gamma through Stat1-dependent and -independent pathways. *EMBO J*. 2000;19(2):263-272.

579. Darnell JE Jr. Transcription factors as targets for cancer therapy. *Nat Rev Cancer* . 2002;2(10):740-749.

580. Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. *Nat Rev Immunol* . 2007;7(1):41-51.

581. Chin YE, Kitagawa M, Su WC, You ZH, Iwamoto Y, Fu XY. Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1. *Science*. 1996;272(5262): 719-722.

582. Su WC, Kitagawa M, Xue N, et al. Activation of Stat1 by mutant fibroblast growth-factor receptor in thanatophoric dysplasia type II dwarfism. *Nature*. 1997;386(6622):288-292.

583. Pfeffer LM, Mullersman JE, Pfeffer SR, Murti A, Shi W, Yang CH. STAT3 as an adapter to couple phosphatidylinositol 3-kinase to the IFNAR1 chain of the type I interferon receptor. *Science*. 1997;276(5317): 1418-1420.

584. Ramana CV, Gil MP, Schreiber RD, Stark GR. Stat1-dependent and -independent pathways in IFN-gamma-dependent signaling. *Trends Immunol*. 2002;23(2):96-101.

585. Zeng R, Spolski R, Cases E, Zhu W, Lew DE, Leonard WJ. The molecular basis of IL-21-mediated proliferation. *Blood*. 2007;109(10): 4135-4142.

586. Dent AL, Shaffer AL, Yu X, Allman D, Staudt LM. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science*. 1997;276(5312):589-592.

587. Brivanlou AH, Darnell JE Jr. Signal transduction and the control of gene expression. *Science*. 2002;295(5556):813-818.

588. Baldwin AS Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Ann Rev Immunol* . 1996;14:649-683.

589. Lee FS, Hagler J, Chen ZJ, Maniatis T. Activation of the IkappaB alpha kinase complex by MEKK1, a kinase of the JNK pathway. *Cell*. 1997;88(2):213-222.

590. Karin M. How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. *Oncogene*. 1999;18(49):6867-6874.

591. Karin M, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* . 2005; 5(10):749-759.

592. Oeckinghaus A, Hayden MS, Ghosh S. Crosstalk in NF-kappaB signaling pathways. *Nat Immunol* . 2011;12(8):695-708.

593. Smale ST. Hierarchies of NF-kappaB target-gene regulation. *Nat Immunol* . 2011;12(8):689-694.

594. Vallabhapurapu S, Karin M. Regulation and function of NF-kappaB transcription factors in the immune system. *Ann Rev Immunol* . 2009;27:693-733.

595. Macian F, Lopez-Rodriguez C, Rao A. Partners in transcription: NFAT and AP-1. *Oncogene*. 2001;20(19):2476-2489.

596. Crabtree GR, Olson EN. NFAT signaling: choreographing the social lives of cells. *Cell*. 2002;109(suppl):S67-S79.

597. Muller M, Rao A. NFAT, immunity and cancer: a transcription factor comes of age. *Nat Rev Immunol* . 2010;10(9):645-656.

598. Takeshita T, Arita T, Higuchi M, et al. STAM, signal transducing adaptor molecule, is associated with Janus kinases and involved in signaling for cell growth and c-myc induction. *Immunity*. 1997;6(4): 449-457.

599. Migone TS, Rodig S, Cacalano NA, Berg M, Schreiber RD, Leonard WJ. Functional cooperation of the interleukin-2 receptor beta chain and Jak1 in phosphatidylinositol 3-kinase recruitment and phosphorylation. *Mol Cell Biol* . 1998;18(11):6416-6422.

600. Savitsky D, Tamura T, Yanai H, Taniguchi T. Regulation of immunity and oncogenesis by the IRF transcription factor family. *Cancer Immunol Immunother* . 2010;59(4):489-510.

601. Tamura T, Yanai H, Savitsky D, Taniguchi T. The IRF family transcription factors in immunity and oncogenesis. *Ann Rev Immunol* . 2008;26:535-584.

602. Elser B, Lohoff M, Kock S, et al. IFN-gamma represses IL-4 expression via IRF-1 and IRF-2. *Immunity*. 2002;17(6):703-712.

603. Horak ID, Gress RE, Lucas PJ, Horak EM, Waldmann TA, Bolen JB. T-lymphocyte interleukin 2-dependent tyrosine protein kinase signal transduction involves the activation of p56lck. *Proc Natl Acad Sci U S A* . 1991;88(5):1996-2000.

604. Hatakeyama M, Kono T, Kobayashi N, et al. Interaction of the IL-2 receptor with the src-family kinase p56lck: identification of novel intermolecular association. *Science*. 1991;252(5012):1523-1528.

605. Kobayashi N, Kono T, Hatakeyama M, et al. Functional coupling of the src-family protein tyrosine kinases p59fyn and p53/56lyn with the interleukin 2 receptor: implications for redundancy and pleiotropism in cytokine signal transduction. *Proc Natl Acad Sci U S A*. 1993;90(9):4201-4205.

606. Torigoe T, Saragovi HU, Reed JC. Interleukin 2 regulates the activity of the lyn protein-tyrosine kinase in a B-cell line. *Proc Natl Acad Sci U S A* . 1992;89(7):2674-2678.

607. Minami Y, Nakagawa Y, Kawahara A, et al. Protein tyrosine kinase Syk is associated with and activated by the IL-2 receptor: possible link with the c-myc induction pathway. *Immunity*. 1995;2(1):89-100.

608. Otani H, Siegel JP, Erdos M, et al. Interleukin (IL)-2 and IL-3 induce distinct but overlapping responses in murine IL-3-dependent 32D cells transduced with human IL-2 receptor beta chain: involvement of tyrosine kinase(s) other than p56lck. *Proc Natl Acad*

Sci U S A. 1992;89(7):2789-2793.

609. Karnitz L, Sutor SL, Torigoe T, et al. Effects of p56lck deficiency on the growth and cytolytic effector function of an interleukin-2-dependent cytotoxic T-cell line. *Mol Cell Biol*. 1992;12(10):4521-4530.

610. Hatakeyama M, Mori H, Doi T, Taniguchi T. A restricted cytoplasmic region of IL-2 receptor beta chain is essential for growth signal transduction but not for ligand binding and internalization. *Cell*. 1989;59(5):837-845.

611. Fujii H, Ogasawara K, Otsuka H, et al. Functional dissection of the cytoplasmic subregions of the IL-2 receptor betac chain in primary lymphocyte populations. *EMBO J*. 1998;17(22):6551-6557.

612. Turner M, Mee PJ, Costello PS, et al. Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature*. 1995;378(6554):298-302.

613. Corey SJ, Burkhardt AL, Bolen JB, Geahlen RL, Tkatch LS, Tweardy DJ. Granulocyte colony-stimulating factor receptor signaling involves the formation of a three-component complex with Lyn and Syk protein-tyrosine kinases. *Proc Natl Acad Sci U S A*. 1994;91(11): 4683-4687.

614. Rao P, Mufson RA. A membrane proximal domain of the human interleukin-3 receptor beta c subunit that signals DNA synthesis in NIH 3T3 cells specifically binds a complex of Src and Janus family tyrosine kinases and phosphatidylinositol 3-kinase. *J Biol Chem*. 1995;270(12):6886-6893.

615. Ernst M, Gearing DP, Dunn AR. Functional and biochemical association of Hck with the LIF/IL-6 receptor signal transducing subunit gp130 in embryonic stem cells. *EMBO J*. 1994;13(7):1574-1584.

616. Matsuda T, Fukada T, Takahashi-Tezuka M, et al. Activation of Fes tyrosine kinase by gp130, an interleukin-6 family cytokine signal transducer, and their association. *J Biol Chem*. 1995;270(19):11037-11039.

617. Matsuda T, Takahasi-Tezuka M, Fukada T, et al. Association and activation of Btk and Tec tyrosine kinases by gp130, a signal transducer of the interleukin-6 family of cytokines. *Blood*. 1995;85(3):627-633.

618. Izuhara K, Feldman RA, Greer P, Harada N. Interaction of the c-fes proto-oncogene product with the interleukin-4 receptor. *J Biol Chem*. 1994;269(28):18623-18629.

619. Burks DJ, White MF. IRS proteins and beta-cell function. *Diabetes*. 2001;50(suppl 1):S140-S145.

620. Wang LM, Myers MG Jr, Sun XJ, Aatonsen SA, White M, Pierce JH. IRS-1: essential for insulin- and IL-4-stimulated mitogenesis in hematopoietic cells. *Science*. 1993;261(5128):1591-1594.

621. Keegan AD, Nelms K, White M, Wang LM, Piece JH, Paul WE. An IL-4 receptor region containing an insulin receptor motif is important for IL-4-mediated IRS-1

phosphorylation and cell growth. *Cell*. 1994;76(5):811-820.

622. Ridderstrale M, Degerman E, Tornqvist H. Growth hormone stimulates the tyrosine phosphorylation of the insulin receptor substrate-1 and its association with phosphatidylinositol 3-kinase in primary adipocytes. *J Biol Chem*. 1995;270(8):3471-3474.

623. Argetsinger LS, Norstedt G, Billestrup N, White MF, Carter-Su C. Growth hormone, interferon-gamma, and leukemia inhibitory factor utilize insulin receptor substrate-2 in intracellular signaling. *J Biol Chem*. 1996;271(46):29415-29421.

624. Johnston JA, Wang LM, Hanson EP, et al. Interleukins 2, 4, 7, and 15 stimulate tyrosine phosphorylation of insulin receptor substrates 1 and 2 in T cells. Potential role of JAK kinases. *J Biol Chem*. 1995; 270(48):28527-28530.

625. Fruman DA, Meyers RE, Cantley LC. Phosphoinositide kinases. *Annu Rev Biochem*. 1998;67:481-507.

626. Jucker M, Feldman RA. Identification of a new adapter protein that may link the common beta subunit of the receptor for granulocyte/macrophage colony-stimulating factor, interleukin (IL)-3, and IL-5 to phosphatidylinositol 3-kinase. *J Biol Chem*. 1995;270(46):27817-27822.

627. Truitt KE, Mills GB, Turck CW, Imboden JB. SH2-dependent association of phosphatidylinositol 3'-kinase 85-kDa regulatory subunit with the interleukin-2 receptor beta chain. *J Biol Chem*. 1994;269(8):5937-5943.

628. Damen JE, Cutler RL, Jiao H, Yi T, Krystal G. Phosphorylation of tyrosine 503 in the erythropoietin receptor (EpR) is essential for binding the P85 subunit of phosphatidylinositol (PI) 3-kinase and for EpR-associated PI 3-kinase activity. *J Biol Chem*. 1995;270(40): 23402-23408.

629. Karnitz LM, Burns LA, Sutor SL, Blenis J, Abraham RT. Interleukin-2 triggers a novel phosphatidylinositol 3-kinase-dependent MEK activation pathway. *Mol Cell Biol*. 1995;15(6):3049-3057.

630. Dong C, Davis RJ, Flavell RA. MAP kinases in the immune response. *Ann Rev Immunol*. 2002;20:55-72.

631. Neel BG, Tonks NK. Protein tyrosine phosphatases in signal transduction. *Curr Opin Cell Biol*. 1997;9(2):193-204.

632. Li L, Dixon JE. Form, function, and regulation of protein tyrosine phosphatases and their involvement in human diseases. *Semin Immunol*. 2000;12(1):75-84.

633. Tsui HW, Siminovitch KA, de Souza L, Tsui FW. Motheaten and viable motheaten mice have mutations in the haematopoietic cell phosphatase gene. *Nat Gen*. 1993;4(2):124-129.

634. Shultz LD, Schweitzer PA, Pajan TV, et al. Mutations at the murine motheaten locus

are within the hematopoietic cell protein-tyrosine phosphatase (Hcph) gene. *Cell*. 1993;73(7):1445-1454.

635. Van Zant G, Shultz L. Hematologic abnormalities of the immunodeficient mouse mutant, viable motheaten (mev). *Exp Hematol*. 1989;17(2):81-87.

636. Klingmuller U, Lorenz U, Cantley LC, Neel BG, Lodish HF. Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. *Cell*. 1995;80(5):729-738.

637. Yi T, Mui AL, Krystal G, Ihle JN. Hematopoietic cell phosphatase associates with the interleukin-3 (IL-3) receptor beta chain and downregulates IL-3-induced tyrosine phosphorylation and mitogenesis. *Mol Cell Biol* . 1993;13(12):7577-7586.

638. Yetter A, Uddin S, Krolewski JJ, Liao H, Yi T, Platanias LC. Association of the interferon-dependent tyrosine kinase Tyk-2 with the hematopoietic cell phosphatase. *J Biol Chem*. 1995;270(31):18179-18182.

639. Jiao H, Berrada K, Yang W, Tabrizi M, Platanias LC, Yi T. Direct association with and dephosphorylation of Jak2 kinase by the SH2-domain-containing protein tyrosine phosphatase SHP-1. *Mol Cell Biol* . 1996;16(12):6985-6992.

640. Yin T, Shen R, Feng GS, Yang YC. Molecular characterization of specific interactions between SHP-2 phosphatase and JAK tyrosine kinases. *J Biol Chem*. 1997;272(2):1032-1037.

641. Haspel RL, Salditt-Georgieff M, Darnell JE Jr. The rapid inactivation of nuclear tyrosine phosphorylated Stat1 depends upon a protein tyrosine phosphatase. *EMBO J*. 1996;15(22):6262-6268.

642. Mertens C, Zhong M, Krishnaraj R, Zou W, Chen X, Darnell JE Jr. Dephosphorylation of phosphotyrosine on STAT1 dimers requires extensive spatial reorientation of the monomers facilitated by the N-terminal domain. *Genes Dev*. 2006;20(24):3372-3381.

643. Krystal G. Lipid phosphatases in the immune system. *Semin Immunol*. 2000;12(4):397-403.

644. Kim TK, Maniatis T. Regulation of interferon-gamma-activated STAT1 by the ubiquitin-proteasome pathway. *Science*. 1996;273(5282): 1717-1719.

645. Yoshimura A, Ohkubo T, Kigucki T, et al. A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. *EMBO J*. 1995;14(12):2816-2826.

646. Matsumoto A, Masuhara M, Mitsui K, et al. CIS, a cytokine inducible SH2 protein, is a target of the JAK-STAT5 pathway and modulates STAT5 activation. *Blood*. 1997;89(9):3148-3154.

647. Naka T, Narazaki M, Hirata M, et al. Structure and function of a new STAT-induced STAT inhibitor. *Nature*. 1997;387(6636):924-929.

648. Starr R, Willson RA, Viney EM, et al. A family of cytokine-inducible inhibitors of signalling. *Nature*. 1997;387(6636):917-921.

649. Endo TA, Masuhara M, Yokouchi M, et al. A new protein containing an SH2 domain that inhibits JAK kinases. *Nature*. 1997;387(6636): 921-924.

650. Krebs DL, Hilton DJ. SOCS proteins: negative regulators of cytokine signaling. *Stem Cells*. 2001;19(5):378-387.

651. Yasukawa H, Sasaki A, Yoshimura A. Negative regulation of cytokine signaling pathways. *Ann Rev Immunol*. 2000;18:143-164.

652. Alexander WS, Hilton DJ. The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. *Ann Rev Immunol*. 2004;22:503-529.

653. Starr R, Hilton DJ. Defining control: regulation of dendritic cell activation and immune homeostasis by SOCS1. *Immunity*. 2003;19(3): 308-309.

654. Cassel SL, Rothman PB. Chapter 3: role of SOCS in allergic and innate immune responses. *Adv Immunol*. 2009;103:49-76.

655. Nakagawa R, Naka T, Tsutsui H, et al. SOCS-1 participates in negative regulation of LPS responses. *Immunity*. 2002;17(5):677-687.

656. Chong MM, Cornish AL, Darwiche R, et al. Suppressor of cytokine signaling-1 is a critical regulator of interleukin-7-dependent CD8+ T cell differentiation. *Immunity*. 2003;18(4):475-487.

657. Knosp CA, Carroll HP, Elliott J, et al. SOCS2 regulates T helper type 2 differentiation and the generation of type 2 allergic responses. *J Exp Med*. 2011;208(7):1523-1531.

658. Croker BA, JMetcalf D, Robb L, et al. SOCS3 is a critical physiological negative regulator of G-CSF signaling and emergency granulopoiesis. *Immunity*. 2004;20(2):153-165.

659. Croker BA, Krebs DL, Zhang JG, et al. SOCS3 negatively regulates IL-6 signaling in vivo. *Nat Immunol*. 2003;4(6):540-545.

660. Lang R, Pauleau AL, Parganas E, et al. SOCS3 regulates the plasticity of gp130 signaling. *Nat Immunol*. 2003;4(6):546-550.

661. Kovanen PE, Leonard WJ. Inhibitors keep cytokines in check. *Curr Biol*. 1999;9(23):R899-R902.

662. Chung CD, Liao J, Liu B, et al. Specific inhibition of Stat3 signal transduction by PIAS3. *Science*. 1997;278(5344):1803-1805.

663. Liu B, Liao J, Rao X, et al. Inhibition of Stat1-mediated gene activation by PIAS1. *Proc Natl Acad Sci U S A*. 1998;95(18):10626-10631.

664. Kile BT, Nicola NA, Alexander WS. Negative regulators of cytokine signaling. *Int J Hematol*. 2001;73(3):292-298.

665. Sachdev S, Bruhn L, Sieber H, Pichler A, Melchior F, Grosschedl R. PIASy, a nuclear matrix-associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies. *Genes Dev*. 2001; 15(23):3088-3103.

666. Jackson PK. A new RING for SUMO: wrestling transcriptional responses into nuclear bodies with PIAS family E3 SUMO ligases. *Genes Dev*. 2001;15(23):3053-3058.

667. Palvimo JJ. PIAS proteins as regulators of small ubiquitin-related modifier (SUMO) modifications and transcription. *Biochem Soc Trans*. 2007;35(pt 6):1405-1408.

668. Gilmour KC, Fujii H, Cranston T, Davies EG, Kinnon C, Gaspar HB. Defective expression of the interleukin-2/interleukin-15 receptor beta subunit leads to a natural killer cell-deficient form of severe combined immunodeficiency. *Blood*. 2001;98(3):877-879.

669. Yang YC, Ricciardi S, Ciarletta A, Calvetti J, Kelleher K, Clark SC. Expression cloning of cDNA encoding a novel human hematopoietic growth factor: human homologue of murine T-cell growth factor P40. *Blood*. 1989;74(6):1880-1884.

670. Altare F, Lammas D, Revy P, et al. Inherited interleukin 12 deficiency in a child with bacille Calmette-Guerin and Salmonella enteritidis disseminated infection. *J Clin Invest*. 1998;102(12):2035-2040.

671. Altare F, Durandy A, Lammas D, et al. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science*. 1998;280(5368):1432-1435.

672. Jouanguy E, Lamhamedi-Cherradi S, Altare F, et al. Partial interferon-gamma receptor 1 deficiency in a child with tuberculoid bacillus Calmette-Guerin infection and a sibling with clinical tuberculosis. *J Clin Invest*. 1997;100(11):2658-2664.

673. Doffinger R, Jouanguy E, Dupuis S, et al. Partial interferon-gamma receptor signaling chain deficiency in a patient with bacille Calmette-Guerin and Mycobacterium abscessus infection. *J Infect Dis*. 2000;181(1):379-384.

674. Dupuis S, Dargemont C, Fieschi C, et al. Impairment of mycobacterial but not viral immunity by a germline human STAT1 mutation. *Science*. 2001;293(5528):300-303.

675. Liu L, Okada S, Kong XF, et al. Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis. *J Exp Med*. 2011;208(8):1635-1648.

676. Pilati C, Amessou M, Bihl MP, et al. Somatic mutations activating STAT3 in human inflammatory hepatocellular adenomas. *J Exp Med*. 2011;208(7):1359-1366.

677. Yoshida H, Hamano S, Senaldi G, et al. WSX-1 is required for the initiation of Th1 responses and resistance to L. major infection. *Immunity*. 2001;15(4):569-578.

678. Chen Q, Chilhardi N, Wang H, et al. Development of Th1-type immune responses

requires the type I cytokine receptor TCCR. *Nature*. 2000; 407(6806):916-920.

679. Hamano S, Himeno K, Miyazaki Y, et al. WSX-1 is required for resistance to Trypanosoma cruzi infection by regulation of proinflammatory cytokine production. *Immunity*. 2003;19(5):657-667.

680. Villarino A, Hibbert L, Lieberman L, et al. The IL-27R (WSX-1) is required to suppress T cell hyperactivity during infection. *Immunity*. 2003;19(5):645-655.

681. Amselem S, Duquesnoy P, Attree O, et al. Laron dwarfism and mutations of the growth hormone-receptor gene. *N Engl J Med* . 1989;321(15):989-995.

682. Dong F, Hoefsloot LH, Schelen AM, et al. Identification of a nonsense mutation in the granulocyte-colony-stimulating factor receptor in severe congenital neutropenia. *Proc Natl Acad Sci U S A*. 1994;91(10):4480-4484.

683. de la Chapelle A, Traskelin AL, Juvonen E. Truncated erythropoietin receptor causes dominantly inherited benign human erythrocytosis. *Proc Natl Acad Sci U S A* . 1993;90(10):4495-4499.

684. Souyri M, Vigon I, Penciolelli JF, Heard JM, Tambourin P, Wendling F. A putative truncated cytokine receptor gene transduced by the myeloproliferative leukemia virus immortalizes hematopoietic progenitors. *Cell*. 1990;63(6):1137-1147.

685. Shochat C, Talet al. Gain-of-function mutations in interleukin-7 receptor-alpha (IL7R) in childhood acute lymphoblastic leukemias. *J Exp Med*. 2011;208(5):901-908.

686. Mullighan CG, Collins-Underwood JR, Phillips LA, et al. Rearrangement of CRLF2 in B-progenitor- and Down syndrome-associated acute lymphoblastic leukemia. *Nat Gen*. 2009;41(11):1243-1246.

687. Russell LJ, Capasso M, Vater I, et al. Deregulated expression of cytokine receptor gene, CRLF2, is involved in lymphoid transformation in B-cell precursor acute lymphoblastic leukemia. *Blood*. 2009;114(13):2688-2698.

688. Hertzberg L, Vendramini E, Ganmore I, et al. Down syndrome acute lymphoblastic leukemia, a highly heterogeneous disease in which aberrant expression of CRLF2 is associated with mutated JAK2: a report from the International BFM Study Group. *Blood*. 2010;115(5):1006-1017.

689. Nishimoto N, Kishimoto T. Inhibition of IL-6 for the treatment of inflammatory diseases. *Curr Opin Pharmacol* . 2004;4(4):386-391.

690. Ding C, Jones G. Technology evaluation: MRA, Chugai. *Curr Opin Mol Ther* . 2003;5(1):64-69.

691. Waldmann TA. The meandering 45-year odyssey of a clinical immunologist. *Ann Rev Immunol*. 2003;21:1-27.

692. Kundig TM, Schorle H, Bachmann MF, Hengartner H, Zinkernagel RM, Horak I.

Immune responses in interleukin-2-deficient mice. *Science*. 1993;262(5136):1059-1061.

693. Sadlack B, Merz H, Schorle H, et al. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell*. 1993;75(2):253-261.

694. Noben-Trauth N, Shultz LD, Brombacher F, Urban JF Jr, Gu H, Paul WE. An interleukin 4 (IL-4)-independent pathway for CD4+ T cell IL-4 production is revealed in IL-4 receptor-deficient mice. *Proc Natl Acad Sci U S A* . 1997;94(20):10838-10843.

695. Brandt EB, Munitz A, Orekov T, et al. Targeting IL-4/IL-13 signaling to alleviate oral allergen-induced diarrhea. *J Allergy Clin Immunol* . 2009;123(1):53-58.

696. Dranoff G, Crawford AD, Sadelain M, et al. Involvement of granulocyte-macrophage colony-stimulating factor in pulmonary homeostasis. *Science*. 1994;264(5159):713-716.

697. Stanley E, Lieschke GJ, Grail D, et al. Granulocyte/macrophage colonystimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *Proc Natl Acad Sci U S A* . 1994;91(12):5592-5596.

698. Nishinakamura R, Nakayama N, Hirabayashi Y, et al. Mice deficient for the IL-3/GM-CSF/IL-5 beta c receptor exhibit lung pathology and impaired immune response, while beta IL3 receptor-deficient mice are normal. *Immunity*. 1995;2(3):211-222.

699. Kopf M, Baumann H, Freer G, et al. Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature*. 1994;368(6469): 339-342.

700. Yoshida K, Taga T, Saito M, et al. Targeted disruption of gp130, a common signal transducer for the interleukin 6 family of cytokines, leads to myocardial and hematological disorders. *Proc Natl Acad Sci U S A* . 1996;93(1):407-411.

701. Akira S, Yoshida K, Tanaka T, Taga T, Kishimoto T. Targeted disruption of the IL-6 related genes: gp130 and NF-IL-6. *Immunol Rev*. 1995;148:221-253.

702. Stewart CL, Kaspar P, Brunet LJ, et al. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature*. 1992;359(6390):76-79.

703. Escary JL, Perreau J, Dumenil D, Ezine S, Brulet P. Leukaemia inhibitory factor is necessary for maintenance of haematopoietic stem cells and thymocyte stimulation. *Nature*. 1993;363(6427):361-364.

704. Rao MS, Sun Y, Escary JL, et al. Leukemia inhibitory factor mediates an injury response but not a target-directed developmental transmitter switch in sympathetic neurons. *Neuron*. 1993;11(6):1175-1185.

705. Ware CB, Horowitz MC, Renshaw BR, et al. Targeted disruption of the low-affinity leukemia inhibitory factor receptor gene causes placental, skeletal, neural and metabolic defects and results in perinatal death. *Development*. 1995;121(5):1283-1299.

706. Li M, Sendtner M, Smith A. Essential function of LIF receptor in motor neurons. *Nature*. 1995;378(6558):724-727.

707. Masu Y, Wolf E, Holtmann B, et al. Disruption of the CNTF gene results in motor neuron degeneration. *Nature*. 1993;365(6441):27-32.

708. DeChiara TM, Vejsada R, Poueymirou WT, et al. Mice lacking the CNTF receptor, unlike mice lacking CNTF, exhibit profound motor neuron deficits at birth. *Cell*. 1995;83(2):313-322.

709. Robb L, Li R, Hartley L, Nandurkar HH, Koentgen F, Begley CG. Infertility in female mice lacking the receptor for interleukin 11 is due to a defective uterine response to implantation. *Nat Med*. 1998;4(3):303-308.

710. Magram J, Connaughton SE, Warrier RR, et al. IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. *Immunity*. 1996;4(5):471-481.

711. Wu C, Ferrante J, Gately MK, Magram J. Characterization of IL-12 receptor beta1 chain (IL-12Rbeta1)-deficient mice: IL-12Rbeta1 is an essential component of the functional mouse IL-12 receptor. *J Immunol*. 1997;159(4):1658-1665.

712. Gran B, Zhang GX, Yu S, et al. IL-12p35-deficient mice are susceptible to experimental autoimmune encephalomyelitis: evidence for redundancy in the IL-12 system in the induction of central nervous system autoimmune demyelination. *J Immunol*. 2002;169(12):7104-7110.

713. Zhang GX, Gran B, Yu S, et al. Induction of experimental autoimmune encephalomyelitis in IL-12 receptor-beta 2-deficient mice: IL-12 responsiveness is not required in the pathogenesis of inflammatory demyelination in the central nervous system. *J Immunol*. 2003;170(4):2153-2160.

714. Ghilardi N, Kljavin N, Chen Q, Lucas S, Gurney AL, De Sauvage FJ. Compromised humoral and delayed-type hypersensitivity responses in IL-23-deficient mice. *J Immunol*. 2004;172(5):2827-2833.

715. Wirtz S, Billmeier U, McHedlidze T, Blumberg RS, Neurath MF. Interleukin-35 mediates mucosal immune responses that protect against T-cell-dependent colitis. *Gastroenterology*. 2011;141(5):1875-1886.

716. Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell*. 1995;83(1):59-67.

717. Lin CS, Lim SK, D'Agati V, Costantini F. Differential effects of an erythropoietin receptor gene disruption on primitive and definitive erythropoiesis. *Genes Dev*. 1996;10(2):154-164.

718. Gurney AL, Carver-Moore K, de Sauvage FJ, Moore MW. Thrombocytopenia in c-mpl-deficient mice. *Science*. 1994;265(5177):1445-1447.

719. Lieschke GJ, Grail D, Hodgson G, et al. Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. *Blood*. 1994;84(6):1737-1746.

720. Yoshida H, Hayashi S, Kunisada T, et al. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature*. 1990;345(6274):442-444.

721. Metcalf D. The granulocyte-macrophage regulators: reappraisal by gene inactivation. *Exp Hematol*. 1995;23(7):569-572.

722. Muller U, Steinhoff U, Reis LF, et al. Functional role of type I and type II interferons in antiviral defense. *Science*. 1994;264(5167):1918-1921.

723. van den Broek MF, Muller U, Huang S, Zinkernagel RM, Aguet M. Immune defence in mice lacking type I and/or type II interferon receptors. *Immunol Rev*. 1995;148:5-18.

724. van den Broek MF, Muller U, Huang S, Aguet M, Zinkernagel RM. Antiviral defense in mice lacking both alpha/beta and gamma interferon receptors. *J Virol*. 1995;69(8):4792-4796.

725. Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A, Stewart TA. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science*. 1993;259(5102):1739-1742.

726. Wang ZE, Reiner SL, Zheng S, Dalton DK, Locksley RM. CD4+ effector cells default to the Th2 pathway in interferon gamma-deficient mice infected with Leishmania major. *J Exp Med*. 1994;179(4): 1367-1371.

727. Scharton-Kersten TM, Wynn TA, Denkers EY, et al. In the absence of endogenous IFN-gamma, mice develop unimpaired IL-12 responses to Toxoplasma gondii while failing to control acute infection. *J Immunol*. 1996;157(9):4045-4054.

728. Huang S, Hendriks W, Althage A, et al. Immune response in mice that lack the interferon-gamma receptor. *Science*. 1993;259(5102): 1742-1745.

729. Azuma YT, Matsuo Y, Kuwamura M, et al. Interleukin-19 protects mice from innate-mediated colonic inflammation. *Inflamm Bowel Dis*. 2010;16(6):1017-1028.

730. Wahl C, Muller W, Leithauser F, et al. IL-20 receptor 2 signaling downregulates antigen-specific T cell responses. *J Immunol*. 2009;182(2): 802-810.

731. Zheng Y, Valdez PA, Danilenko DM, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med*. 2008;14(3):282-289.

732. Ank N, Iversen MB, Bartholdy C, et al. An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. *J Immunol*. 2008;180(4):2474-2485.

733. Parganas E, Wang D, Stravopodis D, et al. Jak2 is essential for signaling through a variety of cytokine receptors. *Cell*. 1998;93(3):385-395.

734. Neubauer H, Cumano A, Muller M, Wu H, Huffstadt U, Pfeffer K. Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. *Cell*. 1998;93(3):397-409.

735. Karaghiosoff M, Meubauer H, Lassnig C, et al. Partial impairment of cytokine responses in Tyk2-deficient mice. *Immunity*. 2000;13(4): 549-560.

736. Shimoda K, Kato K, Aoki K, et al. Tyk2 plays a restricted role in IFN alpha signaling, although it is required for IL-12-mediated T cell function. *Immunity*. 2000;13(4):561-571.

737. Shimoda K, Tsutsui H, Aoki K, et al. Partial impairment of interleukin-12 (IL-12) and IL-18 signaling in Tyk2-deficient mice. *Blood*. 2002;99(6):2094-2099.

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Chapter 26

The Interleukin-1 Family

Charles A. Dinarello

Mihai G. Netea

INTRODUCTION

More than any other cytokine family, the interleukin (IL)-1 family of ligands and receptors is primarily associated with acute and chronic inflammation. Also, more than any other cytokine family, the IL-1 family plays a fundamental role in the nonspecific innate response to infection that facilitates specific immunologic responses such as antibodies and cytotoxic T-lymphocytes. This nonspecific response to infection is now termed the “innate immune response.” The cytosolic segment of each member of the IL-1 receptor family contains the toll-IL-1-receptor (TIR) domain. This domain is also present in each toll-like receptor (TLR), receptors that respond to microbial products, viruses, and nucleic acids. TIR is the functional domain for both the TLR and IL-1 receptor families, as mutations in this domain result in loss of response to IL-1 and TLR agonists. The biologic properties of both IL-1 family ligands and TLR agonists characteristically are proinflammatory and act as adjuvants for specific immune responses to antigen. Thus, the IL-1 family of ligands and receptors is fundamental to innate immunity. Of the 11 members of the IL-1 family, IL-1β has emerged as a therapeutic target for an expanding number of systemic and local inflammatory conditions termed “autoinflammatory” diseases. These diseases are distinct from autoimmune diseases and include rare hereditary conditions. But autoinflammatory diseases are also common diseases such as heart failure, gouty arthritis, and type 2 diabetes. For these, neutralization of IL-1β results in a rapid and sustained reduction in disease severity. Another member of the IL-1 family, IL-1α, is also a mediator of inflammation but is classified as an “alarmin” because the cytokine is present in most cells and readily released upon cell death. Whereas treatment for autoimmune diseases often includes immunosuppressive drugs, neutralization of IL-1β or blocking the IL-1 receptor is mostly anti-inflammatory.

With one exception, all members of the IL-1 family are initially translated as precursors lacking a signal peptide for secretion via the Golgi. The precursors are found in the cytosol and exit the cell following death by necrosis, not apoptosis. For example, once released, IL-1α, IL-33, and IL-36 can be processed extracellularly by neutrophil proteases into active cytokines. Although IL-1β is primarily processed intracellularly by the cysteine protease caspase-1, the IL-1β precursor can also be cleaved extracellularly into an active cytokine by similar serine proteases of neutrophils. The one member of the IL-1 family that is readily secreted is the IL-1 receptor antagonist (IL-1Ra). IL-1Ra is translated with a signal peptide (Fig. 26.1), although an intracellular form also exists.[1] IL-1Ra is produced in health and is

found circulating in mice and humans where the antagonist serves as a brake on inflammation driven by endogenous IL-1α or IL-1β. IL-1Ra binds to the IL-1RI and blocks the receptor from binding to either IL-1α or IL-1β (see Fig. 26.1C). Mice as well as humans born with a deficiency in functional IL-1Ra exhibit increased systemic and local inflammation; in humans, a deficiency in IL-1Ra is lethal. The IL-36 receptor antagonist (IL-36Ra), another member of the IL-1 family, inhibits the activity of endogenous IL-36α, β, and γ. Although IL-36Ra is not readily secreted, individuals with a mutation in IL-36Ra develop a severe form of psoriasis. One may conclude that most members of the IL-1 family primarily promote inflammation and enhance specific acquired immune responses. But there are also members that provide a brake on inflammation. The primary characteristics of the each member of the IL-1 family are depicted in Table 26.1.

INTERLEUKIN-1 FAMILY AND INNATE RESPONSES

Independent of the type of organism or its products, the innate response is one of inflammation in which the host musters its defenses to increase the production and infiltration of phagocytic cells to the area of the invading microbe in an attempt limit infection and kill-off the invader. Systemically, the liver increases the synthesis of acute phase proteins, include antiproteases. Even in humans, in most cases this process protects the subject without the use of antibiotics. For example, a break in the skin allows bacteria to gain access to the dermis and subsequent inflammation provides activation of complement, the release of preformed cytokines from keratinocytes, an increase in vascular wall adhesions molecules, and the extravasation of neutrophils. This response has functioned to battle against invaders for millions of years and can be traced back to fruit flies.

The skin, lung, and intestinal tract each provide a first line of defense against microbial invasion and the lining cells, whether keratinocytes of the skin, the alveolar epithelial cells of the pulmonary tree, or the epithelial cells of the entire gastrointestinal tract, each contain preformed IL-1α, IL-18, and IL-33 as well as the members of the IL-36 subfamily. Because these members of the IL-1 family are each preformed in these cells, their release is a consequence of injury and is immediate. Therefore, they are termed “alarmins” as they alert the host to initiate the response. There are other “alarmins” from the lining cells that participate in defense, for example, defensins, which are directly antimicrobial.

Each of the constitutively present IL-1 family members in lining cells is present as a precursor. In the case of IL-1α, the precursor is fully active; in the case of the other members, the precursors are weakly active at first but are converted to more active cytokines upon the infiltration of neutrophils and processing by extracellular neutrophil proteases. In the end, the infection is contained, the invading microorganism is eliminated, and skin begins its process of repair.

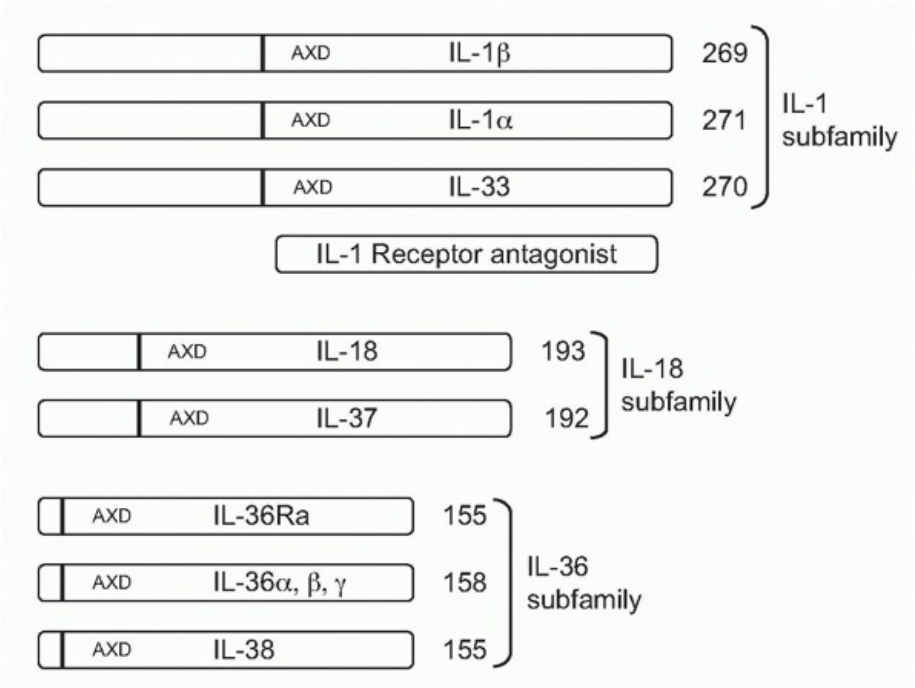

FIG. 26.1. Organization of the Interleukin (IL)-1 Family into Three Subfamilies. The number of amino acids of the fulllength of each member is shown at the C-terminal end. The consensus sequence (A-X-D) is common to all IL-1 family members and serves to locate the N-terminus nine amino acids forward from this site, as shown by the *dark vertical bar*. The N-terminus results in propieces of various lengths. The IL-1Ra has a bonafide signal peptide and is shown by comparison.

Following the cloning of the mouse IL-1 receptor,[2] the cytosolic domain of the IL-1 receptor was found to be homologous to toll of the fruit fly.[3] Moreover, at the same time, the TIR domain for IL-1 signaling (Fig. 26.2A) was shown by Heguy to be required for IL-1 signaling.[4] Toll had been initially studied since its discovery in 1985 because of its central role in establishing dorsal ventral polarity in *Drosophila*. Only since 1996 was toll linked to survival in fruit flies infected with fungi.[5] However, it had already been reported, back in 1988, that a member of the IL-1/TLR family, human IL-1β, protected mice from lethal *Pseudomonas* infection.[6] As noted previously, the TIR domain is essential for both IL-1 receptor family and TLR family signaling; a mutation in the TIR domain severely impairs responses to IL-1 family ligands as well to a large number of microbial products.[7]

TABLE 26.1 Interleukin-1 Family Members

Family Name	Name	Property
IL-1F1	IL-1α	Agonist

IL-1F2	IL-1β	Agonist
IL-1F3	IL-1Ra	Receptor antagonist
IL-1F4	IL-18	Agonist
IL-1F5	IL-36Ra	Receptor antagonist
IL-1F6	IL-36α	Agonist
IL-1F7	IL-37	Anti-inflammatory
IL-1F8	IL-36α	Agonist
IL-1F9	IL-36γ	Agonist
IL-1F10	IL-38	Receptor antagonist
IL-1F11	IL-33	Agonist

IL, interleukin.

The TIR domain binds MyD88 (see Fig. 26.2A and E), itself a TIR domain-containing protein, through TIR/TIR interactions triggering a cascade of kinases that propagate the IL-1 signal and result in transcription of a large number of genes, the majority of which code for other cytokines, chemokines, and a host of inflammatory mediators. Of these is IL-1 and other members of the IL-1 family such as IL-36 and IL-18.

The “innate immune response” regulates to the “acquired immune response.” The late Charles Janeway proposed that the innate response assists the host in mounting an acquired immune response. This relationship between a nonspecific cytokine providing help for a specific response to a microbial antigen is simply the adjuvant property of some cytokines. The adjuvant property of some cytokines functions by upregulating lymphocyte growth factors such as IL-2, IL-4, and IL-6, or lymphocyte receptors resulting in expansion of lymphocyte clones, which will either rid the host of the invading microorganism with neutralizing antibodies or in generation of cytotoxic T cells to eliminate viral infections. In 1979, purified human IL-1β, a nonspecific macrophage product, was shown to augment the T-cell response to specific antigen.[8] It was nearly 20 years later that TLR were identified as inducing IL-1β from monocytes.

ORGANIZATION OF THE INTERLEUKIN-1 FAMILY OF LIGANDS AND THE CONSENSUS SEQUENCE

As depicted in Figure 26.1, the IL-1 family can be divided into subfamilies according to the length of the precursor and the length of the propiece for each precursor. The IL-1 subfamily

is comprised of IL-1α, IL-1β, and IL-33. This subfamily has the longest proteins with the longest propieces. In the case of IL-1β, the propiece is cleaved intracellularly by caspase-1 and then the mature cytokine is secreted. In the case of IL-1α, cleavage appears to be by the membrane protease calpain, but

extracellular neutrophil proteases can also cleave the IL-1α precursor. Extracellular neutrophil proteases account for the cleavage of the propiece of IL-33. The exception in the IL-1 subfamily is IL-1Ra, which contains a signal peptide.

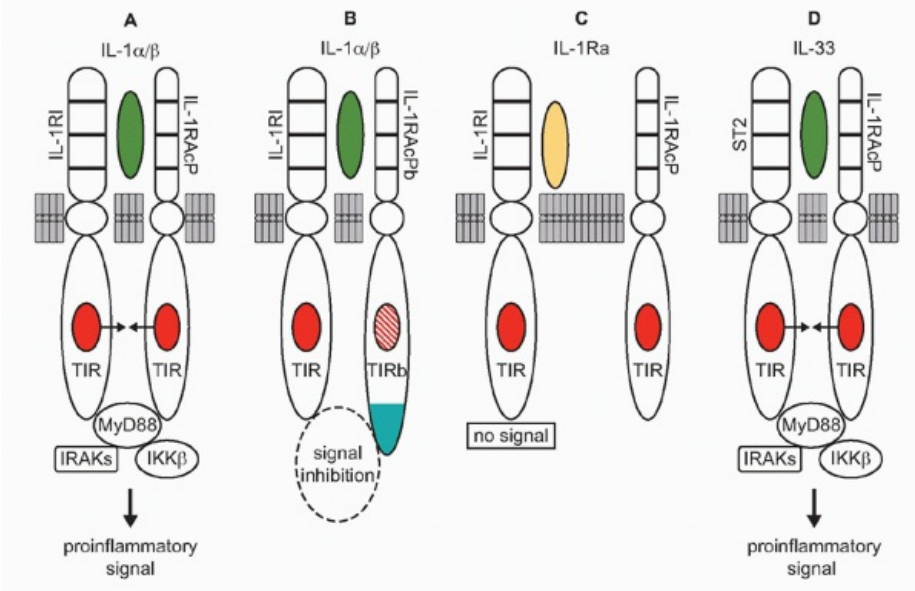

FIG. 26.2. Interleukin (IL)-1 Subfamily. A: IL-1α or IL-1β binds to the IL-1RI and recruits the coreceptor IL-1RAcP. The heterodimeric IL-1 receptor complex results in a close approximation of the toll-IL-1-receptor (TIR) domains on each receptor chain (*arrows*), resulting in the binding of intracellular MyD88 to the complex followed by phosphorylation of MyD88. Subsequent phosphorylations of IRAKs and IKKβ, increased NF-κB, and IL-1R AcP-1 translocation to the nucleus take place followed by expression of proinflammatory genes. **B:** In the central nervous system, IL-1α or IL-1β bind IL-1RI recruiting IL-1RAcP but can also recruit the coreceptor IL-1RAcPb. IL-1RAcPb contains an altered TIR domain, which results in a reduced signal. **C:** IL-1Ra binds to IL-1RI. There is no recruitment of the co-receptor IL-1RAcP, no approximation of the TIR domains, and no signal. **D:** IL-33 binds to its specific receptor, ST2, and recruits the coreceptor IL-1RAcP, the TIR domains approximate, and signal transduction is initiated resulting in the induction of proinflammatory gene profile.

The IL-18 subfamily is comprised of IL-18 and IL-37. By comparison, this subfamily has a smaller propiece. IL-18 requires the cleavage of its propiece by caspase-1 in order to be active. IL-37 is part of the IL-18 subfamily because the cytokine binds to the IL-18Rα chain. It is unclear how the propiece of IL-37 is removed. The IL-36 subfamily comprised of IL-36α, β, and γ, as well as IL-36Ra. In addition, IL-38 likely belongs to this family due to its binding to the IL-36R. The IL-36 subfamily has the shortest propiece.

A consensus sequence in all members of the IL-1 family is A-X-D, where A is an aliphatic amino acid such as isoleucine, methionine, or leucine; X is any amino acid; and D is aspartic acid. The aspartic acid of the consensus sequence is not the aspartic acid of the caspase-1 cleavage recognition site. The A-X-D motif is conserved in the IL-1 family where it plays a role in three-dimensional structure of the active cytokine. The actual N-terminus is often located nine amino acids before the A-X-D site. By eliminating the amino acids before the N-terminus, the first beta-sheet structure common to all members of the IL-1 family can form. For example, with the tenth amino acid before A-X-D consensus site as the N-terminus, the specific activity of the IL-36 subfamily (IL-36α, IL-36β, IL-36γ and the IL-36Ra) is low. However, with the ninth amino acid as the N-terminus, there was a marked increased in the activity.[9] In the case of IL-1β, the ninth amino acid before the A-X-D site coincides exactly with the N-terminal alanine generated by the caspase-1 site.

THE INFLUENCE OF INTERLEUKIN-1 FAMILY ON TH17 RESPONSES

The IL-1 family plays a significant role in interferon (IFN)γ production, which is essential for the defense against intracellular pathogens. On the other hand, Th2 cells are characterized by the production of IL-4 and are important in the host defense against parasitic infections. For more than one decade, the dichotomy between Th1 and Th2 has been the focus of studies on differentiation of cluster of differentiation (CD)4+ T-lymphocytes. More recently, Th17 helper cells have been described and are characterized by their production of IL-17. IL-17 plays a major role in neutrophil recruitment and host defense against extracellular bacteria and fungi. Th17 cells produce a distinct cytokine profile, namely IL-17A, IL-17F, IL-21, and IL-22. The cytokines produced

by Th17 cells, in addition to activating neutrophils, are also crucial for nonimmune cells, for example, induction of defensins by IL-22 in epithelial cells and keratinocytes, which are part of mucosal and skin defenses. It has become apparent that Th17 responses are associated with chronic inflammation and autoimmune diseases such as multiple sclerosis, type 1 diabetes, Crohn disease, and psoriasis. Furthermore, Th17 responses are fundamental for host defense against many microorganisms, although they also contribute to the inflammation during infection.

Whereas IL-4 and IL-12 were the first cytokines described as influencing Th-cell differentiation, cytokines of the IL-1 family also influence cytokine differentiation. IL-18 was initially described as IFNγ-inducing factor due to its strong stimulatory effect on Th1/IFNγ responses.[10] It is now known that IL-18 is, in fact, a crucial cytokine directing the development of Th1 cells, and one role of IL-12 is the induction of the expression of IL-18 receptors. In contrast, binding of IL-33, another member of the IL-1 family, to its ST2 receptor plays a role in inducing Th2 responses,[11] and it thus appeared as if distinct members of the IL-1 family of cytokines directed Th1 versus Th2 differentiation. Considering these effects of IL-18 and IL-33, it came as no surprise that IL-1, the most well-known member of the family, participates in the function of Th cells.

Known for over 30 years that IL-1 enhances T-cell activation and recognition of antigen, one of the early names of IL-1 was lymphocyte activation factor. The specificity of this response was, however, not known. Although initially only IL-23, IL-6, IL-21, and transforming growth factor-β were suggested to play a role in the development of Th17 responses in mice, there

is no dearth of data that a more complex picture exists. Thus, IL-1β, IL-6, and transforming growth factor-β have been reported to induce the development of Th17 cells, whereas IL-23 has been reported to be important for the maintenance of Th17 cells. The combination of IL-23 and IL-1β induce the development of human Th17 cells expressing IL-17A, IL-17F, IL-22, IL-26, the chemokine CCL20, and transcription factor RORγt.[12] Interestingly, these cells also released IFNγ, displaying a phenotype common to both Th17 and Th1 cells.[12] The strong capacity of IL-1 to induce Th17 differentiation has been also linked to its well-known capacity to induce the release of prostaglandins, as reviewed in Dinarello.[13] PGE2 induced by COX-2 is a stimulator of Th17 induction, and inhibitors of cyclooxygenase decrease IL-17 production.[14] On the other hand, engagement of the aryl hydrocarbon receptor, a pathway demonstrated to be crucial for the generation of Th17 cells, has been shown to strongly induce IL-1β.[15] In addition to inducing IL-17 production from the Th17 subset of lymphocytes, IL-1β is required for the production of IL-17 by natural killer (NK) T cells[16] and of IL-22 from NK cells.[17]

Thus, cytokines of the IL-1 family have an important role in the differentiation of the Th subsets, with IL-1β strongly inducing Th17 responses, IL-18 being crucial for the generation of Th1 cells, and IL-33 being important in Th2 responses. Interestingly, reciprocal regulation has been demonstrated between the various Th subsets, with cytokines released by Th2 cells inhibiting Th1 responses, whereas IFNγ release from Th1 cells impairing both Th2 and Th17 responses.

INTERLEUKIN-1α

From an evolutionary viewpoint, IL-1α is the oldest member of the IL-1 family and its primary amino acid sequence is closely related to that of the fibroblast growth factor family. Like fibroblast growth factor, IL-1α does not have signal peptide, binds to nuclear deoxyribonucleic acid (DNA), exits the cell upon death, and binds to its receptor as an unprocessed precursor. As shown in Figure 26.2A, IL-1α binds to the IL-1RI and recruits the IL-1R accessory protein (IL-1RIAcP) to form a heterodimeric complex, which signals to induce inflammation. In health, primary cells contain constitutive levels of the IL-1α precursor but not IL-1β.[18] The IL-1α precursor is present in keratinocytes, thymic epithelium, hepatocytes, endothelial cells, the epithelial cells of mucus membranes, including the entire gastrointestinal tract, and fibroblasts, regardless of their location. The propiece of IL-1α precursor can be cleaved extracellularly by neutrophil proteases, a step that increases its biologic activity. However, IL-1α can also be active as a membrane-associated cytokine. Most cell lines, including tumor cell lines, contain constitutive levels of IL-1α.[19,20,21] Using an epithelial cell line, what was considered to be intrinsic IFNγ activities depended largely on constitutively expressed IL-1α. IFNγ activities were inhibited by antibodies to IL-1α, but not to IL-1β.[20] The concept that IL-1α acts as an autocrine growth factor assumes that the intracellular IL-1α precursor regulates normal cellular differentiation, particularly in epithelial and ectodermal cells. In support of the concept, an antisense oligonucleotide to IL-1α reduces senescence in endothelial cells.[22] In fibroblasts, the constitutive IL-1α precursor binds to HAX-1, a nonreceptor substrate for tyrosine kinases in hematopoietic cells. In fibroblasts, the IL-1α HAX-1 complex translocates to the nucleus.[23] Although the concept is that IL-1α acts as an autocrine growth factor in

fibroblasts or endothelial cells in vitro, the data should be interpreted carefully as mice deficient in IL-1α show no demonstrable defects in growth and development, including skin, fur, epithelium, and gastrointestinal function.[24] However, mice deficient in IL-1α still retain the N-terminal propiece, which functions as nuclear factor.21 In fact, in another study, the N-terminal propiece of IL-1α was shown to bind HAX-1.[25]

Is there is a role for intracellular precursor IL-1α in normal cell function? The IL-1α precursor is present in cells that also contain large amounts of the intracellular form of the IL-1Ra (icIL-1Ra), as reviewed in Arend.[1] This form of the IL-1Ra also binds to the IL-1 receptor and prevents signal transduction. In fact, icIL-1Ra is thought to compete with the intracellular pool of precursor IL-1α for nuclear binding sites.

Membrane-Associated Interleukin-1α

Precursor IL-1α can be found on the surface of several cells, particularly on monocytes and B-lymphocytes, where it is referred to as membrane IL-1α.[26] Membrane IL-1α is biologically active[27]; its biologic activities are neutralized by antibodies to IL-1α but to IL-1β. Endothelial cells undergoing stress-induced apoptosis release membrane apoptotic bodylike particles containing nuclear fragments and histones as well as full-length IL-1α precursor and the processed mature

form.[28] When injected into mice, apoptotic body-like particles containing the IL-1α precursor induce neutrophilic infiltration that was prevented by neutralization of IL-1α but not IL-1β.[28]

Processing and Secretion of Interleukin-1 α

Although the IL-1α precursor is biologically active, the processed form is more active. Furthermore, the binding of IL-1α to the IL-1RI has been modeled using recombinant IL-1α with an N-terminus at 113. The processing of the IL-1α precursor is accomplished by calpain II, a membrane-associated, calcium-dependent cysteine protease.[29] In macrophages treated with hydroquinone, calpain II levels fall and are associated with inhibition of IL-1α precursor processing.[29] Not surprisingly, calcium influx induced IL-1α secretion of the processed form.[30] The secretion of IL-1α requires the presence of IL-1β, as IL-1β-deficient mice do not secrete IL-1α.[31] IL-1α binding to IL-1β has been reported in which IL-1β acts as a chaperone for the secretion mechanism via caspase-1.[31] In another study, IL-1β was shown to bind to, and enhance the activity of, high-mobility group protein B1 (HMGB1).[32] It is thus possible that both IL-1α exits the cell bound to IL-1β and HMGB1.

Biologic Functions of Constitutive Interleukin-1 α: Interleukin-1α and Sterile Inflammation

Large numbers of reports use bacterial and fungal products to induce cytokines as models of inflammatory disease; however, most inflammatory diseases are sterile. For example, the inflammation associated with atherosclerosis, myocardial infarction, stroke, cancer, and renal and liver failure is sterile. The hypoxic insult that takes place in ischemia results in local necrosis and release of cellular contents, including nucleic acids. Members of the IL-1 family contribute to sterile inflammation, and IL-1α plays a significant role in this regard. Upon cell

death by necrosis, the IL-1α precursor is released[33,34] and binds to the IL-1 receptor on nearby tissue macrophages and epithelial cells, triggering a response.[35,36] For example, infiltration of neutrophils occurs first and followed by influx of monocytes.[35] Extracts of tumor cells induce neutrophilic inflammation, which does not occur in mice deficient in IL-1RI and is prevented by neutralization of IL-1α, not neutralization of IL-1β.[37] Sterile inflammation is independent on TLR2 and TLR4.[37]

Thus, IL-1α, either the unprocessed precursor or the calpain cleavage form, is classified as an "alarmin" because the cytokine is preformed and triggers an inflammatory response rapidly. Endothelial cells subjected to nutritional stress release inflammatory apoptotic bodies, which contain both the precursor and processed forms of IL-1α.[28] Inflammatory apoptotic bodies induce chemokine and neutrophilic infiltration into the peritoneal cavity, both of which are IL-1α dependent.[28] Platelets also contain IL-1α as well as IL-1β.[38] Platelet-derived IL-1 induces chemokines such as IL-8 from endothelial cells[39] and monocyte chemotactic protein (MCP-1) from monocytes.[40] Platelet-derived IL-1α is important in brain injury in stroke models[41] and in atherosclerosis.[42]

Studies in Interleukin-1α-Deficient Mice

Mice deficient in IL-1α are born healthy and develop normally. In some models of local inflammatory responses, wild-type and IL-1α-deficient mice develop fever and acutephase proteins, whereas IL-1B-deficient mice do not.[24] In addition, although the inflammation-associated induction of glucocorticoids was suppressed in IL-1β-deficient mice, this suppression was not observed in IL-1α-deficient mice. However, expression of IL-1β messenger ribonucleic acid (mRNA) in the brain decreased 1.5-fold in IL-1α-deficient mice, whereas expression of IL-1α mRNA decreased more than 30-fold in IL-1β-deficient mice. These data suggest that IL-1β exerts greater control over production of IL-1α than does IL-1α over the production of IL-1β. In caspase-1-deficient mice, IL-1α production is also reduced,[43] further suggesting that production of IL-1α is under the control of IL-1β. It is important that caspase-1-deficient mice are also deficient in caspase-11.[44]

In mice fed a high-fat diet, serum amyloid A protein, a marker of inflammation in atherogenesis, was markedly lower in IL-1α-deficient mice compared to wild-type or IL-1β-deficient mice.[45] IL-1α-deficient mice had significantly higher levels of non-high-density lipoprotein cholesterol. The beneficial effect of IL-1α deficiency was due to hematopoietic cells transferred from the bone marrow of IL-1α-deficient mice, resulting in a reduction in aortic lesion size twice that observed in mice transplanted with IL-1β-deficient bone marrow cells. Therefore, IL-1α appears to play a greater role in the pathogenesis of lipid-mediated atherogenesis than IL-1β, and this may be due to an effect of membrane IL-1α.

INTERLEUKIN-1β

Interleukin-1β: The Master Cytokine in the Interleukin-1 Family

More than any other member of the IL-1 family, IL-1β has been the focus of most studies. IL-1β is a highly inflammatory cytokine, particularly in humans, as reviewed in Dinarello.[46] As

shown in Figure 26.2A, IL-1β and IL-1α bind to the same IL-1RI and trigger a proinflammatory signal. The interest in IL-1β is also due, in part, to it being a secreted cytokine from macrophages and to the importance of the macrophage in antigen presentation before the era of dendritic cells. The inactive IL-1β precursor is converted into an active cytokine by the intracellular cysteine protease caspase-1. In particular, persons with activating mutations in one of the key genes that control the activation of caspase-1 can develop life-threatening systemic inflammation, which is reversed by either blocking the IL-1 receptor or through the use of a neutralizing antibody to IL-1β. Other chronic inflammatory diseases are mediated by IL-1β, as neutralizing antibodies have been used to treat a broad spectrum of diseases.

The IL-1β-mediated illnesses fall into the category of "autoinflammatory" diseases, which are to be distinguished from the classic "autoimmune" diseases. Although inflammation is common to both autoinflammatory and autoimmune diseases, in the case of IL-1-mediated disease, there is no evidence for role of adaptive immunity in its induction.

Interleukin-1β is an Inducible Cytokine

Unlike IL-1α, the IL-1β precursor is not present in health. Also unlike IL-1α, IL-1β is primarily a product of monocytes, macrophages, and dendritic cells, as well as B-lymphocytes and NK cells. In health, circulating human blood monocytes or bone marrow cells do not constitutively express mRNA for IL-1β. Endothelial cells, skin keratinocytes, fibroblasts, and epithelial cells contain constitutive IL-1α and constitutive IL-33 as precursors as well as mRNA, but these cells do not express IL-1β mRNA even upon stimulation with TLR ligands. Melanoma cells do express IL-1β as a precursor, and the more aggressive and metastatic the melanoma, the greater the likelihood of active caspase-1 and IL-1β secretion.[47] In the bone marrow neutrophil precursors, IL-1β gene expression is inducible but mature neutrophils in the circulation no longer produce IL-1β. Neutrophil IL-1β plays a pathologic role in the severe inflammation of mice with a mutant form of the phosphatase SHP1.[48] Several malignant tumors do express IL-1β as part of their neoplastic nature, particularly acute myelogenous leukemia, melanoma, multiple myeloma, and juvenile myelogenous leukemia, each of which exhibit constitutive expression of IL-1β. Unlike most cytokine promoters, IL-1β regulatory regions are distributed over several thousand base pairs upstream from the transcriptional start site. In addition to a cAMP response element, there are NF-κB-like and activating protein-1 sites. IL-1β gene regulation has been reviewed in detail.[49] Although steady-state mRNA levels for IL-1β may be present, there is distinct dissociation between transcription and translation of the IL-1β precursor. Non-TLR ligands such as the complement component C5a, hypoxia, adherence to surfaces, or clotting of blood induce the synthesis of large amounts of IL-1β mRNA in monocytic cells without significant translation into the IL-1β protein. In these cells, the IL-1β mRNA assembles into large polyribosomes, but there is no significant elongation of the peptide.[50] This failure to complete the translation into IL-1β protein may be due to the instability element present in the coding region. This instability region is also found in IL-18 and IL-37, and appears to limit the mRNA of these cytokines.[51] However, completion of translation of the mRNA into the respective cytokines can be accomplished by adding low concentrations of TLR ligands or IL-1 itself to the "primed" monocytes.[52]

Processing and Secretion of Interleukin-1 β via the Caspase-1

Nearly all microbial products induce IL-1β via TLR activation; in addition, IL-1 (either IL-1α or IL-1β) induces itself both in vivo and in monocytes in vitro.[53] Other studies supporting this concept of IL-1-induced IL-1 have been reported.[54,55,56,57] Regardless of the stimulus, processing and secretion of IL-1β requires conversion of procaspase-1 to active caspase-1, although in some studies processing of the IL-1β precursor is caspase-1 independent.[58] The activation to active caspase-1 is dependent on a complex of intracellular proteins termed the "inflammasome" by the late Juerg Tschopp.[59,60] The critical component of the inflammasome is NACHT, LRR, and PYD domains containing protein 3 (NLRP3). NLRP3 is also termed cryopyrin as the gene was initially discovered in patients with "familial cold autoin-flammatory syndrome," a genetic disease characterized by constitutional symptoms, fevers, and elevated acute-phase proteins following exposure to cold.[61]

As monocytes exit the bone marrow, they circulate in the bloodstream for approximately 3 days. In the absence of disease, it is likely that these cells do not enter tissues but are destroyed in the spleen or undergo apoptosis. There is no dearth of reports that circulating human blood monocytes release processed IL-1β upon stimulation starting 4 hours after stimulation with TLR agonists and continue to release the cytokine during the following 20 to 40 hours. Following lipopolysaccharide (LPS), IL-1β mRNA levels rise rapidly within 15 minutes but begin to decline after 4 hours due to the short half-life of their mRNA or the action of micro RNA. In contrast, using IL-1 itself as a stimulant, IL-1β mRNA levels are sustained for over 24 hours.[52] Raising intracellular cAMP levels with histamine enhances IL-1-induced IL-1 gene expression and protein synthesis. Monocytes of patients with autoinflammatory diseases such as cryopyrinassociated periodic syndrome (CAPS) and hyper IgΔ syndrome (HIDS) release IL-1β even without TLR stimulation during a 24-hour incubation.[62,63]

When obtained from the venous blood of healthy subjects, human blood monocytes contain active caspase-1. Active caspase-1, as determined by its cleavage into the active dimer, is present even in the absence of stimulation.[64] Active caspase-1 present in freshly obtained monocytes is nevertheless dependent on the presence of the key components of the inflammasome, namely ASC and NLRP3.[64] However, during subsequent incubation, extracellular levels of adenosine triphosphate (ATP) increase in the supernatant as IL-1β also increases and inhibition of ATP by oxidized ATP reduces the secretion of IL-1β.[64] The inhibition of IL-1β secretion by oxidized ATP is consistent with the role of the P2X7 receptor, which binds ATP and opens the potassium channel for release of intracellular potassium. The presence of active caspase-1 in circulating blood monocytes suggests that the rate limiting step in the processing and release of IL-1β is at the level of gene expression.

However, upon differentiation of the same blood monocytes into macrophages in vitro, TLR-induced IL-1β release requires activation of caspase-1 by exogenous ATP.[64] The assembly of the inflammasome components with inactive pro-caspase-1 takes place following a fall in intracellular potassium triggered by ATP binding to the P2X7 receptor. ATP activation of the P2X7 receptor opens the potassium channel, and simultaneously, as potassium levels fall, caspase-1 is activated by the inflammasome.[65,66,67,68,69] Without exogenous ATP, there is little or no processing of the IL-1β precursor in differentiated monocyte-derived macrophages.

Alveolar macrophages obtained from the lungs of healthy human also do not release IL-1β with LPS stimulation unless exogenous ATP is added.[64] In addition to ATP activation of P2X7, activation of IL-1β processing can also take place with a cathelicidin-derived peptide termed LL37, which is released from neutrophils.[69]

The cleavage of the IL-1β precursor by active caspase-1 can take place in the specialized secretory lysosomes or in the cytoplasm. However, more than one pathway seems available for processed IL-1β to exit the cell. These include by exocytosis of the secretory lysosomes,[65,66] shedding of plasma membrane microvesicles, and direct release via transporters or multivesicular bodies containing exosomes.[70] In general, the release of processed IL-1β takes place before there is significant release of lactate dehydrogenase,[71] although in vitro cell death eventually takes place. Pyroptosis is a caspase-1-dependent form of cell death and is induced by certain bacteria using Ipaf, a member of the Nod-like receptor (NLR) family of intracellular receptors.[72] An increase in intracellular calcium is also required for the mature IL-1β to exit the cell and is phospholipase C dependent.[66]

Gain of Function Mutation in Cryopyrin

Diseases associated with single amino acid activating mutations in cryopyrin are termed CAPS. In monocytes from patients with CAPS, activation of caspase-1 occurs without a requirement for a rapid fall in the level of intracellular potassium.[57] Therefore, mutated cryopyrin allows for the assembly of the complex of interacting proteins in the presence of normal intracellular levels of potassium. Although often studied using LPS-induced synthesis of the IL-1β precursor,[73] it is unlikely that LPS plays a role in autoinflammatory diseases. On the other hand, spontaneous secretion of IL-1β from monocytes of patients is due to endogenous IL-1β stimulation. In patients with CAPS, there is a decrease in steady state levels of pro-caspase-1 mRNA with IL-1Ra treatment,[54] suggesting that IL-1β stimulates its own production and processing. Thus, in any disease process that includes an increase in the steady state levels of pro-caspase-1 mRNA, components of the inflammasome or the IL-1β precursor explain the “autoinflammatory” nature of the disease. Type 2 diabetes appears to be an example of an autoinflammatory disease where glucose induces IL-1β production from the insulin-producing beta cell and IL-1β induces the beta cell to produce its own IL-1β .[74]

Polymorphisms in P2X7 and the Activation of the Inflammasome

Patients with classic autoinflammatory diseases such as Familial Mediterranean Fever (FMF) or CAPS have nearly identical clinical parameters, secrete more IL-1β, and respond dramatically to IL-1 receptor blockade yet have no mutation in NALP3. It is therefore possible that mutations in P2X7 itself or regulation of the other genes controlling potassium channels[75] may account for dysfunctional secretion of IL-1β. For example, monocytes from patients with rheumatoid arthritis are more sensitive to release of IL-1β following ATP activation of the P2X7 receptor compared to monocytes from healthy controls.[76] However, monocytes from subjects with a P2X7 Glu496Ala loss-offunction polymorphism secrete

significantly less IL-1β.[77] Monocytes from subjects homozygous for this polymorphism also released significantly less IL-18.[78] Another P2X7 receptor polymorphism is associated with increased mortality in patients undergoing allogenic stem cell transplantation.[79] Bacteremia was documented in 68% of patients with this polymorphism compared to 18% in wild-type control patients.[79]

In mice deficient in P2X7 receptors, inflammation, pain, and IL-1β-mediated IL-6 production are markedly reduced.[80] In addition to a fall in intracellular potassium, ATP triggers formation of peroxynitrite, which is required for caspase-1 activation because peroxynitrite scavengers prevent IL-1β secretion.[81] Pannexin-1, a mammalian protein that functions as a hemichannel for the uptake of dyes, is required for caspase-1 processing and release of IL-1β via the P2X7 receptor.[82] Pannexin-1 can also function for LPS-induced IL-1β synthesis in the absence of TLR4.[83] P2X7 receptor activity is also regulated by "regeneration and tolerance factor."[84]

Non-Caspase-1 Processing of Interleukin-1β

Non-caspase-1 mechanisms also exist to generate active forms of IL-1β. For example, sterile inflammation induces fever, elevated IL-6, and increased production of hepatic acute-phase proteins. These responses are absent in mice deficient in IL-1β but present in mice deficient in caspase-1.[85,86] Sterile inflammation is often associated with neutrophilic infiltration and neutrophils produce IL-1β. Because neutrophils are short-lived cells dying within hours upon emigration, release of the IL-1β precursor from intracellular stores is not unexpected. Processing of the IL-1β precursor extracellularly into an active cytokine has been reported for the common neutrophil protease, proteinase-3.[86,87] Proteinase-3 also contributes to the processing of IL-18.[88] Other proteases such as elastase, matrix metalloprotease 9, and granzyme A process the IL-1β precursor extracellularly. In addition, a mast cell chymase generates active IL-1β.

Mice with a targeted IKKβ deletion in myeloid cells are more susceptible to LPS-induced shock than control mice,[55] and markedly elevated levels of IL-1β are found in the circulation associated with a prominent neutrophilia.[55] The elevated levels of IL-1β are lethal as blockade with IL-1Ra protects these mice from death. The source of the IL-1β in these mice is the neutrophil. When incubated with proteinase-3, cleavage of the IL-1β precursor is observed yielding molecular weights of 25,000 and 15,000 Daltons.[55] Because the cleavage of the IL-1β precursor by proteinase-3, elastase, and cathepsin G are within three amino acids of the caspase-1 cleavage site, the products of the non-caspase-1 cleavage are biologically active.[86,87] Therefore, in inflammatory conditions such as urate crystal arthritis, which is characterized by a prominent neutrophilic infiltration, proteinase-3 cleavage of extracellular IL-1β precursor likely takes place.[89] Mice deficient in caspase-1 are not protected against urate-induced inflammation. Although IL-1Ra is effective in treating gout, IL-1Ra would be equally effective in any disease with extracellular processing of the precursor.[90,91,92] The importance of extracellular processing of the IL-1β precursor by serine proteases may explain, in part, the anti-inflammatory properties of alpha-1-

antitrypsin.[93]

Reactive Oxygen Species and Interleukin-1β Processing

Is there a role for reactive oxygen species (ROS) in the activation of the IL-1β inflammasome? It was reported that uric acid crystals added to human monocytes result in the generation of ROS, which bind to and activate NLRP3 with subsequent secretion of IL-1β.[94] However, mice deficient in ROS production exhibit a proinflammatory phenotype.[95] Humans with chronic granulomatous disease (CGD) due to mutations in p47-phox cannot generate ROS and are severely affected by inflammatory granuloma. Uric acid crystal activation of primary monocytes from persons with CGD produced fourfold higher levels of IL-1β compared to monocytes from unaffected persons.[96] In contrast to previous studies,[94] the small molecule ROS inhibitor diphenyleneiodonium, which reduces the production of IL-1β, does so due to inhibition of IL-1β gene expression rather than decreased caspase-1 activation.[96] Another study identified phagocyte oxidase-defective monocytes from CGD patients as a source of elevated IL-1β.[97] These findings support the concept that ROS likely dampens inflammasome activation and may explain the presence of an inflammatory phenotype characterized by granulomas and inflammatory bowel disease occurring in patients with CGD. In fact, patients with CGD-related inflammatory bowel disease improve upon IL-1 receptor blocking therapy.[98]

Effects in Mice Deficient in Interleukin-1β

After 10 years of continuous breeding, mice deficient in IL-1β exhibit no spontaneous disease. However, upon challenge, IL-1β-deficient mice exhibit specific differences from their wild-type controls. The most dramatic is the response to local inflammation induced by a subcutaneous injection of an irritant. Within the first 24 hours, IL-1β-deficient mice do not manifest an acute-phase response, do not develop anorexia, have no circulating IL-6, and have no fever.[85,99] These findings are consistent with those reported in the same model using anti-IL-1R type I antibodies in wild-type mice.[85,99] IL-1β-deficient mice also have reduced inflammation due to zymosan-induced peritonitis.[85,100] In contrast, IL-1β-deficient mice have elevated febrile responses to LPS, IL-1β, or IL-1α compared to wild-type mice.[101] Nevertheless, IL-1β-deficient mice injected with LPS have little or no expression of leptin mRNA or protein.[102]

Mice deficient in IL-1β were compared to mice deficient in IL-1α after exposure to chemical carcinogens.[103] In IL-1β-deficient mice, tumors developed slower or did not develop in some mice. A deficiency in IL-1α, on the other hand, did not impair tumor development compared to wild-type mice injected with the same carcinogen. In IL-1Ra-deficient mice, tumor development was the most rapid. A leukocyte infiltrate was found at the site of carcinogen injection. The neutrophilic infiltrate was almost absent in IL-1β-deficient mice, whereas in IL-1Ra-deficient mice, a dense neutrophilic infiltrate was observed. In wild-type mice, the leukocytic infiltrate was sparse and the infiltrate that was observed in IL-1α-deficient mice was similar to that of control mice. These findings may reflect the fact that IL-1β is secreted into the microenvironment resulting in the emigration of monocytes and neutrophils, whereas

IL-1α remaining cell-associated is less likely to affect the microenvironment.

Interleukin-1α and Autophagy

Autophagy is an ancient process of recycling cellular components, such as cytosolic organelles and protein aggregates, through degradation mediated by lysosomes. Autophagy is activated in conditions of cell stress, hypoxia, starvation, or growth factor deprivation; it promotes cell survival by generating free metabolites and energy through degradation of the endogenous cellular components.[104] However, in addition to its role in the pathophysiology of cancer, neurodegenerative diseases, or aging, autophagy is also a modulator of inflammation.[105] A role for autophagy in production of proinflammatory cytokines, particularly of IL-1β, has emerged with deletion of ATG16-L1. For example, macrophages from *ATG16L1* -deficient mice produce higher levels of IL-1β and IL-18 after stimulation with TLR4 ligands.[106] The data suggest that higher activation of caspase-1 in the *ATG16L1*-deficient mice accounts for the higher production level.[106] This observation was related to the specific degradation of the IL-1β precursor in autophagosomes in mouse macrophages.[107] Additional studies in the *ATG16L1*-deficient mice point toward a regulatory effect of autophagy on caspase-1 activation through modulation of the NLRP3 inflammasome.[94,108,109]

This role of autophagy in the secretion of IL-1β was also observed in human primary monocytes, in which specific inhibition of autophagy leads to increased production of IL-1β .[110] However, in the same cells, tumor necrosis factor (TNF)α production was decreased by autophagy inhibition. These data suggest divergent effects of autophagy on the production of these two important proinflammatory cytokines. In mice, the increase in IL-1β production is ascribed to increased activation of the inflammasome, but in human cells, it is IL-1β mRNA transcription that is elevated when autophagy was inhibited, whereas no effects were observed on caspase-1 activation.[106,107,110] Despite these differences between mouse and human cells, the inhibition of autophagy increases the production of IL-1β but not TNFα.

The modulation of inflammation by autophagy in humans has been studied in Crohn disease. Genome-wide association studies in large cohorts of patients with Crohn disease have revealed that genetic variants in two autophagy genes, *ATG16L1* and *IRGM*, result in increased susceptibility to the disease. A nonsynonymous polymorphism in *ATG16L1* on chromosome 2q37.1 and two polymorphisms in *IRGM* on chromosome 5q33.1 were significantly associated with Crohn disease risk.[111,112] Another study revealed a significant association of Crohn disease susceptibility with an intronic polymorphism in the autophagy gene *ULK1*.[113] Moreover, autophagy defects have been reported in individuals bearing nucleotide oligomerization domain (*NOD)2* mutations and are consistent with the concept that impaired bacterial clearance and increased bacterial persistence are part of the pathogenesis of Crohn disease.[114]

The mechanism through which polymorphisms in autophagy genes influence susceptibility to Crohn disease appears to involve IL-1β production. The *ATG16L1* 300Ala risk allele was associated with elevated production of IL-1β and IL-6; however, this finding was only observed in cells stimulated with the NOD2 ligand muramyl dipeptide. In contrast, the

expected levels of IL-1β and IL-6 were produced upon stimulation with TLR2 and TLR4 ligands.[115] The increased production of IL-1β was associated with an increase in the steady state levels of IL-1β mRNA rather than increased activation of the inflammasome.[115] Studying the same polymorphism (*ATG16L1* Thr300Ala) in human dendritic cells, Cooney et al. reported defective NOD2-induced, but not TLR-induced, autophagy and antigen presentation.[116] Furthermore, effects of this polymorphism on antibacterial autophagy in epithelial cells have been observed.[117] The specific effect of the *ATG16L1* polymorphism on the NOD2 pathway, and not on TLR-induced stimulation, is likely related to the fact NOD2 and ATG16L1 form a protein complex that is essential for NOD2-induced autophagosome formation.[118] Because the *ATG16L1* Thr300Ala polymorphism affects protein stability,[119] defective induction of autophagy and therefore enhanced IL-1β mRNA transcription upon triggering of NOD2 may be due to the presence of defective complex.

INTERLEUKIN-33

Interleukin-33 as a Member of the Interleukin-1 Subfamily

Formerly termed IL-1F11, IL-33 belongs to the IL-1 subfamily and has been studied for its role in the Th2 paradigm of immune responses. IL-1β is also linked to the Th2 response. The existence of IL-33 was predicted in 1994 following the discovery of a novel member of the IL-1 receptor family termed ST2.[120] ST2 is the ligand binding chain for IL-33 (Table 26.2) and is structurally similar to the ligand binding chain of IL-1α and IL-1β. In addition, the coreceptor for IL-33 is the IL-1RAcP, which is also the coreceptor for IL-1α and IL-1β. It was not until 2005 that IL-33 was reported as the ligand for ST2.[11] ST2 is regulated by the estrogen inducible transcription factor Fos,[120] and this property of estrogens may be related to the large number of studies on the effect of estrogens to regulate IL-1 and inflammation.

TABLE 26.2 Interleukin-1 Receptor Family

Name	Designation	Ligands	Coreceptor
IL-1RI	IL-1R1	IL-1α, IL-1β, IL-1Ra	IL-1RAcP (IL-1R3)
IL-1RII	IL-1R2	IL-1β, IL-1β precursor	IL-1RAcP (IL-1R3)
IL-1RAcP	IL-1R3	IL-1α, IL-1β, IL-33, IL-36	Not applicable
ST2/IL-33Rα	IL-1 R4	IL-33	IL-1RAcP (IL-1R3)

IL-18Rα	IL-1R5	IL-18, IL-37	IL-18Rβ (IL-1R7)
IL-1Rrp-2	IL-1R6	IL-36α,β,γ	IL-1RAcP (IL-1R3)
IL-18Rβ	IL-1R7	IL-18	Not applicable
TIGIRR-2/IL-1RAPL	IL-1R8	Unknown	Unknown
TIGIRR-1	IL-1R9	Unknown	Unknown
SIGIRR	TIR8	Unknown	Unknown

IL, interleukin; SIGIRR, single immunoglobulin IL-1-related receptor; TIGIRR, three immunoglobulin IL-1-related receptor.

Similar to most members of the IL-1 receptor family, ST2 is comprised of three extracellular immunoglobulin domains and an intracellular TIR domain. Although the name ST2 is still used, the correct term is the IL-33 receptor α chain (IL-33Rα). As shown in Figure 26.2D, the IL-33Rα chain similar to the IL-1R1 in that it is the ligand binding chain for IL-33 but requires the IL-1RAcP to signal.[121,122]

Before the discovery of IL-33, several studies suggested that the putative ligand (IL-33) for the ST2 orphan receptor was playing a role in allergic type diseases. It became clear that activation of ST2 was uniquely driving Th2 responses. Structurally, IL-33 is closer to IL-18 than IL-1β. Biologically, IL-33 is closest to IL-1α, as the precursors for IL-1α and IL-33 are constitutively present in all endothelial cells. As discussed in the following, like IL-1α, IL-33 functions as a DNA-binding molecule. The dominant property of IL-33 is the induction of IL-4, IL-5, and IL-13, as well as other properties anticipated for a Th2 type cytokine. Diseases thought to be due to increased immunoglobulin production may also be related to IL-33. IL-33 induces the production of IL-6, IL-1β, and PGE2 from mast cells.

Interleukin-33 and Th2 Responses

The properties of recombinant IL-33 recapitulate much of the existing data that ST2 promotes Th2-type responses. For example, before its discovery, a role for IL-33 in the Th2 response was observed using soluble extracellular forms of ST2.[11] However, IL-33 has properties that go beyond its role in the Th2 paradigm because, similar to IL-1α, IL-1β and IL-36, IL-33 forms a heterodimeric complex with IL-1RAcP for signal transduction.[121,122] Although the IL-1RAcP is expressed on most nucleated cells, ST2 is somewhat restricted to low expression on most cells with the notable exception of mast cells.

There are several mechanisms by which IL-33 favors the Th2 response. Similar to IL-1β, IL-33 induces IL-6, an adjuvant for antibody production. IL-33 induction of IL-6 is prevented by a blocking antibody to IL-1RAcP.[122] IL-33 initiates signal transduction via activation of NF-κB,

is typical of IL-1α, IL-1β and IL-18,[11] but other studies have shown that antibody cross-linking of ST2 does not result in activation of NF-κB but rather activating protein-1. IL-33 treatment also increased serum immunoglobulin A and immunoglobulin E, an expected response for a switch from Th1 to Th2.

Processing of the Interleukin-33 Precursor

Initially, IL-33 was considered closely related to IL-1β and IL-18 because the IL-33 precursor contains a caspase-1 site, which upon activation would cleave the IL-33 precursor and release the active cytokine,[11] similar to that for IL-1β and IL-18. Indeed, the first recombinant forms of IL-33 were produced with an N-terminus at the caspase-1 site.[11] Although recombinant IL-33 was active, the concentrations required for activity were considerably higher than those of other members of the IL-1 family. Indeed, subsequent studies revealed that caspase-1 actually results in loss of IL-33 activity and that the full-length IL-33 precursor binds to ST2 and is active,[123] similar to the ability of the IL-1α precursor to bind to IL-1RI. In addition, it was reported that the caspase-1 cleavage site at 178 is similar to the consensus sequence for caspase-3 and that intracellular IL-33 precursor is a substrate for caspase-3.[123]

Using immobilized IL-33 precursor, neutrophil proteinase 3 (PR3) was isolated from human urinary proteins.[124] Neutrophil PR3 is known to process the IL-1β precursor into an active cytokine.[86] PR3 converted human and mouse precursor IL-33 proteins to biologic active forms; however, increasing the incubation time of PR3 abrogated IL-33 activities.[124] Using the consensus amino acid sequence sites for PR3, six human and mouse recombinant IL-33 proteins were produced and assessed for biologic activities; varying levels of activity were reported.[124] Another study also demonstrated cleavage of the IL-33 precursor by neutrophil proteases such as PR3, neutrophil elastase, and cathepsin G,[125] resulting in the generation of IL-33 with different N-termini and varying levels of activity. These studies support the concept that extracellular IL-33 is released as a precursor, is rapidly processed by neutrophil enzymes, and generates active forms with varying levels of activity. The implications for generation of active IL-33 by neutrophil enzymes for Th2 polarization remain unclear. It may be more relevant to study the effect of proteases from eosinophils in the processing of the IL-33 precursor. Nevertheless, the IL-33 precursor binds to ST2 and recruits the accessory chain for signal transduction, but compared to IL-33 generated by neutrophil proteases, the activity of IL-33 precursor is weak.[124,125]

There was no dearth of studies on ST2 tissue-specific localization, regulation of its expression, effects in transgenic mice overexpressing ST2, as well as deletion, neutralization, and antibody cross-linking of ST2. Elevated levels of the soluble form of ST2 were present in the circulation of patients with a various inflammatory diseases and that exogenous administration of pharmacologic doses of soluble ST2 neutralized endogenous levels of the then putative ligand IL-33 and reduced inflammation.[126] IL-33 activates Th2 lymphocytes, mast cells, basophils, and eosinophils, as well as NK T cells and blood monocytes. One of the most studied properties of IL-33 is the induction of IL-5 and IL-13 and their respective

roles in lung inflammation such as allergic type asthma. For example, instillation of IL-33 into the airways triggers an immediate allergic response in the lung of naïve mice and worsens the response in mice sensitized to antigen peripherally but challenged by exposure of antigen in the lung.[127]

Mice deficient in ST2 do not develop a Th2 response to *Schistosoma* egg antigen. Indeed, several studies have focused on the role of IL-33 in the pathogenesis of helminth worm infections. The Th2 response by the host contributes to the elimination of these worm infestations, which are worldwide and afflict hundreds of millions. The role of IL-33 in the induction of IL-4, IL-5, and IL-13 is of paramount importance in terms of pulmonary and intestinal complications that reduce lifespan. Using mice deficient in IL-33, a crucial role was demonstrated in mice to rid an infection with *Strongyloides venezuelensis*.[128] The infection induces a unique class of cells called natural helper cells or nuocytes, which upon activation by IL-33 produce IL-5 and IL-13, resulting in eosinophilic infiltration into the lungs. In this model, pulmonary inflammation causes damage via eosinophilic infiltration, which is IL-33 and IL-5 dependent.[128]

Mice injected with human IL-33 exhibit impressive pathologic changes in the arterial walls, lungs, and intestinal tissues.[11] Of particular relevance to the concept that IL-33 drives a Th2 response, eosinophilic infiltration was a prominent finding in the lung and in allergic rhinitis as well as allergic conjunctivitis.[129] These initial observations have been confirmed by other reports.[130] Although the interpretation of in vivo effects following the administration of an exogenous cytokine should be conservative, the findings are clearly consistent with IL-33 being a proinflammatory ligand of the IL-1 receptor family. Even before the ability to test IL-33-mediated activation, others had reported that neutralization of the putative ST2 ligand using soluble ST2 markedly reduced joint inflammation, synovial hyperplasia, and joint erosion when given in the therapeutic phase of collagen-induced arthritis in mice.[126]

Interleukin-33 as an Anti-inflammatory Cytokine

Members of the IL-1 family of ligands bind to their specific cell surface receptors and recruit an accessory chain. The IL-1RIAcP is used by IL-1α and IL-1β but also by IL-36 and IL-33. The accessory chain for IL-18 is related to the IL-1RIAcP but is encoded by a distinct gene. We now recognize that other members of the IL-1 receptor family will bind more than one cytokine. The best example is IL-1α and IL-1β. Both bind with similar affinities to IL-1RI, but the three-dimensional structure of IL-1α and IL-1β are hardy identical.[131] The IL-1β precursor binds to IL-1RII as well as a processed form with the first 112 amino acids cleaved from the precursor. IL-37 binds to the IL-18 receptor alpha chain,[132] and both IL-36 and IL-38 bind to the IL-36 receptor.[133]

IL-33 forms a complex with ST2 IL-1RIAcP but also with single immunoglobulin IL-1-related receptor (SIGIRR).[134]

This complex plays a role in the Th2 response by reducing IL-33 signaling,[134] and consistent with these observations, Th2 responses are increased in mice deficient in SIGIRR. Furthermore, there is high expression of SIGIRR in Th2 polarized cells and in models of Th2

antigen sensitization; SIGIRR-deficient mice exhibit a greater Th2 response.[134] The complex with SIGIRR and IL-33 may explain the antiinflammatory properties of IL-33. ST2 can sequester TLR adaptor molecules such as MyD88 and Mal.[135]

In mice deficient in ST2, there is myocardial hypertrophy, ventricle dilation, and fibrosis upon pressure overload, suggesting that IL-33 plays a protective role in the heart.[136] Furthermore, elevated levels of the extracellular domain of ST2 predict outcomes in patients with systolic heart failure or following a myocardial infarction.[136] In a model of cardiomyocyte hypertrophy induced by chronic administration of phenylephrine, administration of recombinant IL-33 inhibited the phosphorylation of IκB and reduced the hypertrophy and fibrosis.[136] One of the more challenging aspects of the properties of IL-33 to act as a Th2 cytokine is its role as an antagonist in the ApoE-deficient mouse model of atherosclerosis. In this model, arterial wall plaques of mice on a high-fat diet contain IL-33 and ST2. In mice treated with IL-33, the atherosclerotic plaques were markedly reduced.[137] In mice treated with soluble ST2 to neutralize IL-33, the disease worsened.[137]

Interleukin-33 as a Transcription Factor

Similar to IL-1α, there is another side to IL-33. Although IL-33 binds to its specific surface receptor, IL-33 is identical to a nuclear factor dominantly expressed in high endothelial venules.[138] This nuclear factor is termed NF-HEV. In addition to endothelial cells, constitutive nuclear localization of IL-33 has been reported in several cell types such as type II lung epithelial cells,[128] epithelial cells,[139] and pancreatic stellate cells.[140] In fact, IL-33 binding to DNA and acting as a nuclear factor is similar to IL-1α binding to chromatin and functioning as a nuclear factor.[21,33,141] A short IL-33 peptide similar to a sequence in Kaposi sarcoma virus binds chromatin.[142] The full-length IL-33 precursor, but not mature IL-33, binds to the N-terminal Rel homology domain of NF-κB p65.[143] In cells overexpressing the IL-33 precursor, there was a reduction in IL-1β-induced TNFα.[143] These data are consistent with other data that IL-33 possesses antiinflammatory properties (see previous discussion) and the mechanism for this property of IL-33 appears to be nuclear sequestration similar to that of IL-1α.[33]

INTERLEUKIN-18 AND INTERLEUKIN-37 SUBFAMILY

Interleukin-18

Background

IL-18 was first described in 1989 as "IFNγ-inducing factor" isolated in the serum of mice following an injection of endotoxin. The mice had been pretreated with *Proprionibacterium acnes*, which stimulates the reticuloendothelial system, particularly the Kupffer cells of the liver. Many investigators concluded that the serum factor was IL-12. With molecular cloning of "IFNγ-inducing factor" in 1995,[144] the name was changed to IL-18. Surprisingly, the new cytokine was related to IL-1 and particularly to IL-1β. Similar to IL-1β, IL-18 lacks a signal peptide, is first synthesized as an inactive precursor, and remains as an intracellular cytokine.

The tertiary structure of the mature form of IL-18 closely resembles that of IL-1β,[144] although the IL-18 precursor is closely related to the IL-37 precursor. Since 1995, many studies have used neutralization of endogenous IL-18 or IL-18-deficient mice to demonstrate the role for this cytokine in promoting inflammation and immune responses.[145] However, the biology of IL-18 is hardly the recapitulation of IL-1β. There are several unique and specific differences between IL-18 and IL-1β. For example, in healthy human subjects and also in healthy mice, gene expression for IL-1β in blood mononuclear cells and hematopoietic cells is absent, and there is no evidence that the IL-1β precursor is constitutively present in epithelial cells.[146] In contrast, in the same blood cells, large amounts of the IL-18 precursor are present. Peritoneal macrophages and mouse spleen contain the IL-18 precursor in the absence of disease.[146] The IL-18 precursor is also present in keratinocytes and nearly all epithelial cells. In this regard, IL-18 is similar to IL-1α and IL-33.

Processing of the Interleukin-18 Precursor

The IL-18 precursor has a molecular weight of 24,000 and is processed by caspase 1 cleavage into a mature molecule of 18,000. Compared to wild-type mice, following an injection of endotoxin into caspase-1-deficient mice, circulating IFNγ is absent. IL-12-induced IFNγ is also absent in caspase-1-deficient mice.[147] Importantly, any phenotypic characteristic of capsase-1-deficient mice must be studied as to whether the deficiency is due to reduced IL-1β or IL-18 activity. For example, the caspase-1-deficient mouse is resistant to colitis,[148] but the IL-1β-deficient mouse is susceptible in the same disease. Because neutralizing antibodies to IL-18 are protective in the colitis model, caspase-1 deficiency appears to prevent processing of IL-18.[148,149] On the other hand, there are examples where caspase-1 processing of IL-18 is not required. For example, Fas ligand stimulation results in release of biologically active IL-18 in caspase-1-deficient murine macrophages.[150] Similar to IL-1β processing, proteinase-3 appears to activate processing to mature IL-18.[88]

Similar to IL-1α and IL-33, the IL-18 precursor is constitutively expressed in endothelial cells, keratinocytes, and intestinal epithelial cells throughout the gastrointestinal tract. Macrophages and dendritic cells are the primary sources for the release of active IL-18, whereas the inactive precursor remains in the intracellular compartment of mesenchymal cells. Also, similar to IL-1α and IL-33, the IL-18 precursor is released from dying cells and processed extracellularly, most likely by neutrophil proteases such as proteinase-3.

The IL-1 family consensus sequence (A-X-D) in IL-18 is I-N-D at amino acid 50, but the N-terminus generated by caspase-1 is 14 amino acids before the consensus sequence rather than 9 amino acids (see Fig. 26.1). Although the IL-18 as well as the IL-1β precursor can be cleaved extracellularly by proteianse-3, there is an additional enzyme that cleaves the IL-18 precursor into an active cytokine. Merprin β is a

member of the merpin family of zinc metalloproteinases, which are expressed on the membrane surface of epithelial cells of the intestine and kidney but also on myeloid cells.[151,152] Merprin α will cut the IL-1β precursor into an active cytokine, and inhibitors of merprin α markedly reduce serum levels of IL-1β in mice subjected to cecal ligation and puncture.[151] Merprin β will cleave the IL-18 precursor and generate an active cytokine on

cells bearing the IL-18R.[153] In a model of dextran sulfate sodium (DSS) colitis, serum IL-18 is elevated on days 3 and 5, during which time the mice develop inflammatory colitis. However, in merprin β-deficient mice, there is a statistically significant reduction in serum IL-18.[153] The role of IL-18 and caspase-1 in DSS colitis is discussed in the following.

Signal Transduction by Interleukin-18

As shown in Figure 26.3A, IL-18 forms a signaling complex by binding to the IL-18 alpha chain (IL-18Rα), which is the ligand binding chain for mature IL-18; however, this binding is of low affinity. In cells that express the coreceptor, termed IL-18 receptor beta chain (IL-18Rβ), a high-affinity complex is formed, which then signals. The complex of IL-18 with the IL-18Rα and IL-18Rβ chains is similar to that formed by other members of the IL-1 family with the coreceptor, the IL-1R accessory chain IL-1RAcP. Following the formation of the heterodimer, the TIR domains approximate, and it appears that the cascade of sequential recruitment of MyD88, the four IRAKs, and TRAF-6 followed by the degradation of IκB and release of NFκB are nearly identical as that for IL-1.[154] There are differences between IL-1 and IL-18 signaling that remains unexplained. With few exceptions, IL-1α or IL-1β are active on cells in the low nanogram/mL range and often in the picogram/mL range. In contrast, IL-18 activation of cells expressing the two IL-18 receptor chains requires 10 to 20 ng/mL and sometimes higher levels.[155,156]

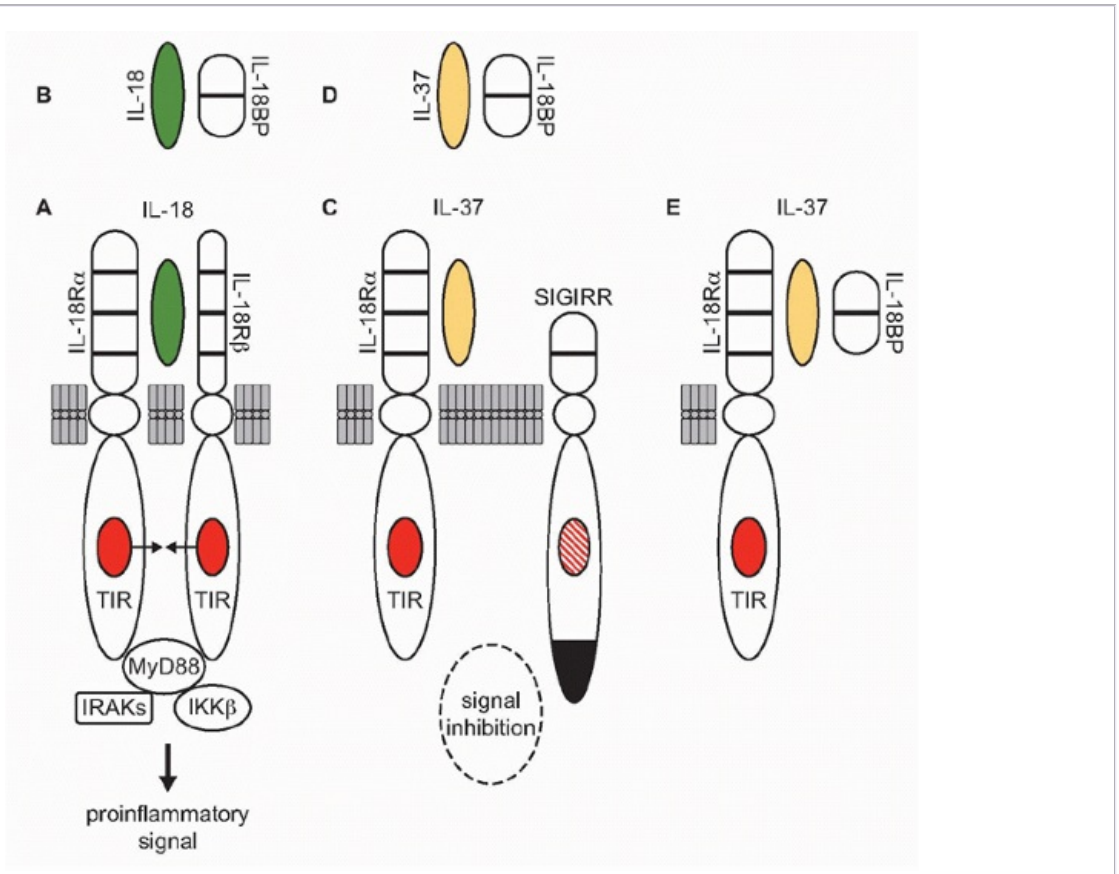

FIG. 26.3. Interleukin (IL)-18 Subfamily. A: IL-18 binds to the IL-18Rα chain and recruits the coreceptor IL-18Rβ. The signaling cascade of the IL-18 receptor complex is nearly the same as that of IL-1α and IL-1β, resulting in the expression of proinflammatory

genes. **B:** The natural occurring IL-18BP binds IL-18, thus neutralizing the activity of the cytokine. **C:** IL-37 also binds to the IL-18Rα but with an affinity lower than that of IL-18 binding to the same receptor. Furthermore, the binding of IL-37 to IL-18Rα does not recruit the coreceptor IL-18Rβ and therefore there is no proinflammatory signal. The anti-inflammatory properties of IL-37 require single immunoglobulin IL-1-related receptor, which may act as a "decoy" for MyD88. **D:** IL-18BP also binds to IL-37, thus preventing binding of IL-37 to the IL-18Rα. **E:** IL-37 binds to IL-18BP forming a complex, which then binds to the IL-18Rα, enhancing the anti-inflammatory property of IL-18BP.

Although nearly all cells express the IL-1RI, not all cells express IL-1RAcP. Similarly, most cells express the IL-18Rα but not all cell express the IL-18Rβ. IL-18Rβ is expressed on T cells and dendritic cells but not commonly expressed in mesenchymal cells. The best example is the A549 cell. This cell line, derived from a lung carcinoma epithelial cell, does not express IL-18Rβ,[157] and there is no signal unless IL-12 is added to induce IL-18Rβ.[10] In the absence of IL-18Rβ, IL-18 binds to IL-18Rα without a proinflammatory signal. In A549 cells transfected with IL-18Rβ, IL-18 induces IL-8 and a large number of genes. One of these genes is the former IL-2-induced gene termed NK4,[158] now termed IL-32.[157] IL-32 is not a member of the IL-1 family but plays an important role in the regulation of cytokines such as IL-1β and TNFα.

Interleukin-18 as an Immunoregulatory Cytokine

Together with IL-12, IL-18 participates in the Th1 paradigm. This property of IL-18 is due to its ability to induce IFNγ either with IL-12 or IL-15. Without IL-12 or IL-15, IL-18 does not induce IFNγ. IL-12 or IL-15 increases the IL-18Rβ, which is essential for IL-18 signal transduction. Without IL-12 or IL-15, IL-18 plays a role in Th2 diseases.[159] The importance of IL-18 as an immunoregulatory cytokine

is derived from its prominent biologic property of inducing IFNγ from NK cells. Macrophage-colony stimulating factor induces human blood monocytes to develop into a subset of macrophages; these cells express a membrane-bound form of IL-18.[160] Membrane IL-18 is expressed in 30% to 40% of macrophage-colony stimulating factor-primed macrophages. In contrast, monocytes, dendritic cells, and monocytes differentiated into M1 macrophages did not express membrane IL-18. Although the expression of membrane IL-18 is caspase-1 dependent,[160] LPS treatment was necessary for the release of membrane IL-18.[160] A major immunoregulating role for IL-18 is on the NK cell. Upon shedding of membrane IL-18 into a soluble form, NK cells expressed CCR7 and produced high levels of IFNγ. As expected, IFNγ production was prevented by neutralization of IL-18. This mechanism may account for the role of IL-18 as major IFNγ-inducing factor from NK cells and the role of NK cells in the pathogenesis of autoimmune diseases.

The induction of FFNγ by IL-18 has been studied with coinducer IL-12. For example, mice injected with the combination of IL-18 plus IL-12 develop high levels of IFNγ and die with hypoglycemia, intestinal inflammation, and inanition.[161] In leptin-deficient mice, IL-18 plus IL-12 induce acute pancreatitis.[162] Several human autoimmune diseases are associated with elevated production of IFNγ and IL-18. Diseases such as systemic lupus erythematosus, rheumatoid arthritis, type 1 diabetes, Crohn disease, psoriasis, and graft-versus-host disease

are thought to be mediated, in part, by IL-18.

Proinflammatory Properties of Interleukin-18

IL-18 exhibits characteristics of other proinflammatory cytokines, such as increases in cell adhesion molecules, nitric oxide synthesis, and chemokine production. Blocking IL-18 activity reduces metastasis in a mouse model of melanoma; this is due to a reduction in IL-18-induced expression of vascular call adhesion molecule-1.[163] A unique property of IL-18 is the induction of Fas ligand (FasL), which may account for the hepatic damage that takes place in macrophage activation syndrome.[150,164] The induction of fever, a well-studied property of IL-1α and IL-1β as well as TNFα and IL-6, is not a property of IL-18. Injection of IL-18 into mice, rabbits, or humans does not produce fever.[165,166] Unlike IL-1 and TNFα, IL-18 does not induce cyclooxygenase-2 and hence there is no production of prostaglandin E2.[156,167] IL-18 has been administered to humans for the treatment of cancer in order to increase the activity and expansion of cytotoxic T cells. Not unexpectedly and similar to several cytokines, the therapeutic focus on IL-18 has shifted from its use as an immune stimulant to inhibition of its activity.[145,168]

Because IL-18 can increase IFNγ production, blocking IL-18 activity in autoimmune diseases is an attractive therapeutic target as anti-IL-12/23 reduces the severity of Crohn disease as well as psoriasis. As discussed subsequently, there appears to be a role for blocking IL-18 in Crohn disease. However, there are several activities of IL-18 that are independent of IFNγ. For example, IL-18 inhibits proteoglycan synthesis in chondrocytes,[169] and proteoglycan synthesis is essential for maintaining healthy cartilage. IL-18 also increases vascular call adhesion molecule-1 expression in endothelial cells independently of IFNγ. Vascular call adhesion molecule-1 plays a major role in multiple sclerosis, other autoimmune diseases, as well as in the metastatic process.[170]

Role of Interleukin-18 in Models of Inflammatory Bowel Disease

Inflammatory bowel disease such as Crohn disease is a complex autoimmune disease. Treatment is initially based on immunosuppressive drugs. Not surprisingly, anticytokines such as neutralizing monoclonal antibodies to TNFα[171] or to IL-12/23 provide effective treatment for many patients.[172,173] The reduction of IFNγ in Crohn disease is linked to the clinical response to these agents.[173] IL-18 is found in affected intestinal lesions from patients with Crohn disease as a mature protein, but the IL-18 precursor form is present in uninvolved intestinal tissues.[174] This observation was confirmed in a similar assessment of mucosal biopsies from patients with Crohn disease.[175] Antisense RNA to IL-18 decreased IFNγ production in lamina propria mononuclear cells.[175]

A commonly used mouse model for colitis is DSS, which is added to the drinking water and which damages the intestinal wall. Thus in DSS-induced colitis, the epithelial barrier defenses against luminal bacterial products are breeched. In this model, reducing IL-18 with a neutralizing antibody is protective and linked to a reduction in IFNγ.[149] Blocking IL-18 with the IL-18 binding protein (IL-18BP) also reduces colitis induced by antigen sensitization.[176] Because generation of active IL-18 requires caspase-1, studies have also been performed in

mice deficient in caspase-1 and subjected to DSS colitis. Nevertheless, despite many studies, the role of caspase-1 in DSS colitis remains unclear. The first study showed that mice deficient in caspase-1 were protected.[148,177] In addition, treatment of mice with a specific caspase-1 inhibitor was also effective in protecting against the colitis.[178,179,180] In both studies, the effect of caspase-1 deficiency was linked to reduced IL-18 activity, whereas reducing IL-1 activity with the IL-1Ra was ineffective.[148] In support of the role of IL-18 in DSS colitis, inhibition of endogenous merprin β to reduce the generation of active IL-18 was protective in DSS colitis.[153]

However, a conundrum has developed whether caspase-1 deficiency is protective or detrimental in DSS colitis. DSS colitis is not the optimal model for Crohn disease, as the model is one of rapid loss of the protective barrier of the intestinal epithelium exposing the lamina propria mononuclear cells to a large amount and variety of bacterial products. Using the same DSS model, mice deficient in the adapter protein inflammasome component ASC experienced increased disease, morbidity, and precancerous lesions compared to wild-type mice exposed to DSS.[181] Similarly, mice deficient in caspase-1 died rapidly from DSS compared to wild-type mice,[182] whereas mice deficient in caspase-12, in which caspase-1 is enhanced, were protected.[182] Administration of exogenous IL-18 restored mucosal healing in caspase-1-deficient mice.[182] Also, mice deficient in NLRP3 were more susceptible to either DSS or 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis and exhibited decreased IL-1β as well as decreased beta-defensins.[183] Macrophages from NLRP3-deficient mice failed to respond

to muramyl dipeptide.[183] Mice deficient in NLRP6 are also more vulnerable to DSS,[37,184] and the susceptibility appears to be due to lack of sufficient IL-18.

How to reconcile these data in mouse models of colitis was addressed by Siegmund[185]? It is likely that IL-18 being constitutive in the intestinal epithelium has a protective role in that the cytokine contributes to maintaining the intestinal barrier. With loss of the barrier, the microbial products stimulate macrophages in the lamina propria and caspase-1-dependent processing of IL-18 results in inflammation. In this model, inhibition of IL-18 production in caspase-1-deficient mice or treatment of wild-type mice with anti-IL-18 antibodies or caspase-1 inhibitors is protective. Worsening of disease in mice deficient in caspase-1 or NLRP3 or NLRP6 may lower the levels of active endogenous IL-18 needed to protect the epithelial barrier. Similarly, active endogenous IL-1β may be needed to protect to maintain the epithelial barrier by inducing growth factors.

Although it remains unclear why caspase-1 deficiency worsens DSS colitis, in humans with Crohn disease, natalizumab, the antibody that blocks the very late antigen-4, is highly effective in treating the disease. Very late antigen-4 is the α4 subunit of the β-1 integrin. Anti-very late antigen-4 binds to the surface of macrophages and other myeloid cells, and prevents the binding of these cells to the very late antigen-4 receptor on endothelial cells known as vascular cell adhesion molecule-1. Thus, the antibody disables the function of vascular cell adhesion molecule-1 and prevents the passage of macrophages and other myeloid cells into tissues such as the intestine in Crohn disease and the brain in multiple sclerosis. Because IL-18 induces vascular cell adhesion molecule-1, blocking IL-18 would

also reduce the passage of cells through the endothelium into to intestine.

Interleukin-18, Hyperphagia, and the Metabolic Syndrome

Whereas there is no constitutive gene expression for IL-1β in freshly obtained human peripheral blood mononuclear cells (PBMC), the same cells express constitutive mRNA for IL-18.[146] In western blot analysis from the same cells, the IL-18 precursor was present but not the IL-1β precursor. Similar observations were also made in mice.[146] These findings suggest that IL-18 may act as regulator of homeostasis. Starting at age 16 weeks of age, IL-18-deficient mice start to overeat, become obese, and exhibit lipid abnormalities; there is increased atherosclerosis, insulin resistance, and diabetes mellitus reminiscent of the metabolic syndrome.[186] IL-18Rα-deficient mice also develop a similar phenotype. The higher body weight is attributed to enhanced food intake, in which the IL-18-deficient mice begin to diverge from wild-type animals at a relatively early age, and to reach values 30% to 40% higher than that of wild-type mice. Others have observed similar findings.[187] A striking finding was an increase of more than 100% in the percent of adipose tissue in the IL-18-deficient animals that was accompanied by fat deposition in the arterial walls. The insulin resistance in these mice is corrected by exogenous recombinant IL-18. Mice deficient in IL-18 respond normally to a challenge with exogenous leptin, suggesting that expression of the leptin receptor is unaffected. The unexpected and unique mechanism is responsible for the higher food intake in the IL-18-deficient animals appears to be due to a central nervous system loss of appetite control. IL-18-deficient mice eat throughout the day, whereas wild-type mice eat once, nocturnally.

Interleukin-18 as a Protected Cytokine

As stated previously, mice deficient in caspase-1 experience increased disease severity when subjected to DSS colitis and that administration of exogenous IL-18 restored mucosal healing in these mice.[182] In addition, mice deficient in NLRP3 were more susceptible to DSS colitis, which is thought to be due to decreased IL-18.[183] Mice deficient in NLRP6 are also more vulnerable to DSS,[37,184] and the susceptibility appears to be due to lack of sufficient IL-18. Thus, there are a growing number of studies that support a protective role for IL-18. The fact that mice deficient in IL-18 develop a metabolic syndrome-like phenotype is consistent with a role for IL-18 in homeostasis. A study in age-related macular degeneration is also consistent with a protective role for IL-18. In that study, drusen, which is mixture of complement-derived and apolipoproteins and lipids, was shown to activate NLRP3 and induce the production of mature IL-1β and IL-18.[188] In a mouse model of “wet” age-related macular degeneration, the disease was worse in mice deficient in NLRP3 but not in IL-1RI-deficient mice.[188] Therefore, IL-18 rather than IL-1α or IL-1β were protective; upon administration of IL-IL-18, the disease severity improved. Taken together, there is a case for IL-18 being a protective rather than inflammatory cytokine.

Interleukin-18 Binding Protein

The discovery of the IL-18BP took place during the search for the soluble receptors for IL-18.[189] IL-18BP is a constitutively secreted protein with an exceptionally high affinity for IL-18

(400 pM) (see Fig. 26.3B). Present in the serum of healthy humans at a 20-fold molar excess compared to IL-18,[190] IL-18BP may contribute to a default mechanism by which a Th1 response to foreign organisms is blunted in order to reduce triggering an autoimmune responses to a routine infection. Although IL-18BP is readily secreted, it falls into the functional category of being a shed soluble receptor. As shown in Figure 26.3B, IL-18BP contains only one immunoglobulin G domain, whereas the type II IL-1 receptor contains three domains. In this regard, the single immunoglobulin G domain of IL-18BP is similar to SIGIRR, which also has a single immunoglobulin G domain and also functions as a decoy receptor. The salient property of IL-18BP in immune responses is in downregulating Th1 responses by binding to IL-18 and thus reducing the induction of IFNγ.[159] Because IL-18 also affects Th2 responses, IL-18BP also has properties controlling a Th2 cytokine response.[159] IL-18BP has a classic signal peptide and therefore is readily secreted. Serum levels in healthy subjects are in the range of 2,000 to 3,000 pg/mL compared to the levels of IL-18 in the same sera of 80 to 120 pg/mL/mL. Moreover, IL-18BP binds IL-18 with an affinity of 3 to 5 nM,[189] an affinity significantly higher than that of IL-18Rα. Because a single IL-18BP molecule binds a single IL-18 molecule, one can calculate bound versus free IL-18 in a mixture of both molecules.[190]

If one examines immunologically mediated diseases where IFNγ plays a pathologic role such as Wegener granulomatosis

and systemic lupus erythematosus, one must consider the level of free IL-18 compared to IL-18 bound to IL-18BP. In fact, in these diseases both IL-18BP and IL-18 are high,[191,192] but the level of IL-18BP is not sufficiently high enough to neutralize IL-18 and therefore, the level of free IL-18 is higher than in healthy subjects. In macrophage activation syndrome where IFNγ plays a pathologic role, both IL-18BP and IL-18 are also high but the clinical and hematologic abnormalities correlate with elevated free IL-18.[164]

A unique property of IL-18BP is that the molecule also binds IL-37[193] and in doing so, enhances the ability of IL-18BP to inhibit the induction of IFNγ by IL-18. IL-37 binds to the IL-18Rα with a very low affinity but in mice expressing human IL-37, a profound anti-inflammatory effect is observed,[194] particularly of LPS-induced cytokines and dendritic cell maturation.[194] Human IL-37-expressing mice are also resistant to colitis.[195] Thus, the anti-inflammatory property of IL-37 can be affected by the concentration of IL-18BP. As the concentration of IL-18BP increases and binds IL-37, there is the possibility that IL-37 becomes less available as an anti-inflammatory cytokine. Indeed, this has been observed in mice injected with IL-18BP. At low dosing of IL-18BP, there is reduced inflammation in a model of rheumatoid arthritis but as the doing of IL-18BP increases, the anti-inflammatory properties of IL-18BP are lost.[196]

IL-18BP is highly regulated at the level of gene expression and unexpectedly, IFNγ increases gene expression and synthesis of IL-18BP.[197,198] Therefore, IFNγ driving an increase in the natural and potent inhibitor of IL-18 falls into the category of a negative feedback loop. The concept is supported by clinical data showing that patients being treated with IFNα for hepatitis have elevated levels of IL-18BP.[199,200] IL-27, like IFNγ, functions as both a pro- as

well as an anti-inflammatory cytokine and both may accomplish their roles as anti-inflammatory cytokines at the level of increased production of IL-18BP. In the skin, IL-27 also acts through a negative feedback loop for inflammation. IL-27 is acting, as is IFNγ, by induction of IL-18BP gene expression and synthesis.[201]

Viral Interleukin-18 Binding Protein

Natural neutralization of human IL-18 by IL-18BP takes place during a common viral infection. In *Molluscum contagiosum* infection, characterized by raised but bland eruptions, there are large numbers of viral particles in the epithelial cells of the skin, but histologically there are few inflammatory or immunologically active cells in or near the lesions. Clearly, the virus fails to elicit an inflammatory or immunologic response. Amino acid similarity exists between human IL-18BP and a gene found in various members of the poxviruses; the greatest degree of homology is found to be expressed by *Molluscum contagiosum* gene.[202] The ability of viral IL-18BP to reduce the activity of mammalian IL-18 likely explains the lack of inflammatory and immune cells in the infected tissues and provides further evidence for the natural ability of IL-18BP to interfere with IL-18 activity.

Interleukin-37

IL-37 was formerly termed IL-1F7. IL-37 lacks a signal peptide and has a caspase-1 site, but the secretion of IL-37 has not been documented with any certainty. It is likely, however, that similar to IL-1α and IL-33, with loss of membrane integrity upon cell death, the IL-37 precursor exits from the cell. The recombinant form of the IL-37 precursor suppresses LPS-induced IL-1β, IL-6, and TNFα. However, this is observed primarily in macrophages that have been differentiated into the M1 phenotype by 5 days in the presence of granulocyte macrophage-colony stimulating factor. There are two consensus sequences (A-X-D) in N-terminal domain of IL-37: IHD and LED. A recombinant form of IL-37 with an N-terminus nine amino acids from the IHD site is active in suppressing LPS-induced TNFα and IL-6. Whether this short form of recombinant IL-37 exists in nature is unclear.

Interleukin-37 Reduces Interleukin-1β and Lipopolysaccharide-Induced Inflammation in Vivo

A mouse homologue for human IL-37 has not been identified. Therefore, to define the in vivo functional role of IL-37, a strain of transgenic mice was generated.[194] The full-length IL-37 complementary DNA was inserted into a vector using the standard cytomegalovirus (CMV) promoter for constitutive expression of the transgene in all cells. Both heterozygous and homozygous IL-37 transgenic mice (IL-37 tg) mice breed normally and exhibit no obvious phenotype. Despite the presence of the CMV promoter, the IL-37 transcript is not constitutively expressed in the tissues of the IL-37 transgenic mice. The failure to express IL-37 is likely due to a functional instability sequence found in IL-37, which limits the half-life of IL-37 mRNA.[51] Nevertheless, upon stimulation with LPS or IL-1β, levels of IL-37 increase after 4 to 24 hours. Once the transcript is present, the IL-37 precursor can be found in peripheral blood cells taken from the transgenic mice.[203]

IL-37 transgenic mice are protected against LPS challenge compared to similarly challenged wild-type mice. IL-37 transgenic mice exhibit significantly less hypothermia, acidosis,

hyperkalemia, hepatitis, and dehydration.[194] In addition, circulating cytokines are significantly reduced as well as cytokines induced in whole blood cultures and in lung and spleen cell homogenates. In addition to LPS-induced cytokines, whole blood cultures from IL-37 transgenic mice produce significantly less IL-6 and TNFα when stimulated by IL-1β or the combination of IL-12 plus IL-18. The antiinflammatory activity of IL-37 was not limited to a reduction of the cytokines and chemokines of innate immunity. Dendritic cells isolated from the spleen of IL-37 transgenic mice upon LPS stimulation revealed a marked reduction (75% and 89%) in expression of CD86 and MHC II, respectively.[194] The total numbers of dendritic cells, macrophages, NK cells, and CD4+ T cells were similar in all strains and experimental conditions.

A Role for Interleukin-37 During Experimental Colitis

IL-37 transgenic mice have been subjected to DSS-induced colitis. Despite the presence of a CMV promoter to drive expression of IL-37, mRNA transcripts were not present in colons in the resting state.[195] Expression was observed only upon disruption of the epithelial barrier, with a six- to sevenfold increase on days 3 and 5 after continuous exposure to DSS. During the development of colitis, clinical disease

scores were reduced by 50% and histologic indices of colitis were one-third less in IL-37 transgenic mice compared with wild-type counterparts. Reduced inflammation was associated with decreased leukocyte recruitment into the colonic lamina propria. In addition, release of IL-1β and TNFα from ex vivo colonic explant tissue was decreased 5- and 13-fold, respectively, compared with wild-type mice, whereas IL-10 was increased 6-fold. However, IL-10 was not required for the anti-inflammatory effects of IL-37 because IL-10 receptor antibody blockade did not reverse IL-37-mediated protection. Mechanistically, IL-37 originating from hematopoietic cells was sufficient to exert anti-inflammatory effects because wild-type mice reconstituted with bone marrow from IL-37 transgenic mice were protected from colitis.

A Nuclear Role for Interleukin-37

In stable transfectants of human IL-37 in RAW macrophages stimulated with LPS, levels of TNFα, IL-1α, IL-6, as well as the chemokine MIP-2 were substantially reduced (72% to 98%) compared with LPS-stimulated cells transfected with the empty plasmid.[204] Similar to IL-1α and IL-33, IL-37 translocates to the nucleus following stimulation.[204] In mouse RAW macrophages stably expressing IL-37, the mature carboxyl-terminal was detected in the nucleus. Furthermore, a specific caspase-1 inhibitor markedly reduced nuclear entry of IL-37.[204] The data demonstrate that IL-37 translocates to the nucleus after caspase-1 processing and may act as a transcriptional modulator reducing the production of LPS-stimulated proinflammatory cytokines, consistent with IL-37 being an anti-inflammatory member of the IL-1 family.

IL-37 was identified in a proteomics-based search for proteins that interacted with Smad3.[205] To test for a functional interaction of Smad3 with IL-37, IL-37 was transfected into A549 cells. IL-37 colocalized with phospho-Smad3 and was found in perinuclear and cytosolic regions, and a IL-37-Smad3 complex was observed.[194] A specific inhibitor of Smad3 reversed the inhibition of IL-6 and IL-1β expression in RAW cells stably transfected with IL-37. In stable

human macrophage lines expressing IL-37, depletion of Smad3 by lentiviral introduction of short hairpin RNA that inhibits Smad3 expression prevented the ability of IL-37 to reduce IL-1β- or LPS-induced production of IL-8, IL-6, and TNF. These in vitro findings were confirmed in vivo. IL-37 transgenic mice were pretreated intranasally with a Smad3 specific small interfering RNA and then challenged with intranasal LPS. The reduction of lung cytokines in IL-37 transgenic mice was reversed in transgenic mice with a lung knockdown of Smad3.[194]

Role of Interleukin-18Rα for Interleukin-37

From the first reports on IL-37, it was observed that the recombinant forms bound to the IL-18Rα.[132,206] This is shown in Figure 26.3C and E. The binding of IL-37 to IL-18Rα has also been observed in cells from IL-37 transgenic mice using immunofluoresence, immunoprecipitation, and fluorescence resonance energy transfer analysis.[207] IL-37 specifically binds to the third domain of the IL-18Rα.[193] Despite these studies showing binding of IL-37 to the IL-18Rα chain, IL-37 does not act as a classical receptor antagonist for IL-18 in that the ability of recombinant IL-18 to induce IFNγ is not inhibited by high concentrations of IL-37. However, in the presence of low concentrations of IL-18BP, recombinant IL-37 modestly reduces IL-18-induced IFNγ.[193] The concept that IL-37 binds to the IL-18Rα and reduces cytokine production is supported, in part, with the finding embryonic fibroblasts from mice deficient in IL-18Rα produce 10-fold more IL-6 in response to IL1β than do wild-type embryonic fibroblasts.[208] In addition, silencing of IL-18Rα in primary human blood monocytes results in a fourfold increase in the secretion of LPS-induced IL-1β, IL-6, IFNγ, and CD40 ligand.[208] Thus, the seemingly paradoxical hyperresponsive state in cells deficient in IL-18Rα supports the concept that IL-18Rα participates in both pro- and anti-inflammatory responses and that the endogenous ligand IL-37 engages the IL-18Rα to deliver an inhibitory signal.

Role of Single Immunoglobulin Interleukin-1-Related Receptor in the Anti-inflammatory Property of Interleukin-37

The mechanism by which an IL-1β or an LPS signal is suppressed by IL-37 requires an understanding of SIGIRR (Fig. 26.4G). The IL-1RAcP serves as the coreceptor for IL-1α, IL-1β, IL-36α, IL-36β, IL-36γ, and IL-33, each a proinflammatory cytokine. However, in the IL-1 family of receptors, three coreceptors contain unusually long intracellular domains. These are SIGIRR (Fig. 26.4G), a variant of the IL-1RAcP termed IL-1RAcPb (Fig. 26.4F) and receptors termed “three immunoglobulin IL-1-related receptors” (TIGIRRs). As shown in Figure 26.4H, there are two TIGIRRs: TIGIRR-1 and TIGIRR-2. IL-1RacPb is expressed only in the brain, and TIGIRR has limited expression. However, SIGIRR is expressed in most cells. The TIR domain of the three coreceptors is also different from that of other members of the IL-1 coreceptor family in that the TIR domain contains an amino acid sequence different from that of wild-type TIR.[209] As shown in Figure 26.2B, IL-1RAcPb forms the expected complex with IL-1 and IL-1R1 but does not recruit MyD88 or phosphorylate IRAK4.[209] Therefore, most IL-1 signaling is arrested. But as some genes are increased in response to the formation of the IL-1RI/IL-1RAcPb complex, partial IL-1 signaling must take place. Nevertheless, IL-1RAcPb functions as an inhibitory receptor chain but only in the brain. Mice deficient in IL-1RAcPb exhibit a normal inflammatory response in the periphery but greater neurodegeneration in the

brain. As such, IL-1RAcPb could play a role in chronic inflammatory responses in the brain by "buffering" IL-1-mediated neurodegeneration.

Like IL-1RAcPb, SIGIRR contains the same amino acids differences in its TIR domain, termed TIRb. Compared to wild-type TIR, TIRb likely has reduced binding of MyD88.[209] In addition to an altered TIR domain, SIGIRR has a carboxyl extension of 140 amino acids. Carboxyl extensions are also present in IL-1RAcPb as well as the two TIGIRRs. TIGIRR-2, which is associated with an X-linked cognitive deficiency, is apparently independent of IL-1 function. Little is known whether these C-terminal segments contribute to the inhibitory properties of these receptors. Nevertheless,

it seems likely that the alternative sequence in the TIRb domain of SIGIRR may act as a partial decoy for MyD88. MyD88 is phosphorylated upon TLR4 as well as IL-1β and IL-18 signaling, and results in downstream phosphorylation of IRAK-4. In cells expressing SIGIRR and activated by IL-37 binding to the IL-18Rα, the signal from either IL-1 or LPS initiates phosphorylation of MyD88. However, the decoy effect by the mutated TIRb of SIGIRR reduces the degree of phosphorylation of MyD88 and thus the phosphorylation of IRAK-4. The reduction, however, is partial. Indeed, the suppression by IL-37 added to blood macrophages is in the range of 20% to 50% and unlike the total loss of activation by a deficiency in MyD88.

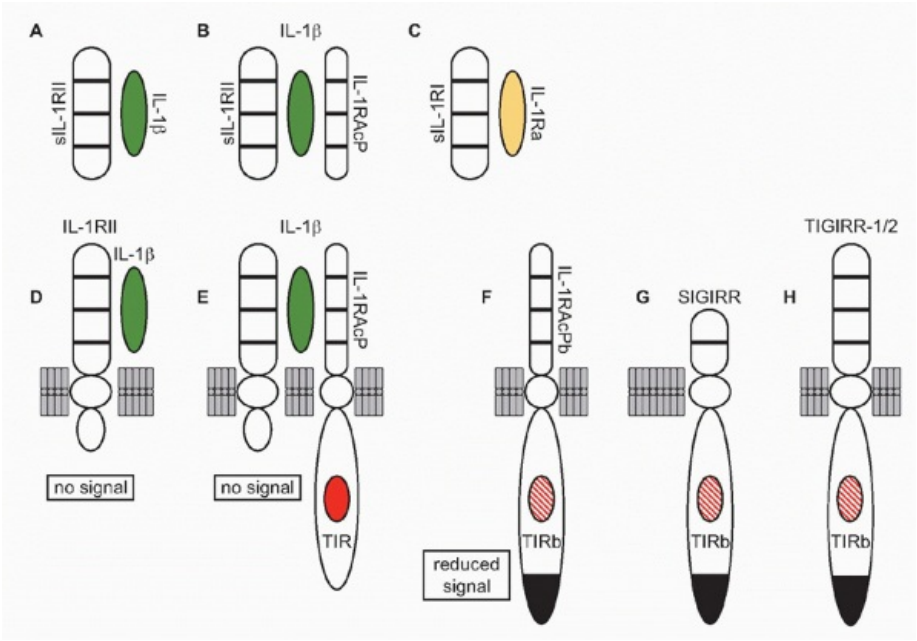

FIG. 26.4. Anti-Inflammatory Receptors of the Interleukin (IL)-1 Receptor Family. **A:** Soluble (extracellular) IL-1RII (sIL-1RII) binds and neutralizes either the precursor or mature form of IL-1β. **B:** A complex of IL-1β, sIL-1RII, and sIL-1RAcP neutralizes IL-1β activity. **C:** Soluble (extracellular) IL-1RI (sIL-1RI) binds IL-1Ra and prevents IL-1Ra from its function as a receptor antagonist. Similarly, IL-1α can bind to the sIL-1RI (not shown). **D:** IL-1RII expressed on the membrane binds mature IL-1β acting as a decoy receptor, preventing IL-1β from binding to its signaling receptor. **E:** A complex of membrane IL-1RII, IL-1β, and membrane IL-1RAcP also prevents IL-1β from signaling. **F:** The IL-1RAcPb is expressed in brain tissue (see Fig. 26.2B). **G:** Single immunoglobulin IL-1-related

receptor is expressed in myeloid cells and epithelial cells and function in damped inflammation due to the TIRb domain. **H:** Three immunoglobulin IL-1-related receptor is also an anti-inflammatory member of the IL-1 due to the toll-IL-1-receptor b domain.

Upon binding to the IL-18Rα, the IL-37 precursor may activate SIGIRR and provide a negative signal (see Fig. 26.3C). An inhibitory signal from IL-37 is enhanced by a low concentration of IL-18BP.[193] As shown in Figure 26.3D, IL-18BP binds IL-37[193] and likely presents the complex of the cytokine with the binding protein to the IL-18Rα (see Fig. 26.3E). Because A549 cells express SIGIRR, it is likely that the inhibitory signal from IL-37 activates SIGIRR or alternatively IL-37 recruits SIGIRR as the accessory chain. The inhibitory signal of SIGIRR is established in several mouse models of inflammation in which SIGIRR-deficient mice exhibit more inflammation compared to wild-type control mice.[210] In differentiated human blood M1 macrophages, recombinant IL-37 suppresses LPS-induced TNFα and IL-6 production 50% to 70%. A source of IL-18BP in this culture may be fetal calf serum. During the differentiation of macrophages into the M1 subset by granulocyte macrophage-colony stimulating factor, it is possible that SIGIRR expression increases whereas the level of IL-18Rβ decreases (see Fig. 26.3A). In the absence of IL-18Rβ, a proinflammatory complex is not formed with IL-18Rα and thus IL-37 binding to IL-18Rα may recruit or activate SIGIRR. Thus, expression of SIGIRR and the absence of IL-18Rβ would best explain the inhibitory properties of recombinant IL-37 reducing the response to LPS induction of IL-6 and TNFα.

INTERLEUKIN-36 SUBFAMILY

The IL-1 family members IL-1F5, IL-1F6, IL-1F8, and IL-1F9 are now termed IL-36Ra, IL-36α, IL-36β, and IL-36γ, respectively.[211] As shown in Figure 26.5A, each member of the IL-36

subfamily binds to the IL-1Rpr2, now termed IL-36R.[212] Because IL-38 also binds to the IL-36R (Fig. 26.5C),[133] IL-38 is included in the IL-36R subfamily. The IL-36 subfamily is closely related to the IL-1 subfamily because, similar to the IL-1α and IL-1β and IL-33, the IL-36R forms a signaling complex with the IL-1RAcP (Fig. 26.5A).[122,212] Thus of the 11 members of the entire IL-1 family,[6] members use IL-1RAcP as the coreceptor for signal transduction.

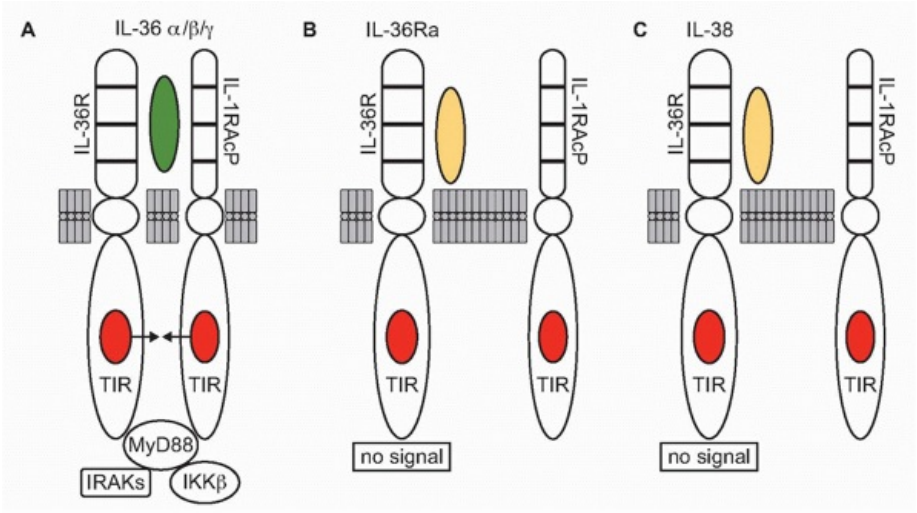

FIG. 26.5. Interleukin (IL)-36 Subfamily. A: IL-36α, IL-36β, or IL-36γ bind to the IL-36R and recruit the coreceptor IL-1RAcP. The heterodimeric IL-36 receptor complex results in a close approximation of the toll-IL-1-receptor (TIR) domains on each receptor chain (*arrows*), resulting in the binding of intracellular MyD88 to the complex with phosphorylation of MyD88. Subsequent phosphorylations of IRAKs and IKKβ, increased NF-κB, and activating protein-1 translocation to the nucleus take place, followed by expression of proinflammatory genes. **B:** IL-36Ra binds to IL-36R. There is no recruitment of the coreceptor IL-1RAcP, no approximation of the TIR domains, and no signal. **C:** IL-38 binds to IL-36R but there is no signal and acts similar to IL-36Ra.

Interleukin-36

The IL-36R is the ligand binding chain and therefore is comparable to the ligand binding IL-1R1 and the IL-18Rα. However, two members of the IL-36 subfamily bind to the IL-36R but do not signal, the IL-36 receptor antagonist (IL-36Ra) and IL-38 (see Fig. 26.5B and C). As such, these function as receptor antagonists.[9,133] An unusual property of IL-38 is that low concentrations (1 to 10 ng/mL) are able to reduce the activity of endogenous IL-36,[133] whereas in the case of IL-1Ra, higher concentrations are required to prevent the activating of endogenous IL-1α or IL-1β.

None of the members of the IL-36 subfamily have a signal peptide, indicating the generation of the N-terminus and secretion via the Golgi. In addition, each member of the IL-36 subfamily has an unusually short propiece compared to those of the IL-1α, IL-1β and IL-33 (see Fig. 26.1). Like IL-1β and IL-18, there is no true caspase-1 cleavage site for generation of an N-terminus in the IL-36 subfamily. It remains unknown which specific proteases generate the various N-termini of the IL-36 subfamily; nevertheless, each member has a unique N-terminus with a different levels of biologic activity.[9] What determines the N-terminus with optimal biologic activity in the IL-36 subfamily? Each member of the IL-36 subfamily contains the IL-1 family consensus sequence of A-X-D. The aspartic acid is not the recognition amino acid for caspase-1 or caspase-3, but rather participates in the stabilization of the first beta sheet of the three-dimensional structure that characterizes the entire IL-1 family.

The “A” of the consensus sequence is for any aliphatic amino acid, for example, leucine or isoleucine. Nine amino acids before the “A” of the consensus sequence is the N-terminal site, which results in the cytokine with the greatest activity.[9] For example, the biologic activity of IL-36γ increases by a factor of 1,000 when the N-terminus is at the site nine amino acids before the consensus sequence and in the case of the IL-36β, there is a 10,000-fold increase.[9] The nine amino acid forward site from the consensus sequence is not only the N-terminus for the agonist members of the IL-36 family but also the IL-36 receptor antagonist (IL-36Ra),[9] which increases from a low level of blocking of IL-36 family ligands to a high degree of blockade. It is unclear what specific protease cleaves at this site as the amino acid is different for each member of the IL-36 subfamily. Moreover, the site for the N-terminus of the IL-36Ra (valine) is but one amino acid from the N-terminal precursor methionine and yet IL-36Ra with an N-terminus at the valine site is 10,000-fold more potent that the IL-36 precursor. It is also an unusual situation that proteases that usually

are inflammatory in processing members of the IL-1 family in that case of IL-36Ra generate an anti-inflammatory molecule.

Interleukin-36α, β, and γ, Proinflammatory Members of the Interleukin-36 Subfamily

IL-36α was highly expressed in the murine model of glomerulonephritis 213 where the cytokine was localized to the kidney epithelium and also in CD3 T cells surrounding the tubules. IL-36α is also found in the kidneys of the MRL/lupus, nephritic syndrome, and streptozotocin-induced diabetic models.[213] IL-36γ increases IL-8, CXCL3, and the Th17 chemokine CCL20 in human lung fibroblasts,[214] and thus may account for acute neutrophilic lung inflammation. In addition to CD4+ T cells, human articular chondrocytes and synovial fibroblasts express the IL-36R.[215] In chondrocytes, there is also constitutive gene expression of IL-36β. Following stimulation with IL-1β or TNFα, levels of the IL-36β precursor rise intracellularly but the cytokine is not secreted. Although IL-36β levels were detected in the joint fluids of patients with rheumatoid arthritis as well as in serum samples, there was no correlation with disease severity.[215] It is likely that IL-36 ligands are functional only when released from dying cells and can be processed extracellularly by enzymes present in inflammatory conditions such as the joints of patients with rheumatoid arthritis. It unclear to what extent IL-36β plays a role in joint disease, although constitutive expression in primary chondrocytes may indicate a role for the cytokine in osteoarthritis.[215]

High levels of this cytokine are found in mouse embryonic tissues rich in epithelial cells.[216] In humans, IL-36α is observed in keratinocytes, not fibroblasts, and is thought to contribute to the inflammation of psoriasis. Upon forming the heterodimer with IL-36R and IL-1RAcP, IL-36α activates NF-κB similar to that of IL-1β.[212] In addition to NF-κB activation, IL-36α also activates MAPK, JNK, and ERK1/2.[212] In the mouse, bone marrow-derived dendritic cells and CD4+ T cells express IL-36 receptors in health. In a comparison with IL-1 as a stimulant, the three IL-36 ligands are more active in inducing IL-1β, IL-6, IL-12, TNFα, and IL-23.[217] In addition, IL-36 ligands induced the production of IFNγ, IL-4, and IL-17 from CD4+ T cells. Not unexpectedly, cytokines induced by IL-36 lands were prevented by 100- to 1,000-fold excess IL-36Ra.[217]

Interleukin-36 in Psoriasis

Several studies implicate IL-36 ligands in the pathogenesis of psoriasis.[218,219,220] IL-36γ is highly expressed in keratinocytes from healthy human skin and increases upon stimulation with TLR polyI:C.[221] Furthermore, polyI:C induced caspase-3, which resulted in cell death and the release of IL-36γ. Unexpectedly, stimulation of IL-36γ gene expression was dependent on caspase-1.[221] The caspase-1 dependency may be due to IL-18 as this cytokine is constitutively present in keratinocytes as is IL-1α. With the release of IL-36γ by polyI:C and the subsequent death of the cell, IL-36γ falls into the category of being an alarmin in the skin, particularly due to infection.[221] There is also a role for IL-36 in the production of IL-17: studies suggest that each of the IL-36 ligands is expressed in the skin

and dependent on IL-22.[222] Furthermore, the expression of IL-36 ligands in the psoriatic skin correlated with IL-17.[222] Similar to other models in the IL-1 family, auto- and coinduction accounts for a role in a pathologic process.

Overexpression of IL-36 in mice results in inflammatory skin lesions that resemble psoriasis in humans, as reviewed in Towne and Sims.[223] Likewise, mice deficient in endogenous IL-36Ra results in a severe lesion similar to that of humans with pustular psoriasis. The role of IL-36 in pustular psoriasis may include IL-1α, as humans with pustular psoriasis responds to an antibody that neutralizes IL-1α. Both IL-36 and IL-1α are found in the keratinocytes in healthy skin.

A Role for Interleukin-36R in Metabolic Regulation

Obesity is characterized by chronic low-grade inflammation originating from expanding adipose tissue. Human adipogenic tissue levels of IL-36α is primarily present in adipose tissue resident macrophages and induced by inflammation; however, IL-36β is absent.[224] IL-36α, but not IL-36β, reduces adipocyte differentiation, as shown by a significant decrease in PPARγ gene expression. Both IL-36α and IL-36β induce inflammatory gene expression in mature adipocytes.[224] Therefore, IL-36α and IL-36β are present in adipose tissue and are involved in the regulation of adipose tissue gene expression. Importantly, IL-36α inhibits PPARγ expression, which may lead to reduced adipocyte differentiation suggesting metabolic effects of this cytokine.

Although IL-36Ra is known to occupy the IL-36R and act as a receptor antagonist, earlier studies revealed that IL-36Ra inhibited the induction of IL-1β by LPS. The role of IL-36Ra was also examined in the brain. IL-36Ra injected into the rat brain induced IL-4 and also in glial cells in vitro.[225] Moreover, the reduction in LPS-induced IL-1β was not observed in cells deficient in IL-4 and also not observed in cell deficient in SIGIRR.[225] However, these unique properties of IL-36Ra were not observed in peripheral monocytes or dendritic cells but only in the brain.

Role of Interleukin-36 in Human Disease

The importance of any cytokine in human biology can be found in mutations that result in a profound clinical picture. In the case IL-1, a mutation in the naturally IL-1Ra (see Fig. 26.5B) results in severe systemic inflammation with erosive bone lesions, sterile meningitis, and death; the syndrome is called deficiency of IL-1Ra.[226,227] In case of the IL-36 family, persons with a mutation in the naturally occurring IL-36Ra suffer with a severe form of pustular psoriasis.[228,229] These human studies are consistent with the data from transgenic mice overexpressing IL-36α in the skin and with the ability of IL-36Ra to suppress the

severity of the inflammation.[218] In mice overexpressing IL-36a in the skin, crossing the mice to generate a strain of mice heterozygous for natural IL-36Ra knockout results in worsening of the skin lesions.[218]

Interleukin-38

IL-38 is the name for the IL-1 family member 10. During the nomenclature revision of the IL-1

family in 2010,[211] the term IL-38 was assigned to IL-1F10 without any known biologic function. Since then, IL-38 has been shown to bind to the IL-36 receptor (formerly IL-1Rrp2) (see Fig. 26.5C).[133] In order to find the receptor for IL-38, each member of the IL-1 receptor family was immobilized and recombinant IL-38 precursor containing 152 amino acids was added and binding assessed using an antibody to the ligand. IL-38 bound only to the IL-36 receptor, as did IL-36Ra.[133] To assess the biologic function of IL-38, heat-killed *Candida albicans* was used to stimulate memory T-lymphocyte cytokine production in freshly obtained human peripheral blood mononuclear cells from healthy subjects. The addition of recombinant IL-38 inhibited the production of T-cell cytokines IL-22 and IL-17. The dose-response suppression of IL-38 as well as that of IL-36Ra of *Candida*-induced IL-22 and IL-17 was not that of the classic IL-1 receptor antagonist, because low concentrations were optimal for inhibiting IL-22 production.[133] These data provide evidence that IL-38 binds to the IL-36R, as does IL-36Ra, and that IL-38 and IL-36Ra have similar biologic effects on immune cells by engaging the IL-36 receptor.

CONCLUSION

In conclusion, IL-1 family of cytokines is one of the most important families of proinflammatory mediators, that have profound local and systemic effects. The importance of these molecules has been demonstrated in homeostasis, as well as in autoinflammatory diseases, and treatment strategies based on modulation of IL-1 cytokines have begun to be employed successfully. Furthermore, important and novel biologic roles are currently being described for the members of IL-1 family of cytokines, and it is to be expected that this will represent one of the most dynamic areas of research in immunology in the coming years.

REFERENCES

1. Arend WP. The balance between IL-1 and IL-1Ra in disease. *Cytokine Growth Factor Rev*. 2002;13:323-340.

2. Sims JE, March CJ, Cosman D, et al. cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. *Science*. 1988;241:585-589.

3. Gay NJ, Keith FJ. Drosophila Toll and IL-1 receptor. *Nature*. 1991;351: 355-356.

4. Heguy A, Baldari CT, Macchia G, et al. Amino acids conserved in interleukin-1 receptors (IL-1Rs) and the Drosophila toll protein are essential for IL-1R signal transduction. *J Biol Chem*. 1992;267:2605-2609.

5. Lemaitre B, Nicolas E, Michaut L, et al. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell*. 1996;86:973-983.

6. van der Meer JWM, Barza M, Wolff SM, et al. A low dose of recombinant interleukin 1 protects granulocytopenic mice from lethal gramnegative infection. *Proc Natl Acad Sci U S A*. 1988;85:1620-1623.

7. O'Neill LA. The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. *Immunol Rev*. 2008;226:10-18.

8. Rosenwasser LJ, Dinarello CA, Rosenthal AS. Adherent cell function in murine T-lymphocyte antigen recognition. IV. Enhancement of murine T-cell antigen recognition by human leukocytic pyrogen. *J Exp Med*. 1979;150:709-714.

9. Towne JE, Renshaw BR, Douangpanya J, et al. Interleukin-36 (IL-36) ligands require processing for full agonist (IL-36alpha, IL-36beta, and IL-36gamma) or antagonist (IL-36Ra) activity. *J Biol Chem*. 2011;286: 42594-42602.

10. Nakanishi K, Yoshimoto T, Tsutsui H, et al. Interleukin-18 regulates both Th1 and Th2 responses. *Ann Rev Immunol*. 2001;19:423-474.

11. Schmitz J, Owyang A, Oldham E, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity*. 2005;23: 479-490.

12. Wilson NJ, Boniface K, Chan JR, et al. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol*. 2007;8:950-957.

13. Dinarello CA. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood*. 2011;117:3720-3732.

14. Chizzolini C, Chicheportiche R, Alvarez M, et al. Prostaglandin E2 synergistically with interleukin-23 favors human Th17 expansion. *Blood*. 2008;112:3696-3703.

15. Henley DV, Bellone CJ, Williams DA, et al. Aryl hydrocarbon receptor-mediated posttranscriptional regulation of IL-1beta. *Arch Biochem Biophys*. 2004;422:42-51.

16. Moreira AP, Cavassani KA, Ismailoglu UB, et al. The protective role of TLR6 in a mouse model of asthma is mediated by IL-23 and IL-17A. *J Clin Invest*. 2011;121:4420-4432.

17. Hughes T, Becknell B, Freud AG, et al. Interleukin-1beta selectively expands and sustains interleukin-22+ immature human natural killer cells in secondary lymphoid tissue. *Immunity*. 2010;32:803-814.

18. Hacham M, Argov S, White RM, et al. Different patterns of interleukin-1alpha and interleukin-1beta expression in organs of normal young and old mice. *Eur Cytokine Netw*. 2002;13:55-65.

19. Lonnemann G, Engler-Blum G, Müller GA, et al. Cytokines in human renal interstitial fibrosis. II. Intrinsic interleukin (IL)-1 synthesis and IL-1-dependent production of IL-6 and

IL-8 by cultured kidney fibroblasts. *Kidney Int*. 1995;47:845-854.

20. Hurgin V, Novick D, Werman A, et al. Antiviral and immunoregulatory activities of IFN-gamma depend on constitutively expressed IL-1alpha. *Proc Natl Acad Sci U S A* . 2007;104:5044-5049.

21. Werman A, Werman-Venkert R, White R, et al. The precursor form of IL-1alpha is an intracrine proinflammatory activator of transcription. *Proc Natl Acad Sci U S A* . 2004;101:2434-2439.

22. Maier JAM, Voulalas P, Roeder D, et al. Extension of the life span of human endothelial cells by an interleukin-1a antisense oligomer. *Science*. 1990;249:1570-1574.

23. Kawaguchi Y, Nishimagi E, Tochimoto A, et al. Intracellular IL-1alphabinding proteins contribute to biological functions of endogenous IL-1alpha in systemic sclerosis fibroblasts. *Proc Natl Acad Sci U S A* . 2006;103:14501-14506.

24. Horai R, Asano M, Sudo K, et al. Production of mice deficient in genes for interleukin (IL)-1a, IL- 1b, IL-1a/b, and IL-1 receptor antagonist shows that IL-1b is crucial in turpentine-induced fever development and glucocorticoid secretion. *J Exp Med*. 1998;187:1463-1475.

25. Yin H, Morioka H, Towle CA, et al. Evidence that HAX-1 is an interleukin-1 alpha N-terminal binding protein. *Cytokine*. 2001;15: 122-137.

26. Kurt-Jones EA, Beller DI, Mizel SB, et al. Identification of a membraneassociated interleukin-1 in macrophages. *Proc Natl Acad Sci U S A* . 1985; 82:1204-1208.

27. Kaplanski G, Farnarier C, Kaplanski S, et al. Interleukin-1 induces interleukin-8 from endothelial cells by a juxacrine mechanism. *Blood*. 1994;84:4242-4248.

28. Berda-Haddad Y, Robert S, Salers P, et al. Sterile inflammation of endothelial cell-derived apoptotic bodies is mediated by interleukin-1alpha. *Proc Natl Acad Sci U S A* . 2011;108:20684-20689.

29. Miller AC, Schattenberg DG, Malkinson AM, et al. Decreased content of the IL1 alpha processing enzyme calpain in murine bone marrowderived macrophages after treatment with the benzene metabolite hydroquinone. *Toxicol Lett*. 1994;74:177-184.

30. Gross O, Yazdi AS, Thomas CJ, et al. Inflammasome activators induce interleukin-1alpha secretion via distinct pathways with differential requirement for the protease function of caspase-1. *Immunity*. 2012;36:388-400.

31. Fettelschoss A, Kistowska M, LeibundGut-Landmann S, et al. Inflammasome activation and IL-1beta target IL-1alpha for secretion as opposed to surface expression.

Proc Natl Acad Sci U S A . 2011; 108:18055-18060.

32. Sha Y, Zmijewski J, Xu Z, et al. HMGB1 develops enhanced proinflammatory activity by binding to cytokines. *J Immunol* . 2008;180:2531-2537.

33. Cohen I, Rider P, Carmi Y, et al. Differential release of chromatinbound IL-1alpha discriminates between necrotic and apoptotic cell death by the ability to induce sterile inflammation. *Proc Natl Acad Sci U S A* . 2010;107:2574-2579.

34. Carmi Y, Voronov E, Dotan S, et al. The role of macrophage-derived IL-1 in induction and maintenance of angiogenesis. *J Immunol* . 2009;183:4705-4714.

35. Rider P, Carmi Y, Guttman O, et al. IL-1α and IL-1β recruit different myeloid cells and promote different stages of sterile inflammation. *J Immunol* . 2011;187:4835-4843.

36. Luheshi NM, Kovacs KJ, Lopez-Castejon G, et al. Interleukin-1alpha expression precedes IL-1beta after ischemic brain injury and is localised to areas of focal neuronal loss and penumbral tissues. *J Neuroinflammation*. 2011;8:186.

37. Chen CJ, Kono H, Golenbock D, et al. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat Med*. 2007;13:851-856.

38. Hawrylowicz CM, Santoro SA, Platt FM, et al. Activated platelets express IL-1 activity. *J Immunol* . 1989;143:4015-4018.

39. Kaplanski G, Porat R, Aiura K, et al. Activated platelets induce endothelial secretion of interleukin-8 in vitro via an interleukin-1-mediated event. *Blood*. 1993;81:2492-2495.

40. Hawrylowicz CM, Howells GL, Feldmann M. Platelet-derived interleukin 1 induces human endothelial adhesion molecule expression and cytokine production. *J Exp Med*. 1991;174:785-790.

41. Thornton P, McColl BW, Greenhalgh A, et al. Platelet interleukin-1alpha drives cerebrovascular inflammation. *Blood*. 2010;115:3632-3639.

42. Gawaz M, Brand K, Dickfeld T, et al. Platelets induce alterations of chemotactic and adhesive properties of endothelial cells mediated through an interleukin-1-dependent mechanism. Implications for atherogenesis. *Atherosclerosis*. 2000;148:75-85.

43. Kuida K, Lippke JA, Ku G, et al. Altered cytokine export and apoptosis in mice deficient in interleukin-1b converting enzyme. *Science*. 1995;267:2000-2003.

44. Kenneth NS, Younger JM, Hughes ED, et al. An inactivating caspase 11 passenger mutation originating from the 129 murine strain in mice targeted for c-IAP1. *Biochem J*. 2012;443:355-359.

45. Kamari Y, Werman-Venkert R, Shaish A, et al. Differential role and tissue specificity of interleukin-1alpha gene expression in atherogenesis and lipid metabolism. *Atherosclerosis*. 2007;195:31-38.

46. Dinarello CA. A clinical perspective of IL-1beta as the gatekeeper of inflammation. *Eur J Immunol* . 2011;41:1203-1217.

47. Okamoto M, Liu W, Luo Y, et al. Constitutively active inflammasome in human melanoma cells mediating autoinflammation via caspase-1 processing and secretion of interleukin-1beta. *J Biol Chem*. 2009;285: 6477-6488.

48. Croker BA, Lewis RS, Babon JJ, et al. Neutrophils require SHP1 to regulate IL-1beta production and prevent inflammatory skin disease. *J Immunol* . 2011;186:1131-1139.

49. Unlu S, Kumar A, Waterman WR, et al. Phosphorylation of IRF8 in a pre-associated complex with Spi-1/PU.1 and non-phosphorylated Stat1 is critical for LPS induction of the IL1B gene. *Mol Immunol* . 2007; 44:3364-3379.

50. Kaspar RL, Gehrke L. Peripheral blood mononuclear cells stimulated with C5a or lipopolysaccharide to synthesize equivalent levels of IL-1b mRNA show unequal IL-1b protein accumulation but similar polyribosome profiles. *J Immunol* . 1994;153:277-286.

51. Bufler P, Gamboni-Robertson F, Azam T, et al. Interleukin-1 homologues IL-1F7b and IL-18 contain functional mRNA instability elements within the coding region responsive to lipopolysaccharide. *Biochem J*. 2004;381:503-510.

52. Schindler R, Clark BD, Dinarello CA. Dissociation between interleukin-1b mRNA and protein synthesis in human peripheral blood mononuclear cells. *J Biol Chem*. 1990;265:10232-10237.

53. Dinarello CA, Ikejima T, Warner SJ, et al. Interleukin 1 induces interleukin 1. I. Induction of circulating interleukin 1 in rabbits in vivo and in human mononuclear cells in vitro. *J Immunol* . 1987;139:1902-1910.

54. Goldbach-Mansky R, Dailey NJ, Canna SW, et al. Neonatal-onset multisystem inflammatory disease responsive to interleukin-1beta inhibition. *N Engl J Med* . 2006;355:581-592.

55. Greten FR, Arkan MC, Bollrath J, et al. NF-kappaB is a negative regulator of IL-1beta secretion as revealed by genetic and pharmacological inhibition of IKKbeta. *Cell*. 2007;130:918-931.

56. Boni-Schnetzler M, Thorne J, Parnaud G, et al. Increased interleukin (IL)-1beta messenger ribonucleic acid expression in beta-cells of individuals with type 2 diabetes

and regulation of IL-1beta in human islets by glucose and autostimulation. *J Clin Endocrinol Metab*. 2008;93:4065-4074.

57. Gattorno M, Tassi S, Carta S, et al. Pattern of interleukin-1beta secretion in response to lipopolysaccharide and ATP before and after interleukin-1 blockade in patients with CIAS1 mutations. *Arthritis Rheum*. 2007;56:3138-3148.

58. Wewers MD, Dare HA, Winnard AV, et al. IL-1b-converting enzyme (ICE) is present and functional in human alveolar macrophages: macrophage IL-1b release limitation is ICE independent. *J Immunol* . 1997;159:5964-5972.

59. Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. *Annu Rev Immunol*. 2009;27:229-265.

60. Agostini L, Martinon F, Burns K, et al. NALP3 forms an IL-1beta processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity*. 2004;20:319-325.

61. Hoffman HM, Mueller JL, Broide DH, et al. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nat Genet*. 2001;29:301-305.

62. Drenth JP, van der Meer JW, Kushner I. Unstimulated peripheral blood mononuclear cells from patients with the hyper-IgD syndrome produce cytokines capable of potent induction of C-reactive protein and serum amyloid A in Hep3B cells. *J Immunol* . 1996;157:400-404.

63. Hoffman HM, Wanderer AA. Inflammasome and IL-1beta-mediated disorders. *Curr Allergy Asthma Rep*. 2011;10:229-235.

64. Netea MG, Nold-Petry CA, Nold MF, et al. Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood*. 2009;113:2324-2335.

65. Andrei C, Dazzi C, Lotti L, et al. The secretory route of the leaderless protein interleukin 1beta involves exocytosis of endolysosome-related vesicles. *Mol Biol Cell* . 1999;10:1463-1475.

66. Andrei C, Margiocco P, Poggi A, et al. Phospholipases C and A2 control lysosome-mediated IL-1 beta secretion: implications for inflammatory processes. *Proc Natl Acad Sci U S A*. 2004;101:9745-9750.

67. Gardella S, Andrei C, Costigliolo S, et al. Secretion of bioactive interleukin-1beta by dendritic cells is modulated by interaction with antigen specific T cells. *Blood*. 2000;95:3809-3815.

68. Perregaux DG, McNiff P, Laliberte R, et al. ATP acts as an agonist to promote stimulus-induced secretion of IL-1 beta and IL-18 in human blood. *J Immunol*. 2000;165:4615-4623.

69. Elssner A, Duncan M, Gavrilin M, et al. A novel P2X7 receptor activator, the human cathelicidin-derived peptide LL37, induces IL-1 beta processing and release. *J Immunol*. 2004;172:4987-4994.

70. Qu Y, Franchi L, Nunez G, et al. Nonclassical IL-1 beta secretion stimulated by P2X7 receptors is dependent on inflammasome activation and correlated with exosome release in murine macrophages. *J Immunol*. 2007;179:1913-1925.

71. Brough D, Rothwell NJ. Caspase-1-dependent processing of prointerleukin-1beta is cytosolic and precedes cell death. *J Cell Sci*. 2007; 120:772-781.

72. Suzuki T, Franchi L, Toma C, et al. Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in Shigellainfected macrophages. *PLoS Pathog*. 2007;3:e111.

73. Kahlenberg JM, Lundberg KC, Kertesy SB, et al. Potentiation of caspase-1 activation by the P2X7 receptor is dependent on TLR signals and requires NF-kappaB-driven protein synthesis. *J Immunol*. 2005; 175:7611-7622.

74. Maedler K, Sergeev P, Ris F, et al. Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. *J Clin Invest*. 2002;110:851-860.

75. Pascual V, Allantaz F, Arce E, et al. Role of interleukin-1 (IL-1) in the pathogenesis of systemic onset juvenile idiopathic arthritis and clinical response to IL-1 blockade. *J Exp Med*. 2005;201:1479-1486.

76. Al-Shukaili A, Al-Kaabi J, Hassan B. A comparative study of interleukin-1beta production and p2x7 expression after ATP stimulation by peripheral blood mononuclear cells isolated from rheumatoid arthritis patients and normal healthy controls. *Inflammation*. 2008;31:84-90.

77. Sluyter R, Shemon AN, Wiley JS. Glu496 to Ala polymorphism in the P2X7 receptor impairs ATP-induced IL-1 beta release from human monocytes. *J Immunol*. 2004;172:3399-3405.

78. Sluyter R, Dalitz JG, Wiley JS. P2X7 receptor polymorphism impairs extracellular adenosine 5′-triphosphate-induced interleukin-18 release from human monocytes. *Genes Immun*. 2004;5:588-591.

79. Lee KH, Park SS, Kim I, et al. P2X7 receptor polymorphism and clinical outcomes in

HLA-matched sibling allogeneic hematopoietic stem cell transplantation. *Haematologica*. 2007;92:651-657.

80. Chessell IP, Hatcher JP, Bountra C, et al. Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain. *Pain*. 2005;114:386-396.

81. Hewinson J, Moore SF, Glover C, et al. A key role for redox signaling in rapid P2X7 receptor-induced IL-1 beta processing in human monocytes. *J Immunol*. 2008;180:8410-8420.

82. Pelegrin P, Surprenant A. Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. *EMBO J*. 2006;25:5071-5082.

83. Kanneganti TD, Lamkanfi M, Kim YG, et al. Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. *Immunity*. 2007;26:433-443.

84. Derks R, Beaman K. Regeneration and tolerance factor modulates the effect of adenosine triphosphate-induced interleukin 1 beta secretion in human macrophages. *Hum Immunol*. 2004;65:676-682.

85. Fantuzzi G, Ku G, Harding MW, et al. Response to local inflammation of IL-1b converting enzyme-deficient mice. *J Immunol*. 1997;158: 1818-1824.

86. Joosten LA, Netea MG, Fantuzzi G, et al. Inflammatory arthritis in caspase 1 gene-deficient mice: contribution of proteinase 3 to caspase 1-independent production of bioactive interleukin-1beta. *Arthritis Rheum*. 2009;60:3651-3662.

87. Coeshott C, Ohnemus C, Pilyavskaya A, et al. Converting enzymeindependent release of TNFa and IL-1b from a stimulated human monocytic cell line in the presence of activated neutrophils or purified proteinase-3. *Proc Natl Acad Sci U S A*. 1999;96:6261-6266.

88. Sugawara S, Uehara A, Nochi T, et al. Neutrophil proteinase 3-mediated induction of bioactive IL-18 secretion by human oral epithelial cells. *J Immunol*. 2001;167:6568-6575.

89. Joosten LA, Netea MG, Mylona E, et al. Engagement of fatty acids with Toll-like receptor 2 drives interleukin-1beta production via the ASC/caspase 1 pathway in monosodium urate monohydrate crystal-induced gouty arthritis. *Arthritis Rheum*. 2010;62:3237-3248.

90. So A, De Smedt T, Revaz S, et al. A pilot study of IL-1 inhibition by anakinra in acute gout. *Arthritis Res Ther*. 2007;9:R28.

91. So A, De Meulemeester M, Pikhlak A, et al. Canakinumab for the treatment of acute

flares in difficult-to-treat gouty arthritis: Results of a multicenter, phase II, dose-ranging study. *Arthritis Rheum*. 2010;62: 3064-3076.

92. Schlesinger N, Mysler E, Lin HY, et al. Canakinumab reduces the risk of acute gouty arthritis flares during initiation of allopurinol treatment: results of a double-blind, randomised study. *Ann Rheum Dis*. 2011;70:1264-1271.

93. Numanami H, Koyama S, Sato E, et al. Serine protease inhibitors modulate chemotactic cytokine production by human lung fibroblasts in vitro. *Am J Physiol Lung Cell Mol Physiol*. 2003;284:L882-890.

94. Zhou R, Tardivel A, Thorens B, et al. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol*. 2010;11:136-140.

95. Meissner F, Molawi K, Zychlinsky A. Superoxide dismutase 1 regulates caspase-1 and endotoxic shock. *Nat Immunol*. 2008;9:866-872.

96. van de Veerdonk FL, Smeekens SP, Joosten LA, et al. Reactive oxygen species-independent activation of the IL-1beta inflammasome in cells from patients with chronic granulomatous disease. *Proc Natl Acad Sci U S A* . 2010;107:3030-3033.

97. Meissner F, Seger RA, Moshous D, et al. Inflammasome activation in NADPH oxidase defective mononuclear phagocytes from patients with chronic granulomatous disease. *Blood*. 2010;116:1570-1573.

98. van de Veerdonk FL, Netea MG, Dinarello CA, et al. Anakinra for the inflammatory complications of chronic granulomatous disease. *Neth J Med*. 2011;69:95.

99. Zheng H, Fletcher D, Kozak W, et al. Resistance to fever induction and impaired acute-phase response in interleukin-1b deficient mice. *Immunity*. 1995;3:9-19.

100. Fantuzzi G, Sacco S, Ghezzi P, et al. Physiological and cytokine responses in interleukin-1b-deficient mice after zymosan-induced inflammation. *Am J Physiol*. 1997;273:R400-R406.

101. Fantuzzi G, Zheng H, Faggioni R, et al. Effect of endotoxin in IL-1bdeficient mice. *J Immunol*. 1996;157:291-296.

102. Faggioni R, Fantuzzi G, Fuller J, et al. IL-1b mediates leptin induction during inflammation. *Am J Physiol*. 1998;274:R204-208.

103. Krelin Y, Voronov E, Dotan S, et al. Interleukin-1beta-driven inflammation promotes the development and invasiveness of chemical carcinogen-induced tumors. *Cancer Res*. 2007;67:1062-1071.

104. Klionsky DJ. Autophagy: from phenomenology to molecular understanding in less than a decade. *Nat Rev Mol Cell Biol* . 2007;8:931-937.

105. Schmid D, Munz C. Innate and adaptive immunity through autophagy. *Immunity*. 2007;27:11-21.

106. Saitoh T, Fujita N, Jang MH, et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature*. 2008;456:264-268.

107. Harris J, Hartman M, Roche C, et al. Autophagy controls IL-1beta secretion by targeting pro-IL-1beta for degradation. *J Biol Chem*. 2011; 286:9587-9597.

108. Nakahira K, Haspel JA, Rathinam VA, et al. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat Immunol* . 2011;12:222-230.

109. Tschopp J, Schroder K. NLRP3 inflammasome activation: the convergence of multiple signalling pathways on ROS production? *Nat Rev Immunol* . 2010;10:210-215.

110. Crisan TO, Plantinga TS, van de Veerdonk FL, et al. Inflammasome-independent modulation of cytokine response by autophagy in human cells. *PLoS One*. 2011;6:e18666.

111. Hampe J, Franke A, Rosenstiel P, et al. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet*. 2007;39:207-211.

112. Rioux JD, Xavier RJ, Taylor KD, et al. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat Genet*. 2007;39:596-604.

113. Henckaerts L, Cleynen I, Brinar M, et al. Genetic variation in the autophagy gene ULK1 and risk of Crohn's disease. *Inflamm Bowel Dis*. 2011;17:1392-1397.

114. Lapaquette P, Glasser AL, Huett A, et al. Crohn's disease-associated adherent-invasive E. coli are selectively favoured by impaired autophagy to replicate intracellularly. *Cell Microbiol* . 2010;12:99-113.

115. Plantinga TS, Crisan TO, Oosting M, et al. Crohn's disease-associated ATG16L1 polymorphism modulates pro-inflammatory cytokine responses selectively upon activation of NOD2. *Gut*. 2011;60:1229-1235.

116. Cooney R, Baker J, Brain O, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med*. 2010;16:90-97.

117. Homer CR, Richmond AL, Rebert NA, et al. ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis. *Gastroenterology*. 2010;139:1630-1641.

118. Travassos LH, Carneiro LA, Ramjeet M, et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat Immunol*. 2010;11:55-62.

119. Kuballa P, Huett A, Rioux JD, et al. Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant. *PLoS One*. 2008;3:e3391.

120. Bergers G, Reikerstorfer A, Braselmann S, et al. Alternative promoter usage of the Fos-responsive gene *Fit-1* generates mRNA isoforms coding for either secreted or membrane-bound proteins related to the IL-1 receptor. *EMBO J*. 1994;13:1176-1188.

121. Chackerian AA, Oldham ER, Murphy EE, et al. IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. *J Immunol*. 2007; 179:2551-2555.

122. Ali S, Huber M, Kollewe C, et al. IL-1 receptor accessory protein is essential for IL-33-induced activation of T lymphocytes and mast cells. *Proc Natl Acad Sci U S A*. 2007;104:18660-18665.

123. Cayrol C, Girard JP. The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1. *Proc Natl Acad Sci U S A*. 2009;106: 9021-9026.

124. Bae S, Kang T, Hong J, et al. Contradictory functions (activation/termination) of neutrophil proteinase 3 enzyme (PR3) in interleukin-33 biological activity. *J Biol Chem*. 2012;287:8205-8213.

125. Lefrancais E, Roga S, Gautier V, et al. IL-33 is processed into mature bioactive forms by neutrophil elastase and cathepsin G. *Proc Natl Acad Sci U S A*. 2012;109:1673-1678.

126. Leung BP, Xu D, Culshaw S, et al. A novel therapy of murine collageninduced arthritis with soluble T1/ST2. *J Immunol*. 2004;173:145-150.

127. Louten J, Rankin AL, Li Y, et al. Endogenous IL-33 enhances Th2 cytokine production and T-cell responses during allergic airway inflammation. *Int Immunol*. 2011;23:307-315.

128. Yasuda K, Muto T, Kawagoe T, et al. Contribution of IL-33-activated type II innate lymphoid cells to pulmonary eosinophilia in intestinal nematode-infected mice. *Proc Natl Acad Sci U S A*. 2012;109: 3451-3456.

129. Matsuba-Kitamura S, Yoshimoto T, Yasuda K, et al. Contribution of IL-33 to induction and augmentation of experimental allergic conjunctivitis. *Int Immunol*. 2010;22:479-489.

130. Kim YH, Yang TY, Park CS, et al. Anti-IL-33 antibody has a therapeutic effect in a murine model of allergic rhinitis. *Allergy*. 2012;67:183-190.

131. Wang D, Zhang S, Li L, et al. Structural insights into the assembly and activation of IL-1beta with its receptors. *Nat Immunol* . 2010;11:905-911.

132. Kumar S, Hanning CR, Brigham-Burke MR, et al. Interleukin-1F7B (IL-1H4/IL-1F7) is processed by caspase-1 and mature IL-1F7B binds to the IL-18 receptor but does not induce IFN-gamma production. *Cytokine*. 2002;18:61-71.

133. van de Veerdonk FL, Stoeckman AK, Wu G, et al. IL-38 binds to the IL-36 receptor and has biological effects on immune cells similar to IL-36 receptor antagonist. *Proc Natl Acad Sci U S A*. 2012;109:3001-3005.

134. Bulek K, Swaidani S, Qin J, et al. The essential role of single Ig IL-1 receptor-related molecule/Toll IL-1R8 in regulation of Th2 immune response. *J Immunol* . 2009;182:2601-2609.

135. Gadina M, Jefferies CA. IL-33: a sheep in wolf's clothing? *Sci STKE*. 2007;2007:pe31.

136. Sanada S, Hakuno D, Higgins LJ, et al. IL-33 and ST2 comprise a critical biomechanically induced and cardioprotective signaling system. *J Clin Invest*. 2007;117:1538-1549.

137. Miller AM, Xu D, Asquith DL, et al. IL-33 reduces the development of atherosclerosis. *J Exp Med*. 2008;205:339-346.

138. Carriere V, Roussel L, Ortega N, et al. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proc Natl Acad Sci U S A* . 2007;104:282-287.

139. Moussion C, Ortega N, Girard JP. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel “alarmin”? *PLoS One*. 2008;3:e3331.

140. Masamune A, Watanabe T, Kikuta K, et al. Nuclear expression of interleukin-33 in pancreatic stellate cells. *Am J Physiol Gastrointest Liver Physiol* . 2010;299:G821-832.

141. Stevenson FT, Turck J, Locksley RM, et al. The N-terminal propiece of interleukin 1 alpha is a transforming nuclear oncoprotein. *Proc Natl Acad Sci U S A* . 1997;94:508-513.

142. Roussel L, Erard M, Cayrol C, et al. Molecular mimicry between IL-33 and KSHV for attachment to chromatin through the H2A-H2B acidic pocket. *EMBO Rep*. 2008;9:1006-

1012.

143. Ali S, Mohs A, Thomas M, et al. The dual function cytokine IL-33 interacts with the transcription factor NF-kappaB to dampen NF-kappaB-stimulated gene transcription. *J Immunol*. 2011;187:1609-1616.

144. Okamura H, Nagata K, Komatsu T, et al. A novel costimulatory factor for gamma interferon induction found in the livers of mice causes endotoxic shock. *Infect Immun*. 1995;63:3966-3972.

145. Dinarello CA. Interleukin-18 and the pathogenesis of inflammatory diseases. *Semin Nephrol*. 2007;27:98-114.

146. Puren AJ, Fantuzzi G, Dinarello CA. Gene expression, synthesis and secretion of IL-1b and IL-18 are differentially regulated in human blood mononuclear cells and mouse spleen cells. *Proc Natl Acad Sci U S A* . 1999;96:2256-2261.

147. Fantuzzi G, Reed DA, Dinarello CA. IL-12-induced IFNg is dependent on caspase-1 processing of the IL-18 precursor. *J Clin Invest*. 1999;104:761-767.

148. Siegmund B, Lehr HA, Fantuzzi G, et al. IL-1beta-converting enzyme (caspase-1) in intestinal inflammation. *Proc Natl Acad Sci U S A* . 2001;98:13249-13254.

149. Siegmund B, Fantuzzi G, Rieder F, et al. Neutralization of interleukin-18 reduces severity in murine colitis and intestinal IFN-g and TNF-a production. *Am J Physiol Regul Integr Comp Physiol*. 2001;281: R1264-1273.

150. Tsutsui H, Matsui K, Okamura H, et al. Pathophysiological roles of interleukin-18 in inflammatory liver diseases. *Immunol Rev*. 2000; 174:192-209.

151. Herzog C, Haun RS, Kaushal V, et al. Meprin A and meprin alpha generate biologically functional IL-1beta from pro-IL-1beta. *Biochem Biophys Res Commun*. 2009;379:904-908.

152. Herzog C, Kaushal GP, Haun RS. Generation of biologically active interleukin-1beta by meprin B. *Cytokine*. 2005;31:394-403.

153. Banerjee S, Bond JS. Prointerleukin-18 is activated by meprin beta in vitro and in vivo in intestinal inflammation. *J Biol Chem*. 2008;283:31371-31377.

154. Weber A, Wasiliew P, Kracht M. Interleukin-1 (IL-1) pathway. *Sci Signal*. 2010;3:cm1.

155. Morel JC, Park CC, Woods JM, et al. A novel role for interleukin-18 in adhesion molecule induction through NFkappa B and phosphatidylinositol (PI) 3-kinase-dependent

signal transduction pathways. *J Biol Chem*. 2001;276:37069-37075.

156. Lee JK, Kim SH, Lewis EC, et al. Differences in signaling pathways by IL-1beta and IL-18. *Proc Natl Acad Sci U S A* . 2004;101:8815-8820.

157. Kim SH, Han SY, Azam T, et al. Interleukin-32: a cytokine and inducer of TNFalpha. *Immunity*. 2005;22:131-142.

158. Dahl CA, Schall RP, He HL, et al. Identification of a novel gene expressed in activated natural killer cells and T cells. *J Immunol* . 1992;148:597-603.

159. Nakanishi K, Yoshimoto T, Tsutsui H, et al. Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu. *Cytokine Growth Factor Rev*. 2001;12:53-72.

160. Bellora F, Castriconi R, Doni A, et al. M-CSF induces the expression of a membrane-bound form of IL-18 in a subset of human monocytes differentiating in vitro toward macrophages. *Eur J Immunol* . 2012;42:1618-1626.

161. Nakamura S, Otani T, Ijiri Y, et al. IFN-gamma-dependent and -independent mechanisms in adverse effects caused by concomitant administration of IL-18 and IL-12. *J Immunol* . 2000;164:3330-3336.

162. Sennello JA, Fayad R, Pini M, et al. Interleukin-18, together with interleukin-12, induces severe acute pancreatitis in obese but not in nonobese leptin-deficient mice. *Proc Natl Acad Sci U S A* . 2008;105: 8085-8090.

163. Vidal-Vanaclocha F, Fantuzzi G, Mendoza L, et al. IL-18 regulates IL-1beta-dependent hepatic melanoma metastasis via vascular cell adhesion molecule-1. *Proc Natl Acad Sci U S A*. 2000;97:734-739.

164. Mazodier K, Marin V, Novick D, et al. Severe imbalance of IL-18/IL-18BP in patients with secondary hemophagocytic syndrome. *Blood*. 2005;106:3483-3489.

165. Gatti S, Beck J, Fantuzzi G, et al. Effect of interleukin-18 on mouse core body temperature. *Am J Physiol Regul Integr Comp Physiol* . 2002;282:R702-709.

166. Li S, Goorha S, Ballou LR, et al. Intracerebroventricular interleukin-6, macrophage inflammatory protein-1 beta and IL-18: pyrogenic and PGE(2)-mediated? *Brain Res*. 2003;992:76-84.

167. Reznikov LL, Kim SH, Westcott JY, et al. IL-18 binding protein increases spontaneous and IL-1-induced prostaglandin production via inhibition of IFN-gamma. *Proc Natl Acad Sci U S A* . 2000;97: 2174-2179.

168. Tak PP, Bacchi M, Bertolino M. Pharmacokinetics of IL-18 binding protein in healthy volunteers and subjects with rheumatoid arthritis or plaque psoriasis. *Eur J Drug Metab Pharmacokinetics*. 2006;31: 109-116.

169. Joosten LA, van De Loo FA, Lubberts E, et al. An IFN-gammaindependent proinflammatory role of IL-18 in murine streptococcal cell wall arthritis. *J Immunol*. 2000;165:6553-6558.

170. Carrascal MT, Mendoza L, Valcarcel M, et al. Interleukin-18 binding protein reduces b16 melanoma hepatic metastasis by neutralizing adhesiveness and growth factors of sinusoidal endothelium. *Cancer Res*. 2003;63:491-497.

171. ten Hove T, van Montfrans C, Peppelenbosch MP, et al. Infliximab treatment induces apoptosis of lamina propria T lymphocytes in Crohn's disease. *Gut*. 2002;50:206-211.

172. Sandborn WJ, Feagan BG, Fedorak RN, et al. A randomized trial of Ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with moderate-to-severe Crohn's disease. *Gastroenterology*. 2008;135:1130-1141.

173. Mannon PJ, Fuss IJ, Mayer L, et al. Anti-interleukin-12 antibody for active Crohn's disease. *N Engl J Med*. 2004;351:2069-2079.

174. Pizarro TT, Michie MH, Bentz M, et al. IL-18, a novel immunoregulatory cytokine, is up-regulated in Crohn's disease: expression and localization in intestinal mucosal cells. *J Immunol*. 1999;162:6829-6835.

175. Monteleone G, Trapasso F, Parrello T, et al. Bioactive IL-18 expression is up-regulated in Crohn's disease. *J Immunol*. 1999;163:143-147.

176. Ten Hove T, Corbaz A, Amitai H, et al. Blockade of endogenous IL-18 ameliorates TNBS-induced colitis by decreasing local TNF-alpha production in mice. *Gastroenterology*. 2001;121:1372-1379.

177. Siegmund B. Interleukin-1b converting enzyme and intestinal inflammation. *Biochem Pharmacol*. 2002;7273:1-8.

178. Bauer C, Duewell P, Mayer C, et al. Colitis induced in mice with dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome. *Gut*. 2010;59:1192-1199.

179. Bauer C, Loher F, Dauer M, et al. The ICE inhibitor pralnacasan prevents DSS-induced colitis in C57BL/6 mice and suppresses IP-10 mRNA but not TNF-alpha mRNA expression. *Dig Dis Sci*. 2007;52: 1642-1652.

180. Loher F, Bauer C, Landauer N, et al. The interleukin-1 beta-converting enzyme inhibitor pralnacasan reduces dextran sulfate sodium-induced murine colitis and T helper

1 T-cell activation. *J Pharmacol Exp Ther* . 2004;308:583-590.

181. Allen IC, TeKippe EM, Woodford RM, et al. The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitisassociated cancer. *J Exp Med*. 2010;207:1045-1056.

182. Dupaul-Chicoine J, Yeretssian G, Doiron K, et al. Control of intestinal homeostasis, colitis, and colitis-associated colorectal cancer by the inflammatory caspases. *Immunity*. 2010;32:367-378.

183. Hirota SA, Ng J, Lueng A, et al. NLRP3 inflammasome plays a key role in the regulation of intestinal homeostasis. *Inflamm Bowel Dis*. 2011;17:1359-1372.

184. Elinav E, Strowig T, Kau AL, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell*. 2011;145:745-757.

185. Siegmund B. Interleukin-18 in intestinal inflammation: friend and foe? *Immunity*. 2010;32:300-302.

186. Netea MG, Joosten LA, Lewis E, et al. Deficiency of interleukin-18 in mice leads to hyperphagia, obesity and insulin resistance. *Nat Med*. 2006;12:650-656.

187. Zorrilla EP, Sanchez-Alavez M, Sugama S, et al. Interleukin-18 controls energy homeostasis by suppressing appetite and feed efficiency. *Proc Natl Acad Sci U S A* . 2007;104:11097-11102.

188. Doyle SL, Campbell M, Ozaki E, et al. NLRP3 has a protective role in age-related macular degeneration through the induction of IL-18 by drusen components. *Nat Med*. 2012;18:791-798.

189. Novick D, Kim S-H, Fantuzzi G, et al. Interleukin-18 binding protein: a novel modulator of the Th1 cytokine response. *Immunity*. 1999;10: 127-136.

190. Novick D, Schwartsburd B, Pinkus R, et al. A novel IL-18BP ELISA shows elevated serum il-18BP in sepsis and extensive decrease of free IL-18. *Cytokine*. 2001;14:334-342.

191. Novick D, Elbirt D, Dinarello CA, et al. Interleukin-18 binding protein in the sera of patients with Wegener's granulomatosis. *J Clin Immunol* . 2009;29:38-45.

192. Novick D, Elbirt D, Miller G, et al. High circulating levels of free interleukin-18 in patients with active SLE in the presence of elevated levels of interleukin-18 binding protein. *J Autoimmun*. 2011;34:121-126.

193. Bufler P, Azam T, Gamboni-Robertson F, et al. A complex of the IL-1 homologue IL-

1F7b and IL-18-binding protein reduces IL-18 activity. *Proc Natl Acad Sci U S A* . 2002;99:13723-13728.

194. Nold MF, Nold-Petry CA, Zepp JA, et al. IL-37 is a fundamental inhibitor of innate immunity. *Nat Immunol* . 2010;11:1014-1022.

195. McNamee EN, Masterson JC, Jedlicka P, et al. Interleukin 37 expression protects mice from colitis. *Proc Natl Acad Sci U S A* . 2011;108: 16711-16716.

196. Banda NK, Vondracek A, Kraus D, et al. Mechanisms of inhibition of collagen-induced arthritis by murine IL-18 binding protein. *J Immunol* . 2003;170:2100-2105.

197. Muhl H, Kampfer H, Bosmann M, et al. Interferon-gamma mediates gene expression of IL-18 binding protein in nonleukocytic cells. *Biochem Biophys Res Commun*. 2000;267:960-963.

198. Hurgin V, Novick D, Rubinstein M. The promoter of IL-18 binding protein: activation by an IFN-gamma-induced complex of IFN regulatory factor 1 and CCAAT/enhancer binding protein beta. *Proc Natl Acad Sci U S A* . 2002;99:16957-16962.

199. Kaser A, Novick D, Rubinstein M, et al. Interferon-alpha induces interleukin-18 binding protein in chronic hepatitis C patients. *Clin Exp Immunol*. 2002;129:332-338.

200. Ludwiczek O, Kaser A, Novick D, et al. Plasma levels of interleukin-18 and interleukin-18 binding protein are elevated in patients with chronic liver disease. *J Clin Immunol*. 2002;22:331-337.

201. Wittmann M, Bachmann M, Doble R, et al. IL-27 regulates IL-18 binding protein in skin resident cells. *PLoS One*.2012;7:e38751.

202. Xiang Y, Moss B. Correspondence of the functional epitopes of poxvirus and human interleukin-18-binding proteins. *J Virol* . 2001;75: 9947-9954.

203. Nold M, Hauser IA, Hofler S, et al. IL-18BPa:Fc cooperates with immunosuppressive drugs in human whole blood. *Biochem Pharmacol* . 2003;66:505-510.

204. Sharma S, Kulk N, Nold MF, et al. The IL-1 family member 7b translocates to the nucleus and down-regulates proinflammatory cytokines. *J Immunol* . 2008;180:5477-5482.

205. Grimsby S, Jaensson H, Dubrovska A, et al. Proteomics-based identification of proteins interacting with Smad3: SREBP-2 forms a complex with Smad3 and inhibits its transcriptional activity. *FEBS Lett*. 2004;577:93-100.

206. Pan G, Risser P, Mao W, et al. IL-1H, an interleukin 1-related protein that binds IL-18

receptor/IL- 1Rrp. *Cytokine*. 2001;13:1-7.

207. Nold MF, Nold-Petry CA, Zepp JA, et al. Interleukin 37 exerts its antiinflammatory functions by associating with IL-18R alpha and SIGIRR. *Cytokine*. 2011;56:12.

208. Nold-Petry CA, Nold MF, Nielsen JW, et al. Increased cytokine production in interleukin-18 receptor alpha-deficient cells is associated with dysregulation of suppressors of cytokine signaling. *J Biol Chem*. 2009;284:25900-25911.

209. Smith DE, Lipsky BP, Russell C, et al. A central nervous system-restricted isoform of the interleukin-1 receptor accessory protein modulates neuronal responses to interleukin-1. *Immunity*. 2009;30:817-831.

210. Garlanda C, Anders HJ, Mantovani A. TIR8/SIGIRR: an IL-1R/TLR family member with regulatory functions in inflammation and T cell polarization. *Trends Immunol*. 2009;30:439-446.

211. Dinarello C, Arend W, Sims J, et al. IL-1 family nomenclature. *Nat Immunol*. 2010;11:973.

212. Towne JE, Garka KE, Renshaw BR, et al. Interleukin (IL)-1F6, IL-1F8, and IL-1F9 signal through IL-1Rrp2 and IL-1RAcP to activate the pathway leading to NF-kappaB and MAPKs. *J Biol Chem*. 2004;279: 13677-13688.

213. Ichii O, Otsuka S, Sasaki N, et al. Local overexpression of interleukin-1 family, member 6 relates to the development of tubulointerstitial lesions. *Lab Invest*. 2010;90:459-475.

214. Chustz RT, Nagarkar DR, Poposki JA, et al. Regulation and function of the IL-1 family cytokine IL-1F9 in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* . 2011;45:145-153.

215. Magne D, Palmer G, Barton JL, et al. The new IL-1 family member IL-1F8 stimulates production of inflammatory mediators by synovial fibroblasts and articular chondrocytes. *Arthritis Res Ther*. 2006; 8:R80.

216. Debets R, Timans JC, Homey B, et al. Two novel IL-1 family members, IL-1 delta and IL-1 epsilon, function as an antagonist and agonist of NF-kappa B activation through the orphan IL-1 receptor-related protein 2. *J Immunol*. 2001;167:1440-1446.

217. Vigne S, Palmer G, Lamacchia C, et al. IL-36R ligands are potent regulators of dendritic and T cells. *Blood*. 2011;118:5813-5823.

218. Blumberg H, Dinh H, Trueblood ES, et al. Opposing activities of two novel members of the IL-1 ligand family regulate skin inflammation. *J Exp Med*. 2007;204:2603-2614.

219. Johnston A, Xing X, Guzman AM, et al. IL-1F5, -F6, -F8, and -F9: a novel IL-1 family signaling system that is active in psoriasis and promotes keratinocyte antimicrobial peptide expression. *J Immunol* . 2011;186:2613-2622.

220. Muhr P, Zeitvogel J, Heitland I, et al. Expression of interleukin (IL)-1 family members upon stimulation with IL-17 differs in keratinocytes derived from patients with psoriasis and healthy donors. *Br J Dermatol* . 2011;165:189-193.

221. Lian LH, Milora KA, Manupipatpong KK, et al. The double-stranded RNA analogue polyinosinic-polycytidylic acid induces keratinocyte pyroptosis and release of IL-36gamma. *J Invest Dermatol* . 2012;132: 1346-1353.

222. Carrier Y, Ma HL, Ramon HE, et al. Inter-regulation of Th17 cytokines and the IL-36 cytokines in vitro and in vivo: implications in psoriasis pathogenesis. *J Invest Dermatol* . 2011;131:2428-2437.

223. Towne J, Sims J. IL-36 in psoriasis. *Curr Opin Pharmacol* . 2012;12(4): 486-490.

224. van Asseldonk EJ, Stienstra R, Koenen TB, et al. The effect of the interleukin-1 cytokine family members IL-1F6 and IL-1F8 on adipocyte differentiation. *Obesity*. 2010;18:2234-2236.

225. Costelloe C, Watson M, Murphy A, et al. IL-1F5 mediates anti-inflammatory activity in the brain through induction of IL-4 following interaction with SIGIRR/TIR8. *J Neurochem*. 2008;105:1960-1969.

226. Aksentijevich I, Masters SL, Ferguson PJ, et al. An autoinflammatory disease with deficiency of the interleukin-1-receptor antagonist. *N Engl J Med* . 2009;360:2426-2437.

227. Reddy S, Jia S, Geoffrey R, et al. An autoinflammatory disease due to homozygous deletion of the IL1RN locus. *N Engl J Med* . 2009;360: 2438-2444.

228. Onoufriadis A, Simpson MA, Pink AE, et al. Mutations in IL36RN/IL1F5 are associated with the severe episodic inflammatory skin disease known as generalized pustular psoriasis. *Am J Hum Genet*. 2011;89:432-437.

229. Marrakchi S, Guigue P, Renshaw BR, et al. Interleukin-36-receptor antagonist deficiency and generalized pustular psoriasis. *N Engl J Med* . 2011;365:620-628.

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Chapter 27

Tumor Necrosis Factor-Related Cytokines in Immunity

Carl F. Ware

INTRODUCTION

Lymphocytes are highly mobile, transiting the vascular and lymphatic circulatory systems with limited stopovers in organized lymphoid tissues (lymph nodes, spleen, and Peyer's patches) in which they commune to initiate immune responses. Cytokines, both secreted and membrane-anchored proteins, serve as the communication media of the immune system. Cytokines in the tumor necrosis factor (TNF) superfamily (TNFSF) help orchestrate the development, homeostasis, and effector actions of cells in the immune system. The diversification of the TNFSF is evidenced by its roles in regulating cells of neuronal, skeletal, and ectodermal origin. This chapter's focus is on members in the TNFSF that are primarily involved in regulating immunity.

The ligands belonging to the TNFSF initiate intercellular communication by binding specific receptors on the surface of the receiving cell. The TNF-related ligands are membrane-bound and require cell-cell contact to initiate signaling, although some ligands may be secreted in soluble form influencing cellular responses at locations distant from the source of production. The TNF receptors (TNFRs) form a corresponding superfamily of cognate membrane proteins that initiate intracellular signaling pathways that influence cellular growth, differentiation, and survival. Each ligandreceptor pair forms a “system” with over 40 distinct ligandreceptor systems. Many of the ligands and receptors engage more than one cognate, thereby forming “circuits” of signaling systems, which often function together as coordinated or integrated networks with other cytokines to regulate a specific cellular process. The signals delivered to the cytosol activate transcription factors such as NF-κB and AP1, which in turn initiate the expression of hundreds of genes that alter cellular differentiation. Additionally, some TNFRs activate cell death pathways, both apoptotic and necrotic, terminating cellular life. Genetic mutations have revealed the importance of individual TNFSF members in human immune responses and development. Moreover, the beneficial effect of TNF antagonists in patients with certain autoimmune diseases brings the need to understand the complexities of the TNFSF into sharp focus.

The nomenclature of the TNFSF is a bit of a morass for students new to the field, although introduction of a numerical system standardizing gene names (www.genenames.org/) helps in accessing genomic databases. However, the use of more common, often colorful, acronyms continues. The name, rank, and genomic identification of each ligand (Table 27.1) and receptor (Table 27.2) is tabulated along with additional pertinent information for human and mouse genes.

TUMOR NECROSIS FACTOR LIGAND FAMILY

The TNF-related cytokines are type II transmembrane proteins (intracellular N-terminus) with a short cytoplasmic tail (15 to 25 residues in length) and a larger extracellular region (˜150 amino acids) containing the signature TNF homology domain where the receptor binding sites are located (Fig. 27.1). The TNF homology domain assembles into trimers, the functional unit of the ligand. Atomic analysis of several members of the family[1,2,3,4,5] revealed the ligands have a highly conserved tertiary structure folding into a β sheet sandwich, yet amino acid sequence conservation is limited to < 35% among the family members. The conserved residues defining this superfamily are primarily located within the internal β strands that form the molecular scaffold, which promote assembly into trimers. The residues in the loops between the external β-strands are variable and in specific loops make contact with the receptor. Although most of the TNF ligands self-assemble into homotrimers, heterotrimers can also form between LTα and LTβ[6] and APRIL and BAFF.[7] The stoichiometry of the LT heterotrimer is 1:2 (LTαβ2), which imparts its distinct receptor specificity from the LTα homotrimer. Interestingly, complement component C1q and several related proteins are structurally related to the TNF family, containing a TNF homology domain, a rather surprising finding given the apparent functional divergence between the complement and TNF systems.[8] Alternate splicing can also generate distinct ligands, such as the splice form joining TWEAK and APRIL (TWE-PRIL),[9] the alternate ligands for ectodysplasin receptor,[10] and a cytosolic form of LIGHT.[11]

The genetic organization of TNF ligands is highly conserved and typically encoded in three or four exons, with the fourth exon encoding most of the extracellular TNF homology domain. The genes encoding TNF, LTβ, and LTα reside adjacent to each other in a compact loci in the class III region of the major histocompatibility complex (MHC) (in humans at chromosome 6p21) sandwiched between the antigenic-peptide presenting MHC proteins encoded by MHC class I and II genes.[6,12] Three other genetic clusters of TNF-related cytokines are found within the corresponding MHC paralogous regions, located on chromosomes 1, 9, and 19. These genetic clusters share a remarkably conserved gene structure and transcriptional orientation, and a similar function linked to regulating cellular immune responses.[11] The evolutionary pressures retaining this genetic configuration of the MHC in general is encompassed in the paralogy theory of genome

evolution.[13] Conservation of gene structure of the TNF ligands outside these MHC paralogs is limited. An evolutionarily conserved pathway in *Drosophila melanogaster*, Eiger-Wengen system, is structurally and functionally related to the TNFSF-related signaling pathway, although this system is predominantly expressed in the nervous system in invertebrates.[14,15,16]

TABLE 27.1 Tumor Necrosis Factor Superfamily Chromosomal Locations

	Chromosomal Location

Gene Name/Alias	Human	Mouse	Ligand Symbol
TNF	6p21.3	ch17 (19.06 cm)	TNFSF1A
LTα	6p21.3	ch17 (19.06 cm)	TNFSF1B
LTβ	6p21.3	ch17 (19.06 cm)	TNFSF3
OX40-L	1q25	ch1 (84.90 cM)	TNFSF4
CD40-L, CD154	Xq26	chX (18.0 cM)	TNFSF5
Fas-L	1q23	ch1 (85.0 cM)	TNFSF6
CD27-L, CD70	19p13	ch17 (20.0 cM)	TNFSF7
CD30-L, CD153	9q33	ch4 (32.20 cM)	TNFSF8
4-1BB-L	19p13	ch17 (20.0 cM)	TNFSF9
TRAIL	3q26	ch3	TNFSF10
RANK-L, TRANCE	13q14	ch14 (45.0 cM)	TNFSF11
TWEAK	17p13	ch11	TNFSF12
APRIL/TALL2	17P13.1	ch13	TNFSF13
BAFF, BLYS, TALL1	13q32-q34	ch8 (3 cM)	TNFSF13B
LIGHT	19p13.3	ch17 (D-E1)	TNFSF14
TL1A	9q33	ch4 (31.80 cM)	TNFSF15
G1TRL, A1TRL	1q23	Unknown	TNFSF18
EDA1	Xq12-q13.1	chX (37.0 cM)	EDA1
EDA2	Xq12-q13.1	cX (37.0 cM)	EDA2

CD, cluster of differentiation; LT, lymphotoxin; TNF, tumor necrosis factor; TNFSF, tumor necrosis factor superfamily; TRAIL, TMF-related apoptosis inducing ligand.

From Ware CF. The TNF superfamily-2008. *Cytokine Growth Factor Rev*. 2008;19:183-186.

TUMOR NECROSIS FACTOR RECEPTOR FAMILY

Members of the TNF receptor superfamily (TNFRSF) include proteins of vertebrate and viral origin. Most of the signaling receptors in the TNFRSF are type I transmembrane glycoproteins (N-terminus exterior to the cell). However, several TNFRSF members lack a membrane-anchor domain, are proteolytically cleaved from the surface, or are anchored via glycolipid linkage (eg, TNF-related apoptosis inducing ligand [TRAIL]R3). These soluble receptors, termed “decoy receptors,” retain their ligand-binding properties and compete with cellular receptors for the specific ligands, thus earning the title of decoy. The structural motifs in the cytoplasmic domains further categorize the TNFR into two groups based on their signaling properties: those contain a death domain (DD) and others that engage TNFR-associated factors (TRAFs).

TABLE 27.2 Tumor Necrosis Factor Receptor Superfamily

	Chromosomal Location		
Gene Name/Aliases	**Human**	**Mouse**	**Gene Symbol**
TNFR-1, p55-60	12p13.2	ch6 (60.55 cM)	TNFRSF1A
TNFR2, p75-80	1p36.3-36.2	ch4 (75.5 cM)	TNFRSF1B
LTβR	12p13	ch6 (60.4 cM)	LTβR
OX40	1p36	ch4 (79.4 cM)	TNFRSF4
CD40	20q12-q13.2	ch2 (97.0 cM)	CD40
FAS, CD95	10q24.1	ch19 (23.0 cM)	TNFRSF6
DcR3	20q13	unknown	TNFRSF6B
CD27	12p13	ch6 (60.35)	TNFRSF7
CD30	1p36	ch4 (75.5 cM)	TNFRSF8
4-1BB	1p36	ch4 (75.5 cM)	TNFRSF9
TRAILR-1, DR4	8p21	Unknown	TNRSF10A

TRAIL-R2, DR5	8p22-p21	ch14 (D1)	TNFRSF10B
TRAILR3, DcR1	8p22-p21	ch7 (69.6 cM)	TNFRSF10C
TRAILR4, DcR2	8p21	ch7 (69.6 cM)	TNFRSF10D
RANK, TRANCE-R	18q22.1	ch1	TNFRSF11A
OPG, TR1	8q24	ch15	TNFRSF11B
FN14	16p13.3	ch17	TNFRSF12A
TRAMP, DR3, LARD	1p36.3	ch4 (E1)	TNFRSF25
TACI	17p11.2	ch11	TNFRSF13B
BAFFR	22q13.1-q13.31	ch15	TNFRSF13C
HVEM, HveA, ATAR	1p36.3-p36.3	ch4	TNFRSF14
P75NTR, NGFR	17q12-q22	ch11 (55.6 cM)	TNFRSF16
BCMA	16p13.1	ch16 (B3)	TNFRSF17
AITR, GITR	1p36.3	ch4 (E)	TNFRSF18
RELT	11q13.2	unknown	TNFRSF19L
TROY, TAJ	13q12.11-q12.3	ch14	TNFRSF19
EDAR	2q11-q13	ch10	EDAR1
EDA2R	Xq11.1	ch X	EDA2R
DR6	6P12.2-21.1	ch17	TNFRSF21
IGFLR1, Tmem149	19q13.12	ch7	IGFLR1

CD, cluster of differentiation; HVEM, herpesvirus entry mediator; LT, lymphotoxin; OPG, osteoprotegerin; TNFR, tumor necrosis factor receptor; TNFRSF, tumor necrosis factor receptor superfamily; TRAIL, TMF-related apoptosis inducing ligand.

From Ware CF. The TNF superfamily-2008. *Cytokine Growth Factor Rev* . 2008;19:183-186.

The cysteine-rich domain (CRD) in the extracellular, ligand-binding region defines membership in the TNFRSF (see Fig. 27.1). Each CRD typically contains six cysteine residues forming three disulfide bonds. The CRD is pseudorepeated in different members ranging from one to six. Based upon the crystal structures solved for several TNFRSF members, the CRD confers an elongated shape and sidedness to the ectodomain.[17,18] The crystal structure of the complex between TNFR1 and one of its ligands, the LTα homotrimer, revealed

that residues in CRD2 and 3 of TNFR1 contact the ligand. Variation in binding interactions has been identified; for example, the receptors for BAFF have one functional CRD.[2,19]

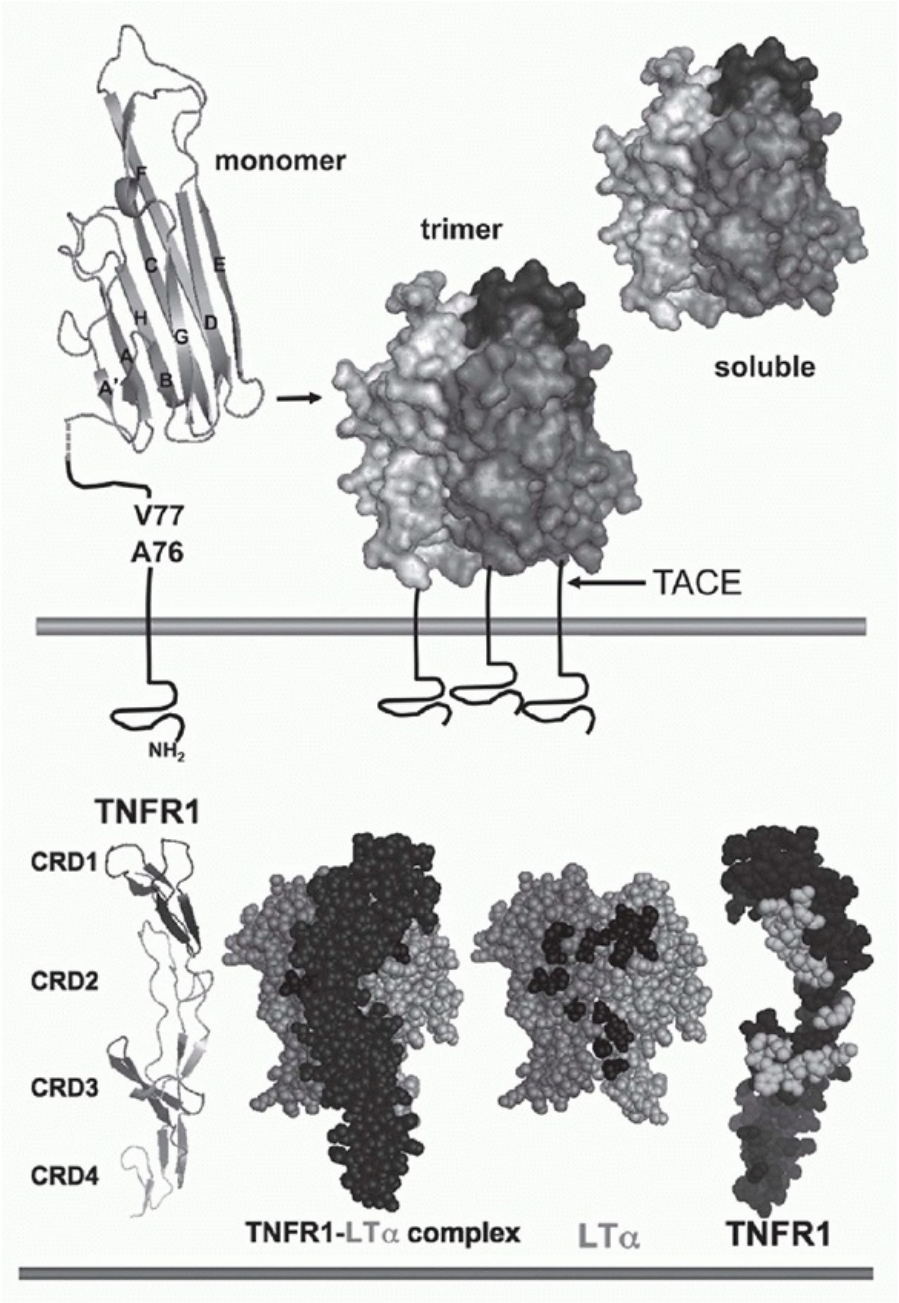

FIG. 27.1. Structure of Tumor Necrosis Factor (TNF) and TNF Receptors (TNFRs). **Top:** TNF. The β-sandwich of the TNF monomer (1A8M.pdf) (shown as a *ribbon diagram*) contains two stacked β-pleated sheets each formed by five antiparallel β strands (*wide ribbons given letter designations, A-G*) that fold into a Greek key or "jelly-roll" topology.[219] The inner β-sheet (strands A, A′, H, C, and F) is involved in contacts between adjacent subunits, which promotes assembly into a trimer. The trimer is formed such that one edge of each subunit is packed against the inner sheet of its neighbor. The outer β sheet (strands B, B′, D, E, and G) is surface exposed. The trimeric structure is characteristic of all TNF-related ligands. The type II configuration of TNF (N-terminus inside the cell) anchors TNF to the membrane. The TNF trimer is ˜60 Å in height with a relatively flat base residing close to the membrane, resembling a bell-shape (shown as surface representation with *different shades of gray* used for each subunit of the trimer). The surface exposed loops between A-A′ and D-E strands are involved in receptor

binding. TNF is cleaved by TNFα-converting enzyme (TACE), a member of the ADAM family of metalloproteinases (ADAM17) involved in processing of many cell surface proteins. TACE is a type 1 transmembrane protein that cleaves TNF between residues Val77 and Ala76, when all three sites in the trimer are cleaved TNF is released from the membrane. **Bottom:** TNFR and ligand complex. The ectodomain of TNFR1 forms an elongated molecule with CRD1 proximal to the N-terminus (*ribbon diagram*). The face of TNFR1 on the left engages LTα. In the ligand-receptor complex, the elongated receptor (*dark*) lies along the cleft formed between adjacent subunits. Shown (space-filling) is a single TNFR1 in complex with two subunits of LTα (*lighter shades*); the receptor N-terminus points upward, closest to the base of the ligand (transorientation). In the exploded view, TNFR1 is removed from in front of the ligand revealing the contact residues in the ligand, which are primarily located in the D-E and the A-A″ β-strands (*dark shade*). TNFR1 is rotated 180 degrees, exposing the contact residues in the receptor (*light shade*). Structures from 1TRN.pdf[17] as visualized with MacPyMOL (www.pymol.org).

RECEPTOR-LIGAND COMPLEX

The binding specificity of the various members of the TNF ligand and receptor superfamilies show monotypic interaction, yet several members interact with multiple partners[20] (Figs. 27.2 and 27.3). The binding interactions between TNF-related ligands and TNFR are typically high affinity, with equilibrium binding constants measured in the high pM to low nM range. The membrane position of the ligands further enhances the binding interaction with their receptors. In the membrane anchored position, the ligands and receptors must be in trans to form a complex. In the LTα-TNFR1 complex, the surface loops between A-A″ and D-E β strands contain many of the amino acid residues that make contact with the receptor, with the receptor binding site formed as a composite of adjoining subunits in the trimeric ligand[17] (see Fig. 27.1).

The trimeric architecture of the TNF ligands, containing three equivalent receptor-binding sites, provides the basis for initiating signaling through aggregation or clustering of receptors. This concept is supported by the finding that receptor-specific bivalent antibodies can act as agonists mimicking the signaling activity of the natural ligand.[21] Indeed, antibodies or peptide mimetic to TNFR can function as antagonists, blocking the ability of the natural ligand to bind the receptor while simultaneously activating the receptor as an agonist.[22] Some ligands such as FasL, TRAIL, and BAFF form higher ordered oligomers of the basic trimer. These higher ordered oligomers promote supraaggregation of receptors, enhancing or sustaining signaling pathways in the receptor-bearing cell.[23,24] Overexpression of TNFR in cells can also lead to ligand-independent signaling, a

feature bestowed in part by the propensity of the cytosolic tails to self-associate.[25] In the physiologic setting, the expression level and compartmentalization of these receptors are tightly controlled. A subregion in the first CRD of TNFR1 known as the preligand assembly domain may restrict the orientation of the unligated receptor to prevent spontaneous activation.[26] It is not known if this mechanism applies to all TNFRSF. Interestingly, some

patients with periodic fever syndrome have mutant TNFR1 that form abnormal disulfide-linked oligomers that are retained intracellullarly and provoke misfolded protein response.[27]

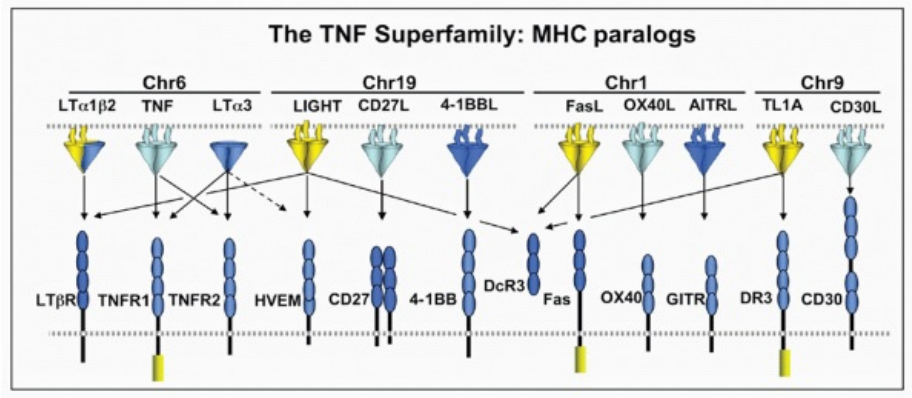

FIG. 27.2. The Tumor Necrosis Factor (TNF) Superfamily-Major Histocompatibility Complex (MHC) Paralogs. Members of the TNF ligand superfamily (*above*) and their corresponding receptors (*below*) are identified by connecting *arrows*. The ligands are grouped according to their chromosomal locations in the MHC paralogous regions. The number of cysteine-rich domains are depicted for each TNF receptor, and TNF receptors containing a death domain are identified by cylinder in the cytoplasmic tail. (Modified from Ware[336]).

Alternate Ligands

There is significant divergence in the ligands and the mechanisms of ligand binding by the TNFR family. A major branch point is exemplified by the ligands for p75 neurotrophin receptor (nerve growth factor and the other neurotrophins), which are structurally unrelated to TNF ligand family. Molecular contacts between NGF and p75NTR occur through two spatially separated binding regions located at the first and second CRD and the junction between the CRD3 and CRD4.[28] The p75NTR functions in complex with two other proteins, Nogo66 and LINGO, to engage myelin-associated inhibitory factors. Taj/TROY can supplant p75NTR in this complex.[29] Like p75 NTR, DR6, ILGF1R, and TROY/Taj do not bind any of the known TNF ligands but do engage other ligands. The pathways activated by p75NTR and TROY/Taj systems show both positive and inhibitory regulation of axonal regeneration.[29,30]

The herpesvirus entry mediator (HVEM; TNFRSF14) provides an example of a TNFR system that binds alternate ligands. Although HVEM engages two TNF-related ligands, LIGHT and LTα, it also engages two members of immunoglobulin (Ig) superfamily, B- and T-lymphocyte attenuator (BTLA)[31] and cluster of differentiation (CD)160.[32] BTLA binding to HVEM occurs in CRD1, on the opposite face of where LIGHT/LTα bind in CRD2 and 3. The BTLA binding site in CRD1 of HVEM is a region also targeted by herpesviruses.[33,34,35] Recent evidence indicates the neurotrophin and chondrocyte growth factor-like protein progranulin interacts with TNFR1 and TNFR2 and competes with TNF for binding.[36]

An insulin growth factor-like protein was recently identified as a ligand for a TNFR-like type 1

transmembrane protein (insulin growth factor-like R, formerly TMEM149).[37] Insulin growth factor-like R has conserved positioning of cysteines delineating a CRD1, but atypical CRD2/3. Insulin growth factor-like messenger ribonucleic acid (mRNA) is expressed in psoriatic skin lesions and the receptor is detected in T cells suggesting a possible role in skin inflammation.

Death receptor-6 (DR6) engages the growth factor-like domain in β-amyloid precursor protein[38,39] and thus may function as a negative regulator more like p75 neurotrophin receptor.[40] Emerging evidence suggests DR6 has a role in neuroimmune function. In a mouse experimental autoimmune encephalomyelitis (EAE) model, DR6-deficiency was shown to protect against central nervous system demyelination and leukocyte infiltration, but also enhanced overall CD4+ T-cell proliferation and T_H2 differentiation, underscoring a role for DR6 in mediating T_H1-specific immune responses in EAE progression.[41] Taj/TROY and RELT also do not bind any of the known TNFSF ligands; however, recent evidence indicates that Taj/TROY functions more like p75NTR, capable of binding myelin inhibitory factors.[29,30] A role for Taj/TROY and RELT in immune function is presently unclear.

The engagement of ligands distinct from TNFSF members implicates a higher level of integration with other signaling pathways.

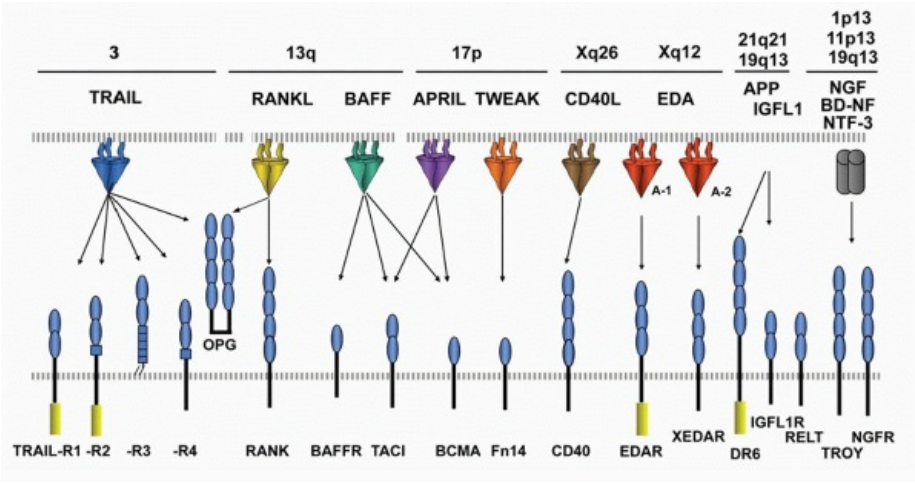

FIG. 27.3. The Tumor Necrosis Factor (TNF) Superfamily—Part 2. Members of the TNF ligand superfamily (*above*) and their corresponding receptors (*below*) are identified by connecting *arrows*. The ligands are grouped according to their chromosomal locations. The number of cysteine-rich domains are depicted for each TNF receptor, and TNF receptors containing a death domain are identified by cylinder in the cytoplasmic tail. (Modified from Ware[336]).

Viral Orthologs

TNFR-like proteins are found in the genomes of several viral pathogens representing captured cellular genes that have evolved as part of that pathogen's immune evasion

strategy (Table 27.3). Poxviruses were the first pathogens identified harboring a version of a cellular TNFR.[43] Poxvirus TNFR displays significant sequence homology to TNFR2 and binds TNF and LTα. The rabbit poxvirus protein T2 is secreted by virally infected cells and contributes to the virulence of infection.[44,45] Smallpox virus, the former scourge of mankind, also harbored viral versions of TNFR2.[46]

TABLE 27.3 Viral Orthologs and Modulators of the Tumor Necrosis Factor Superfamily

Virus	Name/ORF	Ortholog[a]	Mechanism
Poxvirus			
Myxoma	T2	Soluble TNFR2	TNF and LTα decoy
Variola	crm B (G2R)	Soluble TNFR2	TNF and LTα decoy
Cowpox	vCD30	Soluble CD30	CD30 ligand inhibitor
Herpesvirus			
HSV1&2	Glycoprotein D	BTLA	Entry; HVEM blockade
HCMV	UL144	HVEM	BTLA activation
EBV	LMP1	CD40 intracellular	TRAF activation
γHV	vFLIP	FLIP	Caspase 8 blockade
Adenovirus	E3-10.4, 14.5, 6.7	?	Fas and TRAILR downmodulation
Retrovirus			
EIAV	Envelope gp90	?	HVEM entry factor
FIV	Envelope gp95	?	Ox40 entry factor
ASLV	Envelope gp85	?	TRAILR entry factor

Rabies virus	Envelope RVG	?	NTRp75 entry factor

ASLV, avian sarcosis and leukosis virus; BTLA, B- and T-lymphocyte attenuator; CD, cluster of differentiation; EBV, Epstein-Barr virus; HCMV, human cytomegalovirus; HSV, herpes simplex virus; γHV, equine gamma herpesvirus; EIAV, equine infectious anemia virus; FIV, feline immunodeficiency virus; TNFR, tumor necrosis factor receptor; TRAF, TNFR-associated factor; TRAIL, TMF-related apoptosis inducing ligand;?, no homology recognized.

[a] Relationship determined by sequence or structural homology to the indicated TNF superfamily member.

SIGNALING PATHWAYS AND CELLULAR RESPONSES

Regulation

The cellular response activated by a TNF-related cytokine depends on several factors including the temporal patterns of expression of the ligands and receptors on the interacting cells, and the cellular context (the state of differentiation of the responding cell). Regulation is achieved at the level of transcriptional and translational controls, and by modulating the availability of the ligand or receptors (Fig. 27.4). For some ligands, transcriptional activation is a critical feature controlling the duration of mRNA expression. Some ligands exhibit inducible, transient expression of mRNA following signals from antigen receptors or innate receptor recognition systems. The half-life of mRNA

for TNF is short, controlled by an adenylate-uridylate-rich element in the 3′ untranslated region.[47] Deletion of the adenylate-uridylate-rich element in TNF mRNA in mice leads to a profound inflammatory disease.[47,48] TNF mRNA is inducible in macrophages by multiple pathways, particularly innate activation pathways such as toll-like receptor signaling, whereas other ligands like 41BBL and OX40L are constitutively expressed in differentiated antigen-presenting dendritic cells (DCs). T- and B-lymphocytes require activation prior to expression of TNF. In these cells, signals via the antigen receptor utilizing nuclear factor of activated T cells (NFAT) transcription factors are needed for the induction of TNF transcription. The inducible or constitutive patterns of expression are also observed with some receptors as well.

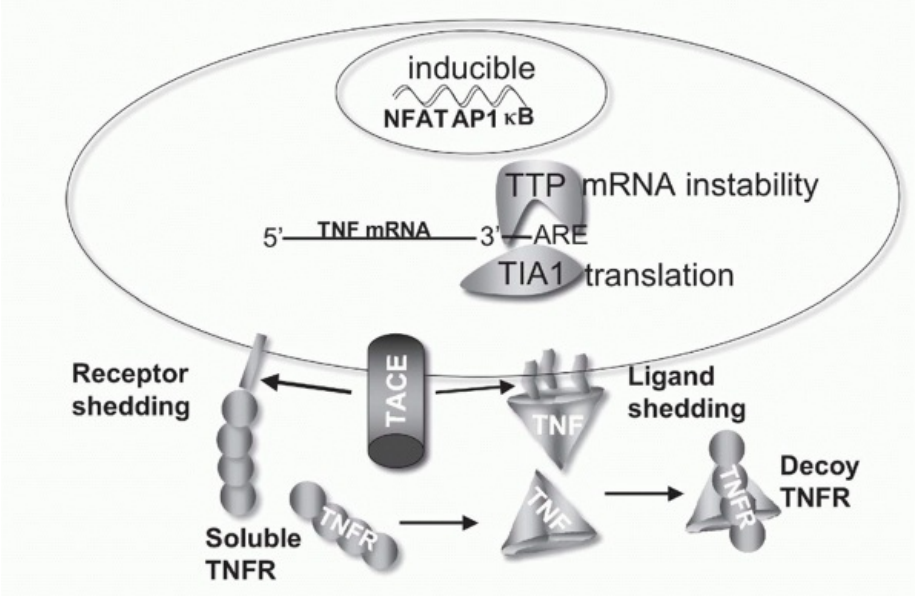

FIG. 27.4. Regulation of Tumor Necrosis Factor (TNF) Bioavailability. The expression of TNF is regulated at the transcriptional and translational levels, and bioavailability by altering its physical location and cellular receptors. TNF transcription is regulated by the action of multiple transcription factors including nuclear factors of activated T cells (NFAT), activated protein-1 (AP1), and NF-κB. NFAT is a predominant acting transcription factor regulating TNF transcription in T- and B-lymphocytes, and NF-κB and AP1 are important in myeloid lineage cells following activation via innate pattern recognition receptors, such as the toll-like receptors. TNF messenger ribonucleic acid (mRNA) stability is controlled by an adenylate-uridylate-rich element (ARE) in the 3′ untranslated region present in many transiently expressed inflammatory genes.[337] Stability of TNF mRNA is decreased by the action of tristetraprolin, and T-cell intracellular antigen silences translation of TNF mRNA through the ARE.[338] TACE proteolytically controls TNF at the membrane, generating the soluble form of TNF. TACE also cleaves TNFR1 and 2,[339] downregulating cell surface receptors and releasing soluble receptors that retain TNF binding activity. Soluble TNFR can stabilize the TNF trimer at sub saturating concentrations, and at higher, saturating concentrations act as decoys competing for TNF binding to cellular receptors.[340]

Posttranslational regulation of signaling is achieved by proteolytic cleavage of the ligand or receptor from the cell's surface, which places the protein into the soluble phase, where its half-life may be dramatically shortened. TNF and Fas ligand, for example, are shed from the surface by membrane proteases. ADAM17 (also known as TNFα-converting enzyme [TACE]), the enzyme that processes TNF into a soluble form, is involved in cleaving multiple cell surface proteins including transforming growth factor α, L-selectin, and TNFR1 and 2. Production of soluble TNFR1 and 2 may be important in regulating TNF bioavailability (see Fig. 27.4).

Most of the TNF-related ligands are expressed by DCs, activated lymphocytes, and myeloid cells, particularly macrophages, but can also be produced by nonlymphoid cells. TNF is an

example of a ligand expressed by many cell types depending on the stimulus. Expression of TNFR is widespread. TNFR1 is expressed on most cells, while TNFR2 is limited to cells of hematopoietic origin and is expressed following activation of B or T cells. Macrophages are a primary source of TNF in response to toll-like receptor signaling, and T cells produce TNF when activated by antigenic stimuli. Fibroblasts also produce TNF in response to virus infection, and nonlymphoid tumor cells may ectopically express TNF. FasL is another example of a ligand with a varied cellular expression pattern. FasL is expressed by effector T cells and natural killer (NK) cells as a component of their cell lytic activity, yet FasL mRNA is also detected in reproductive organs and epithelium of the eye, which may use this TNFSF member to kill organ infiltrating inflammatory cells as a mechanism to dampen inflammation.

Signal Transduction Pathways

TNF receptors initiate signaling pathways that alter gene expression patterns, changing the differentiation status of a cell, as well as apoptotic pathways that terminate cellular life. The propagation of signals from receptor to enzymatically active proteins is mediated by two distinct types of signaling motifs in the cytosolic domain of the TNFR: DDs and TRAF-binding motifs. TNFR that contain a DD include Fas, TNFR1, DR3, and TRAILR1 and 2. Other TNFRs have TRAF recruitment motifs. Three basic schemes are used by TNF receptors to connect to enzymatically-driven signaling pathways (Fig. 27.5A). Adaptor molecules are required to establish the signaling connections between the receptor and signaling enzymes. The DD connects TNFR to cytosolic proteins containing a Death Effector Domain (DED), which in turn link to caspases (cysteine based, aspartic acid specific proteinases). Alternately, the TRAF proteins connect to the cytosolic domain of the TNFR, altering ubiquitin-dependent pathways that regulate key serine kinases that activate NFκB and AP1 transcription factors. The third scheme involves an indirect link between the death domain and TRAFs via the adaptor TNFR-associated death domain (TRADD).

The apoptotic and NF-κB pathways activated by the TNFR family help regulate cellular homeostasis by controlling cell death and survival. In the immune system, apoptosis is essential for homeostasis and for eliminating antigen-bearing cells from the host. Many TNFR can induce activation of survival or death pathways depending in part on the differentiated state of that cell. In all nucleated cells, apoptosis is the default pathway; that is to say, all of the constituents of the pathway are expressed and ready to be activated (Fig. 27.6), whereas cellular survival requires transcriptional control of genes that encode regulatory proteins that suppress the progression of the apoptotic pathway.

Apoptosis

Ligation of TNFR promotes assemble of the death-inducing signaling complex (DISC) that promotes dimerization of procaspase 8 forming an active enzyme complex.[49] Activated caspase 8 acts directly to cleave procaspase 3 and 7, which are known as the executioner caspases as they directly cleave critical cellular substrates leading to apoptotic death. Caspase 8 also cleaves BID, a crucial connector to the mitochondria-associated death mechanism, which greatly amplifies the apoptotic process.[50] A cell must be capable of actively transcribing and translating genes to resist apoptosis signaling. A variety of genes can inhibit apoptosis and cell death pathways. For example, the cellular inhibitor of apoptosis (XIAP) is a direct caspase inhibitor that is regulated at the level of gene expression by

transcription factor NF-κB. Another regulator, FLIP (FLICE inhibitory protein), is also an NF-κB-regulated gene that contains a DED and a pseudocaspase domain that attenuates the apoptotic pathway by blocking conversion of procaspase 8 to the active form.[51] Many viral pathogens parasitize the transcriptional capabilities of the cell and prevent the cell from making new survival proteins, allowing apoptosis to proceed. As expected, viruses have evolved many different strategies to alter proapoptotic pathways (eg, viral orthologs of XIAP and FLIP prevent premature death of the cell) (see Fig. 27.6).

Cell Survival Signaling

Activation of transcription factors by TNFR utilizes the TRAF adaptors. There are seven members of the TRAF family (given numerical designations) that play key roles in regulating TNFR signaling and activation of host defenses. Each TRAF appears to play different roles in modulating signaling. Each TRAF protein contains a TRAF homology domain that binds TNFR and a RING and/or zinc finger domain characteristic of E3 ubiquitin ligases.[52,53,54] Ubiquitination plays an essential role in regulating signal transduction by TNFR[55,56] and in the pathogen recognition receptors as part of innate host defenses.[57]

Clustering of the TNFR promotes the recruitment of TRAFs into a complex with the receptor through two different mechanisms. TRAF2, 3, and 5 bind directly to the receptor's cytosolic domain recognizing a consensus PXQS/T motif,[58] whereas TNFR1 uses TRADD to couple to TRAF2. TRAF3 and TRAF6 are preassociated with different proteins, including protein kinases involved in multiple signaling pathways including the pathogen recognition and interferon (IFN) pathways.[53,59] TRAF adaptors link TNFR directly to protein kinase cascades, which in turn lead to activation of transcription factors including NF-κB and AP1.

The NF-κB family of transcription factors control expression of genes critical for cell survival, inflammatory, and immune responses. In mammalian cells, the NF-κB family consists of five members: RelA, RelB, c-Rel, p50/NFκB B1, and p52/NFκB B2 (proteolytic processing of p105 and p100 yields the active forms p50 and p52, respectively).[60,61] Activation of NF-κB releases inhibitors that restrict nuclear translocation.[62] Homo- and heterodimers of NF-κB family members are held inactive in the cytosol by inhibitors of κB (IκB), such as IκBα, that mask nuclear localization motifs on the NF-κB dimers. A complex consisting of the kinase catalytic subunits IKKα and IKKβ and the regulatory/scaffold subunit IKKγ form the IKK complex that mediates phosphorylation and ubiquitination of IκB, leading to its degradation and release of the active transcription factor. IκB is the common target of a variety of signals that control the activation of the RelA NF-κB transcription factor, which in turn regulates expression of many proinflammatory genes within a signal responsive cell.[63]

A distinct pathway regulates the activation of RelB NF-κB through the NF-κB-inducing kinase (NIK) and IKKα kinases. In unstimulated cells, NIK levels are maintained at vanishing low levels by ubiquitination and proteosome degradation through an E3 ligase complex comprised of TRAF3, TRAF2, and CIAP1/2.[52] Receptor ligation releases the active form of NIK by competitively displacing TRAF3, preventing further ubiquitination and allowing NIK to accumulate.[64] NIK phosphorylates IKKα, which induces the proteosome dependent processing of p100 to p52, degrading the inhibitory domain of p100 and allowing the

RelB/p52 complex to activate gene transcription.

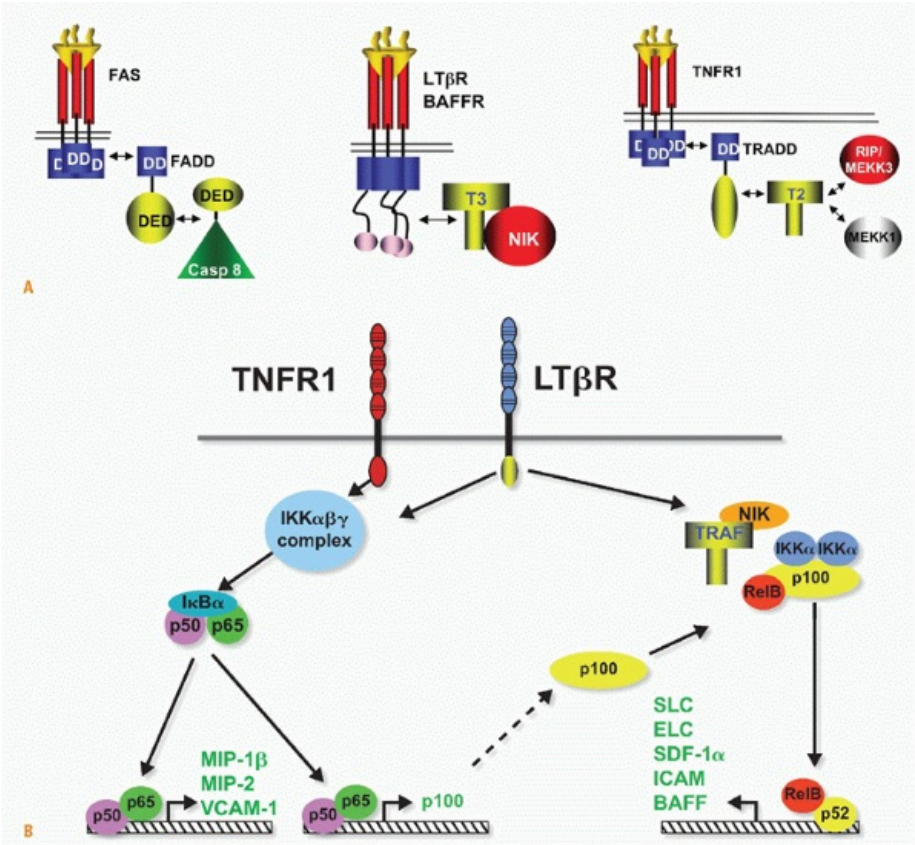

FIG. 27.5. Signaling Pathways and NF-κB. A: Adaptors link tumor necrosis factor receptors (TNFRs) to proteinases and kinases (*upper panel*). Three basic schemes link activated TNFRs to signaling pathways: the death inducing signaling complex formed with Fas is initiated by ligand clustering of Fas, promoting death domain (DD) interactions, which recruits Fas-associated death domain (FADD) (heterotypic interaction). The death effector domain of FADD links to the death effector domain of procaspase 8. The proximity of multiple procaspase 8 domains forms an active enzyme that can process other caspase 8 molecules. By contrast, TNFRs bind TNF-associated factor (TRAF) adaptors via short peptide motifs that release TRAF from associated kinases, such as NF-κB-inducing kinase (NIK). The third scheme is a combination of DD and TRAF recruitment. TNFR1 uses the DD protein TRADD to recruit RIP and TRAF2, promoting the activation of NF-κB. There are currently seven TRAF members with distinct interaction patterns with the TNFR family. TRAF proteins may function as regulators of key kinases. TRAF2, 3, and 6 function as modulators of several different kinases involved in toll-like receptor signaling, induction of type 1 interferon responses, and signaling by some TNFR family members. TRAF proteins contain an N-terminal RING finger motif, a coiled coil domain (isoleucine zipper), and the receptor association domain (TRAF) domain. The TRAF are trimers formed through their TRAF and coiled domains. The TRAF domain can bind to several different TNFR through a relatively short proline-anchored sequence that is responsible for binding directly to the mushroom head of various TRAF molecules.[341] The zinc RING of TRAF6 functions together with Ubc13

and Uev1A as ubiquitin E3 ligase targeting proteins for proteosome degradation. TRAF2, 3 and cIAP form the ubiquitin E3 ligase that targets NIK. Modified with permission from Ware CF. Tumor necrosis factors. In: Bertino JR, ed. *Encyclopedia of Cancer* . San Diego, CA: Academic Press, Inc.: 2002;475-489. **B:** NF-κB activation. TNFR1 and LTβR induce distinct forms of the NF-κB family of transcription factors. TNFR1 signaling is a potent activator of RelA/p50 but does not activate the RelB pathway, whereas lymphotoxin βR can activate both. The RelA and RelB forms of the κB family control transcription of distinct sets of genes; however, the two pathways are interrelated through the control of p100 expression by the RelA/p50 complex. Modified from Dejardin et al.[66]

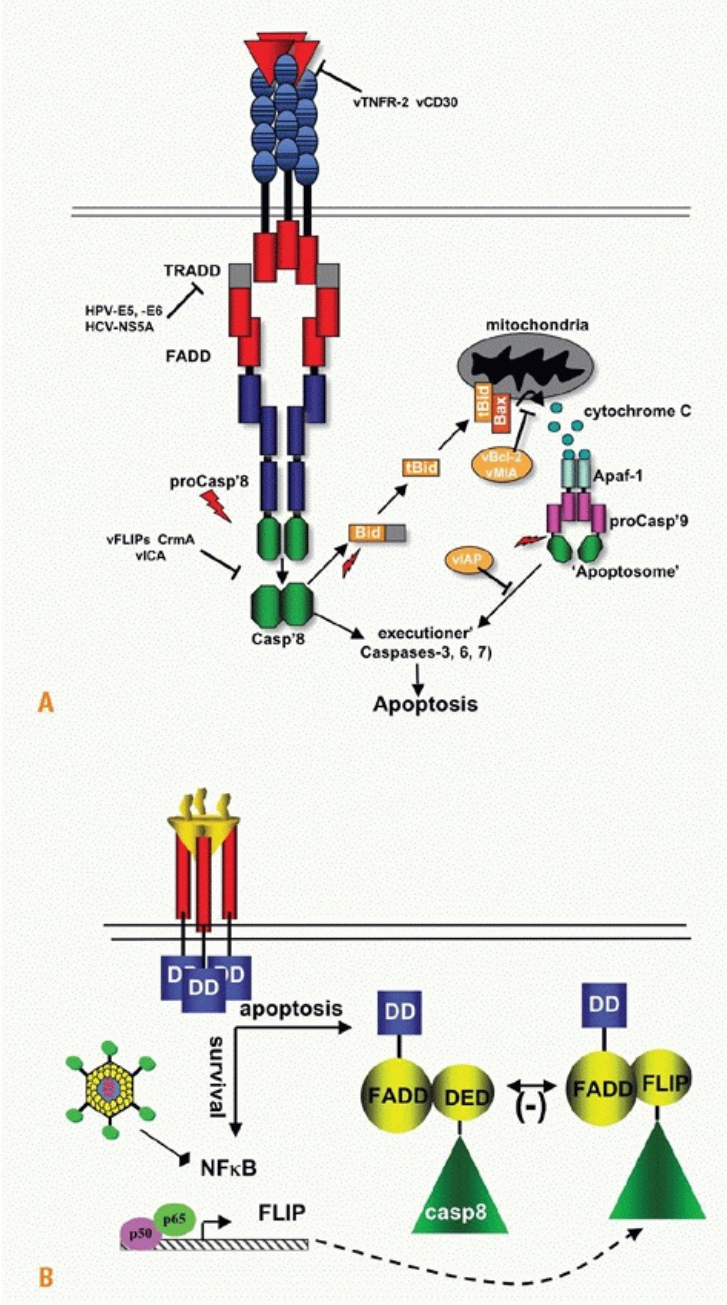

FIG. 27.6. Apoptosis and Survival Signaling. A: Ligation of tumor necrosis factor (TNF) receptor 1, Fas, or TNF-related apoptosis inducing ligand 1 and 2 activates apoptosis pathways. The nascent death-inducing signaling complex can directly cleave the executioner caspases 3, 6, and 7. Caspase 8 also cleaves BID to tBID, activating the mitochondrial regulated apoptotic pathway, dramatically accelerating cellular death. A number of viral proteins interfere with apoptosis induced through TNF pathway including human papilloma virus, hepatitis C virus, herpesvirus vFLIP, and virus orthologs of BCL2 and vMIA act on the mitochrondria. Adapted with permission.111 **B:** The components of the apoptotic pathway are preformed in the cytosol enabling the cell to respond rapidly to death signaling. In contrast, the key regulators of the apoptotic pathway, including Bcl2 and inhibitor of apoptosis, require new gene expression controlled by NF-κB. An important NF-κB-induced gene is cellular FLIP, which contains a pseudocaspase domain. The cFLIP protein interferes with caspase 8 activation, blocking the prodeath pathway. If the cell is transcriptionally inactive or unable to activate NF-κB, which often occurs in a pathogen-infected cell, then the proapoptotic pathway dominates the signals emanating from the TNF receptor 1 signaling complex leading to apoptosis and curtailing parasitism of the cell.

As examples, TNFR1 and LTβR activate separate yet related pathways that lead to distinct forms of NF-κB, the RelA/p50, and RelB/p52 complexes.[65] Each form of NF-κB activates a large number of genes with distinct roles in physiology (see Fig. 27.5B). TNFR1 signaling rapidly mobilizes (within minutes) the RelA/p50 complex, which controls expression of many proinflammatory and survival genes. By contrast, the processing of p100 and accumulation of nuclear RelB/p52 takes several hours after the initial stimulus. RelB-dependent genes are often involved in lymphoid tissue organogenesis and homeostasis. NIK is also required for NF-κB RelB and RelA activation by CD27, CD40, and BAFF-R, but not by TNFRI, which is restricted to activating RelA/p50 complex.[66,67] Components of the NF-κB pathway, including TRAF3, cIAP, NIK, and A20, are frequently mutated in cancer, leading to constitutive expression of survival factors such as the BCL2 family.[68]

THE IMMEDIATE LYMPHOTOXIN AND TUMOR NECROSIS FACTOR FAMILY

TNF (formerly TNFα or TNFSF2) and lymphotoxin (LT)α (formerly TNFβ (or TNFSF1) were originally pursued and characterized as inducers of tumor cell death, holding promise as antitumor therapeutics. However, the potent inflammatory action of TNF, particularly in the cardiovasculature, was quickly realized by the response of cancer patients injected with recombinant protein. We now recognize that TNF and LTα are two components of an interconnected network of “signaling circuits” that include LT-β, LIGHT (TNFSF14), and their specific receptors and regulatory proteins (Fig. 27.7). Each individual pathway has unique and cooperative signaling activities with other members of this immediate family. The immunologic processes controlled by this cytokine network are extensive, ranging from the development and homeostasis of lymphoid organs to the mobilization of innate defense systems to cosignaling activity promoting adaptive immune responses.[69,70,71]

TNF, LTα, LTβ, and LIGHT define the immediate group of TNF-related ligands that bind four cognate cell surface receptors with distinct but shared specificities. TNF and LTα both bind

two distinct receptors, TNFR1 (p55-60, *TNFR1A*) and TNFR2 (p75-80, *TNFR1B*). The heteromeric LTαβ2 complex binds the LTβR, which also binds LIGHT (TNFSF14). LIGHT also engages the HVEM (TNFRSF14), which acts as a ligand for BTLA and CD160,[31] an Ig superfamily member. Two distinct human herpesviruses, herpes simplex virus and cytomegalovirus, target the HVEM-BTLA pathway using different mechanisms[35,72] (see Fig. 27.7).

TNF mediates a diverse range of cellular and physiologic responses linked to acute and chronic inflammatory processes. The diversity of responses is due in part to the broad expression of TNFR1 and the release of TNF as a soluble mediator where it can act in a systemic fashion. TNF production is triggered by many of the innate recognition systems, such as the toll-like receptors and by T and B cells. In chronic inflammation, TNF production can lead to tissue damage and organ failure. Genes expressed in response to TNF signaling

coordinate the physiologic responses during inflammation (Table 27.4). The responses will reflect the characteristic of the inflamed organ, the local or systemic source of TNF, and the duration of TNF signaling. Acute inflammatory responses involve rapid changes in hemodynamics (plasma leakage and edema) and leukocyte adherence, extravasation, and organ infiltration induced by TNF. TNF production during chronic inflammation may contribute to systemic metabolic derangements and wasting (cachexia) or loss of organ structure and function (bone erosion in joints of patients with rheumatoid arthritis [RA]). In some models, TNF can promote an inflammatory environment that promotes tumor formation.[73,74,75]

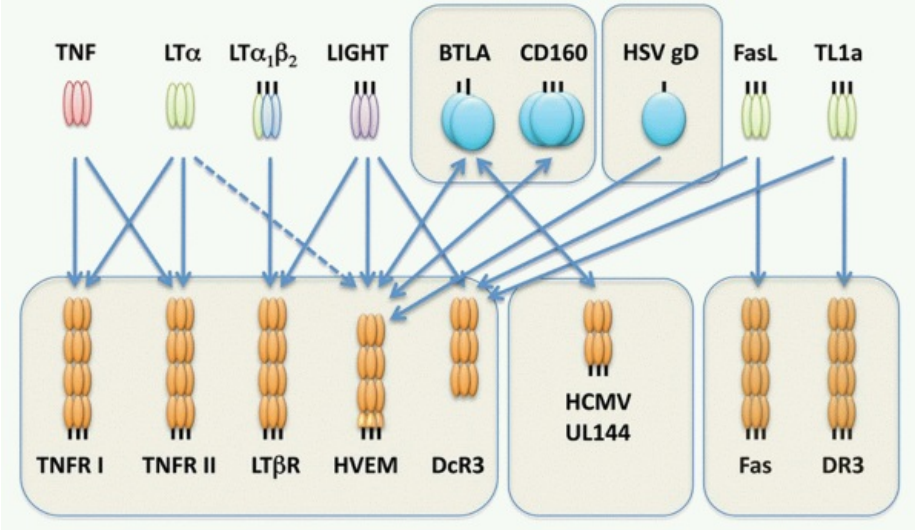

FIG. 27.7. The Immediate Tumor Necrosis Factor (TNF) Lymphotoxin (LT) Network. The cartoon depicts the signaling network formed between TNF, LTα, LTβ, and LIGHT, and their receptors. Each *arrow* indicates a ligand-receptor interaction. The network is defined by the extensive cross-utilization of ligand and receptors. Herpesvirus entry mediator (HVEM) forms a switch between positive cosignaling through LIGHT-HVEM interaction and inhibitory signaling through B- and T-lymphocyte attenuator (BTLA). LIGHT bound to HVEM activates TNF receptor-associated factor-dependent activation of NF-κB, whereas HVEM-BTLA acts through an immunoreceptor tyrosine-based inhibitory motif of BTLA to recruit the phosphatase SHP2, attenuating kinases

activated by T-cell receptor signaling. The herpes simplex viron envelope protein gD attaches to HVEM acting as an entry step for infection. UL144 gene of human cytomegalovirus binds BTLA, but not LIGHT, selectively mimicking the inhibitory pathway of HVEM-BTLA. Fas Ligand and TL1A are included in this network through their interaction with decoy receptor-3.

Elucidation of the functions associated with this TNF/LT signaling network has been aided by studies with genetically modified mice engineered with null or transgene expression of the cytokine or receptor (Table 27.5). Deficiency in TNF or TNFR1, but not TNFR2, have similar phenotypes with alterations in host defense to intracellular bacterial pathogens, like *Listeria monocytogenes* and *Mycobacterium tuberculosis*, but surprisingly modest susceptibility to some viral pathogens. These results demonstrated a role for TNF in acute-phase response of the host defense system. In contrast, LTα-deficient mice showed a failure in formation of peripheral lymphoid organs, a phenotype not observed in mice deficient in either TNFR or TNF, which implicated the LTαβ complex signaling through the LTβR as a key developmental pathway for lymphoid organogenesis.

Lymphotoxinαβ-LymphotoxinβR, A Mammalian Organ Development Pathway

Gene-deficient mice sharing a common phenotype of no lymph nodes revealed the framework of a signaling pathway involved in mammalian organ development. LTα-, LTβ-, or LTβR-deficient mice fail to develop secondary lymphoid tissues.[76,77] Several other knockout mice, including the transcriptional regulators Ikaros, ID2, and RORγt, also lack lymph nodes,[78,79,80,81,82] as do mice deficient in components of the NF-κB

activation pathway, including TRAF6, NIK, IKKα, and Rel B, and their gene targets. Target genes including CXCR5, the receptor for CXCL13, show defective lymphoid organ structure, as do CCR7-/- mice (receptor for CCL19/CCL21). The developmental program of secondary lymphoid organ formation initiates at 9 days post coitus progressing in organized fashion from dorsal to lateral movement, with intestinal Peyer's patches forming during the first postnatal week.[83] The defect is irreversible in that transferring LTαβ-sufficient bone marrow into an adult LT-mutant mouse failed to induce lymph node formation. Accumulating studies indicate that the formation of lymphoid organs involves two distinct cell types, an embryonic LTβR-expressing mesenchymal stromal cell that responds to LTαβ expressed in a cell of hematopoietically derived lineage, termed the lymphoid tissue inducer cell. The lymphoid tissue inducer cell develops separately from lymphocytes and myeloid cells in a pathway dependent on ID2 and RORγt and different cytokines including interleukin (IL)7, RANK ligand (TRANCE, TNFSF11), and TNF. These cytokines induce surface LTαβ in lymphoid tissue inducer cells that differentially engenders formation of lymph nodes and Peyer's patches.[84] Cells of the lymphoid tissue inducer lineage are maintained in the adult tissues at low levels (0.5%), presumably aiding in the homeostasis of lymphoid organs.[85,86]

TABLE 27.4 Physiologic Correlates of Tumor Necrosis Factor-Mediated Gene Induction

Induced Gene	Response
iNOS	Vasodilation, edema
VCAM-1	Leukocyte margination and extravasation
IL-8	Leukocyte chemotaxis
MHC-1	Antigen presentation
Caspase 8 activation	Apoptosis
LPL	Cachexia

Caspase 8, cysteine-dependent aspartic acid specific proteinase-8; IL-8, interleukin-8 (CXC chemokine); iNOS, inducible nitric oxide synthetase; LPL, lipoprotein lipase; MHC-1, major histocompatibility complex-1; VCAM-1, vascular cell adhesion molecule-1.

TABLE 27.5 Phenotypes in Mice Deficient in Lymphotoxin and Tumor Necrosis Factor Immediate Family

	Phenotypes					
Gene Deletion	**LN**[a]	**PP**[b]	**Architecture**[c]	**NK**[d]	**NKT**[e]	**DC**[f]
LTα	-	-	Disrupted	Impaired	Impaired	CD8-DC
LTβ	-	-	Disrupted	Impaired	Impaired	CD8-DC
LIGHT	+	+	+	+	+	+
LTβ-B[g]	+	+	Disrupted	nr	nr	nr
LTβ-T[g]	+	+	+	nr	nr	nr
TNF[h]	+	+	+	+	+	Maturation
LTβR	-	-	Disrupted	-	-	CD8-DC

TNFR1	+	-	Disrupted MZ	+	+	Maturation
TNFR2	+	+	+	+	+	+

-, absent;, +, normal; CD, cluster of differentiation; DC, dendritic cell; LN, lymph node; LT, lymphotoxin; MZ, marginal zone; NK, natural killer; NKT, natural killer T; nr, not reported; PP, Peyer's patches; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor.

[a] LTβ-/- mice have -75% of mesenteric LN; LIGHT/LTβ-/- mice have fewer mesenteric nodes than LTβ-/- mice.76,342,343

[b] See Rutschmann et al.344 and Neumann et al.345

[c] Architecture of the splenic white pulp includes T- and B-zone segregation, MZ, germinal center and follicular DC network.

[d] NK-cell deficiency includes reduced cell numbers and enhanced tumor susceptibility.346

[e] NKT cells Vα14 subset.347

[f] CD8- DC subsets in spleen are diminished from failure to proliferate.95

[g] LTβ conditionally deleted in B cells or T cells.348 LTβ-B cells showed partial disruption in architecture; normal for LTβ-T, but combined knockout in both B and T cells was worse than LTβ-B.

[h] Normal architecture observed in TNF point mutant344; abnormal architecture in TNFR1-/- mice.345

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The microarchitecture of the white pulp in the spleen[87] is disrupted in LT- and TNF-deficient mice.[88] Multiple abnormal features of the architecture are observed in LT- and TNF-deficient mice, including missing macrophages in the marginal sinus and the loss of positional segregation of T and B cells into discrete zones. The segregation of T and B cells into discrete compartments depends on expression of the tissue organizing chemokines CCL19 and CCL21, which attract T cells, and CXCL13, which attracts B cells. CCL19 and CCL21 act through the chemokine receptor CCR7 expressed on T cells, and CXCL13 binds CXCR5 on B cells to promote localization in the follicles. An LT-chemokine circuit is formed by migration

of B cells to LTβR+ stromal cells expressing CXCL13, which in turn induces expression of LTαβ on B cells.[89,90,91] Circulating B cells lack surface LTαβ, but expression is regained upon reentry into the CXCL13 rich microenvironment.[91] The formation of the splenic microarchitecture depends on B-cell expression of LTαβ, which induces differentiation of specialized stromal cells (eg, secretion CCL20 and CXCL13 chemokines) in the spleen during postnatal maturation. Remodeling of the microarchitecture of the secondary lymphoid organs occurs during immune responses, which requires both TNF and LT pathways signaling on fibroblastic reticular cells.[92] A viral pathogen, cytomegalovirus, can induce specific changes in the splenic microenvironment through modulation of CCL21 expression.[93]

The LTβR and TNFR pathways facilitate lymphocyte entry into lymphoid tissues in part by modulating expression of adhesion molecules, such as peripheral node and mucosal addressins on high endothelial venules.[94] LTβR signaling is necessary to maintain networks of follicular DCs involved in capturing antigen and immune complexes that aid in activating B cells. These cellular interactions are further enhanced by LTβR signals that provide growth signals for some conventional myeloid DC (CD8- subsets) within the lymphoid organ, whereas TNF plays a role in differentiation of DC progenitors in bone marrow.[95]

T and B cells can communicate with stromal and myeloid cells via the LTαβ-LTβR pathway and thus modify their immediate microenvironment during the course of an immune response. Nonlymphoid tissues suffering from chronic inflammation associated with autoimmune disease, graft

rejection, or microbial infection often contain organized accumulations of lymphocytes reminiscent of secondary lymphoid organs, called tertiary lymphoid organs. Activated T and B cells provide the source of LTαβ that helps drive the process of forming these structures, but as antigen is cleared, immune responsiveness subsides and these structures resolve. A gradation of features may be found in the tertiary lymphoid organs including presence of DCs, expression of chemokines, high endothelial venules, segregated regions of T and B cells, and germinal centers, but these structures typically lack the permanence of a lymph node. TNF also contributes to formation of granuloma that assists in walling off bacteria.[96] Thus, the LTαβ and TNF pathways operative in embryonic life also play critical roles in the adult in the formation of tertiary lymphoid structures.

Influence of the Lymphotoxinαβ Pathway on Lymphocyte Development

Mounting evidence indicates the LTαβ-LTβR pathway contributes to the ontogeny of unconventional T cells, including γδ T cells and invariant NK T cells, whereas conventional T cell subsets are normal in mice deficient in the TNF and LTβR pathways. The LTβR pathway seems to operate at distinct levels during thymic development.[97] Double positive thymocytes regulate the differentiation of early thymocyte progenitors and γδ T cells via the LTβR pathway,[98] yet the LTβR is not expressed in thymocytes, suggesting an indirect mechanism. In this regard, LTβR signaling is required for the proper formation and function of the thymic stroma, which influences T-cell development.[99] The thymic medulla appears to control the export of invariant NK T cells from the thymus.[100,101] In addition, LTβR signaling in thymic stroma affects central tolerance to peripherally restricted antigens, which may be either

dependent or independent upon the autoimmune regulator *Aire*.[102] Thymic differentiation depends on the LTβR pathway to mediate the cellular communication between lymphoid and stromal compartments.

Autoimmunity

Dysregulated expression of several members of the TNFSF leads to autoimmune-like diseases in humans and animal models. For example, enforced expression of TNF or LIGHT, which overrides the normal transient expression, causes severe autoimmune and inflammatory processes in mice.[103,104,105] LTα or LTαβ transgenic expression in the pancreas leads to insulitis and formation of tertiary lymphoid structures.[106] These types of results have implicated members of the TNF superfamily as immune regulators and support the notion that LTαβ and LIGHT pathways contribute to inflammation and tissue destructive processes.

Infectious Diseases

TNF is a major inflammatory cytokine required for the acutephase response to bacterial infection. For instance, lipopolysaccharide in gram-negative bacteria is a potent inducer of TNF secretion through the TLR4 innate recognition system. In mice, lipopolysaccharide induces a shock syndrome that is rapidly lethal owing to profound changes in blood circulation. However, mice survive lipopolysaccharide in the genetic absence of TNFR1 or if treated with an TNF-neutralizing antibody, indicating that host-derived TNF mediates pathogenesis.[107,108,109] On the other hand, TNFR1 is essential for resistance to infection with a live organism, such as *Listeria monocytogenes*, through multiple processes including enhancement of phagocytosis and bacteriocidal destruction by macrophages. By contrast, T-cell immunity is not overtly impaired in TNFR1-/- mice. Humans treated with TNF inhibitors show some increase in susceptibility to selective pathogens, particularly *Mycobacterium tuberculosis*, reinforcing the role of TNF as a critical host defense system.[110]

In contrast, LT-deficient mice showed significant variability in susceptibility to individual pathogens (Table 27.6). Increased susceptibility resulted from either developmentally controlled aspects of lymphoid organ structure (eg, lymphocytic choriomeningitis virus, *Leishmania*) or a requirement for LTαβ-LTβR pathway as an effector system in innate and adaptive immune systems (eg, murine cytomegalovirus). Viewed from an evolutionary perspective, this variation in the requirement for LT signaling may reflect specific contributions from the pathogen used to evade the broader TNF- and LT-dependent pathways.[35,111]

TUMOR NECROSIS FACTOR SUPERFAMILY AND T-CELL COSIGNALING

Antigen recognition together with cooperating signaling “cosignaling” systems determine the quality of the adaptive immune responses. Lymphocyte responses to antigen are dynamic processes that start with the activation of naïve cells and transition through effector and memory phases. Cosignaling systems assist these phases by promoting more efficient engagement of antigen-binding TCR molecules to enhance initial cell activation and cell division (clonal expansion), augment cell survival (clonal contraction or memory cell differentiation), or induce effector functions such as cytokine secretion or killer function.

Negative signals (inhibitory cosignaling) may also be delivered to T cells depending upon the particular system, which may prevent initial cellular activation or eliminate excess activated cells to dampen inflammation. Cosignaling can be quantitative, modifying thresholds of common signaling intermediates, or qualitative, involving signals distinct from other cosignaling systems or the TCR. Cosignaling receptors and ligands can be up- or downregulated at the transcriptional and protein levels depending on the stage of the T-cell response and the inflammatory milieu. In the absence of cosignaling, T cells may become unresponsive (anergic) or die.

The TNFRSR is one of two major families of cosignaling regulators that modulate T cells. The other cosignaling systems belong to the Ig superfamily, such as CD28,[112] cytotoxic T-lymphocyte (CTL)A-4,[113] ICOS,[114] PD1,[115] and BTLA.[116,117] TNFRSF members involved in T-cell cosignaling include OX40, 41BB, DR3, CD27, CD30, and HVEM,[118,119,120,121,122] whereas CD40 and BAFFR are more involved in cosignaling in B lymphocytes.[123,124] However, considerable crossover of activities in both lymphocyte populations can be demonstrated. Death

receptors such as Fas and TNFR1 are thought to be involved in clonal contraction through apoptosis of activated effector cells, although TNF via TNFR2 also shows costimulatory action in naïve T cells. In tissue culture models, several of the TNF-related signaling pathways show costimulatory activities for T cells, which probably reflects the common induction of NF-κB-dependent survival genes, a common trait of TNFRSF members. However, analyses of physiologic models using genetically deficient mice reveal distinct roles for these molecules in the life cycle of T cells. Cosignaling systems are emerging as important targets to enhance immune responses to tumors or attenuate autoimmune diseases.[114,125,126,127,128,129]

TABLE 27.6 Lymphotoxins in Host Defense: Mouse Models

Pathogen[a]	Mouse Model[b]	Susceptibility	Mechanism	Reference
Herpesvirus				
MHV68	LTα-/-	Minimal	nd	349
HSV-1	LTα-/-	Increased	Decreased effector CD8+ T cells	350
MCMV	LTα-/-;B-LTβ	Increased	IFN response; adaptive immunity lost	351,352,353

MCMV	LTβR-Fc Tg	Increased	Poor innate defenses	354
LCMV	LTβ-/-; LTα-/-	Increased	Defective architecture	354,355,356
LCMV	LTβR-Fc	Decreased	Decreased CD8+/IFNγ	357
Theiler virus	LTα-/- LTβR-Fc	Increased	Defective architecture	358
Influenza	LTα-/-	Minimal	nd	359
Vesicular stomatitis virus	LTβ-/-	Increased	Defective architecture	356
Bacteria and Parasites				
Mycobacterium tuberculosis	LTβR-/-	Increased	NO_2 synthase decreased	360
Mycobacterium tuberculosis	LTα-/-	Increased	No T cells in granuloma	361
Mycobacterium bovis	LTβR-Fc	Increased	Poor granuloma formation	362
Listeria monocytogenes	LTβR-/-	Increased	nd	360
Leishmania major	LTβ-/-	Increased	Defective architecture	363
Leishmania donovani	LIGHT-/-	Increased	Defective T cell	364
Toxoplasma gondii	LTα-/-	Increased	NO_2 synthase decreased	365
			Decreased LTα	

Plasmodium berghei	LTα-/-	Decreased	dependent inflammation	366

CD, cluster of differentiation; HSV, herpes simplex virus; IFN, interferon; LCMV, lymphocytic choriomeningitis virus; LT, lymphotoxin; MCMV, murine cytomegalovirus; MHV murine herpesvirus; nd, not done.

[a] Virus: MHV68, HSV1 (α-herpesvirus), MCMV, LCMV. Bacteria: *Mycobacterium*, *Listeria monocytogenes*. Parasite: *Leishmania major*, *Leishmania donovani*, *Toxoplasma gondii*, *Plasmodium berghei*.

[b] Studies conducted in gene deficient mice (-/-); LTβR-Fc Tg, mice expressing LTβR-Fc as a transgene; LTβR-Fc, mice injected protein.

Modified with permission from Ware.70

Expression and Function of Tumor Necrosis Factor Receptor Superfamily Cosignaling Molecules During Primary T-Cell Responses

The TNFRs encoded on Chr 1p36 share a common function as cosignaling systems for T cells.[120,122] This region (human 1p36.33-1p36.21; syntenic with mouse 4E2-D3) contains the genes encoding GITR, OX40, HVEM, DR3, 41BB, CD30, and TNFR2, and is paralogous to the region on Chr 12p13 where genes for TNFR1, LTβR, and CD27 reside. The TNF-related ligands for these receptors are linked in the MHC paralogs on Chr 1, 6, 9, and 19, reflecting a common functional link as costimulatory molecules modulating T-cell differentiation (see Fig. 27.2).

Expression of TNFRSF members by T cells follows several distinct patterns during T-cell activation: expression on naïve T cells in peripheral lymphoid tissues (eg, CD27 and HVEM) that are upregulated or downregulated, respectively, following activation. OX40, 4-1BB, TNFR2, and CD30 are only expressed by T cells after initial activation[120]; TNFR2 is rapidly downmodulated following cleavage by TACE-generating soluble receptors that modulate TNF availability. The differing time course of expression of these molecules determines the phase of when they act in T-cell differentiation.

Experimental activation of TNFRSF members can be achieved with agonist monoclonal antibodies. Antibodies specific to OX40, 4-1BB, CD30, or CD27 all augment T-cell clonal expansion and TCR-induced cytokine expression in tissue culture and mouse models. For example, CD27, which is expressed at very early stages of T-cell activation, mediates initial cytokine production, cell division, and/or survival,[130,131] whereas OX40 and 4-1BB, which are expressed after cellular activation, promote T-cell survival and proliferation during the later stages of the primary response.[132,133,134] CD30-CD30L interactions promote primary CD8+ T-cell expansion and with OX40 act to promote CD4+ T-cell memory,[135,136] suggesting functional parallels with other TNFRSF molecules. Cowpox virus encodes a specific CD30 mimic to block the action of CD30L as part of its immune evasion strategy (see

Table 27.6).

Death receptor-3 (DR3) engages TL1A, promoting IFNγ expression by proinflammatory TH1 cells in mouse[137] and humans,[138] especially in intestinal tissues.[139] Polymorphisms in TL1A are associated with inflammatory bowel disease.[140,141] The LIGHT-HVEM-LTβR pathway is also associated with T-cell inflammatory processes in mucosal tissues.[104,142] Similar cosignaling activities are reported for GITR,[143] with the potential of these systems to affect different subsets of T cells, such as GITR expression on T-regulatory subsets. The role of TNFRSF in regulating different T-cell subsets is an active area of research.

HVEM appears to play a role as an inhibitor of naïve T-cell activation through engagement of the Ig superfamily members BTLA and CD160.[144,145] In this situation, HVEM acts as a ligand for BTLA, which limits signaling by antigen receptor-associated tyrosine kinases through a phosphatase recruitment domain (immunoreceptor tyrosine-based inhibitory motif) in its cytosolic tail.[146,147] Naïve T cells coexpress HVEM and BTLA, forming an intrinsic complex that limits HVEM activation by its ligands expressed in the surrounding microenvironment. The BTLA binding site on HVEM is distinct from the position occupied by LIGHT, yet membrane LIGHT can disrupt the HVEM-BTLA complex, suggesting that HVEM acts as a regulatory switch between inhibitory and stimulatory cosignaling. Interestingly, like LIGHT, BTLA and CD160 can activate HVEM in trans, promoting cell survival gene expression in cells expressing HVEM.[148] This pathway appears particularly effective in controlling CD4 T-cell activation,[144,149] but it is also important in regulating differentiating memory CD8 T cells.[150]

T-Cell Memory

Cosignaling through several TNFRSF members promotes the differentiation of memory T cells. Mice deficient in OX40-OX40L, CD27-CD70, CD30-CD30L, or HVEM pathways all display defective memory responses to antigen.[131,135,151,152,153,154] The OX40 system is illustrative of the costimulatory signals provided to T cells by these TNFR. Pharmacologic stimulation of OX40 with an agonist antibody or ligand during a primary antigen-specific response in mice enhances the generation of memory CD4 T cells.[155] The enhanced response of T cells includes increased accumulation of antigen-specific effector T cells producing effector cytokines such as IFNγ at the site of inflammation.[156] Moreover, blockade of OX40-OX40L interactions during recall responses with monoclonal antibody specific to Ox40L ameliorates inflammation in models of collagen-induced arthritis[157] and experimental allergic lung inflammation.[158] In OX40-deficient mice, CD8 T cells expand normally, but their accumulation and survival at later times in the primary response was significantly impaired. T cells from OX40-deficient mice also failed to fully differentiate as measured by loss of expression of effector cell surface markers and decreases in synthesis of cytokines and cytotoxic activity. By contrast, the formation of extrafollicular plasma cells, germinal centers, and antibody responses was independent of OX40. OX40 signaling induces transcription of survival genes such as BCL2 and survivin in T cells, which may serve as the key targets that allow memory cell differentiation.[159,160]

The discovery that HVEM can elicit inhibitory signaling in T cells through engagement with the Ig family member BTLA highlights the possibility that, in certain situations, TNFRSF molecules can counter regulate the stimulatory actions during T-cell responses. Indeed, 4-1BB-deficient CD4+ T cells in some models are hyperresponsive,[161] and stimulation of 4-1BB can suppress immune responses in several models of autoimmune disease.[162,163,164,165] Although activation of 4-1BB enhances the expansion of human antiviral memory CD8+ T cells in vitro,[166] several studies demonstrate that the absence of 41BB in mice leads to enhanced T-cell responses,[161,167] reminiscent of the phenotype associated with HVEM-deficient mice.[168,169] Moreover, CD30 signals can, in some circumstances, suppress cytotoxic T-lymphocyte activity.[170] The expression of TNFRSF molecules such as GITR or OX40 on T-regulatory cells suggests that costimulatory pathways may enhance the suppressive function of these cells during immune responses.

Mouse models of autoimmune diseases such as EAE and diabetes uncover critical roles for OX40-Ox40L.[171,172] Blockade of the CD27-CD70 pathway also ameliorates EAE through the suppression of TNF induction, but not T-cell priming.[173] Furthermore, blockade of OX40, 4-1BB, CD27, or CD30 costimulation can all reduce transplant rejection and/or graft-versus-host disease.[174,175,176,177]

Stimulation of these pathways may also be applicable for the treatment of diseases such as cancer and infectious diseases where antigen-specific T-cell responses are ineffective. Stimulation of OX40, 4-1BB, CD30, and CD27 with agonist antibodies augments T-cell-mediated killing of a variety of tumors. The critical roles of the cosignaling TNF-TNFRSF members are discerned in immune responses to infectious pathogens. Similar to the immediate TNF family, the role of a specific cosignaling pathway in mediating protective T-cell responses during infection depends on the pathogen (Table 27.7).

CD40L AND BAFF SYSTEMS

The CD40 and BAFF systems play a major role in coordinating a range of costimulatory signals important to B-cell function, such as affinity maturation, isotype switching, Ig production, and clonal expansion.[123,178,179,180,181] In common with other TNFSF members, the CD40 system (see Fig. 27.3) is a key communication mechanism in host defense, autoimmunity, and cancer. Often, these pathogenic processes are centered on the function of B cells. CD40 ligand maps to the X chromosome and is regulated at the transcriptional level by NFAT, resulting in inducible CD40 ligand expression by T helper cells that promotes humoral immunity. CD40 ligand is also expressed in a variety of other cells including activated B cells, endothelial cells, basophils, mast cells, and platelets. CD40 is expressed on many cells, prominently on B cells, and utilizes both TRAF3 and 6 to activate NF-κB and AP1 transcription factors, among other signaling pathways.[182,183] Epstein-Barr virus, a B-cell-transforming herpesvirus, mimics the CD40 pathway though its LMP1 protein, driving naïve B cells into a memory state.[184,185,186]

TABLE 27.7 Tumor Necrosis Factor Superfamily Cosignaling Pathways in

Infectious Diseases

	Cosignaling Pathway			
Pathogen	**CD27-CD27L**	**Ox40-Ox40L**	**4-1BB-41BBL**	**CD30-CD30L**
Influenza	CD4+ and CD8+131,367	CD4+153,156,368	CD8+151	nd
LCMV	nd	CD4+156	Normal CD8+151	nd
MCMV		CD4+369,370	CD8+371	CD8+372
Leishmania major	No role	CD4+ Th2373	No role	No role
Helminths	nd	CD4+ Th2374	nd	nd
Cryptococcus neoformans	nd	Enhanced CD4 Th2375	nd	nd
Mycobacterium avium	No role376	No role376	No role376	CD4+ and CD8+376
Listeria monocytogenes	CD4+ and CD8+377	nd	CD8+378	CD8+379

CD, cluster of differentiation; LCMV, lymphocytic choriomeningitis virus; MCMV, murine cytomegalovirus; nd, not done.

Unless indicated, the function of the T cells was impaired.

Adapted from Croft.120

CD40 signaling is necessary for induction of activationinduced cytidine deaminase, a key enzyme in class switching and somatic mutation of Ig genes. This process occurs in B cells in contact with T helper cells, which express CD40 ligand. The importance of CD40 system in the activity of B cells was revealed in patients with hyper-IgM syndrome. Several mutated genes underlie this syndrome including CD40 ligand and CD40,[187,188] the RelA NF-κB activating kinase subunit, IKKγ (NEMO), and activation-induced cytidine deaminase, among

others.[189] Patients with hyper-IgM syndrome with mutated CD40L have elevated levels of IgM, often with no serum IgG, IgE, or IgA, and display increased susceptibility to bacterial and opportunistic infections. Treatment of these patients with passive transfer of human immune IgG corrects infection by bacteria, but not opportunistic pathogens, such as *Pneumocystis carinii*. This latter result reflects the importance of CD40 system in T-cell-mediated macrophage activation, which controls these opportunistic pathogens. In some patients with hyper-IgM syndrome, significant amounts of IgA and IgE are present in serum, even in the complete absence of CD40L, a result indicating that another mechanism(s) can induce Ig class switching. Evidence has emerged that the BAFF/APRIL system can induce Ig class switch independent of CD40 system.[190]

Since the discovery of B-cell-activating factor of the TNFSF (BAFF, TNFSF13B; BLyS) and a proliferation-inducing ligand (APRIL, TNFSF13), much has been elucidated with regard to CD40L-independent isotype switching and B-cell development and survival in the periphery, as well as the implications of BAFF-APRIL dysregulation in autoimmune disease and lymphomas.[124,191] Although human *BAFF* maps to Chr 13q34 and APRIL to 17p13.1, the proteins are strikingly more conserved (˜50% homology) than other TNF ligands. TWEAK (TNF-related ligand with weak apoptosis activity) maps adjacent to APRIL, part of a clade of genetically similar members that also includes ectodermal dysplasin (EDA1/EDA2). BAFF and APRIL form homo- and heterotrimers that interact with multiple receptors to form a complex signaling circuit (see Fig. 27.3). Membrane BAFF is shed via a furin protease at the cell surface, while APRIL is processed in the Golgi and secreted only in soluble form,[192] although the receptor binding domain of APRIL can be membrane-anchored via an unusual splice variant with the TWEAK cytosolic and transmembrane domain (TWE-PRIL).[193] The significance of membrane-restricted local BAFF expression to systemic availability of soluble BAFF and APRIL remains to be established, although BAFF-APRIL heterotrimers are detected in serum from patients with systemic rheumatic diseases.[7] BAFF and APRIL are inducibly expressed in monocytes, macrophages, DCs, and T cells in the spleen and lymph nodes in response to certain cytokines in different cell types, such as type I and II IFN, IL-10, and other TNF family members like CD40 and LTβR signaling. BAFF can be expressed in some B-cell-derived chronic lymphocytic leukemia cells, and some epithelial-derived cancer cell lines and primary tumor tissues express APRIL. Stromal cell expression of BAFF maintains the major mature B-cell pool, whereas inducible BAFF expression in myeloid cells aids in local B-cell survival at sites of inflammation.

Three cognate receptors mediate the biological actions of BAFF. BAFFR (TNFRSF13C), transmembrane activator and calcium signal modulating cyclophilin ligand interactor (TACI; TNFRSF13B), and B-cell maturation antigen (BCMA; TNFRSF17) are expressed on B cells and on other cell types. APRIL also engages matrix proteoglycans with weak binding, which may serve to enhance access to TACI and BCMA or function as a distinct receptor-ligand interaction.[194] Signaling through BAFFR and BCMA are required for B-cell survival and differentiation at different stages of B-cell-mediated humoral response. BAFFR-/- mice display an almost complete loss of mature and marginal zone B cells beginning from the late transitional phase of B-cell maturation, whereas BCMA deficiency impairs the survival of long-lived bone marrow plasma cells.[195,196]

By contrast, APRIL-/- mice develop normally but have altered class switching to IgA.[197,198] TACI signaling may antagonize BAFFR and BCMA, as TACI-/- mice have elevated numbers of mature B cells.[199] Both classical and alternative NF-κB pathways have been implicated BAFFR and BCMA survival signals, with BCL2 expression rescuing the B-cell defect in BAFFR-/- mice. BAFFR and TACI engagement

has also been shown to provide the necessary signals for CD40L-independent class switch recombination, to IgG and IgE for BAFFR, and IgG, IgE, and IgA for TACI, though IgA production seems specifically dependent upon APRIL-TACI interaction.[200] BAFFR is also connected to promoting the B-cell coreceptor complex CD19/CD21/CD81, which binds to C3b-opsonized antigens to enhance rapid T-independent antibody responses. Overall impairment of T-independent antibody responses appears to be greatest in TACI-/- mice, and is dependent upon APRIL-TACI interactions in B1 B cells. BAFFR, and not TACI, has also been proven to be important in T cell costimulation in in vitro and in vivo mouse models of allograft rejection response, where BAFFR-/- and BAFF-/-, but not TACI-/- nor BCMA-/-, mouse recipients prolong graft survival due to weakened alloproliferative response.[201]

Elevated levels of BAFF and APRIL have been detected in the serum, and especially the synovial fluid, of patients suffering from inflammatory autoimmune disorders such as systemic lupus erythematosus, RA, Sjörgen syndrome, and multiple sclerosis.[202] These levels tend to correlate with increasing amounts of autoreactive antibodies in serum, and in mouse models of lupus and collagen-induced arthritis, soluble TACI-Ig treatment can lessen the severity of disease symptoms and progression.[203] It is hypothesized that these excess quantities of BAFF and APRIL may contribute to the formation of autoreactive B-lymphocytes and loss of tolerance during development and perpetuating their survival as plasmablasts. Emerging evidence connects the BAFF and APRIL system to the pathology of B-cell-derived cancers like B-cell chronic lymphocytic leukemia, non-Hodgkin lymphoma, and multiple myeloma. Malignant myeloma cells express high levels of BAFF and APRIL and receive ligand-induced survival signals, with upregulation of antiapoptotic Bcl-2 and Mcl-1 molecules.[204] Therefore, BAFF and APRIL may bolster tumor survival via autocrine and paracrine signaling mechanisms.

TWEAK-FN14 SYSTEM

The TWEAK-Fn14 system is emerging as an important signaling system between the immune system and epithelial and stromal tissues.[205] TWEAK is limited to a monogamous interaction with Fn14, its cognate receptor, which is a fibroblast growth factor-2-inducible gene (see Fig. 27.3). Fn14 has one CRD and binds TWEAK homotrimers in a manner similar to BAFF, APRIL, and their receptors.[206,207] TWEAK, like BAFF and APRIL, is cleaved by furin protease to produce an active soluble form.[206] Human and mouse TWEAK share > 90% sequence identity in the receptor binding domain and manifest high affinity cross-species interaction to respective Fn14 homologues.[192] The biologic functions of TWEAK-Fn14 system are broad and perhaps paradoxical, promoting both cell survival and death. TWEAK/Fn14 signaling induces apoptosis in some cancer lines (HT29, HSC3) and primary neurons.[208,209,210] TWEAK is produced primarily by leukocytes, whereas Fn14 expression

is induced during inflammatory and tissue destructive processes. TWEAK-Fn14 system may provide important homeostatic functions to promote regenerative processes that conclude inflammatory responses. These homeostasis-promoting functions include induction of survival and growth in endothelial cells, migration and wound closure in endothelial cells, and angiogenesis.[206,207] Fn14 contributes to the activation of progenitor cell types including liver, skeletal muscle, and mesenchymal lineage progenitors such as osteoblast, chondrocyte and adipocyte, and neuronal progenitors.

TWEAK regulates the shift from innate to adaptive immune responsiveness by repressing innate inflammatory cytokines (IL-12, IFNγ) important in promoting TH1 immunity—a potential counterbalance to the proinflammatory actions of TNF.[208] TWEAK may also have a potentiating influence in chronic tissue inflammation when dysregulated, such as its role in excessive demyelination and ultimate progression to excessive autoimmune encephalomyelitis.[211] In a mouse collagen-induced arthritis model, elevated levels of TWEAK in serum correspond to severe disease progression and enhanced arthritogenic mediator molecules.[212] In obese patients with type 2 diabetes, TWEAK-Fn14 signaling augments proinflammatory cytokine release by adipocytes.[213] In rats, TWEAK may prevent excessive luteinization after gonadotrophin-induced ovulation by controlling progesterone production.[214] The seemingly contradictory functions of TWEAK/Fn14 are less puzzling when TWEAK/Fn14 is viewed as an important signaling system that aids in achieving homeostasis following inflammation, as cell death and survival are essential for homeostasis.

DEATH RECEPTORS AND LIGANDS

Fas Ligand and Fas System

The Fas (CD95, TNFRSF6)-Fas ligand (TNFSF6) system is an example of a direct signaling pathway to apoptotic cell death. The importance of the Fas-FasL system in immune regulation was revealed by the recognition that mice with the autoimmune-like disorders, *lymphoproliferative* (lpr) and *generalized lymphproliferative disorder* (gld) harbored mutations in Fas and Fas ligand and humans with the autoimmune lymphoproliferative syndrome have mutations in Fas.[215,216,217] Mice with *lpr* or *gld* mutations display autoimmune phenotypes with high accumulation of activated T cells in the periphery and CD4-CD8- T cells in the lymph nodes (lymphoadenopathy) and autoantibody production. The Fas-FasL system is involved in proinflammatory responses, tumor survival, and nonimmune tissue homeostasis in osteoclastogenesis and angiogenesis.[218] Activated lymphocytes are particularly sensitive to Fas-induced apoptosis, suggesting a role for Fas in the clonal contraction phase of the immune response. The epithelial cells in the eye express FasL, which may limit inflammation in organs sensitive to immune damage. FasL is thought to be one of several proapoptotic systems used as a killing mechanism by cytotoxic T cells and NK cells. In primates, Fas ligand binds Fas and decoy receptor-3, which also binds LIGHT and TL1A.[219]

FasL can undergo cleavage by matrix metalloproteases to produce soluble FasL, which may induce markedly different events when it engages Fas compared to the membrane-bound form, such as loss in cytotoxicity.[220]

Other findings suggest that soluble FasL may counteract the proinflammatory properties of membrane-associated FasL as an immunosuppressive, particularly in the eye and tumor environments.[221,222] Conversely, Fas has also been linked to lymphoproliferation and lymphocyte activation via NF-κB-signaling pathways.

Essential biologic roles FasL-Fas include regulating lymphocyte homeostasis (via contraction of clonally expanded effector T and NK cells), directing CTL-mediated apoptosis and lysis (immune surveillance), and establishing immune privileged organs and sites. Fas is expressed in a wide variety of tissues such as the thymus, spleen, heart, and liver, whereas FasL can be found on activated T cells, NK cells, tumor cells, immune privileged sites, lung, and other tissues.[218,223,224] In mature activated T and NK cells, Fas-FasL signaling plays a critical role in inducing apoptosis during the latter stages of inflammation, or activation-induced cell death, and shifts lymphocyte population numbers back to baseline.[225] In certain organs like the liver, lung, and small intestine, FasL on stromal cells can be upregulated to control the overabundance of activated T cells after an inflammatory response and ultimately reduce tissue damage. Alternately, dysregulation during hepatitis C infections can lead to death of hepatocytes and acute liver damage.[226] The Fas system has also been implicated in curtailing autoantibody production by damping T cell helper-induced B-cell activation and direct elimination of activated B cells, where CD40 stimulation also upregulates Fas expression.[218]

The Fas-FasL system, together with perforin and granzymes, is a mechanism vital to CTL-mediated destruction and clearance of tumor and virally infected cells. Viruses have evolved multiple mechanisms of suppressing Fas signaling, 111 including downmodulation of Fas[227] or blockade of caspase 8 activation.[228] Tumor genome instability may lead to mutations that aid in escaping or resisting death pathways, conducive to developing the metastatic phenotype.[229] Uterine and breast cancer patients may have elevated levels of soluble FasL and Fas, which may aid in neutralizing CTL- and NK cell-induced lysis or even killing Fas-expressing effector cells. Herpes simplex virus-1 infection in vitro of human neonatal neutrophils upregulates Fas and FasL surface expression, leading to apoptosis, exemplifying a theme of pathogenic modulation of apoptotic signaling events to evade or attenuate immune response.[230] FasL is constitutively expressed in the eye, central nervous system, testis, fetal trophoblast, and placenta, where any lymphocyte infiltration could lead to unrestricted, irreversible bystander damage to these tissues.[231] The implications of immune privilege have been applied to tissue transplantation rejection therapies; however, conflicting results as to whether FasL expression reduces likelihood of allograft rejection[232,233] or potentates effector responses[234,235] emphasize that induction of immune privilege by the Fas L system is complex. Fas-FasL interactions are at the core of graft-versus-host disease, where contaminating donor effector T cells in an allograft expand to damage the recipient's tissues. In a mouse model for acute graft-versus-host disease, p53-dependent upregulation of Fas on host stem cells leads to subsequent bone marrow depletion through FasL-mediated apoptosis.[236] With regard to tissue homeostasis, FasL has been reported to enhance RANKL-mediated osteoclastogenesis and differentiation in mouse bone marrow-

derived macrophages.[237] Fas is also implicated in pulmonary inflammatory diseases resulting in fibrosis after acute lung injury.[223]

TRAIL RECEPTOR SYSTEM

TRAIL (TNFSF10) is closely related to FasL and TNF based on sequence homology. Interest in TRAIL was spurred by its ability to selectively induce apoptosis in tumor cells.[238] TRAIL binds five different receptors: TRAILR1 (DR4, TNFRSF10A), TRAILR2 (DR5, TNFRSF10B), TRAILR3 (DcR1, TNFRSF10C), TRAILR4 (DcR2, TNFRSF10D), and osteoprotegerin (OPG, OCIF, TNFRSF11B) (see Fig. 27.3). TRAILR1, 2, and 4 are type I transmembrane proteins and are membrane associated, whereas the more distantly related OPG is a dimeric, soluble decoy receptor. TRAILR3 is a type III transmembrane protein.[239] TRAILR1 and 2 contain highly homologous DD in their cytoplasmic tails that form the death-inducing signaling complex[240,241] responsible for apoptosis. TRAILR3 is anchored to the membrane via a glycophosphatidyl inositol tail, whereas TRAILR4 has a truncated, nonfunctional DD.[219,242,243] The lack of a functional DD indicates TRAILR3 and 4 antagonize TRAIL-induced apoptosis. Alternative splicing is a common feature amongst TRAIL and its receptors, and is believed to regulate programmed cell death.[244,245,246] The genetic organization of the TRAIL receptor loci in the mouse differs from that of the human. The mouse genome has TRAILR2, 3, and 4 homologues, but the TRAILR4 homologue does not bind TRAIL.

TRAIL-TRAILR signaling system induces apoptosis and to a lesser degree NF-κB activation. For example, the apoptotic function of TRAIL system is necessary for regulating the response of memory CD8+ T cells to rechallenge with antigen. In the absence of CD4 T-cell help, CD8 T cells undergo apoptosis mediated by TRAIL.[247,248] In much the same way as Fas, TRAILR death domains are thought to recruit FADD and initiator caspases 8 and/or 10 into the DISC to activate downstream effector caspases in apoptosis. Caspase 8 contribution to this process is well defined, but caspase 10 involvement was less so until recent studies in patients with autoimmune lymphoproliferative syndrome II and some carcinoma cell lines found that mutant caspase 10 ablates TRAIL-induced apoptosis.[242] In some cell types, the extrinsic mitochondrial pathway is important in amplifying DISC signals in TRAIL-induced apoptosis, which is analogous to mechanisms for Fas-FasL signaling.[249] TRAIL is being intensely pursued as an anticancer therapeutic and has proved particularly potent in combination with chemotherapy, because TRAILR2 expression can be increased in response to deoxyribonucleic acid damage.[250]

TRAIL seems most involved in regulating immune homeostasis and immune surveillance, or clearing virally infected and cancerous cells. IFNγ treatment of tumor cells increases sensitization to TRAIL-induced death. This has been observed in melanoma and ovarian carcinoma cells,[242] and is analogous to Fas-FasL apoptotic signaling in the human colorectal carcinoma line, HT-29. IFNα possesses

powerful antiviral and antitumor properties as well, and have been shown to upregulate TRAIL via JAK-STAT pathways in stimulated human multiple myeloma in vitro. TRAIL is also upregulated on NK cells in response to IFNγ, enhancing antitumor effector function.[251]

TL1A-DR3 SYSTEM

The TL1A-DR3 system provides essential signals that promote the expansion of effector T cells at the sites of inflammation. 139 However, the broad tissue expression profile of DR3 indicates its involvement is a range of cellular responses beyond T cells. TL1A (TNFSF15) is a paralog of FasL, LIGHT, and LTβ, and its receptor, DR3, is a homolog of TNFR1. TL1A also binds decoy receptor-3, potentially limiting any TL1A signaling mediated by DR3. Gene deletion experiments of TL1A or DR3 in mice revealed poor T-cell responses, but no overt developmental abnormalities.[137,252,253,254] Specific mutations in the ligand-binding domain of DR3 are linked to RA and interestingly appear to parallel other TNFRSF members and associated autoimmune pathologies.[255]

RANK LIGAND, RANK, AND OSTEOPROTEGERIN SYSTEM

OPG (TNFRSF11B) was initially identified as key mechanism regulating bone density[256] (see Fig. 27.3). As a soluble receptor, OPG is a regulator of the RANK (receptor activator of NF-κB, TNFRSF11A) and RANK ligand (also known as TRANCE) signaling pathway involved in the differentiation of bone-resorptive osteoclasts from hematopoietic progenitors (osteoclastogenesis).[257,258,259] However, this system is also crucial for development of mammary glands,[260] lymph nodes, and DC-T-cell interactions.[261,262] Bone remodeling needed to sustain skeletal integrity and calcium homeostasis is regulated by a dynamic equilibrium between osteoblasts (bone forming) and osteoclasts (bone resorbing), which is regulated by RANKL-RANK-OPG system. Dysregulation of the RANKL-RANK-OPG system has consequences in inflammatory, osteologic, and cancer pathophysiology, such as bone loss in inflammatory autoimmune diseases, preferential breast and prostate tumor metastasis to the bone, and gender bias in osteoporosis.

OPG functions as a decoy receptor that binds RANKL with high affinity, and also binds TRAIL, albeit at low affinity in comparison to RANKL.[263,264] Despite such a weak binding, in vitro studies show that OPG can block TRAIL-mediated apoptosis, which suggests the OPG-TRAIL system contributes to osteoclastogenesis. RANK interacts with TRAF2, 5, and 6. The membrane-proximal TRAF site is highly specific for TRAF6, and several studies have shown TRAF6 to be essential in RANKL-RANK signaling in osteoclastogenesis and lymph node genesis.[265,266] Several “osteotropic” factors have been reported to regulate RANKL and OPG expression in osteoblast and osteoclast lineages, such as transforming growth factor β, IL-1, TNF, estrogen, prostaglandin E2, glucocorticoids, and vitamin D3.[265,267] In a few cases, OPG and RANKL are differentially modulated by the same factor; for example, parathyroid-related protein increases RANKL while decreasing OPG mRNA expression in osteoclast-like odontoclasts.[268] The components in the RANK signaling triad are widely expressed. RANK is upregulated on CD40L-stimulated maturing DCs, while RANKL is upregulated during T-cell activation and is constitutively expressed in some tumors and mammary gland epithelial cells.

RANK-/- and TRAF6-/- mice lack NF-κB activation in osteoclasts and exhibit abnormally high bone density (osteopetrosis).[269,270] Osteoblasts and stromal cells stimulate differentiation via direct cell contact with osteoclast precursors. Macrophage colony stimulating factor cooperates with RANKL-expressing osteoblasts to transmit positive signals through c-Fms

and RANK expressed on precursors promoting their differentiation,[259] whereas OPG negatively regulates by competitively binding RANKL, thus ablating all signals through RANK. RANK-/- and RANKL-/- mice phenocopy each other, displaying severe osteopetrosis due to the lack of osteoclasts.[271] Complementing the observations in RANKL and RANK deficiencies, OPG blocks osteoclast differentiation in a dose-dependent manner. OPG overexpression in transgenic mice causes acute osteopetrosis, while OPG-deficient mice suffer from osteoporosis due to excessive osteoclastogenesis. In humans, OPG and RANK mutations are linked to juvenile Paget disease, familial expansile osteolysis, and other osteolytic disorders.[258,265]

The RANKL-RANK system is proving to be an important enhancer of cell-mediated immune responses by promoting DC survival and naïve T-cell proliferation. RANKL stimulates DCs to produce proinflammatory cytokines like IL-12.[272,273] The RANK pathway may serve to bolster CD40-CD40L interaction between DC and T cells after T-cell receptor stimulation, or prolong survival of activated DCs to ensure the establishment of T-cell memory. For instance, in CD40L-/- mice, RANKL-RANK costimulation is protective against *Leishmania* infection by inducing IL-12 secretion and consequent T_H1 immune response.[274]

Bone loss in autoimmune and infectious models of inflammatory disease is mediated through slightly different mechanisms. In recent studies, activated, CD4+RANKL+ T cells have been shown to support osteoclastogenesis and mediate bone loss in humanized mouse models of *Actinobacillus*-specific periodontal disease, suggesting a role for T_H1 immunity in inflammatory bone destruction.[275,276] In patients with human immunodeficiency virus, the viron envelop gp120 induces disease-related osteoporosis via induction of RANKL on CD4+ T cells and augmentation of osteoclastogenesis.[277]

In mouse models of tumor metastasis, high OPG and RANK levels are risk factors for metastasis to the bone and subsequent osteolysis, especially in breast tumors.[278] OPG from human bone marrow stroma can protect prostate tumors from TRAIL-mediated apoptosis,[279] and treatment with anti-TRAIL neutralizing antibodies reduces osteoclastogenesis in mixed lymphocyte-bone marrow cultures from patients with multiple myeloma, presumably by interfering with OPG-TRAIL complexes formed by activated T cells expressing TRAIL, OPG, and RANKL.[280] The

RANKL-RANK-OPG system has also been implicated in diabetes-associated osteopenia, as serum levels of OPG are higher in patients with type I and II diabetes.[281] Interestingly, these patients are at greater risk for developing atherosclerotic plaques, which is perhaps reflected in the phenotype of OPG-deficient mice. These mice suffer from arterial calcification,[282] presumably from unrestricted bone resorption and calcium release. Although elevated endogenous OPG levels are considered a risk factor in certain disease manifestations, the complexity of the cytokine networks that regulate RANKL-RANK-OPG interactions and vice versa render the clinical data difficult to interpret.

TABLE 27.8 Human Genetic Diseases Associated with the Tumor Necrosis Factor

Superfamily

SYSTEM	Disease	Mutation
TNFR1	Familial periodic fever	Mutation in first cysteine-rich domain
CD40 Ligand	Hyper-IgM syndrome	Multiple mutations affecting receptor binding and processing
EDA	X-linked hypohidrotic ectodermal dysplasia	Multiple mutations affecting receptor binding, trimerization, and secretion
RANK	Familial expansile osteolysis	Mutation in the signal peptide
Fas	Autoimmune lymphoproliferative syndrome	Multiple mutations/deletion in Fas

CD, cluster of differentiation; EDA, ectodermal dysplasin A; Ig, immunoglobulin; TNFR, tumor necrosis factor receptor.

TABLE 27.9 Tumor Necrosis Factor Superfamily Therapeutics

TNFRSF Member	Drug	Therapeutic Designation	Indication	Status
TNF	Chimeric Ab	Remicade (infliximab)	RA, Ps, IBD	Approved
TNF LTα	TNFR-Fc	Enbrel (etanercept)	RA, Ps	Approved
TNF	Hu Fab-PEG	Cimzia (certolizumab)	RA, Ps, IBD	Approved
TNF	Hu mAb	Humira (adalimumab)	RA, Ps, IBD	Approved
TNF	Hu mAb	Simponi (golimumab)	RA, Ps, IBD	Approved

BAFF	Hu mAb	Benlysta (belimumab)	Lupus	Approved
CD40 L	chimeric mAb	ruplizumab	Lupus	Stopped
″	Hu Fab-PEG	CDP7657	Lupus	In trials
RANK L	Hu mAb	Prolia (denosumab)	Osteoporosis	Approved
″	Hu mAb	Xgeva (denosumab)	Bone metastasis	Approved
TRAIL	Recom cytokine		Cancer	In trials
TRAILR1	Hu mAb	mapatumumab	Cancer	In trials
TRAILR2	Hu mAb	conatumumab	Cancer	In trials
LTβR	LTβR-Fc	baminercept	Autoimmune diseases	In trials
LTα	Hu mAb	MLTA3698A	Autoimmune disease	In trials
OX40	chimeric mAb	9B12	Cancer	In trials
TWEAK	chimeric mAb	BIIB 023	Lupus nephritis	In trials

Fab-PEG, fragment of antibody polyethyleneglycoylated; IBD, inflammatory bowel diseases; LT, lymphotoxin; Ps, psoriasis; RA, rheumatoid arthritis; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TNFRSF, tumor necrosis factor receptor superfamily.

TUMOR NECROSIS FACTOR SUPERFAMILY IN THE THERAPY OF HUMAN DISEASE

Tumor Necrosis Factor Inhibitors

The TNFSF is associated with a wide range of human diseases, evidenced by genetic deficiencies or mutations in individual components of the TNFSF and in the therapeutic efficacy of TNF inhibitors in autoimmune diseases (Table 27.8). Although cancer therapy was the original motivation in developing TNF, the success of TNF inhibitors in limiting symptoms in several autoimmune diseases provides significant motivation to explore therapeutics targeting other members of the TNFSF, currently an active area of research. Research into the function of TNFSF in human diseases is providing strong rationale for testing both inhibitors and agonists of these cytokines (Table 27.9).

TNF inhibitors are specific antibodies or soluble receptors and are classified as biologics (protein-based drugs). These biologic-based drugs provide specificity and desirable pharmacodynamic properties (Fig. 27.8). Both antibody- and receptor-based drugs function as competitive antagonists, sterically hindering the binding of ligand to receptor. The approved antibody-based drugs include a partially humanized mouse monoclonal antibody to human TNF (infliximab), two fully human antibodies (adalimumab and golimumab), and a monovalent Fab fragment of a TNF antibody (certolizumab pegol). Certolizumab is derivatized with polyethylene glycol to enhance half-life. To create a soluble “decoy” receptor, the extracellular domain of TNFR-2 was genetically linked to the Fc region of human IgG1, forming a bivalent molecule (etanercept) that has higher avidity for TNF than naturally occurring soluble receptors. Etanercept binds both TNF and LTα, whereas anti-TNF antibodies are specific for TNF and do not cross-react with LTα.

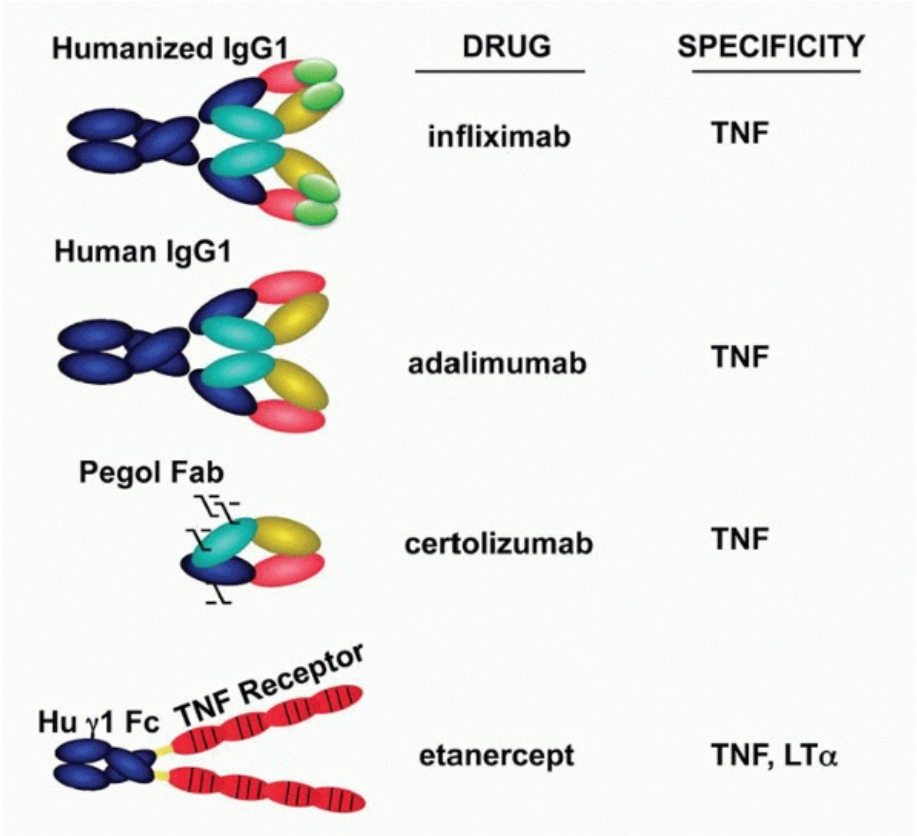

FIG. 27.8. Tumor Necrosis Factor (TNF) Inhibitors. The diagram depicts the structural features of the antibody- and receptor-based drugs targeted at TNF that are approved

for use in patients with autoimmune diseases. Monoclonal antibody specific for human TNF derived from mice and partially humanized (infliximab), a fully human antibody (adalimumab) containing both heavy and light immunoglobulin chains, and an antibody-binding fragment (Fab) of a human anti-TNF antibody (certolizumab). Certolizumab is monovalent and derivatized with polyethyleneglycol to extend half-life in vivo. TNF decoy receptor (etanercept) created as a genetic fusion protein of the ectodomain of TNF receptor 2 with the Fc region of human immunoglobulin G1 forms a disulfide-linked dimer. Both immunoglobulin and receptor-based inhibitors are approved for use in treating autoimmune diseases: rheumatoid arthritis, psoriasis, and inflammatory bowel diseases.

Bivalent antibodies directed to TNFRs can function as agonists, mimicking the ligand but specific for a single receptor, a feature that may distinguish an antibody from ligand such as TRAIL, which has multiple receptors. Moreover, antibodies are inherently more stable than the native ligand, providing better pharmacodynamics (serum half-life and bioavailability). Agonist receptor antibodies (eg, anti-TRAILR, anti-Ox40) are in preclinical studies and clinical trials.

Impact of Tumor Necrosis Factor Blockade in Inflammatory Diseases

TNF inhibitors are approved for use in the treatment of RA, psoriatic arthritis, ankylosing spondylitis, juvenile rheumatoid arthritis, Crohn disease, and ulcerative colitis. RA is a chronic autoimmune joint disease that occurs in genetically predisposed individuals. Treatment of RA is usually initiated with disease-modifying antirheumatic drugs such as methotrexate or corticosteroids to improve symptoms and reduce joint damage. The American College of Rheumatology uses clinical and laboratory measures to assess improvement in response to therapy in RA. A patient with a 70% or greater improvement according to these parameters is designated as having an ACR-70 response. TNF inhibitors in combination with methotrexate (the most widely used disease-modifying antirheumatic drugs) produce ACR-70 responses in 33% to 40% of patients with early RA and in 10% to 27% in established RA. In comparison to patients receiving methotrexate alone, the ACR-70 responses occurred in 19% to 21% of early RA patients and fewer than 5% of patients with established disease.[283] In RA, joint damage begins early in the disease course as articular erosions when visualized by radiographic techniques in 40% of patients in the first year of disease and 90% by the second year.[284] TNF inhibitors slow radiographic progression in RA.[285] Interestingly, in a randomized controlled trial of 428 patients with active RA, the combination of infliximab and methotrexate reduced the radiographic progression.[286]

The inflammatory bowel diseases, ulcerative colitis (limited to colon and bowel), and Crohn disease (entire intestine) are chronic inflammatory conditions where TNF is disproportionately expressed when the disease is active. Monocytes from patients with inflammatory bowel disease produce significantly higher levels of TNF after stimulation by lipopolysaccharide.[287] The formation of fistulating inflammatory granulomas is a pathologic feature of Crohn disease. Clinical trials demonstrated an initial response in 59% to 69% of patients, and a dose-dependent clinical remission that ranged between 28% to 38% in nonfistulating Crohn disease and 64% to 97% of patients with complete fistula disease, when categorized by

location.[288,289] In ulcerative colitis, controlled trials (800 patients with active ulcerative colitis) showed an initial response in 8 weeks with 65% response in patients treated with anti-TNF (infliximab) versus 35% in the placebo group, with remission achieved in 35% and 15% in patients treated with infliximab and placebo, respectively.[290] Anti-TNF (adalimumab) was efficacious in controlled trials in patients with moderate to severe Crohn disease.[288] By contrast, soluble TNFR2-Fc (etanercept) at doses known to be effective in RA failed to produce benefit in a controlled trial for Crohn disease.[291]

Erythrosquamous lesions containing infiltrating leukocytes and epidermal hypertrophy characterize psoriasis, a chronic inflammatory skin disease. Skin lesions of psoriatic patients contain elevated levels of TNF, and following treatment with TNF inhibitors, serum and skin levels of TNF decrease with remarkable clinical response.[292,293] A significant subset of patients with psoriasis develops inflammatory, erosive arthritis.[294] In clinical trials, both etanercept and infliximab significantly inhibited disease activity and improved the quality of life in patients with psoriatic arthritis.[295,296,297,298,299]

Serious Adverse Effects

TNF inhibitors are inherently safe because of their specificity. However, side effects occur with the use of TNF inhibitors and include lupus-like syndrome, aplastic anemia, hepatotoxicity, interstitial lung disease, optic neuritis, and exacerbations of quiescent multiple sclerosis. TNF inhibition is associated with diminished host defense, perhaps expected based on the role of TNF in innate and adaptive immune responses. The rate of serious skin and soft-tissue infections is higher in treated patients, and there is an increase risk of intracellular bacterial infections, primary tuberculosis and reactivation of latent

Mycobacterium tuberculosis among patients treated with TNF inhibitors.[300,301] Risk due to infection can be minimized and controlled (eg, a tuberculosis skin test prior to treatment in patients that have not been previously vaccinated for *Mycobacterium*). The contribution of anti-TNF therapy to the increase the risk of cancer is still unclear. A systematic review of randomized clinical trials using infliximab and adalimumab found a dose-related increase of malignancies in patients with RA patients.[302] Some of the increase risk of malignancy (ie, lymphoma) is difficult to dissect due to the increased association of lymphoma with RA.[301] TNF has been implicated in the pathogenesis of cardiac dysfunction, in part due to the negative ionotropic activities in vitro and in vivo; however, TNF blockade significantly increased the incidence of cardiac death. This observation led to the recommendation that TNF inhibitors be discontinued in patients with cardiac dysfunction.[303,304,305] Limiting adverse effects have been observed with biologics targeting other TNFSF members. Although interference with CD40L ameliorated autoimmune disease and allograft rejection in animal models,[306,307,308,309,310] anti-CD40L had an unacceptable impact on platelet function in transplantation and autoimmune diseases. The use of monovalent Fab of anti-CD40L may avoid these problems.[311,312,313] Another TNFRSF member, OPG, was dropped out of clinical investigation because the recombinant protein induced antibody responses that limited its therapeutic effects. Thus, dose- or mechanism-limiting toxicities can impact physiology in unanticipated ways, limiting successful clinical outcomes.

Anti-RANK Ligand

The central function of RANK-RANKL system in osteoclastogenesis has led to the development of anti-RANKL antibodies to treat bone-resorbing conditions. Human anti-RANKL monoclonal antibody (denosumab) to disrupt RANKL-RANK signaling has been approved for the treatment of osteoporosis in postmenopausal women and for treatment of skeletal changes from metastasis of solid tumors. Anti-RANKL therapy reduces osteoclastic bone resorption, thereby increasing bone mineral density at both the lumbar spine and the hip. Treatment with anti-RANKL for 36 months decreased the risk of vertebral, nonvertebral, and hip fractures.[314] There is no apparent increased risk of cancer or serious infection associated with anti-RANKL therapy in subjects with osteoporosis, although longer study periods are needed.

Treatment of patients with prostate cancer with androgen deprivation therapy and patients with breast cancer with aromatase inhibitors leads to increased bone loss and increased risk of fracture. Anti-RANKL is prescribed to increase bone mass both of these patient populations.[315,316] In a double-blind phase III clinical trial, women with nonmetastatic breast cancer who received anti-RANKL therapy had an increased lumbar spine bone mineral density of 7.6% after 24 months of treatment compared to placebo.[316] Similarly, patients with prostate cancer on androgen deprivation therapy that were treated with anti-RANKL in a phase III clinical trial had increased bone mineral density at all measured sites (hip, femoral neck, radius, and whole body) compared to placebo.[315] Anti-RANKL reduced the overall risk of bone fracture in the prostate cancer trial, while the phase III trial of patients with breast cancer treated with aromatase inhibitors was not designed to evaluate risk of fracture.[315,316]

In addition to patients with prostate and patients with breast cancer undergoing hormone deprivation therapy, anti-RANKL aids in prevention of skeletal-related events in patients with bone metastasis from solid tumors.[317,318,319] Three independent phase III clinical trials compared anti-RANKL with zoledronic acid, a drug prescribed to alleviate bone pain and reduce fracture risk in cancer patients with bone metastasis.[317,318,319] Anti-RANKL therapy delayed the time to the first skeletal changes in patients with metastatic breast cancer and metastatic, castration-resistant prostate cancer.[317,319] In a third phase III trial of patients with solid tumors (excluding breast and prostate) or multiple myeloma, anti-RANKL was equivalent to zoledronic acid in reducing fracture risk.[318]

Anti-BAFF

Systemic lupus erythematous is characterized by hyperreactive B cells, production of antideoxyribonucleic acid antibodies, and overproduction of BAFF.[320,321] The anti-BAFF antibody (belimumab) was approved in 2011 for the treatment of systemic lupus erythematosus, becoming the first new drug to gain approval for the condition in 56 years. In a phase III clinical trial, anti-BAFF decreased the rate of disease flares and increased the time between disease flares compared with placebo.[322] Anti-BAFF treatment increased systemic lupus erythematosus responder index (a reduction of SELENA-SLEDAI score of four or more points) compared to placebo.[322]

Tumor Necrosis Factor Superfamily in Cancer Therapy

Tumor angiogenesis, the formation of vessels to support cancer growth, is a process essential for tumor growth. The ability of TNF to induce apoptosis of tumor-associated endothelial cells can result in the impairment of the tumor vasculature leading to necrosis of the tumor. This mechanism involves perturbation of cell-cell adhesive junctions and inhibition of αvβ3-integrin signaling in tumor-associated vessels.[323] However, clinical phase I and II studies in cancer patients receiving recombinant TNF reported "septic shock-like syndrome," a dose-limiting toxicity in quantities 10 times lower than the calculated antitumor dose extrapolated from animal studies.[324,325] Animal studies also revealed the strong hemodynamic effects of TNF in the mediation of septic shock.[326] Although there are anecdotic reports of tumor regression following TNF administration,[327] due to the induction of vasoplegia, TNF use as an antitumor agent has been limited to treatment of locally advanced tumors by isolated limb perfusion, which limits systemic toxicity of TNF.[328]

A number of studies are in progress to determine whether TRAIL and its death receptors can be used in treatment of

cancer.[329] In phase 1/2 studies, TRAIL and agonist mAb to TRAILR1 or R2 showed no dose-limiting toxicities.[330,331,332,333]

Early clinical trials are in progress for other members of the TNFSF. For example, antibodies to LTα and LTβR-Fc decoy are in clinical trials for suppressing inflammation in autoimmune diseases. Agonist antibodies to OX40 appear promising for induction of tumor immunity.[334] Redirecting T cells using a retroviral-based vector incorporating 41BB can eliminate leukemic cells.[335] It is not surprising that several other TNFSF systems are being targeted to modify inflammatory, infectious, and malignant diseases.

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REFERENCES

1. Hymowitz SG, Compaan DM, Yan M, et al. The crystal structures of EDA-A1 and EDA-A2: splice variants with distinct receptor specificity. *Structure*. 2003;11:1513-1520.

2. Gordon NC, Pan B, Hymowitz SG, et al. BAFF/BLyS receptor 3 comprises a minimal TNF receptor-like module that encodes a highly focused ligand-binding site. *Biochemistry*. 2003;42:5977-5983.

3. Cha SS, Kim MS, Choi YH, et al. 2.8 A resolution crystal structure of human TRAIL, a cytokine with selective antitumor activity. *Immunity.* 1999;11:253-261.

4. Jones EY, Stuart DI, Walker NP. Crystal structure of TNF. *Immunol Ser.* 1992;56:93-127.

5. Eck MJ, Ultsch M, Rinderknecht E, de Vos AM, Sprang SR. The structure of human lymphotoxin (tumor necrosis factor-b) at 1.9-A resolution. *J Biol Chem.* 1992;267:2119-2122.

6. Browning JL, Ngam-ek A, Lawton P, et al. Lymphotoxin b, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. *Cell*. 1993;72:847-856.

7. Roschke V, Sosnovtseva S, Ward CD, et al. BLyS and APRIL form biologically active heterotrimers that are expressed in patients with systemic immune-based rheumatic diseases. *J Immunol*. 2002;169:4314-4321.

8. Kishore U, Gaboriaud C, Waters P, et al. C1q and tumor necrosis factor superfamily: modularity and versatility. *Trends Immunol*. 2004;25:551-561.

9. Pradet-Balade B, Medema JP, Lopez-Fraga M, et al. An endogenous hybrid mRNA encodes TWE-PRIL, a functional cell surface TWEAK-APRIL fusion protein. *EMBO J*. 2002;21: 5711-5720.

10. Yan M, Wang LC, Hymowitz SG, et al. Two-amino acid molecular switch in an epithelial morphogen that regulates binding to two distinct receptors. *Science*. 2000;523-527.

11. Granger SW, Butrovich KD, Houshmand P, Edwards WR, Ware CF. Genomic characterization of LIGHT reveals linkage to an immune response locus on chromosome 19p13.3 and distinct isoforms generated by alternate splicing or proteolysis. *J Immunol*. 2001;167:5122-5128.

12. Muller U, Jongeneel CV, Nedospasov SA, Lindahl KF, Steinmetz M. Tumour necrosis factor and lymphotoxin genes map close to H-2D in the mouse major histocompatibility complex. *Nature*. 1987;325:265-267.

13. Flajnik MF, Kasahara M. Comparative genomics of the MHC: glimpses into the evolution of the adaptive immune system. *Immunity*. 2001;15:351-362.

14. Igaki T, Kanda H, Yamamoto-Goto Y, et al. Eiger, a TNF superfamily ligand that triggers the Drosophila JNK pathway. *EMBO J*. 2002;21: 3009-3018.

15. Kauppila S, Maaty WS, Chen P, et al. Eiger and its receptor, Wengen, comprise a TNF-like system in Drosophila. *Oncogene*. 2003;22:4860-4867.

16. Moreno E, Yan M, Basler K. Evolution of TNF signaling mechanisms: JNK-dependent

apoptosis triggered by Eiger, the Drosophila homolog of the TNF superfamily. *Curr Biol* . 2002;12:1263-1268.

17. Banner DW, D'Arcy A, Janes W, et al. Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell*. 1993;73:431-445.

18. Carfi A, Willis SH, Whitbeck JC, et al. Herpes simplex virus glycoprotein D bound to the human receptor HveA. *Molecular Cell* . 2001;8:169-179.

19. Hymowitz SG, Patel DR, Wallweber HJ, et al. Structures of APRIL-receptor complexes: like BCMA, TACI employs only a single cysteine-rich domain for high affinity ligand binding. *J Biol Chem*. 2005;280: 7218-7227.

20. Bossen C, Ingold K, Tardivel A, et al. Interactions of tumor necrosis factor (TNF) and TNF receptor family members in the mouse and human. *J Biol Chem*. 2006;281:13964-13971.

21. Engelmann H, Holtmann H, Brakebusch C, et al. Antibodies to a soluble form of a tumor necrosis factor (TNF) receptor have TNF-like activity. *J Biol Chem.* 1990;265:14497-14504.

22. Li B, Russell SJ, Compaan DM, et al. Activation of the proapoptotic death receptor DR5 by oligomeric peptide and antibody agonists. *J Mol Biol* . 2006;361:522-536.

23. Holler N, Tardivel A, Kovacsovics-Bankowski M, et al. Two adjacent trimeric Fas ligands are required for Fas signaling and formation of a death-inducing signaling complex. *Mol Cell Biol* . 2003;23:1428-1440.

24. Liu Y, Hong X, Kappler J, et al. Ligand-receptor binding revealed by the TNF family member TALL-1. *Nature*. 2003;423:49-56.

25. Boldin MP, Mett IL, Varfolomeev EE, et al. Self-association of the “Death domains” of the p55 tumor necrosis factor (TNF) receptor and Fas/APO1 prompts signaling for TNF and Fas/APO1 effects. *J Biol Chem.* 1995;270:387-391.

26. Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, Lenardo MJ. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science*. 2000;288:2351-2354.

27. Lobito AA, Kimberley FC, Muppidi JR, et al. Abnormal disulfidelinked oligomerization results in ER retention and altered signaling by TNFR1 mutants in TNFR1-associated periodic fever syndrome (TRAPS). *Blood*. 2006;108:1320-1327.

28. He XL, Garcia KC. Structure of nerve growth factor complexed with the shared

neurotrophin receptor p75. *Science*. 2004;304:870-875.

29. Shao Z, Browning JL, Lee X, et al. TAJ/TROY, an orphan TNF receptor family member, binds Nogo-66 receptor 1 and regulates axonal regeneration. *Neuron*. 2005;45:353-359.

30. Park JB, Yiu G, Kaneko S, et al. A TNF receptor family member, TROY, is a coreceptor with Nogo receptor in mediating the inhibitory activity of myelin inhibitors. *Neuron*. 2005;45:345-351.

31. Sedy JR, Gavrieli M, Potter KG, et al. B and T lymphocyte attenuator regulates T cell activation through interaction with herpesvirus entry mediator. *Nat Immunol*. 2005;6:90-98.

32. Cai G, Anumanthan A, Brown JA, Greenfield EA, Zhu B, Freeman GJ. CD160 inhibits activation of human CD4+ T cells through interaction with herpesvirus entry mediator. *Nat Immunol*. 2008;9:176-185.

33. Montgomery RI, Warner MS, Lum B, Spear PG. Herpes simplex virus 1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell*. 1996;87:427-436.

34. Compaan DM, Gonzalez LC, Tom I, Loyet KM, Eaton D, Hymowitz SG. Attenuating lymphocyte activity: the crystal structure of the BTLA-HVEM complex. *J Biol Chem*. 2005;280:39553-39561.

35. Cheung TC, Humphreys IR, Potter KG, et al. Evolutionarily divergent herpesviruses modulate T cell activation by targeting the herpesvirus entry mediator cosignaling pathway. *Proc Natl Acad Sci U S A* . 2005;102:13218-13223.

36. Tang W, Lu Y, Tian QY, et al. The growth factor progranulin binds to TNF receptors and is therapeutic against inflammatory arthritis in mice. *Science*. 2011;332:478-484.

37. Lobito AA, Ramani SR, Tom I, et al. Murine insulin growth factor-like (IGFL) and human IGFL1 proteins are induced in inflammatory skin conditions and bind to a novel tumor necrosis factor receptor family member, IGFLR1. *J Biol Chem*. 2011;286:18969-18981.

38. Nikolaev A, McLaughlin T, O'Leary DD, Tessier-Lavigne M. APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. *Nature*. 2009;457:981-989.

39. Ponomarev SY, Audie J. Computational prediction and analysis of the DR6-NAPP interaction. *Proteins*. 2011;79:1376-1395.

40. Mi S, Lee X, Hu Y, et al. Death receptor 6 negatively regulates oligodendrocyte survival, maturation and myelination. *Nat Med*. 2011;17:816-821.

41. Schmidt CS, Zhao J, Chain J, et al. Resistance to myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis by death receptor 6-deficient mice. *J Immunol* . 2005;175:2286-2292.

42. Benedict CA. Viruses and the TNF-related cytokines, an evolving battle. *Cytokine Growth Factor Rev*. 2003;14:349-357.

43. Smith CA, Davis T, Anderson D, et al. A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. *Science*. 1990;248:1019-1023.

44. Upton C, Macen J, Schreiber M, McFadden G. Myxoma virus expresses a secreted protein with homology to the tumor necrosis factor receptor gene family that contributes to viral virulence. *Virology*. 1991;184: 370-382.

45. Rahman MM, McFadden G. Modulation of tumor necrosis factor by microbial pathogens. *PLoS Pathog*. 2006;2:e4.

46. Massung RF, Esposito JJ, Liu LI, et al. Potential virulence determinants in terminal regions of variola smallpox virus genome [see comments]. *Nature*. 1993;366:748-751.

47. Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F, Kollias G. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU- rich elements: implications for joint and gut-associated immunopathologies. *Immunity*. 1999;10:387-398.

48. Kontoyiannis D, Boulougouris G, Manoloukos M, et al. Genetic dissection of the cellular pathways and signaling mechanisms in modeled tumor necrosis factor-induced Crohn's-like inflammatory bowel disease. *J Exp Med*. 2002;196:1563-1574.

49. Boatright KM, Salvesen GS. Mechanisms of caspase activation. *Curr Opin Cell Biol* . 2003;15:725-731.

50. Green DR, Galluzzi L, Kroemer G. Mitochondria and the autophagy-inflammation-cell death axis in organismal aging. *Science*. 2011;333: 1109-1112.

51. Scott FL, Stec B, Pop C, et al. The Fas-FADD death domain complex structure unravels signalling by receptor clustering. *Nature*. 2009;457: 1019-1022.

52. Sun SC. Non-canonical NF-kappaB signaling pathway. *Cell Res*. 2011; 21:71-85.

53. Hacker H, Tseng PH, Karin M. Expanding TRAF function: TRAF3 as a tri-faced immune regulator. *Nat Rev Immunol* . 2011;11:457-468.

54. Zheng C, Kabaleeswaran V, Wang Y, Cheng G, Wu H. Crystal structures of the TRAF2: cIAP2 and the TRAF1: TRAF2: cIAP2 complexes: affinity, specificity, and

regulation. *Mol Cell*. 2010;38:101-113.

55. Walczak H. TNF and ubiquitin at the crossroads of gene activation, cell death, inflammation, and cancer. *Immunol Rev*. 2011;244:9-28.

56. Silke J. The regulation of TNF signalling: what a tangled web we weave. *Curr Opin Immunol*. 2011;23:620-626.

57. Belgnaoui SM, Paz S, Hiscott J. Orchestrating the interferon antiviral response through the mitochondrial antiviral signaling (MAVS) adapter. *Curr Opin Immunol* . 2011;23:564-572.

58. Ely KR, Li C. Structurally adaptive hot spots at a protein interaction interface on TRAF3. *J Mol Recognit*. 2002;15:286-290.

59. Oganesyan G, Saha SK, Guo B, et al. Critical role of TRAF3 in the Tolllike receptor-dependent and -independent antiviral response. *Nature*. 2006;439:208-211.

60. Ghosh S, May M, Kopp E. NF-kB and REL proteins: evolutionarily conserved mediators of immune responses. *Ann Rev Immunol.* 1998;16: 225-260.

61. Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol*. 2000;18:621-663.

62. Schrofelbauer B, Hoffmann A. How do pleiotropic kinase hubs mediate specific signaling by TNFR superfamily members? *Immunol Rev*. 2011;244:29-43.

63. Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. *Cell*. 2002;109(Supp l):S81-S96.

64. Sanjo H, Zajonc DM, Braden R, Norris PS, Ware CF. Allosteric regulation of the ubiquitin:NIK and ubiquitin:TRAF3 E3 ligases by the lymphotoxin-β receptor. *J Biol Chem*. 2010;285:17148-17155.

65. Pomerantz JL, Baltimore D. Two pathways to NF-kappaB. *Mol Cell*. 2002;10:693-695.

66. Dejardin E, Droin NM, Delhase M, et al. The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways. *Immunity*. 2002;17:525-535.

67. Ramakrishnan P, Wang W, Wallach D. Receptor-specific signaling for both the alternative and the canonical NF-kappaB activation pathways by NF-kappaB-inducing kinase. *Immunity*. 2004;21:477-489.

68. Staudt LM. Oncogenic activation of NF-kappaB. *Cold Spring Harb Perspect Biol* . 2010;2:a000109.

69. Kollias G. TNF pathophysiology in murine models of chronic inflammation and autoimmunity. *Semin Arthritis Rheum*. 2005;34:3-6.

70. Ware CF. Network communications: lymphotoxins, LIGHT, and TNF. *Annu Rev Immunol*. 2005;23:787-819.

71. McCarthy DD, Summers-Deluca L, Vu F, Chiu S, Gao Y, Gommerman JL. The lymphotoxin pathway: beyond lymph node development. *Immunol Res*. 2006;35:41-54.

72. Sedy JR, Spear PG, Ware CF. Cross-regulation between herpesviruses and the TNF superfamily members. *Nat Rev Immunol* . 2008;8: 861-873.

73. Kulbe H, Thompson R, Wilson JL, et al. The inflammatory cytokine tumor necrosis factor-alpha generates an autocrine tumor-promoting network in epithelial ovarian cancer cells. *Cancer Res*. 2007;67:585-592.

74. Balkwill F. TNF-alpha in promotion and progression of cancer. *Cancer Metastasis Rev*. 2006;25:409-416.

75. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature*. 2006;441:431-436.

76. De Togni P, Goellner J, Ruddle NH, et al. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science*. 1994;264:703-706.

77. Cupedo T, Mebius RE. Cellular interactions in lymph node development. *J Immunol* . 2005;174:21-25.

78. Georgopoulos K, Bigby M, Wang J, et al. The Ikaros gene is required for the development of all lymphoid lineages. *Cell*. 1994;79:143-156.

79. Yokota Y, Mansouri A, Mori S, et al. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature*. 1999;397:702-706.

80. Sun Z, Unutmaz D, Zou YR, et al. Requirement for RORgamma in thymocyte survival and lymphoid organ development. *Science*. 2000;288:2369-2373.

81. Kurebayashi S, Ueda E, Sakaue M, et al. Retinoid-related orphan receptor gamma (RORgamma) is essential for lymphoid organogenesis and controls apoptosis during thymopoiesis. *Proc Natl Acad Sci U S A* . 2000;97:10132-10137.

82. Eberl G, Marmon S, Sunshine MJ, Rennert PD, Choi Y, Littman DR. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. *Nat Immunol*. 2004;5:64-73.

83. Rennert PD, Browning JL, Mebius R, Mackay F, Hochman PS. Surface lymphotoxin a/b complex is required for the development of peripheral lymphoid organs. *J Exp Med.* 1996;184:1999-2006.

84. Nishikawa S, Honda K, Vieira P, Yoshida H. Organogenesis of peripheral lymphoid organs. *Immunol Rev*. 2003;195:72-80.

85. Lane PJ, Gaspal FM, Kim MY. Two sides of a cellular coin: CD4(+) CD3- cells regulate memory responses and lymph-node organization. *Nat Rev Immunol*. 2005;5:655-660.

86. Eberl G. Inducible lymphoid tissues in the adult gut: recapitulation of a fetal developmental pathway? *Nat Rev Immunol*. 2005;5:413-420.

87. Mebius RE, Kraal G. Structure and function of the spleen. *Nat Rev Immunol*. 2005;5:606-616.

88. Cyster JG. Lymphoid organ development and cell migration. *Immunol Rev*. 2003;195:5-14.

89. Ngo VN, Korner H, Gunn MD, et al. Lymphotoxin alpha/beta and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. *J Exp Med.* 1999;189:403-412.

90. Luther SA, Bidgol A, Hargreaves DC, et al. Differing activities of homeostatic chemokines CCL19, CCL21, and CXCL12 in lymphocyte and dendritic cell recruitment and lymphoid neogenesis. *J Immunol*. 2002;169:424-433.

91. Ansel KM, Ngo VN, Hyman PL, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature*. 2000;406:309-314.

92. Katakai T, Hara T, Sugai M, Gonda H, Shimizu A. Lymph node fibroblastic reticular cells construct the stromal reticulum via contact with lymphocytes. *J Exp Med*. 2004;200:783-795.

93. Benedict CA, De Trez C, Schneider K, Ha S, Patterson G, Ware CF. Specific remodeling of splenic architecture by cytomegalovirus. *PLoS Pathog*. 2006;2:164-174.

94. Drayton DL, Liao S, Mounzer RH, Ruddle NH. Lymphoid organ development: from ontogeny to neogenesis. *Nat Immunol*. 2006;7:344-353.

95. Kabashima K, Banks TA, Ansel KM, Lu TT, Ware CF, Cyster JG. Intrinsic lymphotoxin-beta receptor requirement for homeostasis of lymphoid tissue dendritic cells. *Immunity*. 2005;22:439-450.

96. Kindler V, Sappino AP, Grau GE, Piguet PF, Vassalli P. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. *Cell*. 1989;56:731-740.

97. Elewaut D, Ware CF. The unconventional role of LTalphabeta in T cell differentiation. *Trends Immunol*. 2007;28(4):169-175.

98. Silva-Santos B, Pennington DJ, Hayday AC. Lymphotoxin-mediated regulation of gammadelta cell differentiation by alphabeta T cell progenitors. *Science*. 2005;307:925-928.

99. Boehm T, Scheu S, Pfeffer K, Bleul CC. Thymic medullary epithelial cell differentiation, thymocyte emigration, and the control of autoimmunity require lympho-epithelial cross talk via LTbetaR. *J Exp Med*. 2003;198:757-769.

100. Elewaut D, Brossay L, Santee SM, et al. Membrane lymphotoxin is required early in ontogeny for development of different subpopulations of NK T cells. *J Immunol*. 2000;165:671-679.

101. Franki AS, Van Beneden K, Dewint P, et al. A unique lymphotoxin α beta-dependent pathway regulates thymic emigration of Vα14 invariant natural killer T cells. *Proc Natl Acad Sci U S A*. 2006;103:9160-9165.

102. Chin RK, Zhu M, Christiansen PA, et al. Lymphotoxin pathway-directed, autoimmune regulator-independent central tolerance to arthritogenic collagen. *J Immunol*. 2006;177:290-297.

103. Keffer J, Probert L, Cazlaris H, et al. Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. *EMBO J.* 1991;10:4025-4031.

104. Shaikh R, Santee S, Granger SW, et al. Constitutive expression of LIGHT on T cells leads to lymphocyte activation, inflammation and tissue destruction. *J Immunol*. 2001;167:6330-6337.

105. Wang J, Chun T, Lo JC, et al. The critical role of LIGHT, a TNF family member, in T cell development. *J Immunol*. 2001;167:5099-5105.

106. Drayton DL, Ying X, Lee J, Lesslauer W, Ruddle NH. Ectopic LT alpha beta directs lymphoid organ neogenesis with concomitant expression of peripheral node addressin and a HEV-restricted sulfotransferase. *J Exp Med*. 2003;197:1153-1163.

107. Beutler B, Cerami A. The biology of cachectin/TNF—a primary mediator of the host response. *Annu Rev Immunol.* 1989;7:625-655.

108. Pfeffer K, Matsuyama T, Kundig TM, et al. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. *Cell*. 1993;73:457-467.

109. Rothe J, Lesslauer W, Lotscher H, et al. Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. *Nature*. 1993;364:798-802.

110. Wallis RS, Broder MS, Wong JY, Hanson MA, Beenhouwer DO. Granulomatous infectious diseases associated with tumor necrosis factor antagonists. *Clin Infect Dis*. 2004;38:1261-1265.

111. Benedict CA, Banks TA, Ware CF. Death and survival: viral regulation of TNF signaling pathways. *Curr Opin Immunol* . 2003;15:59-65.

112. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. *Nat Rev Immunol* . 2002;2:116-126.

113. Egen JG, Kuhns MS, Allison JP. CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nat Immunol* . 2002;3:611-618.

114. Riley JL, June CH. The CD28 family: a T-cell rheostat for therapeutic control of T-cell activation. *Blood*. 2005;105:13-21.

115. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 Family Revisited. *Annu Rev Immunol* . 2005;23:515-548.

116. Watanabe N, Gavrieli M, Sedy JR, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat Immunol* . 2003;4:670-679.

117. Han P, Goularte OD, Rufner K, Wilkinson B, Kaye J. An inhibitory Ig superfamily protein expressed by lymphocytes and APCs is also an early marker of thymocyte positive selection. *J Immunol* . 2004;172:5931-5939.

118. Granger SW, Ware CF. Commentary: turning on LIGHT. *J Clin Investigation.* 2001;108:1741-1742.

119. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* . 2001;104:487-501.

120. Croft M. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? *Nat Rev Immunol* . 2003;3:609-620.

121. Croft M. Costimulation of T cells by OX40, 4-1BB, and CD27. *Cytokine Growth Factor Rev*. 2003;14:265-273.

122. Watts TH. TNF/TNFR family members in costimulation of T cell responses. *Annu Rev Immunol*. 2005;23:23-68.

123. Toubi E, Shoenfeld Y. The role of CD40-CD154 interactions in autoimmunity and the benefit of disrupting this pathway. *Autoimmunity*. 2004;37:457-464.

124. Schneider P. The role of APRIL and BAFF in lymphocyte activation. *Curr Opin Immunol*. 2005;17:282-289.

125. Vonderheide RH, June CH. A translational bridge to cancer immunotherapy: exploiting costimulation and target antigens for active and passive T cell immunotherapy. *Immunol Res*. 2003;27:341-356.

126. Appleman LJ, Boussiotis VA. T cell anergy and costimulation. *Immunol Rev*. 2003;192:161-180.

127. Saito T, Yamasaki S. Negative feedback of T cell activation through inhibitory adapters and costimulatory receptors. *Immunol Rev*. 2003;192:143-160.

128. Subudhi SK, Alegre ML, Fu YX. The balance of immune responses: costimulation verse coinhibition. *J Mol Med*. 2005;83:193-202.

129. Yu P, Lee Y, Liu W, et al. Priming of naive T cells inside tumors leads to eradication of established tumors. *Nat Immunol*. 2004;5:141-149.

130. Hintzen RQ, Lens SM, Lammers K, Kuiper H, Beckmann MP, van Lier RA. Engagement of CD27 with its ligand CD70 provides a second signal for T cell activation. *J Immunol*. 1995;154:2612-2623.

131. Hendriks J, Gravestein LA, Tesselaar K, van Lier RA, Schumacher TN, Borst J. CD27 is required for generation and long-term maintenance of T cell immunity. *Nat Immunol*. 2000;1:433-440.

132. Bansal-Pakala P, Halteman BS, Cheng MH, Croft M. Costimulation of CD8 T cell responses by OX40. *J Immunol*. 2004;172:4821-4825.

133. Rogers PR, Song J, Gramaglia I, Killeen N, Croft M. OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. *Immunity*. 2001;15:445-455.

134. Cooper D, Bansal-Pakala P, Croft M. 4-1BB (CD137) controls the clonal expansion

and survival of CD8 T cells in vivo but does not contribute to the development of cytotoxicity. *Eur J Immunol* . 2002;32:521-529.

135. Podack ER, Strbo N, Sotosec V, Muta H. CD30-governor of memory T cells? *Ann N Y Acad Sci*. 2002;975:101-113.

136. Withers DR, Gaspal FM, Bekiaris V, et al. OX40 and CD30 signals in CD4(+) T-cell effector and memory function: a distinct role for lymphoid tissue inducer cells in maintaining CD4(+) T-cell memory but not effector function. *Immunol Rev*. 2011;244:134-148.

137. Bamias G, Mishina M, Nyce M, et al. Role of TL1A and its receptor DR3 in two models of chronic murine ileitis. *Proc Natl Acad Sci U S A* . 2006;103:8441-8446.

138. Papadakis KA, Prehn JL, Landers C, et al. TL1A synergizes with IL-12 and IL-18 to enhance IFN-gamma production in human T cells and NK cells. *J Immunol*. 2004;172:7002-7007.

139. Meylan F, Richard AC, Siegel RM. TL1A and DR3, a TNF family ligandreceptor pair that promotes lymphocyte costimulation, mucosal hyperplasia, and autoimmune inflammation. *Immunol Rev*. 2011;244:188-196.

140. Dubois PC, Trynka G, Franke L, et al. Multiple common variants for celiac disease influencing immune gene expression. *Nat Genet*. 2010;42:295-302.

141. Barrett JC, Hansoul S, Nicolae DL, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet*. 2008;40:955-962.

142. Wang J, Anders RA, Wang Y, et al. The critical role of LIGHT in promoting intestinal inflammation and Crohn's disease. *J Immunol*. 2003;174:8173-8182.

143. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25+CD4+ regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol.* 2002;135-142.

144. Steinberg MW, Cheung TC, Ware CF. The signaling networks of the herpesvirus entry mediator (TNFRSF14) in immune regulation. *Immunol Rev*. 2011;244:169-187.

145. Murphy TL, Murphy KM. Slow down and survive: enigmatic immunoregulation by BTLA and HVEM. *Annu Rev Immunol*. 2010;28:389-411.

146. Chemnitz JM, Lanfranco AR, Braunstein I, Riley JL. B and T lymphocyte attenuator-mediated signal transduction provides a potent inhibitory signal to primary human CD4 T cells that can be initiated by multiple phosphotyrosine motifs. *J Immunol*. 2006;176:6603-6614.

147. Vendel AC, Calemine-Fenaux J, Izrael-Tomasevic A, Chauhan V, Arnott D, Eaton DL. B and T lymphocyte attenuator regulates B cell receptor signaling by targeting Syk and BLNK. *J Immunol*. 2009;182:1509-1517.

148. Ware CF, Sedy JR. TNF superfamily networks: bidirectional and interference pathways of the herpesvirus entry mediator (TNFSF14). *Curr Opin Immunol*. 2011;23(5):627-631.

149. Krieg C, Han P, Stone R, Goularte OD, Kaye J. Functional analysis of B and T lymphocyte attenuator engagement on CD4+ and CD8+ T cells. *J Immunol*. 2005;175:6420-6427.

150. Krieg C, Boyman O, Fu YX, Kaye J. B and T lymphocyte attenuator regulates CD8(+) T cell-intrinsic homeostasis and memory cell generation. *Nat Immunol*. 2007;8:162-171.

151. DeBenedette MA, Wen T, Bachmann MF, et al. Analysis of 4-1BB ligand (4-1BBL)-deficient mice and of mice lacking both 4-1BBL and CD28 reveals a role for 4-1BBL in skin allograft rejection and in the cytotoxic T cell response to influenza virus. *J Immunol*. 1999;163:4833-4841.

152. Tan JT, Whitmire JK, Murali-Krishna K, et al. 4-1BB costimulation is required for protective anti-viral immunity after peptide vaccination. *J Immunol*. 2000;164:2320-2325.

153. Dawicki W, Bertram EM, Sharpe AH, Watts TH. 4-1BB and OX40 act independently to facilitate robust CD8 and CD4 recall responses. *J Immunol*. 2004;173:5944-5951.

154. Soroosh P, Doherty TA, So T, et al. Herpesvirus entry mediator (TNFRSF14) regulates the persistence of T helper memory cell populations. *J Exp Med*. 2011;208:797-809.

155. Croft M. Control of immunity by the TNFR-related molecule OX40 (CD134). *Annu Rev Immunol*. 2010;28:57-78.

156. Kopf M, Ruedl C, Schmitz N, et al. OX40-deficient mice are defective in Th cell proliferation but are competent in generating B cell and CTL responses after virus infection. *Immunity*. 1999;11:699-708.

157. Yoshioka T, Nakajima A, Akiba H, et al. Contribution of OX40/OX40 ligand interaction to the pathogenesis of rheumatoid arthritis. *Eur J Immunol*. 2000;30:2815-2823.

158. Salek-Ardakani S, Song J, Halteman BS, et al. OX40 (CD134) controls memory T helper 2 cells that drive lung inflammation. *J Exp Med*. 2003;198:315-324.

159. Song A, Tang X, Harms KM, Croft M. OX40 and Bcl-xL promote the persistence of CD8 T cells to recall tumor-associated antigen. *J Immunol*. 2005;175:3534-3541.

160. Song J, So T, Cheng M, Tang X, Croft M. Sustained survivin expression from OX40 costimulatory signals drives T cell clonal expansion. *Immunity*. 2005;22:621-631.

161. Lee SW, Vella AT, Kwon BS, Croft M. Enhanced CD4 T cell responsiveness in the absence of 4-1BB. *J Immunol*. 2005;174:6803-6808.

162. Sun Y, Lin X, Chen HM, et al. Administration of agonistic anti-4-1BB monoclonal antibody leads to the amelioration of experimental autoimmune encephalomyelitis. *J Immunol*. 2002;168:1457-1465.

163. Sun Y, Chen HM, Subudhi SK, et al. Costimulatory molecule-targeted antibody therapy of a spontaneous autoimmune disease. *Nat Med*. 2002;8:1405-1413.

164. Foell J, Strahotin S, O'Neil SP, et al. CD137 costimulatory T cell receptor engagement reverses acute disease in lupus-prone NZB x NZW F1 mice. *J Clin Invest*. 2003;111:1505-1518.

165. Seo SK, Choi JH, Kim YH, et al. 4-1BB-mediated immunotherapy of rheumatoid arthritis. *Nat Med*. 2004;10:1088-1094.

166. Serghides L, Bukczynski J, Wen T, et al. Evaluation of OX40 ligand as a costimulator of human antiviral memory CD8 T cell responses: comparison with B7.1 and 4-1BBL. *J Immunol*. 2005;175:6368-6377.

167. Lee SW, Park Y, Song A, Cheroutre H, Kwon BS, Croft M. Functional dichotomy between OX40 and 4-1BB in modulating effector CD8 T cell responses. *J Immunol*. 2006;177:4464-4472.

168. Wang Y, Subudhi SK, Anders RA, et al. The role of herpesvirus entry mediator as a negative regulator of T cell-mediated responses. *J Clin Invest*. 2005;115:711-717.

169. Truong W, Hancock WW, Anderson CC, Merani S, Shapiro AM. Coinhibitory T-cell signaling in islet allograft rejection and tolerance. *Cell Transplant*. 2006;15:105-119.

170. Kurts C, Carbone FR, Krummel MF, Koch KM, Miller JF, Heath WR. Signalling through CD30 protects against autoimmune diabetes mediated by CD8 T cells. *Nature*. 1999;398:341-344.

171. Weinberg AD, Wegmann KW, Funatake C, Whitham RH. Blocking OX-40/OX-40 ligand interaction in vitro and in vivo leads to decreased T cell function and amelioration of experimental allergic encephalomyelitis. *J Immunol*. 1999;162:1818-1826.

172. Pakala SV, Bansal-Pakala P, Halteman BS, Croft M. Prevention of diabetes in NOD mice at a late stage by targeting OX40/OX40 ligand interactions. *Eur J Immunol* . 2004;34:3039-3046.

173. Nakajima A, Oshima H, Nohara C, et al. Involvement of CD70-CD27 interactions in the induction of experimental autoimmune encephalomyelitis. *J Neuroimmunol* . 2000;109:188-196.

174. Blazar BR, Kwon BS, Panoskaltsis-Mortari A, Kwak KB, Peschon JJ, Taylor PA. Ligation of 4-1BB (CDw137) regulates graft-versus-host disease, graft-versus-leukemia, and graft rejection in allogeneic bone marrow transplant recipients. *J Immunol* . 2001;166:3174-3183.

175. Tsukada N, Akiba H, Kobata T, Aizawa Y, Yagita H, Okumura K. Blockade of CD134 (OX40)-CD134L interaction ameliorates lethal acute graft-versus-host disease in a murine model of allogeneic bone marrow transplantation. *Blood*. 2000;95:2434-2439.

176. Blazar BR, Sharpe AH, Chen Al, et al. Ligation of OX40 (CD134) regulates graft-versus-host disease (GVHD) and graft rejection in allogeneic bone marrow transplant recipients. *Blood*. 2003;101:3741-3748.

177. Blazar BR, Levy RB, Mak TW, et al. CD30/CD30 ligand (CD153) interaction regulates CD4+ T cell-mediated graft-versus-host disease. *J Immunol* . 2004;173:2933-2941.

178. Banchereau J, Rousset F. Growing human B lymphocytes in the CD40 system. *Nature*. 1991;353:678-679.

179. Clark EA, Craxton A. A CD40 bridge between innate and adaptive immunity. *Immunity*. 2003;18:724-725.

180. Mackey MF, Barth RJ Jr, Noelle RJ. The role of CD40/CD154 interactions in the priming, differentiation, and effector function of helper and cytotoxic T cells. *J Leukoc Biol.* 1998;63:418-428.

181. Quezada SA, Jarvinen LZ, Lind EF, Noelle RJ. CD40/CD154 interactions at the interface of tolerance and immunity. *Annu Rev Immunol* . 2004;22:307-328.

182. Hildebrand JM, Yi Z, Buchta CM, Poovassery J, Stunz LL, Bishop GA. Roles of tumor necrosis factor receptor associated factor 3 (TRAF3) and TRAF5 in immune cell functions. *Immunol Rev*. 2011;244:55-74.

183. Craxton A, Otipoby KL, Jiang A, Clark EA. Signal transduction pathways that regulate the fate of B lymphocytes. *Adv Immunol* . 1999;73: 79-152.

184. Uchida J, Yasui T, Takaoka-Shichijo Y, et al. Mimicry of CD40 signals by Epstein-Barr virus LMP1 in B lymphocyte responses. *Science*. 1999;286:300-303.

185. Thorley-Lawson DA. Epstein-Barr virus: exploiting the immune system. *Nat Rev Immunol*. 2001;1:75-82.

186. Luftig M, Yasui T, Soni V, et al. Epstein-Barr virus latent infection membrane protein 1 TRAF-binding site induces NIK/IKKα-dependent noncanonical NF-κB activation. *Proc Natl Acad Sci USA*. 2004;101: 141-146.

187. Aruffo A, Farrington M, Hollenbaugh D, et al. The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. *Cell*. 1993;72:291-300.

188. Allen RC, Armitage RJ, Conley ME, et al. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome [see comments]. *Science*. 1993;259:990-993.

189. Durandy A, Revy P, Fischer A. Human models of inherited immunoglobulin class switch recombination and somatic hypermutation defects (hyper-IgM syndromes). *Adv Immunol*. 2004;82:295-330.

190. Litinskiy MB, Nardelli B, Hilbert DM, et al. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. *Nat Immunol*. 2002;3:822-829.

191. Mackay F, Leung H. The role of the BAFF/APRIL system on T cell function. *Semin Immunol*. 2006;18:284-289.

192. Bossen C, Schneider P. BAFF, APRIL and their receptors: Structure, function and signaling. *Semin Immunol*. 2006;18:263-275.

193. Kolfschoten GM, Pradet-Balade B, Hahne M, Medema JP. TWE-PRIL; a fusion protein of TWEAK and APRIL. *Biochem Pharmacol*. 2003;66:1427-1432.

194. Ingold K, Zumsteg A, Tardivel A, et al. Identification of proteoglycans as the APRIL-specific binding partners. *J Exp Med*. 2005;201:1375-1383.

195. O'Connor BP, Raman VS, Erickson LD, et al. BCMA is essential for the survival of long-lived bone marrow plasma cells. *J Exp Med*. 2004;199:91-98.

196. Sasaki Y, Casola S, Kutok JL, Rajewsky K, Schmidt-Supprian M. TNF family member B cell-activating factor (BAFF) receptor-dependent and -independent roles for BAFF in B cell physiology. *J Immunol*. 2004;173:2245-2252.

197. Varfolomeev E, Kischkel F, Martin F, et al. APRIL-deficient mice have normal immune system development. *Mol Cell Biol*. 2004;24:997-1006.

198. Castigli E, Scott S, Dedeoglu F, et al. Impaired IgA class switching in APRIL-deficient mice. *Proc Natl Acad Sci U S A* . 2004;101:3903-3908.

199. Yan M, Wang H, Chan B, et al. Activation and accumulation of B cells in TACI-deficient mice. *Nat Immuno*. 2001; 2:638-643.

200. Castigli E, Wilson SA, Scott S, et al. TACI and BAFF-R mediate isotype switching in B cells. *J Exp Med*. 2005;201:35-39.

201. Ye Q, Wang L, Wells AD, et al. BAFF binding to T cell-expressed BAFF-R costimulates T cell proliferation and alloresponses. *Eur J Immunol* . 2004;34:2750-2759.

202. Sutherland AP, Mackay F, Mackay CR. Targeting BAFF: immunomodulation for autoimmune diseases and lymphomas. *Pharmacol Ther* . 2006; 112:774-786.

203. Dillon SR, Gross JA, Ansell SM, Novak AJ. An APRIL to remember: novel TNF ligands as therapeutic targets. *Nat Rev Drug Discov* . 2006; 5:235-246.

204. Moreaux J, Legouffe E, Jourdan E, et al. BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone. *Blood*. 2004;103:3148-3157.

205. Burkly LC, Michaelson JS, Zheng TS. TWEAK/Fn14 pathway: an immunological switch for shaping tissue responses. *Immunol Rev*. 2011; 244:99-114.

206. Wiley SR, Winkles JA. TWEAK, a member of the TNF superfamily, is a multifunctional cytokine that binds the TweakR/Fn14 receptor. *Cytokine Growth Factor Rev*. 2003;14:241-249.

207. Vince JE, Silke J. TWEAK shall inherit the earth. *Cell Death Differ* . 2006;13(11):1842-1844.

208. Maecker H, Varfolomeev E, Kischkel F, et al. TWEAK attenuates the transition from innate to adaptive immunity. *Cell*. 2005;123:931-944.

209. Potrovita I, Zhang W, Burkly L, et al. Tumor necrosis factor-like weak inducer of apoptosis-induced neurodegeneration. *J Neurosci* . 2004;24: 8237-8244.

210. Nakayama M, Ishidoh K, Kayagaki N, et al. Multiple pathways of TWEAK-induced cell death. *J Immunol* . 2002;168:734-743.

211. Aktas O, Prozorovski T, Zipp F. Death ligands and autoimmune demyelination. *Neuroscientist*. 2006;12:305-316.

212. Perper SJ, Browning B, Burkly LC, et al. TWEAK is a novel arthritogenic mediator. *J Immunol*. 2006;177:2610-2620.

213. Chacon MR, Richart C, Gomez JM, et al. Expression of TWEAK and its receptor Fn14 in human subcutaneous adipose tissue. Relationship with other inflammatory cytokines in obesity. *Cytokine*. 2006;33:129-137.

214. De A, Park JI, Kawamura K, et al. Intraovarian TWEAK/Fn14 ligandreceptor system limits ovarian preovulatory follicles from excessive luteinization. *Mol Endocrinol*. 2006;20(10):2528-2538.

215. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature*. 1992;356:314-317.

216. Takahashi T, Tanaka M, Brannan CI, et al. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell*. 1994;76:969-976.

217. Fisher GH, Rosenberg FJ, Straus SE, et al. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell*. 1995;935-946.

218. Lee HO, Ferguson TA. Biology of FasL. *Cytokine Growth Factor Rev*. 2003;14:325-335.

219. Bodmer JL, Schneider P, Tschopp J. The molecular architecture of the TNF superfamily. *Trends Biochem Sci*. 2002;27:19-26.

220. Schneider P, Holler N, Bodmer JL, et al. Conversion of membranebound Fas(CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity. *J Exp Med*. 1998;187:1205-1213.

221. Gregory MS, Repp AC, Holhbaum AM, Saff RR, Marshak-Rothstein A, Ksander BR. Membrane Fas ligand activates innate immunity and terminates ocular immune privilege. *J Immunol*. 2002;169:2727-2735.

222. Wajant H, Pfizenmaier K, Scheurich P. Non-apoptotic Fas signaling. *Cytokine Growth Factor Rev*. 2003;14:53-66.

223. Dosreis GA, Borges VM, Zin WA. The central role of Fas-ligand cell signaling in inflammatory lung diseases. *J Cell Mol Med*. 2004;8:285-293.

224. Houston A, O'Connell J. The Fas signalling pathway and its role in the pathogenesis of cancer. *Curr Opin Pharmacol*. 2004;4:321-326.

225. Green DR, Droin N, Pinkoski M. Activation-induced cell death in T cells. *Immunol Rev*. 2003;193:70-81.

226. Hayashi N, Mita E. Involvement of Fas system-mediated apoptosis in pathogenesis of viral hepatitis. *J Viral Hepat*. 1999;6:357-365.

227. Benedict CA, Norris PS, Prigozy TI, et al. Three adenovirus E3 proteins cooperate to evade apoptosis by tumor necrosis factor-related apoptosisinducing ligand receptor-1 and -2. *J Biol Chem*. 2001;276:3270-3278.

228. Thome M, Schneider P, Hofmann K, et al. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature*. 1997;386:517-521.

229. Kim R, Emi M, Tanabe K, Arihiro K. Tumor-driven evolution of immunosuppressive networks during malignant progression. *Cancer Res*. 2006;66:5527-5536.

230. Ennaciri J, Menezes J, Proulx F, Toledano BJ. Induction of apoptosis by herpes simplex virus-1 in neonatal, but not adult, neutrophils. *Pediatr Res*. 2006;59:7-12.

231. Niederkorn JY. See no evil, hear no evil, do no evil: the lessons of immune privilege. *Nat Immunol*. 2006;7:354-359.

232. Wang XY, Sun J, Wang C, France M, Sheil AG. Effect of liver transplantation on islet allografts: up-regulation of Fas ligand and apoptosis of T lymphocytes are associated with islet graft tolerance. *Transplantation*. 2001;71:102-111.

233. Stuart PM, Yin X, Plambeck S, Pan F, Ferguson TA. The role of Fas ligand as an effector molecule in corneal graft rejection. *Eur J Immunol* . 2005;35:2591-2597.

234. Turvey SE, Gonzalez-Nicolini V, Kingsley CI, et al. Fas ligand-transfected myoblasts and islet cell transplantation. *Transplantation*. 2000;69:1972-1976.

235. Allison J, Georgiou HM, Strasser A, Vaux DL. Transgenic expression of CD95 ligand on islet beta cells induces a granulocytic infiltration but does not confer immune privilege upon islet allografts. *Proc Natl Acad Sci USA*. 1997;94:3943-3947.

236. Yada S, Takamura N, Inagaki-Ohara K, et al. The role of p53 and Fas in a model of acute murine graft-versus-host disease. *J Immunol*. 2005;174:1291-1297.

237. Park H, Jung YK, Park OJ, et al. Interaction of Fas ligand and Fas expressed on osteoclast precursors increases osteoclastogenesis. *J Immunol*. 2005;175:7193-7201.

238. Wiley SR, Schooley K, Smolak PJ, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity*. 1995;3:673-682.

239. Zauli G, Secchiero P. The role of the TRAIL/TRAIL receptors system in hematopoiesis and endothelial cell biology. *Cytokine Growth Factor Rev* . 2006;17:245-257.

240. Schneider P, Tschopp J. Apoptosis induced by death receptors. *Pharm Acta Helv*. 2000;74:281-286.

241. Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ, Ashkenazi A. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity*. 2000;12:611-620.

242. Almasan A, Ashkenazi A. Apo2L/TRAIL: apoptosis signaling, biology, and potential for cancer therapy. *Cytokine Growth Factor Rev* . 2003;14: 337-348.

243. Yang X, Wang J, Liu C, et al. Cleavage of p53-vimentin complex enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis of rheumatoid arthritis synovial fibroblasts. *Am J Pathol* . 2005;167:705-719.

244. Krieg A, Krieg T, Wenzel M, et al. TRAIL-beta and TRAIL-gamma: two novel splice variants of the human TNF-related apoptosis-inducing ligand (TRAIL) without apoptotic potential. *Br J Cancer* . 2003;88:918-927.

245. Kimberley FC, Screaton GR. Following a TRAIL: update on a ligand and its five receptors. *Cell Res*. 2004;14:359-372.

246. Krieg A, Schulte Am Esch J 2nd, Ramp U, et al. TRAIL-R4-beta: a new splice variant of TRAIL-receptor 4 lacking the cysteine rich domain 1. *Biochem Biophys Res Commun*. 2006;349:115-121.

247. Hamilton SE, Wolkers MC, Schoenberger SP, Jameson SC. The generation of protective memory-like CD8+ T cells during homeostatic proliferation requires CD4+ T cells. *Nat Immunol* . 2006;7:475-481.

248. Janssen EM, Droin NM, Lemmens EE, et al. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature*. 2005;434:88-93.

249. Barnhart BC, Alappat EC, Peter ME. The CD95 type I/type II model. *Semin Immunol*. 2003;15:185-193.

250. Duiker EW, Mom CH, de Jong S, et al. The clinical trail of TRAIL. *Eur J Cancer* . 2006;42:2233-2240.

251. Sato K, Hida S, Takayanagi H, et al. Antiviral response by natural killer cells through TRAIL gene induction by IFN-alpha/beta. *Eur J Immunol*. 2001;31:3138-3146.

252. Papadakis KA, Zhu D, Prehn JL, et al. Dominant role for TL1A/DR3 pathway in IL-12 plus IL-18-induced IFN-gamma production by peripheral blood and mucosal CCR9+ T lymphocytes. *J Immunol*. 2005;174:4985-4990.

253. Zhai Y, Ni J, Jiang GW, et al. VEGI, a novel cytokine of the tumor necrosis factor family, is an angiogenesis inhibitor that suppresses the growth of colon carcinomas in vivo. *FASEB J*. 1999;13:181-189.

254. Kim S, Zhang L. Identification of naturally secreted soluble form of TL1A, a TNF-like cytokine. *J Immunol Methods*. 2005;298:1-8.

255. Borysenko CW, Furey WF, Blair HC. Comparative modeling of TNFRSF25 (DR3) predicts receptor destabilization by a mutation linked to rheumatoid arthritis. *Biochem Biophys Res Commun*. 2005;328: 794-799.

256. Simonet WS, Lacey DL, Dunstan CR, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell*. 1997; 89:309-319.

257. Walsh MC, Kim N, Kadono Y, et al. Osteoimmunology: interplay between the immune system and bone metabolism. *Annu Rev Immunol*. 2006;24:33-63.

258. Tanaka S, Nakamura K, Takahasi N, Suda T. Role of RANKL in physiological and pathological bone resorption and therapeutics targeting the RANKL-RANK signaling system. *Immunol Rev*. 2005;208:30-49.

259. Xing L, Schwarz EM, Boyce BF. Osteoclast precursors, RANKL/RANK, and immunology. *Immunol Rev*. 2005;208:19-29.

260. Kong YY, Yoshida H, Sarosi I, et al. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature*. 1999;397:315-323.

261. Anderson DM, Maraskovsky E, Billingsley WL, et al. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendriticcell function. *Nature*. 1997;390:175-179.

262. Fata JE, Kong YY, Li J, et al. The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. *Cell*. 2000;103:41-50.

263. Emery JG, McDonnell P, Burke MB, et al. Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. *J Biol Chem*. 1998;273:14363-14367.

264. Miyashita T, Kawakami A, Nakashima T, et al. Osteoprotegerin (OPG) acts as an endogenous decoy receptor in tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis of fibroblast-like synovial cells. *Clin Exp Immunol*. 2004;137:430-436.

265. Wada T, Nakashima T, Hiroshi N, Penninger JM. RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med*. 2006;12:17-25.

266. Bharti AC, Aggarwal BB. Ranking the role of RANK ligand in apoptosis. *Apoptosis*. 2004;9:677-690.

267. Walsh MC, Choi Y. Biology of the TRANCE axis. *Cytokine Growth Factor Rev*. 2003;14:251-263.

268. Fukushima H, Jimi E, Kajiya H, Motokawa W, Okabe K. Parathyroidhormone-related protein induces expression of receptor activator of NF-κB ligand in human periodontal ligament cells via a cAMP/protein kinase A-independent pathway. *J Dent Res*. 2005;84:329-334.

269. Lomaga MA, Yeh WC, Sarosi I, et al. TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev*. 1999;13:1015-1024.

270. Naito A, Azuma S, Tanaka S, et al. Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *Genes Cells*. 1999;4:353-362.

271. Dougall WC, Glaccum M, Charrier K, et al. RANK is essential for osteoclast and lymph node development. *Genes Dev*. 1999;13:2412-2424.

272. Josien R, Li HL, Ingulli E, et al. TRANCE, a tumor necrosis factor family member, enhances the longevity and adjuvant properties of dendritic cells in vivo. *J Exp Med*. 2000;191:495-502.

273. Josien R, Wong BR, Li HL, Steinman RM, Choi Y. TRANCE, a TNF family member, is differentially expressed on T cell subsets and induces cytokine production in dendritic cells. *J Immunol*. 1999;162: 2562-2568.

274. Padigel UM, Kim N, Choi Y, Farrell JP. TRANCE-RANK costimulation is required for IL-12 production and the initiation of a Th1-type response to Leishmania major infection in CD40L-deficient mice. *J Immunol*. 2003;171:5437-5441.

275. Kotake S, Nanke Y, Mogi M, et al. IFN-gamma-producing human T cells directly induce osteoclastogenesis from human monocytes via the expression of RANKL. *Eur J Immunol*. 2005;35:3353-3363.

276. Teng YT, Mahamed D, Singh B. Gamma interferon positively modulates Actinobacillus actinomycetemcomitans-specific RANKL+ CD4+ Th-cell-mediated alveolar bone destruction in vivo. *Infect Immun*. 2005;73:3453-3461.

277. Fakruddin JM, Laurence J. HIV envelope gp120-mediated regulation of osteoclastogenesis via receptor activator of nuclear factor kappa B ligand (RANKL) secretion and its modulation by certain HIV protease inhibitors through interferon-gamma/RANKL cross-talk. *J Biol Chem*. 2003;278:48251-48258.

278. Fisher JL, Thomas-Mudge RJ, Elliott J, et al. Osteoprotegerin overexpression by breast cancer cells enhances orthotopic and osseous tumor growth and contrasts with that delivered therapeutically. *Cancer Res* . 2006;66:3620-3628.

279. Nyambo R, Cross N, Lippitt J, et al. Human bone marrow stromal cells protect prostate cancer cells from TRAIL-induced apoptosis. *J Bone Miner Res* . 2004;19:1712-1721.

280. Colucci S, Brunetti G, Rizzi R, et al. T cells support osteoclastogenesis in an in vitro model derived from human multiple myeloma bone disease: the role of the OPG/TRAIL interaction. *Blood*. 2004;104: 3722-3730.

281. Galluzzi F, Stagi S, Salti R, et al. Osteoprotegerin serum levels in children with type 1 diabetes: a potential modulating role in bone status. *Eur J Endocrinol* . 2005;153:879-885.

282. Bucay N, Sarosi I, Dunstan CR, et al. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev*. 1998;12:1260-1268.

283. Scott DL, Kingsley GH. Tumor necrosis factor inhibitors for rheumatoid arthritis. *N Engl J Med*. 2006;355:704-712.

284. van der Heijde DM, van Leeuwen MA, van Riel PL, et al. Biannual radiographic assessments of hands and feet in a three-year prospective followup of patients with early rheumatoid arthritis. *Arthritis Rheum*. 1992;35:26-34.

285. van der Heijde DM. Overview of radiologic efficacy of new treatments. *Rheum Dis Clin North Am*. 2004;30:285-293, vi.

286. Lipsky PE, van der Heijde DM, St Clair EW, et al. Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. *N Engl J Med* . 2000;343:1594-1602.

287. Reinecker HC, Steffen M, Witthoeft T, et al. Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. *Clin Exp Immunol*. 1993;94:174-181.

288. Hanauer SB, Sandborn WJ, Rutgeerts P, et al. Human anti-tumor necrosis factor monoclonal antibody (adalimumab) in Crohn's disease: the CLASSIC-I trial. *Gastroenterology*. 2006;130:323-333; quiz 591.

289. Sands BE, Anderson FH, Bernstein CN, et al. Infliximab maintenance therapy for fistulizing Crohn's disease. *N Engl J Med* . 2004;350: 876-885.

290. Rutgeerts P, Sandborn WJ, Feagan BG, et al. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med* . 2005;353: 2462-2476.

291. Sandborn WJ, Targan SR. Biologic therapy of inflammatory bowel disease. *Gastroenterology*. 2002;122:1592-1608.

292. Bonifati C, Ameglio F. Cytokines in psoriasis. *Int J Dermatol* . 1999;38: 241-251.

293. Schon MP, Boehncke WH. Psoriasis. *N Engl J Med* . 2005;352:1899-1912.

294. Mease PJ. Psoriatic arthritis therapy advances. *Curr Opin Rheumatol* . 2005;17:426-432.

295. Antoni CE, Kavanaugh A, Kirkham B, et al. Sustained benefits of infliximab therapy for dermatologic and articular manifestations of psoriatic arthritis: results from the infliximab multinational psoriatic arthritis controlled trial (IMPACT). *Arthritis Rheum*. 2005;52:1227-1236.

296. Gordon KB, Langley RG, Leonardi C, et al. Clinical response to adalimumab treatment in patients with moderate to severe psoriasis: double-blind, randomized controlled trial and open-label extension study. *J Am Acad Dermatol* . 2006;55:598-606.

297. Leonardi CL, Powers JL, Matheson RT, et al. Etanercept as monotherapy in patients with psoriasis. *N Engl J Med* . 2003;349:2014-2022.

298. Papp KA, Tyring S, Lahfa M, et al. A global phase III randomized controlled trial of etanercept in psoriasis: safety, efficacy, and effect of dose reduction. *Br J Dermatol* . 2005;152:1304-1312.

299. Tobin AM, Kirby B. TNF alpha inhibitors in the treatment of psoriasis and psoriatic arthritis. *BioDrugs*. 2005;19:47-57.

300. Dixon WG, Watson K, Lunt M, Hyrich KL, Silman AJ, Symmons DP. Rates of serious infection, including site-specific and bacterial intracellular infection, in rheumatoid arthritis patients receiving antitumor necrosis factor therapy: results from the British Society for Rheumatology Biologics Register. *Arthritis Rheum*. 2006;54:2368-2376.

301. Hochberg MC, Lebwohl MG, Plevy SE, Hobbs KF, Yocum DE. The benefit/risk profile of TNF-blocking agents: findings of a consensus panel. *Semin Arthritis Rheum*. 2005;34:819-836.

302. Bongartz T, Sutton AJ, Sweeting MJ, Buchan I, Matteson EL, Montori V. Anti-TNF antibody therapy in rheumatoid arthritis and the risk of serious infections and malignancies: systematic review and metaanalysis of rare harmful effects in randomized controlled trials. *JAMA*. 2006;295:2275-2285.

303. von Haehling S, Jankowska EA, Anker SD. Tumour necrosis factoralpha and the failing heart—pathophysiology and therapeutic implications. *Basic Res Cardiol*. 2004;99:18-28.

304. Anker SD, Coats AJ. How to RECOVER from RENAISSANCE? The significance of the results of RECOVER, RENAISSANCE, RENEWAL and ATTACH. *Int J Cardiol* . 2002;86:123-130.

305. Chung ES, Packer M, Lo KH, Fasanmade AA, Willerson JT. Randomized, double-blind, placebo-controlled, pilot trial of infliximab, a chimeric monoclonal antibody to tumor necrosis factor-alpha, in patients with moderate-to-severe heart failure: results of the anti-TNF Therapy Against Congestive Heart Failure (ATTACH) trial. *Circulation*. 2003;107:3133-3140.

306. Mohan C, Shi Y, Laman JD, Datta SK. Interaction between CD40 and its ligand gp39 in the development of murine lupus nephritis. *J Immunol* . 1995;154:1470-1480.

307. Kalled SL, Cutler AH, Datta SK, Thomas DW. Anti-CD40 ligand antibody treatment of SNF1 mice with established nephritis: preservation of kidney function. *J Immunol* . 1998;160:2158-2165.

308. Early GS, Zhao W, Burns CM. Anti-CD40 ligand antibody treatment prevents the development of lupus-like nephritis in a subset of New Zealand black x New Zealand white mice. Response correlates with the absence of an anti-antibody response. *J Immunol* . 1996;157:3159-3164.

309. Parker DC, Greiner DL, Phillips NE, et al. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. *Proc Natl Acad Sci U S A* . 1995;92:9560-9564.

310. Larsen CP, Alexander DZ, Hollenbaugh D, et al. CD40-gp39 interactions play a critical role during allograft rejection. Suppression of allograft rejection by blockade of the CD40-gp39 pathway. *Transplantation*. 1996;61:4-9.

311. Kalunian KC, Davis JC Jr, Merrill JT, Totoritis MC, Wofsy D. Treatment of systemic lupus erythematosus by inhibition of T cell costimulation with anti-CD154: a randomized, double-blind, placebocontrolled trial. *Arthritis Rheum*. 2002;46:3251-3258.

312. Boumpas DT, Furie R, Manzi S, et al. A short course of BG9588 (anti-CD40 ligand antibody) improves serologic activity and decreases hematuria in patients with

proliferative lupus glomerulonephritis. *Arthritis Rheum*. 2003;48:719-727.

313. Bussel JB. Overview of idiopathic thrombocytopenic purpura: new approach to refractory patients. *Semin Oncol*. 2000;27:91-98.

314. Cummings SR, San Martin J, McClung MR, et al. Denosumab for prevention of fractures in postmenopausal women with osteoporosis. *New Eng J Med* . 2009;361:756-765.

315. Smith MR, Egerdie B, Hernandez Toriz N, et al. Denosumab in men receiving androgen-deprivation therapy for prostate cancer. *New Eng J Med* . 2009;361:745-755.

316. Ellis GK, Bone HG, Chlebowski R, et al. Randomized trial of denosumab in patients receiving adjuvant aromatase inhibitors for nonmetastatic breast cancer. *J Clin Oncol.* 2008;26: 4875-4882.

317. Stopeck AT, Lipton A, Body JJ, et al. Denosumab compared with zoledronic acid for the treatment of bone metastases in patients with advanced breast cancer: a randomized, double-blind study. *J Clin Oncol.* 2010;28:5132-5139.

318. Henry DH, Costa L, Goldwasser F, et al. Randomized, double-blind study of denosumab versus zoledronic acid in the treatment of bone metastases in patients with advanced cancer (excluding breast and prostate cancer) or multiple myeloma. *J Clin Oncol.* 2011;29:1125-1132.

319. Fizazi K, Carducci M, Smith M, et al. Denosumab versus zoledronic acid for treatment of bone metastases in men with castration-resistant prostate cancer: a randomised, double-blind study. *Lancet*. 2011;377: 813-822.

320. Cheema GS, Roschke V, Hilbert DM, Stohl W. Elevated serum B lymphocyte stimulator levels in patients with systemic immune-based rheumatic diseases. *Arthritis Rheum*. 2001;44:1313-1319.

321. Zhang J, Roschke V, Baker KP, et al. Cutting edge: a role for B lymphocyte stimulator in systemic lupus erythematosus. *J Immunol* . 2001;166: 6-10.

322. Navarra SV, Guzman RM, Gallacher AE, et al. Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial. *Lancet*. 2011;377:721-731.

323. Ruegg C, Yilmaz A, Bieler G, Bamat J, Chaubert P, Lejeune FJ. Evidence for the involvement of endothelial cell integrin alphaVbeta3 in the disruption of the tumor vasculature induced by TNF and IFN-gamma. *Nat Med*. 1998;4:408-414.

324. Taguchi T, Sohmura Y. Clinical studies with TNF. *Biotherapy*. 1991;3: 177-186.

325. Skillings J, Wierzbicki R, Eisenhauer E, et al. A phase II study of recombinant tumor necrosis factor in renal cell carcinoma: a study of the National Cancer Institute of Canada Clinical Trials Group. *J Immunother* . 1992;11:67-70.

326. Tracey KJ, Lowry SF, Cerami A. The pathophysiologic role of cachectin/TNF in septic shock and cachexia. *Ann Inst Pasteur Immunol* . 1998;139:311-317.

327. Maruno M, Yoshimine T, Nakata H, Nishioka K, Kato A, Hayakawa T. Complete regression of anaplastic astrocytoma by intravenous tumor necrosis factor-alpha (TNF alpha) after recurrence: a case report. *Surg Neurol* . 1994;41:482-485.

328. Lejeune FJ, Lienard D, Matter M, Ruegg C. Efficiency of recombinant human TNF in human cancer therapy. *Cancer Immun* . 2006;6:6.

329. Abdulghani J, El-Deiry WS. TRAIL receptor signaling and therapeutics. *Expert Opin Ther Targets*. 2010;14:1091-1108.

330. Younes A, Vose JM, Zelenetz AD, et al. A Phase 1b/2 trial of mapatumumab in patients with relapsed/refractory non-Hodgkin's lymphoma. *Br J Cancer* . 2010;103:1783-1787.

331. Herbst RS, Eckhardt SG, Kurzrock R, et al. Phase I dose-escalation study of recombinant human Apo2L/TRAIL, a dual proapoptotic receptor agonist, in patients with advanced cancer. *J Clin Oncol* . 2010;28: 2839-2846.

332. Forero-Torres A, Shah J, Wood T, et al. Phase I trial of weekly tigatuzumab, an agonistic humanized monoclonal antibody targeting death receptor 5 (DR5). *Cancer Biother Radiopharm*. 2010;25:13-19.

333. Rosevear HM, Lightfoot AJ, Griffith TS. Conatumumab, a fully human mAb against death receptor 5 for the treatment of cancer. *Curr Opin Investig Drugs* . 2010;11:688-698.

334. Weinberg AD, Morris NP, Kovacsovics-Bankowski M, Urba WJ, Curti BD. Science gone translational: the OX40 agonist story. *Immunol Rev*. 2011;244:218-231.

335. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med* . 2011;365(8):725-733.

336. Ware CF. The TNF superfamily. *Cytokine Growth Factor Rev* . 2003; 14:181-184.

337. Seko Y, Cole S, Kasprzak W, Shapiro BA, Ragheb JA. The role of cytokine mRNA stability in the pathogenesis of autoimmune disease. *Autoimmun Rev*. 2006;5:299-305.

338. Zhang T, Kruys V, Huez G, Gueydan C. AU-rich element-mediated translational

control: complexity and multiple activities of trans-activating factors. *Biochem Soc Trans*. 2002;30:952-958.

339. Black RA, Doedens JR, Mahimkar R, et al. Substrate specificity and inducibility of TACE (tumour necrosis factor alpha-converting enzyme) revisited: the Ala-Val preference, and induced intrinsic activity. *Biochem Soc Symp*. 2003;39-52.

340. Aderka D, Engelmann H, Maor Y, Brakebusch C, Wallach D. Stabilization of the bioactivity of tumor necrosis factor by its soluble receptors. *J Exp Med*. 1992;175:323-329.

341. Li C, Norris PS, Ni CZ, et al. Structurally distinct recognition motifs in lymphotoxin-beta receptor and CD40 for tumor necrosis factor receptor-associated factor (TRAF)-mediated signaling. *J Biol Chem*. 2003;278:50523-50529.

342. Koni PA, Sacca R, Lawton P, Browning JL, Ruddle NH, Flavell RA. Distinct roles in lymphoid organogenesis for lymphotoxins a and b revealed in lymphotoxin b-deficient mice. *Immunity*. 1997;6:491-500.

343. Wang J, Foster A, Chin R, et al. The complementation of lymphotoxin deficiency with LIGHT, a newly discovered TNF family member, for the restoration of secondary lymphoid structure and function. *Eur J Immunol*. 2002;32:1969-1979.

344. Rutschmann S, Hoebe K, Zalevsky J, et al. PanR1, a dominant negative missense allele of the gene encoding TNF-alpha (Tnf), does not impair lymphoid development. *J Immunol*. 2006;176:7525-7532.

345. Neumann B, Luz A, Pfeffer K, Holzmann B. Defective Peyer's patch organogenesis in mice lacking the 55-kD receptor for tumor necrosis factor. *J Exp Med*. 1996;184:259-264.

346. Iizuka K, Chaplin DD, Wang Y, et al. Requirement for membrane lymphotoxin in natural killer cell development. *Proc Natl Acad Sci U S A* . 1995;96:6336-6340.

347. Elewaut D, Shaikh RB, Hammond KJ, et al. NIK-dependent RelB activation defines a unique signaling pathway for the development of V alpha 14i NKT cells. *J Exp Med*. 2003;197:1623-1633.

348. Tumanov AV, Grivennikov SI, Shakhov AN, et al. Dissecting the role of lymphotoxin in lymphoid organs by conditional targeting. *Immunol Rev*. 2003;195:106-116.

349. Lee BJ, Santee S, Von Gesjen S, Ware CF, Sarawar SR. Lymphotoxin α-/- mice can clear a productive infection with murine gammaherpevirus-68 (MHV-68) but fail to develop splenomegaly or lymphocytosis. *J Virol*. 2000;74:2786-2792.

350. Kumaraguru U, Davis IA, Deshpande S, Tevethia SS, Rouse BT. Lymphotoxin alpha(-/-) mice develop functionally impaired CD8(+) T cell responses and fail to contain virus infection of the central nervous system. *J Immunol.* 2001;166:1066-1074.

351. Benedict CA, Banks TA, Senderowicz L, et al. Lymphotoxins and cytomegalovirus cooperatively induce interferon-β, establishing host-virus détente. *Immunity*. 2001;15:617-626.

352. Banks TA, Rickert S, Benedict CA, et al. A lymphotoxin-IFN-beta axis essential for lymphocyte survival revealed during cytomegalovirus infection. *J Immunol*. 2005;174:7217-7225.

353. Schneider K, Loewendorf A, De Trez C, et al. Lymphotoxin-mediated crosstalk between B cells and splenic stroma promotes the initial type I interferon response to cytomegalovirus. *Cell Host Microbe*. 2008;3: 67-76.

354. Berger DP, Naniche D, Crowley MT, Koni PA, Flavell RA, Oldstone MB. Lymphotoxin-b-deficient mice show defective antiviral immunity. *Virol.* 1999;260:136-147.

355. Suresh M, Lanier G, Large MK, et al. Role of lymphotoxin alpha in T-cell responses during an acute viral infection. *J Virol.* 2002;76:3943-3951.

356. Junt T, Tumanov AV, Harris N, et al. Expression of lymphotoxin beta governs immunity at two distinct levels. *Eur J Immunol.* 2006;36: 2061-2075.

357. Puglielli MT, Browning JL, Brewer AW, et al. Reversal of virus-induced systemic shock and respiratory failure by blockade of the lymphotoxin pathway. *Nat Med*. 1999;5:1370-1374.

358. Lin X, Ma X, Rodriguez M, et al. Membrane lymphotoxin is required for resistance to Theiler's virus infection. *Int Immunol*. 2003;15:955-962.

359. Lund FE, Partida-Sanchez S, Lee BO, et al. Lymphotoxin-alphadeficient mice make delayed, but effective, T and B cell responses to influenza. *J Immunol*. 2002;169:5236-5243.

360. Ehlers S, Holscher C, Scheu S, et al. The lymphotoxin beta receptor is critically involved in controlling infections with the intracellular pathogens Mycobacterium tuberculosis and Listeria monocytogenes. *J Immunol*. 2003;170:5210-5218.

361. Roach DR, Briscoe H, Saunders B, France MP, Riminton S, Britton WJ. Secreted lymphotoxin-alpha is essential for the control of an intracellular bacterial infection. *J Exp Med*. 2001;193:239-246.

362. Lucas R, Tacchini-Cottier F, Guler R, et al. A role for lymphotoxin beta receptor in

host defense against Mycobacterium bovis BCG infection. *Eur J Immunol* . 1999;29:4002-4010.

363. Wilhelm P, Riminton DS, Ritter U, et al. Membrane lymphotoxin contributes to anti-leishmanial immunity by controlling structural integrity of lymphoid organs. *Eur J Immunol.* 2002;32:1993-2003.

364. Stanley AC, de Labastida Rivera F, Haque A, et al. Critical roles for LIGHT and its receptors in generating T cell-mediated immunity during Leishmania donovani infection. *PLoS Pathog*. 2011;7:e1002279.

365. Schluter D, Kwok LY, Lutjen S, et al. Both lymphotoxin-alpha and TNF are crucial for control of Toxoplasma gondii in the central nervous system. *J Immunol*. 2003;170:6172-6182.

366. Engwerda CR, Mynott TL, Sawhney S, De Souza JB, Bickle QD, Kaye PM. Locally up-regulated lymphotoxin alpha, not systemic tumor necrosis factor alpha, is the principle mediator of murine cerebral malaria. *J Exp Med*. 2002;195:1371-1377.

367. Hendriks J, Xiao Y, Rossen JW, et al. During viral infection of the respiratory tract, CD27, 4-1BB, and OX40 collectively determine formation of CD8+ memory T cells and their capacity for secondary expansion. *J Immunol*. 2005;175:1665-1676.

368. Humphreys IR, Walzl G, Edwards L, Rae A, Hill S, Hussell T. A critical role for OX40 in T cell-mediated immunopathology during lung viral infection. *J Exp Med*. 2003;198:1237-1242.

369. Humphreys IR, de Trez C, Kinkade A, Benedict CA, Croft M, Ware CF. Cytomegalovirus exploits IL-10-mediated immune regulation in the salivary glands. *J Exp Med*. 2007;204:1217-1225.

370. Humphreys IR, Loewendorf A, de Trez C, et al. OX40 costimulation promotes persistence of cytomegalovirus-specific CD8 T cells: a CD4- dependent mechanism. *J Immunol*. 2007;179:2195-2202.

371. Humphreys IR, Lee SW, Jones M, et al. Biphasic role of 4-1BB in the regulation of mouse cytomegalovirus-specific CD8(+) T cells. *Eur J Immunol* . 2010;40(10):2762-2768.

372. Bekiaris V, Gaspal F, Kim MY, et al. Synergistic OX40 and CD30 signals sustain CD8+ T cells during antigenic challenge. *Eur J Immunol* . 2009;39:2120-2125.

373. Akiba H, Miyahira Y, Atsuta M, et al. Critical contribution of OX40 ligand to T helper cell type 2 differentiation in experimental leishmaniasis. *J Exp Med*. 2000;191:375-380.

374. Ekkens MJ, Liu Z, Liu Q, et al. The role of OX40 ligand interactions in the

development of the Th2 response to the gastrointestinal nematode parasite Heligmosomoides polygyrus. *J Immunol*. 2003;170:384-393.

375. Humphreys IR, Edwards L, Walzl G, et al. OX40 ligation on activated T cells enhances the control of Cryptococcus neoformans and reduces pulmonary eosinophilia. *J Immunol*. 2003;170:6125-6132.

376. Florido M, Borges M, Yagita H, Appelberg R. Contribution of CD30/CD153 but not of CD27/CD70, CD134/OX40L, or CD137/4-1BBL to the optimal induction of protective immunity to Mycobacterium avium. *J Leukoc Biol*. 2004;76:1039-1046.

377. Laouar A, Haridas V, Vargas D, et al. CD70+ antigen-presenting cells control the proliferation and differentiation of T cells in the intestinal mucosa. *Nat Immunol*. 2005;6:698-706.

378. Shedlock DJ, Whitmire JK, Tan J, MacDonald AS, Ahmed R, Shen H. Role of CD4 T cell help and costimulation in CD8 T cell responses during Listeria monocytogenes infection. *J Immunol*. 2003;170: 2053-2063.

379. Nishimura H, Yajima T, Muta H, Podack ER, Tani K, Yoshikai Y. A novel role of CD30/CD30 ligand signaling in the generation of longlived memory CD8+ T cells. *J Immunol*. 2005;175:4627-4634.

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Chapter 28

Chemokines

Philip M. Murphy

INTRODUCTION

Chemokines constitute a large specialized family of cytokines that regulate immune responses by activating specific G protein-coupled receptors (GPCRs) on leukocytes.[1,2,3,4,5,6,7,8,9,10,11,12,13,14] Chemokines may act at multiple levels, including immune system development,[15] leukocyte transendothelial migration,[15,16,17,18] leukocyte positioning within microenvironments,[19,20,21,22,23] and both phagocyte and lymphocyte activation.[24,25,26] They may function beneficially, for example in the setting of antimicrobial host defense and tissue repair, or harmfully, for example in the setting of cancer, chronic inflammation, autoimmunity, and infectious disease (Fig. 28.1). Many pathogens produce broad-spectrum chemokine blocking agents, attesting to the importance of chemokines in host defense,[27,28,29] and some pathogens, most notably human immunodeficiency virus (HIV) and *Plasmodium vivax*, exploit host chemokine receptors as essential cell entry factors.[30,31,32] Chemokines may also have nonimmunologic functions, including regulation of organ development.[33,34,35] Together, these attributes have suggested potential chemokine-based therapeutic opportunities, and there are now two drugs targeting chemokine receptors approved by the U.S. Food and Drug Administration (FDA), the CCR5 antagonist maraviroc (Pfizer) in HIV,[36,37] and the CXCR4 antagonist plerixafor (Sanofi-Aventis) in hematopoietic stem cell mobilization for transplantation in cancer.[38]

To date,[48] chemokines and 23 chemokine receptors have been discovered in man. The goal of this chapter is to delineate their molecular properties and provide an overview of current knowledge of how they coordinate innate and adaptive immune responses, focusing on mouse and human. In this regard, it is important to note that the chemokine system may differ substantially among species, even at the genomic level.[39,40,41] In addition, despite adopting a standard nomenclature in the year 2000,[42,43,44] competing nonstandard aliases continue to be used in the primary literature, confusing even experts in the field. To address these challenges to clear communication, three illustrations are provided. The first defines the chemokine system repertoire at the genomic level in mouse and human, allowing comprehensive and facile identification of orthologues and species-specific elements (Fig. 28.2); the second is a dendrogram depicting the evolutionary relationship of chemokine and chemokine receptor protein sequences in mouse and man (Fig. 28.3); and the third provides the standard nomenclatures approved by the International Union of Immunological Societies for chemokine proteins and by the International Union of Pharmacology for chemokine receptors, linked to nonstandard aliases and protein sequence accession numbers (Tables 28.1 and 28.2). The names assigned by the HUGO Gene Nomenclature Committee are used for the corresponding chemokine system genes (see Fig. 28.2). In most cases, but not all, the approved gene and protein names are the same.

MOLECULAR ORGANIZATION OF THE CHEMOKINE SYSTEM

Chemokine Structure and Classification

The word "chemokine" is a contraction of " *chemo*tactic cyto*kine*," which conveys the main function possessed by members of the family. Nevertheless, chemokines are defined by structure, not function, and may have other functions besides inducing cell movement. At the level of primary structure, chemokines occupy a common sector of sequence space bounded by ~20% identity for any pairwise comparison, and after processing most are 66 to 111 amino acids in length. At the level of tertiary structure, chemokines fold in a highly conserved manner constrained in part by conservatively spaced disulfide-bonded cysteines.[45] The family is subclassified based on the number and location of these cysteines. All chemokines have at least two cysteines, and all but two have at least four cysteines (Fig. 28.4A). In the four cysteine group, the first two are either adjacent (CC motif) or separated by either one (CXC motif) or three (CX3C motif) amino acids. C chemokines have only two cysteines, corresponding to the location of Cys-2 and Cys-4 in the other groups. Most chemokines are members of either the CC or CXC group; there is only one CX3C chemokine and one (mouse) or two (human) C chemokines. The group motifs are used as roots followed by the letter "L" (for "ligand") and a number to create standard chemokine names (see Tables 28.1 and 28.2, and Fig. 28.2).[44] In human names, all letters are capitalized; in the corresponding mouse name, only the first letter is capitalized. Amino acid sequence identity is < 30% between members of the four major chemokine groups, but ranges from ~30% to 99% among members of the same group, indicating separate evolutionary histories. Although chemokine genes are located on 10 different human chromosomes, most are found in two large clusters, one for CXC chemokine genes on chromosome 4q21 and one for CC chemokine genes on chromosome 17q12 (see Fig. 28.2). These two clusters contain most of the chemokines that mediate inflammatory immune responses, whereas other chemokines, scattered throughout the genome either alone or in

small clusters on chromosomes 1, 2, 5, 7, 9, 10, 16, 17, and 19, mainly subserve homeostatic immune functions. The strongest functional correlate of the four major chemokine groups is receptor specificity: that is, most chemokine receptors bind more than one chemokine but are restricted by group (Table 28.3). With regard to leukocyte specificity, most neutrophil-targeted chemokines are in the CXC group and most of the monocyte-/macrophage-targeted chemokines are in the CC group. Major T and B cell-targeted chemokines can be found in both groups. The leukocyte target specificity of a chemokine may be narrow or broad and is defined by the expression pattern of its cognate receptor(s) (Table 28.4).

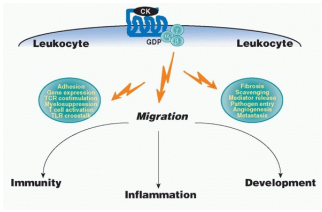

FIG. 28.1. Chemokines Coordinate Immune System Development and Immune Responses in Large Part by Inducing Leukocyte Migration Through Activation of G Protein-Coupled Receptors. Other functions induced by chemokines are listed in the *green spaces*. CK, chemokine.

CC and CXC chemokines can be further subclassified. The ELR subgroup of CXC chemokines is defined by the amino acid motif glu-leu-arg *N*-terminal to Cys-1 and is encoded by a cluster of genes on human chromosome 4q21 (see Fig. 28.2). A major species difference is the presence of the major neutrophil-targeted chemokine CXCL8 in human, but not in mouse. ELR is required for function of these chemokines, which are all > 40% identical at the amino acid level (see Fig. 28.3), attract neutrophils, bind the same receptor CXCR2, and are angiogenic.[46,47] Of the ELR-negative CXC chemokines, only CXCL12 is angiogenic and attracts neutrophils, but by activating CXCR4.[48] The so-called IP-10 subgroup, comprised of CXCL9-11, is encoded by a distinct cluster of related genes on human chromosome 4q21. Members share the receptor CXCR3, but are angiostatic, not angiogenic.[46,49]

The CC group is comprised of multiple subgroups. The two largest are the monocyte chemoattractant protein and macrophage inflammatory protein (MIP) clusters on human chromosome 17q12, which typically chemoattract monocytes/macrophages, plus other differential cell targets[50] (see Fig. 28.3). CC chemokines may also be subclassified by the presence or absence of additional cysteines (see Fig. 28.4). Although in separate groups as defined by cysteine motifs, CXCL16 and CX3CL1 also form a unique multimodular subgroup[51,52] (see Fig. 28.4). In addition to a typical chemokine domain, both these chemokines have a mucin-like stalk, a transmembrane domain, and a *C*-terminal cytoplasmic module, and can exist as a membrane-bound or shed form, mediating direct G protein-

independent cell-cell adhesion and chemotaxis, respectively.[53] The discovery of CXCL17 by sequence threading techniques, not by primary sequence homology, provides proof of principle that the boundaries of chemokine sequence space could expand substantially as protein structures are determined or modeled.[54]

All chemokines fold into a highly conserved and compact three-dimensional structure. Disulfide bonds link Cys-1 to Cys-3 and Cys-2 to Cys-4.[45,55] The core, which contains three β sheets arranged in the shape of a Greek key, is overlaid by a C-terminal α-helical domain and is flanked by an *N*-terminal domain that lacks order (see Fig. 28.4). Forced chemokine monomers are active,[56,57] but dimer and tetramer structures may occur, and complex quaternary structures bound to glycosaminoglycans (GAGs) on the surface of cells may be important for function in vivo.[4,58,59,60,61,62] Location of the GAG binding domains is variable and depends in part on the highly basic nature typical of most chemokines. Chemokine heterodimers have been described, both CC/CC and CC/CXC, and some may form preferentially over homodimers in a GAG-dependent manner; native heterodimers have also been identified.[63,64,65,66,67,68]

Chemokine Receptor Structure and Classification

Chemokine receptors are defined as mediators that activate cellular responses upon binding chemokines. All 23 known subtypes, together with receptors for the classical lipid and peptide chemoattractant receptors, are members of the rhodopsin-like seven-transmembrane (7TM) domain superfamily of GPCRs[8,42,43] (see Fig. 28.3; www.gpcr.org/7tm). The leukocyte chemoattractant receptors comprise the second largest subfamily of GPCRs (see Fig. 28.3). Chemokine binding, membrane anchoring, and signaling domains are formed from a single polypeptide chain ~340 to 370 aa long. CXCR4 is the only chemokine receptor, and one of only four 7TM receptors, whose structure has been determined. When bound to small molecule and cyclic

peptide antagonists, it is a dimer, in general agreement with previous biochemical data describing ligand-independent CXCR4 dimerization[69,70] (Fig. 28.5). Viral GPCRs have been proposed to hijack human receptors such as CXCR4 by heterodimerization.[71] Evidence for homo- and heterodimerization has been reported for other chemokine receptors, such as CCR2, CCR5, and CX3CR1, but the precise physiologic forms remain undefined.[72,73,74]

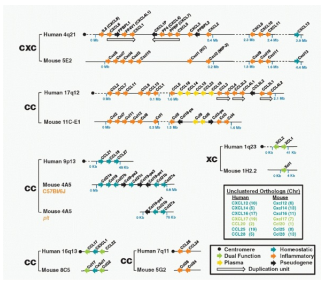

FIG. 28.2. Chemokine Gene Repertoires in Mouse and Human. Data are from www.ncbi.nlm.nih.gov/genome Builds 37.2 for *Homo sapiens* and *Mus musculus*. *Brackets* pair regions of conserved synteny between the indicated mouse and human chromosomes. *Bracket labels* denote chemokine groups (see text). Gene names are from the HUGO Gene Nomenclature Committee assignments. Protein names that differ from gene names are given in parentheses. *Arrows*, gene location and orientation relative to centromere; Mb, megabases; plasma, chemokines found at high concentrations constitutively in plasma. Other codes are in box at lower right. The figure layout is based on a previously published design and helpful advice from Hisayuki Nomiyama of Kumamoto University.

Standard receptor names are based on the property of chemokine group restriction, as follows: receptor name = ligand group root + R (for "receptor") + number, in order of discovery. An exception is the C chemokine receptor XCR1, where "X" distinguishes it from CR1, the previously assigned name for complement receptor 1. For consistency, the XCR1 ligands are named XCL1 and XCL2. All but six chemokines have had their receptors identified (see Table 28.3). Chemokines have unique receptor specificity profiles, and chemokine receptors have unique chemokine specificity profiles. Ligands that share a common receptor interact with the same receptor chain, in contrast to many other types of cytokines that share receptors via specialized subunits.[75] Pharmacologically, almost all chemokines are agonists, but a few are agonists at one receptor and antagonists at another (see Table 28.3).

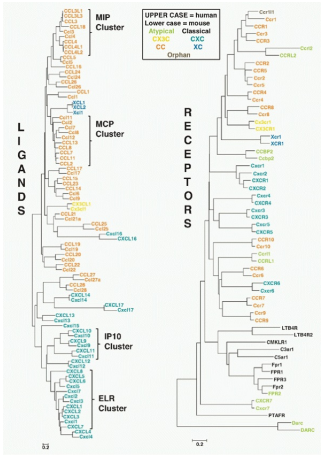

FIG. 28.3. Chemokine and Chemokine Receptor Family Trees in Mouse and Human. Branch distances are calibrated by the bar at the bottom of each tree. The dendrograms were created in collaboration with Steven Tsang, National Institute for Allergy and Infectious Diseases.

Atypical Chemokine System Components

There are at least six categories of atypical chemokine system components. The first includes the chemokines and 7TM chemokine receptors encoded by poxviruses and herpesviruses, the probable result of "molecular piracy" of their hosts.[29,71,76,77] This group includes chemokine scavengers and chemokine receptor antagonists thought to function in immune evasion; additional functions include modulation of host cell proliferation and movement. The second is a heterogeneous group of host 7TM chemokine-binding proteins that signal anomalously or not at all.[78,79,80,81] Included in this group are Duffy antigen receptor for chemokines (DARC), CXCR7, CCBP2 (also known as D6), CCRL1, and CCRL2. CXCR7, DARC, and CCBP2 are the best studied. CXCR7 has been included in the standard nomenclature system due to evidence of β-arrestin- but not G-protein-mediated signaling.[82] Its functions will be discussed in a later section with CXCR4, with which it shares a common ligand, CXCL12. DARC is nonsignaling and is expressed on red cells, lymphatic endothelium,

and cerebellar Purkinje cells, but not

P.685

P.686

P.687

P.688

P.689

leukocytes. It is an exception to the ligand rule as it binds many, but not all, CC and CXC chemokines.[83] DARC mediates transcytosis of chemokines across endothelial cells[84] and modulates inflammatory responses.[85] CCBP2 is expressed on leukocytes but at low levels. More prominently expressed on placental trophoblasts[86] and lymphatic endothelium,[87] CCBP2 scavenges inflammatory CC chemokines by a process of rapid internalization and recycling to plasma membrane,[88] and affects embryo survival, inflammatory responses, immune activation, and antimicrobial resistance in infection and cancer models.[89] One theory is that nonsignaling chemokine binding proteins function as antiinflammatory chemokine buffers, and accordingly they have been referred to as chemokine scavengers, interceptors, and silent or decoy receptors. A third category includes structurally unique broad-spectrum anti-inflammatory chemokine

P.690

binding proteins encoded by microbes, including several herpesviruses and poxviruses,[90] as well as *Schistosoma mansoni*[91] and ectoparasitic ticks.[92]

TABLE 28.1 Chemokine Nomenclature

			Accession Number	
Standard Name	**Common Aliases**	**Other Names**	**Human**	**Mouse**
CXCL1	GROα, MGSA Mouse: KC	SCYB1, NAP-3, FSP, GRO1, N51	P09341	P12850
CXCL2	Groβ; MIP-2α Mouse: MIP-2	SCYB2, CINC-2a, GRO2, MGSA-β	P19875	P10889
CXCL3	Groγ, MIP-2β ,	CINC-2b, GRO3, SCYB3 Mouse: Dcip1	P19876	Q6W5C0
CXCL4	Platelet factor-4	SCYB4	P02776	Q9Z126
CXCL4L1	PF4V1	CXCL4V1,SCYB4V1,	P10720	
CXCL5	ENA-78 Mouse: LIX	SCYB5	P42830	P50228
CXCL6	GCP-2	SCYB6, CKA-3	P80162	NA
CXCL7	NAP-2	*THBGB1*; PBP => CTAP-III => β-TG => NAP-2[a] SCYB7, β-TG1, Beta-TG, CTAP3, LA-PF4, LDGF, MDGF	P02775	Q9EQI5
CXCL8	IL-8	3-10C, AMCF-I, β-ENAP, GCP-1, K60, LECT, LUCT, MDNCF, MONAP, NAF, NAP-1, SCYB8, TSG-1	P10145	NA
CXCL9	Mig	SCYB9, crg-10, Humig Mouse: CRG-1	Q07325	P18340
CXCL10	γIP-10	SCYB10, C7, crg-2, IFI10, mob-1 Mouse: CRG-2	P02778	P17515
CXCL11	I-TAC	IP9, H174, SCYB11	O14625	Q8R392
CXCL12	SDF-1α	SDF-1β[b], PBSF, SCYB12, TPAR1, TLSF	P48061	P40224
CXCL13	BLC	BCA-1, SCYB13, ANGIE, ANGIE2, BLR1L	O43927	O55038
CXCL14	BRAK	bolekine, SCYB14, BMAC, Kec, KS1, MIP-2γ, NJAC	O95715	Q6AXC2
Cxcl15	Lungkine		NA	Q9WVL7
CXCL16	SR-PSOX		Q9H2A7	Q8BSU2
CXCL17		Dcip1, DMC, UNQ473, VCC1	Q6UXB2	Q8R3U6
CCL1	I-309	P500, SISε Mouse: TCA-3	P22362 P10146	
CCL2	MCP-1 Mouse: JE	MCAF, HC11, SCYA2, SMC-CF, GDCF-2	P13500 P10148	
CCL3	MIP-1α	MIP-1αS, LD78α, GOS19-1; PAT 464.1; TY-5; SISα	P10147	P10855
CCL3L1		MIP-1αP; LD78β SCYA3L, SCYA3L1, G0S19-2; PAT 464.2	P16619	P10855
CCL3L3		MGC12815	P16619	
CCL4	MIP-1β	ACT-2, PAT 744, H400; SIS-γ, LAG-1, HC21, G-26, MAD-5, AT744.1	P13236	P14097
CCL4L1		AT744.2, LAG-1	Q8NHW4	NA
CCL4L2			Q8NHW4	NA
CCL5	RANTES	SIS-δ, MGC17164, p288, TCP228	P13501	P30882
Ccl6	C10, MRP-1		NA	P27784
CCL7	MCP-3	NC28, FIC Mouse: MARC	P80098	Q03366
CCL8	MCP-2	HC14	P80075	Q9Z121
Ccl9	MRP-2, MIP-1γ	CCF18	NA	P51670
CCL10 (reserved)			NA	NA

CCL11	Eotaxin	SCYA11, eotaxin-1, MGC22554	P51671	P48298
Ccl12	Mcp-5		NA	Q62401
CCL13	MCP-4	Ckβ10; NCC-1, MGC17134, SCYL1, SCYA13	Q99616	NA
CCL14	HCC-1	CC-1, NCC-2, CCCK-1/CCCK-3, Ckβ1, MCIF, HCC-3, SCYL2, SCYA14	Q16627	NA
CCL15	HCC-2	Leukotactin-1 (Lkn-1), MIP-5, CC-2, NCC-3, MIP-1δ, HMRP-2B, SCYL3, SCYA15	Q16663	NA
CCL16	HCC-4	LEC, NCC-4, LMC, monotactin-1 (Mtn-1), LCC-1, ILINCK, CKβ12, SCYL4, SCYA16	O15467	NA
CCL17	TARC	STCP-1, SCYA17	Q92583	Q9WUZ6
CCL18	PARC	DC-CK-1, MIP-4, AMAC-1, ckβ7, SCYA18	P55774	NA
CCL19	ELC	MIP-3β, Exodus-3, ckβ11, SCYA19	Q99731	O70460
CCL20	MIP-3α, LARC	Exodus-1, CKβ4, SCYA20 Mouse: ST38	P78556	O89093
CCL21	SLC	6Ckine, exodus-2, TCA4, ckβ9, ECL, SCYA21	O00585	P84444
CCL22	MDC	STCP-1, SCYA22 Mouse: abcd-1, dc/b-ck	O00626	O88430
CCL23	MPIF-1	MIP-3, ckβ8-1, SCYA23	P55773	NA
CCL24	Eotaxin-2	MPIF-2, ckβ6, SCYA24	O00175	Q9JKC0
CCL25	TECK	ckβ15, SCYA25	O15444	O35903
CCL26	Eotaxin-3	MIP-4α, IMAC, N1, TSC-1, SCYA26	Q9Y258	Q5C9Q0
CCL27	CTACK	ILC, SCYA27, ESkine, PESKY, skinkine mouse: ALP	Q9Y4X3	Q9Z1X0
CCL28	MEC	SCYA28, CCK1	Q9NRJ3	Q9JIL2
XCL1	Lymphotactin α	SCM-1α, ATAC[a], SCYC1, LPTN	P47992	P47993
XCL2	Lymphotactin β	SCM-1β, ATAC, SCYC2	Q9UBD3	NA
CX$_3$CL1	Fractalkine	SCYD1, ABCD-3, C3Xkine, CXC3, CXC3C, Mouse: neurotactin	P78423	O35188

6Ckine, chemokine with 6 cysteines; AMAC, alternative macrophage activation-associated CC-chemokine; ATAC, activation-induced, chemokine-related molecule exclusively expressed in CD8+ T lymphocytes; BCA-1, B cell activating chemokine-1; BLC, B lymphocyte chemoattractant; BRAK, Breast and kidney chemokine; CC-#, CC chemokine-#; ckb#, CC chemokine #; CD, cluster of differentiation; CTACK, cutaneous T cell attracting chemokine; CTAP, connective tissue activating peptide; DC, dendritic cell; dc/b-ck, dendritic cell beta chemokine-1; ELC, Epstein-Barr virus-induced receptor ligand chemokine; ENA-78, epithelial cell-derived neutrophil-activating factor, 78 amino acids; FIC, fibroblast inducible cytokine; GCP-#, granulocyte chemoattractant protein-#; Gro, growth-related oncogene; HCC-#, hemofiltrate CC chemokine-#; IL-8, interleukin-8; IP-10, interferon-inducible protein-10; I-TAC, IFN-inducible T cell alpha chemoattractant; LARC, liver and activation-related chemokine; LCC, liver CC chemokine; LEC, liver expressed chemokine; LMC, lymphocyte and monocyte chemoattractant CC chemokine; LYNAP, lymphocyte-derived neutrophil-activating peptide; MARC, mast cell activation related chemokine; MCAF, monocyte chemoattractant and activating factor; MCIF, macrophage colony inhibitory factor; MCP, monocyte chemoattractant protein; MDC, macrophagederived chemokine; MDNCF, monocyte-derived neutrophil chemotactic factor; MEC, mucosa-associated epithelial cell chemokine; MGSA, melanoma growth-stimulatory activity; Mig, monokine induced by gamma interferon; MIP, macrophage inflammatory protein; MPIF-#, myeloid progenitor inhibitory factor-#; MRP-#, MIP-related protein-#; Mtn-1, monotactin-1; NAF, neutrophil-activating factor; NAP-#, neutrophil-activating protein-#; NCC-#, novel CC chemokine-#; PARC, pulmonary and activation-regulated chemokine; PBP, platelet basic protein; PBSF, pre B cell stimulatory factor; PF4, platelet factor 4; RANTES, regulated upon activation normal T cell-expressed and secreted; SCM, single cysteine motif-1; SCY#, small cytokine #; SDF-1, stromal cell-derived factor-1; SIS-#, small inducible secreted protein-#; SLC, secondary lymphoid tissue chemokine; SR-PSOX, scavenger receptor for phosphatidylserine and oxidized lipoprotein; TARC, thymus and activation-related chemokine; TCA-#, T-cell activation protein-#; TECK, thymus expressed chemokine; TG, thromboglobulin; TLSF, thymic lymphoma cell stimulating factor; TPAR, TPA repressed protein.

[a]Sequential N-terminal truncation of PBP produces the chemokines shown. Only NAP-2 has leukocyte chemoattractant activity, specifically for neutrophils.

[b]SDF-1α and β are splice variants of the same human gene.

TABLE 28.2 Chemokine Receptor Nomenclature

			Accession Number	
Name	**CD#**	**Previous Names**	**Human**	**Mouse**
CXCR1	CD181	IL8R$_A$, IL-8R-I, CDw128a, CKR-1, IL-8Rα	P25024	Q810W6
CXCR2	CD182	IL8R$_B$, IL-8R-II, CMKAR2, IL-8R β	P25025	P35343
CXCR3	CD183	IP10/Mig R, GPR9, CKR-L2, CMKAR3, IP10-R, MigR	P49682	O88410
CXCR4	CD184	HUMSTSR, LESTR, fusin, HM89, LCR1, NPYR, D2S201E, D2S201E, fusin, HSY3RR, NPY3R,	P61073	P70658
CXCR5	CD185	BLR-1, MDR15	P32302	Q04683

CXCR6	CD186	BONZO, STRL33, TYMSTR	O00574	Q9EQ16
CXCR7		RDC1,GPR159, CMKOR1	P25106	P56485
CCR1	CD191	CKR1, CC CKR1, MIP-1α/RANTES, CMKBR1	P32246	P51675
CCR2	CD192	CKR2, CC CK2, CC CKR2, MCP-1, CMKBR2 CC-CKR-2, FLJ78302, MCP-1-R	P41597	P51683
CCR3	CD193	CKR3, CC CKR3, Eotaxin receptor, CMKBR3	P51677	P51678
CCR4	CD194	CKR4, CC CKR4, K5-5, CMKBR4, CHEMR1	P51679	P51680
CCR5	CD195	CKR5, CC CKR5, ChemR13, CMKBR5, IDDM22	P51681	P51682
CCR6	CD196	GPR-CY4, CKR-L3, STRL-22, DRY-6, DCR2, BN-1, GPR29, CMKBR6	P51684	O54689
CCR7	CD197	EBI-1, BLR-2, CMKBR7	P32248	P47774
CCR8	CDw198	TER1, CKR-L1, GPR-CY6, CMKBR8, CKR-L1	P51685	P56484
CCR9	CDw199	GPR28, GPR 9-6	P51686	Q9WUT7
CCR10		GPR2	P46092	Q9JL21
XCR1		GPR5	P46094	Q9R0M1
CX$_3$CR1		Fractalkine receptor, GPR13, V28, CMKBRL1, CCRL1, CMKDR1	P49238	Q9Z0D9
DARC	CD234	Duffy; glycoprotein D, CCBP1, Dfy, GPD, FY	Q16570	Q9QUI6
CCBP2		D6, CCR9 (unofficial), CCR10 (unofficial)	O00590	Y12879
CCRL1		CCX-CKR, CCBP2, CCR11, PPR1, VSHK1	Q9NPB9	Q924I3
CCRL2		CKRX, CRAM-A, L-CCR, CRAM-B, HCR, CCR11 (unofficial)	O00421	O35457

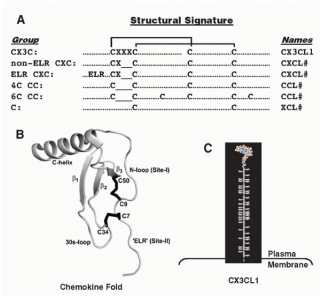

FIG. 28.4. Chemokine Classification, Nomenclature, and Structure. A: Chemokine groups are defined by the number and arrangement of conserved cysteines, as shown. *Brackets* link cysteines that form disulfide bonds. ELR, glu-leu-arg; X, an amino acid other than cysteine. The *underscore* is a spacer used to optimize the alignment. The *N-* and *C-* termini can vary considerably in length (not illustrated). For the molecules with four cysteines, there are approximately 24 amino acids between Cys-2 and Cys-3 and 15 amino acids between Cys-3 and Cys-4. At right is listed the nomenclature system. **B:** The chemokine fold illustrated by monomeric CXCL8. **C:** Model of transmembrane chemokine CX3CL1. See text for details.

TABLE 28.3 Chemokine Specificities for Human Seven-Transmembrane Chemokine Receptors

		CXC						CC										XC	CX3C	Atypical				
		CXCR1	CXCR2	CXCR3	CXCR4	CXCR5	CXCR6	CCR1	CCR2	CCR3	CCR4	CCR5	CCR6	CCR7	CCR8	CCR9	CCR10	XCR1	CX3CR1	DARC	CCBP2	CCRL1	CCRL2	CXCR7
CXC	CXCL1/Groα		+																	+				
	CXCL2/Groβ		+																					
	CXCL3/Groγ		+																					
	CXCL4/PF-4																							
	CXCL4L1																							
	CXCL5/ENA-78	+	+																					
	CXCL6/GCP-2	+	+																					
	CXCL7/NAP-2		+																	+				
	CXCL8/IL-8	+	+																	+				
	CXCL9/Mig			+						**A**														
	CXCL10/γIP-10			+						**A**														
	CXCL11/I-TAC			+						**A**														+
	CXCL12/SDF-1				+																			+
	CXCL13/BCA1			+		+																		
	CXCL14/BRAK																							
	*Cxcl15/lungkine																							
	CXCL16						+																	

	CXCL17																							
CC	CCL1/I-309														+									
	CCL2/MCP-1							+	+											+	+			
	CCL3/MIP-1α							+				+												
	CCL4/MIP-1β							**A**				+									+			
	CCL5/RANTES							+		+		+								+	+			
	*Ccl6/MRP-1							+																
	CCL7/MCP-3							+	+	+		**A**									+			
	CCL8/MCP-2											+			+						+			
	*Ccl9/MRP-2							+																
	CCL11/eotaxin			**A**					**A**	+		+									+			
	*Ccl12/MCP-5								+															
	CCL13/MCP-4							+	+	+											+			
	CCL14a/HCC-1							+			+									+				
	CCL14b/HCC-3																							
	CCL15/HCC-2							+		+														
	CCL16/HCC-4							+	+			+												
	CCL17/TARC										+													
	CCL18/PARC																							
	CCL19/ELC													+								+	+	
	CCL20/LARC												+											
	CCL21/SLC			+										+								+		
	CCL22/MDC										+													
	CCL23/MPIF-1							+																
	CCL24/eotaxin-2									+														
	CCL25/TECK															+						+		
	CCL26/eotaxin-3									+														
	CCL27/CTACK																+							
	CCL28/MEC									+							+							
XC	XCL1/lymphotactin α																	+						
	XCL2/lymphotactin β																	+						
CX3C	CX3CL1/fractalkine																		+					

MCP, monocyte chemoattractant protein; NAP, neutrophil-activating protein.

TABLE 28.4 Chemokine Receptor Distribution on Some Hematopoietic Cells

		CXC						CC										XC	CX3C	Atypical				
		CXCR1	CXCR2	CXCR3	CXCR4	CXCR5	CXCR6	CCR1	CCR2	CCR3	CCR4	CCR5	CCR6	CCR7	CCR8	CCR9	CCR10	XCR1	CX3CR1	DARC	CCBP2	CCRL1	CCRL2	CXCR7
	CD34+ HSC				+																			
	Erythrocytes																					+		
	Megakaryocytes																						+	
	Platelets	+			+			+		+	+													
Granulocytes	Neutrophils	+	+		+			+	+															
	Eosinophils			+	+			+		+														
	Basophils		+		+			++		+		+												
Mononuclear Phagocytes	CD14+ Monocytes	+	+		+			+	+										+					
	CD16+ Monocytes				+																			
	Immature DC	+	+		+			+	+			+	+								+			
	Mature DC			+								+	++				+							
	NK Cells	$CD56^{dim}$ CD16+ NK	+		+	+	+	+	+			+	+	+			+			+				
	$CD56^{bright}$ CD16- NK	+		+	+	+	+	+				+	+	+		+								
NK-T cells	CD4 NK-T			+	+				+		+	+												
	CD8 NK-T			+	+		+	+	+			+	+											
	CD4-CD8- NK-T			+	+		+	+	+			+	+											
B cells	Marginal Zone B Cells																							+
	Plasma cells				+		+			+							+							
	IgA Ab-secreting cells				+											+	+							

T cells	Naïve T cells		+									+					
	Follicular help T cells			+													
	Central memory T cells	+							+		+	+					
	Effector memory T cells					+		+		+							
	Th1 Effector T cells	+	+		+		+			+		+					+
	Th2 Effector T cells		+					+	+			+	+				
	α4β7+ Gut-homing memory T cells	+					+			+				+			
	CLA+ Skin-homing memory T cells		+						+		+	+			+		
	CD4+ CD25+Foxp3 + Regulatory T cells								+	+	+		+				

CD, cluster of differentiation; DC, dendritic cell; Ig, immunoglobulin; HSC, hematopoietic stem cell.

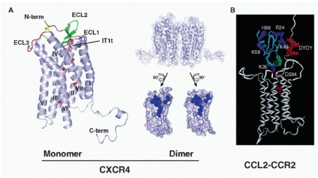

FIG. 28.5. Chemokine Receptor Structure. A: Structure of CXCR4 bound to ligand IT1t, at left[70]; CXCR4 dimer, at right, illustrating the dimer contact surface at the bottom right.[70] **B:** CCL2 monomer docking to model of CCR2.[135]

The fourth group of atypical components includes endogenous nonchemokine ligands that bind chemokine receptors. Examples include aminoacyl transfer ribonucleic acid synthetases, some of which function as autoantigens in autoimmune disorders[93,94]; the antimicrobial peptide β defensin 2, which activates CCR6 on immature dendritic cells (DCs), possibly linking innate to adaptive immunity[95]; and cluster of differentiation (CD)82 (KAI1), a tetraspanin expressed on leukocytes and cancer cells that binds to endothelial cell-expressed DARC, triggering senescence in tumor cells and suppression of metastasis.[96] The fifth group includes chemokines that bind scavenger receptor ligands, such as oxidized LDL, through their receptor binding domains.[97] The prototype is CXCL16, which was originally called scavenger receptor for phosphatidyl serine and oxidized LDL, or SR-PSOX. CXCL16 has also been shown to function as a transporter for CpG dinucleotides across the plasma membrane for action at toll-like receptor 9, as well as to mediate bacterial phagocytosis by antigen-presenting cells.[98]

In contrast, a truncated form of CCL23, unlike full-length CCL23, has not been shown to activate any chemokine receptors, yet is an agonist at FPR2, a member of the classical chemoattractant fMet-Leu-Phe receptor subfamily.[99] Perhaps the most surprising example of an alternative chemokine receptor agonist is the tripeptide Pro-Gly-Pro, a breakdown product of collagen from extracellular matrix that is found in bronchoalveolar lavage samples from patients with chronic obstructive pulmonary disease.[100] This peptide shares structural homology with a domain in CXC chemokines and has been shown to recruit neutrophils via CXCR2 activation in a mouse model of lung inflammation. It has been reported to be a chemotactic agonist at CXCR2, but this has not been confirmed.[101]

A final category of atypical components includes nonchemokine ligands for chemokine receptors (agonists or antagonists) produced by pathogens. For example, HIV Tat is an antagonist at CXCR4,[102] and CCR5 can be activated by viral (HIV gp120), bacterial (*Mycobacterium tuberculosis* Hsp70), and protozoan (*Toxoplasma gondii* immunophilin) factors.[103,104,105,106] *M. tuberculosis* Hsp70 activation of CCR5 induces DC aggregation, T-DC immune synapse formation, and effector immune responses.

Chemokine System Genes and Evolution

The chemokine system appears to have originated in teleost fish (for updates and phylogenies, see the Cytokine Family cDNA database at http://cytokine.medic.kumamoto-u.ac.jp). Comparative whole genome analysis has now shown that the chemokine repertoire differs dramatically in size throughout phylogeny with zebrafish having 63 chemokine genes compared to 48 in human.[40,41,107,108] Only human CXCL12 and CXCL14 have unambiguous orthologues in fish, and none of the other mammalian CXC chemokines has clear orthologues in any other vertebrate class, including birds. In addition, gene copy number may differ among closely related species (see Fig. 28.2 for human and mouse) and among individuals of the same species (eg, the triplicated MIP cluster in humans). A model of chemokine system evolution has been proposed involving lineage-specific en bloc and tandem duplications with expansion and functional specialization accompanying the origin of adaptive immunity.[107]

Chemokine genes typically are ˜4 kb long and usually have four exons in the case of CXC chemokines and three exons in the case of CC chemokines. The promoter is immediately upstream of exon 1, which encodes a leader sequence and a few amino acids of the mature peptide. Alternative

P.691

splicing has been reported for several chemokines, but the significance is not well defined. CCL14 and CCL15 are an exceptional example of neighboring genes that give rise to a family of mono- and bicistronic transcripts.[109]

The 23 chemokine receptor genes can be divided by chromosomal location into three groups: a large cluster on human chromosome 3p21-23 including multiple CCRs, CX3CR1, XCR1, and CXCR6, plus CCBP2 and CCRL1; CXCR1, CXCR2, and one receptor pseudogene clustered on 2q34-q35; and the rest unclustered. With the exception of CCR9, genes in the two clustered groups lack introns in the open reading frame (ORF) but have at least one and as many as 10 introns separating the promoter from the ORF. In contrast, an intron divides the *N*-terminus of the majority of unclustered receptor genes (CXCR3, 4, and 5; CCR6 and 10). Several of these undergo alternative splicing but the products appear to function similarly. CCR2 undergoes alternative splicing of a virtual intron in the *C*-terminal region of the ORF, but the two products have similar function and CCR2B appears to be the major expressed form.

With regard to horizontal evolution, variation in gene copy number among individuals has been observed for only a few chemokines. *CCL3* is an example of this in human,[110] and the *plt* locus (*p*aucity of *l*ymph node *T* cells), which will be discussed in a later section, is an immunologically important example in mouse[111] (see Fig. 28.2). In contrast, variation in gene sequence is common among individuals for most chemokine and chemokine receptors. However, the degree of polymorphism varies greatly among different genes. The most extreme and important example is the *CCR2-CCR5* locus in human, in which combinations of common dimorphic single nucleotide polymorphisms in the CCR5 promoter with a single nucleotide polymorphism in the ORF of *CCR2* named *CCR2 V64I* and a 32 base pair deletion in the ORF of *CCR5* named *CCR5Δ32* together form at least eight distinct haplotypes and in some cases affect HIV disease susceptibility.[112,113,114]

TABLE 28.5 Sources and Main Immunologic Functions of Human CXC, CX3C, and C Chemokines

Chemokine	Main Source	Main Immunologic Roles
CXCL1	Inducible in most hematopoietic and tissue cells	Neutrophil trafficking
CXCL2	Many tumors Neutrophil	trafficking
CXCL3		Neutrophil trafficking
CXCL4	Preformed in platelets	Procoagulant
CXCL4L1	Preformed in platelets	ND
CXCL5	Induced in epithelial cells of gut and lung; N, Mo, Plts, EC	Neutrophil trafficking
CXCL6	Induced in lung microvascular EC; Mo; alveolar epithelial cells, mesothelial cells, EC and MΦ	Neutrophil trafficking
CXCL7	Preformed in platelets	Neutrophil trafficking
CXCL8	Induced in most cell types	Neutrophil trafficking
CXCL9	Induced in PMN, MΦ, T cells, astrocytes, microglial cells, hepatocytes, EC, fibroblasts, keratinocytes, thymic stromal cells	Th1 response
CXCL10	Induced in ECs, Mo, keratinocytes, respiratory & intestinal epithelial cells, astrocytes, microglia, mesangial cells, smooth muscle cells	Th1 response
CXCL11	ECs, Mo	Th1 response
CXCL12	Constitutive in bone marrow stromal cells; most tissues	Myelopoiesis HPC, neutrophil homing to BM B lymphopoiesis
CXCL13	Constitutive in follicular HEV of secondary lymphoid tissue	Naïve B- and T-cell homing to follicles B1 cell homing to peritoneum Natural Ab production
CXCL14	Constitutive in most tissues, breast and kidney tumors	Macrophage migration
(CXCL15)	Constitutive in lung epithelial cells	Neutrophil trafficking
CXCL16	Constitutive in spleen; DCs of the T zone	T-cell, NK and DC trafficking
CX3CL1	EC, neurons, Mo, DC	NK, Monocyte, MΦ, and Th1 cell migration; neuroprotection
XCL1	γδ epidermal T cells, NK, NK-T, activated CD8+ and Th1 CD4+ T cells	Cross presentation of antigen by XCR1+ CD8+ DC to CD8+ T cells
XCL2		

Immunologic Classification of the Chemokine System

Chemokines and chemokine receptors have differential leukocyte specificity but together regulate all leukocyte subsets (Tables 28.5 and 28.6). As such, they can be loosely divided into two main functional systems, *homeostatic* and

P.692

P.693

inflammatory. Homeostatic chemokines are differentially and constitutively expressed primarily in specific microenvironments of primary and secondary immune organs, and coordinate migration of hematopoietic precursor cells, mature DCs, and naïve and central memory lymphocyte subsets via constitutively expressed receptors.[115,116,117,118,119] Inflammatory chemokines are induced by noxious stimuli in tissue cells and leukocytes. Inflammatory chemokine receptors tend to be constitutively expressed on myeloid cells, natural killer (NK) cells, and effector but not naïve lymphocytes.[120,121] Dynamic shifts in receptor expression occur during leukocyte differentiation, maturation, and activation.[115,122,123,124,125] This is not an absolute classification, because constitutive chemokines may be further induced, and chemokines that are highly inducible in some cell types may be constitutively expressed in others. In addition, there may be differences between mouse and human leukocyte subsets. Table 28.4 condenses a small portion of this vast subject available from the literature. Additional information can be obtained from the Immunologic Genome Project (www.immgen.org), which is a consortium systematically evaluating surface molecule expression in over 250 leukocyte subsets.

TABLE 28.6 Sources and Main Immunologic Functions of Human CC Chemokines

Chemokine	Sources	Main Immunologic Roles
CCL1	Inducible in Mo and CD4+ and CD8+ αβ and CD4-CD8- γδ T cells	Th2 response
CCL2	Inducible in Mo, fibroblasts, keratinocytes, EC, PMN, synoviocytes, mesangial cells, astrocytes, lung epithelial cells and MΦ Constitutively made in splenic arteriolar lymphatic sheath and medullary region of lymph node, many tumors, and arterial plaque EC Innate immunity	Th2 response CD4+ T cell differentiation
CCL3	Inducible in Mo/MΦ, CD8+ T cells, B cells, plts, PMN, Eo, Ba, DC, NK, mast cells, keratinocytes, fibroblasts, mesangial cells, astrocytes, microglial cells, epithelial cells	Innate immunity Th1 response CD4 T-cell differentiation
CCL3L1	Similar to CCL3	Probably similar to CCL3
CCL4	Similar to CCL3	Innate immunity Th1 response
CCL5	Inducible in EC, T cells, epithelial cells, Mo,	Innate

	fibroblasts, mesangial cells, NK cells Constitutively expressed and stored in plt and Eo granules	immunity Th1 and Th2 response
(Ccl6)	Inducible in bone marrow and peritoneal-derived MΦ	ND
CCL7	Inducible in Mo, plts, fibroblasts, EC, skin, bronchial epithelial cells, astrocytes	Th2 response
CCL8	Inducible in fibroblasts, PMN, astrocytes Constitutively expressed in colon, small intest, heart, lung, thymus, pancreas, spinal cord, ovary, placenta	Th2 response
(Ccl9/10)	Constitutively expressed in all mouse organs except brain; highest in lung, liver and thymus Induced in heart and lung	ND
CCL11	Epithelial cells, EC, smooth muscle, cardiac muscle, Eo, dermal fibroblasts, mast cells, MΦ, Reed-Sternberg cells	Th2 response Eosinophil trafficking Mast cell trafficking Basophil trafficking, degranulation
(Ccl12)	—Inducible in lung alveolar MΦ smooth muscle cells; spinal cord. Constitutive expression in lymph node and thymic stromal cells	Allergic inflammation
CCL13	Inducible in nasal and bronchial epithelial cells; dermal fibroblasts; PBMCs; atherosclerotic plaque EC and MΦ —Constitutively expressed in small intestine, colon, thymus, heart and placenta	Th2 response
CCL14a	Constitutively expressed in most organs; high plasma levels	ND
CCL14b	Same as CCL14b except absent from skeletal muscle and pancreas	ND
CCL15	Inducible in Mo and DC Constitutive RNA expression in liver, gut, heart and skeletal muscle, adrenal gland and lung leukocytes.	ND
CCL16	Constitutively expressed in liver, possibly many other organs. Also, Mo, T cells, and NK cells express mRNA.	DC maturation factor
CCL17	Constitutive in normal DC and Reed-Sternberg cells of Hodgkin's Disease	Th2 response
CCL18	Constitutive in Mo/MΦ, germinal center DC	DC attraction of naïve T cells Hematopoiesis
CCL19	Constitutive on interdigitating DC in secondary lymphoid tissue	Naïve and memory T cell & DC homing to lymph node
CCL20	Constitutive in follicle-associated epithelium overlying GALT inductive sites (Peyer's patches and isolated lymphoid follicles) Inducible in GALT	GALT development B and DC homing to GALT IgA humoral response in gut
CCL21	Constitutive in lymphatic EC, HEV, and interdigitating DC in T areas of 2° lymphoid tissue, thymic medullary epithelial cells and EC	Naïve and memory T cell and DC homing to lymph node
CCL22	Constitutive in DC and MΩ Inducible in Mo, T, and B cells	Th2 response
CCL23	Constitutive in pancreas and skeletal muscle	ND
CCL24	Inducible in Mo	Eosinophil migration
CCL25	Constitutive in thymic stromal cells and small intestine	Thymocyte migration Homing of memory T cells to gut
CCL26	Constitutive in heart and ovary Inducible on dermal fibroblastsand EC	Th2 response
CCL27	Constitutive in placenta, keratinocytes, testis, and brain	Homing of memory and effector T cells to skin
CCL28	Constitutive in epith cells of gut, airway	Homing of T cells to mucosal surfaces

The Issue of Chemokine Redundancy

Two chemokines that bind to the same receptor may still have highly specific biologic roles, due to differential expression in time and space,[126,127,128,129] activation of the same receptor via different signal transduction pathways or the same pathway with differential efficacy, or activation of additional differentially expressed receptors. A good example of differential expression is CXCL7 (neutrophil-activating protein-2) and CXCL8. Both are agonists for neutrophil CXCR2, but CXCL7 is stored in platelet α granules and released upon platelet activation,[130] whereas CXCL8 is made by many human cell types upon gene induction by proinflammatory stimuli. There are few inflammatory processes in which only one chemokine is involved. Instead, inflammatory chemokines typically act cooperatively, on the same or separate cell types, but in a hierarchical manner through promiscuous chemokine receptors sometimes in concert with classical chemoattractant receptors. The net result is that there may be enough functional redundancy in the whole system such that loss of a single inflammatory chemokine or chemokine receptor, for example in a knockout mouse, does not cause altered susceptibility to naturally acquired infections or other diseases, yet there may not be sufficient redundancy to handle a stronger stress when one particularly important component is missing. In contrast, homeostatic chemokines act much less redundantly, with typically only one or two ligands per receptor, and their loss has been associated with major defects in development and leukocyte trafficking and in some cases,

death.

Chemokine Presentation Mechanisms

Chemokines typically act locally and are thought to be concentrated for cell activation by tethering to endothelial cells via GAGs or transmembrane domains, or to matrix.[22,59] GAG-dependent oligomerization has been shown to be important for in vivo activity of CCL5 and CXCL10.[62,131] The tethering cell may have produced the chemokine or else imported it by transcytosis from neighbors.[132,133] The ligand binding site includes 1) the receptor *N*-terminus and one or more extracellular loops, depending on the specific chemokine, which allow docking of the chemokine *N*-loop domain, and 2) the 7TM domains, which then accept the chemokine's *N*-terminus and are critical for triggering[134,135] (see Fig. 28.5C). Tyrosine sulfation on the receptor *N*-terminus has been shown to facilitate chemokine receptor binding to both chemokine and nonchemokine ligands (eg, HIV gp120).[136,137]

LEUKOCYTE RESPONSES TO CHEMOKINES

Both homeostatic and inflammatory leukocyte trafficking requires transendothelial migration, a multistep process involving an endothelium- and leukocyte-specific molecular code, in which chemokines regulate at least two steps.[16,18] In an initial chemokine-independent step, leukocytes roll on inflamed endothelium in a selectin-dependent manner. Next, chemokines posted on endothelium stimulate rolling leukocytes to express activated β2 integrins, which mediate shear-resistant adhesion via endothelial intercellular adhesion molecules. Leukocytes respond to chemokine gradients by changing their shape to produce a front and a back and by crawling toward the front.[138,139] Motion involves sheardependent coordinated cytoskeletal remodeling, involving expansion of the front (lamellipodium), myosin-based contraction at the back (uropod), release of the uropod from substrate, and membrane lipid movement. Navigation through tissue may require relays of chemokines and adhesion molecules. Fine navigation within tissue microenvironments has also been shown to be directed by specific chemokines up to the point of immunologic synapse formation.[26,140,141,142] There is now evidence that CCR5 and CXCR4 may accumulate in the immunologic synapse formed by CD4+ T cells and DCs in vitro, and that this may provide chemokine-dependent costimulatory signals.[142,143]

Chemokines and their receptors have increasingly been implicated in other cell functions, including regulation of proliferation and apoptosis,[34,144,145,146,147,148,149,150,151,152,153,154] regulation of antigen sampling by immature DCs (eg, CCR7 and CX3CR1), maturation of DCs (CCR7), and inhibition of mature DC endocytosis (CCR7).[155,156] Inflammatory chemokines may induce mediator release (eg, defensins, proteases, perforins, histamine, eicosanoids) resulting in cytotoxic or vasomotor responses.[121,157] Some chemokines even have direct antibacterial activity,[158,159] which could explain why some of them can be found constitutively at high concentrations in body fluids such as blood and sweat.[109]

CHEMOKINE SIGNALING PATHWAYS

Gi-Dependent Effectors

Classically, ligand-bound chemokine receptors, possibly in the dimer state,[72] act as guanine nucleotide exchange factors, especially for G_{i2} (Fig. 28.6).[160] G protein activation presumably

involves a conformational change in the transmembrane domains of the receptor and binding of the G protein to multiple intracellular domains, including the C-tail, intracellular loop 3 and the DRY motif at the end of TMD2. The latter is highly conserved in classic chemokine receptors but mutated in atypical chemokine receptors, which do not couple to G protein-dependent signaling. Activation of G proteins involves exchange of GDP for GTP by the α subunit, leading to dissociation of the trimer into α and βγsubunits. The duration of G protein signaling is regulated in part by intrinsic GTPase activity possessed by the α subunit which can be markedly increased by GTPase activating proteins known as RGS proteins (*r*egulators of *G* protein signaling).[160,161] Signaling appears to be mainly mediated by βγ ,[162,163] which activates diverse effectors, including phospholipases A2, C (subtypes β2 and β3) and D, phosphatidylinositol-3-kinase γ (PI3Kγ), protein tyrosine kinases and phosphatases, low molecular weight GTPases, and mitogen-activated protein kinases. The requirements of these enzymes for chemotaxis are both chemoattractant- and celltype dependent, and the complex mechanistic details underlying specificity remain to be elucidated.

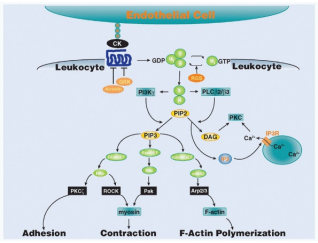

FIG. 28.6. Chemokine Signal Transduction in Chemotaxis. Depicted are key steps in two of the main pathways induced by most chemokines. The PI3Kγ pathway is particularly important for cell migration. Chemokines are able to activate other pathways as well, including non-Gitype G proteins, protein tyrosine kinases and MAP kinases, resulting in effects on cell proliferation and activation. Model is modified from the Alliance for Cell Signaling (www.signaling-gateway.org). CK, chemokine; DAG, diacylglycerol; GAG, glycosaminoglycan; GEF, guanine nucleotide exchange factor; GRK, G protein-coupled receptor kinase; IP3, inositol trisphosphate; PI3K, phosphatidylinositol-3-kinase; PIP, phosphatidylinsol phosphate; PKC, protein kinase C; PLC, phospholipase C; RGS, regulator of G protein signaling.

PLC hydrolyzes PI(4,5) bisphosphate (PIP_2) to form 1,2-diacylglycerol and inositol-1,4,5-trisphosphate.[164,165] Inositol-1,4,5-trisphosphate induces calcium release from intracellular stores, which acts with diacylglycerol to activate protein kinase C. PI3Kγ phosphorylates PIP_2 to form PIP_3, which recruits proteins containing pleckstrin homology or Phox domains to lamellipodium, thereby converting shallow extracellular chemokine gradients to steep intracellular effector gradients.[166,167] Four pleckstrin homology domain-containing targets —Akt, and guanine nucleotide exchange factors for Rac, Rho, and cdc42—modulate distinct phases of cell movement in various model systems. Rho regulates cell adhesion and chemotaxis, and myosin contraction. Rac and cdc42 control lamellipodia and filipodia formation, respectively. Downstream targets of Rac

include PAK1, which also regulates myosin contraction. In neutrophils, signal amplification, gradient sensing, and cell orientation have been shown to be further amplified and reinforced by ATP release at the leading edge, which provides autocrine feedback signaling through P2Y2 nucleotide receptors. This is further amplified by conversion of ATP to extracellular adenosine, which signals through A3 receptors mobilized to the leading edge.[168] Motility, phagocytosis, and immunologic synapse formation are all critically dependent on local polymerization of actin, coordinated by actinbinding proteins.

Gi-Independent Effectors

Many chemokine receptors couple to G proteins other than Gi in vitro; however, the role of these proteins in vivo is not well defined. There is in vitro evidence from studies of the fMet-Leu-Phe receptor that G12/13 through activation of Rho may be important in formation of the uropod.[169] Mice lacking both G12 and G13 selectively in B cells have reduced numbers of splenic marginal zone B cells, resulting in a delay of antibody production in response to thymus-independent antigens. However, responsiveness of these cells to chemokines is normal. Instead, the phenotype may be due to impaired sphingosine-1-phosphate signaling.[170]

Chemokines may activate effectors such as MAP kinases and nonreceptor protein tyrosine kinases by a variety of Gi-independent mechanisms. An example is CXCR4, which has been shown to physically associate with T-cell receptors (TCRs) in a CXCL12-dependent manner, resulting in activation of ZAP-70 and downstream effectors, including ERK, calcium flux, AP-1, and costimulation of cytokine secretion.[143] β-arrestin signaling has been reported for

chemokine receptors, including the atypical receptor CCBP2/D6.[88] Constitutive activity has also been reported, most notably for the HHV8 receptor encoded by ORF74.[171] Signaling through the Jak-Stat pathway has also been proposed and debated.[72,172]

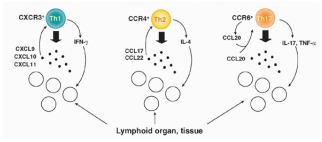

FIG. 28.7. Chemokine-Cytokine Feedback Loops in T Helper Cell Polarization. *Dots* denote gradients of the chemokines indicated to the left. *Thick arrows* denote the direction of T helper cell movement. *Afferent thin arrows*, release of the indicated factors from the indicated cell; *efferent thin arrows*, action of the indicated factor on the indicated cell. The figure was created and generously provided by Joshua Farber, National Institute for Allergy and Infectious Diseases.

REGULATION OF CHEMOKINE ACTION

Virtually all nucleated mouse and human cell types are able to produce chemokines, but in a differential and highly regulated manner (see Tables 28.5 and 28.6). Chemokine and chemokine receptor expression may be positively or negatively regulated at the transcriptional level by diverse factors, including proinflammatory cytokines, hypoxia, viruses, bacterial products, cell adhesion, shear stress, antigen uptake, T-cell costimulation, and diverse transcription factors. Proinflammatory cytokines such as interleukin (IL)-1, tumor necrosis factor, and IL-15 induce expression of many of the inflammatory chemokines involved in innate immunity such as CXCL8, whereas immunoregulatory cytokines such as interferon (IFN)γ, IL-4, and IL-17 are more tightly focused on Th1 (eg, CXCL9-11)-, Th2 (eg, CCL11)-, and Th17 (CCL20)-selective chemokines, respectively (Fig. 28.7). IFNs, glucocorticoids, and anti-inflammatory cytokines (eg, IL-10) may inhibit inflammatory chemokine gene expression. Chemokines may also be regulated at the level of messenger ribonucleic acid stability and cell type-specific polyubiquitinylation and proteasomal degradation.[173,174]

A chemokine gene may generate families of proteins varying dramatically in activity and potency by alternative splicing and posttranslational modification, especially *N*- and *C*-terminal proteolytic trimming. Proteases may target many chemokines (eg, CD26 [dipeptidyl peptidase IV] and matrix metalloproteinases),[175,176,177,178] or few or only one (eg, TACE [the tumor necrosis factor α converting enzyme],[179] plasmin, urokinase plasminogen activator, and cathepsin G), and the cleaved forms may be the dominant forms in biologic fluids, as shown for several CCR1 ligands.[180] Chemokine action may be blocked by receptor decoys (eg, DARC),[181] endogenous receptor antagonists, and autoantibodies. In addition, cytokines may convert a signaling receptor into a decoy (eg, IL-10 inactivates CCR2 and other receptors on monocytes/macrophages).[182] Also, a receptor may have different functions on different cell types.

P.696

In addition to RGS proteins, chemokine receptor signaling may be regulated by homologous and heterologous desensitization, which involves phosphorylation by G protein-coupled receptor kinases or protein kinase C and protein kinase A, respectively, and internalization by clathrin-dependent and -independent mechanisms.[183] Release mechanisms may vary dramatically for different chemokines. CXCL4 is stored in platelet α granules, whereas the highly related CXCL4L1 is continuously produced and released.[184] CCL5 is released from T-cell granules preassociated with GAGs.[185] CCL3 is released multidirectionally from activated T cells, presumably to create chemotactic gradients, unlike IL-2 and IFNγ which are secreted directly into the immunologic synapse.[186]

CHEMOKINE REGULATION OF HEMATOPOIESIS

For the next two sections, Figure 28.8 provides an overview of chemokine receptors that regulate movement of specific leukocyte subsets between compartments of the hematopoietic system during immune system development and during the immune response. Table 28.7 contains a list of major phenotypes observed in chemokine receptor knockout mice.

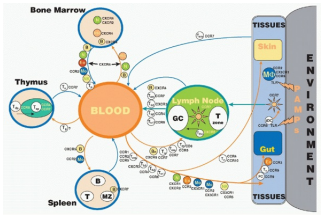

FIG. 28.8. Chemokine Control of Leukocyte Trafficking. Shown are routes among primary and secondary immune organs and the periphery, leukocyte subtypes trafficking on those routes and some of the chemokine receptors that appear to be most important in regulating each route. Eo, eosinophil; iDC, immature dendritic cells; GC, germinal center; HSC, hematopoietic stem cell; MΦ, macrophage; Mo, monocyte; MZ, marginal zone; N, neutrophil; NK, natural killer cell; PAMP, pathogen-associated molecular patterns; PC, plasma cell; Tcm, central memory T cells; Tem, effector memory T cells; T_{FH}, follicular help T cells; TLR, Toll-like receptor; Tm, memory T cells; Tn, naïve T cells; Tp, precursor T cells. Model is based primarily on studies in knockout mice.

CXCR2, CXCR4, and CXCR7

Originally identified as a stromal cell-derived pre-B cell stimulatory factor that also enhanced hematopoietic progenitor cell (HPC) colony formation ex vivo, the homeostatic chemokine CXCL12 is the most abundant chemokine produced in bone marrow. Its production is under the control of granulocyte-colony stimulating factor and adrenergic neurotransmission, among other factors.[34,187] CXCL12 is the only chemokine ligand known for CXCR4 but also binds to the atypical receptors DARC and CXCR7.[188,189,190,191,192,193,194,195,196,197,198,199,200] CXCL12, CXCR4, and CXCR7 are unusual among chemokine elements in being extremely highly conserved phylogenetically from fish to man, yet CXCR4 and CXCR7, despite binding the same ligand, are very distantly related (see Fig. 28.3). The three molecules also have the shared property of being essential for life in mice, the only chemokine system components tested to date for which this is true.[35,201] Yet their biologic roles are very different.

Loss of Cxcl12 or Cxcr4 in knockout mice results in the same dramatic and fatal multisystem developmental phenotype: ventricular septal defect, defective gastric vascularization, defective cerebellar development, and severe defects

P.697

P.698

in bone marrow myelopoiesis and B-cell lymphopoiesis. The phenotype is consistent with the widespread constitutive expression of both CXCL12 and CXCR4 on both hematopoietic and vascular cells. In bone marrow, CXCR4 activation by CXCL12 is thought to induce β2 integrin-mediated retention of neutrophils, hematopoietic stem cells, and probably other hematopoietic cell types, which normally regulate leukocyte distribution among hematopoietic compartments.[189,202,203] The balance of CXCR2 and CXCR4 signaling is thought to be particularly important in regulating neutrophil distribution between blood and bone marrow.[204,205] Immature neutrophils express high levels of CXCR4 and low levels of CXCR2. As they mature, there is a reciprocal switch in expression of these two receptors, and the cells leave the marrow in response in part to CXCR2 signaling. As the cells senesce in the periphery and undergo apoptosis, CXCR4 expression increases and the cells home back to marrow in response to CXCL12 activation for destruction by macrophages. The importance of CXCR4 in this process is dramatically illustrated by patients with WHIM syndrome (*w*arts, *h*ypogammaglobulinemia, *i*nfections, and *m*yelokathexis syndrome), a primary immunodeficiency that will be described in the section on disease.

TABLE 28.7 Some Phenotypes of Chemokine Receptor Knockout Mice

Receptor	Phenotype of Knockout Mouse
Cxcr2	• Increased susceptibility to *Toxoplasma gondii*, brain abscess, *Onchocerca volvulus*, *Escherichia coli* pyelonephritis • Reduced: wound healing, *Aspergillus fumigatus* AHR, urate crystal-induced synovitis • Expansion of neutrophils and B cells in blood, marrow, and lymphoid organs (not seen when derived in germ-free environment)
Cxcr3	• Increased susceptibility to MTb • Delayed cardiac allograft rejection
Cxcr4	• Perinatal lethality • Defective: ventricular septum, bone marrow myelopoiesis, B-cell lymphopoiesis, gastric vascularization, cerebellar granule cell migration
Cxcr5	• Few Peyer's patches, no inguinal LN; defective germinal centers and B-cell homing to LN
Cxcr7	• Defective cardiac valve development
Ccr1	• Increased susceptibility to *T. gondii*, Pneumonia virus of mice, *A. fumigatus* • Increased:Th1 response and glomerular injury in nephrotoxic nephritis model, Th2 response to SEA • Reduced: neutrophilic alveolitis in pancreatitis-acute respiratory distress syndrome model, airway remodeling and Th2 response in *A. fumigatus* model; Th1 response and resistance to EAE; Th1 response to PPD; delayed cardiac allograft rejection
Ccr2	• Monocytopenia; enhanced myeloid progenitor cell cycling and apoptosis • Increased susceptibility to *Cryptococcus neoformans*; mouse hepatitis virus; *Leishmania monocytogenes*; *Leishmania major*; *Mycobacterium tuberculosis* • Resistance to *Leishmania donovani*, influenza A • Decreased: EAE; DVT resolution; response to PPD; atherosclerosis; cardiac allograft rejection; AHR to CRA; dextran sulfate-mediated colitis; thioglycollate-induced peritonitis; FITC and bleomycin induced pulmonary fibrosis; MΦ recruited to injured nerve; DTH; ⁺-monocyte extravasation • Increased: AHR to OVA and to *A. fumigatus*; glomerulonephritis in antiglomerular basement membrane antibody model
Ccr3	• ip OVA sensitization → OVA challenge: increased AHR and airway mast cells; decreased airway eos, trapped between elastic lamina and endothelial cells • Epicutaneous OVA sensitization → OVA challenge: protection from allergic skin inflammation and AHR. Eos and mast cells not recruited to skin or lung. • Decreased IL-13-induced pulmonary eosinophilia and remodeling
Ccr4	• Increased susceptibility to Ips
Ccr5	• Increased susceptibility to *L. monocytogenes*; *C. neoformans*; *T. gondii*; influenza A; *L. major* • Resistance to *L. donovani* • Decreased: dextran sulfate-mediated colitis; Lps toxicity; mouse hepatitis virus-induced demyelination due to decreased MΦ recruitment to CNS; cardiac allograft rejection • Increased: DTH; humoral response to T-dependent Ag
Ccr6	• Defective CRA-induced allergic airway inflammation • Increased contact hypersensitivity to 2,4-dinitrofluorobenzene • Resistance to DTH to allogeneic splenocytes • Absent myeloid CD11b+ CD11c+ dendritic-cells in subepithelial dome of Peyer's patches • Increased T cells in intestinal mucosa • Impaired humoral immune response to orally administered antigen and to rotavirus • Protection from IL-22-induced psoriasoform lesions in skin
Ccr7	• Defective: humoral responses; contact sensitivity; DTH; renal fibrosis and fibrocyte accumulation in unilateral ureteral obstruction; lymphocyte and DC migration to LN • Undeveloped T-cell zones; impaired trafficking of T cells and DC to LN
Ccr8	• Defective SEA-induced granuloma formation; • Decreased OVA- and CRA-induced allergic airway inflammation
Ccr9	• Decreased: preproB cells, but nl T and B cells; ratio of gut intraepithelial T-cell-to-epithelial cell ratio due to decreased γδ T cells
Cx3cr1	• Increased susceptibility to *Salmonella* infection and to retinal degeneration • Resistance to atherosclerosis and cardiac allograft rejection in cyclosporin A treated animals
Darc	• Increased or decreased neutrophil mobilization, depending on the model
Ccbp2	• Increased CFA- or TPA-induced psoriasiform dermatitis • Reduced EAE

AHR, airway hyperreactivity; CRA, cockroach antigen; DC, dendritic cell; DTH, delayed type hypersensitivity; EAE, experimental allergic encephalitis; LN, lymph node; NR, not reported; OVA, ovalbumin; SEA, *Schistosoma mansoni* soluble egg antigen.

A developmental role for CXCR4 has also been found in zebrafish, where the orthologue *cxcr4b* mediates gonadal germ cell migration and self-organizing tissue migration in the lateral line during morphogenesis.[206] Consistent with a more general role in vascular biology, CXCR4 is expressed on vascular and cancer stem cells under the control of oxygen tension. These cells home to vascular niches under hypoxic conditions that occur during wound healing and in tumors. Hypoxia induces reduction of pVHL, which derepresses transcription factor HIF-1, a strong inducer of *CXCR4* expression.[207,208,209]

In contrast to CXCR4, CXCR7 is expressed in a more restricted manner, on marginal zone B cells, possibly on some T-cell subsets and on endothelial cells of all four cardiac valves.[35,188,195] It has been reported to couple to β-arrestin-but not G protein-dependent signaling, and to modulate cell survival, adhesion, and tumorigenesis.[195] It may function as a chemokine scavenger in vitro, and in zebrafish has been shown to modulate gonadal germ cell migration by shaping gradients of CXCL12 acting at CXCR4 as the signaling receptor.[21] In mice, it plays an essential role in development of all four cardiac valves, but the mechanism is not precisely defined.[35]

Myelosuppressive Chemokines

When added to bone marrow culture systems ex vivo, many chemokines are able to suppress

growth factor-dependent colony formation, apparently by acting directly on stem cells and early progenitors. The biologic significance of this remains unclear except for CXCR2. *Cxcr2*-/- mice develop massive neutrophilia, splenomegaly, myeloid hyperplasia, and expansion of HPCs.[210] CXCR2-dependent myelosuppression may be important for opposing overstimulation of hematopoiesis induced by environmental flora, as this phenotype is absent in mice derived in germ-free conditions. These mice also develop lymph node B-cell hyperplasia, which may occur by an indirect mechanism.

T Lymphopoiesis

During development, T cells must migrate from thymic cortex to medulla. CCR9 and its ligand CCL25 appear to be important as competitive transplantation of *Ccr9*-/- bone marrow is less efficient than normal marrow at repopulating the thymus of lethally irradiated *Rag-1*-/- mice.[211,212] CCL25 is expressed by medullary DCs and both cortical and medullary epithelial cells, and CCR9 is expressed on the majority of immature CD4+CD8+ thymocytes, but is downregulated during transition to the CD4+ or CD8+ single-positive stage.[213] Just before thymic egress, thymocytes become CCR9 negative and upregulate L-selectin. Transition from CD4+CD8+ thymocytes in the cortex to CD4 or CD8 single-positive thymocytes in the medulla is also associated with upregulation of CCR4 and CCR7, receptors for CCL22, and CCL19 and CCL21, respectively, which are expressed on endothelial cells of medullary venules. Studies with knockout mice have suggested that CCR7-dependent cortex to medulla migration of positively selected thymocytes may be essential for establishing central tolerance but not for maturation or export of thymocytes in vivo.[214]

Hematopoietic Cell Positioning in Peripheral Tissue

CXCL14 has been reported to have antitumor activity. It is a highly selective chemoattractant for monocytes and DCs, and may be important for their positioning in certain tumors.[215,216] CCL11 and its receptor CCR3 are important for migration of eosinophils to the gastrointestinal tract (and spleen).[23] CCL20 and its receptor CCR6 regulate positioning of immature myeloid CD11c+CD11b+ DC in the subepithelial dome of Peyer's patches, which may explain in part why humoral immune responses within the gut mucosa and defense against enteroinvasive pathogens are abnormal in *Ccr6*-/- mice.[217,218] CCR6 is also expressed on gut-homing B and T cells, which may become rapidly activated by CCR6+ DCs. CX3CR1 also regulates location of myeloid DCs in Peyer's patches and may promote dendrite protrusion into the lumen as a method of antigen sampling. Consistent with this, CX3CR1 is important for controlling *Salmonella* infection after oral challenge.[155] CCL25/CCR9 signaling regulates gut cryptopatch formation and subsequent positioning of intraepithelial lymphocytes.[218]

CHEMOKINE REGULATION OF THE IMMUNE RESPONSE

Innate Immunity

Platelet-Derived Chemokines

Made primarily during platelet development, stored in platelet α granules and rapidly released during platelet degranulation, CXCL4, CXCL4L1, and CXCL7 may be among the first chemokines to appear at sites of tissue injury

and infection, particularly when there is hemorrhage and vascular damage, and may reach high concentrations.[130,219] CXCL4 aggregates to form tetramers critical for binding to chondroitin sulfate proteoglycans. In contrast, CXCL7 is activated by sequential proteolysis of its *N*-terminus. The preproppeptide form, named platelet basic protein (92 aa), is trimmed during platelet maturation to produce the 85 aa major stored form, named connective tissue activating peptide-III, which is inactive on neutrophils. Connective tissue activating peptide-III is further processed during degranulation to 81 aa β-thromboglobulin, also inactive. This is then cleaved by a cell surface-bound, cathepsin G-like enzyme on neutrophils to form 70 aa CXCL7, which has high homology to CXCL4 (70% aa identity). Thus, CXCL7 may function as an immediate-early mediator of neutrophil recruitment released from platelets at sites of inflammation. Although it is not a prominent leukocyte chemoattractant and does not induce degranulation of neutrophil lysosomal enzymes, CXCL4 is able to induce secondary granule exocytosis and release matrix-degrading enzymes that may facilitate neutrophil penetration of infected or injured tissues. Both CXCL4 and CXCL4L1, the product of the highly homologous non-allelic variant gene of CXCL4, *PF4V1*, are strong angiostatic factors.[220,221] In contrast, another plateletderived chemokine, CXCL12, may be a major determinant of revascularization, as suggested by an ischemic hindlimb mouse model, where its release could be regulated by Kit ligand and thrombopoietin and it functioned to recruit CXCR4+ VEGFR+ hemangiocytes to the site of injury.[222] The ultimate response in inflammation and revascularization may depend on the relative concentration and activities of these counterregulatory chemokines.

Neutrophil-Targeted Chemokines

All seven ELR+ CXC chemokines preferentially recruit neutrophils in vitro by binding to CXCR2. One of these, CXCL8, is also a potent agonist at CXCR1, which is coexpressed at similar levels on neutrophils.[223] These seven chemokines are rapidly inducible but may differ biologically due to temporal and spatial differences in expression, which may provide a mechanism for graded navigation of neutrophils through tissue. Blocking studies in multiple animal models have demonstrated the importance of CXCL8 and CXCR2 in neutrophil accumulation in response to infectious and noninfectious stimuli.[224,225] The role of CXCR1 in vivo has not yet been defined and is difficult to analyze because its major human agonist, CXCL8, is not found in the mouse. In a human blister model, endogenous CXCL8 peaks at ~24 hours, whereas C5a and leukotriene B4, which also recruit neutrophils, appear earlier. Thus the primary role of CXCL8 may be to amplify recruitment of neutrophils initiated by other chemoattractants.

Intradermal injection of CXCL8 in man causes rapid (<30 minutes) and selective accumulation of large numbers of neutrophils in perivascular regions of the skin without causing edema. Tissue-specific transgenic overexpression of mouse CXCL8 paralogues Cxcl1 (KC) and Cxcl2 (MIP-2) results in a similar picture, suggesting that in vivo these factors may recruit cells but not independently activate cytotoxic mechanisms. In vitro, the ability of CXC chemokines to activate neutrophil cytotoxic responses is modest. Instead, they may prolong survival of neutrophils in the setting of bacterial infection and paradoxically provide an intracellular niche in which at least some bacterial species such as *Staphylococcus aureus* may persist.[226] Studies in mice have revealed that αβ CD4+ T cells are critical in providing CXC chemokines that foster abscess formation in several models of experimental *S. aureus* infection and that the absence of these cells results paradoxically in smaller/fewer abscesses with reduced bacterial burden. In addition, pathogens such as *Streptococci* may actively interfere with the action of CXC chemokines by degrading them with specific proteases.[227] Thus, while there is no question that neutrophils are critical in defense against bacterial and fungal pathogens and that CXCR2 agonists are critical for neutrophil trafficking in the setting of infectious disease, the ultimate outcome of infection may be determined by a balance between CXCR2-dependent neutrophil recruitment, niche development, and negative regulatory mechanisms.

CXCL1, 2, 3, 7, and 8 have also been reported to induce basophil chemotaxis and histamine release in vitro, which together with other factors such as complement-derived anaphylatoxins may promote vasodilatation during early stages of the innate immune response.[157] Some CC chemokines can also activate human neutrophils, but this typically requires prestimulation of the cells with IFNγ and other factors able to induce expression of appropriate receptors. Conversely, late apoptotic neutrophils can actively promote the resolution phase of acute inflammation by terminating chemokine signaling through scavenging by CCR5, which is expressed in a manner dependent on caspases and antiinflammatory lipid mediators.[228] In the mouse, CCR1 is an important neutrophil chemotactic receptor and has been demonstrated to function in a sequential manner with the LTB4 receptor for neutrophil recruitment in a model of rheumatoid arthritis.[229]

Monocyte/Macrophage-Targeted Chemokines

Circulating blood monocytes can be divided into two principal subsets with distinct migratory properties based on chemokine receptor expression.[230] *Inflammatory* monocytes are short-lived CX3CR1^{lo}CCR2+Ly6C^{hi} cells actively recruited to inflamed tissues in the mouse. These cells correspond to human CD14^{hi}CD16- monocytes. *Resident* monocytes are long-lived CX3CR1^{hi}CCR2-Ly6C^{lo} cells in the mouse, characterized by CX3CR1-dependent engraftment and recruitment to noninflamed tissues. These cells correspond to human CD14^{lo}CD16+ monocytes that are CX3CR1^{hi} but do not express inflammatory chemokine receptors. Both subsets can differentiate into DCs in vivo that stimulate naïve T cells. There are numerous examples of the functional importance of these receptors in the monocyte/macrophage lineage in vivo. Monocyte CCR2 is required for normal bone marrow egress of monocytes to blood under homeostatic conditions in mice, as well as after infection with pathogens such as *Listeria* or West Nile virus.[231,232,233] Both receptors have been linked to human disease, as will be detailed in the

disease section. Monocytes express additional inflammatory chemokine receptors including CCR1, CCR5, CXCR1, and CXCR2. Differentiation in vitro to macrophages is associated with downregulation of CCR2 and induction of CCR5 and CX3CR1. Polarized M1 and M2 subsets of macrophages have been described in keeping with the Th1/Th2 polarization paradigm for T cells. In this regard, the classic anti-inflammatory Th2 cytokine IL-10 is able to downregulate signaling by inflammatory chemokine receptors on macrophages.[234]

Natural Killer Cell-Targeted Chemokines

Human NK cell subsets express unique repertoires of chemokine receptors.[122] The $CD56^{dim}CD16+$ subset, which is associated with high cytotoxic capacity and low cytokine production, expresses primarily CXCR1 and CX3CR1, whereas the minor subset of $CD56^{bright}CD16^{dim}$ cells, which produce large amounts of cytokines but have low killing capacity, preferentially express CCR7.[120] The exact profile of chemokine receptor expression can be modulated by adherence and stimulation ex vivo with IL-2.[121] Cognate chemokines chemoattract NK cells and promote degranulation and killing. The importance of chemokines in NK cell function in vivo is well illustrated by mouse cytomegalovirus infection, a cause of hepatitis. Mouse cytomegalovirus induces CCL3 production in the liver, which is required for recruitment of NK cells. NK cells are the major source of IFNγ in this model, and IFNγ induces CXCL9, which is required for protection. Thus, a cytokine to chemokine to cytokine cascade is required for NK cell-mediated host defense against this pathogen.[235]

Dendritic Cells and Transition to the Adaptive Immune Response

Transition from innate to adaptive phases of the immune response involves antigen uptake by antigen-presenting cells, especially DCs, mediated by Fc and complement phagocytic receptors, as well as pattern recognition receptors, including DC-specific intercellular adhesion molecule and toll-like receptors.[236] Through specific pattern recognition receptor ligands, pathogens may shape the nature and magnitude of the immune response in a specific direction, in part by inducing production of specific sets of chemokines with distinct leukocyte specificities.[6,237]

Chemokine receptor expression and function depends not only on the nature of the inflammatory stimulus, but also on the type of DC. For example, blood-derived plasmacytoid and myeloid DCs express a similar repertoire of inflammatory chemoattractant receptors but they are functional only on myeloid DCs. CCL3, CCL4, and CCL5 may be particularly important for recruiting additional mononuclear phagocytes and DCs to sites of infection. This can amplify the late stage of the innate immune response, and in the extreme may devolve into endotoxic shock. Consistent with this idea, genetic disruption of the CCL3/CCL4/CCL5 receptor CCR5 renders mice relatively resistant to lipopolysaccharide-induced endotoxemia. CCR4-/- mice are also resistant to endotoxic shock; this is associated with reduced macrophage extravasation to the peritoneum and production of the CCR4 ligand CCL17, which can be produced by DCs.[238]

Adaptive Immunity

Afferent Trafficking to Secondary Lymphoid Tissue

The homeostatic receptors CXCR4, CXCR5, and CCR7 and their ligands are major regulators of lymphoid development and the adaptive immune response.[20,123,239,240,241,242] DC maturation in peripheral tissues is associated with downregulation of inflammatory receptors, which may be important for recruitment, migration, and retention in the periphery, and reciprocal upregulation of CCR7, which mediates mature DC migration via afferent lymphatics to draining lymph nodes in response to CCL21 expressed on lymphatic endothelial cells. Inflammatory receptors may also contribute as demonstrated for CCR2 on Langerhans cells in a mouse model of *Leishmania* infection.[243]

With regard to lymphocytes, CCR7, and to a lesser extent CXCR4, mediate both B- and T-cell entry from blood into lymph node. In lymph node and Peyer's patch T zones, CCL21 is produced by high endothelial venule (HEV) endothelial cells, whereas CCL19 (the second CCR7 ligand) and the CXCR4 ligand CXCL12 are produced by adjacent stromal cells. All three chemokines are displayed on the luminal and abluminal sides of the HEV appropriately positioned to mediate transendothelial migration from the blood of naïve B- and T-lymphocytes, which both coexpress CCR7 and CXCR4.[244,245] CCR7 plays a dominant role in mediating B- and T-cell adhesion, but CXCR4 also contributes. *Ccr7-/-* mice and the *plt* mouse, which is naturally deficient in Ccl19 and the Ccl21 isoforms expressed in secondary lymphoid organs (see Fig. 28.2 for *plt* locus description),[111] have similar phenotypes: atrophic T-cell zones populated by a paucity of naïve T cells. This plus the failure of activated DCs to migrate to lymph node from the skin of these mice, explains why contact sensitivity, delayed type hypersensitivity and antibody production are severely impaired.

CXCR5 is expressed on all peripheral blood and tonsillar B cells, but only on a fraction of bone marrow B cells. Its ligand CXCL13, produced selectively by HEV endothelial cells and follicular stromal cells, is constitutively displayed on follicular HEV. The CXCL13/CXCR5 axis supports ~50% of the signaling required for B cell entry from blood to Peyer's patch; CXCR4 may also contribute.[244,245] In *Cxcr5-/-* mice, B cells do not migrate to lymph node, Peyer's patches are abnormal, and inguinal lymph nodes are absent. CXCL13 is also required for B1 cell homing, natural antibody production, body cavity immunity, and lymphoneogenesis in the setting of autoimmunity.[246,247] *Cxcr5-/-* mice still can produce antibody, perhaps in part because B cells and follicular DCs, by an unknown mechanism, are able to form ectopic germinal centers within T zones of the periarteriolar lymphocyte sheath of spleen. In Peyer's patches, B-cell entry is also dependent on CCR7 and CXCR4.

CXCR5 and CCR7 are probably not the only chemokine receptors responsible for afferent trafficking of leukocytes to lymph node. CCL9 has been reported to mediate monocyte homing.[248] In addition, inflamed peripheral tissues

may exert "remote control" for monocyte homing to draining lymph nodes from the blood by "projecting" their local chemokine profile to HEVs of the draining lymph node.[249] Migration of T cells to splenic red pulp may involve local production of CXCL16.[52] NK-T cells and activated CD4+ and CD8+ T cells are found in this area and express the CXCL16 receptor CXCR6. CXCL16 is also made by DCs in the T zone, and CXCR6 is also found on intraepithelial lymphocytes. Thus, CXCL16 may function in T cell-DC interactions and in regulating movements of activated T cells in the splenic red pulp, and in peripheral tissues.

Migration within Lymph Node Microenvironments

CXCR5 and CCR7 also appear to be important for lymphocyte movement within the lymphoid microenvironment. So-called follicular help T cells, a CD57+ subset, lack CCR7 but express CXCR5, which appears to facilitate migration from the T zone following activation to the follicles in response to CXCL13, where they provide help for B-cell maturation and antibody production. Reciprocally, B cells activated by antigen in the follicles upregulate CCR7 and move toward the T zone in response to CCL21.[250] Thus B-T interaction may be facilitated by reciprocal movement of these cells, which may be determined in part by the balance of chemokines made in adjacent lymphoid zones.

CXCR4 signaling may also be important in naïve and memory B cell trafficking to germinal centers. An active chemokine-dependent process has also been identified for CD4+ T cell-dependent activation of rare antigen-specific naïve CD8+ T cells.[141] After immunization in a TCR-transgenic mouse model, but before antigen recognition, naïve CD8+ T cells in immunogen-draining lymph nodes upregulate CCR5, permitting the cells to home to sites of CD4+ T cell-DC interaction in lymph nodes, where CCL3 and CCL4 are produced. Interference with this process inhibits antigen-specific memory CD8+ T-cell generation. There is also evidence that T-cell zone chemokines such as CCL21 are bound to the surface of lymph node DCs in vivo and function to capture and prime naïve T cells for activation by peptide-MHC. Thus T cells are costimulated "in trans" and sequentially after initial engagement with their chemokine-rich environment.[251]

DCs appear to swarm to T-cell zones in lymph node by using immobilized CCL21 for adhesive random migration and soluble CCL21 for chemotactic steering, both acting through CCR7 activation.[252,253] CCL21 is produced by fibroblastic reticular cells in T-cell zones and is immobilized on the reticular network by GAG interaction with its extended C-terminal domain. The chemokine domain appears to be cleaved from the GAG binding domain by an undefined protease. CCL19, the other CCR7 ligand, lacks a C-terminal domain extension and may preferentially function chemotactically as a soluble ligand, not haptotactically bound to surface.

Efferent Trafficking

Critical determinants of lymphocyte egress from lymph node are sphingosine-1-phosphate and its receptor S1P.[19,20] Naïve lymphocytes that do not encounter antigen continue to recirculate between the blood and secondary lymphoid tissue without acquiring any tissue-specific homing properties. In contrast, most antigen-stimulated T cells die by apoptosis. The survivors may be divided into functionally distinct subsets that preferentially express certain chemokine receptors. Within the CD4+ subpopulation, at least three memory subsets and two primary effector subsets can be distinguished. The memory subsets include follicular help T

cells, described previously, and effector memory (T_{EM}) and central memory (T_{CM}) cells.[254] Memory cells classically express CD45RO. In addition to this, classic T_{CM} cells also express L-selectin (CD62L) and CCR7, which are thought to mediate homing of the cells from blood to secondary lymphoid organs across HEVs; these cells are not polarized and lack immediate effector function. Instead, they are thought to interact with DCs in lymph node and differentiate into T_{EM} upon secondary stimulation. T_{EM} cells lack L-selectin and CCR7, and were originally proposed to differentiate from T_{CM} and to traffic through peripheral tissues as immune surveillance cells, rapidly releasing cytokines in response to activation by recall antigens.

It is increasingly clear that the original T_{CM}/T_{EM} paradigm requires revision, in part because CCR7+ effector memory cells have been detected in inflamed tissues and CCR7 facilitates their exit into lymphatics.[255,256,257,258] Also, rather than a dualism with a single precursor-product relationship, analysis of freshly isolated human peripheral blood CD4+ T cells has suggested that memory cell differentiation may be a continuum with multiple branch points arising probabilistically, giving rise to many subpopulations that preferentially express certain chemokine receptors.[259]

Upon activation, the classic polarized effector subsets, Th1 and Th2, downregulate CXCR5 and CCR7 and upregulate inflammatory chemokine receptors.[260] This switch facilitates exit from lymph node via efferent lymphatics and homing to inflamed sites. In vivo, Th1 cells, which by the simplest definition secrete IFNγ but not IL-4 and control cellular and humoral immunity to intracellular pathogens, more frequently express CXCR3, CXCR6, CCR2, CCR5, and CX3CR1 than Th2 cells. In contrast, Th2 cells, which express IL-4 but not IFNγ and are associated with cellular and humoral immunity to extracellular pathogens and allergic inflammation, more frequently express CCR3, CCR4, and CCR8 than Th1 cells. CXCR3 expression is dependent on T-bet expression and has been most consistently associated with Th1 immune responses and Th1-associated disease. Consistent with this, its agonists CXCL9-11 are highly induced by IFNγ but not by IL-4. Thus, in Th1 immunity there is the potential for a positive feedback loop in which IFNγ induces production of CXCL9-11 that then recruits CXCR3+ Th1 cells that produce IFNγ (see Fig. 28.7). The "Th1 chemokines" may also help maintain Th1 dominance in part through their ability to block CCR3. Specific cytokines, microenvironments, and inflammatory stresses may differentially regulate CXCL9-11 expression, which may account for specialized biologic roles. For example, though all three chemokines are inducible by IFNγ, CXCL10 and CXCL11 but not CXCL9 are also inducible by type I IFN. There is evidence that Th1 and Th2 cell recruitment into tissue is

differentially regulated by Stat1 and Stat6, respectively, and that Stat1 controls recruitment of Th1 cells through the induction of CXCR3 ligands and CXCL16.[261]

Conversely, research on chemokine expression and targets has led to a model of Th2 immunity in which IL-4 and IL-13 made at inflamed sites in the periphery may induce production of CCL7, CCL11, and other CCR3 ligands, the CCR4 ligands CCL17 and CCL22, and the CCR8 ligand CCL1. CCR3 is expressed on a subset of Th2 T-lymphocytes as well as on eosinophils and basophils, the three major cell types associated with Th2-type allergic inflammation and Th2 cells are associated with CCR4 expression. Arrival of Th2 cells amplifies a positive feedback loop through secretion of additional IL-4. Moreover, CCL7 and CCL11 may block Th1 responses by antagonizing CCR2, CXCR3, and CCR5. Many chemokine receptors have been identified on CD4+ regulatory T cells, including CCR4, CCR5, and CCR8. CCR5 has been shown to play an important role in homing of these cells to skin during *Leishmania major* infection, favoring pathogen persistence.[262]

All Th17 cells express CCR6 controlled by the signature Th17 transcription factor Rorγt; CCR6 may also participate in a positive feedback loop with tissue cells and Th17 cells themselves expressing its ligand CCL20.[263,264] The CCR6+ subset of human CD4+ T cells is much larger, however, and includes IL-22-producing, IL-17-negative skinhoming memory T cells and IL-10-producing, autoreactive memory T cells with context-dependent regulatory function.[265,266]

Some chemokines appear to regulate not just trafficking but also T-cell polarization at least in certain contexts. CCL2 and its receptor CCR2 have been most extensively studied in this regard, but the results are complex and appear at first glance contradictory as in vivo CCR2 is strongly associated with Th1 immune responses and CCL2 is associated with Th2 responses.[126] CCL2 appears to promote Th2 polarization directly, by inhibiting IL-12 production in monocytes, and by enhancing IL-4 but not IFNγ production in memory and activated T cells. Thus, CCL2 influences both innate immunity, through effects on monocyte trafficking, and adaptive immunity, through control of T helper cell trafficking and potentially T-cell polarization. In the case of aerosol challenge with *M. tuberculosis* in *Ccr2-/-* mice, DC migration to draining lymph node is markedly impaired which preempts any direct effects of CCL2 on T cells. Why CCL2 and CCR2 have opposite effects on Th polarization is unclear but is most likely due to ligand-receptor promiscuity.

Tissue-Specific Lymphocyte Homing

CLA+ T-lymphocytes, which home to skin, preferentially express CCR4 and CCR10.[267] The CCR4 ligand CCL22 is made by resident dermal macrophages and DCs, whereas the CCR10 ligand CCL27 is made by keratinocytes. Blocking both of these pathways, but not either one alone, has been reported to inhibit lymphocyte recruitment to the skin in a DTH model, implying that in this model these two molecules act redundantly and independently of inflammatory chemokines.[268]

Homing to small intestine is determined in part by T-lymphocyte expression of the integrin $\alpha_4\beta_7$ and CCR9. The $\alpha_4\beta_7$ ligand MAdCAM-1 and the CCR9 ligand CCL25 colocalize on normal and inflamed small intestinal endothelium, and most T cells in the intraepithelial and lamina propria zones of the small intestine express CCR9. These cells, which are mainly γδ TCR(+) or CD8αβ(+)αβTCR(+), are reduced in number in small intestine from *Ccr9-/-* mice. CCL20 and its receptor CCR6 are also important in gut mucosal immune responses. CCL20 is constitutively expressed in follicle-associated epithelial cells overlying gutassociated lymphoid tissue (GALT) inductive sites (Peyer's patches and isolated lymphoid follicles), but can also be induced in GALT under inflammatory conditions. It is important for GALT development and for B and DC homing to GALT. CCR6+ DCs recruited to the dome region of Peyer's patches upon invasion of follicle-associated epithelial cells by an enteric pathogen have been shown to mediate rapid activation of pathogen-specific T cells.[269]

As B cells differentiate into plasma cells, they also downregulate CXCR5 and CCR7 and exit the lymph node. B immunoblasts expressing immunoglobulin (Ig)G coordinately upregulate CXCR4, which promotes homing to the bone marrow, whereas B immunoblasts expressing IgA specifically migrate to mucosal sites.[270] Like gut-homing T cells, gut homing IgA-secreting B immunoblasts express $\alpha_4\beta_7$ integrin and CCR9 and respond to CCL25. Intestinal DCs may help shape mucosal immunity by generating gut-homing IgA-secreting B cells through the synergistic action of several mediators, including retinoic acid, IL-5, and IL-6.[271,272]

CHEMOKINES AND DISEASE

There is a vast literature correlating the presence of chemokines with diverse human diseases and delineating diverse phenotypes of chemokine and chemokine receptor knockout mice (see Table 28.7). Due to space limitations, this section will address a small subset of these diseases, including ones with strong evidence in humans for prominent chemokine involvement (Table 28.8).

Human Immunodeficiency Virus

HIV infection requires fusion of the viral envelope to a target cell membrane, a process that is initiated by direct binding of the HIV envelope glycoprotein gp120 to both CD4 and a coreceptor on the target cell.[30,32,273] Although many chemokine receptors and related GPCRs can function as coreceptors in vitro, CCR5 and CXCR4 appear to be the most relevant on primary cells (Fig. 28.9). Their interaction with CD4 may be constitutive and stabilized by gp120. The usage of these receptors is so important that HIV strains are now functionally classified and named according to their specificity for CXCR4 (X4 strains), CCR5 (R5 strains), or both (R5X4 strains).

The importance of CCR5 in clinical HIV/acquired immunodeficiency syndrome (AIDS) was established at the population level by the seminal discovery that *CCR5Δ32*, a nonfunctional allele founded more than 5000 years ago in Caucasians and now present in ~20% of North American

Caucasians, is a strong AIDS restriction factor, protecting homozygotes from initial infection and slowing the rate of progression to AIDS in infected heterozygotes.[274,275,276] Additional genetic risk factors affecting the rate of HIV disease progression have been identified in the CCR5 signaling system, including single nucleotide polymorphisms in the promoters for *CCR5* and its ligand *CCL5*, and variation in the gene copy number of the CCR5 ligand CCL3L1.[110,114,277] The pattern of genetic variation at *CCR5Δ32* is most consistent with neutral evolution.[278] *CCR5Δ32* homozygotes appear healthy as do unstressed CCR5 knockout mice. These observations provided proof-of-principle for the central role of CCR5 in

HIV transmission and progression and for development of the FDA-approved CCR5 antagonist maraviroc in HIV/AIDS[36,37] (Fig. 28.10). In addition, a patient with HIV and leukemia was fortuitously transplanted after cytoreductive therapy with bone marrow from a *CCR5Δ32* homozygous donor in February 2007 and as of January 2012 had remained well off antiretroviral therapy with undetectable viral load.[279,280] This has fortified efforts to develop zinc finger nuclease and other genetic methods to block CCR5 in patients.

TABLE 28.8 Chemokines and Human Disease

Molecule	Disease	Mechanism	Mutation
CCR5	HIV/AIDS	Cell entry	*CCR5Δ32*
	West Nile virus infection	Immune control	
	Rheumatoid arthritis	Inflammation	
	Chronic renal allograft rejection	Inflammation	
CXCR4	HIV/AIDS	Cell entry	None
	WHIM syndrome	Myelokathexis	Autosomal Dominant
		HPV proliferation	C-terminal truncation
CX3CR1	Cardiovascular disease	Undefined	*CX3CR1-M280*
	Age-related macular degeneration	Microglial cell activation	
DARC	*Plasmodium vivax* malaria	Cell entry	*Fy*
CCL26	Eosinophilic esophagitis	Eosinophil trafficking	SNP
HHV8 ORF74	Kaposi's sarcoma	Angiogenesis	None defined
		Inflammation	
CXCL4	Heparin-induced thrombocytopenia	Autoantibody to CXCL4-heparin complex	Not defined

AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; HPV, human papillomavirus; SNP, single nucleotide polymorphism.

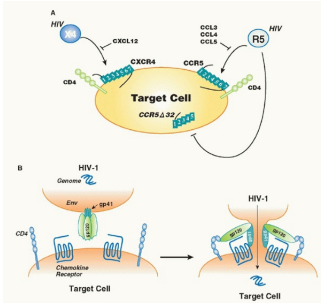

FIG. 28.9. Model of Human Immunodeficiency Virus (HIV) Coreceptor Activity. A: HIV cytotropism is explained by differential usage of CXCR4 and CCR5 as coreceptors. The mutant receptor CCR5Δ32 is not expressed on the cell surface and cannot be used for cell entry by HIV. **B:** Chemokine receptors in a proposed mechanism of HIV entry.

CCR5 is also important in the neuropathogenesis of West Nile virus infection, in both humans and in a mouse model of disease, but unlike HIV, it plays a protective role by facilitating antiviral leukocyte trafficking to the brain.[281,282,283] CCR5-blocking agents could theoretically increase the risk of symptomatic West Nile virus disease in infected patients, particularly in the setting of HIV/AIDS where the immune system is already compromised. Pathogenesis in the mouse model of West Nile virus is also regulated by neuronal CXCL10 through recruitment of CD8+ T cells to the brain.[284] CXCL10 also controls pathogenesis in a mouse model of the related flavivirus dengue virus, both by regulating T-cell recruitment through CXCR3 and by competitively inhibiting viral binding to cell surface heparan sulfate.[285]

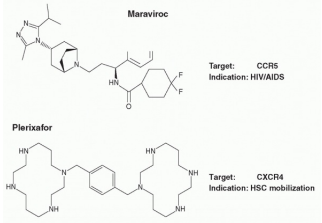

FIG. 28.10. U.S. Food and Drug Administration-Approved Chemokine Receptor Antagonists. Plerixafor was developed by Genzyme, Inc., and is now marketed by Sanofi-Aventis; maraviroc was developed and is marketed by Pfizer.

X4 HIV strains typically do not transmit disease, but instead appear to evolve from R5 quasispecies in a minority of patients late in infection during the transition to clinical disease. Consistent with this, these strains appear more virulent than R5 strains in vitro. The reason is not fully defined; however, it is important to note that the percentage of CD4+ T cells expressing CXCR4 exceeds that expressing CCR5 in vagina, blood, and rectum. Moreover, the X4 envelope protein expressed on CD4+ T cells has been shown to induce autophagic programmed cell death of uninfected bystander cells by binding to CXCR4 on the cell membrane, signaling in a non-Gi-, noncaspase 3-dependent manner.[286] An unresolved question is why X4 strains do not readily transmit disease, given the broad expression of CXCR4 on macrophages and CD4+ T cells.

Malaria

Analagous to CCR5 and HIV, DARC, the 7TM promiscuous chemokine binding protein and minor blood group antigen, is required for infection of erythrocytes by *P. vivax*, a major cause

of malaria. The parasite ligand, which is named the *P. vivax* Duffy binding protein, is expressed in micronemes of merozoites and binds to the *N*-terminal domain of Duffy via a cysteine-rich domain.[287] This interaction is required for junction formation during invasion but not for initial binding or parasite orientation. DARC deficiency, which is due mainly to an inherited single nucleotide substitution named -46C at an erythroid-specific GATA motif in the DARC promoter,[288] is fixed in sub-Saharan Africa but not in other malaria-endemic regions of the world. Accordingly, *P. vivax* malaria is rare in sub-Saharan Africa but common in Central and South America, India, and Southeast Asia. Fixation of the mutation in Africa presumably occurred because of positive selective pressure from malaria. Together with *CCR5Δ32*, DARC -46C is the strongest genetic resistance factor known

for any infectious disease in man. Chemokine control of *P. falciparum* malaria is less well studied although, in an intriguing development, endothelial cell CX3CL1 was reported to bind parasite-infected erythrocytes in vitro,[289] suggesting the parasite expresses a mimic for the CX3CL1 receptor CX3CR1. Conversely, the G glycoprotein of respiratory syncytial virus has been reported to function as a CX3CL1 mimic binding to CX3CR1 to induce chemotaxis and possibly to promote viral entry[290]; human cytomegalovirus encodes US28, a chemokine receptor able to bind CX3CL1, possibly to mediate viral attachment to target cells. Together, these examples show the rich dialogue between hosts and pathogens using the chemokine system as a preferred method of communication.

WHIM Syndrome

WHIM syndrome is a rare combined immunodeficiency disorder caused by autosomal dominant inheritance of gainof-function mutations truncating the C-tail of CXCR4.[291,292] Enhanced function appears to result from CXCR4 dimerization and β-arrestin signaling plus impaired receptor desensitization and internalization.[293,294] This causes exaggeration of CXCR4's normal neutrophil and hematopoietic stem cell retention function in the bone marrow resulting in myelokathexis (neutropenia without maturation arrest). Patients typically have panleukocytopenia but suffer mainly from recurrent sinopulmonary and skin infections caused by pyogenic organisms typically controlled by neutrophils. Hypogammaglobulinemia probably also contributes; however, pathogens typically associated with T-cell deficiency are not a problem. Neutrophils can be mobilized in the setting of infection by unknown mechanisms, so that the condition is debilitating but usually not acutely life-threatening. Patients also develop large numbers of warts due to human papillomavirus, the signature pathogen in the disease and the only problematic virus. This may be caused by virus-induced CXCR4 expression and signaling directly in keratinocytes.[295]

Administration of the drug plerixafor (Mozobil, AMD 3100) (see Fig. 28.10), a selective CXCR4 antagonist, results in mobilization of almost all leukocyte subpopulations, but not erythrocytes or platelets, to the blood in healthy human subjects as well as in patients with WHIM syndrome.[296,297] This provides proof-of-concept for development of CXCR4 inhibitors as mechanism-based therapy, with the goal of restoring signaling to normal, not blocking it entirely, which would heighten safety concerns. Plerixafor was originally developed in combination with granulocyte-colony stimulating factor for mobilization of hematopoietic stem cells for transplantation in patients with multiple myeloma or non-Hodgkins lymphoma receiving cytoreductive therapy, and is now FDA-approved for those indications.[38,298,299] Its use in chronic therapy, as would be necessary in WHIM syndrome, is limited by a very short half-life, parenteral administration, and high cost.

In G6PC3 deficiency, a recently described second rare primary immunodeficiency disorder characterized by neutropenia/myelokathexis and recurrent bacterial infections, the mechanism may also involve increased CXCR4 signaling.[300,301] In this case, however, CXCR4 is indirectly affected by metabolic stress caused by the total absence of glucose-6-phosphatase activity in neutrophils. This increases neutrophil apoptosis and induces high expression of wild-type CXCR4, which is thought to drive the cells back to the bone marrow for destruction, contributing to the neutropenia picture. Unlike WHIM syndrome, G6PC3 patients do not have deficiencies in other leukocyte lineages, due to expression of other glucose-6-phosphatase isoforms in those cells.

Kaposi's Sarcoma

A full discussion of the many virally encoded chemokines and chemokine receptors is beyond the scope of this chapter, but can be found in recent reviews.[76,77] HHV8 ORF74 deserves special mention however. HHV8 encodes three CC chemokines, vMIP-I, II, and III, as well as a constitutively active CC/CXC chemokine receptor named vGPCR encoded by ORF74.[171,302] All of these factors are angiogenic and may contribute to the pathogenesis of Kaposi's sarcoma, a highly vascular multicentric nonclonal tumor caused by HHV8 typically in the setting of immunosuppression such as in HIV/AIDS. Consistent with this, vGPCR induces Kaposi's sarcoma-like tumors when expressed constitutively or inducibly in transgenic mice on endothelial cells.[303] The mechanism may involve signaling through diverse pathways including the nonconventional G protein Gq and phosphorylation of tuberin, which promotes activation of mTOR (mammalian target of rapamycin) through direct and paracrine mechanisms.[304] The latter may include activation of NF-κB and induction of angiogenic factors such as vascular endothelial growth factor and proinflammatory chemokines and cytokines.[305] Thus, this virus appears to have converted a hijacked receptor, probably CXCR2, into a regulator of gene expression. The model identifies rapamycin as a candidate therapeutic in Kaposi's sarcoma.

Atherosclerosis

There is now compelling genetic evidence from knockout mice and human disease cohorts that CX3CR1 is a significant proatherogenic factor. The mechanism remains unclear but may involve induction of CX3CL1 on vascular smooth muscle cells, and recruitment and retention of CX3CR1-positive monocytes to plaque through the dual chemotactic and adhesive functions of this ligand-receptor pair.[306] The evidence in man is based in part on consistent association of a defective receptor variant named CX3CR1 M280 with reduced risk of cardiovascular disease endpoints in multiple cohort studies; mice lacking Cx3cr1 on the *ApoE-/-* background fed a Western diet are also at reduced risk of developing vascular lesions.[307,308,309,310,311] There is also extensive evidence for a proatherogenic role for CCL2 and its receptor CCR2.[312,313,314]

Adoptive transfer studies of bone marrow from knockout mice have also revealed a role for CCR5 and CXCR2 in mouse models of atherosclerosis. CXCR2 may work by promoting monocyte adhesion to early atherosclerotic endothelium, through interaction with its mouse ligand KC and activation of the VLA-4/vascular cell adhesion molecule-1 adhesion system.[315,316] Immunoneutralization experiments have revealed

a role for CCR7 on foam cells in a mouse model of atheroregression, and statins may be protective in part through this pathway.[317,318] Surprisingly, CCR1 and CXCL16 deficiency appeared to increase inflammation in atherosclerosis models.[319] CXCL10 has also been implicated in atherogenesis in a mouse model, possibly acting by recruitment of CXCR3+ Th1 cells.[320] Lack of CXCL10 was also associated with a relative increase of regulatory T cells in plaque. CCR6 has also been implicated as a proatherogenic factor possibly acting at the level of monocyte distribution and trafficking.[321]

Age-Related Macular Degeneration

CX3CR1 is particularly important in microglial cell migration and activation in the inflamed central nervous system.[3] Mice lacking Cx3cr1 develop retinal lesions similar to macular degeneration. Human age-related macular degeneration is associated with a loss-of-function mutant allele in *CX3CR1*,[322,323,324,325] The mechanism appears to involve dysregulated activation of microglial cells in the retina. CCR3 has also been detected in human age-related macular degeneration, and targeting Ccr3 was effective in a mouse model of age-related macular degeneration.[326]

Heparin-Induced Thrombocytopenia

Heparin-induced thrombocytopenia is a human autoimmune disease directly linked mechanistically to chemokines.[327] An established risk factor for thromboembolic complications of heparin therapy, heparin-induced thrombocytopenia occurs in 1% to 5% of patients treated with heparin and is the result of autoantibodies that bind specifically to CXCL4-heparin complexes in plasma.

In general, T cell-dependent autoimmune diseases in man such as psoriasis, multiple sclerosis, rheumatoid arthritis, and type I diabetes mellitus are associated with inflammatory chemokines and tissue infiltration by T-lymphocytes and monocytes expressing inflammatory chemokine receptors. There is an extensive literature exploring chemokine mechanisms in mouse models of these diseases.[128,198,323,328,329,330,331,332,333,334,335,336] Rheumatoid arthritis has been consistently negatively associated with homozygous *CCR5Δ32*, suggesting that CCR5 is important in pathogenesis in humans.[337]

The eosinophil selective CCL26/CCR3 axis has been implicated in eosinophilic esophagitis, based on high expression of CCL26 in the esophagus from patients, association of a

polymorphism in *CCL26* with human disease, and resistance of *Ccr3*-/- mice to experimental eosinophilic esophagitis.[338,339]

Acute Neutrophil-mediated Inflammatory Disorders

Many neutrophil-mediated human diseases have been associated with the presence of CXCL8, including psoriasis, gout, acute glomerulonephritis, chronic obstructive pulmonary disease, actue respiratory distress syndrome, rheumatoid arthritis, and ischemia-reperfusion injury. Systemic administration of neutralizing anti-CXCL8 antibodies is protective in diverse models of neutrophil-mediated acute inflammation in the rabbit (skin, airway, pleura, glomeruli), providing proof-of-concept that CXCL8 is a nonredundant mediator of innate immunity and acute pathologic inflammation in these settings.[224,225] Moreover, CXCR2 knockout mice are less susceptible to acute urate crystal-induced gouty synovitis,[340] and SB-265610, a nonpeptide small molecule antagonist with exquisite selectivity for CXCR2, prevents neutrophil accumulation in the lungs of hyperoxia-exposed newborn rats.[341] Together, the results identify CXCL8 and its receptors as candidate drug targets for diseases mediated by acute neutrophilic inflammation. CXCR2 knockout mice also have delayed wound healing.[342]

Transplant Rejection

An advantage of transplant rejection over other animal models of human disease is that, in both the human and animal situation, the time of antigenic challenge is precisely known. The most extensive analysis of the role of chemokines in transplant rejection has been carried out in a major histocompatibility complex class I/II-mismatched cardiac allograft rejection model in the mouse, which is mediated by a Th1 immune response.[343] Similar sets of inflammatory chemokines are found in the mouse model as in the human disease. Analysis of knockout mice has demonstrated that while multiple chemokine receptors contribute to rejection in this model, there is a marked rank order: CXCR3 >> CCR5 > CCR1 = CX3CR1 = CCR2. Most impressively, rejection and graft arteriosclerosis do not occur if the recipient mouse, treated with a brief, subtherapeutic course of cyclosporin A, is *Cxcr3*-/- or if the donor heart is *Cxcl10*-/-, identifying this axis as a potential drug target.[344] Neutralization of Cxcl9, a Cxcr3 ligand that appears later than Cxcl10, can also prolong cardiac allograft survival and delay T-cell infiltration and acute rejection in class II major histocompatibility complex-disparate skin allografts.[345] In man, CCR5 may be important in chronic kidney allograft rejection, as individuals homozygous for *CCR5Δ32* are underrepresented among patients with this outcome in a large German kidney transplantation cohort.[346]

Allergic Airway Disease

Chemokine receptors associated with asthma include CXCR2, CXCR4, CCR3, CCR4, and CCR8.[347,348,349] CCR3 is present on eosinophils, basophils, mast cells, and some Th2 T cells, and CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. Ccr8 knockout mice have reduced allergic airway inflammation in response to three different Th2-polarizing antigens: *Schistosoma mansoni* soluble egg antigen, ovalbumin, and cockroach antigen.[350] A role for the CCR3 axis in asthma has been supported by Ccl11 neutralization in guinea pig and Ccr3 gene knockout in mouse. The net effect of *Ccr3* knockout was expected to be more profound; however, the exact phenotype depends dramatically on the specific method of sensitization and challenge due to complex and opposite effects on eosinophil and mast cell trafficking.[351,352] CCR3 and its ligands have also been clearly shown to play a critical role in an *Aspergillus*-induced chronic allergic airway disease mouse model, in part due to failure of eosinophils to traffic to the lung.[353] CCR6 also appears to play a role, as *Ccr6*-/- mice have decreased allergic airway inflammation in response to sensitization and challenge with cockroach antigen, which

is consistent with the induction of its ligand CCL20 in this model. The CXCL12/CXCR4 axis may be more than a homeostatic regulator since neutralization of either ligand or receptor resulted in decreased lung eosinophilia and airway hyperreactivity in a mouse model of allergic airway disease.[354]

Cancer

Many chemokines have been detected in situ in tumors, and cancer cells have been shown to produce chemokines and express chemokine receptors.[355] However, the exact role played by endogenous tumor-associated chemokines in recruiting tumor-infiltrating lymphocytes and tumorassociated macrophages and in promoting an antitumor immune response has not been delineated. On the contrary, there are data from mouse models suggesting that the overall effect may often be to promote tumorigenesis through additional effects on cell growth, angiogenesis, apoptosis, immune evasion, and metastasis.[234,356] Controlling the balance of angiogenic and angiostatic chemokines may be particularly important.[357] This has been shown in several instances, including human non-small cell lung carcinoma, in which the ratio of ELR to non-ELR CXC chemokine expression is high, and where in a *Scid* mouse model neutralization of endogenous tumor-derived CXCL8 (angiogenic) could inhibit tumor growth and metastasis by about 50% through a decrease in tumor-derived vessel density, without directly affecting tumor cell proliferation. Chemokine receptors on tumor cells have been shown to directly mediate chemokine-dependent proliferation, for example in the case of CXCL1 in melanoma, and metastasis, in the case of CXCR4 in a mouse model of breast cancer and glioblastoma, and CXCR4 in human breast cancer cells, responding to CXCL12 produced by carcinoma-associated fibroblasts.[358] Moreover, chemokines may function in cancer by reprogramming cancer-associated stromal fibroblasts to a senescent protumorigenic state, as shown in ovarian cancer for CXCL1.[359] It remains to be seen how general and important these effects are for human cancer.

THERAPEUTIC APPLICATIONS

Chemokines and Chemokine Receptors as Targets for Drug Development

The two FDA-approved drugs targeting the chemokine system, plerixafor (CXCR4) and maraviroc (CCR5), are both small molecules developed for surprising indications, hematopoietic stem cell mobilization from bone marrow in cancer and HIV/AIDS (see Fig. 28.10), respectively, as discussed previously.[36,37,38] Despite major investment, there has not been success in therapeutically targeting the chemokine system for expected indications, such as acute and chronic inflammation or cancer. For these areas, despite the ability of the pharmaceutical industry to develop many orally available high potency small molecule chemokine receptor antagonists and other chemokine-blocking agents, there are major obstacles to clinical application, including the issue of inflammatory chemokine redundancy, difficulties in optimizing bioavailability and timing in patients, and the unreliability of many preclinical disease models in predicting the best indications and targets.[360]

Chemokines as Biologic Response Modifiers

Both inflammatory and homeostatic chemokines are being evaluated for therapeutic potential as biological response modifiers, acting mainly as immunomodulators or as regulators of angiogenesis.[361,362,363,364,365,366,367,368,369] Studies to date have not revealed major problems with toxicity, and efficacy has been observed in animal models of cancer, inflammation, and infection. To date, clinical trials in cancer and stem cell protection have been disappointing. Chemokines are also being developed as vaccine adjuvants, delivered either as pure protein, immunomodulatory plasmid, or as recombinant protein within antigen-pulsed DCs. Chemokine gene administration has also been shown to induce neutralizing antibody against the encoded chemokine and has successfully blocked immune responses and improved experimental autoimmune encephalomyelitis and arthritis scores in rodent models.

Many chemokines, when delivered pharmacologically as recombinant proteins or by plasmid deoxyribonucleic acid or in transfected tumor cells, are able to induce immunologically mediated antitumor effects in mouse models and could be clinically useful. Mechanisms may differ depending on the model but may involve recruitment of monocytes, NK cells, and CD8+ cytotoxic T cells to tumor. Chemokines may also function as adjuvants in tumor antigen vaccines. Chemokine-tumor antigen fusion proteins represent a novel twist on this approach that facilitates uptake of tumor antigens by antigen-presenting cells via the normal process of ligand-receptor internalization. Non-ELR CXC chemokines such as CXCL4 also exert antitumor effects through angiostatic mechanisms.

CONCLUSION

The immune system is a system in motion, and the study of chemokines has provided major new insights into how order is maintained as cells enter a tissue, traffic to targets, and in some cases leave and recirculate. In addition, chemokines are increasingly appreciated as multifunctional cytokines, playing large roles in some cases such as CXCL12 in development, and perhaps smaller or less well-appreciated ones in immune activation processes. A major challenge for the future is to deepen basic knowledge of chemokine-mediated immunoregulation as a way to advance new therapeutics for immunologically mediated disease.

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REFERENCES

1. Baggiolini M, Dewald B, Moser B. Human chemokines: an update. *Annu Rev Immunol*. 1997;15:675-705.

2. Campbell DJ, Kim CH, Butcher EC. Chemokines in the systemic organization of immunity. *Immunol Rev*. 2003;195:58-71.

3. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med*. 2006;354(6):610-621.

4. Lau EK, Allen S, Hsu AR, Handel TM. Chemokine-receptor interactions: GPCRs, glycosaminoglycans and viral chemokine binding proteins. *Adv Protein Chem*. 2004;68:351-.91.

5. Luster AD. Chemokines—chemotactic cytokines that mediate inflammation. *N Engl J Med*. 1998;338(7):436-445.

6. Luster AD. The role of chemokines in linking innate and adaptive immunity. *Curr Opin Immunol*. 2002;14(1):129-135.

7. Mantovani A. The chemokine system: redundancy for robust outputs. *Immunol Today*. 1999;20(6):254-257.

8. Murphy PM. The molecular biology of leukocyte chemoattractant receptors. *Annu Rev Immunol*. 1994;12:593-633.

9. Oppenheim JJ, Zachariae CO, Mukaida N, Matsushima K. Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu Rev Immunol*. 1991;9:617-648.

10. Premack BA, Schall TJ. Chemokine receptors: gateways to inflammation and infection. *Nat Med*. 1996;2(11):1174-1178.

11. Rollins BJ. Chemokines. *Blood*. 1997;90(3):909-928.

12. Schall TJ. Biology of the RANTES/SIS cytokine family. *Cytokine*. 1991; 3(3):165-183.

13. Wolpe SD, Cerami A. Macrophage inflammatory proteins 1 and 2: members of a novel superfamily of cytokines. *FASEB J*. 1989;3(14):2565-2573.

14. Yoshie O, Imai T, Nomiyama H. Chemokines in immunity. *Adv Immunol*. 2001;78:57-110.

15. Muller G, Hopken UE, Lipp M. The impact of CCR7 and CXCR5 on lymphoid organ development and systemic immunity. *Immunol Rev*. 2003;195:117-135.

16. Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. *Science*. 1996;272(5258):60-66.

17. Laudanna C, Alon R. Right on the spot. Chemokine triggering of integrinmediated arrest of rolling leukocytes. *Thromb Haemost*. 2006;95(1):5-11.

18. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell*. 1994;76(2):301-314.

19. Cyster JG. Shining a light on germinal center B cells. *Cell*. 2012;143(4): 503-505.

20. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu Rev Immunol*. 2005;23:127-159.

21. Raz E, Mahabaleshwar H. Chemokine signaling in embryonic cell migration: a fisheye view. *Development*. 2009;136(8):1223-1229.

22. Rot A, von Andrian UH. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. *Annu Rev Immunol*. 2004;22:891-928.

23. Rothenberg ME, Mishra A, Brandt EB, Hogan SP. Gastrointestinal eosinophils. *Immunol Rev*. 2001;179:139-155.

24. Bromley SK, Burack WR, Johnson KG, et al. The immunological synapse. *Annu Rev Immunol*. 2001;19:375-396.

25. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol*. 2004;25(12):677-686.

26. Molon B, Gri G, Bettella M, et al. T cell costimulation by chemokine receptors. *Nat Immunol*. 2005;6(5):465-471.

27. Alcami A, Lira SA. Modulation of chemokine activity by viruses. *Curr Opin Immunol*. 2010;22(4):482-487.

28. Lalani AS, McFadden G. Evasion and exploitation of chemokines by viruses. *Cytokine Growth Factor Rev*. 1999;10(3-4):219-233.

29. Murphy PM. Viral exploitation and subversion of the immune system through chemokine mimicry. *Nat Immunol*. 2001;2(2):116-122.

30. Berger EA, Murphy PM, Farber JM. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu Rev Immunol*. 1999;17:657-700.

31. Horuk R, Chitnis CE, Darbonne WC, et al. A receptor for the malarial parasite Plasmodium vivax: the erythrocyte chemokine receptor. *Science*. 1993;261(5125):1182-1184.

32. Lusso P. HIV and the chemokine system: 10 years later. *EMBO J*. 2006;25(3):447-456.

33. Doitsidou M, Reichman-Fried M, Stebler J, et al. Guidance of primordial germ cell migration by the chemokine SDF-1. *Cell*. 2002;111(5): 647-659.

34. Nagasawa T, Tachibana K, Kishimoto T. A novel CXC chemokine PBSF/SDF-1 and its receptor CXCR4: their functions in development, hematopoiesis and HIV infection. *Semin Immunol*. 1998;10(3):179-185.

35. Sierro F, Biben C, Martinez-Munoz L, et al. Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proc Natl Acad Sci U S A*. 2007; 104(37):14759-14764.

36. Meanwell NA, Kadow JF. Maraviroc, a chemokine CCR5 receptor antagonist for the treatment of HIV infection and AIDS. *Curr Opin Investig Drugs*. 2007;8(8):669-681.

37. Troia-Cancio P, Asmuth DM. Lessons from maraviroc clinical trials. *Expert Rev Anti Infect Ther*. 2011;9(6):649-651.

38. De Clercq E. The AMD3100 story: the path to the discovery of a stem cell mobilizer (Mozobil). *Biochem Pharmacol*. 2009;77(11):1655-1664.

39. Nomiyama H, Mera A, Ohneda O, Miura R, Suda T, Yoshie O. Organization of the chemokine genes in the human and mouse major clusters of CC and CXC chemokines: diversification between the two species. *Genes Immun*. 2001;2(2):110-113.

40. Nomiyama H, Osada N, Yoshie O. A family tree of vertebrate chemokine receptors for

a unified nomenclature. *Dev Comp Immunol*. 2011;35(7):705-715.

41. Nomiyama H, Osada N, Yoshie O. The evolution of mammalian chemokine genes. *Cytokine Growth Factor Rev*. 2010;21(4):253-262.

42. Murphy PM. International Union of Pharmacology. Update on chemokine receptor nomenclature. *Pharmacol Rev*. 2002;54(2):227-229.

43. Murphy PM, Baggiolini M, Charo IF, et al. International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev*. 2000;52(1):145-176.

44. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. *Immunity*. 2000;12(2):121-127.

45. Clore GM, Gronenborn AM. Three-dimensional structures of alpha and beta chemokines. *FASEB J*. 1995;9(1):57-62.

46. Belperio JA, Keane MP, Arenberg DA, et al. CXC chemokines in angiogenesis. *J Leukoc Biol*. 2000;68(1):1-8.

47. Clark-Lewis I, Kim KS, Rajarathnam K, et al. Structure-activity relationships of chemokines. *J Leukoc Biol*. 1995;57(5):703-711.

48. Salcedo R, Oppenheim JJ. Role of chemokines in angiogenesis: CXCL12/SDF-1 and CXCR4 interaction, a key regulator of endothelial cell responses. *Microcirculation*. 2003;10(3-4):359-370.

49. Farber JM. Mig and IP-10: CXC chemokines that target lymphocytes. *J Leukoc Biol*. 1997;61(3):246-257.

50. Van Coillie E, Van Damme J, Opdenakker G. The MCP/eotaxin subfamily of CC chemokines. *Cytokine Growth Factor Rev*. 1999;10(1):61-86.

51. Bazan JF, Bacon KB, Hardiman G, et al. A new class of membrane-bound chemokine with a CX3C motif. *Nature*. 1997;385(6617):640-644.

52. Matloubian M, David A, Engel S, Ryan JE, Cyster JG. A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. *Nat Immunol*. 2000;1(4):298-304.

53. Haskell CA, Cleary MD, Charo IF. Molecular uncoupling of fractalkine-mediated cell adhesion and signal transduction. Rapid flow arrest of CX3CR1-expressing cells is independent of G-protein activation. *J Biol Chem*. 1999;274(15):10053-10058.

54. Pisabarro MT, Leung B, Kwong M, et al. Cutting edge: novel human dendritic cell- and monocyte-attracting chemokine-like protein identified by fold recognition methods. *J Immunol*. 2006;176(4):2069-2073.

55. Kuloglu ES, McCaslin DR, Kitabwalla M, Pauza CD, Markley JL, Volkman BF. Monomeric solution structure of the prototypical "C" chemokine lymphotactin. *Biochemistry*. 2001;40(42):12486-12496.

56. Ali S, Palmer ACV, Fritchley SJ, Maley Y, Kirby JA. Multimerization of monocyte chemoattractant protein-1 is not required for glycosaminoglycan-dependent transendothelial chemotaxis. *Biochemical J*. 2001; 358:737-745.

57. Rajarathnam K, Sykes BD, Kay CM, Dewald B, Geiser T, Baggiolini M, et al. Neutrophil activation by monomeric interleukin-8. *Science*. 1994;264(5155):90-92.

58. Lodi PJ, Garrett DS, Kuszewski J, et al. High-resolution solution structure of the beta chemokine hMIP-1 beta by multidimensional NMR. *Science*. 1994;263(5154):1762-1767.

59. Handel TM, Johnson Z, Crown SE, Lau EK, Proudfoot AE. Regulation of protein function by glycosaminoglycans—as exemplified by chemokines. *Annu Rev Biochem*. 2005;74:385-410.

60. Johnson Z, Proudfoot AE, Handel TM. Interaction of chemokines and glycosaminoglycans: a new twist in the regulation of chemokine function with opportunities for therapeutic intervention. *Cytokine Growth Factor Rev*. 2005;16(6):625-636.

61. Proudfoot AE, Fritchley S, Borlat F, et al. The BBXB motif of RANTES is the principal site for heparin binding and controls receptor selectivity. *J Biol Chem*. 2001;276(14):10620-10626.

62. Proudfoot AE, Handel TM, Johnson Z, et al. Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc Natl Acad Sci U S A*. 2003;100(4):1885-1890.

63. Crown SE, Yu Y, Sweeney MD, Leary JA, Handel TM. Heterodimerization of CCR2 chemokines and regulation by glycosaminoglycan binding. *J Biol Chem*. 2006;281(35):25438-25446.

64. Dudek AZ, Nesmelova I, Mayo K, Verfaillie CM, Pitchford S, Slungaard A. Platelet factor 4 promotes adhesion of hematopoietic progenitor cells and binds IL-8: novel mechanisms for modulation of hematopoiesis. *Blood*. 2003;101(12):4687-4694.

65. Guan E, Wang J, Norcross MA. Identification of human macrophage inflammatory proteins 1alpha and 1beta as a native secreted heterodimer. *J Biol Chem*. 2001;276(15):12404-12409.

66. Nesmelova IV, Sham Y, Dudek AZ, et al. Platelet factor 4 and interleukin-8 CXC chemokine heterodimer formation modulates function at the quaternary structural level. *J Biol Chem*. 2005;280(6):4948-4958.

67. Nesmelova IV, Sham Y, Gao J, Mayo KH. CXC and CC chemokines form mixed heterodimers: association free energies from molecular dynamics simulations and experimental correlations. *J Biol Chem*. 2008;283(35):24155-24166.

68. Weber C, Koenen RR. Fine-tuning leukocyte responses: towards a chemokine "interactome." *Trends Immunol*. 2006;27(6):268-273.

69. Babcock GJ, Farzan M, Sodroski J. Ligand-independent dimerization of CXCR4, a principal HIV-1 coreceptor. *J Biol Chem*. 2003;278(5): 3378-3385.

70. Wu B, Chien EY, Mol CD, et al. Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science*. 2010;330(6007):1066-1071.

71. Vischer HF, Nijmeijer S, Smit MJ, Leurs R. Viral hijacking of human receptors through heterodimerization. *Biochem Biophys Res Commun*. 2008;377(1):93-97.

72. Mellado M, Rodriguez-Frade JM, Manes S, Martinez AC. Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation. *Annu Rev Immunol*. 2001;19:397-421.

73. Springael JY, Urizar E, Parmentier M. Dimerization of chemokine receptors and its functional consequences. *Cytokine Growth Factor Rev*. 2005;16(6):611-623.

74. Thelen M, Baggiolini M. Is dimerization of chemokine receptors functionally relevant? *Sci STKE*. 2001;2001(104):E34.

75. Waldmann TA. T-cell receptors for cytokines: targets for immunotherapy of leukemia/lymphoma. *Ann Oncol*. 2000;11(suppl 1):101-106.

76. Rosenkilde MM, Smit MJ, Waldhoer M. Structure, function and physiological consequences of virally encoded chemokine seven transmembrane receptors. *Br J Pharmacol*. 2008;153(suppl 1):S154-S166.

77. Slinger E, Langemeijer E, Siderius M, Vischer HF, Smit MJ. Herpesvirusencoded GPCRs rewire cellular signaling. *Mol Cell Endocrinol*. 2011; 331(2):179-184.

78. Graham GJ. D6 and the atypical chemokine receptor family: novel regulators of immune and inflammatory processes. *Eur J Immunol* . 2009; 39(2):342-351.

79. Locati M, Torre YM, Galliera E, et al. Silent chemoattractant receptors: D6 as a decoy and scavenger receptor for inflammatory CC chemokines. *Cytokine Growth Factor Rev* . 2005;16(6):679-686.

80. Bonecchi R, Savino B, Borroni EM, Mantovani A, Locati M. Chemokine decoy receptors: structure-function and biological properties. *Curr Top Microbiol Immunol* . 2010;341:15-36.

81. Gosling J, Dairaghi DJ, Wang Y, et al. Cutting edge: identification of a novel chemokine receptor that binds dendritic cell- and T cell-active chemokines including ELC, SLC, and TECK. *J Immunol* . 2000;164(6):2851-2856.

82. Rajagopal S, Kim J, Ahn S, et al. Beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. *Proc Natl Acad Sci U S A* . 2010;107(2):628-632.

83. Szabo MC, Soo KS, Zlotnik A, Schall TJ. Chemokine class differences in binding to the Duffy antigen-erythrocyte chemokine receptor. *J Biol Chem* . 1995;270(43):25348-25351.

84. Pruenster M, Mudde L, Bombosi P, et al. The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity. *Nat Immunol* . 2009;10(1):101-108.

85. Dawson TC, Lentsch AB, Wang Z, et al. Exaggerated response to endotoxin in mice lacking the Duffy antigen/receptor for chemokines (DARC). *Blood* . 2000;96(5):1681-1684.

86. Madigan J, Freeman DJ, Menzies F, et al. Chemokine scavenger D6 is expressed by trophoblasts and aids the survival of mouse embryos transferred into allogeneic recipients. *J Immunol* . 2010;184(6):3202-3212.

87. Nibbs RJ, Kriehuber E, Ponath PD, et al. The beta-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors. *Am J Pathol* . 2001;158(3):867-877.

88. Galliera E, Jala VR, Trent JO, et al. beta-Arrestin-dependent constitutive internalization of the human chemokine decoy receptor D6. *J Biol Chem* . 2004;279(24):25590-25597.

89. Di Liberto D, Locati M, Caccamo N, et al. Role of the chemokine decoy receptor D6 in balancing inflammation, immune activation, and antimicrobial resistance in Mycobacterium tuberculosis infection. *J Exp Med* . 2008;205(9):2075-2084.

90. van Berkel V, Barrett J, Tiffany HL, et al. Identification of a gammaherpesvirus selective chemokine binding protein that inhibits chemokine action. *J Virol* . 2000;74(15):6741-6747.

91. Smith P, Fallon RE, Mangan NE, et al. Schistosoma mansoni secretes a chemokine binding protein with antiinflammatory activity. *J Exp Med* . 2005;202(10):1319-1325.

92. Deruaz M, Frauenschuh A, Alessandri AL, et al. Ticks produce highly selective chemokine binding proteins with antiinflammatory activity. *J Exp Med* . 2008;205(9):2019-2031.

93. Howard OZ. Autoantigen signalling through chemokine receptors. *Curr Opin Rheumatol* . 2006;18(6):642-646.

94. Wakasugi K, Schimmel P. Two distinct cytokines released from a human aminoacyl-tRNA synthetase. *Science.* 1999;284(5411):147-151.

95. Yang D, Chertov O, Bykovskaia SN, et al. Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science.* 1999;286(5439):525-528.

96. Bandyopadhyay S, Zhan R, Chaudhuri A, et al. Interaction of KAI1 on tumor cells with DARC on vascular endothelium leads to metastasis suppression. *Nat Med* . 2006;12(8):933-938.

97. Shimaoka T, Nakayama T, Hieshima K, et al. Chemokines generally exhibit scavenger receptor activity through their receptor-binding domain. *J Biol Chem* . 2004;279(26):26807-26810.

98. Gursel M, Gursel I, Mostowski HS, Klinman DM. CXCL16 influences the nature and specificity of CpG-induced immune activation. *J Immunol* . 2006;177(3):1575-1580.

99. Elagoz A, Henderson D, Babu PS, et al. A truncated form of CKbeta8-1 is a potent agonist for human formyl peptide-receptor-like 1 receptor. *Br J Pharmacol* . 2004;141(1):37-46.

100. Weathington NM, van Houwelingen AH, Noerager BD, et al. A novel peptide CXCR ligand derived from extracellular matrix degradation during airway inflammation. *Nat Med* . 2006;12(3):317-323.

101. de Kruijf P, Lim HD, Overbeek SA, et al. The collagen-breakdown product N-acetyl-Proline-Glycine-Proline (N-alpha-PGP) does not interact directly with human CXCR1 and CXCR2. *Eur J Pharmacol* . 2010;643(1):29-33.

102. Xiao H, Neuveut C, Tiffany HL, et al. Selective CXCR4 antagonism by Tat: implications for in vivo expansion of coreceptor use by HIV-1. *Proc Natl Acad Sci U S A* . 2000;97(21):11466-11471.

103. Aliberti J, Valenzuela JG, Carruthers VB, et al. Molecular mimicry of a CCR5 binding-domain in the microbial activation of dendritic cells. *Nat Immunol* . 2003;4(5):485-490.

104. Arthos J, Rubbert A, Rabin RL, et al. CCR5 signal transduction in macrophages by human immunodeficiency virus and simian immunodeficiency virus envelopes. *J Virol* . 2000;74(14):6418-6424.

105. Floto RA, MacAry PA, Boname JM, et al. Dendritic cell stimulation by mycobacterial Hsp70 is mediated through CCR5. *Science.* 2006;314(5798):454-458.

106. Weissman D, Rabin RL, Arthos J, et al. Macrophage-tropic HIV and SIV envelope proteins induce a signal through the CCR5 chemokine receptor. *Nature.* 1997;389(6654):981-985.

107. DeVries ME, Kelvin AA, Xu L, Ran L, Robinson J, Kelvin DJ. Defining the origins and evolution of the chemokine/chemokine receptor system. *J Immunol* . 2006;176(1):401-415.

108. Kaiser P, Poh TY, Rothwell L, et al. A genomic analysis of chicken cytokines and chemokines. *J Interferon Cytokine Res* . 2005;25(8):467-484.

109. Forssmann U, Magert HJ, Adermann K, Escher SE, Forssmann WG. Hemofiltrate CC chemokines with unique biochemical properties: HCC-1/CCL14a and HCC-2/CCL15. *J Leukoc Biol* . 2001;70(3):357-366.

110. Gonzalez E, Kulkarni H, Bolivar H, et al. The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. *Science.* 2005;307(5714):1434-1440.

111. Nakano H, Gunn MD. Gene duplications at the chemokine locus on mouse chromosome 4: multiple strain-specific haplotypes and the deletion of secondary lymphoid-organ chemokine and EBI-1 ligand chemokine genes in the plt mutation. *J Immunol* . 2001;166(1):361-369.

112. Gonzalez E, Dhanda R, Bamshad M, et al. Global survey of genetic variation in CCR5, RANTES, and MIP-1alpha: impact on the epidemiology of the HIV-1 pandemic. *Proc Natl Acad Sci U S A* . 2001;98(9):5199-5204.

113. Mummidi S, Bamshad M, Ahuja SS, et al. Evolution of human and nonhuman primate CC chemokine receptor 5 gene and mRNA. Potential roles for haplotype and mRNA diversity, differential haplotype-specific transcriptional activity, and altered transcription factor binding to polymorphic nucleotides in the pathogenesis of HIV-1 and simian immunodeficiency virus. *J Biol Chem*. 2000;275(25):18946-18961.

114. O'Brien SJ, Moore JP. The effect of genetic variation in chemokines and their receptors on HIV transmission and progression to AIDS. *Immunol Rev*. 2000;177:99-111.

115. Allavena P, Sica A, Vecchi A, Locati M, Sozzani S, Mantovani A. The chemokine receptor switch paradigm and dendritic cell migration: its significance in tumor tissues. *Immunol Rev*. 2000;177:141-149.

116. Campbell JJ, Pan J, Butcher EC. Cutting edge: developmental switches in chemokine responses during T cell maturation. *J Immunol*. 1999; 163(5):2353-2357.

117. Hargreaves DC, Hyman PL, Lu TT, et al. A coordinated change in chemokine responsiveness guides plasma cell movements. *J Exp Med*. 2001;194(1):45-56.

118. Kim CH, Rott L, Kunkel EJ, et al. Rules of chemokine receptor association with T cell polarization in vivo. *J Clin Invest*. 2001;108(9): 1331-1339.

119. Kunkel EJ, Boisvert J, Murphy K, et al. Expression of the chemokine receptors CCR4, CCR5, and CXCR3 by human tissue-infiltrating lymphocytes. *Am J Pathol*. 2002;160(1):347-355.

120. Campbell JJ, Qin S, Unutmaz D, et al. Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. *J Immunol*. 2001;166(11):6477-6482.

121. Robertson MJ. Role of chemokines in the biology of natural killer cells. *J Leukoc Biol*. 2002;71(2):173-183.

122. Berahovich RD, Lai NL, Wei Z, Lanier LL, Schall TJ. Evidence for NK cell subsets based on chemokine receptor expression. *J Immunol*. 2006;177(11):7833-7840.

123. Sallusto F, Mackay CR. Chemoattractants and their receptors in homeostasis and inflammation. *Curr Opin Immunol*. 2004;16(6):724-731.

124. Mantovani A, Garlanda C, Locati M. Macrophage diversity and polarization in atherosclerosis: a question of balance. *Arterioscler Thromb Vasc Biol*. 2009;29(10):1419-1423.

125. Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. *Immunity*. 2005;23(4):344-346.

126. Gerard C, Rollins BJ. Chemokines and disease. *Nat Immunol*. 2001; 2(2):108-115.

127. Gutierrez-Ramos JC, Lloyd C, Kapsenberg ML, Gonzalo JA, Coyle AJ. Non-redundant functional groups of chemokines operate in a coordinate manner during the inflammatory response in the lung. *Immunol Rev*. 2000;177:31-42.

128. Karpus WJ, Ransohoff RM. Chemokine regulation of experimental autoimmune encephalomyelitis: temporal and spatial expression patterns govern disease pathogenesis. *J Immunol*. 1998;161(6):2667-2671.

129. Qiu B, Frait KA, Reich F, Komuniecki E, Chensue SW. Chemokine expression dynamics in mycobacterial (type-1) and schistosomal (type-2) antigen-elicited pulmonary granuloma formation. *Am J Pathol*. 2001;158(4):1503-1515.

130. Brandt E, Petersen F, Ludwig A, Ehlert JE, Bock L, Flad HD. The beta-thromboglobulins and platelet factor 4: blood platelet-derived CXC chemokines with divergent roles in early neutrophil regulation. *J Leukoc Biol*. 2000;67(4):471-478.

131. Campanella GS, Grimm J, Manice LA, et al. Oligomerization of CXCL10 is necessary for endothelial cell presentation and in vivo activity. *J Immunol*. 2006;177(10):6991-6998.

132. Middleton J, Patterson AM, Gardner L, Schmutz C, Ashton BA. Leukocyte extravasation: chemokine transport and presentation by the endothelium. *Blood*. 2002;100(12):3853-3860.

133. Baekkevold ES, Yamanaka T, Palframan RT, et al. The CCR7 ligand elc (CCL19) is transcytosed in high endothelial venules and mediates T cell recruitment. *J Exp Med*. 2001;193(9):1105-1112.

134. Berson JF, Doms RW. Structure-function studies of the HIV-1 coreceptors. *Semin Immunol*. 1998;10(3):237-248.

135. Hemmerich S, Paavola C, Bloom A, et al. Identification of residues in the monocyte chemotactic protein-1 that contact the MCP-1 receptor, CCR2. *Biochemistry*. 1999;38(40):13013-13015.

136. Farzan M, Mirzabekov T, Kolchinsky P, et al. Tyrosine sulfation of the amino terminus of CCR5 facilitates HIV-1 entry. *Cell*. 1999;96(5): 667-676.

137. Kehoe JW, Bertozzi CR. Tyrosine sulfation: a modulator of extracellular protein-protein interactions. *Chem Biol*. 2000;7(3):R57-R61.

138. Manes S, Gomez-Mouton C, Lacalle RA, Jimenez-Baranda S, Mira E, Martinez AC. Mastering time and space: immune cell polarization and chemotaxis. *Semin Immunol*. 2005;17(1):77-86.

139. Van Haastert PJ, Devreotes PN. Chemotaxis: signalling the way forward. *Nat Rev Mol Cell Biol*. 2004;5(8):626-634.

140. Castellino F, Germain RN. Chemokine-guided CD4+ T cell help enhances generation of IL-6RalphahighIL-7Ralpha high prememory CD8+ T cells. *J Immunol*. 2007;178(2):778-787.

141. Castellino F, Huang AY, Altan-Bonnet G, Stoll S, Scheinecker C, Germain RN. Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. *Nature*. 2006; 440(7086):890-895.

142. Viola A, Contento RL, Molon B. T cells and their partners: the chemokine dating agency. *Trends Immunol*. 2006;27(9):421-427.

143. Kumar A, Humphreys TD, Kremer KN, et al. CXCR4 physically associates with the T cell receptor to signal in T cells. *Immunity*. 2006; 25(2):213-224.

144. Cerdan C, Devilard E, Xerri L, Olive D. The C-class chemokine lymphotactin costimulates the apoptosis of human CD4(+) T cells. *Blood*. 2001;97(8):2205-2212.

145. Colamussi ML, Secchiero P, Gonelli A, Marchisio M, Zauli G, Capitani S. Stromal derived factor-1 alpha (SDF-1 alpha) induces CD4+ T cell apoptosis via the functional up-regulation of the Fas (CD95)/Fas ligand (CD95L) pathway. *J Leukoc Biol*. 2001;69(2):263-270.

146. Flad HD, Grage-Griebenow E, Petersen F, et al. The role of cytokines in monocyte apoptosis. *Pathobiology*. 1999;67(5-6):291-293.

147. Gu L, Tseng S, Horner RM, Tam C, Loda M, Rollins BJ. Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature*. 2000;404(6776):407-411.

148. Lataillade JJ, Clay D, Bourin P, et al. Stromal cell-derived factor 1 regulates primitive hematopoiesis by suppressing apoptosis and by promoting G(0)/G(1) transition in CD34(+) cells: evidence for an autocrine/paracrine mechanism. *Blood*. 2002;99(4):1117-1129.

149. Nanki T, Lipsky PE. Cutting edge: stromal cell-derived factor-1 is a costimulator for CD4+ T cell activation. *J Immunol*. 2000;164(10): 5010-5014.

150. Youn BS, Kim YJ, Mantel C, Yu KY, Broxmeyer HE. Blocking of c-FLIP(L) —independent cycloheximide-induced apoptosis or Fasmediated apoptosis by the CC chemokine receptor 9/TECK interaction. *Blood*. 2001;98(4):925-933.

151. Boehme SA, Lio FM, Maciejewski-Lenoir D, Bacon KB, Conlon PJ. The chemokine fractalkine inhibits Fas-mediated cell death of brain microglia. *J Immunol*. 2000;165(1):397-403.

152. Grayson MH, Holtzman MJ. Chemokine signaling regulates apoptosis as well as immune cell traffic in host defense. *Cell Cycle*. 2006;5(4):380-383.

153. Perfettini JL, Castedo M, Roumier T, et al. Mechanisms of apoptosis induction by the HIV-1 envelope. *Cell Death Differ*. 2005;12 (suppl 1):916-923.

154. Sanchez-Sanchez N, Riol-Blanco L, de la Rosa G, et al. Chemokine receptor CCR7 induces intracellular signaling that inhibits apoptosis of mature dendritic cells. *Blood*. 2004;104(3):619-625.

155. Niess JH, Brand S, Gu X, et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science*. 2005; 307(5707):254-258.

156. Sanchez-Sanchez N, Riol-Blanco L, Rodriguez-Fernandez JL. The multiple personalities of the chemokine receptor CCR7 in dendritic cells. *J Immunol*. 2006;176(9):5153-5159.

157. Kaplan AP. Chemokines, chemokine receptors and allergy. *Int Arch Allergy Immunol*. 2001;124(4):423-431.

158. Cole AM, Ganz T, Liese AM, Burdick MD, Liu L, Strieter RM. Cutting edge: IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity. *J Immunol*. 2001;167(2):623-627.

159. Yung SC, Parenti D, Murphy PM. Host chemokines bind to Staphylococcus aureus and stimulate protein A release. *J Biol Chem*. 2011; 286(7):5069-5077.

160. Kehrl JH. Chemoattractant receptor signaling and the control of lymphocyte migration. *Immunol Res*. 2006;34(3):211-227.

161. Kehrl JH. Heterotrimeric G protein signaling: roles in immune function and fine-tuning by RGS proteins. *Immunity*. 1998;8(1):1-10.

162. Neptune ER, Bourne HR. Receptors induce chemotaxis by releasing the betagamma subunit of Gi, not by activating Gq or Gs. *Proc Natl Acad Sci U S A*. 1997;94(26):14489-14494.

163. Neptune ER, Iiri T, Bourne HR. Galphai is not required for chemotaxis mediated by Gi-coupled receptors. *J Biol Chem*. 1999;274(5):2824-2828.

164. Rickert P, Weiner OD, Wang F, Bourne HR, Servant G. Leukocytes navigate by compass: roles of PI3Kgamma and its lipid products. *Trends Cell Biol*. 2000;10(11):466-473.

165. Wu D, Huang CK, Jiang H. Roles of phospholipid signaling in chemoattractant-induced responses. *J Cell Sci*. 2000;113(pt 17):2935-2940.

166. Parent CA, Devreotes PN. A cell's sense of direction. *Science*. 1999; 284(5415):765-770.

167. Wang F, Herzmark P, Weiner OD, Srinivasan S, Servant G, Bourne HR. Lipid products of PI(3)Ks maintain persistent cell polarity and directed motility in neutrophils. *Nat Cell Biol*. 2002;4(7):513-518.

168. Chen Y, Corriden R, Inoue Y, et al. ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science*. 2006;314(5806):1792-1795.

169. Xu J, Wang F, Van Keymeulen A, et al. Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils. *Cell*. 2003;114(2):201-214.

170. Rieken S, Sassmann A, Herroeder S, et al. G12/G13 family G proteins regulate marginal zone B cell maturation, migration, and polarization. *J Immunol*. 2006;177(5):2985-2993.

171. Arvanitakis L, Geras-Raaka E, Varma A, Gershengorn MC, Cesarman E. Human herpesvirus KSHV encodes a constitutively active G-proteincoupled receptor linked to cell proliferation. *Nature*. 1997;385(6614): 347-350.

172. Moriguchi M, Hissong BD, Gadina M, et al. CXCL12 signaling is independent of Jak2 and Jak3. *J Biol Chem*. 2005;280(17):17408-14714.

173. Marchese A, Benovic JL. Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. *J Biol Chem*. 2001;276(49):45509-45512.

174. Peterson FC, Thorpe JA, Harder AG, Volkman BF, Schwarze SR. Structural determinants involved in the regulation of CXCL14/BRAK expression by the 26 S proteasome. *J Mol Biol*. 2006;363(4):813-822.

175. Overall CM, McQuibban GA, Clark-Lewis I. Discovery of chemokine substrates for matrix metalloproteinases by exosite scanning: a new tool for degradomics. *Biol Chem*. 2002;383(7-8):1059-1066.

176. Struyf S, Proost P, Van Damme J. Regulation of the immune response by the interaction of chemokines and proteases. *Adv Immunol*. 2003; 81:1-44.

177. McQuibban GA, Butler GS, Gong JH, et al. Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell-derived factor-1. *J Biol Chem*. 2001;276(47):43503-43508.

178. McQuibban GA, Gong JH, Tam EM, McCulloch CA, Clark-Lewis I, Overall CM. Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science*. 2000;289(5482):1202-1206.

179. Tsou CL, Haskell CA, Charo IF. Tumor necrosis factor-alpha-converting enzyme mediates the inducible cleavage of fractalkine. *J Biol Chem*. 2001;276(48):44622-44626.

180. Berahovich RD, Miao Z, Wang Y, Premack B, Howard MC, Schall TJ. Proteolytic activation of alternative CCR1 ligands in inflammation. *J Immunol*. 2005;174(11):7341-7351.

181. Mantovani A, Locati M, Vecchi A, Sozzani S, Allavena P. Decoy receptors: a strategy to regulate inflammatory cytokines and chemokines. *Trends Immunol*. 2001;22(6):328-336.

182. Mantovani A, Bonecchi R, Locati M. Tuning inflammation and immunity by chemokine sequestration: decoys and more. *Nat Rev Immunol*. 2006;6(12):907-918.

183. Neel NF, Schutyser E, Sai J, Fan GH, Richmond A. Chemokine receptor internalization and intracellular trafficking. *Cytokine Growth Factor Rev*. 2005;16(6):637-658.

184. Lasagni L, Grepin R, Mazzinghi B, et al. PF-4/CXCL4 and CXCL4L1 exhibit distinct subcellular localization and a differentially regulated mechanism of secretion. *Blood*. 2007;109(10):4127-4134.

185. Wagner L, Yang OO, Garcia-Zepeda EA, et al. Beta-chemokines are released from HIV-1-specific cytolytic T-cell granules complexed to proteoglycans. *Nature*. 1998;391(6670):908-911.

186. Huse M, Lillemeier BF, Kuhns MS, Chen DS, Davis MM. T cells use two directionally distinct pathways for cytokine secretion. *Nat Immunol*. 2006;7(3):247-255.

187. Katayama Y, Battista M, Kao WM, et al. Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell*. 2006;124(2):407-421.

188. Balabanian K, Lagane B, Infantino S, et al. The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *J Biol Chem*. 2005;280(42):35760-35766.

189. Hartmann TN, Grabovsky V, Pasvolsky R, et al. A crosstalk between intracellular CXCR7 and CXCR4 involved in rapid CXCL12-triggered integrin activation but not in chemokine-triggered motility of human T lymphocytes and CD34+ cells. *J Leukoc Biol*. 2008;84(4):1130-1140.

190. Infantino S, Moepps B, Thelen M. Expression and regulation of the orphan receptor RDC1 and its putative ligand in human dendritic and B cells. *J Immunol*. 2006;176(4):2197-2207.

191. Naumann U, Cameroni E, Pruenster M, et al. CXCR7 functions as a scavenger for CXCL12 and CXCL11. *PLoS One*. 2010;5(2):e9175.

192. Thelen M, Thelen S. CXCR7, CXCR4 and CXCL12: an eccentric trio? *J Neuroimmunol*. 2008;198(1-2):9-13.

193. Berahovich RD, Penfold ME, Schall TJ. Nonspecific CXCR7 antibodies. *Immunol Lett*. 2010;133(2):112-114.

194. Berahovich RD, Zabel BA, Penfold ME, et al. CXCR7 protein is not expressed on human or mouse leukocytes. *J Immunol*. 2010;185(9): 5130-5139.

195. Burns JM, Summers BC, Wang Y, et al. A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *J Exp Med*. 2006;203(9):2201-2213.

196. Hattermann K, Held-Feindt J, Lucius R, et al. The chemokine receptor CXCR7 is highly expressed in human glioma cells and mediates antiapoptotic effects. *Cancer Res*. 2010;70(8):3299-3308.

197. Miao Z, Luker KE, Summers BC, et al. CXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature. *Proc Natl Acad Sci U S A*. 2007;104(40):15735-15740.

198. Watanabe K, Penfold ME, Matsuda A, et al. Pathogenic role of CXCR7 in rheumatoid arthritis. *Arthritis Rheum*. 2010;62(11):3211-3220.

199. Zabel BA, Lewen S, Berahovich RD, Jaen JC, Schall TJ. The novel chemokine receptor CXCR7 regulates trans-endothelial migration of cancer cells. *Mol Cancer*. 2011;10:73.

200. Zabel BA, Wang Y, Lewen S, et al. Elucidation of CXCR7-mediated signaling events and inhibition of CXCR4-mediated tumor cell transendothelial migration by CXCR7 ligands. *J Immunol*. 2009;183(5): 3204-3211.

201. Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature*. 1998;393(6685):595-599.

202. Dar A, Kollet O, Lapidot T. Mutual, reciprocal SDF-1/CXCR4 interactions between hematopoietic and bone marrow stromal cells regulate human stem cell migration and development in NOD/SCID chimeric mice. *Exp Hematol*. 2006;34(8):967-975.

203. Peled A, Petit I, Kollet O, et al. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. *Science*. 1999;283(5403):845-848.

204. Martin C, Burdon PC, Bridger G, Gutierrez-Ramos JC, Williams TJ, Rankin SM. Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity*. 2003;19(4):583-593.

205. Pelus LM, Fukuda S. Peripheral blood stem cell mobilization: the CXCR2 ligand GRObeta rapidly mobilizes hematopoietic stem cells with enhanced engraftment properties. *Exp Hematol*. 2006;34(8):1010-1020.

206. Haas P, Gilmour D. Chemokine signaling mediates self-organizing tissue migration in the zebrafish lateral line. *Dev Cell*. 2006;10(5): 673-680.

207. Ceradini DJ, Gurtner GC. Homing to hypoxia: HIF-1 as a mediator of progenitor cell recruitment to injured tissue. *Trends Cardiovasc Med*. 2005;15(2):57-63.

208. Jin DK, Shido K, Kopp HG, et al. Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes. *Nat Med*. 2006;12(5):557-567.

209. Zagzag D, Lukyanov Y, Lan L, et al. Hypoxia-inducible factor 1 and VEGF upregulate CXCR4 in glioblastoma: implications for angiogenesis and glioma cell invasion. *Lab Invest*. 2006;86(12):1221-1232.

210. Cacalano G, Lee J, Kikly K, et al. Neutrophil and B cell expansion in mice that lack the murine IL-8 receptor homolog. *Science*. 1994; 265(5172):682-684.

211. Uehara S, Grinberg A, Farber JM, Love PE. A role for CCR9 in T lymphocyte development and migration. *J Immunol*. 2002;168(6): 2811-2819.

212. Uehara S, Song K, Farber JM, Love PE. Characterization of CCR9 expression and CCL25/thymus-expressed chemokine responsiveness during T cell development: CD3(high)CD69+ thymocytes and gammadeltaTCR+ thymocytes preferentially respond to CCL25. *J Immunol*. 2002;168(1):134-142.

213. Zabel BA, Agace WW, Campbell JJ, et al. Human G protein-coupled receptor GPR-9-6/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis. *J Exp Med*. 1999;190(9):1241-1256.

214. Kurobe H, Liu C, Ueno T, et al. CCR7-dependent cortex-to-medulla migration of positively selected thymocytes is essential for establishing central tolerance. *Immunity*. 2006;24(2):165-177.

215. Kurth I, Willimann K, Schaerli P, Hunziker T, Clark-Lewis I, Moser B. Monocyte selectivity and tissue localization suggests a role for breast and kidney-expressed chemokine (BRAK) in macrophage development. *J Exp Med*. 2001;194(6):855-861.

216. Shurin GV, Ferris RL, Tourkova IL, et al. Loss of new chemokine CXCL14 in tumor tissue is associated with low infiltration by dendritic cells (DC), while restoration of human CXCL14 expression in tumor cells causes attraction of DC both in vitro and in vivo. *J Immunol*. 2005;174(9):5490-5498.

217. Rescigno M. CCR6(+) dendritic cells: the gut tactical-response unit. *Immunity*. 2006;24(5):508-510.

218. Onai N, Kitabatake M, Zhang YY, Ishikawa H, Ishikawa S, Matsushima K. Pivotal role of CCL25 (TECK)-CCR9 in the formation of gut cryptopatches and consequent appearance of intestinal intraepithelial T lymphocytes. *Int Immunol*. 2002;14(7):687-694.

219. Gear AR, Camerini D. Platelet chemokines and chemokine receptors: linking hemostasis, inflammation, and host defense. *Microcirculation*. 2003;10(3-4):335-350.

220. Struyf S, Burdick MD, Peeters E, et al. Platelet factor-4 variant chemokine CXCL4L1 inhibits melanoma and lung carcinoma growth and metastasis by preventing

angiogenesis. *Cancer Res*. 2007;67(12): 5940-5948.

221. Struyf S, Burdick MD, Proost P, Van Damme J, Strieter RM. Platelets release CXCL4L1, a nonallelic variant of the chemokine platelet factor-4/CXCL4 and potent inhibitor of angiogenesis. *Circ Res*. 2004; 95(9):855-857.

222. Massberg S, Konrad I, Schurzinger K, et al. Platelets secrete stromal cell-derived factor 1alpha and recruit bone marrow-derived progenitor cells to arterial thrombi in vivo. *J Exp Med*. 2006;203(5):1221-1233.

223. Bizzarri C, Beccari AR, Bertini R, Cavicchia MR, Giorgini S, Allegretti M. ELR+ CXC chemokines and their receptors (CXC chemokine receptor 1 and CXC chemokine receptor 2) as new therapeutic targets. *Pharmacol Ther*. 2006;112(1):139-149.

224. Harada A, Mukaida N, Matsushima K. Interleukin 8 as a novel target for intervention therapy in acute inflammatory diseases. *Mol Med Today*. 1996;2(11):482-489.

225. Harada A, Sekido N, Akahoshi T, Wada T, Mukaida N, Matsushima K. Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J Leukoc Biol*. 1994;56(5):559-564.

226. McLoughlin RM, Solinga RM, Rich J, et al. CD4+ T cells and CXC chemokines modulate the pathogenesis of Staphylococcus aureus wound infections. *Proc Natl Acad Sci U S A*. 2006;103(27):10408-10413.

227. Hidalgo-Grass C, Mishalian I, Dan-Goor M, et al. A streptococcal protease that degrades CXC chemokines and impairs bacterial clearance from infected tissues. *EMBO J*. 2006;25(19):4628-4637.

228. Ariel A, Fredman G, Sun YP, et al. Apoptotic neutrophils and T cells sequester chemokines during immune response resolution through modulation of CCR5 expression. *Nat Immunol*. 2006;7(11):1209-1216.

229. Chou RC, Kim ND, Sadik CD, et al. Lipid-cytokine-chemokine cascade drives neutrophil recruitment in a murine model of inflammatory arthritis. *Immunity*. 2010;33(2):266-278.

230. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*. 2003; 19(1):71-82.

231. Lim JK, Obara CJ, Rivollier A, Pletnev AG, Kelsall BL, Murphy PM. Chemokine receptor Ccr2 is critical for monocyte accumulation and survival in West Nile virus encephalitis. *J Immunol*. 2011;186(1):471-478.

232. Serbina NV, Pamer EG. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol*. 2006;7(3):311-317.

233. Serbina NV, Jia T, Hohl TM, Pamer EG. Monocyte-mediated defense against microbial pathogens. *Annu Rev Immunol*. 2008;26:421-452.

234. Mantovani A, Allavena P, Sozzani S, Vecchi A, Locati M, Sica A. Chemokines in the recruitment and shaping of the leukocyte infiltrate of tumors. *Semin Cancer Biol*. 2004;14(3):155-160.

235. Salazar-Mather TP, Hamilton TA, Biron CA. A chemokine-to-cytokineto-chemokine cascade critical in antiviral defense. *J Clin Invest*. 2000; 105(7):985-993.

236. Medzhitov R, Janeway C Jr. Innate immune recognition: mechanisms and pathways. *Immunol Rev*. 2000;173:89-97.

237. Coelho AL, Hogaboam CM, Kunkel SL. Chemokines provide the sustained inflammatory bridge between innate and acquired immunity. *Cytokine Growth Factor Rev*. 2005;16(6):553-560.

238. Ness TL, Ewing JL, Hogaboam CM, Kunkel SL. CCR4 is a key modulator of innate immune responses. *J Immunol*. 2006;177(11):7531-7539.

239. Moser K, Tokoyoda K, Radbruch A, MacLennan I, Manz RA. Stromal niches, plasma cell differentiation and survival. *Curr Opin Immunol*. 2006;18(3):265-270.

240. Randolph GJ, Sanchez-Schmitz G, Angeli V. Factors and signals that govern the migration of dendritic cells via lymphatics: recent advances. *Springer Semin Immunopathol*. 2005;26(3):273-287.

241. Schaerli P, Moser B. Chemokines: control of primary and memory T-cell traffic. *Immunol Res*. 2005;31(1):57-74.

242. Weninger W, von Andrian UH. Chemokine regulation of naive T cell traffic in health and disease. *Semin Immunol*. 2003;15(5):257-270.

243. Sato N, Ahuja SK, Quinones M, et al. CC chemokine receptor (CCR)2 is required for langerhans cell migration and localization of T helper cell type 1 (Th1)-inducing dendritic cells. Absence of CCR2 shifts the Leishmania major-resistant phenotype to a susceptible state dominated by Th2 cytokines, b cell outgrowth, and sustained neutrophilic inflammation. *J Exp Med*. 2000;192(2):205-218.

244. Okada T, Miller MJ, Parker I, et al. Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS Biol*. 2005;3(6):e150.

245. Okada T, Ngo VN, Ekland EH, et al. Chemokine requirements for B cell entry to lymph nodes and Peyer's patches. *J Exp Med*. 2002;196(1): 65-75.

246. Ansel KM, Harris RBS, Cyster JG. CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. *Immunity*. 2002;16(1):67-76.

247. Luther SA, Lopez T, Bai W, Hanahan D, Cyster JG. BLC expression in pancreatic islets causes B cell recruitment and lymphotoxin-dependent lymphoid neogenesis. *Immunity*. 2000;12(5):471-481.

248. Janatpour MJ, Hudak S, Sathe M, Sedgwick JD, McEvoy LM. Tumor necrosis factor-dependent segmental control of MIG expression by high endothelial venules in inflamed lymph nodes regulates monocyte recruitment. *J Exp Med*. 2001;194(9):1375-1384.

249. Palframan RT, Jung S, Cheng CY, et al. Inflammatory chemokine transport and presentation in HEV: a remote control mechanism for monocyte recruitment to lymph nodes in inflamed tissues. *J Exp Med*. 2001;194(9):1361-73.

250. Reif K, Ekland EH, Ohl L, et al. Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature*. 2002;416(6876):94-99.

251. Friedman RS, Jacobelli J, Krummel MF. Surface-bound chemokines capture and prime T cells for synapse formation. *Nat Immunol*. 2006;7 (10):1101-1108.

252. Schumann K, Lammermann T, Bruckner M, et al. Immobilized chemokine fields and soluble chemokine gradients cooperatively shape migration patterns of dendritic cells. *Immunity*. 2010;32(5):703-713.

253. Wendland M, Willenzon S, Kocks J, et al. Lymph node T cell homeostasis relies on steady state homing of dendritic cells. *Immunity*. 2011; 35(6):945-957.

254. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*. 1999;401(6754):708-712.

255. Bromley SK, Thomas SY, Luster AD. Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics. *Nat Immunol*. 2005;6(9):895-901.

256. Debes GF, Arnold CN, Young AJ, et al. Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. *Nat Immunol*. 2005;6(9):889-894.

257. Forster R, Davalos-Misslitz AC, Rot A. CCR7 and its ligands: balancing immunity and tolerance. *Nat Rev Immunol*. 2008;8(5):362-371.

258. Schneider MA, Meingassner JG, Lipp M, Moore HD, Rot A. CCR7 is required for the in vivo function of CD4+ CD25+ regulatory T cells. *J Exp Med*. 2007;204(4):735-745.

259. Song K, Rabin RL, Hill BJ, et al. Characterization of subsets of CD4+ memory T cells reveals early branched pathways of T cell differentiation in humans. *Proc Natl Acad Sci U S A*. 2005;102(22):7916-7921.

260. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J Exp Med*. 1998;187(6):875-883.

261. Mikhak Z, Fleming CM, Medoff BD, et al. STAT1 in peripheral tissue differentially regulates homing of antigen-specific Th1 and Th2 cells. *J Immunol*. 2006;176(8):4959-4967.

262. Yurchenko E, Tritt M, Hay V, Shevach EM, Belkaid Y, Piccirillo CA. CCR5-dependent homing of naturally occurring CD4+ regulatory T cells to sites of Leishmania major infection favors pathogen persistence. *J Exp Med*. 2006;203(11):2451-2460.

263. Acosta-Rodriguez EV, Rivino L, Geginat J, et al. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol*. 2007;8(6):639-646.

264. Singh SP, Zhang HH, Foley JF, Hedrick MN, Farber JM. Human T cells that are able to produce IL-17 express the chemokine receptor CCR6. *J Immunol*. 2008;180(1):214-221.

265. Duhen T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat Immunol*. 2009;10(8):857-863.

266. Rivino L, Gruarin P, Haringer B, et al. CCR6 is expressed on an IL-10-producing, autoreactive memory T cell population with contextdependent regulatory function. *J Exp Med*. 2010;207(3):565-577.

267. Kunkel EJ, Butcher EC. Chemokines and the tissue-specific migration of lymphocytes. *Immunity*. 2002;16(1):1-4.

268. Homey B, Alenius H, Muller A, et al. CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. *Nat Med*. 2002;8(2):157-165.

269. Salazar-Gonzalez RM, Niess JH, Zammit DJ, et al. CCR6-mediated dendritic cell activation of pathogen-specific T cells in Peyer's patches. *Immunity*. 2006;24(5):623-632.

270. Bowman EP, Kuklin NA, Youngman KR, et al. The intestinal chemokine thymus-expressed chemokine (CCL25) attracts IgA antibody-secreting cells. *J Exp Med*. 2002;195(2):269-275.

271. Mora JR, Bono MR, Manjunath N, et al. Selective imprinting of guthoming T cells by Peyer's patch dendritic cells. *Nature*. 2003;424(6944): 88-93.

272. Mora JR, von Andrian UH. Differentiation and homing of IgA-secreting cells. *Mucosal Immunol*. 2008;1(2):96-109.

273. Feng Y, Broder CC, Kennedy PE, Berger EA. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science*. 1996;272(5263):872-877.

274. Dean M, Carrington M, Winkler C, et al. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science*. 1996;273(5283):1856-1862.

275. Liu R, Paxton WA, Choe S, et al. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell*. 1996;86(3):367-377.

276. Zimmerman PA, Buckler-White A, Alkhatib G, et al. Inherited resistance to HIV-1 conferred by an inactivating mutation in CC chemokine receptor 5: studies in populations with contrasting clinical phenotypes, defined racial background, and quantified risk. *Mol Med*. 1997;3(1):23-36.

277. Arenzana-Seisdedos F, Parmentier M. Genetics of resistance to HIV infection: Role of co-receptors and co-receptor ligands. *Semin Immunol*. 2006;18(6):387-403.

278. Sabeti PC, Walsh E, Schaffner SF, et al. The case for selection at CCR5-Delta32. *PLoS Biol*. 2005;3(11):e378.

279. Allers K, Hutter G, Hofmann J, et al. Evidence for the cure of HIV infection by CCR5Delta32/Delta32 stem cell transplantation. *Blood*. 2011;117(10):2791-2799.

280. Hutter G, Nowak D, Mossner M, et al. Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *N Engl J Med*. 2009;360(7):692-698.

281. Glass WG, Lim JK, Cholera R, Pletnev AG, Gao JL, Murphy PM. Chemokine receptor CCR5 promotes leukocyte trafficking to the brain and survival in West Nile virus infection. *J Exp Med*. 2005;202(8):1087-1098.

282. Glass WG, McDermott DH, Lim JK, et al. CCR5 deficiency increases risk of symptomatic West Nile virus infection. *J Exp Med*. 2006;203(1): 35-40.

283. Lim JK, McDermott DH, Lisco A, et al. CCR5 deficiency is a risk factor for early clinical manifestations of West Nile virus infection but not for viral transmission. *J Infect Dis*. 2010;201(2):178-185.

284. Klein RS, Lin E, Zhang B, et al. Neuronal CXCL10 directs CD8+ T-cell recruitment and control of West Nile virus encephalitis. *J Virol*. 2005;79(17):11457-11466.

285. Hsieh MF, Lai SL, Chen JP, et al. Both CXCR3 and CXCL10/IFN-inducible protein 10 are required for resistance to primary infection by dengue virus. *J Immunol*. 2006;177(3):1855-1863.

286. Levine B, Sodora DL. HIV and CXCR4 in a kiss of autophagic death. *J Clin Invest*. 2006;116(8):2078-2080.

287. Gaur D, Mayer DC, Miller LH. Parasite ligand-host receptor interactions during invasion of erythrocytes by Plasmodium merozoites. *Int J Parasitol*. 2004;34(13-14):1413-1429.

288. Tournamille C, Colin Y, Cartron JP, Le Van Kim C. Disruption of a GATA motif in the Duffy gene promoter abolishes erythroid gene expression in Duffy-negative individuals. *Nat Genet*. 1995;10(2):224-228.

289. Hatabu T, Kawazu S, Aikawa M, Kano S. Binding of Plasmodium falciparum-infected erythrocytes to the membrane-bound form of Fractalkine/CX3CL1. *Proc Natl Acad Sci U S A*. 2003;100(26):15942-15946.

290. Tripp RA, Jones LP, Haynes LM, Zheng H, Murphy PM, Anderson LJ. CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. *Nat Immunol*. 2001;2(8):732-738.

291. Diaz GA. CXCR4 mutations in WHIM syndrome: a misguided immune system? *Immunol Rev*. 2005;203:235-243.

292. Gorlin RJ, Gelb B, Diaz GA, Lofsness KG, Pittelkow MR, Fenyk JR Jr. WHIM syndrome, an autosomal dominant disorder: clinical, hematological, and molecular studies. *Am J Med Genet* . 2000;91(5):368-376.

293. Lagane B, Chow KY, Balabanian K, et al. CXCR4 dimerization and beta-arrestin-mediated signaling account for the enhanced chemotaxis to CXCL12 in WHIM syndrome. *Blood*. 2008;112(1):34-44.

294. McDermott DH, Lopez J, Deng F, et al. AMD3100 is a potent antagonist at CXCR4(R334X), a hyperfunctional mutant chemokine receptor and cause of WHIM syndrome. *J Cell Mol Med* . 2011;15(10):2071-2081.

295. Chow KY, Brotin E, Ben Khalifa Y, et al. A pivotal role for CXCL12 signaling in HPV-mediated transformation of keratinocytes: clues to understanding HPV-pathogenesis in WHIM syndrome. *Cell Host Microbe* . 2010;8(6):523-533.

296. Dale DC, Bolyard AA, Kelley ML, et al. The CXCR4 antagonist plerixafor is a potential therapy for myelokathexis, WHIM syndrome. *Blood*. 2011;118(18):4963-4966.

297. McDermott DH, Liu Q, Ulrick J, et al. The CXCR4 antagonist plerixafor corrects panleukopenia in patients with WHIM syndrome. *Blood*. 2011;118(18):4957-4962.

298. Pusic I, DiPersio JF. Update on clinical experience with AMD3100, an SDF-1/CXCL12-CXCR4 inhibitor, in mobilization of hematopoietic stem and progenitor cells. *Curr Opin Hematol* . 2010;17(4):319-326.

299. DiPersio JF, Micallef IN, Stiff PJ, et al. Phase III prospective randomized double-blind placebo-controlled trial of plerixafor plus granulocyte colony-stimulating factor compared with placebo plus granulocyte colony-stimulating factor for autologous stem-cell mobilization and transplantation for patients with non-Hodgkin's lymphoma. *J Clin Oncol* . 2009;27(28):4767-4773.

300. Boztug K, Appaswamy G, Ashikov A, et al. A syndrome with congenital neutropenia and mutations in G6PC3. *N Engl J Med* . 2009;360(1):32-43.

301. McDermott DH, De Ravin SS, Jun HS, et al. Severe congenital neutropenia resulting from G6PC3 deficiency with increased neutrophil CXCR4 expression and myelokathexis. *Blood*. 2010;116(15):2793-2802.

302. Jensen KK, Lira SA. Chemokines and Kaposi's sarcoma. *Semin Cancer Biol* . 2004;14(3):187-194.

303. Holst PJ, Rosenkilde MM, Manfra D, et al. Tumorigenesis induced by the HHV8-encoded chemokine receptor requires ligand modulation of high constitutive activity. *J Clin Invest*. 2001;108(12):1789-1796.

304. Sodhi A, Chaisuparat R, Hu J, et al. The TSC2/mTOR pathway drives endothelial cell transformation induced by the Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor. *Cancer Cell* . 2006;10(2):133-143.

305. Schwarz M, Murphy PM. Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor constitutively activates NF-kappa B and induces proinflammatory cytokine via a C-terminal signaling and chemokine production determinant. *J Immunol* . 2001;167(1):505-513.

306. Barlic J, Zhang Y, Foley JF, Murphy PM. Oxidized lipid-driven chemokine receptor switch, CCR2 to CX3CR1, mediates adhesion of human macrophages to coronary artery smooth muscle cells through a peroxisome proliferator-activated receptor gamma-dependent pathway. *Circulation*. 2006;114(8):807-819.

307. McDermott DH, Halcox JP, Schenke WH, et al. Association between polymorphism in the chemokine receptor CX3CR1 and coronary vascular endothelial dysfunction and atherosclerosis. *Circ Res*. 2001; 89(5):401-407.

308. Moatti D, Faure S, Fumeron F, et al. Polymorphism in the fractalkine receptor CX3CR1 as a genetic risk factor for coronary artery disease. *Blood*. 2001;97(7):1925-1928.

309. Combadiere C, Potteaux S, Gao JL, et al. Decreased atherosclerotic lesion formation in CX3CR1/apolipoprotein E double knockout mice. *Circulation*. 2003;107(7):1009-1016.

310. McDermott DH, Fong AM, Yang Q, et al. Chemokine receptor mutant CX3CR1-M280 has impaired adhesive function and correlates with protection from cardiovascular disease in humans. *J Clin Invest*. 2003; 111(8):1241-1250.

311. Lesnik P, Haskell CA, Charo IF. Decreased atherosclerosis in CX3CR1-/- mice reveals a role for fractalkine in atherogenesis. *J Clin Invest*. 2003;111(3):333-340.

312. Aiello RJ, Bourassa PA, Lindsey S, et al. Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*. 1999;19(6):1518-1525.

313. Boring L, Gosling J, Cleary M, Charo IF. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature*. 1998;394(6696):894-897.

314. Gosling J, Slaymaker S, Gu L, et al. MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. *J Clin Invest*. 1999;103(6):773-778.

315. Gerszten RE, Garcia-Zepeda EA, Lim YC, et al. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature*. 1999;398(6729):718-723.

316. Smith DF, Galkina E, Ley K, Huo Y. GRO family chemokines are specialized for monocyte arrest from flow. *Am J Physiol Heart Circ Physiol* . 2005;289(5):H1976-H1984.

317. Feig JE, Shang Y, Rotllan N, et al. Statins promote the regression of atherosclerosis via activation of the CCR7-dependent emigration pathway in macrophages. *PLoS One*. 2011;6(12):e28534.

318. Trogan E, Feig JE, Dogan S, et al. Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice. *Proc Natl Acad Sci U S A* . 2006;103(10):3781-3786.

319. Aslanian AM, Charo IF. Targeted disruption of the scavenger receptor and chemokine CXCL16 accelerates atherosclerosis. *Circulation*. 2006; 114(6):583-590.

320. Heller EA, Liu E, Tager AM, et al. Chemokine CXCL10 promotes atherogenesis by modulating the local balance of effector and regulatory T cells. *Circulation*. 2006;113(19):2301-2312.

321. Wan W, Lim JK, Lionakis MS, et al. Genetic deletion of chemokine receptor Ccr6 decreases atherogenesis in ApoE-deficient mice. *Circ Res*. 2011109(4):374-381.

322. Combadiere C, Feumi C, Raoul W, et al. CX3CR1-dependent subretinal microglia cell accumulation is associated with cardinal features of age-related macular degeneration. *J Clin Invest*. 2007;117(10):2920-2928.

323. Rebenko-Moll NM, Liu L, Cardona A, Ransohoff RM. Chemokines, mononuclear cells and the nervous system: heaven (or hell) is in the details. *Curr Opin Immunol* . 2006;18(6):683-689.

324. Tuo J, Bojanowski CM, Zhou M, et al. Murine ccl2/cx3cr1 deficiency results in retinal lesions mimicking human age-related macular degeneration. *Invest Ophthalmol Vis Sci* . 2007;48(8):3827-3836.

325. Tuo J, Smith BC, Bojanowski CM, et al. The involvement of sequence variation and expression of CX3CR1 in the pathogenesis of age-related macular degeneration. *FASEB J*. 2004;18(11):1297-1299.

326. Takeda A, Baffi JZ, Kleinman ME, et al. CCR3 is a target for age-related macular degeneration diagnosis and therapy. *Nature*. 2009;460 (7252):225-230.

327. Aster RH. Drug-induced immune thrombocytopenia: an overview of pathogenesis. *Semin Hematol*. 1999;36(1)(suppl 1):2-6.

328. Plater-Zyberk C, Hoogewerf AJ, Proudfoot AE, Power CA, Wells TN. Effect of a CC chemokine receptor antagonist on collagen induced arthritis in DBA/1 mice. *Immunol Lett*. 1997;57(1-3):117-120.

329. Barcellos LF, Schito AM, Rimmler JB, et al. CC-chemokine receptor 5 polymorphism and age of onset in familial multiple sclerosis. Multiple Sclerosis Genetics Group. *Immunogenetics*. 2000;51(4-5):281-288.

330. Fiten P, Vandenbroeck K, Dubois B, et al. Microsatellite polymorphisms in the gene promoter of monocyte chemotactic protein-3 and analysis of the association between monocyte chemotactic protein-3 alleles and multiple sclerosis development. *J Neuroimmunol*. 1999;95(1-2): 195-201.

331. Huang D, Han Y, Rani MR, et al. Chemokines and chemokine receptors in inflammation of the nervous system: manifold roles and exquisite regulation. *Immunol Rev*. 2000;177:52-67.

332. Karpus WJ, Lukacs NW, McRae BL, Strieter RM, Kunkel SL, Miller SD. An important role for the chemokine macrophage inflammatory protein-1 alpha in the pathogenesis of the T cell-mediated autoimmune disease, experimental autoimmune encephalomyelitis. *J Immunol*. 1995;155(10): 5003-5010.

333. Kennedy KJ, Karpus WJ. Role of chemokines in the regulation of Th1/Th2 and autoimmune encephalomyelitis. *J Clin Immunol*. 1999; 19(5):273-279.

334. Liu MT, Keirstead HS, Lane TE. Neutralization of the chemokine CXCL10 reduces inflammatory cell invasion and demyelination and improves neurological function in a viral model of multiple sclerosis. *J Immunol*. 2001;167(7):4091-4097.

335. Teuscher C, Butterfield RJ, Ma RZ, Zachary JF, Doerge RW, Blankenhorn EP. Sequence polymorphisms in the chemokines Scya1 (TCA-3), Scya2 (monocyte chemoattractant protein (MCP)-1), and Scya12 (MCP-5) are candidates for eae7, a locus controlling susceptibility to monophasic remitting/nonrelapsing experimental allergic encephalomyelitis. *J Immunol*. 1999;163(4):2262-2266.

336. Youssef S, Wildbaum G, Maor G, et al. Long-lasting protective immunity to experimental autoimmune encephalomyelitis following vaccination with naked DNA encoding C-C chemokines. *J Immunol*. 1998;161(8):3870-3879.

337. Prahalad S. Negative association between the chemokine receptor CCR5-Delta32 polymorphism and rheumatoid arthritis: a meta-analysis. *Genes Immun*. 2006;7(3):264-268.

338. Blanchard C, Stucke EM, Rodriguez-Jimenez B, et al. A striking local esophageal cytokine expression profile in eosinophilic esophagitis. *J Allergy Clin Immunol*. 2011;127(1):208-217, 217e1-7.

339. Blanchard C, Wang N, Stringer KF, et al. Eotaxin-3 and a uniquely conserved gene-expression profile in eosinophilic esophagitis. *J Clin Invest*. 2006;116(2):536-547.

340. Terkeltaub R, Baird S, Sears P, Santiago R, Boisvert W. The murine homolog of the interleukin-8 receptor CXCR-2 is essential for the occurrence of neutrophilic inflammation in the air pouch model of acute urate crystal-induced gouty synovitis. *Arthritis Rheum*. 1998;41(5):900-909.

341. Auten RL, Richardson RM, White JR, Mason SN, Vozzelli MA, Whorton MH. Nonpeptide CXCR2 antagonist prevents neutrophil accumulation in hyperoxia-exposed newborn rats. *J Pharmacol Exp Ther*. 2001;299(1):90-95.

342. Devalaraja RM, Nanney LB, Du J, et al. Delayed wound healing in CXCR2 knockout mice. *J Invest Dermatol*. 2000;115(2):234-244.

343. Hancock WW. Chemokine receptor-dependent alloresponses. *Immunol Rev*. 2003;196:37-50.

344. Hancock WW, Lu B, Gao W, et al. Requirement of the chemokine receptor CXCR3 for acute allograft rejection. *J Exp Med*. 2000;192(10):1515-1520.

345. Miura M, Morita K, Kobayashi H, et al. Monokine induced by IFN-gamma is a dominant factor directing T cells into murine cardiac allografts during acute rejection. *J Immunol*. 2001;167(6):3494-3504.

346. Fischereder M, Luckow B, Hocher B, et al. CC chemokine receptor 5 and renal-transplant survival. *Lancet*. 2001;357(9270):1758-1761.

347. Fukunaga K, Asano K, Mao XQ, et al. Genetic polymorphisms of CC chemokine receptor 3 in Japanese and British asthmatics. *Eur Respir J*. 2001;17(1):59-63.

348. Panina-Bordignon P, Papi A, Mariani M, et al. The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. *J Clin Invest*. 2001;107(11):1357-1364.

349. Schuh JM, Blease K, Hogaboam CM. CXCR2 is necessary for the development and persistence of chronic fungal asthma in mice. *J Immunol*. 2002;168(3):1447-1456.

350. Lukacs NW, Miller AL, Hogaboam CM. Chemokine receptors in asthma: searching for the correct immune targets. *J Immunol*. 2003;171(1):11-15.

351. Humbles AA, Lu B, Friend DS, et al. The murine CCR3 receptor regulates both the role of eosinophils and mast cells in allergen-induced airway inflammation and hyperresponsiveness. *Proc Natl Acad Sci U S A*. 2002;99(3):1479-1484.

352. Ma W, Bryce PJ, Humbles AA, et al. CCR3 is essential for skin eosinophilia and airway hyperresponsiveness in a murine model of allergic skin inflammation. *J Clin Invest*. 2002;109(5):621-628.

353. Fulkerson PC, Fischetti CA, McBride ML, Hassman LM, Hogan SP, Rothenberg ME. A central regulatory role for eosinophils and the eotaxin/CCR3 axis in chronic experimental allergic airway inflammation. *Proc Natl Acad Sci U S A*. 2006;103(44):16418-16423.

354. Gonzalo JA, Lloyd CM, Peled A, Delaney T, Coyle AJ, Gutierrez-Ramos JC. Critical involvement of the chemotactic axis CXCR4/stromal cellderived factor-1 alpha in the inflammatory component of allergic airway disease. *J Immunol*. 2000;165(1):499-508.

355. Balkwill F. Cancer and the chemokine network. *Nat Rev Cancer*. 2004; 4(7):540-550.

356. Mantovani A, Savino B, Locati M, Zammataro L, Allavena P, Bonecchi R. The chemokine system in cancer biology and therapy. *Cytokine Growth Factor Rev*. 2010;21(1):27-39.

357. Strieter RM, Burdick MD, Mestas J, Gomperts B, Keane MP, Belperio JA. Cancer CXC chemokine networks and tumour angiogenesis. *Eur J Cancer*. 2006;42(6):768-778.

358. Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell*. 2005;121(3): 335-348.

359. Yang G, Rosen DG, Zhang Z, et al. The chemokine growth-regulated oncogene 1

(Gro-1) links RAS signaling to the senescence of stromal fibroblasts and ovarian tumorigenesis. *Proc Natl Acad Sci U S A* . 2006; 103(44):16472-16477.

360. Schall TJ, Proudfoot AE. Overcoming hurdles in developing successful drugs targeting chemokine receptors. *Nat Rev Immunol* . 2011; 11(5):355-363.

361. Braun SE, Chen K, Foster RG, et al. The CC chemokine CK beta-11/MIP-3 beta/ELC/Exodus 3 mediates tumor rejection of murine breast cancer cells through NK cells. *J Immunol* . 2000;164(8):4025-4031.

362. Eo SK, Lee S, Chun S, Rouse BT. Modulation of immunity against herpes simplex virus infection via mucosal genetic transfer of plasmid DNA encoding chemokines. *J Virol*. 2001;75(2):569-578.

363. Eo SK, Lee S, Kumaraguru U, Rouse BT. Immunopotentiation of DNA vaccine against herpes simplex virus via co-delivery of plasmid DNA expressing CCR7 ligands. *Vaccine*. 2001;19(32):4685-4693.

364. Homey B, Muller A, Zlotnik A. Chemokines: agents for the immunotherapy of cancer? *Nat Rev Immunol* . 2002;2(3):175-184.

365. Kim JJ, Yang JS, Dentchev T, Dang K, Weiner DB. Chemokine gene adjuvants can modulate immune responses induced by DNA vaccines. *J Interferon Cytokine Res*. 2000;20(5):487-498.

366. Lu Y, Xin KQ, Hamajima K, et al. Macrophage inflammatory protein-1alpha (MIP-1alpha) expression plasmid enhances DNA vaccineinduced immune response against HIV-1. *Clin Exp Immunol*. 1999; 115(2):335-341.

367. Sin J, Kim JJ, Pachuk C, Satishchandran C, Weiner DB. DNA vaccines encoding interleukin-8 and RANTES enhance antigen-specific Th1-type CD4(+) T-cell-mediated protective immunity against herpes simplex virus type 2 in vivo. *J Virol* . 2000;74(23):11173-11180.

368. Xin KQ, Lu Y, Hamajima K, et al. Immunization of RANTES expression plasmid with a DNA vaccine enhances HIV-1-specific immunity. *Clin Immunol*. 1999;92(1):90-96.

369. Youssef S, Maor G, Wildbaum G, Grabie N, Gour-Lavie A, Karin N. C-C chemokine-encoding DNA vaccines enhance breakdown of tolerance to their gene products and treat ongoing adjuvant arthritis. *J Clin Invest*. 2000;106(3):361-371.

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Chapter 29

Helper T-Cell Differentiation and Plasticity

John J. O'Shea

INTRODUCTION

Cluster of differentiation (CD)4 T cells are critical for the elimination of microbial pathogens, but different types of microbes demand distinct responses. To effectively combat these microbes, CD4 T cells differentiate to form subsets of activated cells with specific characteristics. These discrete subsets of CD4 T cells in turn cause the recruitment of different types of inflammatory cells. Each type of inflammatory cell is adapted to eliminate particular types of microorganisms, and this cooperation of T-lymphocytes with other effector cells links adaptive and innate responses for optimal host defense.

In this respect, the action of CD4 T cells role is largely indirect in that they primarily exert their effect by acting on other cells. For this reason, CD4 T cells are referred to as “helper cells,” which can influence macrophages, B cells, CD8 T cells, natural killer (NK) cells, and other effector cells. The “help” provided by CD4 comes largely in the form of cytokines; however, the production of cytokines is a tightly controlled process: distinct subsets of CD4 T cells secrete characteristic repertoires of cytokines. That is, after activation, CD4 T cells divide, differentiate, and adopt restricted patterns of gene expression, which depend upon the nature of the offending microbial pathogen. Consequently, depending upon whether the pathogen is an intracellular microbe, a worm, or an extracellular bacteria or fungus, different types of activated CD4 T cells are generated. Sometimes though, helper cells are not so helpful insofar as CD4 T cells are also important drivers of autoimmunity.

In this chapter, the different fates available to CD4 T cells will be discussed, as will the molecular mechanisms underlying their specification. The degree to which T cells exhibit characteristics of terminal differentiation versus plasticity will also be considered, as will the mechanisms underlying commitment versus flexibility. Finally, the contribution of different helper T-cell subsets to immune-mediated disease will also be discussed.

FROM THE THYMUS TO THE PERIPHERY

Following maturation and selection in thymus, single positive CD4 T cells exit the thymus and migrate through blood to secondary lymphoid tissues including the spleen, lymph nodes, and mucosal-associated lymphoid tissues.[1,2] These tissues and organs are composed of zones that are enriched with T- or B-lymphocytes: in the lymph node, the paracortical region contains T cells and dendritic cells (DCs), whereas in the spleen, T cells and DCs reside within the white pulp in the periarteriolar lymphoid sheath.

The trafficking and retention of CD4 T cells in secondary lymphoid tissue is dependent upon selective expression of chemokine receptors and adhesion molecules.[3] In lymph nodes and Peyer patches, T cells enter the tissue via specialized blood vessels termed high endothelial venules (HEVs). HEVs express L-selectin and CCL21. Naive T cells, T cells that have yet to encounter foreign antigen, express ligands for L-selectin and CCL21, namely CD62L and CCR7. These ligands promote the entry of naïve T cells into these tissues.[4,5,6] Extravasation of naïve lymphocytes through HEVs is dependent upon intercellular adhesion molecules that bind the integrin lymphocyte function-associated antigen-1. Within secondary lymphoid organs, naïve T cells are retained in the T-cell-rich zones by the chemokines CCL21 and CCL19.

If no antigen is encountered, naïve T cells exit the lymph node through the efferent lymphatic vessels and then return to the bloodstream via the thoracic duct. Egress from lymph nodes (and the thymus) requires the seven transmembrane receptor sphingosine 1-phosphate receptor-1.[7,8] The concentration of sphingosine 1-phosphate is higher in blood, and this provides a concentration gradient for emerging lymphocytes to follow. The immunomodulatory drug fingolimod,[1] a partial agonist of S1P1, acts by blocking egress of T cells from the thymus and lymph nodes.[8] Expression of S1P1 is under the control of the transcription factor KLF2, a factor that promotes the naïve state.[7,9,10] The transcription factor Foxp1 and miR-125b are two additional factor that preserve T-cell naïveté.[11]

In the spleen, naïve T cells enter through terminal arterioles, not HEVs; as a result, expression of CD62L is not required. Migration to the splenic white pulp does require integrin and chemokine receptor signaling. Exit from the spleen is thought to occur by migration from white pulp to red pulp with subsequent entry into blood.

Naïve T cells spend relatively short times in the blood before finding a new secondary lymphoid organ to enter. In the absence of encountering cognate antigen, T cells repeat this

cycle many times over their lifespan. In this stage, they receive survival signals from low-level, or “tonic,” signaling through the T-cell receptor (TCR) provided by self-peptide/major histocompatibility complex (MHC) complexes and interleukin (IL)-7.

ENCOUNTERING ANTIGEN: NAÏVETÉ LOST

Free antigen can travel by blood to the spleen or by lymph via the afferent lymphatic vessels to lymph nodes. Alternatively, pathogen-associated inflammatory signals activate nonlymphoid resident tissue DCs, causing them to mature. Activated DCs express the receptor CCR7 and migrate to lymph nodes in a CCL21-dependent manner. B cells can also serve as antigen-presenting cells (APCs). Within the lymph nodes, APCs activate naïve T cells bearing the appropriate TCRs in a process that involves direct physical contact involving an array of receptor/ligand interactions including: specific foreign peptide/MHC complex recognized by the TCR; costimulatory molecules CD80, CD86 (ligands for CD28), and adhesion molecules such as CD2; and lymphocyte function-associated antigen-1. In this phase of activation, T cells have prolonged interactions with APCs that last up to several hours.[12,13] The ability of CD4 T cells to promote contacts with DCs appears to involve the chemokines CCL3 and CCL4, and ligation of CD40. The adapter molecule SAP (encoded by *SH2D1*), which regulates signaling by signaling lymphocytic activation molecule-family molecules, promotes stable interactions between T and cognate B cells in nascent germinal centers in the lymph node.[14]

Within the first 24 hours of encountering antigen, T cells secrete cytokines such as IL-2 and tumor necrosis factor (TNF)α. Transcription factors activated by TCR signals include NF-κB family members, NFAT proteins, and AP-1, which are critical for cytokine production. At approximately 25 to 30 hours after initial antigen presentation, T cells begin to divide and proliferate. This is followed by the hallmark of lymphocyte activation: clonal expansion of T cells expressing TCRs that recognize cognate peptide.

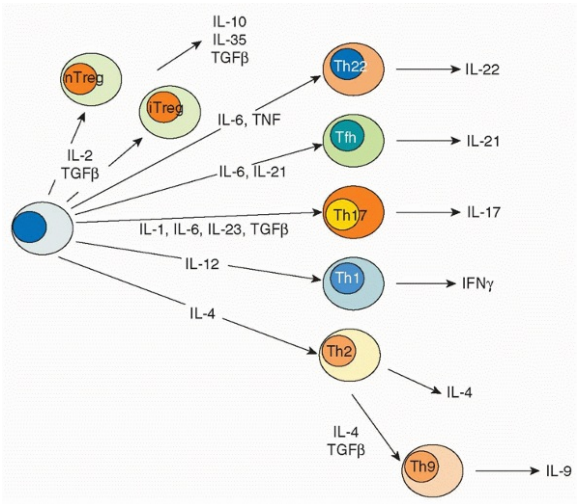

FIG. 29.1. Helper T Cell Fates. In response to the distinct cytokine milieux elicited by different microbial pathogens, naïve T cells become specified to produce selective cytokine repertoires. For instance, intracellular pathogens can induce production of interleukin (IL)-12, which acts via STAT4 to promote differentiation of Thelper (Th)1 cells that selectively produce interferon-γ and not other cytokines. Likewise, helminthes induce IL-4 production, which acts via STAT6 to drive cells toward a Th2 phenotype characterized by IL-4 secretion. Fungi and extracellular bacteria elicit secretion of IL-23 and IL-6, which activate STAT3 and produce CD4 cells that produce IL-17, so-called Th17 cells. Each of these effector cells express transcription factors that enforce commitment to these distinctive phenotypes. These transcription factors can be referred to as “master regulators.” Other cluster of differentiation (CD)4 T cells reside in germinal centers and provide help to B cells. This subset of CD4 T cells is termed follicular helper cells. Another subset of CD4 T cells arises in the thymus and expresses the transcription factor Foxp3. These cells are termed natural regulatory T cells. Foxp3 can also be induced in cells in the periphery and these cells are termed induced Treg cells. Foxp3+ regulatory T cells are critical for constraining immune responses.

Next, T cells must migrate to infected tissues to exert their effect. This occurs by the loss of homing receptors for the secondary lymphoid organs, CD62L and CCR7, and acquisition of new homing molecules such PSGL-1, CD44, CCR5, and CXCR3.[15] At this time, T cells transiently downregulate S1P1. Ligation of the TCR induces expression of the integrins, VLA-4 and VLA-5, which bind to fibronectin in extracellular matrices, Activated T cells also upregulate CD44, which binds to hyaluronan. Consequently, T cells that have encountered

antigen are retained at the extravascular site where the offending antigen is present.

MANY FATES FOR ACTIVATED CLUSTER OF DIFFERENTATION 4 T CELLS

After initial activation, CD4 T cells can differentiate further such that they begin to selectively produce certain cytokines and not others. A major driver of the selective differentiation of CD4 T cells to populations of cells with distinctive cytokine production is the cytokine milieu generated by DCs and other innate cells. The resultant T cells tend to preserve their phenotype and with time maintain their distinctive phenotypes in a cell-autonomous manner. In this respect, the acquisition of the distinct fates can be thought of as a process of lineage commitment, although the extent to which activated T cells are terminally differentiated or retain a flexible repertoire is the topic of intense ongoing research.

Classically, T cells were viewed as having two major fates: Thelper1 (Th1) cells, which selectively produce interferon (IFN)-γ, and Th2 cells, which produce IL-4[16,17,18,19,20] (Fig. 29.1 and Table 29.1). In addition to a stereotypic pattern of cytokine production, these lineages express a "master regulator"

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P.711

transcription factor responsible for the distinctive pattern of gene expression.[21,22] Recently, though, newer fates for helper T cells have been recognized, and they are designated based on expression of their eponymous, signature cytokines: Th17, Th22, and Th9 cells.[23] These newly appreciated subsets can also express characteristic, lineage-defining transcription factors; however, as will be discussed, the expression of these master regulators is now recognized to be more promiscuous than previously appreciated. In addition to Th1, Th2, Th17, Th9, and Th22 cells subsets are T cells that reside in proximity to B cells in germinal centers, so-called follicular helper T (Tfh) cells. However, the distinction between Tfh cells and cytokine-secreting effector subsets is also a topic of intense investigation. Finally, there are various types of regulatory T cells. Each subset will be briefly discussed with respect to their characteristics features, inductive factors, key transcription factors, and negative regulators.

TABLE 29.1 Th Subsets

Subset	Inducing Cytokines	Sensors	Master Regulator Transcription Factor	Other Transcription Factors	Signature Cytokine	Other products	Inhibitors	Function	Host Defense Against:
Th1	IL-12, IFN-γ Also IL-18, IFNα/β, IL-2, IL-27	STAT4, STAT1	T-bet	Hlx Runx3 Ets family Eomes	IFN-γ	TNF IL-12Rβ1 IL-12Rβ2, IL-18R, CXCR3, CCR5	IL-4	Activation of macrophages, enhanced antigen presentation, B-cell class switching to IgG1, IgG3(human), IgG2α, IgG3 (mouse)	Intracellular microbial pathogens (eg, *Mycobacteria*, *Listeria monocytogenes*, *Toxoplasmosis gondii*, viruses
Th2	IL-4 Also IL-2, IL-25, IL-33, TSLP	STAT6, STAT5	GATA3	Maf STAT3, Notch, IRF-4, Gfi-1	IL-4	IL-5, IL-13, IL-24, IL-31, CCR3, CCR4, CCR8, ECM1	IFN-γ Mina	Activation and mobilization of mast cells, basophils, eosinophils, Alternative macrophage activation, barrier function, mucus production, B-cell class switching (IgE)	Helminths
Th17	IL-6, IL-23, TGFβ, IL-1, TLR2	STAT3	Rorγt	Ahr, Batf, IKBζ, IRF-4, Runx1, HIF1α,	IL-17a, IL-17f	IL-21, IL-22, CCR6, CCL20	IL-2, IFN-γ, IL-4, Foxp3, T-bet, Ets-1, Tcf-1	Induction of neutrophilia and recruitment of neutrophils, germinal	Extracellular bacteria: *Staphylococcus aureus*, *Klebsiella pneumoniae*

								center formation	Candida
Th22	IL-6, TNF	STAT3	?	Ahr, Rorgt, Notch	IL-22	CCR10, CCR6, CCR4	TGFβ	Induction of defensins	*Klebsiella pneumoniae*
Th9	IL-4, TGFβ IL-2 IL-25			Pu.1, IRF-4				Mucus production	
Tfh	IL-6, IL-21	STAT3 Also STAT4	Bcl6		PD-1, CXCR5, BTLA, PSGL-1, ICOS		IL-2 Blimp1	B-cell maturation, plasma cell differentiation, Ig class switching	
Treg	IL-2, TGFβ, retinoic acids	STAT5	Foxp3	Runx/CBF, Nrfa2, Foxo1, Foxo3, Eos, Helios	IL-10, TGFβ IL-35		IL-6 Roryt gamma, HIF1a		Maintenance of peripheral tolerance
Tr1	IL-27, IFNα/β, ICOS		?	Ahr					

IFN, interferon; Ig, immunoglobulin; IL, interleukin; TNF, tumor necrosis factor.

As indicated, activated CD4 T cells help eliminate pathogens by recruiting different types of effector cells (macrophages, neutrophils, eosinophils, NK cells, B cells, and CD8 T cells). However, activated, differentiated CD4 T cells are themselves referred to as *effector* cells. While this terminology may be confusing, it refers to the ability of activated CD4 T cells to rapidly produce maximal levels of their signature cytokines. The limitation of the terminology is that it does not refer to an exact, precisely defined step in maturation, but is rather used largely to distinguish them from naïve and "memory" cells. Unlike CD8 T cells, CD4 T cells are much more heterogeneous; not surprisingly, CD4 memory T cells are also more heterogeneous and our understanding of the transition of CD4 effector cells to memory cells is poorly understood. Memory CD4 cells can be broadly classified as effector memory (Tem) and central memory (Tcm).[24,25,26] Tcm express CCR7 and recirculate through lymphoid organs. Tem do not express CCR7 and home to nonlymphoid tissues. Tem rapidly produce cytokines following secondary stimulation, but have limited proliferative capacity. Tcm, by contrast, produce IL-2 and are able to proliferate.

Th1 Cells

Th1 cells are defined by the selective secretion of IFN-γ. IFN-γ activates macrophages to kill phagocytosed microbes. It enhances antigen presentation, by upregulating MHC and costimulatory molecules, amplifying T-cell-dependent immune responses. IFN-γ also further promotes the differentiation of CD4+ T cells to the T_H1 subset and activates NK and CD8 T cells, thereby further enhancing production of IFN-γ and inhibiting T_H2 and T_H17 cell differentiation. IFN-γ promotes B-cell class switching to immunoglobulin (Ig)G1 and IgG3 (IgG2a or IgG3 in mice) and inhibits switching to IL-4-dependent isotypes (eg, IgE). In this manner, Th1 cells effectively protect the host against intracellular pathogens including: *Mycobacteria*, *Toxoplasma*, *Listeria*, and viruses.

In addition to expressing their signature cytokine IFN-γ, T_H1 cells also express high levels of IL-12 receptor β1 and β2 subunits, IL-18 receptor, E-selectin, P-selectin, CXCR3, and CCR5.

T_H1 differentiation is stimulated by intracellular bacteria, viruses, and adjuvants that elicit production of IL-12, IL-18, and type I IFN. IL-12 and to some extent type I IFNs activate the transcription factor STAT4, which has thousands of targets in developing Th1 cells, including the genes encoding IFN-γ, and IL-12 and IL-18 receptors.[27] IFN-γ, acting via STAT1, reinforces Th1 differentiation. STAT4 and STAT1 induce expression of T-bet (encoded by the *Tbx21* gene), which has been termed the master regulator of Th1 cells.[28,29,30,31] Conversely, though, the transcriptional repressor Twist1 is also target of STAT4. Twist1 limits the expression of cytokines including IFN-γ, IL-2, and TNF.[32] Similarly, the protease Furin, which processes the anti-inflammatory cytokine transforming growth factor (TGF)-β, is also regulated by STAT4.[27,33] Thus, STAT4 simultaneously promotes and constrains T-cell activation and thereby limits Th1-mediated immunopathology.

T-bet regulates thousands of genes in Th1 cells.[34,35] It binds to the *Ifng* gene and promotes its expression.[36,37,38] T-bet also directly binds and inhibits expression of the *Socs1*, *Socs3*,

and *Tcf7* (which encodes TCF-1) genes. Interestingly, it can also associate with the repressor Bcl6 and inhibit IFN-γ expression. T-bet also inhibits expression of Rorγt, the master regulator of Th17 cells, by blocking the action of Runx1.[39] T-bet inhibits *Il4* gene expression in a Runx3-dependent manner.[36] T-bet is phosphorylated by Itk, and phosphorylated T-bet binds and inhibits the action of GATA3, the master regulator of Th2 cells.[40] T-bet is also important for T-cell trafficking and promotes the differentiation of T cells to an effector phenotype.[41] As will be discussed, T-bet antagonizes the Tfh characteristics.[42,43]

It is important to bear in mind that Th1 differentiation can occur in the absence of either T-bet or STAT4, indicating that there are alternative means of regulating IFN-γ. In CD8 cells, a related T-box family member, Eomes, is the major regulator of IFN-γ production. In addition, other transcription factors are preferentially expressed in Th1 cells including Hlx, Runx3, and Ets family members; they also promote IFN-γ production and repress IL-4 transcription. Another factor that influences Th1 specification is the "strength" of TCR signaling, with strong signaling favoring TCR signaling. The mechanisms underlying this are relatively poorly characterized. A downstream signaling molecule engaged following TCR ligation and cytokine receptor occupancy is mammalian target of rapamycin (mTor). Deficiency of mTor inhibits effector cell differentiation including generation of Th1 cells.[44] Its regulators include Tor complex (Torc)1 and Torc2. Inhibition of Torc1 signaling inhibits Th1 and Th17 differentiation.[45,46,47]

Th2 Cells

The signature cytokines for Th2 cells are IL-4, IL-13, and IL-5, which are important for barrier defense at mucosal and epithelial surfaces. IL-4 stimulates the development of T_H2 cells and functions as an autocrine growth factor for differentiated T_H2 cells. It also stimulates B-cell immunoglobulin class switching to IgG4 in humans (IgG1 in mice) and IgE in both species. Conversely, IL-4 inhibits switching to the IgG2a

and IgG3 isotypes in mice, both of which are stimulated by IFN-γ. IL-4, together with IL-13, promotes the formation of "alternatively activated" macrophages, suppresses IFN-γ-mediated classical macrophage activation and thus inhibits defense against intracellular microbes. IL-4, IL-13, and IL-5 mobilize eosinophils, basophils, and mast cells, and IL-13 increases mucus secretion from airway and gut epithelial cells. These actions contribute to elimination of microbes at epithelial surfaces. In this manner, Th2 cells are important for host defense against helminths and other parasites, but are also the major drivers of allergy and asthma.

T_H2 cells also produce IL-24 and IL-31, and express the chemokine receptors CCR3, CCR4, and CCR8.[48,49] Th2 cells also expression extracellular matrix protein-1, which regulates cell migration and expression of S1P1.[50]

For Th2 cells, a major driver of differentiation is IL-4 itself. IL-4 can be produced by an array of innate cells including: NK T cells, mast cells, and basophils.[51,52,53,54,55] More recently recognized are innate cells designated natural helper cells that reside in fat associated lymphoid clusters.[56,57,58] IL-25, an IL-17-related cytokine, IL-33, an IL-1-related cytokine, and thymic stromal lymphopoietin all promote Th2 responses, principally by driving IL-4 production in innate cells.[51,57,59,60,61,62]

IL-4 stimulates T_H2 development by activating STAT6, which together with TCR-dependent signals, induces expression of the Th2 master regulator, GATA-3. Like STAT4, STAT6 also regulates thousands of other genes in Th2 cells including pivotal transcription factors, cytokines and cytokine receptors, and chemokines and chemokine receptors.[27,63,64,65] IL-2 also contributes to Th2 differentiation by activating STAT5, which positively regulates expression of both IL-4 and the IL-4 receptor.[66,67,68,69] STAT5's targets in Th2 cells include both the *Il4* and the *Il4r* gene.[70] Additionally, STAT3 has been reported to be a contributor to Th2 differentiation.[71]

Gata-3 is a critical factor for lineage commitment of Th2 cells, although it also plays essential roles in thymic differentiation. Gata-3 binds to many key Th2 genes including the *Il4*, *Il5*, *Il13*, *Gata3*, and other genes. 72 GATA3 also actively represses RUNX3, which in turn leads to reduced IFN-γ production.[73] GATA3 also induces the transcription factor c-Maf, which can aid in Th2 differentiation; however, c-Maf deficiency does not abrogate production of Th2 cytokines.[74] GATA3 also has important roles in thymic development, regulating key factors such as Th-POK, Notch, and TCR subunits.[72]

The transcription factors Notch, IRF-4, and growth factor independent (Gfi)-1 also aid in Th2 differentiation. Notch has been reported to promote Gata3 expression and to do so in a Stat6-independent manner.[75] IRF-4 is a contributor to Th2 differentiation, but it is also important for other subsets.[76] Gfi is induced by IL-4 and regulates Th2-cell proliferation.[77,78,79] Whereas "strong" TCR signals favor Th1 differentiation, weak TCR signaling favors Th2 differentiation; again, the signaling intermediates responsible remain unclear. In addition, deletion of Torc2 inhibits Th2 differentiation.[45]

Finally, Mina, a member of the jumonji C (JmjC) protein family, binds and represses the *Il4* promoter. Conversely, reducing levels of Mina in T cells leads to *Il4* de-repression.[80] Eomesodermin (Eomes), a key regulator of IFN-γproduction in CD8 T cells, is important for limiting IL-5 production in Th2 memory cells.[48] Eomes acts by limiting GATA3 binding to the *Il5* promoter.

Th17 Cells

The appreciation that the Th1/Th2 paradigm failed to explain a good deal about immunity and autoimmunity,[81,82,83] and the recognition of the existence of a subset of T cells that preferentially produces IL-17 led to a small renaissance in CD4 T-cell biology. This newly appreciated subset, termed Th17 cells, was noted to produce IL-17A, IL-17F, IL-21, and IL-22.[84,85,86,87,88,89,90,91,92]

IL-17 enhances neutrophil generation by increasing granulocyte macrophage colony stimulating factor production and promotes neutrophil recruitment by production of chemokines.[93,94,95] Because neutrophils are a major defense mechanism against extracellular bacteria and fungi, T_H17 cells play an especially important role in defense in such infections including *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Candida albicans*.[49,96] The IL-23/IL-17 axis also contributes to host defense against *Mycobacteria tuberculosis*.[97] Additionally, IL-17 stimulates the production of antimicrobial substances, including defensins. IL-17 also contributes to germinal center formation, as well as lymphoid structures induced during inflammation.[98,99] As will be discussed, Th17 cells are also very important in the pathogenesis of a variety of autoimmune and immunemediated diseases.

Th17 cells typically express the chemokine receptor CCR6, along with its ligand, CCL20.[100] Cytokines that promote Th17 differentiation include IL-6, IL-23, IL-1, and TGF-β.[91,101,102,103,104,105,106,107,108,109,110,111,112] IL-6 and IL-23 activate Stat3, which directly regulates many genes that contribute to the phenotype of Th17 cells including *Il17a/f*, *Il23r*, and *Il21*.[113] Stat3 also regulates *Rorc*, which encodes Rorγt, the master regulator for Th17 cells and other transcription factors expressed by Th17 cells.[114,115] In the mouse, absence of *Stat3* in T cells blocks Th17 differentiation.[116,117] Th17 cells are abundant in the gut and are regulated by intestinal flora, but the precise mechanisms responsible for driving IL-17 production have not been elucidated.[114]

Rorγ is an orphan retinoid receptor that is ubiquitously expressed.[118] One splice variant, Rorγt, is selectively expressed in hematopoietic cells. In addition to serving as "master regulator" of a helper-cell subset, like GATA3, Rorγt has important functions in thymic development, in the development of lymphoid tissue inducer cells, and in development of IL-22-producing NK cells.[119,120,121,122] In addition to Rorγt, Rorα is also a contributor to Th17 differentiation.[123]

Other transcription factors expressed by Th17 include the aryl hydrocarbon receptor, Batf, I kappaB zeta, IRF-4, and Runx1; absence of any of these impairs IL-17 expression.[39,124,125,126,127,128,129] The key metabolic sensor hypoxia-inducible factor (HIF)-1[130] is also a positive regulator of Th17 differentiation. HIF-1 associates with Rorγt and recruits the acetyl transferase p300 to the *Il17* gene.[130,131] TLR2

signaling also promotes Th17 differentiation.[132] As indicated previously, mTor signaling via mTorc1 also promotes Th17 differentiation.[45]

IL-2 acting via STAT5 is a potent negative regulator of IL-17.[133] Regulatory T (T_{reg}) cells can promote Th17 differentiation, and they appear to do so by limiting IL-2.[134,135] IL-2 activates STAT5, which binds to the *Il17a/f* locus and displaces STAT3.[136] IL-4 and IFN-γ also inhibit Th17 differentiation.[84]

Foxp3 negatively regulates Th17 differentiation and does so by directly binding Rorγt and inhibiting its function.[137] The transcription factors Gfi-1, Ets-1, T-bet, and Tcf-1 also inhibit Th17 cell differentiation.[39,138,139,140,141]

Th22 Cells

IL-22 is an IL-10-related cytokine located in proximity to the *IFNG* gene, but regulated in a distinctive manner.[142] It acts on epithelial barrier cells, having both pro- and anti-inflammatory effects.[143,144,145] It is important for host defense against *Klebsiella pneumonia*.[49] Although IL-22 is produced by Th17 cells, some cells produce IL-22 and not IL-17.[146,147,148,149] This is especially prominent in skin-homing cells that express the chemokine receptors CCR10, CCR6, and CCR4.[150,151] IL-22 is also produced by mucosal innate immune cells including lymphoid tissue inducer and so-called NK-22 cells.[59,119,152,153,154,155,156]

Production of IL-6 and TNF by plasmacytoid DCs promotes IL-22 production without inducing

IL-17 and IFN-γ. Unlike other Th subsets, no clear master regulator that directs this phenotype has been identified, but the aryl hydrocarbon receptor[157] and Roryt are important for IL-22 production.[125,158] Notch drives IL-22 production by enhancing Ahr expression.[159] Conversely, Tgfβ inhibits IL-22; it does so by inducing Maf, which directly represses IL-22.[160]

Th9 Cells

Adding TGF-β to cultures of T cells incubated with IL-4 generates a population of cells that produce IL-9 but not IL-4. Such cells have been referred to as Th9 cells.[161,162,163,164] IL-2 promotes IL-9 production.[165] In addition, Th9 cells express the receptor for IL-25 (IL-17RB) and IL-25 promotes IL-9 production.[166] Innate cells are major sources of IL-9.[167]

IL-9 acts on mucosal cells to enhance mucus production.[168,169] IL-9 has also been reported to have variable effects on Th17 differentiation and Th17-dependent pathology.[170,171] The transcription factors PU.1 and IRF-4 are required for development of IL-9-producing T cells and allergic inflammation.[164,172,173]

Follicular Helper T Cells

For the subsets of CD4 T cells discussed, activation and instruction by cytokines is associated with the acquisition of particular chemokine receptors that promote exit from lymph nodes and recruitment to sites of inflammation. However, some antigen-experienced CD4 T cells, called follicular helper T cells (Tfh), stay in the lymph nodes and provide "help" to B cells.[174,175,176] The signature cytokine for Tfh cells is IL-21. However, this cytokine is not exclusively produced by Tfh cells; it is also produced by other subsets including Th1 and T_H17 cells. IL-21 promotes germinal center development and also contributes to the generation of plasma cells in the germinal center reaction.[177] Tfh cells promote B-cell Ig class switching and maturation of B cells to plasma cells.

Tfh cells also selectively express PD-1, CXCR5, BTLA, PSGL-1, and high levels of the costimulatory molecule inducible costimulator (ICOS). Other molecular interactions including signaling lymphocytic activation molecule-associated protein/signaling lymphocytic activation molecule and CD40/CD40L are also important for germinal center reactions.

The mechanisms that drive the development of Tfh cells from CD4+ cells are not fully understood. IL-6 and IL-21 promote Tfh cell differentiation via activation of STAT3; however, these factors are not absolutely required for Tfh differentiation. In fact, IL-12 acting via STAT4 also promotes Tfh differentiation.[43,178] ICOS promotes expression of c-Maf, which regulates IL-21 production.[179] Complete differentiation of Tfh is thought to require a signal derived from B cells in the follicle.[176]

The master regulator of Tfh cells is the transcriptional repressor Bcl6, which is induced by IL-6 and IL-21 via activation of STAT3.[180,181,182,183] Bcl-6 promotes expression of CXCR5, CXCR4, and PD-1. It also binds and represses *Tbx21* and *Rorc* genes (that encode T-bet and Rorgt, respectively). Bcl-6 also represses IL-4, IFN-γ, and IL-17 production.[183,184] As discussed subsequently, the extent to which Tfh should be considered a distinct lineage versus a flexible state is still a topic of debate. Tfh cells can secrete other cytokines that are characteristic of T_H1, T_H2, and T_H17 cells, and as indicated these cytokines regulate isotype switching.

Factors that negatively regulate Tfh characteristics of cells include *PRDM1*, which encodes the transcriptional repressor Blimp1.[182] In addition, T-bet negatively regulates IL-21 and Bcl6 expression.[42,43,185,186]

Regulatory T Cells

Foxp3+ Regulatory T Cells

Although multiple T-cell populations possess various suppressive activities, the major professional CD4 cell with essential regulatory activity is the FoxP3+ $CD25_{high}$ T_{reg} cell.[187] In the mouse, expression of Foxp3 is a reliable indicator of suppressive function, but this is not necessarily the case in humans.[188] Nonetheless, in both species absence of *Foxp3* (*FOXP3*) leads to fatal autoimmunity. Foxp3+ T_{reg} cells produce IL-10 and TGF-β, but low levels of other cytokines. However, the production of IL-10 and TGF-β is by no means unique to T_{reg} cells as other subsets also produce these cytokines. FoxP3+ T_{reg} cells also typically express high levels of CTLA-4, which is required for their proper function.

T_{reg} cells that arise in the thymus are designated natural T_{reg} (nT_{reg}) cells, whereas CD4 T cells that acquire Foxp3 expression in the periphery are termed induced T_{reg} (iT_{reg}) cells. IL-2 and TGF-β are important for the growth and survival of Foxp3+ T_{reg} cells.[189,190,191] Retinoic acid also induces expression of Foxp3.[192,193,194,195,196]

Activated by IL-2, Stat5 plays a pivotal role in regulating T_{reg} differentiation by directly

binding the *Foxp3* gene and inducing its expression. Foxp3, in turn, directly regulates many genes involved in the program of T_{reg} cells.[197] One action of FoxP3 is to bind Roryt and interfere with Th17-cell development.[137] A trimolecular complex comprising Runx1, Roryt, and Foxp3 can form with the net result being inhibition of IL-17 production.[129]

Runx/CBF also promotes expression of Foxp3,[198] and the transcription factors Nrfa2, Foxo1, and Foxo3 also promote T_{reg}-cell differentiation.[199,200] Eos, a member of the Ikaros transcription factor family, associates with Foxp3 and helps repress gene expression. Helios is expressed by iT_{reg} but not nT_{reg} cells, but its function is not clear and can be induced in other helper-cell subsets.[201,202] Lack of mTor or inhibition with rapamycin favors T_{reg}-cell generation.[44]

Cytokines, like IL-6, that activate STAT3 inhibit Foxp3 expression.[89,203,204] HIF-1 also binds Foxp3 and promotes proteasomal degradation.[130]

Other Cluster of Differentation 4 Subsets with Regulatory Activities

Other CD4 T-cell subsets that have immunosuppressive properties include IL-10- and TGFβ-producing regulatory T cells. Such cells have been referred to Tr1 and Th3 cells.[205,206] These cells are induced in oral tolerance models and do not necessarily express Foxp3. Type I IFN, IL-27, and ICOS induce c-Maf expression, which promotes differentiation of IL-10-producing cells.[207,208,209,210,211,212,213,214,215] Similarly, arylhydrocarbon ligands also reported to induce this subset.[216,217] As discussed in the following, though, it is now appreciated that IL-10 is widely expressed by various CD4 T cells and innate immune cells; whether IL-10-producing cells should be viewed as a separate lineage is therefore debatable.

T_{reg} cells also express the cytokine IL-35.[218] This is a dimeric cytokine that comprises the EBI3 and IL-12α (p35) subunits. IL-35 has suppressive activity and induces its own expression in CD4 T cells.[219,220] However, IL-35 expression does not require coexpression of Foxp3. No "master regulator" has been described for IL-35-producing cells.

EPIGENETIC REGULATION OF HELPER-CELL DIFFERENTIATION

Once cells have attained a differentiated fate, they tend to maintain that phenotype through multiple rounds of cell division without continued instructive signals. Equally important for the acquisition of a differentiated state is the active silencing of genes that contribute to alternative fates.

An important determinant in gene expression is whether the gene is accessible or not. Deoxyribonucleic acid (DNA) is associated with octameric histone molecules forming nucleosomes, which may be in a condensed (heterochromatin) or open (euchromatin) configuration. Genes residing within regions of condensed chromatin are inaccessible and are therefore silenced, whereas genes that reside in open chromatin are readily expressed. The accessibility of genes is influenced by adenosine-triphosphate-dependent remodeling of nucleosome complexes, as well as covalent modifications of histone tails (including acetylation, methylation, and phosphorylation). Another important factor that influences gene expression is DNA methylation. These various modifications contribute to epigenetic control of gene expression and when measured on a genomewide scale are referred to as the "epigenome."

Epigenetic modifications, especially DNA methylation, are relatively stable and may persist in the absence of continued exogenous signals and even the transcription factors that initially induced them. This allows a cell to "remember" its distinctive transcriptional profile and, by extension, its cellular identity.

The differentiation of CD4 T cell has long been recognized to be associated with the dismantling of condensed chromatin structure and the acquisition of accessible chromatin structures at the signature cytokine loci and other key genes expressed by effector CD4 T cells.[221,222,223,224,225,226] This makes sense as the preservation of phenotype would be desirable with the massive clonal expansion that accompanies T-cell activation. Extensive profiling of histone marks in CD4 T cells has been now been accomplished, and the epigenomic view of T cells confirms early work regarding accessible and repressive marks of signature cytokine genes.[227,228,229] Once this remodeling occurs, it tends to persist in daughter cells, and the accessibility of these genes helps explain the ability differentiated CD4 to rapidly express selective cytokine genes. Equally, the silencing of other cytokine genes help to explain why genes can be effectively shut off and CD4 T cells can behave like "lineages" in terms of phenotypic stability. However, as will be discussed in the following, the epigenetic regulation of master regulator genes is more complicated and allows for flexibility in expression.

As might be expected, genetic deletion or knocking down expression of factors involved in histone modifications, nucleosome positioning, or DNA methylation impact CD4 differentiation.[230] For instance, impaired expression of constituents of the Trithorax complex, which is responsible for histone 3 lysine 4 trimethylation, a mark of accessible chromatin, is associated with aberrant Th2 differentiation.[231,232] Mice that lack components of polycomb

repressor complexes, which are responsible for histone3 lysine 27 trimethylation, have impaired helper-cell differentiation.[233,234,235] Chromatin remodeling complexes, which contain Brahma-related gene 1 (Brg1), displace nucleosomes. This is important for regulating the accessibility of the *Ifng* promoter in Th1 cells and knocking down *Brg1* results in decreased IFN-γ production.[236] The cohesin complex of proteins is also important for maintenance of gene architecture and knocking down the expression of one component of this complex, *RAD21*, also reduces *IFNG* transcription.[237] Finally, conditional ablation of the DNA methyl-transferase, DNMT1, or methyl-CpG binding domain protein 2 leads to increased expression of IFN-γ and Th2-type cytokines and inability of Th1 or Th2 cells to properly silence the expression of opposing cytokine genes.[238,239,240]

Like cytokine genes, the expression of key transcription factors can also be regulated by epigenetic modifications. For instance, a conserved CpG-rich region resides upstream of the *FoxP3* promoter. This region is fully demethylated in nT_{reg} cells. In contrast, there is residual methylation of

this region in iT_{reg} cells.[241,242,243] This correlates well with the finding that nT_{reg} cells tend to be more stable in their phenotype than their induced cousins.

Cis regulatory elements are the DNA elements that control gene expression and constitute an integral part of gene structure.[222] These elements may be located in promoters, introns, or in distal enhancers; are enriched for transcription factor-binding sites; and are sites of epigenetic modification. In differentiating Th cells, much attention has been focused on the structure of lineage-defining cytokine genes including their distal cis regulatory elements. In fact, the structure of many cytokine genes is remarkably complex. The *IFNG*/*Ifng* locus encompasses approximately 200 kb, with multiple distal enhancers.[222,244,245,246,247,248,249] Similarly, the *IL4 Il4* locus comprises multiple enhancers as well as a silencer element (HS-IV), which binds Runx/CBFbeta.[250,251,252,253] Likewise, the *Foxp3* gene also has a complicated structure, which is critical for appropriate, stable expression.[254]

Taken together, there is strong evidence that epigenetic regulation is a key aspect of helper-cell differentiation; however, it should also be clear that epigenetics refers to a diverse group of mechanisms and that not all of the genes expressed in helper cells are regulated in the same manner.

LINEAGE COMMITMENT VERSUS PLASTICITY AND FLEXIBILITY

With the initial discovery of Th1 and Th2 cells, the impression was that these cells tended to have reasonably stable phenotypes and thus began to be viewed as distinct lineages in much the same way that we think of CD4 and CD8 cells as being distinct and phenotypically stable. T_{reg} cells can be stable in terms of their phenotype, so they too came to be viewed as another helper-cell lineage. With the rapid discovery of new fates for CD4 T cells delineated previously, many were also elevated to the status of new lineages; however, their phenotypic stability has not been as firmly established. On the contrary, recent research also points to substantial phenotypic flexibility of the “newer” helper T cells and indeed, previously indentified subsets also appear to be more flexible than initially thought.[255,256,257,258,259,260] Attention will now be turned to the ways in which helper subsets exhibit flexibility and some of the mechanisms responsible. This is not to say that older views should be abandoned; rather, one needs to have an open mind regarding what CD4 subsets can and cannot do as they continue to surprise us.

Some Cytokines are Made by Many Subsets of T Cells

Originally thought to be a Th2 cytokine, IL-10 is now known to be made by Th1, Th2, T_{reg} cells, and a variety of innate immune cells.[261,262] Likewise, TGF-β is made by both effector cells and regulatory cells. Conversely, some suppressive cells can express IL-17.[92,263] Conversely, granulocyte macrophage colony stimulating factor made by Th1 and Th2 cells is also made by Th17 cells and is important in immune-mediated disease.[264,265]

IL-21, the “signature cytokine” of Tfh, can also be made by Th17 and Th1 cells.[27,43,178,266] Likewise, despite the nomenclature, IL-9 and IL-22 are made by cells other than their namesake “lineage.”

Even for the classical subsets, Th1 and Th2 cells, it is recognized that IL-4-producing cells are present early in the Th1 response against *Leishmania*. While the cassette of genes encoding IL-4, IL-13, and IL-5 are typically coordinately regulated in Th2 differentiation, there are circumstances in which Th1 cells can produce IL-13 but not IL-4. The transcription factor NF-IL3 (E4BP4) appears to regulate IL-13 independently of IL-4.[267]

Helper Cells can Change Their Phenotype

Although IL-17-secreting Th cells were suggested to represent a new lineage because they did not make other lineage-defining cytokines like IFN-γ and IL-4, it is now well recognized that Th17 cells often become IFN-γ producers.[268,269,270,271] Although the subject is far

from resolved, T_{reg} cells can lose FoxP3 expression and acquire the capacity to produce proinflammatory cytokines.[228,272,273] Even committed GATA-3+ Th2 cells can be reprogrammed to express T-bet+ and IFN-γ+ in the setting of viral infection.[274]

Tfh cells are perhaps the most fluid subset.[174,275] In vitro, Tfh cells can become Th1, Th2, and Th17 cells, and conversely, Th1, Th2, and Th17 can acquire attributes of Tfh cells.[276,277,278] In vivo isolated Tfh cells can also express cytokines characteristic of other Th cells.[174,276,279] In Peyer patches, FoxP3+ T cells can convert to Tfh cells.[280] Thus, it remains an open question whether Tfh cells should be viewed a distinct lineage parallel to other subsets of helper T cells or as a metastable state of differentiation that can be superimposed upon Th1, Th2, Th17, or T_{reg} cells.

Collectively, the data indicate that although helper cells may exhibit features of phenotypic stability, it is clear that they also have the capacity for considerable plasticity. This then raises the broader question of what factors preserve the phenotype of specialized cells.

Mechanistic Explanations

Helper T Cells can Express More than One "Master Regulator"

Implicit in the notion of lineage-defining master regulators is that their expression dictates a distinctive phenotype. In the strictest sense, expression of a master regulator in a given subset is equally distinct; the expectation is that specific lineages express one lineage-defining master regulator whose expression equates with the distinctive phenotype and does not overlap among the different subsets. However, this is not the case for CD4 T cells. For example, Foxp3+ cells can express T-bet, GATA3, STAT3, IRF4, Rorγt, and Bcl6, all of which are functionally relevant.

Expression of the Th1 master regulator, T-bet, in Foxp3+ cells is important for trafficking of the cells to sites of Th1-associated inflammation.[281] The Th2 master regulator, GATA3, is expressed in T_{reg} cells that reside in barrier sites such as the gastrointestinal tract and the skin, but is also induced by TCR and IL-2 stimulation.[282,283] Although not required for basal T_{reg}-cell homeostasis and function,

T_{reg}-cell expression of GATA3 is critical during inflammatory responses where it maintains Foxp3 expression and limits T_{reg}-cell conversion to an effector T-cell phenotype. Considerable numbers of human CD4+ T cells coexpress FOXP3 and RORGT. These T cells express CCR6 and produce IL-17 upon activation, but also have suppressive activity.[284] Although IRF4 is important for Th2 and Th17 differentiation, deletion of this factor in T_{reg} cells leads to autoimmunity associated with overproduction of Th2 cytokines.[285] Similarly, T_{reg} cell-specific deletion of Stat3, a factor that drives Th17 and Tfh differentiation, also leads to autoimmunity.[286] Finally, T_{reg}cells present in the GC that express Bcl6 function to constrain B-cell responses.[287,288]

Likewise, inflammatory Th17 cells can be generated that express both Rorγt and T-bet and GATA3.[110,136,289,290] In experimental autoimmune encephalomyelitis, T-bet+, Rorgt+ cells are highly pathogenic, and in this model expression of T-bet is associated with pathogenicity, even when IFN-γ is not.

Bcl6 and T-bet can be coexpressed and in fact, can cooperate to repress gene expression.[291] GATA3 and Bcl6 are coexpressed in the setting of helminth infestation[279]; in fact, Bcl6 is broadly expressed in CD4 T cells and not just expressed by bona fide Tfh cells. Moveover, even though IL-6 and IL-21 acting via STAT3 can drive Tfh-cell generation, they are not essential. Tfh can differentiation can occur in the absence of these factors.[292,293] This can be explained by the fact that IL-12 is capable of driving IL-21 production and Bcl6 expression.[43,178] So, some of the "master regulator" transcription factors have diverse stimuli that induce their expression.

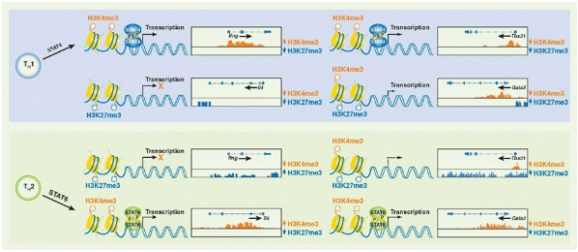

FIG. 29.2. Epigenetic Regulation of Helper-Cell Phenotype. Once differentiated, the distinct T-cell subsets have a propensity to maintain their distinctive patterns of gene expression. The tendency to "remember" this phenotype is controlled by the selective expression of key transcription factors, but also the chromatin configuration of the target

genes. Deoxyribonucleic acid forms nucleosomes, which can be compacted and inaccessible (heterochromatin) or open and accessible (euchromatin). Specific modifications of the histone molecules that make up the nucleosomes are associated with accessibility. That is, genes that are accessible exhibit histone 3 lysine 4 trimethylation (H3K4me3). In contrast, genes that are silenced and inaccessible exhibit histone 3 lysine 27 trimethylation (H3K27me3). Typically, cytokine genes exhibit accessible (H3K4me3) marks in the subsets in which they are expressed (eg, *Ifng* in Th1 cells and *Il4* in Th2 cells) and repressive (H3K27me3) in subsets in which they silenced (eg, *Ifng* in Th2 cells and *Il4* in Th1 cells). In contrast though, master regulators genes such as *Tbx21* (which encodes T-bet) and *Gata3* can exhibit both marks indicative of the fact that they can have more flexible expression. Although they are not necessarily expressed, they may not be completely silenced.

Thus, for all these reasons, it should be clear that there is not a simple relationship between phenotype and master regulator expression and that expression of more than one master regulator is frequent event.[258]

Epigenetic Explanations

As discussed, consistent with the standard "lineage commitment" model of helper-cell differentiation, cytokine genes typically show permissive (H3K4me3) marks on lineage-appropriate cytokines in the corresponding lineages (eg, *Ifng* in Th1 cells) and are accompanied by repressive (H3K27me3) marks in other subsets (Fig. 29.2). However, this simple scenario is not always the case. After the recognition of Th17 cells as a new subset, it was quickly realized that these cells are intrinsically unstable.[268,269] Indeed, this instability is correlated with the rapid remodeling of the *Ifng* locus when Th17 cells are stimulated in the presence of IL-12.[294]

Even more striking is that the histone methylation patterns of genes encoding several master regulators do not confirm to this simple "on or off" mode of regulation (Fig. 29.2). They exhibit both repressive and accessible marks, even when these transcription factors are not expressed.[228] For example, the *Tbx21* gene is associated with accessible H3K4me3 marks in Th1 cells. Although, repressive H3K27me3 marks are present in Th2 and Th17 cells, H3K4me3 marks are also present. Such regions, marked by both chromatin modifications, are termed bivalent domains and have been seen in genes poised for expression in stem

cells.[295,296] This then can help explain how T-bet might be induced in T_{reg}, Th17, and even Th2 cells. Similarly, the *Bcl6* locus has H3K4me3 marks in polarized Th1, Th2, and Th17 cells, and conversely, the *Tbx21*, *Rorc*, and *Gata3* genes have accessible marks in Tfh cells.[278]

Micro-Ribonucleic Acids and Phenotypic Stability

Micro-ribonucleic acids (RNAs) are small RNAs that bind to target messenger RNAs (mRNAs) and lead to inhibition of translation or mRNA degradation.[297,298] Drosha and Dicer are two key components of the machinery responsible for microRNA generation. Loss of these factors is associated with autoimmune diseases and loss of T_{reg}-cell stability.[299,300] Our understanding of how microRNAs regulate helper-cell differentiation is expanding rapidly. It is clear miR-146a is important for suppressive function of T_{reg},[301] whereas miR-155 influences Th1, Th2 differentiation, and T_{reg}-cell development.[302,303,304,305,306,307] By contrast, miR-326 enhances T_H17 differentiation, whereas miR-29 regulates T_H1 differentiation.[308,309,310] Bcl-6 represses expression of many microRNAs predicted to control the Tfh cell signature, including miR-17-92, which represses CXCR5 expression.[183] Yet another microRNA, miR-125b, contributes to maintaining the naïve state of helper T cells and miR-182 promotes clonal expansion.[311,312] This is surely an area that will continue to provide new insights into control of CD4 phenotypic stability.

In summary, although there are aspects of helper-cell behavior in which these cells appear to be stable subsets, there is also no shortage of molecular mechanisms that permit flexibility in their repertoire of responses.

HELPER T CELLS IN IMMUNE-MEDIATED DISEASE AND CANCER

There are increasing numbers of examples in which what we have learned about CD4 helper cells has direct bearing on health and disease in humans.

Immunodeficiency

The critical importance of CD4 T cells is exemplified by the range of infections seen in human immunodeficiency virus and acquired immunodeficiency syndrome. In addition, other immunodeficiency disorders are also linked to derangement in the function of specific subsets of Th cells. For example, STAT3 is critical for Th17 differentiation in humans and accordingly, dominant negative mutations of *STAT3* underlie the human disorder hyperimmunoglobulinemia E, or Job syndrome. The failure to generate Th17 cells is an

important aspect of this disorder that likely contributes to *Staphylococcal* and fungal infections.[313,314,315,316,317]

IFN-γ inhibits Th17 differentiation and activates STAT1; interestingly, gain-of-function mutations of *STAT1* result in the disorder chronic mucocutaneous candidiasis, which is associated with impaired Th17 differentiation.[318,319,320] Conversely, loss of function of *STAT1* mutations are associated with atypical mycobacterial infection.[321]

Recently, production of anticytokine autoantibodies has come be recognized as an underlying cause of susceptibility to various infectious diseases.[322,323,324]

Autoimmunity

Coupled with the discovery of IL-23, the recognition in mouse models that a simple Th1/Th2 paradigm did not adequately explain autoimmunity led to important insights into the role of Th17 cells in immune-mediated disease[81,82,325]; in reality, different types of helper T cells can elicit different types of pathology.[326]

Importantly, the lessons learned in mice appear to be very relevant to human disease. First, a number of human diseases are associated with increased levels of IL-17.[90] These include a wide range of autoimmune diseases including systemic lupus erythematosus, rheumatoid arthritis, psoriasis, multiple sclerosis, and other disorders.[327,328,329,330,331] It should be noted, however, that autoimmune disease in humans is often not a pure "Th17" disease and that IL-17, IFN-γ, and other cytokines are overproduced. Moreover, dysregulated Tfh cells can cause systemic autoimmunity and autoantibody production or contribute to T cell-mediated organ-specific autoimmunity.[177,332]

In addition, there are strong genetic clues linking these pathways with human autoimmunity. Polymorphisms of *IL23R*, *STAT3*, and *JAK2* are associated with inflammatory bowel disease, spondyloarthropathy, and Behcet disease.[333,334,335,336] Polymorphisms of *STAT4* are associated with systemic lupus erythematosus and rheumatoid arthritis.[337] Mutations of *FOXP3* underlie the disorder immune polyendocrinopathy and endocrinopathy syndrome. As is the mouse, this is associated with loss of T_{reg} cells resulting in severe systemic autoimmune disease.[338] A critical role for STAT5 is regulation of FOXP3 and accordingly, *STAT5* mutations are also associated with autoimmunity due to impaired T_{reg} function.[339]

Perhaps most importantly, targeting IL-12 and IL-23 with the drug ustekinumab is efficacious in inflammatory bowel disease and psoriasis, but curiously not multiple sclerosis.[340,341,342] Anti-IL-17 (secukinumab) is currently being studied in a variety of human autoimmune diseases.[343] Interestingly, the drug digoxin appears to target Rorγt and inhibits Th17 differentiation[344]; whether it will be feasible to target Th17 cells with small molecule inhibitors remains to be determined.

Asthma and Allergy

In contrast to perplexing relationship between Th1 cells and autoimmunity, the connection between Th2 cells and allergies and asthma was more straightforward. Nonetheless, as we learn more about the complexities of helper-cell differentiation, even this straightforward association starts to fray at the edges. For example, IL-17 and IL-22 appear to be important players in allergic disease[345]; in fact, asthma severity correlates with serum IL-17 levels.[346,347,348] Likewise, IL-9 and IL-21 are also relevant components to allergic and Th2-mediated disease.[349,350,351]

Cancer

The importance of IFNs, including IFN-γ, in tumor surveillance and cancer immunoediting is well appreciated.[352,353] In adoptive transfer models though, Th17 cells appear to be more effective than Th1 cells in eliminating cancer cells.[354,355] However, given the role of IL-17 in driving inflammation, there are also data suggesting that IL-17 promotes oncogenesis; clearly, this will be an important area to follow closely.[354,356]

REFERENCES

1. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu Rev Immunol*. 2005;23:127-159.

2. Weinreich MA, Hogquist KA. Thymic emigration: when and how T cells leave home. *J Immunol*. 2008;181:2265-2270.

3. Bromley SK, Mempel TR, Luster AD. Orchestrating the orchestrators: chemokines in control of T cell traffic. *Nat Immunol*. 2008;9:970-980.

4. Bromley SK, Thomas SY, Luster AD. Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics. *Nat Immunol*. 2005;6:895-901.

5. Henrickson SE, von Andrian UH. Single-cell dynamics of T-cell priming. *Curr Opin Immunol*. 2007;19:249-58.

6. Worbs T, Mempel TR, Bolter J, von Andrian UH, Forster R. CCR7 ligands stimulate the intranodal motility of T lymphocytes in vivo. *J Exp Med*. 2007;204:489-495.

7. Carlson CM, Endrizzi BT, Wu J, et al. Kruppel-like factor 2 regulates thymocyte and T-cell migration. *Nature*. 2006;442:299-302.

8. Schwab SR, Cyster JG. Finding a way out: lymphocyte egress from lymphoid organs. *Nat Immunol*. 2007;8:1295-1301.

9. Weinreich MA, Takada K, Skon C, Reiner SL, Jameson SC, Hogquist KA. KLF2 transcription-factor deficiency in T cells results in unrestrained cytokine production and upregulation of bystander chemokine receptors. *Immunity*. 2009;31:122-130.

10. Takada K, Wang X, Hart GT, et al. Kruppel-like factor 2 is required for trafficking but not quiescence in postactivated T cells. *J Immunol*. 2011;186:775-783.

11. Feng X, Wang H, Takata H, Day TJ, Willen J, Hu H. Transcription factor Foxp1 exerts essential cell-intrinsic regulation of the quiescence of naive T cells. *Nat Immunol*. 2011;12:544-550.

12. Huang AY, Qi H, Germain RN. Illuminating the landscape of in vivo immunity: insights from dynamic in situ imaging of secondary lymphoid tissues. *Immunity*. 2004;21:331-339.

13. Germain RN, Jenkins MK. In vivo antigen presentation. *Curr Opin Immunol* . 2004;16:120-125.

14. Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature*. 2008;455:764-769.

15. Mora JR, von Andrian UH. T-cell homing specificity and plasticity: new concepts and future challenges. *Trends Immunol*. 2006;27:235-243.

16. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*. 1986;136:2348-2357.

17. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol*. 1989;7:145-173.

18. Paul WE, Seder RA. Lymphocyte responses and cytokines. *Cell*. 1994;76:241-251.

19. Seder RA, Paul WE. Acquisition of lymphokine-producing phenotype by CD4+ T cells. *Annu Rev Immunol*. 1994;12:635-673.

20. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature*. 1996;383:787-793.

21. Szabo SJ, Sullivan BM, Peng SL, Glimcher LH. Molecular mechanisms regulating Th1 immune responses. *Annu Rev Immunol*. 2003;21:713-758.

22. Rincon M, Flavell RA. T-cell subsets: transcriptional control in the Th1/Th2 decision. *Curr Biol* . 1997;7:R729-R732.

23. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol*. 2010;28:445-489.

24. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol*. 2004;22:745-763.

25. Pepper M, Jenkins MK. Origins of CD4(+) effector and central memory T cells. *Nat Immunol*. 2011;12:467-471.

26. Ballesteros-Tato A, Randall TD. Memory: the incomplete unhappening of differentiation. *Immunity*. 2011;35:496-498.

27. Wei L, Vahedi G, Sun HW, et al. Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell

differentiation. *Immunity*. 2010;32: 840-851.

28. Szabo SJ, Sullivan BM, Peng SL, Glimcher LH. Molecular mechanisms regulating Th1 immune responses. *Annu Rev Immunol*. 2003;21: 713-758.

29. Lazarevic V, Glimcher LH. T-bet in disease. *Nat Immunol*. 2011;12: 597-606.

30. Lighvani AA, Frucht DM, Jankovic D, et al. T-bet is rapidly induced by interferon-Œ≥ in lymphoid and myeloid cells. *Proc Natl Acad Sci U S A*. 2001;98:15137-15142.

31. Thieu VT, Yu Q, Chang HC, et al. Signal transducer and activator of transcription 4 is required for the transcription factor T-bet to promote T helper 1 cell-fate determination. *Immunity*. 2008;29:679-690.

32. Niesner U, Albrecht I, Janke M, et al. Autoregulation of Th1-mediated inflammation by twist1. *J Exp Med*. 2008;205:1889-1901.

33. Pesu M, Watford WT, Wei L, et al. T-cell-expressed proprotein convertase furin is essential for maintenance of peripheral immune tolerance. *Nature*. 2008;455:246-250.

34. Beima KM, Miazgowicz MM, Lewis MD, Yan PS, Huang TH, Weinmann AS. T-bet binding to newly identified target gene promoters is cell type-independent but results in variable context-dependent functional effects. *J Biol Chem*. 2006;281:11992-12000.

35. Miller SA, Weinmann AS. Molecular mechanisms by which T-bet regulates T-helper cell commitment. *Immunol Rev*. 2010;238:233-246.

36. Djuretic IM, Levanon D, Negreanu V, Groner Y, Rao A, Ansel KM. Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in T helper type 1 cells. *Nat Immunol*. 2007;8:145-153.

37. Sekimata M, Perez-Melgosa M, Miller SA, et al. CCCTC-binding factor and the transcription factor T-bet orchestrate T helper 1 cell-specific structure and function at the interferon-gamma locus. *Immunity*. 2009;31:551-564.

38. Kao C, Oestreich KJ, Paley MA, et al. Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virusspecific CD8+ T cell responses during chronic infection. *Nat Immunol*. 2011;12:663-671.

39. Lazarevic V, Chen X, Shim JH, et al. T-bet represses T(H)17 differentiation by preventing Runx1-mediated activation of the gene encoding RORgammat. *Nat Immunol*. 2011;12:96-104.

40. Hwang ES, Szabo SJ, Schwartzberg PL, Glimcher LH. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science*. 2005;307:430-433.

41. Lord GM, Rao RM, Choe H, et al. T-bet is required for optimal proinflammatory CD4+ T-cell trafficking. *Blood*. 2005;106:3432-3439.

42. Marshall HD, Chandele A, Jung YW, et al. Differential expression of Ly6C and T-bet distinguish effector and memory Th1 CD4(+) cell properties during viral infection. *Immunity*. 2011;35:633-646.

43. Nakayamada S, Kanno, Y, Takayashi, H, et al. Early T-helper 1 differentiation is marked by a follicular helper-like transition: differential roles of STAT4 and T-bet. *Immunity*. 2011. In press.

44. Delgoffe GM, Kole TP, Zheng Y, et al. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity*. 2009;30:832-844.

45. Delgoffe GM, Pollizzi KN, Waickman AT, et al. The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. *Nat Immunol*. 2011;12:295-303.

46. Lee K, Gudapati P, Dragovic S, et al. Mammalian target of rapamycin protein complex 2 regulates differentiation of Th1 and Th2 cell subsets via distinct signaling pathways. *Immunity*. 2010;32:743-753.

47. Powell JD, Pollizzi KN, Heikamp EB, Horton MR. Regulation of immune responses by mTOR. *Annu Rev Immunol*. 2012;30:39-68.

48. Endo Y, Iwamura C, Kuwahara M, et al. Eomesodermin controls interleukin-5 production in memory T helper 2 cells through inhibition of activity of the transcription factor GATA3. *Immunity*. 2011;35:733-745.

49. Aujla SJ, Chan YR, Zheng M, et al. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat Med*. 2008;14: 275-281.

50. Li Z, Zhang Y, Liu Z, et al. ECM1 controls T(H)2 cell egress from lymph nodes through re-expression of S1P(1). *Nat Immunol*. 2011;12: 178-185.

51. Siracusa MC, Saenz SA, Hill DA, et al. TSLP promotes interleukin-3-independent basophil haematopoiesis and type 2 inflammation. *Nature*. 2011;477:229-233.

52. Karasuyama H, Mukai K, Obata K, Tsujimura Y, Wada T. Nonredundant roles of basophils in immunity. *Annu Rev Immunol*. 2011;29:45-69.

53. Paul WE, Zhu J. How are T(H)2-type immune responses initiated and amplified? *Nat Rev Immunol*. 2010;10:225-235.

54. Ziegler SF, Artis D. Sensing the outside world: TSLP regulates barrier immunity. *Nat Immunol*. 2010;11:289-293.

55. Sokol CL, Medzhitov R. Role of basophils in the initiation of Th2 responses. *Curr Opin Immunol*. 2010;22:73-77.

56. Moro K, Yamada T, Tanabe M, et al. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature*. 2010;463:540-544.

57. Saenz SA, Siracusa MC, Perrigoue JG, et al. IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature*. 2010;464:1362-1366.

58. Barlow JL, McKenzie AN. Nuocytes: expanding the innate cell repertoire in type-2 immunity. *J Leukoc Biol*. 2011;90:867-874.

59. Mjosberg JM, Trifari S, Crellin NK, et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol*. 2011;12:1055-1062.

60. Schmitz J, Owyang A, Oldham E, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity*. 2005;23:479-490.

61. Arend WP, Palmer G, Gabay C. IL-1, IL-18, and IL-33 families of cytokines. *Immunol Rev*. 2008;223:20-38.

62. Palmer G, Gabay C. Interleukin-33 biology with potential insights into human diseases. *Nat Rev Rheumatol*. 2011;7:321-329.

63. Lund R, Ahlfors H, Kainonen E, Lahesmaa AM, Dixon C, Lahesmaa R. Identification of genes involved in the initiation of human Th1 or Th2 cell commitment. *Eur J Immunol*. 2005;35:3307-3319.

64. Elo LL, Jarvenpaa H, Tuomela S, et al. Genome-wide profiling of interleukin-4 and STAT6 transcription factor regulation of human Th2 cell programming. *Immunity*. 2010;32:852-862.

65. O'Shea JJ, Lahesmaa R, Vahedi G, Laurence A, Kanno Y. Genomic views of STAT function in CD4+ T helper cell differentiation. *Nat Rev Immunol*. 2011;11:239-250.

66. Cote-Sierra J, Foucras G, Guo L, et al. Interleukin 2 plays a central role in Th2 differentiation. *Proc Natl Acad Sci U S A*. 2004;101:3880-3885.

67. Zhu J, Cote-Sierra J, Guo L, Paul WE. Stat5 activation plays a critical role in Th2 differentiation. *Immunity*. 2003;19:739-748.

68. Yamane H, Zhu J, Paul WE. Independent roles for IL-2 and GATA-3 in stimulating naive CD4+ T cells to generate a Th2-inducing cytokine environment. *J Exp Med*. 2005;202:793-804.

69. Liao W, Lin JX, Wang L, Li P, Leonard WJ. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. *Nat Immunol*. 2011;12:551-

559.

70. Liao W, Schones DE, Oh J, et al. Priming for T helper type 2 differentiation by interleukin 2-mediated induction of interleukin 4 receptor Œ±-chain expression. *Nat Immunol*. 2008;9:1288-1296.

71. Stritesky GL, Muthukrishnan R, Sehra S, et al. The transcription factor STAT3 is required for T helper 2 cell development. *Immunity*. 2011;34:39-49.

72. Wei G, Abraham BJ, Yagi R, et al. Genome-wide analyses of transcription factor GATA3-mediated gene regulation in distinct T cell types. *Immunity*. 2011;35:299-311.

73. Yagi R, Junttila IS, Wei G, et al. The transcription factor GATA3 actively represses RUNX3 protein-regulated production of interferongamma. *Immunity*. 2010;32:507-517.

74. Kim JI, Ho IC, Grusby MJ, Glimcher LH. The transcription factor c-Maf controls the production of interleukin-4 but not other Th2 cytokines. *Immunity*. 1999;10:745-751.

75. Amsen D, Blander JM, Lee GR, Tanigaki K, Honjo T, Flavell RA. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell*. 2004;117:515-526.

76. Lohoff M, Mittrucker HW, Prechtl S, et al. Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. *Proc Natl Acad Sci U S A* . 2002;99:11808-11812.

77. Zhu J, Guo L, Min B, et al. Growth factor independent-1 induced by IL-4 regulates Th2 cell proliferation. *Immunity*. 2002;16:733-744.

78. Zhu J, Min B, Hu-Li J, et al. Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. *Nat Immunol*. 2004;5:1157-1165.

79. Zhu J, Jankovic D, Grinberg A, Guo L, Paul WE. Gfi-1 plays an important role in IL-2-mediated Th2 cell expansion. *Proc Natl Acad Sci U S A* . 2006;103:18214-18219.

80. Okamoto M, Van Stry M, Chung L, et al. Mina, an Il4 repressor, controls T helper type 2 bias. *Nat Immunol*. 2009;10:872-879.

81. Gor DO, Rose NR, Greenspan NS. TH1-TH2: a procrustean paradigm. *Nat Immunol*. 2003;4:503-505.

82. Cua DJ, Sherlock J, Chen Y, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature*. 2003;421:744-748.

83. Langrish CL, Chen Y, Blumenschein WM, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med*. 2005;201:233-240.

84. Harrington LE, Hatton RD, Mangan PR, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol*. 2005;6:1123-1132.

85. Park H, Li Z, Yang XO, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol*. 2005;6:1133-1141.

86. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity*. 2006;24:677-688.

87. Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol*. 2007;25:821-852.

88. Stockinger B, Veldhoen M. Differentiation and function of Th17 T cells. *Curr Opin Immunol*. 2007;19:281-286.

89. Bettelli E, Korn T, Oukka M, Kuchroo VK. Induction and effector functions of T(H)17 cells. *Nature*. 2008;453:1051-1057.

90. Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. *N Engl J Med*. 2009;361:888-898.

91. Annunziato F, Cosmi L, Liotta F, Maggi E, Romagnani S. Type 17 T helper cells-origins, features and possible roles in rheumatic disease. *Nat Rev Rheumatol*.

2009;5:325-331.

92. Peters A, Lee Y, Kuchroo VK. The many faces of Th17 cells. *Curr Opin Immunol* . 2011;23(6):702-706.

93. Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity*. 2004;21:467-476.

94. Kolls JK, Khader SA. The role of Th17 cytokines in primary mucosal immunity. *Cytokine Growth Factor Rev* . 2010;21:443-448.

95. Onishi RM, Gaffen SL. Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. *Immunology*. 2010;129:311-321.

96. Kolls JK, Khader SA. The role of Th17 cytokines in primary mucosal immunity. *Cytokine Growth Factor Rev* . 2010;21:443-448.

97. Khader SA, Guglani L, Rangel-Moreno J, et al. IL-23 is required for long-term control of Mycobacterium tuberculosis and B cell follicle formation in the infected lung. *J Immunol*. 2011;187:5402-5407.

98. Hsu HC, Yang P, Wang J, et al. Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice. *Nat Immunol*. 2008;9:166-175.

99. Rangel-Moreno J, Carragher DM, de la Luz Garcia-Hernandez M, et al. The development of inducible bronchus-associated lymphoid tissue depends on IL-17. *Nat Immunol*. 2011;12:639-646.

100. Acosta-Rodriguez EV, Rivino L, Geginat J, et al. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* . 2007;8:639-646.

101. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity*. 2006;24:179-189.

102. Mangan PR, Harrington LE, O'Quinn DB, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature*. 2006;441:231-234.

103. Wilson NJ, Boniface K, Chan JR, et al. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* . 2007;8:950-957.

104. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* . 2007;8:942-949.

105. Chen Z, Tato CM, Muul L, Laurence A, O'Shea JJ. Distinct regulation of interleukin-17 in human T helper lymphocytes. *Arthritis Rheum*. 2007;56:2936-2946.

106. Zhou L, Ivanov II, Spolski R, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol* . 2007;8:967-974.

107. Manel N, Unutmaz D, Littman DR. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat Immunol* . 2008;9:641-649.

108. Romagnani S, Maggi E, Liotta F, Cosmi L, Annunziato F. Properties and origin of human Th17 cells. *Mol Immunol* . 2009;47(1):3-7.

109. Ghoreschi K, Laurence A, Yang XP, et al. Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. *Nature*. 2010;467:967-971.

110. Ghoreschi K, Laurence A, Yang XP, Hirahara K, O'Shea JJ. T helper 17 cell heterogeneity and pathogenicity in autoimmune disease. *Trends Immunol*. 2011;32:395-401.

111. Annunziato F, Cosmi L, Santarlasci V, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med*. 2007;204:1849-1861.

112. Annunziato F, Romagnani S. Mouse T helper 17 phenotype: not so different than in man after all. *Cytokine*. 2011;56:112-115.

113. Durant L, Watford WT, Ramos HL, et al. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity*. 2010;32:605-615.

114. Ivanov II, McKenzie BS, Zhou L, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+T helper cells. *Cell*. 2006;126:1121-1133.

115. Ivanov II, Zhou L, Littman DR. Transcriptional regulation of Th17 cell differentiation. *Semin Immunol*. 2007;19:409-417.

116. Chen Z, Laurence A, Kanno Y, et al. Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc Natl Acad Sci U S A* . 2006;103:8137-8142.

117. Yang XO, Panopoulos AD, Nurieva R, et al. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem*. 2007;282:9358-9363.

118. Zhou L, Littman DR. Transcriptional regulatory networks in Th17 cell differentiation. *Curr Opin Immunol* . 2009;21:146-152.

119. Takatori H, Kanno Y, Watford WT, et al. Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. *J Exp Med*. 2009;206:35-41.

120. Luci C, Reynders A, Ivanov II, et al. Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin. *Nat Immunol*. 2009;10:75-82.

121. Sawa S, Cherrier M, Lochner M, et al. Lineage relationship analysis of RORgammat+ innate lymphoid cells. *Science*. 2010;330:665-669.

122. Sawa S, Lochner M, Satoh-Takayama N, et al. RORgammat+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. *Nat Immunol*. 2011;12:320-326.

123. Yang XO, Pappu BP, Nurieva R, et al. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity*. 2008;28:29-39.

124. Quintana FJ, Basso AS, Iglesias AH, et al. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature*. 2008;453:65-71.

125. Veldhoen M, Hirota K, Westendorf AM, et al. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature*. 2008;453:106-109.

126. Schraml BU, Hildner K, Ise W, et al. The AP-1 transcription factor Batf controls T(H)17 differentiation. *Nature*. 2009;460:405-409.

127. Okamoto K, Iwai Y, Oh-Hora M, et al. IkappaBzeta regulates T(H)17 development by cooperating with ROR nuclear receptors. *Nature*. 2010;464:1381-1385.

128. Huber M, Brustle A, Reinhard K, et al. IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype. *Proc Natl Acad Sci U S A* . 2008;105:20846-20851.

129. Zhang F, Meng G, Strober W. Interactions among the transcription factors Runx1, RORgammat and Foxp3 regulate the differentiation of interleukin 17-producing T cells. *Nat Immunol*. 2008;9:1297-1306.

130. Dang EV, Barbi J, Yang HY, et al. Control of T(H)17/T(reg) balance by hypoxia-inducible factor 1. *Cell*. 2011;146:772-784.

131. Shi LZ, Wang R, Huang G, et al. HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J Exp Med*. 2011;208:1367-1376.

132. Reynolds JM, Pappu BP, Peng J, et al. Toll-like receptor 2 signaling in CD4(+) T lymphocytes promotes T helper 17 responses and regulates the pathogenesis of autoimmune disease. *Immunity*. 2010;32:692-702.

133. Laurence A, Tato CM, Davidson TS, et al. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity*. 2007;26: 371-381.

134. Pandiyan P, Conti HR, Zheng L, et al. CD4(+)CD25(+)Foxp3(+) regulatory T cells promote Th17 cells in vitro and enhance host resistance in mouse Candida albicans Th17 cell infection model. *Immunity*. 2011;34:422-434.

135. Chen Q, Kim YC, Laurence A, Punkosdy GA, Shevach EM. IL-2 controls the stability of Foxp3 expression in TGF-{beta}-induced Foxp3+ T cells in vivo. *J Immunol*. 2011;1836(11):6329-6337.

136. Yang XP, Ghoreschi K, Steward-Tharp SM, et al. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. *Nat Immunol*. 2011;12:247-254.

137. Zhou L, Lopes JE, Chong MM, et al. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature*. 2008;453:236-240.

138. Moisan J, Grenningloh R, Bettelli E, Oukka M, Ho IC. Ets-1 is a negative regulator of Th17 differentiation. *J Exp Med*. 2007;204: 2825-2835.

139. Zhu J, Davidson TS, Wei G, et al. Down-regulation of Gfi-1 expression by TGF-beta is important for differentiation of Th17 and CD103+ inducible regulatory T cells. *J Exp Med*. 2009;206:329-341.

140. Ma J, Wang R, Fang X, Ding Y, Sun Z. Critical role of TCF-1 in repression of the IL-17 gene. *PLoS One*. 2011;6:e24768.

141. Yu Q, Sharma A, Ghosh A, Sen JM. T cell factor-1 negatively regulates expression of IL-17 family of cytokines and protects mice from experimental autoimmune encephalomyelitis. *J Immunol*. 2011;186: 3946-3952.

142. Ouyang W, Rutz S, Crellin NK, Valdez PA, Hymowitz SG. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu Rev Immunol*. 2011;29:71-109.

143. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Stevens S, Flavell RA. Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. *Immunity*. 2008;29:947-957.

144. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Karow M, Flavell RA. Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation. *Immunity*. 2007;27: 647-659.

145. Zenewicz LA, Flavell RA. Recent advances in IL-22 biology. *Int Immunol*. 2011;23:159-163.

146. Zheng Y, Danilenko DM, Valdez P, et al. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature*. 2007;445:648-651.

147. Zheng Y, Valdez PA, Danilenko DM, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med*. 2008;14:282-289.

148. Rutz S, Ouyang W. Regulation of interleukin-10 and interleukin-22 expression in T helper cells. *Curr Opin Immunol*. 2011;23:605-612.

149. Eyerich S, Eyerich K, Pennino D, et al. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J Clin Invest*. 2009;119:3573-3585.

150. Duhen T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F. Production of interleukin 22 but not interleukin 17 by a subset of human skinhoming memory T cells. *Nat Immunol*. 2009;10:857-863.

151. Trifari S, Kaplan CD, Tran EH, Crellin NK, Spits H. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. *Nat Immunol*. 2009;10:864-871.

152. Crellin NK, Trifari S, Kaplan CD, Satoh-Takayama N, Di Santo JP, Spits H. Regulation of cytokine secretion in human CD127(+) LTilike innate lymphoid cells by toll-like receptor 2. *Immunity*. 2010;33: 752-764.

153. Crellin NK, Trifari S, Kaplan CD, Cupedo T, Spits H. Human NKp44+IL-22+ cells and LTi-like cells constitute a stable RORC+ lineage distinct from conventional natural killer cells. *J Exp Med*. 2010;207:281-290.

154. Cella M, Fuchs A, Vermi W, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature*. 2009;457:722-725.

155. Vivier E, Spits H, Cupedo T. Interleukin-22-producing innate immune cells: new players in mucosal immunity and tissue repair? *Nat Rev Immunol*. 2009;9:229-234.

156. Spits H, Di Santo JP. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. *Nat Immunol*. 2011;12:21-27.

157. Ambrosi A, Dzikaite V, Park J, et al. Anti-Ro52 monoclonal antibodies specific for amino acid 200-239, but not other Ro52 epitopes, induce congenital heart block in a rat model. *Ann Rheum Dis*. 2011;71(3): 448-454.

158. Sanos SL, Bui VL, Mortha A, et al. RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nat Immunol*. 2009;10:83-91.

159. Alam MS, Maekawa Y, Kitamura A, et al. Notch signaling drives IL-22 secretion in CD4+ T cells by stimulating the aryl hydrocarbon receptor. *Proc Natl Acad Sci U S A*. 2010;107:5943-5948.

160. Rutz S, Noubade R, Eidenschenk C, et al. Transcription factor c-Maf mediates the TGF-beta-dependent suppression of IL-22 production in T(H)17 cells. *Nat Immunol*. 2011;12(12):1238-1245.

161. Dardalhon V, Awasthi A, Kwon H, et al. IL-4 inhibits TGF-betainduced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat Immunol*. 2008;9:1347-1355.

162. Veldhoen M, Uyttenhove C, van Snick J, et al. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol*. 2008;9:1341-1346.

163. Beriou G, Bradshaw EM, Lozano E, et al. TGF-beta induces IL-9 production from human Th17 cells. *J Immunol*. 2010;185:46-54.

164. Perumal NB, Kaplan MH. Regulating Il9 transcription in T helper cells. *Trends Immunol*. 2011;32:146-150.

165. Schmitt E, Germann T, Goedert S, et al. IL-9 production of naive CD4+ T cells depends on IL-2, is synergistically enhanced by a combination of TGF-beta and IL-4, and is inhibited by IFN-gamma. *J Immunol*. 1994;153:3989-3996.

166. Angkasekwinai P, Chang SH, Thapa M, Watarai H, Dong C. Regulation of IL-9 expression by IL-25 signaling. *Nat Immunol*. 2010;11:250-256.

167. Wilhelm C, Hirota K, Stieglitz B, et al. An IL-9 fate reporter demonstrates the induction of an innate IL-9 response in lung inflammation. *Nat Immunol*. 2011;12:1071-1077.

168. Reynolds JM, Angkasekwinai P, Dong C. IL-17 family member cytokines: regulation and function in innate immunity. *Cytokine Growth Factor Rev*. 2010;21:413-423.

169. Goswami R, Kaplan MH. A brief history of IL-9. *J Immunol*. 2011; 186:3283-3288.

170. Nowak EC, Weaver CT, Turner H, et al. IL-9 as a mediator of Th17-driven inflammatory disease. *J Exp Med*. 2009;206:1653-1660.

171. Elyaman W, Bradshaw EM, Uyttenhove C, et al. IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells. *Proc Natl Acad Sci U S A*. 2009;106:12885-12890.

172. Chang HC, Sehra S, Goswami R, et al. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat Immunol*. 2010;11:527-534.

173. Staudt V, Bothur E, Klein M, et al. Interferon-regulatory factor 4 is essential for the

developmental program of T helper 9 cells. *Immunity*. 2010;33:192-202.

174. Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG. Follicular helper T cells: lineage and location. *Immunity*. 2009;30: 324-335.

175. Yu D, Vinuesa CG. The elusive identity of T follicular helper cells. *Trends Immunol*. 2010;31:377-383.

176. Crotty S. Follicular helper CD4 T cells (T(FH)). *Annu Rev Immunol*. 2011;29:621-663.

177. Spolski R, Leonard WJ. IL-21 and T follicular helper cells. *Int Immunol*. 2010;22:7-12.

178. Schmitt N, Morita R, Bourdery L, et al. Human dendritic cells induce the differentiation of interleukin-21-producing T follicular helper-like cells through interleukin-12. *Immunity*. 2009;31:158-169.

179. Bauquet AT, Jin H, Paterson AM, et al. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat Immunol*. 2009;10: 167-175.

180. Dent AL, Shaffer AL, Yu X, Allman D, Staudt LM. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science*. 1997;276:589-592.

181. Nurieva RI, Chung Y, Martinez GJ, et al. Bcl6 mediates the development of T follicular helper cells. *Science*. 2009;325:1001-1005.

182. Johnston RJ, Poholek AC, DiToro D, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science*. 2009;325:1006-1010.

183. Yu D, Rao S, Tsai LM, et al. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity*. 2009;31: 457-468.

184. Dent AL, Hu-Li J, Paul WE, Staudt LM. T helper type 2 inflammatory disease in the absence of interleukin 4 and transcription factor STAT6. *Proc Natl Acad Sci U S A*. 1998;95:13823-13828.

185. Mehta DS, Wurster AL, Weinmann AS, Grusby MJ. NFATc2 and T-bet contribute to T-helper-cell-subset-specific regulation of IL-21 expression. *Proc Natl Acad Sci U S A*. 2005;102:2016-2021.

186. Pepper M, Pagan AJ, Igyarto BZ, Taylor JJ, Jenkins MK. Opposing signals from the bcl6 transcription factor and the interleukin-2 receptor generate T helper 1 central and effector memory cells. *Immunity*. 2011;35:583-595.

187. Rudensky AY. Regulatory T cells and Foxp3. *Immunol Rev*. 2011;241: 260-268.

188. Miyara M, Sakaguchi S. Human FoxP3(+)CD4(+) regulatory T cells: their knowns and unknowns. *Immunol Cell Biol*. 2011;89:346-351.

189. Liu Y, Zhang P, Li J, Kulkarni AB, Perruche S, Chen W. A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. *Nat Immunol*. 2008;9:632-640.

190. Maruyama T, Konkel JE, Zamarron BF, Chen W. The molecular mechanisms of Foxp3 gene regulation. *Semin Immunol*. 2011;23(6):418-423.

191. Malek TR. The biology of interleukin-2. *Annu Rev Immunol*. 2008;26: 453-479.

192. Mucida D, Park Y, Kim G, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science*. 2007;317:256-260.

193. Mucida D, Pino-Lagos K, Kim G, et al. Retinoic acid can directly promote TGF-beta-mediated Foxp3(+) Treg cell conversion of naive T cells. *Immunity*. 2009;30:471-472; author reply 2-3.

194. Elias KM, Laurence A, Davidson TS, et al. Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. *Blood*. 2008;111:1013-1020.

195. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med*. 2007;204:1757-1764.

196. Sun CM, Hall JA, Blank RB, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med*. 2007;204:1775-1785.

197. Zheng Y, Josefowicz SZ, Kas A, Chu TT, Gavin MA, Rudensky AY. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature*. 2007;445:936-940.

198. Rudra D, Egawa T, Chong MM, Treuting P, Littman DR, Rudensky AY. Runx-CBFbeta complexes control expression of the transcription factor Foxp3 in regulatory T cells. *Nat Immunol*. 2009;10:1170-1177.

199. Sekiya T, Kashiwagi I, Inoue N, et al. The nuclear orphan receptor Nr4a2 induces Foxp3 and regulates differentiation of CD4+ T cells. *Nat Commun*. 2011;2:269.

200. Kerdiles YM, Stone EL, Beisner DR, et al. Foxo transcription factors control regulatory T cell development and function. *Immunity*. 2010;33:890-904.

201. Getnet D, Grosso JF, Goldberg MV, et al. A role for the transcription factor Helios in human CD4(+)CD25(+) regulatory T cells. *Mol Immunol*. 2010;47:1595-1600.

202. Akimova T, Beier UH, Wang L, Levine MH, Hancock WW. Helios expression is a marker of T cell activation and proliferation. *PLoS One*. 2011;6:e24226.

203. Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*. 2006;441:235-238.

204. Laurence A. Stat3 promotes instability of nTreg cells and limits generation of iTreg cells in acute graft versus host disease. *Immunity*. 2012. In press.

205. Weiner HL. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol Rev*. 2001;182:207-214.

206. Weiner HL. The mucosal milieu creates tolerogenic dendritic cells and T(R)1 and T(H)3 regulatory cells. *Nat Immunol*. 2001;2:671-672.

207. Groux H, O'Garra A, Bigler M, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature*. 1997;389:737-742.

208. Levings MK, Sangregorio R, Galbiati F, Squadrone S, de Waal Malefyt R, Roncarolo MG. IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. *J Immunol*. 2001;166:5530-5539.

209. Levings MK, Sangregorio R, Roncarolo MG. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med*. 2001;193:1295-1302.

210. Pot C, Apetoh L, Awasthi A, Kuchroo VK. Induction of regulatory Tr1 cells and inhibition of T(H)17 cells by IL-27. *Semin Immunol*. 2011;23(6):438-445.

211. Pot C, Apetoh L, Kuchroo VK. Type 1 regulatory T cells (Tr1) in autoimmunity. *Semin Immunol*. 2011;23:202-208.

212. Pot C, Jin H, Awasthi A, et al. Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. *J Immunol*. 2009;183:797-801.

213. Stumhofer JS, Silver JS, Laurence A, et al. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat Immunol*. 2007;8:1363-1371.

214. Awasthi A, Carrier Y, Peron JP, et al. A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. *Nat Immunol*. 2007;8:1380-1389.

215. Murugaiyan G, Mittal A, Lopez-Diego R, Maier LM, Anderson DE, Weiner HL. IL-27 is a key regulator of IL-10 and IL-17 production by human CD4+ T cells. *J Immunol*.

2009;183:2435-2443.

216. Apetoh L, Quintana FJ, Pot C, et al. The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. *Nat Immunol* 2010;11:854-861.

217. Wu HY, Quintana FJ, da Cunha AP, et al. In vivo induction of Tr1 cells via mucosal dendritic cells and AHR signaling. *PLoS One*. 2011;6:e23618.

218. Collison LW, Vignali DA. Interleukin-35: odd one out or part of the family? *Immunol Rev*. 2008;226:248-262.

219. Collison LW, Workman CJ, Kuo TT, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature*. 2007;450:566-569.

220. Collison LW, Chaturvedi V, Henderson AL, et al. IL-35-mediated induction of a potent regulatory T cell population. *Nat Immunol* . 2010;11:1093-1101.

221. Amsen D, Spilianakis CG, Flavell RA. How are T(H)1 and T(H)2 effector cells made? *Curr Opin Immunol* . 2009;21:153-160.

222. Aune TM, Collins PL, Chang S. Epigenetics and T helper 1 differentiation. *Immunology*. 2009;126:299-305.

223. Wilson CB, Rowell E, Sekimata M. Epigenetic control of T-helper-cell differentiation. *Nat Rev Immunol* . 2009;9:91-105.

224. Ansel KM, Lee DU, Rao A. An epigenetic view of helper T cell differentiation. *Nat Immunol*. 2003;4:616-623.

225. O'Shea J J, Kanno Y. Epigenetics T cells something or other. *Annu Rev Immunol* . 2012.

226. Oestreich KJ, Weinmann AS. Encoding stability versus flexibility: lessons learned from examining epigenetics in T helper cell differentiation. *Curr Top Microbiol Immunol* . 2012;356:145-164.

227. Barski A, Cuddapah S, Cui K, et al. High-resolution profiling of histone methylations in the human genome. *Cell*. 2007;129:823-837.

228. Wei G, Wei L, Zhu J, et al. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity*. 2009;30:155-167.

229. Cuddapah S, Barski A, Zhao K. Epigenomics of T cell activation, differentiation, and memory. *Curr Opin Immunol* . 2010;22:341-347.

230. Nakayama T, Yamashita M. Initiation and maintenance of Th2 cell identity. *Curr Opin Immunol*. 2008;20:265-271.

231. Yamashita M, Hirahara K, Shinnakasu R, et al. Crucial role of MLL for the maintenance of memory T helper type 2 cell responses. *Immunity*. 2006;24:611-622.

232. Onodera A, Yamashita M, Endo Y, et al. STAT6-mediated displacement of polycomb by trithorax complex establishes long-term maintenance of GATA3 expression in T helper type 2 cells. *J Exp Med*. 2010;207:2493-2506.

233. Kimura M, Koseki Y, Yamashita M, et al. Regulation of Th2 cell differentiation by mel-18, a mammalian Polycomb group gene. *Immunity*. 2001;15:275-287.

234. Yamashita M, Kuwahara M, Suzuki A, et al. Bmi1 regulates memory CD4 T cell survival via repression of the Noxa gene. *J Exp Med*. 2008;205:1109-1120.

235. Koyanagi M, Baguet A, Martens J, Margueron R, Jenuwein T, Bix M. EZH2 and histone 3 trimethyl lysine 27 associated with Il4 and Il13 gene silencing in Th1 cells. *J Biol Chem*. 2005;280:31470-34177.

236. Zhang F, Boothby M. T helper type 1-specific Brg1 recruitment and remodeling of nucleosomes positioned at the IFN-gamma promoter are Stat4 dependent. *J Exp Med*. 2006;203:1493-1505.

237. Hadjur S, Williams LM, Ryan NK, et al. Cohesins form chromosomal cis-interactions at the developmentally regulated IFNG locus. *Nature*. 2009;460:410-413.

238. Lee PP, Fitzpatrick DR, Beard C, et al. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity*. 2001;15:763-774.

239. Makar KW, Pérez-Melgosa M, Shnyreva M, Weaver WM, Fitzpatrick DR, Wilson CB. Active recruitment of DNA methyltransferases regulates interleukin 4 in thymocytes and T cells. *Nat Immunol*. 2003;4:1183-1190.

240. Hutchins AS, Mullen AC, Lee HW, et al. Gene silencing quantitatively controls the function of a developmental trans-activator. *Molecular Cell*. 2002;10:81-91.

241. Floess S, Freyer J, Siewert C, et al. Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol*. 2007;5:e38.

242. Huehn J, Polansky JK, Hamann A. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? *Nat Rev Immunol*. 2009;9:83-89.

243. Kim HP, Leonard WJ. CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. *J Exp Med*. 2007;204:1543-1551.

244. Balasubramani A, Mukasa R, Hatton RD, Weaver CT. Regulation of the Ifng locus in the context of T-lineage specification and plasticity. *Immunol Rev*. 2010;238:216-232.

245. Balasubramani A, Shibata Y, Crawford GE, Baldwin AS, Hatton RD, Weaver CT. Modular utilization of distal cis-regulatory elements controls Ifng gene expression in T cells activated by distinct stimuli. *Immunity*. 2010;33:35-47.

246. Aune TM, Penix LA, Rincon MR, Flavell RA. Differential transcription directed by discrete gamma interferon promoter elements in naive and memory (effector) CD4 T cells and CD8 T cells. *Mol Cell Biol*. 1997;17:199-208.

247. Soutto M, Zhou W, Aune TM. Cutting edge: distal regulatory elements are required to achieve selective expression of IFN-Œ≥ in Th1/Tc1 effector cells. *J Immunol*. 2002;169:6664-6667.

248. Chang S, Aune TM. Dynamic changes in histone-methylation 'marks' across the locus encoding interferon-Œ≥ during the differentiation of T helper type 2 cells. *Nat Immunol*. 2007;8:723-731.

249. Collins PL, Chang S, Henderson M, et al. Distal regions of the human IFNG locus direct cell type-specific expression. *J Immunol*. 2010;185:1492-1501.

250. Lee DU, Agarwal S, Rao A. Th2 lineage commitment and efficient IL-4 production involves extended demethylation of the IL-4 gene. *Immunity*. 2002;16:649-660.

251. Ansel KM, Djuretic I, Tanasa B, Rao A. Regulation of Th2 differentiation and Il4 locus accessibility. *Annu Rev Immunol*. 2006;24: 607-656.

252. Ansel KM, Greenwald RJ, Agarwal S, et al. Deletion of a conserved Il4 silencer impairs T helper type 1-mediated immunity. *Nat Immunol*. 2004;5:1251-1259.

253. Naoe Y, Setoguchi R, Akiyama K, et al. Repression of interleukin-4 in T helper type 1 cells by Runx/Cbf beta binding to the Il4 silencer. *J Exp Med*. 2007;204:1749-1755.

254. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature*. 2010;463:808-812.

255. Bluestone JA, Mackay CR, O'Shea JJ, Stockinger B. The functional plasticity of T cell subsets. *Nat Rev Immunol*. 2009;9:811-816.

256. Locksley RM. Nine lives: plasticity among T helper cell subsets. *J Exp Med*. 2009;206:1643-1646.

257. Zhou L, Chong MM, Littman DR. Plasticity of CD4+ T cell lineage differentiation. *Immunity*. 2009;30:646-655.

258. O'Shea J, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science*. 2010;327:1098-1102.

259. Zhu J, Paul WE. Heterogeneity and plasticity of T helper cells. *Cell Re*. 2010;20:4-12.

260. Murphy KM, Stockinger B. Effector T cell plasticity: flexibility in the face of changing circumstances. *Nat Immunol*. 2010;11:674-680.

261. O'Garra A, Vieira P. T(H)1 cells control themselves by producing interleukin-10. *Nat Rev Immunol*. 2007;7:425-428.

262. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. *Nat Rev Immunol*. 2010;10:170-181.

263. Esplugues E, Huber S, Gagliani N, et al. Control of TH17 cells occurs in the small intestine. *Nature*. 2011;475:514-518.

264. El-Behi M, Ciric B, Dai H, et al. The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat Immunol*. 2011;12:568-575.

265. Codarri L, Gyulveszi G, Tosevski V, et al. RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat Immunol*. 2011;12:560-567.

266. Suto A, Kashiwakuma D, Kagami S, et al. Development and characterization of IL-21-producing CD4+ T cells. *J Exp Med*. 2008;205:1369-1379.

267. Motomura Y, Kitamura H, Hijikata A, et al. The transcription factor E4BP4 regulates the production of IL-10 and IL-13 in CD4+ T cells. *Nat Immunol*. 2011;12:450-459.

268. Lee YK, Turner H, Maynard CL, et al. Late developmental plasticity in the T helper 17 lineage. *Immunity*. 2009;30:92-107.

269. Shi G, Cox CA, Vistica BP, Tan C, Wawrousek EF, Gery I. Phenotype switching by inflammation-inducing polarized Th17 cells, but not by Th1 cells. *J Immunol*. 2008;181:7205-7213.

270. Muranski P, Boni A, Antony PA, et al. Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood*. 2008;112:362-373.

271. Hirota K, Duarte JH, Veldhoen M, et al. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nat Immunol*. 2011;12: 255-263.

272. Oldenhove G, Bouladoux N, Wohlfert EA, et al. Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity*. 2009;31:772-786.

273. Feng T, Cao AT, Weaver CT, Elson CO, Cong Y. IL-12 converts Foxp3 + regulatory T cells to Foxp3 + IFN-gamma + T cells with inhibitory functions during induction of colitis. *Gastroenterology*. 2011.

274. Hegazy AN, Peine M, Helmstetter C, et al. Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions. *Immunity*. 2010;32:116-128.

275. King C, Tangye SG, Mackay CR. T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annu Rev Immunol*. 2008;26:741-766.

276. Reinhardt RL, Liang HE, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol*. 2009;10:385-393.

277. Nurieva RI, Chung Y, Hwang D, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity*. 2008;29:138-149.

278. Lu KT, Kanno Y, Cannons JL, et al. Functional and epigenetic studies reveal multistep differentiation and plasticity of in vitro-generated and in vivo-derived follicular T helper cells. *Immunity*. 2011;35:622-632.

279. Zaretsky AG, Taylor JJ, King IL, Marshall FA, Mohrs M, Pearce EJ. T follicular helper cells differentiate from Th2 cells in response to helminth antigens. *J Exp Med*. 2009;206:991-999.

280. Tsuji M, Komatsu N, Kawamoto S, et al. Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. *Science*. 2009;323:1488-1492.

281. Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol*. 2009;10:595-602.

282. Wohlfert EA, Grainger JR, Bouladoux N, et al. GATA3 controls Foxp3+ regulatory T cell fate during inflammation in mice. *J Clin Invest*. 2011;121:4503-4515.

283. Wang Y, Su MA, Wan YY. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. *Immunity*. 2011;35: 337-348.

284. Voo KS, Wang YH, Santori FR, et al. Identification of IL-17-producing FOXP3+regulatory T cells in humans. *Proc Natl Acad Sci U S A* . 2009;106: 4793-4798.

285. Zheng Y, Chaudhry A, Kas A, et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature*. 2009;458:351-356.

286. Chaudhry A, Rudra D, Treuting P, et al. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science*. 2009;326: 986-991.

287. Linterman MA, Pierson W, Lee SK, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med*. 2011;17: 975-982.

288. Chung Y, Tanaka S, Chu F, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med*. 2011;17:983-988.

289. Yeh WI, McWilliams IL, Harrington LE. Autoreactive Tbet-positive CD4 T cells develop independent of classic Th1 cytokine signaling during experimental autoimmune encephalomyelitis. *J Immunol*. 2011;187:4998-5006.

290. Wang YH, Voo KS, Liu B, et al. A novel subset of CD4(+) T(H)2 memory/effector cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic allergic asthma. *J Exp Med*. 2010;207:2479-2491.

291. Oestreich KJ, Huang AC, Weinmann AS. The lineage-defining factors T-bet and Bcl-6 collaborate to regulate Th1 gene expression patterns. *J Exp Med*. 2011;208:1001-1013.

292. Poholek AC, Hansen K, Hernandez SG, et al. In vivo regulation of Bcl6 and T follicular helper cell development. *J Immunol*. 2010;185: 313-326.

293. Eto D, Lao C, DiToro D, Barnett B, et al. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. *PLoS One*. 2011;6:e17739.

294. Mukasa R, Balasubramani A, Lee YK, et al. Epigenetic instability of cytokine and transcription factor gene loci underlies plasticity of the T helper 17 cell lineage. *Immunity*. 2010;32:616-627.

295. Bernstein BE, Kamal M, Lindblad-Toh K, et al. Genomic maps and comparative analysis of histone modifications in human and mouse. *Cell*. 2005;120:169-181.

296. Zhou VW, Goren A, Bernstein BE. Charting histone modifications and the functional organization of mammalian genomes. *Nat Rev Genet*. 2011;12:7-18.

297. Baltimore D, Boldin MP, O'Connell RM, Rao DS, Taganov KD. MicroRNAs: new regulators of immune cell development and function. *Nat Immunol*. 2008;9:839-845.

298. O'Connell RM, Rao DS, Chaudhuri AA, Baltimore D. Physiological and pathological roles for microRNAs in the immune system. *Nat Rev Immunol*. 2010;10:111-122.

299. Muljo SA, Ansel KM, Kanellopoulou C, Livingston DM, Rao A, Rajewsky K. Aberrant T cell differentiation in the absence of Dicer. *J Exp Med*. 2005;202:261-269.

300. Zhou X, Jeker LT, Fife BT, et al. Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. *J Exp Med*. 2008;205: 1983-1991.

301. Lu LF, Boldin MP, Chaudhry A, et al. Function of miR-146a in controlling Treg cell-

mediated regulation of Th1 responses. *Cell*. 2010;142:914-929.

302. Rodriguez A, Vigorito E, Clare S, et al. Requirement of bic/microRNA-155 for normal immune function. *Science*. 2007;316:608-611.

303. Thai TH, Calado DP, Casola S, et al. Regulation of the germinal center response by microRNA-155. *Science*. 2007;316:604-608.

304. Lu LF, Thai TH, Calado DP, et al. Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. *Immunity*. 2009;30:80-91.

305. Kohlhaas S, Garden OA, Scudamore C, Turner M, Okkenhaug K, Vigorito E. Cutting edge: the Foxp3 target miR-155 contributes to the development of regulatory T cells. *J Immunol*. 2009;182:2578-2582.

306. Banerjee A, Schambach F, DeJong CS, Hammond SM, Reiner SL. Micro-RNA-155 inhibits IFN-gamma signaling in CD4+ T cells. *Eur J Immunol* . 2010;40:225-231.

307. O'Connell RM, Kahn D, Gibson WS, et al. MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development. *Immunity*. 2010;33:607-619.

308. Du C, Liu C, Kang J, et al. MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis. *Nat Immunol*. 2009;10:1252-1259.

309. Steiner DF, Thomas MF, Hu JK, et al. MicroRNA-29 regulates T-box transcription factors and interferon-gamma production in helper T cells. *Immunity*. 2011;35:169-181.

310. Ma F, Xu S, Liu X, et al. The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-gamma. *Nat Immunol*. 2011;12:861-869.

311. Rossi RL, Rossetti G, Wenandy L, et al. Distinct microRNA signatures in human lymphocyte subsets and enforcement of the naive state in CD4+ T cells by the microRNA miR-125b. *Nat Immunol*. 2011;12:796-803.

312. Stittrich AB, Haftmann C, Sgouroudis E, et al. The microRNA miR-182 is induced by IL-2 and promotes clonal expansion of activated helper T lymphocytes. *Nat Immunol*. 2010;11:1057-1062.

313. Holland SM, DeLeo FR, Elloumi HZ, et al. STAT3 mutations in the hyper-IgE syndrome. *N Engl J Med* . 2007;357:1608-1619.

314. Minegishi Y, Saito M, Tsuchiya S, et al. Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. *Nature*. 2007;448:1058-1062.

315. Ma CS, Chew GY, Simpson N, et al. Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J Exp Med*. 2008;205: 1551-1557.

316. Milner JD, Brenchley JM, Laurence A, et al. Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature*. 2008;452:773-776.

317. de Beaucoudrey L, Puel A, Filipe-Santos O, et al. Mutations in STAT3 and IL12RB1 impair the development of human IL-17-producing T cells. *J Exp Med*. 2008;205:1543-1550.

318. Puel A, Cypowyj S, Bustamante J, et al. Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science*. 2011;332:65-68.

319. van de Veerdonk FL, Plantinga TS, Hoischen A, et al. STAT1 mutations in autosomal dominant chronic mucocutaneous candidiasis. *N Engl J Med* . 2011;365:54-61.

320. Liu L, Okada S, Kong XF, et al. Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis. *J Exp Med*. 2011;208:1635-1648.

321. Rosenzweig SD, Holland SM. Defects in the interferon-gamma and interleukin-12 pathways. *Immunol Rev*. 2005;203:38-47.

322. Burbelo PD, Browne SK, Sampaio EP, et al. Anti-cytokine autoantibodies are

associated with opportunistic infection in patients with thymic neoplasia. *Blood*. 2010;116:4848-4858.

323. Browne SK, Holland SM. Anti-cytokine autoantibodies explain some chronic mucocutaneous candidiasis. *Immunol Cell Biol* . 2010;88:614-615.

324. Browne SK, Holland SM. Anticytokine autoantibodies in infectious diseases: pathogenesis and mechanisms. *Lancet Infect Dis*. 2010;10:875-885.

325. Watford WT, O'Shea JJ. Autoimmunity: a case of mistaken identity. *Nature*. 2003;421:706-708.

326. Jager A, Dardalhon V, Sobel RA, Bettelli E, Kuchroo VK. Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *J Immunol* . 2009;183:7169-7177.

327. Apostolidis SA, Crispin JC, Tsokos GC. IL-17-producing T cells in lupus nephritis. *Lupus*. 2011;20:120-124.

328. Gaffen SL. The role of interleukin-17 in the pathogenesis of rheumatoid arthritis. *Curr Rheumatol Rep* . 2009;11:365-370.

329. Lowes MA, Bowcock AM, Krueger JG. Pathogenesis and therapy of psoriasis. *Nature*. 2007;445:866-873.

330. Goverman J. Autoimmune T cell responses in the central nervous system. *Nat Rev Immunol*. 2009;9:393-407.

331. Nestle FO, Kaplan DH, Barker J. Psoriasis. *N Engl J Med* . 2009;361: 496-509.

332. Sarra M, Pallone F, Macdonald TT, Monteleone G. Targeting interleukin-21 in immune-mediated pathologies. *Curr Drug Targets* . 2010;11:645-649.

333. Burton PR, Clayton DG, Cardon LR, et al. Association scan of 14,500 nonsynonymous SNPs in four diseases identifies autoimmunity variants. *Nat Genet*. 2007;39:1329-1337.

334. Danoy P, Pryce K, Hadler J, et al. Association of variants at 1q32 and STAT3 with ankylosing spondylitis suggests genetic overlap with Crohn's disease. *PLoS Genet*. 2010;6:e1001195.

335. Reveille JD, Sims AM, Danoy P, et al. Genome-wide association study of ankylosing spondylitis identifies non-MHC susceptibility loci. *Nat Genet*. 2010;42:123-127.

336. Remmers EF, Cosan F, Kirino Y, et al. Genome-wide association study identifies variants in the MHC class I, IL10, and IL23R-IL12RB2 regions associated with Behcet's disease. *Nat Genet*. 2010;42:698-702.

337. Remmers EF, Plenge RM, Lee AT, et al. STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. *N Engl J Med* . 2007;357:977-986.

338. Moraes-Vasconcelos D, Costa-Carvalho BT, Torgerson TR, Ochs HD. Primary immune deficiency disorders presenting as autoimmune diseases: IPEX and APECED. *J Clin Immunol*. 2008;28(suppl 1):S11-S19.

339. Cohen AC, Nadeau KC, Tu W, et al. Cutting edge: decreased accumulation and regulatory function of CD4+ CD25(high) T cells in human STAT5b deficiency. *J Immunol* . 2006;177:2770-2774.

340. Laws PM, Warren RB. Ustekinumab for the treatment of psoriasis. *Expert Rev Clin Immunol*. 2011;7:155-164.

341. Segal BM, Constantinescu CS, Raychaudhuri A, Kim L, Fidelus-Gort R, Kasper LH. Repeated subcutaneous injections of IL12/23 p40 neutralising antibody, ustekinumab, in patients with relapsing-remitting multiple sclerosis: a phase II, double-blind, placebo-controlled, randomised, dose-ranging study. *Lancet Neurol* . 2008;7:796-804.

342. Steinman L. Mixed results with modulation of TH-17 cells in human autoimmune diseases. *Nat Immunol* . 2010;11:41-44.

343. Hueber W, Patel DD, Dryja T, et al. Effects of AIN457, a fully human antibody to

interleukin-17A, on psoriasis, rheumatoid arthritis, and uveitis. *Sci Transl Med*. 2010;2:52ra72.

344. Huh JR, Leung MW, Huang P, et al. Digoxin and its derivatives suppress TH17 cell differentiation by antagonizing RORgammat activity. *Nature*. 2011;472:486-490.

345. Souwer Y, Szegedi K, Kapsenberg ML, de Jong EC. IL-17 and IL-22 in atopic allergic disease. *Curr Opin Immunol* . 2010;22:821-826.

346. Molet S, Hamid Q, Davoine F, et al. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol* . 2001;108:430-438.

347. Chakir J, Shannon J, Molet S, et al. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol* . 2003;111:1293-1298.

348. Wang YH, Wills-Karp M. The potential role of interleukin-17 in severe asthma. *Curr Allergy Asthma Rep*. 2011;11:388-394.

349. Soroosh P, Doherty TA. Th9 and allergic disease. *Immunology*. 2009;127:450-458.

350. Jin H, Oyoshi MK, Le Y, et al. IL-21R is essential for epicutaneous sensitization and allergic skin inflammation in humans and mice. *J Clin Invest*. 2009;119:47-60.

351. Pesce J, Kaviratne M, Ramalingam TR, et al. The IL-21 receptor augments Th2 effector function and alternative macrophage activation. *J Clin Invest*. 2006;116:2044-2055.

352. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science*. 2011;331:1565-1570.

353. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. Natural innate and adaptive immunity to cancer. *Annu Rev Immunol* . 2011;29: 235-271.

354. Martin-Orozco N, Muranski P, Chung Y, et al. T helper 17 cells promote cytotoxic T cell activation in tumor immunity. *Immunity*. 2009;31:787-798.

355. Zou W, Restifo NP. T(H)17 cells in tumour immunity and immunotherapy. *Nat Rev Immunol*. 2010;10:248-256.

356. Murugaiyan G, Saha B. Protumor vs antitumor functions of IL-17. *J Immunol* . 2009;183:4169-4175.

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Chapter 30

Programmed Cell Death

Andrew L. Snow

Michael J. Lenardo

INTRODUCTION

Nontransformed cells have a finite lifespan. After a characteristic number of divisions, cells generally undergo senescence, stop dividing, and subsequently die. Considering adult vertebrates, and their internal organs, generally stay a constant size, a form of homeostasis is required.[1] In certain organs, this involves a constantly fluctuating dynamic equilibrium of cell proliferation and death. A prime example is the immune system, which employs cell renewal, expansion, and elimination in carrying out its functions.[2,3,4,5] These changes can be systemic or localized to anatomic sites proximate to an antigen stimulus, and may affect specific subsets of immune cells as dictated by the conditions.

Programmed cell death (PCD) denotes a set of internal biochemical mechanisms that cause specific cells to die under defined conditions that are typically advantageous to the organism.[6] Reasons for cell elimination include cell excess, improper cell differentiation, cell transformation, genetic damage to cells, and infection. In addition to this operational definition, considering a death event “programmed” usually means that one or more genes are required. This chapter has been organized to start with a broad overview of general immunoregulatory principles and then cover details of the molecules involved in PCD in specific sections. Although there are conceptual antecedents from the 19th century, the selection of cells for survival or death by specific external stimuli was introduced by Levi-Montalcini for neural cells in the 1940s and later by Burnet for lymphocytes. Investigation into the molecular mechanisms of PCD began in the mid-1980s and accelerated rapidly. Deoxyribonucleic acid (DNA) sequence databases permitted the rapid identification molecules involved in PCD.[7] Although PCD is a large and contentious area of cell biology research, molecular advances have established a firm and tractable theoretical foundation. Remarkably, much of what we will discuss was almost completely unknown two decades ago. Yet these pathways are at work every day in our bodies to control responses to infectious agents, establish cellular homeostasis, prevent autoimmunity, and avert lymphoid malignancies.

OVERVIEW: CELLULAR HOMEOSTASIS AND PROGRAMMED CELL DEATH IN MULTICELLULAR ORGANISMS

Internal programs of death exist in all mammalian cells that likely require constant and active

suppression.[8] For experimental investigation, it is thus important to discriminate between accidental cell death and that initiated or "programmed" by an internal genetically determined biochemical mechanism. The term "*apoptosis*," a Greek word meaning "falling off," as in leaves from a tree, was introduced in 1972 by Andrew Wyllie and coworkers to describe the normal, presumably programmed, attrition of cells.[9] It was defined microscopically as cell shrinkage with nuclear and cytoplasmic condensation within a cell membrane that forms blebs but stays intact until late in the death process[10] (Fig. 30.1). This cell phenotype has long been associated with cell death.[11] Apoptosis is now mainly identified by the biochemical effects of the caspase family of proteases.[12] Caspases are important in two respects. First, they are a feature of most, if not all, apoptosis pathways. Second, once highly activated, they usually represent a commitment to apoptosis that is not reversible, although we now recognize that low-level caspase activation participates in lymphocyte activation.[13,14] For these reasons, caspases have been regarded as a final common pathway of apoptosis. In fact, the concept of PCD was significantly illuminated by the identification of caspases and other molecules that regulate these internal biochemical death pathways. In general, the molecular components of the death mechanism are preassembled and available without new gene transcription or protein synthesis.[6] For example, caspases are constitutively expressed in the cytoplasm of the cell as zymogens. Once proteolytically activated, caspases carry out specific protein cleavages leading to the morphologic changes of apoptosis. The proteolytic substrates of caspases are highly selective because most proteins remain uncleaved. A panoply of molecular events entrained to caspases includes cleavage of chromosomal DNA, nuclear chromatin condensation, exposure of phosphatidylserine on the exterior of the cell membrane, proteolysis of specific proteins including other caspases, and mitochondrial changes. These processes are detectable by simple assays in tissue culture cells in vitro or, in some cases, in vivo. Protocols for these assays have been well described.[15,16]

Cells that die without the characteristics of apoptosis typically undergo what is usually called *necrosis*. While necrosis is often associated with accidental cell death such as physical or chemical trauma, it may also result from programmed mechanisms. In certain cases, nonapoptotic mechanisms of cell death can be promoted by caspase inhibition.[17] The appearance of a necrotic cell differs dramatically from that of an apoptotic cell. Necrotic cells swell and lose the integrity of internal organelles with early plasma

membrane breakdown, giving an enlarged "fractured" appearance under the microscope (see Fig. 30.1D). Recently, various research groups have begun to define molecular programs resulting in necrotic death.[18] Several varieties of "programmed" necrosis that share the hallmarks of early loss of plasma membrane integrity and excessive reactive oxygen species (ROS) have been described. Programmed necrosis that involves receptor-interacting protein (RIP) serine/threonine kinases has been well documented in lymphocytes, especially under conditions of caspase inhibition.[17,18] A closely related variant of this cell death program termed "necroptosis" is inhibitable by necrostatin, a compound that inhibits RIP kinases.[19] Finally, the induction of autophagy has been genetically linked to a necrotic program of death.[20] Autophagy is induced by starvation or other stimuli and involves internal membrane rearrangement leading to engulfment of portions of the cytoplasm, which are then degraded

by fusion with lysosomes. Under death conditions, this mechanism leads to membrane damage and necrosis by ROS, which overaccumulate because of accelerated catalase degradation.[21] The precise role of these necrosis programs, especially autophagic cell death, in immune function has not yet been worked out. However, they are often provoked by the inhibition of caspase-dependent apoptosis. Caspase inhibition, perhaps by blocking constitutive low-level caspase activity, can actually trigger these necrotic death programs.[20]

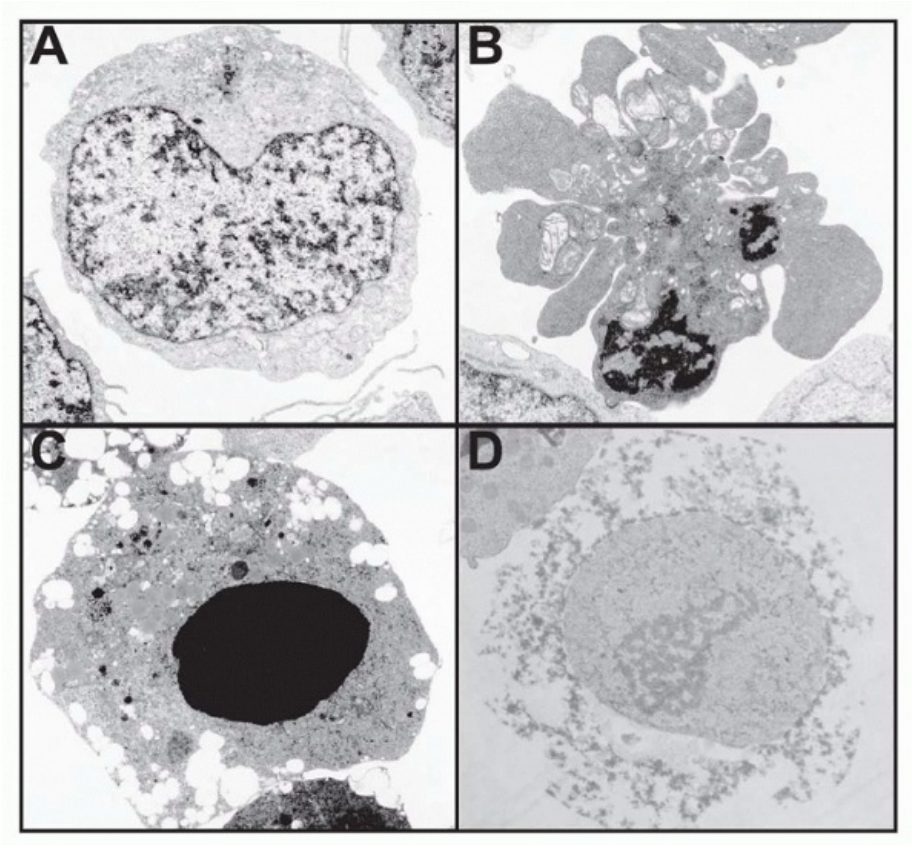

FIG. 30.1. Electron Microscopy Elucidates the Morphology of Different Forms of Programmed Cell Death. A: A normal, unstimulated Jurkat leukemia cell. **B:** Jurkat cell undergoing apoptosis in response to Fas receptor stimulation. Notice the condensed chromatin and the blebbing with maintenance of plasma membrane integrity. **C:** Jurkat cell undergoing apoptosis in response to staurosporine treatment. Note the prominent nuclear condensation. **D:** Necrosis of human peripheral blood T-lymphocyte infected with the human immunodeficiency virus. Notice the general loss of cellular integrity as well as the lack of chromatin condensation in the dying cell. (Figure courtesy of Diane Bolton and Jan Orenstein.)

Thus, it is important to distinguish between “programmed” necrosis and necrosis due to accidental causes as these involve very different molecular events. It has been generally argued that apoptosis, which preserves membrane integrity, prevents inflammation from released cellular contents, whereas necrosis results in total cellular breakdown and content release, which causes an inflammatory response.[22] Recent evidence suggests this paradigm is oversimplified and that the immunogenicity of a dying cell varies with cell type and

activation status, the nature of the phagocytic cell that engulfs it, and/or the specific death stimulus. The release of inflammatory damage-associated molecular patterns that may accompany apoptosis or necrosis of stressed cells (eg, heat shock proteins, high-mobility group box 1, oligonucleotides, uric acid) may be particularly important for invoking a strong immune response.[23] These distinct immunologic effects due to apoptosis and necrosis remain the subject of continued experimental exploration.

As we will argue in the following, the necessity of cellular homeostasis as well as the acute need to eliminate cells that are harmful or nonfunctional led to the early emergence of conserved cell death mechanisms during evolution.[11]. Work by Horvitz and colleagues genetically identified several molecules essential for the death of specific cells during the development of the roundworm *Caenorhabditis elegans* that have subsequently been found to be homologues for mammalian PCD genes.[24] It is clear from this simplified system that the molecular logic of one form of cell death was likely established early in evolving multicellular organisms.[25] PCD mechanisms are now evident in most contemporary multicellular organisms from plants to humans, although it is not clear whether convergent evolution or conservation of function is responsible. However, the molecular pathways in worms and other simple organisms are rudimentary compared with the complexity found in mammalian PCD systems. To understand how these mechanisms contribute to immunity, we focus on mice and humans, which are the subjects of most immunologic research. Also, while we focus mainly on research carried out on B- and T-lymphocytes, similar themes and mechanisms govern death programs for other hematopoietic cells including dendritic cells (DCs), macrophages, natural killer cells, granulocytes, and mast cells, among others.

PROGRAMMED CELL DEATH AND IMMUNE REGULATION

The fundamental unit of immune responsiveness is the cell. Lymphocytes, the major adaptive immune cells, are distinguished by expressing a unique clonotypic antigen receptor. A large number of lymphocytes with different receptor specificities are generated during ontogeny, providing a diverse “immune repertoire,” and these cells can be programmed to expand or die throughout the life of the organism. Somatic hypermutation during B-cell expansion in the germinal center can expand the repertoire. Although the level of antigen presentation, the degree of lymphocyte responsiveness (versus unresponsiveness or anergy), and other factors also play important roles, the presence or absence of cells with

specific recognition properties at any given time is a primary determinant of the quantitative response to any antigenic stimulus. The homeostasis of major lymphocyte populations is independently regulated such that deficits in B cells, T cells, or even major T-cell subpopulations (αβ versus γδ or cluster of differentiation [CD]4 versus CD8) may qualitatively alter but do not prevent the normal homeostasis of the remaining populations. During thymic development, lymphocytes respond to the antigenic environment with either survival or death.[26] Developmental PCD eliminates lymphocytes that cannot recognize antigen appropriately or have potentially dangerous self-reactivity.[26,27] In the mature immune system, death is principally a negative feedback response that counterbalances proliferative responses to antigen.[28] Although the clonal selection theory of F.M. Burnet encompassed clonal elimination during ontogeny, it allowed only selective expansion of antigen-stimulated

lymphocytes in the mature immune system. However, antigen-specific regulation of mature lymphocyte survival also powerfully controls immune responses and tolerance.[4,28,29] Because the organism encounters an unpredictable universe of antigens in a lifetime, it is essential that there is feedback regulation of adaptive immune responses. Feedback is an essential element of any dynamic system in which final outcomes cannot be predicted from the starting conditions.[30] To achieve measured immune responses, proliferation and death are coordinated by feedback regulation to control the number of responsive immune cells. Feedback death mechanisms can also eliminate potentially harmful specificities that increase unexpectedly during immune reactions. Hence, homeostasis of both lymphocyte numbers and reactivities can be continuously maintained.

Thymic Deletion: Positive and Negative Selection

Thymic selection represents an intriguing example of apoptosis in which the same receptor —the clonotypic T-cell receptor (TCR)—can lead to diametrically opposite outcomes depending on the level of stimulation.[26,27] During development, when thymocytes (ie, T-cell progenitors in the thymus) express the TCR and both the CD4 and CD8 coreceptors (the "double positive" stage), thymocytes will undergo apoptosis if they receive no TCR stimulation. This process, called "death by neglect," will eliminate thymocytes that have not productively rearranged the TCR genes or have no capacity to recognize antigen in the context of self-major histocompatibility complex.[31] "Low-level" stimulation of the TCR antagonizes death by neglect. This protective event insures major histocompatibility complex-specific antigen recognition by T cells and is called *positive selection*.[32] While weak TCR signals can deliver an antiapoptotic stimulus, strong TCR engagement of double positive thymocytes delivers a proapoptotic signal. This event, termed *negative selection*, prevents the emergence of strongly autoreactive lymphocytes from the thymus.[31] This deletion step is a major mechanism of central tolerance and the prevention of autoimmunity.[33] These processes of selection employ caspase-dependent apoptosis and rapid phagocytosis of the dead thymocytes.[34,35] Hence, thymocytes travel a narrow bridge of TCR avidity during development and will die if they deviate from it.

The differences between the neglect (or null), weak, and strong signals that result in life or death appear to be determined at early stages of TCR signaling.[36,37] Death receptors (DRs; see the following discussion) appear not to be crucial; instead, there is a direct connection of the TCR signaling apparatus to mitochondrial death pathways.[38,39,40,41] Though there is not complete certainty how the TCR dictates life or death at specific antigen levels, the answer to this puzzle will almost certainly reside in the complex signal pathways emanating from this receptor. TCR engagement that causes transient induction of the Erk kinase is associated with positive selection, whereas slow but constant Erk activity is associated with negative selection.[42,43,44] Other distinctions in TCR signaling have been identified. Signaling through phosphotidylinositol-3 kinase, the antiapoptotic Akt kinase, and the retinoid orphan receptor-gamma may promote thymocyte survival.[36,45] Genetically modified mice have revealed that several transcription factors, such as E2A, Nur77, Id3, and IRF-1, can affect thymic cellularity, indicating that differential signaling may trigger transcriptional events that regulate cell survival.[46,47,48,49,50] Finally, Andreas Strasser has emphasized that

various forms of physiologic cell death are likely to involve the subset of BH3-only proteins in the Bcl-2 family.[51,52] The activation of proapoptotic BH3-only proteins, such as Bak, Bid, and especially Bim, by TCR signals initiates mitochondrial apoptosis during thymic selection processes. Thus, early TCR-induced signaling differences directly entrain transcriptional events that modulate BH3-only regulators of mitochondrial apoptosis. These distinct signals are tied to specific microenvironments within the thymus in which positive and negative selection occur.[53] Hence, by intracellular communication with distinct apoptosis regulatory molecules, the TCR has a remarkable ability to signal life or death by the apparent strength of stimulus it receives.

Programmed Cell Death and the Homeostasis of Peripheral T Cells

Death of peripheral T cells differs markedly from thymocyte selection in that PCD of mature lymphocytes occurs mainly in cells that have proven usefulness (ie, they have been already selected in the thymus and activated by antigen). This is because PCD of mature T cells is employed primarily to counter antigen-driven proliferation of activated T cells, including those that could be autoreactive. In general, most naïve lymphocytes survive and circulate in the body in a resting state (G_0). The survival of such resting T cells is constitutively maintained by the presence of contact with major histocompatibility complex, the cytokine interleukin (IL)-7, and expression of the antiapoptotic protein Bcl-2.[33,54,55,56] During an active immune response, T-lymphocyte proliferation can involve as much as a 10,000-fold expansion within days. Such explosive proliferation is necessitated by the extraordinarily rapid propagation of microbial pathogens. However, these activated and cycling T cells are potentially damaging due to toxic effector functions and potential

cross-reactivity with self-antigens. Activated T-cell expansion does not go unchecked and is subject to negative feedback in the form of cell death. However, because immune responses are directed at specific antigens, they must be independently regulated because some responses might expand while others contract. The immune system has developed propriocidal mechanisms to control independent populations of activated T cells. *Propriocidal regulation* refers to the various negative feedback death mechanisms that maintain homeostasis of mature peripheral T cells in an antigen-specific manner. These potently restrain antigen-activated T cells and tightly control lymphocyte numbers during and at the conclusion of immune responses.

Propriocidal regulation of T cells is triggered by remarkably simple attributes of T-cell activation: the level of cell cycling and the level of antigen restimulation.[28,54] These two features are ideally suited for triggering negative feedback death because they provide both a “sensing” mechanism for the level of active T-cell proliferation and a negative response mechanism sensitive to the level of antigen stimulation. There are essentially two different mechanisms: 1) active or antigen-stimulated PCD and 2) passive or lymphokine withdrawal PCD (Fig. 30.2).[4,57,58] These have different roles and occur at different times, as will be described in detail in the following. In some respects, it is paradoxical that lymphocytes that respond well to foreign antigen and presumably could have protective value are actively eliminated. However, it is vital to constrain the number of activated T cells to prevent unhealthy effector or autoimmune reactions. During a robust immune response, lymphocytes that cross-react with self-antigens may also proliferate. The propriocidal mechanisms that

cause these cells to die upon encountering self-antigens could be an important mechanism of preserving self-tolerance. By this formulation, tolerance is a quantitative effect that is due to the low number of significantly self-reactive lymphocytes in the naïve organism. Clonal expansion during immune responses can unleash dormant or infrequent self-reactive clones, called “forbidden clones” by Burnet, creating an autoimmune diathesis. Propriocidal death reduces these clones and thereby promotes tolerance. Active antigeninduced propriocidal death, which is induced by high or repeated doses of antigen, is especially well suited for the elimination of self-reactive clones because self-antigen is likely to be present in continuous and potentially high amounts.[28,54]

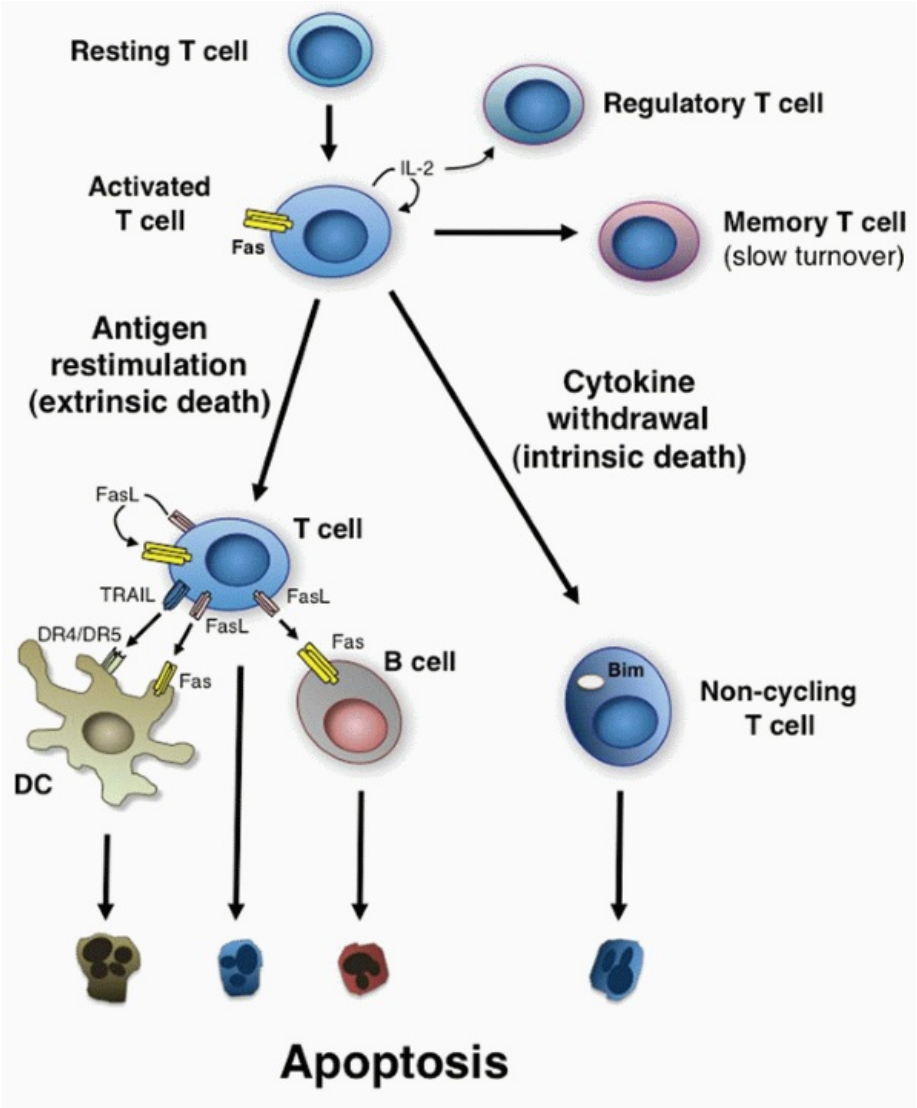

FIG. 30.2. Propriocidal Regulation of Immune Cells. Shown are the apoptosis pathways that govern T-lymphocyte homeostasis by the antigen-restimulated (extrinsic death) and no antigen (cytokine withdrawal [intrinsic death]) pathways of apoptosis. Also shown is the regulation of B cells by FasL expressed by T cells, dendritic cells by Fas ligand/tumor necrosis factor-related apoptosis-inducing ligand expressed by T cells, and T cells by regulatory T cells competing for cytokines.

Extrinsic or Antigen-stimulated T-Lymphocyte Death

The extrinsic death mechanism involves apoptosis of mature T cells in response to antigen stimulation. This requires the T cells to be activated and cycling at the time that they undergo strong antigenic restimulation.[28,54] The death is indirect in the sense that it requires the antigen-induced secretion of death ligands that engage specific apoptosisinducing DRs in the tumor necrosis factor (TNF) receptor (TNFR) and ligand superfamilies (see Fig. 30.2).[59,60] Current evidence suggests that Fas ligand (FasL) as well as TNF are the key death ligands that mediate this process in mature CD4+ and CD8+ T cells.[4,5,61] However, DR-independent apoptosis mediators also contribute in specific contexts. For example, Th2-differentiated CD4+ cells are less sensitive than their Th1-differentiated counterparts to Fas-mediated killing.[62] Instead, they preferentially die through a suicidal pathway that triggers internal release of granzyme B.[63,64] Nagata originally observed that the lymphoproliferative and autoimmune phenotype of *lpr* mice was due to genetic alterations of Fas and a similar disease was due to a mutation in FasL.[65,66] Defects in Fas (CD95) or FasL in mice and humans cause severe derangements of lymphocyte homeostasis and tolerance, which will be detailed subsequently. However, activated Fas-deficient T cells can be induced to die through other apoptotic signals, including cytotoxic granules containing perforin and granzymes, and/or TCR-induced upregulation of Bim. These alternative mechanisms may be particularly relevant to clearance of activated CD8+ T cells.[67,68,69,70,71,72,73] Often, these forms of death are called “activation-induced cell death,” but this is a misnomer.[74] Activation-induced cell death has been used to describe any form of death of activated T cells, thus causing confusion among investigators working on molecularly distinct death pathways.[4,75] Activation per se

does not directly cause cell death; instead, extrinsic death induction requires antigenic restimulation of activated T cells (ie, TCR reengagement after initial activation). For resting T cells, antigen encounter under costimulatory conditions leads to activation with very little cell death. Obviously, if the initial activation directly induced death, this would preclude immune responses. On the contrary, activated, cycling T cells do not spontaneously die by FasL or TNF unless they are strongly restimulated by antigen in the activated state, which causes upregulation of the genes for these death cytokines and responsiveness to them.[76] For these reasons, we prefer to use the term “restimulation-induced cell death” (RICD) to clarify this important distinction.[77,78]

Extrinsic death is a negative feedback mechanism, which explains why it is triggered by lymphokine-induced cell cycling (usually caused by IL-2) and reengagement of the TCR. IL-2-induced cell cycling indicates that there has been a productive antigen response and the T cells are multiplying. Because T cells can expand rapidly to great numbers, a large fraction of cycling T cells dictates a need to downregulate the response to any further antigen exposure. The presence of repeated or continuously high amounts of antigen would be a powerful stimulus to greater proliferation. Under these conditions, the system programs a fraction of the restimulated cells to undergo apoptosis via RICD. RICD sensitivity is dictated in part by the relative strength of the TCR restimulation signal, which must meet a certain “threshold” to fully activate proximal signaling proteins like CD3ζ and induce sufficient expression of

proapoptotic molecules.[79,80] It remains unclear how this apoptosis threshold is calibrated in any given T cell, although additional signals provided through certain signaling lymphocyte activation molecule receptors and the small SH2 adaptor signaling lymphocytic activation molecule-associated protein appear critical for potentiating TCR signal strength in RICD.[81] Another key determinant of death in this context is T-cell phenotype. Specifically, among CD4+ T cells, those with an effector memory phenotype are highly sensitive to Fas- and TCR-induced apoptosis, whereas central memory and activated naïve CD4+ T cells are comparatively resistant.[82] The molecular basis of this sensitivity to Fas-induced apoptosis depends on the enrichment of Fas in lipid raft microdomains in the plasma membrane of effector memory T cells, which leads to more effective nucleation of the Fas signaling complex. Thus, Fas-/TCR-induced apoptosis can specifically cull extraneous effector memory T cells without diminishing subsequent immune responses by a simple and specific feedback loop. Like most negative feedback systems, the propriocidal response directly reverses the ongoing process of proliferation by eliminating activated T cells. Antigeninduced death provides an explanation for many historical observations in the literature that show that reapplication or continuous presence of high concentrations of antigen can lead to specific suppression rather than augmentation of an immune response.[54]

Sensitivity to this extrinsic death mechanism is also directly connected to the effect of IL-2 in inducing cell cycle progression into late G_1 or S phase, which confers susceptibility to death.[83,84,85] The requirement for cell cycle progression has not been fully explained but appears to be necessary for apoptosis induced by TCR engagement or with direct Fas stimulation. Other cytokines that augment T-cell cycling, such as IL-4, IL-7, and IL-15, can also promote cell death to some degree but none with the potency of IL-2.[76] Hence, the theory of propriocidal regulation advanced the concept that IL-2 would have an important regulatory role in the elimination of activated T cells in addition to its previously known role in lymphocyte proliferation.[54] This concept was later validated when genetic deficiencies of IL-2 and IL-2 receptor in mice were found to have defective apoptosis of activated T cells and autoimmunity.[86,87] Although the immunopathology in these animals is also attributed to loss of T regulatory (T_{reg}) cells, the latter constitutes another form of polyclonal deletion by cytokine competition, which we explain in detail subsequently. This surprising property of IL-2 is important to consider in the use of IL-2 as a therapeutic agent or vaccine adjuvant. It also underscores an important feature of feedback regulation: that to achieve a maximal response for, say, a vaccine, more stimulation is not necessarily better. In a variety of test situations, extrinsic antigen-induced death decreases the number of T cells but does not completely eliminate the T-cell immune response.[28,29] In certain extraordinary conditions, such as high levels of a noncytopathic or chronic virus, essentially all responding T cells can be eliminated.[88] FasL expressed on T cells also causes the death of B cells, which do not themselves express FasL.[89] This causes a parallel regulation of B-cell proliferation by Fas-induced death that can be antagonized by B-cell receptor engagement (see Fig. 30.2). Antigen-induced death therefore provides a mechanism to eliminate specific antigen-reactive lymphocytes under chronic stimulatory conditions when they might cause the host more harm than good.

Antigen-induced expression of death ligands and other proapoptotic molecules shunts a

proportionate fraction of the antigen-specific activated cells, but not bystander cells, into the death pathway. The activated T cells still carry out their effector function when restimulated by antigen, but their ultimate fate is death instead of proliferation.[76,88] Because the primary agents of T-cell death, Fas and other TNFRs, have no inherent antigen specificity, it is important to consider how the clonal specificity of antigen-induced apoptosis is achieved.[28] For the death of activated T cells, the simple engagement of Fas by its ligand is insufficient.[90,91] Efficient death induction also requires a "competency" signal from the TCR delivered at the same time FasL binds Fas.[90,92] However, these signals do not require new protein synthesis and are delivered rapidly in a few hours or less.[90,91] The requirement for simultaneous engagement of Fas and TCR plays a critical role in establishing the antigen specificity of death. For example, it was shown that TCR stimulation of a specific subpopulation within a pool of Fas-expressing T-cell blasts, such as with agonistic anti-Vβ8 antibody, leads only to the death of that subpopulation despite the apparent exposure of other subtypes of T cells to the Vβ8-expressing cells that have been induced to express FasL.[28,90] Also, deletion in vivo is antigen-specific.[93]

The molecular nature of the competency signal is presently unknown.

Intrinsic Growth Factor Withdrawal T-Cell Death

As important as it is to avert cellular overexpansion during an immune response through extrinsic, TCR-induced death, it is equally important for the immune system to downmodulate the immune reaction after successful elimination of the pathogen. Lymphokine withdrawal death is a form of T-cell apoptosis that occurs naturally at the end of an immune response when the accumulation of effector cells becomes unnecessary and potentially damaging.[94] When the trophic cytokine for activated T cells, typically IL-2, decreases because of reduced antigen stimulation, the excess T cells undergo apoptosis.[95] This form of negative feedback death may affect specific classes of T cells, for example, "effector" versus "memory" cells, though this distinction may be difficult to discern experimentally. As most T cells in the expanded population are antigen-specific, this represents clonotype-specific T-cell propriocidal regulation controlled by antigen and cell cycle progression. The activated cells can "sense" decreased antigen drive and decreased trophic cytokine, which programs them for apoptosis. The elimination of the expanded pool of activated cells, except for a small amount of memory cells, reestablishes homeostasis in T-cell numbers. It has been shown that if IL-2 is exogenously delivered during a proliferative response to superantigen, the reactive T cells persist as long as lymphokine is provided.[96] Therefore, the lack of IL-2 is a key element in the feedback regulation of the cellular response. Antigen and IL-2 therefore mediate propriocidal regulation, in the midst of an immune response and at its conclusion to reduce T-cell numbers.

Genetic studies reveal that the molecular mechanism of lymphokine withdrawal death is different than antigen-induced apoptosis.[57,58] Although this event is often confused with extrinsic cell death mediated by Fas, DRs are not involved. Rather, cytokine withdrawal for as little as 2 to 4 hours commits the cell to a death pathway requiring new protein synthesis.[97] Apoptosis is initiated through the mitochondrial pathway and is orchestrated by the Bcl-2

family of proteins. In fact, the ratio between the pro- and the antiapoptotic Bcl-2 family members is believed to determine the fate of the cells. In the presence of growth factors, the antiapoptotic Bcl-2, Bcl-X_L, and Mcl-1 proteins maintain viability via retrotranslocation of the executioner molecules Bax and Bak from the mitochondria into the cytosol, precluding oligomerization and permeabilization of the mitochondrial outer membrane (see following section).[98] Proapoptotic, "BH3-only" activators like Bim and Bid can bind to antiapoptotic Bcl-2 family proteins and disrupt this process to allow Bax-/Bak-mediated mitochondria depolarization. Bim is an essential regulator upstream of Bax and Bak during cytokine withdrawal-induced apoptosis in lymphocytes.[99] CD4+ and CD8+ T cells from mice homozygously deficient for Bim survive much longer than their wild-type counterparts following in vitro and in vivo activation with the superantigen *Staphylococcus aureus* enterotoxin B.[100] Moreover, Bim is critical for cell death of T cells induced by both IL-2 and IL-7 withdrawal.[51,100,101] Bim dysregulation also underlies the overaccumulation of lymphocytes due to failed cytokine deprivation apoptosis in the lymphoproliferation disorder caused by activating mutations of Ras.[102] Cytokine deprivation rapidly upregulates Bim messenger ribonucleic acid levels through activation of the forkheadlike transcription factor FOXO3A. Bim is also subject to sophisticated posttranscriptional regulation in T cells. For example, removal of IL-2 shuts down the Ras-ERK signaling cascade emanating from the IL-2 receptor, which normally promotes Bim protein degradation and destabilization of Bim messenger ribonucleic acid.[103,104] PUMA, another BH3-only protein, functions synergistically with Bim downstream of FOXO3A and accounts for Bim-independent death.[105,106] When the trophic cytokine is present, the Ser/Thr kinase Akt also phosphorylates key substrates and prevents mitochondrial collapse. For example, Akt suppresses FOXO3A activity. Akt also phosphorylates the pro-apoptotic Bad protein, which is then sequestered by the 14-3-3 scaffold protein.[107,108] Another target inactivated by Akt is glycogen synthase kinase-3, which phosphorylates Mcl-1 on residue S159 when cytokines are removed. The rapid degradation of phosphorylated Mcl-1 by the ubiquitin-proteasome pathway disrupts the fragile equilibrium between the pro- and the antiapoptotic Bcl-2 family members, and promotes cell death.[109] Thus, Akt can preserve viability by targeting appropriate Bcl-2 family members. Ultimately, the relative balance of proapoptotic (Bim, Puma) versus antiapoptotic (Bcl-2, Mcl-1, etc.) proteins expressed regulates mitochondrial stability and apoptosis sensitivity in response to cytokine withdrawal.

T Regulatory Cells

Research on the intriguing subset of CD4+ T cells known as T_{reg} cells, and their role in immune tolerance, continues to expand vigorously. These cells express the forkhead box P3 transcription factor and suppress the expression of many TCR-induced genes including cytokines and cytokine receptors.[110,111] Similar to other subsets of T cells in their "activated" state, T_{reg}s require common gamma chain cytokines for survival. In particular, they characteristically express the IL-2 alpha chain receptor (CD25) and require IL-2 to persist in the periphery.[112] IL-7 can also act as a survival factor for T_{reg} cells.[113] The mechanisms by which T_{reg} cells "suppress" immune responses have remained somewhat obscure. However, recent evidence suggests that the direct T_{reg}-mediated suppression of

other T cells can be attributed in large part to their large appetite for survival cytokines, which T_{reg}s themselves cannot produce.[114,115] T_{reg}s absorb significant amounts of trophic cytokines like IL-2 at a site of an immune reaction, which induces Bim-dependent, intrinsic lymphokine withdrawal death of nearby activated and cytokine-requiring T cells.[116] Consistent with historical observations, this phenomenon is not antigen-specific but requires T_{reg}s to be in close proximity to IL-2-producing T cells to effectively consume the cytokine and preclude autocrine/paracrine signaling in

targeted responder T cells.[115,117] T_{reg}s can also consume other common gamma chain cytokines involved in lymphocyte survival and effector function, such as IL-4, IL-7, IL-15, and IL-21.[118] Therefore, this form of T_{reg}-mediated polyclonal deletion constitutes a form of dominant tolerance dependent on intrinsic PCD. This mechanism allows a productive immune response to unfold while the ratio of effector T cells to T_{reg}s remains high. Subsequent IL-2-mediated expansion of T_{reg}s then establishes a natural brake on effector cell expansion via competition for available cytokines and culling of excess effectors deprived of IL-2. As polyclonal deletion is enforced, T_{reg}s may also utilize alternative strategies for functional suppression of remaining responder T cells or antigen-presenting cells, including the production of suppressive cytokines (eg, transforming growth factor-β, IL-10) and cytotoxic T-lymphocyte antigen-4 effects on costimulatory molecules (CD80, CD86).[119]

T-Cell Memory

T cells, once activated, can persist as “memory” cells. One view is that this process involves an escape from propriocidal mechanisms of apoptosis.[35,120,121,122] Increasing evidence supports the concept that memory is due to the long-term survival of antigen-specific T cells even without further antigen exposure. Several means to achieve such survival are possible. To escape killing by Fas and other death receptors, T cells could express cellular FLICE inhibitory protein (FLIP), which is a homologue of caspase-8 and -10 located in the same genetic locus as these caspases that has no enzymatic function but can interpose itself into the death receptor complex and block caspase activation.[123] This type of inhibition has been demonstrated in B cells by the ability of B-cell receptor (BCR) stimulation, which upregulates cellular FLIP,[124,125] to block Fas killing.[126] Specialized T-cell subsets, including proinflammatory Th17 cells, may escape Fas-mediated RICD through cellular FLIP overexpression.[127] Various “inhibitor of apoptosis proteins” (IAPs) might also interfere with DR-mediated killing.[128,129] Furthermore, the mitochondrial death pathway could be attenuated by upregulation of Bcl-2 and Bcl-X_L. These antiapoptotic molecules, which are upregulated in CD8+ and CD4+ memory cells,[122,130] have been shown to block lymphokine withdrawal apoptosis.[131] The necessity of these inhibitory molecules for the persistence of a memory population of T cells is unknown. Moreover, it is mainly the central memory rather than effector memory T cells that become refractory to RICD.[82]

Another view is that long-term survival in a nonproliferative “resting” state characteristic of “virgin” (naïve) T cells is never reestablished by memory cells. Work by Phillipa Marrack, Rafi Ahmed, and others reveals a surprisingly dynamic T-cell memory pool. Their study proposes

a constant, low-level proliferation of memory CD8+ T cells that is antigen-dependent and maintained by IL-15 and to a lesser extent by IL-7.[132,133,134] Also, the fraction of memory cells remains constant over time, indicating that death is continuously maintaining a balance. Hence, the balance of slow proliferation and slow death ensures memory cell maintenance. As the molecular mechanism of the memory state is further elucidated, the differing views of "memory" are likely to be reconciled.

The mitochondrial pathway of death is likely to play a prominent role in the generation of memory cells. In addition to their proliferative effects, the cytokines IL-15 and IL-7 appear to promote survival of memory cells through their induction of antiapoptotic molecules such as Bcl-2.[135,136,137] This countervailing effect is consistent with a recently demonstrated Bim-dependent mechanism for limiting memory CD8+ T cells.[138] In addition, a Bim-independent mechanism for expunging memory T cells was shown.[138] The nonmitochondrial pathways of death contributing to memory cell generation may involve death receptors. For instance, CD4+ T-cell help is required for CD8+ T-cell memory. In the absence of CD4+ help, TNF-related apoptosis-inducing ligand (TRAIL) is expressed on and mediates apoptosis of CD8+ cells upon antigen restimulation.[139] TRAIL deficiency, however, only delays the loss of CD8+ memory cells, revealing that non-TRAIL mechanisms also contribute to the homeostasis of CD8+ memory cells.[140] Further studies are needed to dissect the PCD mechanisms contributing to T memory cell generation and homeostasis.

B-Cell Homeostasis

We have focused most of our attention on T-lymphocyte apoptosis thus far because it has received the greatest experimental examination and more details are known. However, PCD also governs B-cell homeostasis and is regulated in ways that have similarities and differences with T cells. Both DR triggering and withdrawal of trophic stimuli contribute to B-cell elimination. Similar to T cells, B cells developing in the bone marrow undergo a series of proliferative expansion and apoptotic contraction events to shape the final B-cell repertoire.[141] Immunoglobulin (Ig) gene rearrangement starting at the pro-B-cell stage generates the BCR. The cytokine IL-7, which promotes the survival of developing thymocytes,[142,143] is also crucial for sustaining survival at the pro-B/pre-B juncture.[144,145] IL-7 induces transcription of the antiapoptotic molecule Mcl-1, which selectively binds to and antagonizes the proapoptotic Bcl-2 family member Bim.[146,147] Then selection for B cells occurs in two developmental steps with successive gene rearrangements at the heavy and light chain Ig loci. B cells that fail to generate a productive BCR, due to abortive Ig gene rearrangements, are eliminated by PCD. At the other end of the spectrum, B cells that express BCRs directed against self-antigens are eliminated by apoptosis or undergo receptor editing to acquire new antigen specificity.[148] The survival and antigen responsiveness of immature B cells can also be increased by lipopolysaccharide exposure, presumably to enhance B-cell production during infections.[149] Fas, TNFR-1, and perforin are not involved in the death of developing B cells.[150] Rather, death appears to be a consequence of a direct signaling event generated by the BCR.[151]

In contrast to B cells in development, mature B cells rely heavily on TNFR family receptors

such as CD40, Fas, and BAFF for regulating their survival and death. It is also

clear that T cells control PCD of mature B cells. Christopher Goodnow and colleagues have illustrated the importance of CD40 and Fas in the balance of life and death in B cells using the hen egg lysozyme (HEL)-transgenic and HEL-specific TCR transgenic mice.[152,153] Naïve, HEL-specific B cells undergo proliferation in the presence of HEL and HEL-specific CD4+ T-cell help. Hence, proper B-cell activation requires triggering of the BCR as well as "help" in the form of CD40L expressed on activated T cells. The primary function of CD40L is to prevent anergy and/or death of activated B cells in the germinal center, allowing further differentiation and function.[154] However, anergic HEL-specific B cells from HEL transgenic animals undergo apoptosis in the presence of the same HEL-specific CD4+ T-cell help. This antigen-specific B cell death is absent in Fas-deficient B cells,[152] thus suggesting a role for Fas in causing the death of anergic antigen-specific B cells. Fas killing of mature B cells can also be abrogated by BCR engagement and IL-4, which promote antibody responses.[155,156] An imbalance of CD40 and Fas signals might contribute to autoantibody production consequent to Fas and FasL mutations in both human and mouse. Indeed, ablation of Fas in murine B cells alone recapitulates the same autoimmune and lymphoproliferative phenotypes noted in *lpr/gld* mice, illustrating the importance of Fas in enforcing B-cell tolerance.[157]

More recent studies using similar transgenic systems suggest that whereas Fas can mediate apoptosis of activated B cells by engaging CD4+ T cells, BCR-induced death of immature or resting mature B cells is primarily Fas-independent. As in T cells, differential expression of pro- and antiapoptotic Bcl-2 family members control mature B-cell fate at different stages. For example, Bcl-2 and Bcl-xL are critical for naïve B-cell survival, whereas Mcl-1 sustains germinal center B cells that persist into memory B cells.[158] As for T cells, knockout mouse experiments reveal that both Bim and Puma are central regulators of mature B cell PCD.[159,160,161,162] The critical B-cell survival and maturation factor BAFF, in combination with BCR signaling, ultimately controls mature B-cell homeostasis via NF-κB-mediated induction of antiapoptotic genes like A1 and Bcl-xL.[163] BAFF signaling also promotes naïve B-cell survival by sustaining the MAPK signals that block Bim accumulation.[164]

The attenuation of B-cell responses at the end of an immune reaction is likely to involve cytokine withdrawal death similar to that of T cells, but this phenomenon is less well characterized. Deprivation of cytokines such as IL-7 and IL-15, which enhance cellular survival through the upregulation of Bcl-2, Bcl-X_L, or Mcl-1, may be responsible.[147,165] Lymphokine withdrawal death in B cells, like T cells, requires de novo ribonucleic acid/protein synthesis.[166] An interesting observation is that a secreted lipocalin, identified through microarray analysis of an IL-3-dependent pro-B-cell line, was implicated as a potential mediator for IL-3 withdrawal death.[167] This protein appears to regulate intracellular iron levels, which may modulate Bim levels for apoptosis induction.[168] However, it remains to be seen whether lipocalins or other similar molecules are involved in lymphokine withdrawal death in primary mature B or T cells. As we will discuss later in this chapter, survival genes such as Bcl-2 can protect cells against lymphokine withdrawal death and therefore play an important role in mature B-cell homeostasis. Moreover, Bcl-2 can contribute to the survival of

B lymphoma cells as revealed by the t(14:18) translocation of Bcl-2 to the Ig locus in follicular B-cell lymphomas.[169]

Dendritic Cell Homeostasis

Understanding the cell fate regulation of DCs is still in its infancy. However, emerging evidence suggests that these highly efficient antigen-presenting cells are also subject to homeostatic regulation by PCD. The natural turnover of DCs was demonstrated in the mouse by Jenkins and coworkers.[170] Using elegant cell-labeling experiments, they showed that antigen-laden DCs stimulate the formation of a cluster of activated antigen-specific T cells and then disappear. This process was antigen- and T cell-dependent. Later, it was shown that the TNF homologue TRAIL, which is produced by activated T cells, could induce apoptosis in DCs and that this mechanism could be defective in patients harboring mutations in caspase-10.[171] Together, these data introduced the concept that there is homeostatic regulation of DCs involving differentiation and recruitment followed by their active elimination by stimulated T cells. Early removal of DCs has the benefit of allowing the activation of T cells but avoiding T-cell restimulation and propriocidal death too soon in the response to antigen. Recent work in mice genetically engineered to selectively express the p35 apoptosis inhibitory protein in DCs reveals that apoptotic removal of DCs is essential to preserve tolerance and prevent autoimmunity.[172] Moreover, forced p35 expression in DCs accelerated autoimmunity on the autoimmune-prone *MRL* background, similar to what is observed for T- and B-cell apoptosis defects. Thus, impaired DC apoptosis can collaborate with other genetic and environmental factors to powerfully predispose to autoimmunity. Similar to B cells, specific ablation of Fas in DCs also gives rise to autoimmunity and excessive lymphocyte proliferation in mice.[157]

On the other hand, PCD of DCs may also serve to promote inflammation and clearance of intracellular pathogens. This is achieved through a distinct pathway of PCD known as "pyroptosis."[173] This pathway is selectively induced in DCs and macrophages upon caspase-1 activation in inflammasomes, cytosolic protein complexes designed to detect microbial components and other "danger" signals. Although it shares some morphologic features with apoptosis, pyroptosis is a distinct proinflammatory, lytic process designed to expose intracellular pathogens and recruit neutrophils. Thus pyroptosis appears necessary for effective clearance of intracellular pathogens that would otherwise replicate and avoid immunosurveillance.

Impaired Programmed Cell Death in Lymphoproliferative Disease and Autoimmunity

Genetically modified mice continue to provide invaluable insights into the molecular underpinnings of PCD in immune cells. The recent description of Bim-deficient *lpr*

mice provides overwhelming evidence that Fas and Bim are the most important proapoptotic genes governing immune homeostasis.[174,175,176] Loss of either Fas or Bim function in isolation manifests in lymphoproliferative disease and autoimmunity. Loss of both results in strikingly accelerated fatal lymphocyte accumulation, early-onset lupus-like autoimmune disease, and failed contraction of antiviral T-cell responses. These findings indicate that Fas

and Bim must cooperate in ensuring immune homeostasis through proper PCD. This corroborates earlier work showing that *lpr* combined with Bcl-2 overexpression caused a synergistic increase in lymphadenopathy and autoimmunity.[57,58]

The study of inherited human lymphoproliferative diseases has further cemented the importance of FAS- and BIM-mediated PCD in maintaining immune homeostasis. Autoimmune lymphoproliferative syndrome (ALPS) was the first genetic disorder of impaired lymphocyte apoptosis (and perhaps the first inherited disease of apoptosis ever described) initially linked to debilitating mutations in FAS. We now appreciate the existence of several forms of ALPS caused by germline or somatic mutations in FAS, FASL, and caspase-10.[177] A similar syndrome known as RAS-associated autoimmune leukoproliferative disease is caused by somatic gain-of-function mutations in NRAS, which impair lymphokine withdrawal apoptosis by suppressing upregulation of BIM.[102] Patients with ALPS typically present early in life with lymphadenopathy and splenomegaly, including the unique expansion of atypical CD4- CD8- "doublenegative" T cells. Autoimmune cytopenias and increased incidence of lymphoma are also characteristic of ALPS and RAS-associated autoimmune leukoproliferative disease, underscoring the significance of Fas- and Bim-induced apoptosis for culling excess lymphocytes to maintain tolerance and prevent malignancy.

Other lymphoproliferative disorders with ALPS-like features have been linked to faulty PCD. The recently recognized immunodeficiency linked to loss of caspase-8 (caspase-8 deficiency syndrome) also features abnormal lymphocytosis due to impaired Fas-induced apoptosis. The unexpected requirement for caspase-8 in lymphocyte activation explains why patients with caspase-8 deficiency syndrome suffer from recurrent infections rather than autoimmune manifestations.[178] Similarly, lymphoproliferation and increased susceptibility to bacterial and viral infections characterizes with a recently-identified family with homozygous FADD-deficiency, reflecting a critical function for FADD in Fas-independent immune cell effector functions.[179] Excessive accumulation of T cells deficient in signaling lymphocytic activation molecule-associated protein in X-linked lymphoproliferative disease is also ultimately the result of impaired RICD, explained by insufficient induction of FASL and BIM.[81] Defective RICD may partially explain why many patients with X-linked lymphoproliferative disease succumb to fulminant infectious mononucleosis upon Epstein-Barr virus infection, marked by lethal overexpansion of activated, Epstein-Barr virus-specific CD8+ T cells that fail to die after repeated encounters with Epstein-Barr virus-infected B cells. Furthermore, defective PCD may be partially responsible for T-cell hyperproliferation and multiorgan infiltration in patients with immunodysregulation, polyendocrinopathy, and enteropathy, X-linked syndrome, and the equivalent "scurfy" mouse model. In this disease, T_{reg} cells are lost due to Foxp3 mutations, hampering polyclonal, Bim-directed T-cell apoptosis induced by T_{reg}-mediated consumption of IL-2.[116] As new pathologic mutations are rapidly uncovered by rapid whole-genome sequencing, similar disorders featuring unwanted accumulation of activated lymphocytes, including self-reactive autoimmune clones, may eventually be tied to related defects in cell death programs. The investigation of these disorders of apoptosis and immune homeostasis has provided useful lessons on contemporary human genetic investigation which have been summarized elsewhere.[180]

Programmed Cell Death and Development of Lymphoid Malignancy

Here we return to the question of why the mature immune system assiduously eliminates activated lymphocytes that have proven their utility in recognizing and responding to specific antigen. Why can't the immune system adopt a laissez-faire economy of previously activated cells, which might have great value if the same pathogen is re-encountered? Several lines of evidence strongly suggest that in addition to the potential loss of tolerance and autoimmunity, lymphoid malignancy is also promoted by defective apoptosis. Hence, the accumulation of immune cells that have a propensity to proliferate and can undergo additional genetic changes may be deleterious to the organism. The association of translocations of the Bcl-2 gene with diffuse large B cell lymphoma suggested that somatic aberrations in apoptosis pathways might be important in the transformation process. Since then, somatic changes in a variety of apoptosis molecules including Fas, caspases, and Bcl-2 family members have been documented in lymphoid tumors.[181] By contrast, the well-known inheritable apoptosis defect in p53 in the Li-Fraumeni syndrome seems to cause predominantly solid tumors but not lymphomas. Examination of large kindreds of patients with ALPS has revealed a 15-fold greater incidence of lymphomas relative to the general population. It is striking that these families have several different classes of lymphoma, suggesting that Fas protects against transformation not for a single cell type but generally for B- and T-lymphocytes.[182] Moreover, Fas stimulation not only results in death induction, but also stimulation of signals for survival and proliferation such as NF-κB and MAPK activation; these dichotomous signals may have different thresholds that can contribute to lymphomagenesis.[183] Patients with X-linked lymphoproliferative disease are also at greater risk for developing B-cell lymphomas, which may be linked to impaired B-cell PCD attributed to poor T or natural killer cell cytotoxic capability, including hampered FasL induction.[184] Thus, besides autoimmune manifestations, we can infer that protection against lymphoid malignancy demands strict control over the accumulation of activated lymphocytes.

Programmed Cell Death as an Immune Effector Mechanism

Although this topic will be covered authoritatively elsewhere in the book, it is important to recognize that the same pathways that participate in homeostatic cell death also are used as immune effector mechanisms. The Fas pathway is the principal calcium-independent pathway of cytotoxic T-lymphocyte killing.[68] Fas ligand displayed by either CD4+ or CD8+ T cells can eliminate Fas-bearing cells that may be infected or malignant. Similarly, the calciumdependent cytotoxic T-lymphocyte killing mechanism involving perforin and granzymes also can induce PCD in target cells. The two main proteases are granzyme A and granzyme B, the latter being the only serine protease capable of processing caspases.[185,186,187,188,189] Following endocytosis into a target cell, perforin forms pores in endosomal membranes through which granzyme B gains access to the cytoplasm and proteolytically activates caspases, leading to apoptosis.[190] Though this cytoplasmic pathway of death does not emanate from the mitochondria, the latter may amplify the death signal by Bid cleavage.[191,192,193,194,195] These parallels may provide an insight into why lymphocytes have evolved mechanisms in trans, involving the surface interaction of FasL and Fas or perforin/granzyme release to homeostatically control their numbers by apoptosis. This permits the T cell to simultaneously regulate itself and other immune cells, and to expunge nonlymphoid cells that require immune

elimination such as those that are infected or malignant. Natural killer cells may also use PCD mechanisms for selfregulation and expunging infected or malignant cells.[196,197] Hence, for mature T cells, the same molecules can subserve several death functions. By contrast, a more direct connection of the TCR or BCR to death pathways is present in developing lymphocytes, which have no use for effector mechanisms or propriocidal regulation.

Programmed Cell Death as Immune Therapy

Because of its exquisite antigen specificity, the possibility of using antigen-induced death, particularly of T-lymphocytes, for the treatment of immunologic diseases has been suggested.[198,199] In particular, the T-cell components of graft rejection, autoimmune diseases, and allergic reactions could potentially be suppressed by antigen-induced elimination. This concept has been tested in both mice and monkeys with clearly beneficial effects on disease outcome. For example, it was found that the repetitive administration of a myelin basic protein antigen could suppress experimental allergic encephalomyelitis in mice by deleting the diseasecausing T cells.[93] This suggests that antigen-specific therapy is achievable if antigens relevant to the disease process are sufficiently well-defined and death of the culpable T cells can be triggered without exacerbation of symptoms.[29]

Because these requirements have limited further evaluation of antigen-specific therapy in human clinical trials, other ways of triggering death have been explored. Fas or TNFR1 agonist administration results in systemic toxicities limiting their potential utility.[200,201] However, TRAIL receptor agonists potentially induce death of neoplastic cells preferentially.[202,203] Intracellular apoptosis signaling molecules targeted in preclinical or clinical trials include caspases, IAPs, SMAC, and proapoptotic or antiapoptotic Bcl-2 family members.[204,205,206,207] Many of these are rationally designed small molecules or peptidomimetics, which have demonstrated promising results, particularly against lymphoid and other malignancies.

CELLULAR AND MOLECULAR MECHANISMS OF PROGRAMMED CELL DEATH

Apoptosis Initiation Mediated by Caspase Complexes: Two Principal Pathways

Caspases must be highly active within the cytoplasm to cause apoptosis. Like all proteases, these potentially destructive proteins are first produced as zymogens that are then proteolytically activated. Elucidating these complex mammalian pathways provides a window into the myriad of molecular abnormalities of apoptosis that contribute to immunologic diseases and cancer (Fig. 30.3). Also, it is necessary to understand the important nonapoptotic functions assigned to caspases including cellular activation, suppression of necrotic pathways, and cytokine processing as key enzymatic functions of inflammasomes. Fortunately, mammalian apoptosis can be understood with a few key concepts. Most importantly, the processing enzymes that activate caspases are mainly caspases themselves. As explained in the following, activation and autoprocessing occur when caspase zymogens are brought into specific signaling complexes. In general, there are two main forms of caspases. Those with long prodomains that have protein interaction domains capable of bringing them into activating platforms for autoprocessing are called initiators, upstream, or apical caspases. Those with short prodomains that must be cleaved by other proteases

(caspases or granzyme B) are called effector, downstream, or executioner caspases. Key adaptor molecules harbor domains that specifically recruit caspases into complexes: the death effector domains (DEDs) or the caspase recruitment domains (CARDs).[208,209] The DEDs and CARDs are protein-protein interaction domains found in the caspase prodomains and other adapter molecules that generate the specific intermolecular assemblies. In contrast to executioner caspases, which require processing to become active, initiator caspases are activated by multimerization.[210,211] When juxtaposed at the receptor complex, initiator caspases become proteolytically active and will cleave one another. This "proximity-driven model" posits that specific intra- and intermolecular cleavage triggered by procaspase dimerization drives initiator caspase activation.[212] This leads to the maturation of the enzyme through separation of the large and small enzymatic subunits. Autoprocessing also cleaves the enzymatic units from the prodomain, thereby liberating the highly active enzyme from the receptor complex. As discussed later in the chapter, structural studies suggest that active caspases are tetrameric species composed of two small and two large subunits.

To initiate the caspase cascade, the enzyme must dock onto the appropriate adapter molecules. The adapter/caspase complex presumably provides a conformation that facilitates

the initial autoprocessing step of the caspase zymogen. Adapter/caspase complexes originate from two principal sites in the cell: 1) the plasma membrane (through DRs), also known as the extrinsic pathway or 2) the mitochondrion, or intrinsic pathway (see Fig. 30.3). We will employ this primary dichotomy of extrinsic and intrinsic pathways as it recapitulates the two major forms of propriocidal apoptosis: antigen-induced and lymphokine withdrawal, respectively.

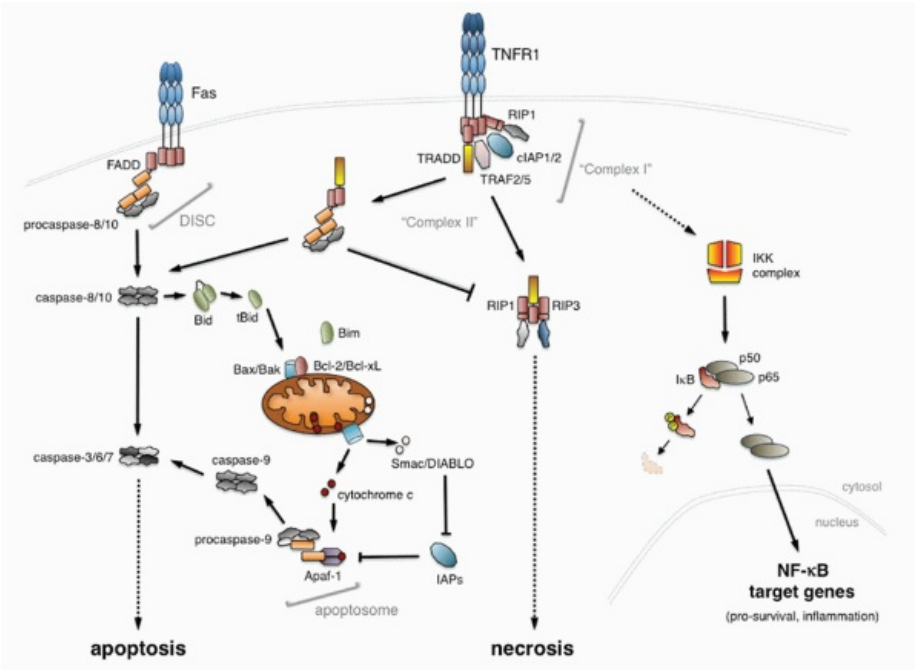

FIG. 30.3. Signal Transduction Pathways of Death Receptors. Shown are the two principal apoptosis pathways associated with death receptors (Fas and tumor necrosis factor receptor-1) and the mitochondrial pathway of caspase-9 activation. The *solid arrows* indicate direct association of the steps involved, whereas the *dashed arrows*

indicate that multiple steps are involved. Inhibitory interactions are shown by a *barred line*.

DR engagement by cognate ligand causes the formation of the death-inducing signaling complex (DISC), which comprises the cytoplasmic tail of the receptor, the FADD adapter protein, and caspase-8 or -10.[213,214] The recruitment of caspase-8 or -10 into the DISC triggers the processing of these proteases into their active form. An 80-amino acid death domain (DD) present in the Fas cytoplasmic tail and FADD causes their interaction. The DD contains a "hexahelical bundle" that nucleates this complex, as described in the following. FADD then recruits caspase-8 or -10 by homotypic interaction between DEDs present in each of these molecules. The DED has a hexahelical bundle homologous but not identical to DDs. Sensitive energy transfer techniques have demonstrated that both caspases can enter the same receptor complex.[215] Recent work shows that the signaling likely involves an asymmetric oligomeric complex of five to seven Fas DDs and five FADD DDs, forming a flexible helical structure that may adjust to the level of agonist simulation.[216,217] Molecular modeling studies show that the Fas pathway appears to be governed by a threshold of signal required for the commitment to death.[218] This may arise from the oligomeric assembly of each individually liganded Fas complex that then form larger assemblies of engaged Fas receptors, leading to the formation of microscopically visible clusters of Fas termed signaling protein oligomerization transduction structures. These structures enhance caspase-8 recruitment and activation.[219] Fas receptor capping and internalization in endosomal vesicles follow signaling protein oligomerization transduction structure formation and may promote the killing process.[220]

The signal complex for TNFR-1 is different from that for Fas as it includes the DD-containing adapter TRADD in addition to FADD and caspase-8 and -10 (see the following discussion). Similar signaling complexes are likely formed with other DD-containing members of the TNFR superfamily including DR3, TRAIL receptors DR4 and DR5, and DR6.[221,222,223] Our knowledge of the physiologic significance of apoptosis-inducing receptors besides Fas and TNFR-1 in immune regulation remains incomplete. DR3 was once shown to participate in negative selection of thymocytes,[224] although DR3 is better known for its proinflammatory, pathogenic role in autoimmune disorders.[225] DR6 has been implicated in attenuating Th2 responses.[226,227] It has been proposed that DR4 and DR5 may mediate the preferential killing of tumor cells (including lymphoid tumors) through TRAIL.[202]

Activation of the mitochondrial pathway of apoptosis during lymphokine withdrawal or during development achieves the same end as DRs: caspase activation. However, signal complex formation occurs in a different way. Intrinsic signals converge on the mitochondria, resulting in mitochondrial fission and outer membrane permeabilization and the release of the proapoptotic molecules from the intermembrane space (IMS) into the cytosol.[228] Cytochrome c, a protein involved in the electron transport chain, is liberated into the cytosol where it binds in a heptameric complex with the apoptotic protease-activating factor 1 (Apaf-1) in the presence of ATP/dATP, often referred to as the "apoptosome."[229,230] This complex

nucleates caspase-9 recruitment and activation.[231,232,233] There are specialized regulatory proteins for caspase-9 that provide additional levels of control: X-linked IAP (XIAP), which inhibits caspase-9, and Smac/Diablo which counteracts XIAP.[234,235] These mitochondrial events are tightly regulated as caspase-9 activation commits the cell to die.

The Central Role of the Mitochondrion

A special role for the mitochondrion in apoptosis was first suggested by the finding that Bcl-2 and related molecules were anchored predominantly in the outer mitochondrial membrane (OMM). The inner mitochondrial membrane (IMM) is devoted to energy conversion and adenosine triphosphate generation, but the OMM has emerged as a primary regulator of cell viability. A diversity of death inducers, including trophic factor withdrawal, drugs such as staurosporine or steroids, or DNA damage, can all generate signals that converge on the mitochondrion. Their principle effect is to induce mitochondrial fission and increase OMM permeability to large proteins, leading to the release of apoptogenic factors into the cytosol.[228] The critical role of cytochrome c release was elegantly demonstrated by generating “knock in” mice expressing a mutant cytochrome c (K72A).[236] This mutant version of cytochrome c retains its electron transfer function but cannot activate Apaf-1. The mutant mice display developmental abnormalities similar to Apaf-1- or caspase-9-deficient animals, and the surviving mice exhibit impaired lymphocyte homeostasis. In contrast to Apaf-1-/- cells, thymocytes from mice with mutant cytochrome c were normally sensitive to intrinsic stimuli such as γ-irradiation, dexamethasone, and etoposide. This reveals a cytochrome c- and apoptosome-independent but Apaf-1-dependent mechanism of caspase activation and cell death.[236]

Interestingly, 85% of cytochrome c is sequestered inside the intracristae region formed by the convoluted folds of IMM, with limited access to the IMS.[237] Therefore, the sudden “all-or-nothing” release of cytochrome c requires two steps: 1) a BH3-only protein-dependent remodeling of the cristae structure allowing cytochrome c to access the IMS and 2) the Bax and Bak-dependent permeabilization of the OMM. The cristae structure was recently shown to be controlled by the IMM presenilin-associated rhomboidlike protein (PARL) and by the IMS resident, dynaminrelated optic atrophy 1.[238,239] In yeast, homologues of these proteins, Rbd1 and Mgm1, respectively, regulate mitochondrial fusion, a process of mitochondrial metabolism. Mice deficient in PARL die between 8 and 12 weeks of age due to progressive cachexia, and their lymphocytes undergo massive apoptosis. Moreover, mouse embryonic fibroblasts derived from these animals were significantly more sensitive to intrinsic stimuli, demonstrating the antiapoptotic function of PARL. Normally, PARL cleaves optic atrophy 1, and both cleaved and uncleaved optic atrophy 1 form heterooligomers that maintain a tight bottleneck configuration of the cristae, keeping cytochrome c sequestered.

How the OMM releases its mortal poison is still unclear, and two models have been proposed. In one, the proapoptotic Bcl-2 family proteins Bax and Bak selectively permeabilize the OMM, without affecting the IMM. In the second model, a pore in the IMM known as the mitochondrial permeability transition complex opens and allows water and solutes up to 1.5 kDa to accumulate into the matrix. The matrix swells as water enters and the OMM bursts.[240,241] Cells from mice lacking cyclophilin D, a critical component of the mitochondrial permeability transition complex, remain sensitive to intrinsic death insults,

favoring the first model. Moreover, the first model fits with recent data that the mitochondrial fission leads to weakening of the outer membrane and can be controlled by Bcl-2 family proteins.[228] However, the mitochondrial permeability transition complex appears to be essential for stimuli promoting necrosis such as calcium overload and ROS. Perhaps this accounts for the fact that cyclophilin D knockout mice were resistant to ischemia/reperfusion-induced cardiac injury.[242,243] As detailed in the following, OMM permeability is regulated by the large family of proteins related to Bcl-2.

In addition to cytochrome c, several other proapoptotic proteins from the IMS are released outside the mitochondria during apoptosis. The flavoprotein "apoptosis-inducing factor" (AIF) translocates from the mitochondria to the nucleus, causing DNA fragmentation and chromatin condensation in response to apoptotic stimuli.[244,245] Genetic deficiency of this protein in mice inhibits the death of embryonic cells in response to serum starvation and appears to be responsible for embryonic morphogenesis and cavitation of the embryonic cell mass. The observation that these processes did not depend on caspase-3, caspase-9, or Apaf-1,

showing that unlike *C. elegans*, not all programmed death in mammalian cells depends on caspases.[246] Further work will be needed to determine if AIF has any role in immunity. Another mitochondrial protein important in apoptosis is endonuclease G, a mitochondrial nuclease that is released and translocates to the nucleus in response to death stimuli. Once situated in the nucleus, endonuclease G cleaves DNA into nucleosomal sizes independent of caspases, differing from the caspase-dependent activation of another apoptotic nuclease, caspase-activated DNase[247] (see subsequent discussion). Other mitochondrial proteins released into the cytosol, Smac/DIABLO and Omi/HtrA2, neutralize the IAPs and thus promote caspase activation.

Studies on the mitochondrion have also focused on caspaseindependent apoptosis, which AIF, endonuclease G, and Omi/HtrA2 were proposed to mediate. In general, this concept stemmed from examples of apoptosis that could not be blocked by small peptide caspase inhibitors such as zVAD.[248,249] However, conclusions drawn from such experiments are limited by the short half-life of such inhibitors and the fact that they do not block all caspases. Also, kinetic experiments show that AIF release is slower than that of cytochrome c, Smac/DIABLO, and Omi/HtrA2.[250,251] Finally, cells from caspase-3 and -7 knockout mice exhibit a delayed translocation of cytochrome c and AIF, indicating that caspases act as an amplification loop necessary to fully permeabilize the mitochondria.[252] Thus, it is uncertain if a truly caspase-independent form of apoptosis exists.

Programmed Necrosis

While PCD has generally been equated with apoptosis, recent evidence suggests that necrotic or alternative forms of death may also result from internal death programs.[248,249,253,254] This is different from secondary necrosis that occurs in the late phase of apoptosis when membrane integrity is lost. Rather, PCD leading to a necrotic morphology without any intermediate stage of apoptosis or caspase activation is now well documented.[18,246] For example, it has been found that DRs can trigger necrotic death rather than apoptotic death under certain circumstances.[248,249,253] Although TNF

stimulation through TNFR-1 can trigger caspase-dependent, classical apoptosis, necrosis may well be the dominant pathway for TNFR-1, at least in certain cell types.[248,253] By contrast, Fas predominantly triggers apoptosis but can also induce necrosis.[18] A shift from apoptosis to necrosis can be induced by tetrapeptide caspase inhibitors, suggesting that necrosis causes cell elimination when apoptosis fails or is blocked.[249] Moreover, the discovery of a specific inhibitor of necrosis but not apoptosis points to a distinct biochemical pathway for programmed necrosis.[19] These observations have physiologic relevance as propriocidal death of mature T cells is partly refractory to inhibition by caspase blockers and can manifest features of necrosis.[18] For these reasons, we utilize the term "programmed necrosis" to describe death by necrosis (or at least a clearly nonapoptotic phenotype) that results from a specific molecular pathway and appears to be advantageous to the host. "Necroptosis" may be considered an equivalent moniker used more often in recent literature.[255]

Programmed necrosis can be triggered through Fas and other DR stimulation in a process that requires RIP (also known as RIP1).[18] Originally identified by a yeast two-hybrid interaction screen using the DD of Fas as bait,[256] RIP1 was later identified as a component of the TNFR-1 signaling complex that could induce the antiapoptotic transcription factor NF-κB.[257,258,259] Besides a carboxyl-terminal DD that is required for homotypic binding to the receptor-signaling complex, RIP also contains an amino-terminal serine/threonine kinase domain that is dispensable to its apoptosis-inducing activity but is essential for its necrotic function. Both direct Fas engagement or TCR stimulation (which presumably indirectly triggers Fas by FasL induction) can stimulate necrosis in vitro.[18] Although Fas-induced PCD has not been investigated extensively in the RIP1-deficient animals, the physiological role of RIP1-dependent programmed necrosis is gradually becoming clear. It appears to enhance inflammatory processes and may restrain replication of certain classes of viruses such as poxviruses and cytomegaloviruses.[260]

A similar form of RIP-dependent, caspase-independent necrosis is now well described for TNFR-1.[17,255,261,262] Initial binding of TNF induces a membrane-proximal "complex I" containing TRADD, RIP1, and cellular IAP 1 and 2. cIAPs in complex 1 are E3 ligases that mediate ubiquitination of RIP1, a required step for TNFR1-induced NF-κB activation. Once bound TNFR1 is internalized, however, cylindromatosis enzyme deubiqitinates RIP1 and allows it to incorporate into a cytosolic "complex II" containing TRADD, FADD, caspase 8, and RIP3. Active caspase-8 triggers apoptosis and is capable of directly cleaving RIP1 and RIP3. New data suggest heterodimers of unprocessed procaspase 8 and cellular FLIP, which display attenuated proteolytic activity, are essential for blocking RIP3-dependent necrosis.[263] Thus, caspase-8 prevents TNFR-mediated necrosis. However, inhibition of caspase-8 catalytic activity permits phosphorylation of RIP1 and RIP3 and subsequent stimulation of necroptosis, which requires RIP1 kinase activity. Small molecule inhibitors of programmed necrosis, known as necrostatin-1 and -3, allosterically block RIP1 kinase activity.[19] Thus, RIP1 appears to be a bifunctional signaling molecule with stimulatory and necrosis-inducing effects. Paradoxically, RIP1-deficient mice are severely runted and die shortly after birth, apparently due to increased TNF-induced death from impaired NF-κB induction.[264]

Nevertheless, RIP1 appears to be the clearest talisman of a molecular pathway of programmed necrosis. Several groups recently reported that RIP3 is also essential for programmed necrosis, and may be capable of inducing necrosis in the absence of RIP1.[265,266,267] Signals downstream of the RIP1-RIP3-associated "necrosome" are only now being elucidated, but are distinct from the TRADD-dependent, caspase-dependent apoptotic TNF pathway[262] and likely involve oxidative stress, cytosolic calcium flux, and substantial alterations in mitochondrial bioenergetics.[255,262,268]

What is the potential role of DR-induced necrosis in immunity? Many viruses, including vaccinia and other poxviruses, encode inhibitors of caspases.[17,269] Infection by poxviruses blocks caspases and apoptosis, sensitizing cells to TNF-induced necrosis.[270] Therefore, programmed necrosis may counteract viral antiapoptotic mechanisms by

eliminating virally infected cells. RIP3-deficient mice succumb to uncontrolled vaccinia infection linked to defective programmed necrosis.[265] Also, vaccinia infection induces extensive inflammation and necrosis in visceral fat pads, which primes the antiviral adaptive immune response. From an immunologic standpoint, necrotic cells often have a superior stimulatory activity than apoptotic cells for DC maturation.[271,272] During viral infection, TNFinduced necrosis may indirectly enhance the cytotoxic T-lymphocyte response to the virus by promoting DC maturation. Inhibition of programmed necrosis by expression of virally encoded FLIPs and/or inhibitors of the RIP1/RIP3 necrosome complex (eg, murine cytomegalovirus protein M45) may represent a strategy for evading these immune responses.[17,273]

Another important reason to further understand necrosis in the context of the immune system is that it may be responsible for the pathogenesis of viruses such as human immunodeficiency virus. Although many claims have been made that human immunodeficiency virus causes apoptosis of either directly infected or bystander T cells, most dying infected cells do not manifest hallmarks of apoptosis. Furthermore, these cells undergo cell cycle blockade and appear necrotic when examined by electron microscopy (see Fig. 30.1).[274,275] Hence, apoptosis does not appear to be the major mode of death. Human immunodeficiency virus-induced necrosis is not impaired by the absence of RIP1, thus distinguishing it from DR-induced necrosis. Further studies on identifying the molecules involved in virally induced necrosis are needed to determine whether it involves a specific molecular program or simply lethal cell injury.

FAMILIES OF MOLECULES PROVIDE PRECISE REGULATION OF PROGRAMMED CELL DEATH

Caspases

PCD was so important to the successful evolution of multicellular organisms that a specific set of genes was dedicated to the task. Chief among these genes were those encoding caspases. Junying Yuan and H. Robert Horvitz in 1993 first observed that a gene crucial for PCD in *C. elegans*, ced-3, was related to a mammalian caspase, IL-1β-converting enzyme, thus implicating specific proteolysis in the death program.[276] The "caspase" moniker is a

rubric indicating that these enzymes contain an active site *c*ysteine, cleave substrates on the carboxyl side of *asp*artate residues, and are prote *ases*. Caspases have been called the "executioners" of apoptosis. Once activated, their cleavage of various cellular substrates, including other caspases, results in the morphologic features of apoptotic cell death. Earnshaw et al.[277] authoritatively discussed the detailed biochemical features of caspases and their substrates. There are 15 caspases in mammals, all of which can induce apoptosis.[278] Although caspase-1 and caspase-4 (caspase-11 in the mouse) also proteolytically process proinflammatory cytokines such as IL-1β into their mature forms, they cause pyroptosis and endoplasmic reticulum stress-induced apoptosis, respectively.[173,279] In addition, caspase-12 was recently proposed to inhibit the proinflammatory functions of caspase-1. Indeed, caspase-12 functions as a FLIP, and directly binds to caspase-1 to suppress its activity. Functionally, mice deficient in caspase-12 are resistant to sepsis and clear bacterial infection more efficiently, whereas humans with a caspase-12 polymorphism demonstrate hyporesponsiveness to lipopolysaccharide and increased susceptibility to sepsis, due to an attenuated proinflammatory innate immune response.[280,281]

Much early work equated caspase enzymatic activity with processing. While true for the so-called downstream "effector" caspases, recent studies have established that cleavage is neither necessary nor sufficient for activation of initiator caspases.[210] Rather, oligomerization of procaspases-8 or -10 within the DISC, procaspase-9 within the apoptosome, or procaspase-2 within an Apaf-1-independent complex is sufficient for activity.[282,283,284,285] We now understand that full-length caspase-8 becomes partly active upon antigen receptor engagement on lymphocytes, which is required for downstream NF-κB activation.[178,286] The proximity-induced dimerization of the initiator caspases due to their long N-terminal prodomains allows them to form multimeric complexes that generate active proteases (as described subsequently).

Because of the thermodynamic irreversibility of proteolysis, downstream caspase activation is a commitment to death that cannot be undone. Hence, caspases are tightly regulated. This regulation is achieved by three principal means: 1) caspases are zymogens requiring proteolytic processing; 2) caspase "prodomains" control access to complexes with adaptor molecules; and 3) specific inhibitors exist.[6] How these mechanisms work is clear from the structures of caspases (Fig. 30.4). Caspases have an NH_2-terminal "prodomain" that is removed in the active enzyme. The COOH-terminal protease domain comprises two subunits of the mature enzyme that are denoted by their processed molecular weights, for example, p20 and p10. The processing sites between these parts occur at short specific tetrapeptide sequences ending in aspartate residues that dictate that the major processing enzymes are caspases themselves.[277] For caspases with short prodomains, namely caspases-3, -6, and -7, this event is believed to be the primary mode of regulation. Those with long prodomains harbor protein-interaction domains that allow them to enter activating complexes for specific death pathways, as described previously. Caspase-8 and -10 have DEDs and caspases-1, -2, -4/-11, -5, -9, -12, and -13 have CARD domains. Upon recruitment to relevant activating platforms, the proenzyme chains adopt a specific stoichiometric conformation that facilitates autocatalytic processing. For example, homodimerization of procaspase-8 is sufficient for enzymatic competency, with the dimer being more susceptible to processing than the

monomer.[287] In contrast, caspase-9 engineered to constitutively dimerize exhibits far less proteolytic activity than Apaf-1-activated caspase-9, suggesting an induced conformation model applies to caspase-9 activation within the apoptosome.[288] Following these initial cleavage events, the liberated subunits form a heterotetramer of two large and two small enzyme subunits as reflected in the crystal structures of processed caspases.[289,290,291,292,293] The consequences of subunit cleavage are dramatic: there is a 180-degree shift of the NH_2-terminus of the small subunit to bring it into

apposition with the catalytic cleft.[294] Once a processed heterotetramer is formed, it will be thermodynamically stable and can move away from the activating complex to find apoptotic substrates within the cell.

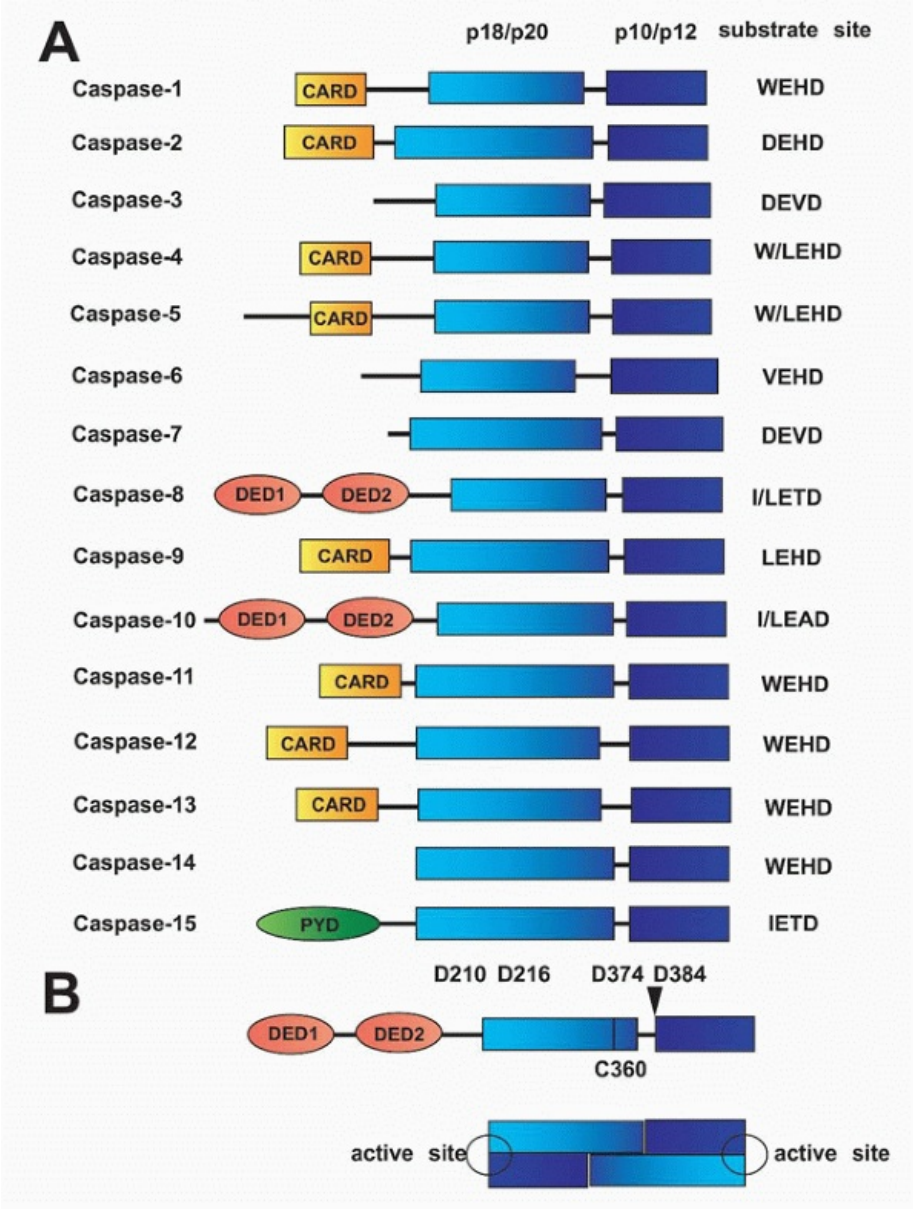

FIG. 30.4. Mammalian Caspases. A: The structures of the 15 known mammalian caspases are shown. Many of the caspases contain a caspase recruitment domain at

their NH2-terminus. Caspase-8 and caspase-10 contain a tandem copy of the death effector domain at the NH2-terminal end of the proenzyme, which is essential for recruitment to the death-inducing signaling complex. Caspase-15 contains a pyrin (PYD) domain at the NH2-terminus. The large (p18/p20) and small (p10/p12) subunits near the COOH-termini are also shown. The optimal tetrapeptide substrate specificity of each caspase is shown on the right-hand column. Notice that the caspase-4 and caspase-11 are human and mouse homologues, respectively (*asterisks*). **B:** Proximity-induced oligomerization is crucial in activating initiator proenzymes, which also undergo autoproteolytic cleavage. Shown in the diagram by the *arrows* are the proteolytic cleavage sites of caspase-8 at aspartate residues 210, 216, 374, and 384, as well as the active site cysteine (C360), indicated by a *bar*. Two of each of the large and small subunits of the enzyme form the active enzyme in a head-to-tail conformation, resulting in a tetramer that contains two catalytic sites at the two ends of the molecule. The active sites of the enzyme, which are made up of residues from both the large and small subunits, are designated by the *circles* at the ends of the processed enzyme.

Proteolytic activation can be regulated by a variety of proteins. These inhibitors, including the CrmA protein, its homologs found in other viruses, and IAPs, interact with the fully formed enzyme and remain bound as competitive inhibitors.[269,295] Other viral proteins that harbor DEDs can enter the DR signaling complexes and inhibit cleavage of caspases-8 and -10.[123,296] Initially, it was thought that these inhibitors, termed viral FLIPs, inhibit apoptosis by competing with caspase-8 and -10 for entry into the DR complex. However, it is now clear that the inhibitor and the caspase join the complex together. Resolution of the crystal structure of MC159, a viral FLIP from the poxvirus *Molluscum contagiosum* virus, reveals that it cooperatively assembles with FADD and Fas through an extensive charge surface encompassing a conserved charge triad. This prevents FADD self-aggregation and disrupts higher order oligomerization of Fas signaling complexes.[297] Cellular FLIP is structurally homologous to caspase-8 and -10 (indeed, it is encoded in the same locus on human chromosome 2 as these caspases) but has multiple amino acid differences that inactivate its protease function.[125] It also enters Fas signaling complexes and prevents caspase activation.[297,298] For example, the ability of antigen receptor stimulation to block Fas-induced death in B cells is regulated in part by cellular FLIP.[126] However, cellular FLIP can also induce apoptosis under certain conditions, which may be due to its ability to heterodimerize with and thus activate procaspase-8.[299] Recent studies suggest that under physiologic conditions, cellular FLIP heterodimerization with caspase-8 triggers sufficient catalytic activity for NF-κB activation and/or suppression of RIP-dependent necrosis but disrupts caspase-8 homodimerization required for full processing and cleavage of apoptotic substrates.[263,300]

Another class of caspase regulators is the cellular IAPs that can directly inactivate mature caspases to avert the deleterious effects of inadvertent caspase activation.[128,129] IAPs are characterized by the presence of up to three baculovirus IAP repeats (BIRs). Originally identified in baculovirus, the BIR domains are characterized by the presence of cysteine and histidine residues in defined spacing arrangements ($Cx_2Cx_6Wx_3Dx_5Hx_6C$).[128] The

mechanisms of inhibition by IAPs on different caspases are quite distinct. For caspase-9, inhibition by XIAP depends on binding of the BIR3 domain with the tetrapeptide sequence ATPF at the NH_2-terminal of the p12 subunit that is exposed only in the mature enzyme. Hence, XIAP specifically inhibits active caspase-9 but not procaspase-9.[234] By contrast, XIAP inhibits caspase-7 and -3 by forming contacts in the catalytic groove with little involvement of the BIR domains.[301] Interestingly, this mechanism of XIAP association is adopted by Smac/Diablo to inhibit the antiapoptotic function of XIAP during the onset of mitochondrial PCD pathways.[235] Smac/Diablo is a protein that resides in the intermembrane space of the mitochondria. It functions as an elongated dimer of α helices that adopts the shape of an arch. Mutation of a specific alanine to methionine abolished the binding and inhibitory activity of Smac/Diablo on XIAP.[234,235] This IAP-binding motif is also shared by other IAP antagonists (Omi/HtrA2 and GSPT1/eRF3) and by caspase-9, suggesting that IAP antagonists and caspase-9 compete for binding to XIAP.[234] The action of XIAP is flexible in order to carry out diverse functions. Instead of the BIR3 domain, XIAP uses the linker region between

BIR1 and BIR2 to interact with and inhibit the function of caspases-3 and -7. In this case, the linker blocks the catalytic groove of the caspase. While BIR2 does not directly participate in the inhibitory activity, it may stabilize the association by making other contacts with caspase-3.[290,302,303]

The death-inducing effect of caspases involves a limited set of targets because most proteins in the dying cell remain uncleaved. Caspases have an absolute requirement for aspartate at the amino side of its cleavage sites (P1 site). Further specificity is dictated in part by the three amino acids preceding the obligatory aspartate in the substrate (P2 to P4 positions).[277] Preferred tetrapeptides have been identified for each of the mammalian caspases so that, for example, caspase-3 is known to preferentially cleave at the sequence DEVD whereas caspase-8 prefers IETD.[12,304,305] Nicholson and coworkers have developed valuable tetrapeptide substrates and inhibitors based on these preferences, but they emphasize that these are not absolutely specific.[12,277] In addition to primary amino acid sequence, additional secondary structural features of target proteins may be recognized. For example, many proteins harboring a DEVD sequence may not be cleaved at that sequence by caspase-3.[129] Thus, the tetrapeptide recognition sequences are required but not sufficient for apoptotic protein cleavage. Nevertheless, model caspase substrates and inhibitors based on short recognition peptides, such as zVAD- or DVED-fmk (*f*luoro*m*ethyl *k*etone), have been useful in assessing caspase function in vitro and in vivo.

Caspase substrates in dying cells have been grouped according to apparent functional importance: 1) cytoskeletal proteins such as actin, gelsolin, and α-fodrin, among others; 2) nuclear structure proteins, especially lamins A and B; 3) DNA metabolism and repair proteins such as PARP; 4) protein kinases such as various isoforms of PKC; and 5) signal transduction proteins such as STAT1, SREBP-1, and phospholipase C-γ1.[277] As PCD typically involves the elimination of somatic cells, there may be little evolutionary constraint on random cleavage sites. Hence, it has been difficult to distinguish functional versus adventitious sites. Key caspase substrates that have unequivocal roles in apoptosis include

caspases themselves, Bcl-2 and Bcl-X_L, the inhibitor of caspase activated deoxyribonuclease of the caspase-activated DNase, which is one enzyme causing apoptotic nuclear fragmentation, and the nuclear lamins.[306,307] Cleavage of the nuclear lamins was shown by Eileen White to be responsible for certain nuclear changes in apoptosis by experiments in which the aspartate cleavage sites were modified and the apoptotic changes were abrogated.[308] This stringent test has been applied to very few proteins cleaved during apoptosis. In fact, evidence weighs against the role of many caspase substrates in apoptosis. For example, the knockout of the PARP gene (a caspase substrate important for DNA repair) in mice revealed no abnormality of development, immunity, or apoptosis.[309] Hence, further work is necessary to determine the importance of specific proteins cleaved during apoptosis.

Genetic analyses of human caspases have provided important information about their function. By contrast, homozygous deficiencies in mice have been associated with embryonic lethality or neurological defects but have not yielded specific immunologic phenotypes.[277] The first two diseases involving inherited mutations of caspases in humans cause prominent effects in the immune system. The first, an inherited mutation in caspase-10, was detected in the human disease ALPS type IIa, now referred to as ALPS-CASP10.[171,310] Individuals with caspase-10 mutations exhibited defects in apoptosis triggered by multiple DRs affecting the homeostasis of T cells, B cells, and DCs. The abnormal accumulation of immune cells leads to the formation of a variety of autoantibodies that cause autoimmune conditions including hemolytic anemia, thrombocytopenia, and others. The second involves mutations in caspase-8, which cause abnormal lymphocyte apoptosis and lymphocyte accumulation in secondary lymphoid tissues.[311] However, unlike a caspase-10 mutant individual, the lack of caspase-8 leads to immunodeficiency manifested as recurrent sinopulmonary and viral infections with minimal autoimmunity. Biochemical analyses show that caspase-8 is required for NF-κB induction by the TCR, BCR, and Fc receptor, which accounts for the T-, B-, and natural killer-lymphocyte activation defects. These patients represent a novel clinical entity, termed caspase-8 deficiency state.[178] The clinical differences between humans having caspase-10 or caspase-8 mutations may reflect differences in substrate specificities of these two closely related enzymes.[204,215]

Tumor Necrosis Factor/Tumor Necrosis Factor Receptor Superfamily Members

The TNF/TNF receptor superfamily contains many key regulators of immunity (Fig. 30.5). These receptors and their cognate ligands facilitate communication between immune cells in the orchestration of immune responses. They also comprise a major class of cellular sensors to external physiologic cues that regulate PCD.[312] The hallmark of these receptors is the presence of “cysteine-rich domains” (CRDs) in the extracellular region, which are required for receptor assembly and ligand binding. Each receptor contains between one (BCMA and TWEAK-R)[313,314] and six (CD30) CRDs. The receptors can be further subdivided into two classes based on sequence homology within the cytoplasmic signaling domain. The DRs contain an 80-residue long DD that is essential for death signaling.[213,315] The majority of the receptors lack a DD and therefore are not called DRs, but have a region that interacts with the TNFR-associated factor (TRAF) proteins.[316] Interestingly, some of the DRs,

including TNFR-1, can indirectly recruit TRAF proteins, which may explain cross-talk between certain DD-containing and DD-lacking receptors.[317,318,319] Signals transduced by the non-DDcontaining receptors are frequently prosurvival while that of the DRs are typically proapoptotic. In all, over 20 members of the TNFR family in human have been identified.[59] With a few exceptions (EDAR and XEDAR), essentially all TNFRs play important regulatory roles in the immune system.

Signaling by the TNFRs is initiated when the cognate ligands, which are obligate trimers, contact the preformed receptor complex.[317,320] Downstream signaling of DRs requires the recruitment of DD-containing and/or

P.734

DED-containing proteins. For Fas, receptor engagement results in rapid recruitment of FADD and caspases-8 or -10 within the DISC, as detailed previously.[214] Other DRs such as TNFR-1 may require recruitment of an additional adaptor molecule like TRADD prior to the docking of FADD.[321] These events rapidly culminate in caspase activation. The non-DD-containing receptors mediate NF-κB induction and the activation of c-Jun kinases through the recruitment of TRAF proteins, a property that is shared by some DRs such as TNFR-1. Recruitment of TRAF proteins by DRs may counter the proapoptotic response through their interactions with cellular IAPs. “Knock-out” analyses in mice reveal that many of these signaling intermediates, including TRAF2,[322] RIP,[258] and components of the NF-κB activation pathway,[323,324,325,326,327] are essential for conferring protection against apoptosis induced by DR ligands. Deficiency of

P.735

NF-κB tends to sensitize cells to TNF-induced death.[328,329,330] TRAF2 and NF-κB induction through RIP may act in concert to promote survival in response to TNF.[331] Interestingly, unlike the TNFR-1- and Fas-deficient mice, which are viable, knockout of the signaling components such as FADD or TRADD often results in embryonic lethality. The discordant results in knockout animals imply that TNFR signaling intermediates are involved in ontogenetic processes other than PCD. Alternatively, other TNFRs may mediate critical PCD events during development using the same set of signaling molecules. However, many other TNFR-deficient animals, including Fas, DR6, or multiply deficient animals (TNFR1-/-, TNFR2-/-, and $Fas^{lpr/lpr}$ together) are viable, thus arguing against the latter hypothesis.[226,227,261]

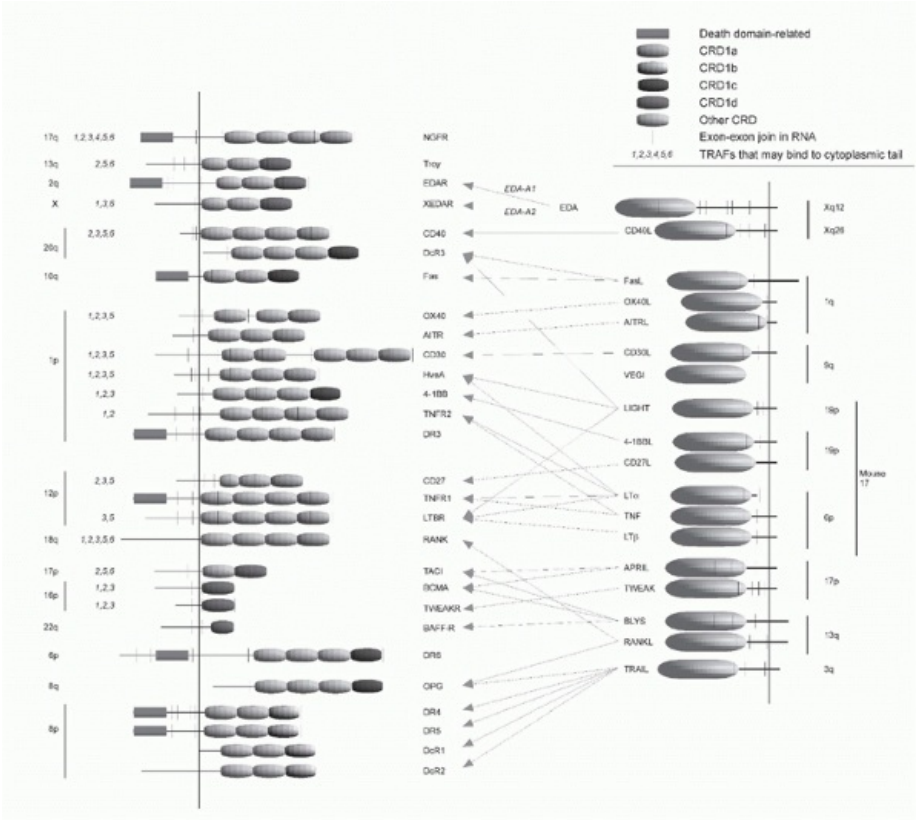

FIG. 30.5. Interacting Proteins of the Tumore Necrosis Factor (TNF)/TNF Receptor (TNFR) Superfamily. TNFR- and TNF-related proteins are shown at the left and right of the figure, respectively, with *arrows* connecting ligand-receptor pairs. Cysteine-rich domains (CRDs) are shown as *small ovals*. The NH2-terminal CRDs (CRD1a, 1b, 1c, 1d) are grouped on the basis of sequence similarity as indicated by the use of *colors* in the figure. *Small vertical lines* denote the locations of intron excision sites from the ribonucliec acids that encode the proteins (this information was not available for RANK, DcR1, and DcR2). *Red boxes* mark the locations of death domain-related sequences in the cytoplasmic regions of the TNFR-related proteins. *Numbers* to the immediate left of the TNFR cytoplasmic regions denote known or inferred interactions with the indicated TRAFs. The locations of the human genes that encode the proteins are provided at the extreme left and right of the figure; the mouse cluster on chromosome 17 is also noted. (Figure adapted from Locksley et al.[59] and Nagata and Golstein,[60] with permission from Cell Press by Nigel Killeen.)

Bcl-2 Gene Family

Bcl-2 and Bcl-X_L are the prototypes of a diverse family of apoptosis-regulatory proteins whose familial relationships are conferred by four impressively short homology regions, termed BH-1 to BH-4 (Fig. 30.6).[332,333] Proteins in this family can be classified into three groups: 1) the "multidomain" antiapoptotic proteins (Bcl-2, Bcl-X_L, Mcl-1, Bcl-w, A1, and BOO), which contain the four BH domains, 2) the "multidomain" proapoptotic Bax, Bak, and Bok that possess BH1-3 domains, and 3) the "BH3-only" proteins (Bid, Bad, Bim, Bik, Noxa, and Puma). Bcl-2 was identified at the chromosomal breakpoint of t(14;18)-bearing follicular

B-cell lymphomas. Vaux and Cory demonstrated that the oncogenic effect of Bcl-2 could be attributed to enhanced cell survival rather than proliferation.[169,334] Consequently, Bcl-2 represented a new class of death-preventing oncogenes that collaborates with growth-promoting oncogenes, such as Myc.[335] Abnormal overexpression of Bcl-2 is common in lymphoid and nonlymphoid malignancies.[336] Bcl-2 and Bcl-X_L regulate various types of immune cell death caused by lymphokine withdrawal, γ-irradiation, and chemical death inducers such as glucocorticoids, phorbol esters, DNA-damaging agents, and ionomycin, all of which provoke mitochondrial apoptosis.[337] Apoptosis induction is one means by which cancer chemotherapeutics exert an antitumor effect. Thus, as apoptosis antagonism may be as important in transformation as mitogenesis, new therapeutic strategies aimed at antagonizing Bcl-2 may be effective in cancer therapy (see following discussion).

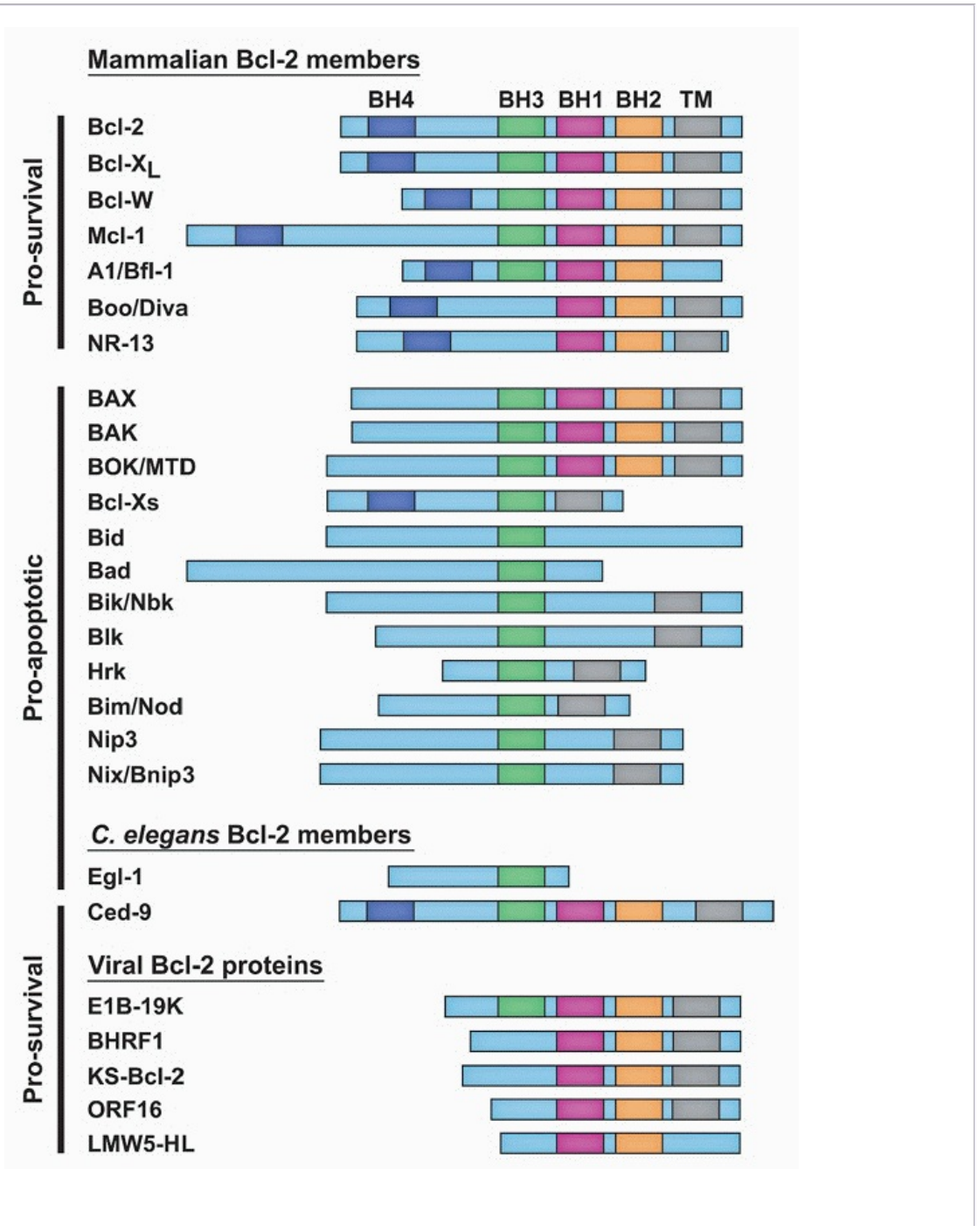

FIG. 30.6. Schematic Diagram of Members of the Bcl-2 Family. The *dark blue, green, red, and orange boxes* represent the different Bcl-2 homology domains, and the *gray boxes* designate the hydrophobic transmembrane region that is required for insertion into the mitochondrial membrane.

Mechanisms by which Bcl-2 family proteins regulate cell death remain unclear, but several regulatory principles have been delineated.[332] Key family members, including Bcl-2, Bcl-X_L, Bak and Bax, associate with intracellular membranes, especially the OMM, via a carboxy-terminal hydrophobic domain.[338,339,340,341] Death regulation by the Bcl-2 family ultimately involves controlling the permeability and integrity of the OMM. Bax and Bak directly participate in the formation of an "apoptotic pore" upon activation and oligomerization, which permeabilizes the OMM. This critical step results in mitochondrial release of cytochrome c, activation of cytosolic caspases, and commitment of the cell to apoptosis.[342] Bcl-2 family members form homotypic and heterotypic dimer complexes. The balance between pro- and antiapoptotic members determines cell fate probably through direct interactions with each other.[343,344,345,346,347,348] The precise stoichiometry of these associations that leads to survival or death has not been defined. For example, it remains unclear whether BH3-only proteins bind and directly activate Bax/Bak, or instead serve to displace prosurvival Bcl-2 proteins to facilitate indirect activation.[349] Recent data demonstrates that prosurvival Bcl-2 proteins prevent Bax oligomerization by directing constant retrotranslocation of Bax from mitochondrial membranes into the cytosol, lending credence to the latter model.[98] Regulation by these proteins occurs "upstream" of caspase activation, but they may also control caspase-independent forms of cell death including autophagic cell death.[246] These proteins have their most important effects on mitochondrial death pathways and comparatively less effect on DR-initiated pathways. Diverse pathways, such as transcriptional induction (Bim, Puma, Noxa, and Bcl-X_L), phosphorylation (Bad), processing (Bid), conformational changes (Bax and Bak), or organelle translocation (Bim, Bid, Bax), control the presence and biologic activity of Bcl-2 family members.[108,350] Each of these

principles has reported exceptions, but they constitute the current basis for our understanding of the Bcl-2 family of proteins.

The biologic function of Bcl-2 family members has been examined in genetically engineered mice and tissue culture cells.[99] Bcl-X_L-deficient mice die around embryonic day 13 due to massive neuronal apoptosis.[351] By contrast, Bcl-2-deficient mice survive a few weeks postnatally but then develop polycystic kidney disease, hypopigmentation due to decreased melanocyte survival, and most importantly, massive apoptotic loss of all B- and T-lymphocytes.[352] Thus, while Bcl-2 is not necessary for lymphocyte maturation, it is indispensable for the maintenance of mature lymphocytes. On the other hand, expression of Bcl-X_L is required for the survival of DP (CD4+CD8+) thymocytes, B220+ bone marrow cells, and mature B cells.[332] Mcl-1 deficiency results in peri-implantation lethality.[353] A conditional knockout in the T-cell compartment increases apoptosis at the double negative 2 stage and arrests the development of both B- and T-lymphocytes. Mcl-1 is also essential for mature

lymphocyte survival, and experimental elimination of this molecule triggers their rapid loss.[147] Transgenic experiments in which Bcl-2 or Bcl-X_L were overexpressed suggest, however, that the two proteins can functionally substitute for one another and govern a final common death pathway.[332] Selective transgenic overexpression of Bcl-2 in B or T cells leads to increased numbers of those cells and the late onset of lymphomas.[354,355] Bcl-2 overexpression also blocks Apaf-1- and caspase-9-independent pathways of apoptosis, demonstrating the broad inhibitory effect of Bcl-2.[356] Furthermore, Bcl-2 was recently shown to suppress Beclin 1 autophagic cell death, a novel form of nonapoptotic cell death.[20,357] Deficiency of the proapoptotic Bax protein has a surprisingly mild phenotype in mice, causing only abnormalities in testis development and sterility.[358] Deficiency of the proapoptotic Bak protein also causes very little effect in mice. However, a combined deficiency of Bax and Bak causes multiple organ abnormalities and perinatal death due to failed apoptosis.[359] This includes an accumulation of lymphocytes and other hematopoietic cells, underscoring the importance of the Bax and Bak proteins for normal immune homeostasis.

Recent work has focused on the BH-3-only subset (see Fig. 30.6). Structural analysis has revealed that Bcl-2 family members interact through a hydrophobic pocket formed by the confluence of the BH-1, BH-2, and BH-3 helices. The interactions between Bcl-2 family members have a characteristic selectivity and hierarchy that may depend principally on binding to the BH-3 helix.[360] The BH-3-only proteins may selectively regulate the other family members through interactions at the hydrophobic pocket. Genetic ablation experiments have demonstrated the critical role of this family during developmentally PCD as well as stressinduced death.[99] So far, only one BH-3-only protein, called egg laying defective 1, has been characterized in *C. elegans*. In mammals, the BH-3-only subset includes Bad, Bik, Bid, HRK, Bim, Noxa, Puma, Bmf, and perhaps others as the BH-3 motif is only a 9 to 16 amino acid long of alpha helix. Induction of cell death by BH3-only proteins requires Bax and Bak, indicating that BH3 proteins act upon or upstream of proapoptotic relatives.[361,362] The activity of BH-3-only proteins is governed transcriptionally and posttranscriptionally in a highly regulated fashion according to their function. Bid is cleaved by caspase-8 yielding a 15 kDa fragment that is myristoylated, which can insert into the mitochondrial membrane. This triggers the oligomerization of Bax and Bak thereby connecting the DRs to the mitochondrial pathway of death.[191,195,342,363,364,365] Phosphorylation of Bad and Bim can control their association with 14-3-3 proteins or the microtubule-associated dynein motor complex, respectively.[108] When released, Bad and Bim can induce apoptosis through the mitochondrial pathway. Interestingly, most of Bim is bound to Bcl-2 and Bcl-X_L at the mitochondria in both resting and activated T-lymphocytes.[366] Genetic deficiency of Bim protects against a variety of death inducers, such as cytokine deprivation, γ-radiation, glucocorticoids, ionomycin, DNA damage, etoposide, and Taxol, but not FasL.[51] Deficiency of Bim also significantly impairs TCRinduced thymocyte selection. These diverse forms of regulation provide a mechanism for a variety of different apoptosis inducers to converge on the mitochondrial pathway of death.

The mechanism by which the BH3-only proteins control cell death is still an intense field of research. Studies carried out with peptides corresponding to the α-helical BH3 domains

distinguish two different subgroups of BH3-only proteins.[367,368,369,370] The “activating BH3” proteins (including Bim and Bid) can directly trigger death by Bax and Bak besides counteracting Bcl-2 and Bcl-X_L. The second group includes Bad and Bik and is called “derepressor BH3” or “sensitizing BH3.” These molecules suppress the antiapoptotic activity of Bcl-2 and Bcl-X_L but fail to activate the executioners Bax and Bak. They thus sensitize the mitochondria by increasing OMM permeability but require the intervention of an additional activator of Bax to induce apoptosis. Although it was originally thought that Bax/Bak induced mitochondrial fission and outer membrane permeabilization through the so-called “permeability transition pore,” later studies have contested its role.[371] In general, intrinsic apoptosis is always accompanied by fission of mitochondria, which participates in the release of various inducers of death, including cytochrome c.[228] In fact, fission intermediates may play a direct role in Bax oligomerization and mitochondrial fission and outer membrane permeabilization.[372] Continued study of the mechanism by which these proteins interface with mitochondrial fission should yield further insights into their mode of action.

The development of drugs mimicking “sensitizing BH3” protein associations have been developed to modulate the antiapoptotic Bcl-2 proteins during cancer treatment.[336] A small organic molecule (ABT-737) with mechanistic similarity to Bad is a potent inhibitor of the antiapoptotic molecules Bcl-2, Bcl-X_L, and Bcl-w. Resembling a Bad-derived peptide, ABT-737 can bind antiapoptotic Bcl-2 proteins to block cytosolic retrotranslocation of Bax, but does not directly activate Bax and Bak.[98] ABT-737 exhibits synergistic cytotoxicity with chemotherapeutics and radiation in vitro and in vivo. It displays single-agent activity against lymphoma, small-cell lung carcinoma, primary patient-derived cells, and promotes the regression of solid tumors.[206]

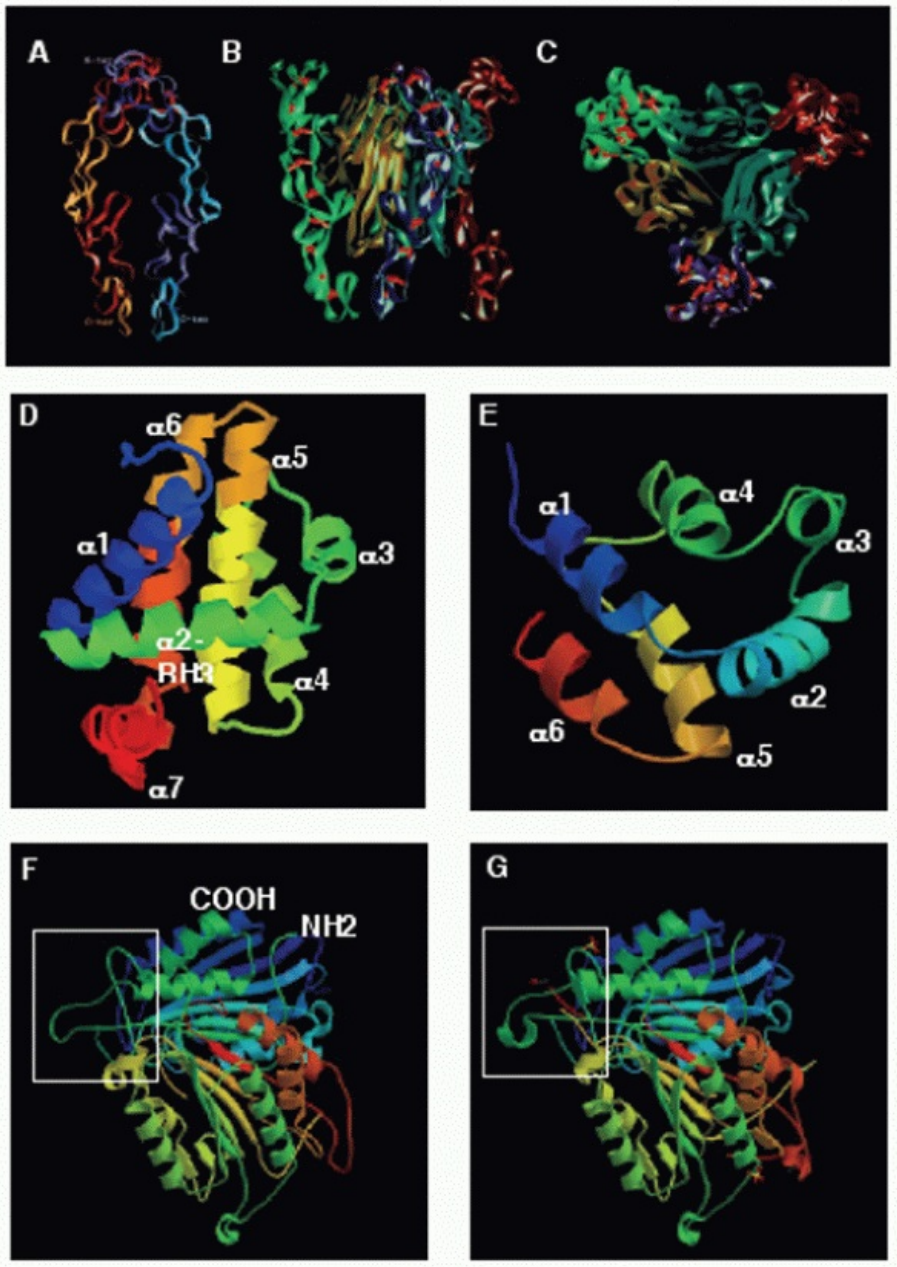

FIG. 30.7. Structural Biology of Apoptosis. A: Structure of the unliganded tumor necrosis factor (TNF) receptor (TNFR)-1. **B:** Side view of the TNFβ-bound TNFR-1 looking from the plane of the membrane. Conserved disulfide bonds that are structurally essential are shown in *red*. **C:** Bird's eye view of the TNF/TNFR-1 structure looking down at the plane of the membrane. **D:** NMR structure of Bcl-X_L. The BH3 (α2) domain runs perpendicularly across the molecule and the two central hydrophobic helices (α5 and α6) are buried in the middle portion of the molecule. **E:** Structure of the Fas DD, a prototypical hexahelical bundle. The bundle is composed of six helices, a structural scaffold that is conserved in also the death effector domain and the caspase recruitment domain. The position of each of the helices is indicated. **F,G:** Structures of caspase-7 before (*F*) and after (*G*) proteolytic autoprocessing. Notice that the catalytic site (*box*) in the proenzyme is relatively "loose" (*F*). Processing of the proenzyme results in substantial "tightening" of the catalytic loops. The *red-colored strand inside the boxed area* in (*G*) denotes the position of the pseudosubstrate tetrapeptide DEVD.

Apoptotic Protease-Activating Factor Proteins

Mammalian Apaf-1 is critical for the mitochondrial pathway of apoptosis. Apaf-1 is structurally and functionally homologous to the *C. elegans* Ced-4 protein.[373] Both Apaf-1 and Ced-4 contain an NH_2-terminal CARD domain followed by a nucleotide-binding oligomerization domain (NOD). Ced-4 can complex with caspase-9 via CARD domains present in both molecules. The NOD domain is essential for binding adenosine triphosphate and homooligomerization. In addition, Apaf-1 also contains WD-40 repeats at the carboxy-terminus, which bind cytochrome c released from damaged mitochondria. Interestingly, *C. elegans* Ced-4 contains no carboxy-terminal WD-40 repeats and does not respond to mitochondrial insults.[374] Apaf-1, cytochrome c, and adenosine triphosphate are all required to properly activate procaspase-9.[230,375] Genetic evidence from knockout animals revealed that Apaf-1 and caspase-9 are obligatory for the apoptosis response through p53 to DNA damage and other mitochondrial death events.[376,377,378]

Other proteins similar to Apaf-1/Ced-4 have been identified, most of which contain regions of homology to the NOD domain. Overexpression studies suggest that several of these (NOD1, NOD2, IPAF, and cryopyrin) may modulate apoptosis but most evidence indicates they chiefly mediate proinflammatory responses. These proteins initiate intracellular responses to peptidoglycan or other bacterial antigens, through NF-κB induction and/or caspase-1 maturation.[379] One of these proteins, NOD2, has been mapped as a susceptibility gene for Crohn disease.[380,381] The CARD domain of NOD2 and related inflammasome components likely involves these proteins in apoptosis as well as inflammatory responses. The adaptor protein ASC, which contains both CARD and pyrin domain, also coordinates divergent inflammasome proteins for caspase-1 processing and pyroptosis induction.[173] Hence, combinations of protein interaction motifs like the CARD, NOD, and other signaling domains in single adapter proteins promotes bifurcation of biological responses from the same receptor.

STRUCTURAL REGULATION OF PROGRAMMED CELL DEATH

Tumor Necrosis Factor/Tumor Necrosis Factor Receptor Structure

The recent determination of structures of components of the PCD pathways has led to a better physical sense of how these death programs work (Fig. 30.7). In almost all cases, the formation of specific stoichiometric protein complexes is crucial for apoptosis signaling. Receptor-mediated apoptosis by DRs within the TNFR superfamily is triggered by receptor-specific ligands within the TNF superfamily.[59] The defining structural motif of the TNFR superfamily, the CRD, is a 40-amino acid cluster of β strands folded back on themselves and pinned in place by three disulfide bonds formed by six cysteines in highly conserved positions (see Fig. 30.7A). Most of the TNFRs have multiple CRDs, and these serve two primary functions depending on location. The membrane-proximal or central CRDs mediate ligand binding. For example, in TNFR-1, the two central CRDs, CRD2 and 3, provide the key ligand-binding contacts. The membrane distal CRD mediates receptor preassembly on the cell

surface.[382,383] This membrane-distal CRD along with adjacent amino-terminal sequences is called the “preligand assembly domain” (PLAD). It promotes the oligomerization of like receptor chains prior to ligand engagement. Preassembly of receptor chains into trimers or perhaps other oligomeric complexes is obligatory for both ligand binding and signal transduction. The PLAD has been identified in both DRs such as Fas, TNFR-1, and TRAIL receptors as well as in non-DD receptors such as TNFR-2.[382,383] The orientation of the receptor chains is likely to change drastically between the unliganded and liganded complexes as reflected in the extant crystal structures for unliganded and liganded TNFR-1 (see Fig. 30.7A and B). The receptor chains are initially attached at the membrane distal CRD, and then these interactions fall apart as the receptor admits the ligand between three receptor chains to interact with the CRDs closer to the membrane. The discovery of the PLAD overturned the prevailing model that TNFR family members signal by the ligand “cross-linking” solitary receptor chains into a trimeric complex. Instead, the ligand will bind to and change the configuration of preassembled receptor complexes. Hence, CRDs can regulate both intra- or intermolecular receptor associations.

The ligands for TNFRs are typically obligate homotrimers that adopt a compact “jelly-roll” conformation with a hydrophobic face where the individual subunits stably interact with each other.[59,384] Certain exceptions, such as LTα and LTβ, can form heterotrimers. The ligand subunits are usually synthesized as type II transmembrane proteins and, following trimerization, may remain membranebound or undergo metalloproteinase cleavage to a soluble form.[385] Binding of the preassembled receptor complex with the trimeric ligand, forms a symmetric three-to-three ligand-receptor complex that is evident in the crystal structures of the liganded TNFR1 and TRAIL receptors.[301,386,387,388,389] Functionally, the ligand interaction appears to reorient the receptor chains so that the intracellular domains adopt a specific juxtaposition favoring the formation of intracellular signaling complexes. The recently solved structure of the TRAIL trimer differs from the TNF structure in that it contains a loop insertion in the first β-strand that is critical for receptor association.[301,386,387,388,389] In both the TNFR-1 and TRAIL-R2 crystals, ligand contacts appear to be restricted to the second and third CRDs. The extensive receptor-receptor interaction in the PLAD region found in the unliganded TNFR-1 receptor structure is absent in the ligand-bound receptor crystal structures.[390,391,392,393] How the ligand initially contacts the preformed receptor complex and initiates chain rearrangement are important unresolved questions.

The fundamental three-fold symmetry of the TNF superfamily ligands and receptors appears to be conserved among the proximal signaling complexes, including the receptor-TRAF2 interaction[394,395] and the TRADD/TRAF2 interaction.[396,397] This not true of the Fas/FADD interaction, however, based on recent structural studies that show that the Fas-FADD DDs associate in an asymmetric oligomeric complex of five to seven Fas DD and five FADD DD in a layered helical

structure comparable to the PIDD-RAIDD DD complex.[217] This interesting oligomeric structure apparently requires hexameric or membrane-bound FasL for assembly during Fas signaling and explains the potent dominant-negative effects observed for Fas mutations occurring in patients with ALPS (see following). The structure also reveals how the FADD

DED can interact with the caspase-8/-10 DEDs for caspase recruitment and activation through aggregation. This helical symmetry common to DD-containing signaling assemblies may regulate the strength of signal transduction by permitting a variable number of binding partners with specificity, while establishing a threshold for signaling through aggregation. 216 The importance of the trifold symmetry in the receptor signaling apparatus is highlighted by genetic mutations found in both human and mouse. For instance, heterozygous mutations in the cytoplasmic DD of Fas found in the *lpr*cg (mouse) or Fas-defective form of ALPS resulted in a greater than 50% reduction in receptor signaling.[398,399,400] This is likely due to the fact that inclusion of at least one bad subunit in the receptor trimer abrogates signaling.[400]

The genetic effect by which a mutant allele encodes a protein that interferes with the same protein encoded by a wild-type allele is called *dominant interference*. Dominant interference is usually observed in proteins that function by forming multiprotein complexes, which is now known to be a common feature in PCD pathways. A dominant-interfering disease mechanism may be especially important in PCD disorders in outbred human populations, where most gene loci are heterozygous rather than homozygous. However, disruptive mutations described in the Fas DD for patients with ALPS-FAS did not localize to interfaces with FADD in the first Fas-FADD DD complex structure. Hence, a structural explanation of dominant interference remained poorly understood.

The recent solution of a new and revised protein complex structure containing the Fas and FADD DDs, crystallized under more physiologic conditions, clarifies this question and underscores the potency of dominant interfering mutations associated with ALPS-FAS.[217] Wu and colleagues demonstrated that the Fas-FADD DD complex contains five to seven Fas DDs and five FADD DDs in a layered structure comparable to the PIDD-RAIDD DD complex.[401] In this structure, debilitating ALPS-associated mutations are localized to specific interfaces between these layered DDs. The severity of the Fas DD mutation correlates with the number of affected interfaces containing it, and typically precludes complex formation with FADD DDs. Based on 5:5 stoichiometry of Fas and FADD DDs, the proportion of wild-type Fas signaling complexes in a patient with heterozygous ALPS-FAS, assuming equal expression of wild-type and mutant alleles, is less than $(1/2)^5$ or ~3% of total signaling complexes. This explains the disproportionately severe effect of heterozygous mutations on function. The new crystallographic information about the Fas signaling complex also implies that two trimers of FasL are required to form this complex in vivo by bringing six Fas DDs in close proximity. These findings are congruent with previous work demonstrating that two FasL trimers, normally presented on an adjacent cell in membrane-bound form, constitute the minimal, competent ligand component for initiating Fas-induced apoptosis.[402,403] Thus, our understanding of the Fas receptor reveals that while higher order oligomeric signaling complexes provide cooperative, rapid, and regulatable signaling for apoptosis induction, they also create a vulnerability to genetic disease. A dominant-interfering disease mechanism may be especially important in PCD disorders in outbred human populations, where most gene loci are heterozygous rather than homozygous.

Bcl-2 Homology Structures

Bcl-X_L has the best-characterized structure, and it suggests interesting hypotheses regarding the function of Bcl-2 family members.[209] It contains a conserved nest of alpha helices

including a backbone of two hydrophobic helices that are encircled by several amphipathic helices including the BH4 helix, which is a key interaction site for members of the Bcl-2 family (see Fig. 30.7D). The structure has revealed a tantalizing similarity to pore-forming domains of bacterial toxins. This has stimulated a wealth of research into whether membrane pore formation is a key element of apoptosis modulation by Bcl-2 family proteins.[404,405] It is interesting to consider that pore-forming toxins of bacteria have homology to regulatory proteins whose primary site of action is the mitochondria (which is believed to have evolved as a bacterialike organism subsumed into eukaryotic cells). Structurally, Bcl-2 homology regions, BH1, BH2, and BH3, are widely spaced in the primary sequence but closely apposed in a hydrophobic pocket. Through this pocket, the proapoptotic and antiapoptotic members of the Bcl-2 family interact and cross-regulate one another. Proteins with only an amphipathic BH3 helix can also interact with this pocket in the antiapoptotic Bcl-2 family members and potentially nullify their protective function.[209] The specific amino acids contained in this pocket dictate a select hierarchy of interactions between various members of the Bcl-2 family. Hence, the structure reveals a possible functional interaction with the mitochondrial membrane and explains associations between members of the Bcl-2 family that determine survival or death.

The Hexahelical Bundle

The DDs, DEDs, and CARDs are critical protein-protein interaction motifs in DR-mediated PCD. Interestingly, all three domains adopt a similar structural scaffold of a compact bundle of six antiparallel α-helices, which we will refer to as the "hexahelical bundle" (see Fig. 30.7E). This structure is an ancient one that is related to the "ankyrin repeat," a protein interaction motif occuring in a plethora of proteins and conserved throughout phylogeny.[59,209] Despite remarkable overall similarity, significant differences exist among the DDs, DEDs, and CARDs, which likely account for their binding specificities. They usually form homotypic associations. For example, the surface of Fas DD is made up of mostly charged residues,[406] whereas the FADD DED contains hydrophobic patches that are important for binding to caspase-8.[208] The six helices in the CARD domain of RAIDD form an acidic surface on one side of the molecule and a basic region on the other side of the molecule, which

may be important for binding of the corresponding CARD domain in caspase-2.[407] Structural and mutagenesis data corroborate the notion that helices 2 and 3 are critical contact regions for homophilic DD and DED interactions. In addition to α2 and α3, homophilic CARD/CARD interactions appear to involve electric dipole interactions between α2, α3, α5 on one molecule and α1, α4 on the partner. Examination of the CARD domain from Apaf-1 yields similar observations.[408,409] Thus, specific molecular pathways are determined by defining residues in DDs, DEDs, or CARDs presented on a common scaffold. It is also important to recognize that DDs, DEDs, and CARDs are primarily protein-protein interaction domains, and although they were initially discovered in apoptosis pathways, homologues of these motifs may be utilized by nature to associate proteins in pathways unrelated to apoptosis. In fact, a class of proteins containing the pyrin domain also appears to use a hexahelical bundle structural scaffold.[410,411,412] The pyrin domain was originally identified in a protein mutated in

patients with familial Mediterranean fever, thus providing a molecular link between apoptosis and inflammatory diseases.

An intriguing feature of hexahelical bundles is that they often occur in two tandem copies in proteins that promote or inhibit apoptosis. For example, the tandem DEDs in caspase-8 or -10 can augment apoptosis, whereas those in viral or cellular FLIPs inhibit apoptosis. The recent solution of the crystal structure of a viral FLIP, MC159, at 1.2 Angstroms resolution revealed a noncanonical fold of DEDs with a rigid dumbbell-shaped structure. Each DED played a unique role in the overall structure to generate an asymmetric interaction with DD proteins over an extensive surface.[297] MC159 assembles into the DISC with Fas and FADD via a surface topology that differs from proapoptotic molecules. This blocks FADD self-association, which disrupts the higherorder oligomerization of the Fas receptors on the cell surface that is required for caspase activation.

Caspase Structure

The recruitment of DD-, DED-, or CARD-containing proteins eventually leads to the complex formation and activation of caspases. The structures of caspase-1, -3, -7, -8, and -9 have been determined.[235,289,290,292,293,294,302,303] Each caspase is composed of small and large subunits of approximately 10 and 20 kDa (generally referred as p10 and p20, respectively). The catalytic subunits of all caspases are similarly comprised of two p10/p20 heterodimers. Each p10/p20 heterodimer is folded into a compact cylindrical structure of six β strands and five α helices. The active site of the enzyme is formed by loops contributed by both the p10 and p20 subunits that come together at the top of the cylinder of β strands. The two heterodimers are aligned in an antiparallel fashion where the β sheet forms the vertical axis of the tetrameric complex (see Figs. 30.6 and 30.7F,G). Despite the overall structural similarity, distinct differences, especially in loops surrounding the catalytic active site, can explain the respective substrate preferences between different caspases.

Regulation of caspases by viral inhibitors has also been elucidated by structural studies. The baculovirus p35 is a potent inhibitor of many caspases that requires cleavage at its caspase recognition residue D87 to manifest inhibitory activity. Crystallographic studies reveal the formation of a thioester bond between the active site cysteine of caspase-8 (C360) and the aspartate of the amino-terminal cleavage product of p35.[413] Lu et al. showed that following caspase-8 processing, the buried p35 NH_2-terminus portion is liberated and undergoes a native chemical ligation reaction between residues D87 and C2. This mechanism, also used by selfsplicing proteins, leads to the formation of a circular NH2-terminal p35 peptide, which is kept bound to the caspase-8 active site to ensure its long-lasting inhibitory effect.[414]

CONCLUSION

The study of PCD has provided insights into many aspects of immune function, particularly in the establishment of central and peripheral tolerance and the numerical homeostasis of immune cells. PCD plays a vital part in the clonal composition, both qualitatively and quantitatively, of the immune repertoire. Immune cells utilize conserved mechanisms of apoptosis and necrosis that are common to perhaps all mammalian cells. Indeed, the immune system has been one of the most instructive model systems for understanding how these networks function. Finally, death is regulated by cues from the environment. For developing lymphocytes, the antigen environment determines cell survival and elimination. For mature

cells, antigen and growth cytokines determine life or death. In this manner, the immune system can develop a wide repertoire of reactive cells, select the most useful members of the repertoire, and then expand and contract specific clonotypes as needed for specific immune responses. Such homeostatic control allows rapid cell proliferation in protective responses while preserving tolerance and avoiding autoimmunity and immune cell malignancies. There are still important challenges in this area of investigation. The molecular mechanisms of antigen receptor-induced apoptosis and necrosis are still not fully understood. Fundamental questions, such as what proteolytic substrates of caspases are crucial for apoptosis, necrosis, or proinflammatory signaling, remain unanswered. Also, the genetic or environmental influences that cause autoimmunity in certain individuals with Fas defects but not others await definition. Finally, harnessing our understanding of PCD in the immune system for the diagnosis, prevention, and treatment of immunologic diseases is an exciting frontier for the future.

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REFERENCES

1. Conlon I, Raff M. Size control in animal development. *Cell*. 1999;96(2): 235-244.

2. Cohen JJ. Apoptosis: mechanisms of life and death in the immune system. *J Allergy Clin Immunol*. 1999;103(4):548-554.

3. Green DR, Cotter TG. Introduction: apoptosis in the immune system. *Semin Immunol*. 1992;4(6):355-362.

4. Lenardo M, Chan KM, Hornung F, et al. Mature T lymphocyte apoptosis—immune regulation in a dynamic and unpredictable antigenic environment. *Annu Rev Immunol*. 1999;17:221-253.

5. Zheng L, Fisher G, Combadiere B, et al. Mature T lymphocyte apoptosis in the healthy and diseased immune system. *Adv Exp Med Biol*. 1996;406:229-239.

6. Raff M. Cell suicide for beginners. *Nature*. 1998;396(6707):119-122.

7. Boguski MS, Tolstoshev CM, Bassett DE Jr. Gene discovery in dbEST. *Science*. 1994;265(5181):1993-1994.

8. Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y, Jacobson MD. Programmed cell death and the control of cell survival. *Philos Trans R Soc Lond B Biol Sci* . 1994;345(1313):265-268.

9. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* . 1972;26(4):239-257.

10. Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* . 1980;68:251-306.

11. Clarke PG, Clarke S. Nineteenth century research on naturally occurring cell death and related phenomena. *Anat Embryol (Berl)* . 1996;193(2):81-99.

12. Nicholson D, Thornberry N. Caspases: killer proteases. *TIBS*. 1997;22: 299-306.

13. Alam A, Cohen LY, Aouad S, Sekaly RP. Early activation of caspases during T lymphocyte stimulation results in selective substrate cleavage in nonapoptotic cells. *J Exp Med*. 1999;190(12):1879-190.

14. Kennedy NJ, Kataoka T, Tschopp J, Budd RC. Caspase activation is required for T cell proliferation. *J Exp Med*. 1999;190(12):1891-1896.

15. Coligan JE, ed. *Current Protocols in Immunology*. New York, NY: Wiley; 1991.

16. Reed JC, ed. *Apoptosis*. New York, NY: Academic Press; 2000.

17. Chan FK, Shisler J, Bixby JG, et al. A role for tumor necrosis factor receptor-2 and receptor-interacting protein in programmed necrosis and antiviral responses. *J Biol Chem*. 2003;278(51):51613-51621.

18. Holler N, Zaru R, Micheau O, et al. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat Immunol* . 2000;1(6):489-495.

19. Degterev A, Huang Z, Boyce M, et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat Chem Biol* . 2005;1(2):112-119.

20. Yu L, Alva A, Su H, et al. Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science*. 2004;304(5676):1500-1502.

21. Yu L, Wan F, Dutta S, et al. Autophagic programmed cell death by selective catalase degradation. *Proc Natl Acad Sci U S A* . 2006;103(13): 4952-4957.

22. Thompson CB. Apoptosis. In: Paul WE, ed. *Fundamental Immunology*. 4th ed. Philadelphia, PA: Lippincott-Raven; 1999.

23. Green DR, Ferguson T, Zitvogel L, Kroemer G. Immunogenic and tolerogenic cell death. *Nat Rev Immunol* . 2009;9(5):353-363.

24. Metzstein MM, Horvitz HR. The C. elegans cell death specification gene ces-1 encodes a snail family zinc finger protein. *Mol Cell* . 1999;4(3):309-319.

25. Lodish H, Berk A, Zipursky L, Matsudaira P, Baltimore D, Darnell J. *Molecular Cell Biology*. 4th ed. New York, NY: W.H. Freeman; 2000.

26. Ashton-Rickardt PG, Tonegawa S. A differential-avidity model for T-cell selection. *Immunol Today*. 1994;15(8):362-366.

27. Ashton-Rickardt PG, Bandeira A, Delaney JR, et al. Evidence for a differential avidity model of T cell selection in the thymus [see comments]. *Cell*. 1994;76(4):651-663.

28. Lenardo MJ. Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. *Nature*. 1991;353(6347):858-861.

29. Critchfield JM, Lenardo MJ. Antigen-induced programmed T cell death as a new approach to immune therapy. *Clin Immunol Immunopathol* . 1995;75(1):13-19.

30. Weiner NL. *Cybernetics, Control and Communication in the Animal and Machine* . New York, NY: M.I.T. Press; 1961.

31. von Boehmer H, Teh HS, Kisielow P. The thymus selects the useful, neglects the useless and destroys the harmful. *Immunol Today*. 1989;10(2):57-61.

32. Jameson SC, Hogquist KA, Bevan MJ. Positive selection of thymocytes. *Annu Rev Immunol*. 1995;13:93-126.

33. Sprent J, Kishimoto H. The thymus and central tolerance. *Philos Trans R Soc Lond B Biol Sci*. 2001;356(1409):609-616.

34. Jiang D, Zheng L, Lenardo MJ. Caspases in T-cell receptor-induced thymocyte apoptosis. *Cell Death Differ* . 1999;6(5):402-411.

35. Sprent J, Tough DF. T cell death and memory. *Science*. 2001;293(5528): 245-248.

36. Berg LJ, Kang J. Molecular determinants of TCR expression and selection. *Curr Opin Immunol*. 2001;13(2):232-241.

37. Germain RN. The T cell receptor for antigen: signaling and ligand discrimination. *J Biol Chem*. 2001;276(38):35223-35226.

38. Conte D, Liston P, Wong JW, Wright KE, Korneluk RG. Thymocytetargeted overexpression of xiap transgene disrupts T lymphoid apoptosis and maturation. *Proc Natl Acad Sci U S A*. 2001;98(9):5049-5054.

39. DeYoung AL, Duramad O, Winoto A. The TNF receptor family member CD30 is not essential for negative selection. *J Immunol*. 2000;165(11):6170-6173.

40. Page DM, Roberts EM, Peschon JJ, Hedrick SM. TNF receptor-deficient mice reveal striking differences between several models of thymocyte negative selection. *J Immunol*. 1998;160(1):120-133.

41. Sidman CL, Marshall JD, Von Boehmer H. Transgenic T cell receptor interactions in the lymphoproliferative and autoimmune syndromes of lpr and gld mutant mice. *Eur J Immunol*. 1992;22(2):499-504.

42. Alberola-Ila J, Forbush KA, Seger R, Krebs EG, Perlmutter RM. Selective requirement for MAP kinase activation in thymocyte differentiation. *Nature*. 1995;373(6515):620-623.

43. Mariathasan S, Jones RG, Ohashi PS. Signals involved in thymocyte positive and negative selection. *Semin Immunol*. 1999;11(4):263-272.

44. Werlen G, Hausmann B, Palmer E. A motif in the alphabeta T-cell receptor controls positive selection by modulating ERK activity. *Nature*. 2000;406(6794):422-426.

45. Kruisbeek AM, Amsen D. Mechanisms underlying T-cell tolerance. *Curr Opin Immunol*. 1996;8(2):233-244.

46. Bain G, Engel I, Robanus Maandag EC, et al. E2A deficiency leads to abnormalities in alphabeta T-cell development and to rapid development of T-cell lymphomas. *Mol Cell Biol*. 1997;17(8):4782-4791.

47. Penninger JM, Sirard C, Mittrucker HW, et al. The interferon regulatory transcription factor IRF-1 controls positive and negative selection of CD8+ thymocytes. *Immunity*. 1997;7(2):243-254.

48. Rivera RR, Johns CP, Quan J, Johnson RS, Murre C. Thymocyte selection is regulated by the helix-loop-helix inhibitor protein, Id3. *Immunity*. 2000;12(1):17-26.

49. Williams O, Brady HJ. The role of molecules that mediate apoptosis in T-cell selection. *Trends Immunol*. 2001;22(2):107-111.

50. Zhou T, Cheng J, Yang P, et al. Inhibition of Nur77/Nurr1 leads to inefficient clonal deletion of self-reactive T cells. *J Exp Med*. 1996; 183(4):1879-1892.

51. Bouillet P, Metcalf D, Huang DC, et al. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science*. 1999;286(5445):1735-1738.

52. Huang DC, Strasser A. BH3-Only proteins-essential initiators of apoptotic cell death. *Cell*. 2000;103(6):839-842.

53. Ladi E, Yin X, Chtanova T, Robey EA. Thymic microenvironments for T cell differentiation and selection. *Nat Immunol*. 2006;7(4):338-343.

54. Critchfield JM, Boehme SA, Lenardo MJ. The regulation of antigeninduced apoptosis in mature T lymphocytes. In: Gregory CC, ed. *Apoptosis and the Immune Response*. New York, NY: Wiley-Liss, Inc.; 1995:55-114.

55. Tan JT, Dudl E, LeRoy E, et al. IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc Natl Acad Sci U S A* . 2001;98(15):8732-8737.

56 Tanchot C, Lemonnier FA, Perarnau B, Freitas AA, Rocha B. Differential requirements for survival and proliferation of CD8 naive or memory T cells. *Science*. 1997;276(5321):2057-2062.

57. Reap EA, Felix NJ, Wolthusen PA, Kotzin BL, Cohen PL, Eisenberg RA. bcl-2 transgenic Lpr mice show profound enhancement of lymphadenopathy. *J Immunol*. 1995;155(11):5455-5462.

58. Strasser A, Harris AW, Huang DC, Krammer PH, Cory S. Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. *EMBO J*. 1995;14(24):6136-6147.

59. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell*. 2001;104(4):487-501.

60. Nagata S, Golstein P. The Fas death factor. *Science*. 1995;267(5203): 1449-1456.

61. Zheng L, Fisher G, Miller RE, Peschon J, Lynch DH, Lenardo MJ. Induction of apoptosis in mature T cells by tumour necrosis factor. *Nature*. 1995;377(6547):348-351.

62. Oberg HH, Lengl-Janssen B, Kabelitz D, Janssen O. Activationinduced T cell death: resistance or susceptibility correlate with cell surface fas ligand expression and T helper phenotype. *Cell Immunol*. 1997;181(1):93-100.

63. Devadas S, Das J, Liu C, et al. Granzyme B is critical for T cell receptor-induced cell death of type 2 helper T cells. *Immunity*. 2006;25(2): 237-247.

64. Sharma V, Delgado M, Ganea D. Granzyme B, a new player in activation-induced cell death, is down-regulated by vasoactive intestinal peptide in Th2 but not Th1 effectors. *J*

Immunol. 2006;176(1):97-110.

65. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature*. 1992;356(6367):314-317.

66. Watanabe-Fukunaga R, Brannan CI, Itoh N, et al. The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. *J Immunol*. 1992;148(4):1274-1279.

67. Kagi D, Odermatt B, Mak TW. Homeostatic regulation of CD8+ T cells by perforin. *Eur J Immunol* . 1999;29(10):3262-3272.

68. Kagi D, Vignaux F, Ledermann B, et al. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science*. 1994; 265(5171):528-530.

69. Mateo V, Menager M, de Saint-Basile G, et al. Perforin-dependent apoptosis functionally compensates Fas deficiency in activation-induced cell death of human T lymphocytes. *Blood*. 2007;110(13):4285-4292.

70. Sandalova E, Wei CH, Masucci MG, Levitsky V. Regulation of expression of Bcl-2 protein family member Bim by T cell receptor triggering. *Proc Natl Acad Sci U S A* . 2004;101(9):3011-3016.

71. Snow AL, Oliveira JB, Zheng L, Dale JK, Fleisher TA, Lenardo MJ. Critical role for BIM in T cell receptor restimulation-induced death. *Biol Direct*. 2008;3:34.

72. Spaner D, Raju K, Rabinovich B, Miller RG. A role for perforin in activation-induced T cell death in vivo: increased expansion of allogeneic perforin-deficient T cells in SCID mice. *J Immunol*. 1999;162(2):1192-1199.

73. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science*. 1999;286(5446):1957-1959.

74. Budd RC. Activation-induced cell death. *Curr Opin Immunol* . 2001; 13(3):356-362.

75. Lenardo MJ. The molecular regulation of lymphocyte apoptosis. *Semin Immunol*. 1997;9(1):1-5.

76. Zheng L, Trageser CL, Willerford DM, Lenardo MJ. T cell growth cytokines cause the superinduction of molecules mediating antigeninduced T lymphocyte death. *J Immunol*. 1998;160(2):763-769.

77. Snow AL, Pandiyan P, Zheng L, Krummey SM, Lenardo MJ. The power and the promise of restimulation-induced cell death in human immune diseases. *Immunol Rev*.

2010;236:68-82.

78. Strasser A, Jost PJ, Nagata S. The many roles of FAS receptor signaling in the immune system. *Immunity*. 2009;30(2):180-192.

79. Combadiere B, Freedman M, Chen L, Shores EW, Love P, Lenardo MJ. Qualitative and quantitative contributions of the T cell receptor zeta chain to mature T cell apoptosis. *J Exp Med*. 1996;183(5): 2109-2117.

80. She J, Matsui K, Terhorst C, Ju ST. Activation-induced apoptosis of mature T cells is dependent upon the level of surface TCR but not on the presence of the CD3 zeta ITAM. *Int Immunol* . 1998;10(11):1733-1740.

81. Snow AL, Marsh RA, Krummey SM, et al. Restimulation-induced apoptosis of T cells is impaired in patients with X-linked lymphoproliferative disease caused by SAP deficiency. *J Clin Invest*. 2009;119(10): 2976-2989.

82. Ramaswamy M, Cruz AC, Cleland SY, et al. Specific elimination of effector memory CD4+ T cells due to enhanced Fas signaling complex formation and association with lipid raft microdomains. *Cell Death Differ* . 2011;18(4):712-720.

83. Boehme SA, Lenardo MJ. Propriocidal apoptosis of mature T lymphocytes occurs at S phase of the cell cycle. *Eur J Immunol* . 1993;23(7):1552-1560.

84. Li QS, Tanaka S, Kisenge RR, Toyoda H, Azuma E, Komada Y. Activation-induced T cell death occurs at G1A phase of the cell cycle. *Eur J Immunol* . 2000;30(11):3329-3337.

85. Lissy NA, Davis PK, Irwin M, Kaelin WG, Dowdy SF. A common E2F-1 and p73 pathway mediates cell death induced by TCR activation. *Nature*. 2000;407(6804):642-645.

86. Hunig T, Schimpl A. Systemic autoimmune disease as a consequence of defective lymphocyte death. *Curr Opin Immunol* . 1997;9(6):826-830.

87. Willerford DM, Chen J, Ferry JA, Davidson L, Ma A, Alt FW. Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. *Immunity*. 1995;3(4):521-530.

88. Moskophidis D, Lechner F, Pircher H, Zinkernagel RM. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature*. 1993;362(6422):758-761.

89. Rothstein TL, Wang JK, Panka DJ, et al. Protection against Fasdependent Th1-mediated apoptosis by antigen receptor engagement in B cells. *Nature*. 1995;374(6518):163-165.

90. Hornung F, Zheng L, Lenardo MJ. Maintenance of clonotype specificity in CD95/Apo-1/Fas-mediated apoptosis of mature T lymphocytes. *J Immunol*. 1997;159(8):3816-3822.

91. Wong B, Arron J, Choi Y. T cell receptor signals enhance susceptibility to Fas-mediated apoptosis. *J Exp Med*. 1997;186(11):1939-1944.

92. Combadiere B, Reis e Sousa C, Trageser C, Zheng LX, Kim CR, Lenardo MJ. Differential TCR signaling regulates apoptosis and immunopathology during antigen responses in vivo. *Immunity*. 1998;9(3): 305-313.

93. Critchfield JM, Racke MK, Zuniga-Pflucker JC, et al. T cell deletion in high antigen dose therapy of autoimmune encephalomyelitis. *Science*. 1994;263(5150):1139-1143.

94. Nahill SR, Welsh RM. High frequency of cross-reactive cytotoxic T lymphocytes elicited during the virus-induced polyclonal cytotoxic T lymphocyte response. *J Exp Med*. 1993;177(2):317-327.

95. Razvi ES, Jiang Z, Woda BA, Welsh RM. Lymphocyte apoptosis during the silencing of the immune response to acute viral infections in normal, lpr, and Bcl-2-transgenic mice. *Am J Pathol*. 1995;147(1):79-91.

96. Kuroda K, Yagi J, Imanishi K, et al. Implantation of IL-2-containing osmotic pump prolongs the survival of superantigen-reactive T cells expanded in mice injected with bacterial superantigen. *J Immunol*. 1996;157(4):1422-1431.

97. Duke RC, Cohen JJ. IL-2 addiction: withdrawal of growth factor activates a suicide program in dependent T cells. *Lymphokine Res*. 1986;5(4): 289-299.

98. Edlich F, Banerjee S, Suzuki M, et al. Bcl-x(L) retrotranslocates Bax from the mitochondria into the cytosol. *Cell*. 2011;145(1):104-116.

99. Strasser A. The role of BH3-only proteins in the immune system. *Nat Rev Immunol*. 2005;5(3):189-200.

100. Hildeman DA, Zhu Y, Mitchell TC, et al. Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. *Immunity*. 2002;16(6):759-67.

101. Pellegrini M, Belz G, Bouillet P, Strasser A. Shutdown of an acute T cell immune response to viral infection is mediated by the proapoptotic Bcl-2 homology 3-only protein Bim. *Proc Natl Acad Sci U S A*. 2003;100(24):14175-14180.

102. Oliveira JB, Bidere N, Niemela JE, et al. NRAS mutation causes a human autoimmune lymphoproliferative syndrome. *Proc Natl Acad Sci U S A*. 2007;104(21):8953-8958.

103. Ley R, Balmanno K, Hadfield K, Weston C, Cook SJ. Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim. *J Biol Chem*. 2003;278(21):18811-11816.

104. Matsui H, Asou H, Inaba T. Cytokines direct the regulation of Bim mRNA stability by heat-shock cognate protein 70. *Mol Cell*. 2007;25(1):99-112.

105. Bauer A, Villunger A, Labi V, et al. The NF-kappaB regulator Bcl-3 and the BH3-only proteins Bim and Puma control the death of activated T cells. *Proc Natl Acad Sci U S A* . 2006;103(29):10979-10984.

106. You H, Pellegrini M, Tsuchihara K, et al. FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. *J Exp Med*. 2006;203(7):1657-1663.

107. Kelly E, Won A, Refaeli Y, Van Parijs L. IL-2 and related cytokines can promote T cell survival by activating AKT. *J Immunol*. 2002;168(2): 597-603.

108. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell*. 1996;87(4):619-628.

109. Maurer U, Charvet C, Wagman AS, Dejardin E, Green DR. Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. *Mol Cell*. 2006;21(6): 749-760.

110. Li B, Samanta A, Song X, et al. FOXP3 ensembles in T-cell regulation. *Immunol Rev*. 2006;212:99-113.

111. Wing K, Fehervari Z, Sakaguchi S. Emerging possibilities in the development and function of regulatory T cells. *Int Immunol*. 2006;18(7): 991-1000.

112. Maloy KJ, Powrie F. Fueling regulation: IL-2 keeps CD4+ Treg cells fit. *Nat Immunol*. 2005;6(11):1071-1072.

113. Harnaha J, Machen J, Wright M, et al. Interleukin-7 is a survival factor for CD4+ CD25+ T-cells and is expressed by diabetes-suppressive dendritic cells. *Diabetes*. 2006;55(1):158-170.

114. Barthlott T, Moncrieffe H, Veldhoen M, et al. CD25+ CD4+ T cells compete with naive CD4+ T cells for IL-2 and exploit it for the induction of IL-10 production. *Int Immunol*. 2005;17(3):279-288.

115. Scheffold A, Huhn J, Hofer T. Regulation of CD4+CD25+ regulatory T cell activity: it takes (IL-)two to tango. *Eur J Immunol* . 2005;35(5): 1336-1341.

116. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apotosis of effector CD4+ T cells. *Nat Immunol*. 2007;8(12):1353-1362.

117. Thornton AM, Shevach EM. Suppressor effector function of CD4+ CD25+ immunoregulatory T cells is antigen nonspecific. *J Immunol*. 2000;164(1):183-190.

118. Pandiyan P, Lenardo MJ. The control of CD4+CD25+Foxp3+ regulatory T cell survival. *Biol Direct*. 2008;3:6.

119. Qureshi OS, Zheng Y, Nakamura K, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science*. 2011;332(6029):600-603.

120. Callan MF, Fazou C, Yang H, et al. CD8(+) T-cell selection, function, and death in the primary immune response in vivo. *J Clin Invest*. 2000;106(10):1251-1261.

121. Opferman JT, Ober BT, Ashton-Rickardt PG. Linear differentiation of cytotoxic effectors into memory T lymphocytes. *Science*. 1999; 283(5408):1745-1748.

122. Grayson JM, Harrington LE, Lanier JG, Wherry EJ, Ahmed R. Differential sensitivity of naive and memory CD8+ T cells to apoptosis in vivo. *J Immunol*. 2002;169(7):3760-3770.

123. Thome M, Schneider P, Hofmann K, et al. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature*. 1997;386(6624):517-521.

124. Hu S, Vincenz C, Ni J, Gentz R, Dixit VM. I-FLICE, a novel inhibitor of tumor necrosis factor receptor-1- and CD-95-induced apoptosis. *J Biol Chem*. 1997;272(28):17255-17257.

125. Irmler M, Thome M, Hahne M, et al. Inhibition of death receptor signals by cellular FLIP. *Nature*. 1997;388(6638):190-195.

126. Wang J, Lobito AA, Shen F, Hornung F, Winoto A, Lenardo MJ. Inhibition of Fas-mediated apoptosis by the B cell antigen receptor through c-FLIP. *Eur J Immunol* . 2000;30(1):155-163.

127. Yu Y, Iclozan C, Yamazaki T, et al. Abundant c-Fas-associated death domain-like interleukin-1-converting enzyme inhibitory protein expression determines resistance of T helper 17 cells to activation-induced cell death. *Blood*. 2009;114(5):1026-1028.

128. Deveraux QL, Reed JC. IAP family proteins—suppressors of apoptosis. *Genes Dev*. 1999;13(3):239-252.

129. Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *EMBO J*. 1997;16(23):6914-6925.

130. Garcia S, DiSanto J, Stockinger B. Following the development of a CD4 T cell response in vivo: from activation to memory formation. *Immunity*. 1999;11(2):163-171.

131. Chao DT, Linette GP, Boise LH, White LS, Thompson CB, Korsmeyer SJ. Bcl-XL and Bcl-2 repress a common pathway of cell death. *J Exp Med*. 1995;182(3):821-828.

132. Ku CC, Murakami M, Sakamoto A, Kappler J, Marrack P. Control of homeostasis of CD8+ memory T cells by opposing cytokines. *Science*. 2000;288(5466):675-678.

133. Murali-Krishna K, Lau LL, Sambhara S, Lemonnier F, Altman J, Ahmed R. Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science*. 1999;286(5443):1377-1381.

134. Marrack P, Kappler J. Control of T cell viability. *Annu Rev Immunol*. 2004;22:765-787.

135. Kondrack RM, Harbertson J, Tan JT, McBreen ME, Surh CD, Bradley LM. Interleukin 7 regulates the survival and generation of memory CD4 cells. *J Exp Med*. 2003;198(12):1797-1806.

136. Li J, Huston G, Swain SL. IL-7 promotes the transition of CD4 effectors to persistent memory cells. *J Exp Med*. 2003;198(12):1807-1815.

137. Wu TS, Lee JM, Lai YG, et al. Reduced expression of Bcl-2 in CD8 + T cells deficient in the IL-15 receptor alpha-chain. *J Immunol*. 2002;168(2):705-12.

138. Wojciechowski S, Jordan MB, Zhu Y, White J, Zajac AJ, Hildeman DA. Bim mediates apoptosis of CD127(lo) effector T cells and limits T cell memory. *Eur J Immunol*. 2006;36(7):1694-1706.

139. Janssen EM, Droin NM, Lemmens EE, et al. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature*. 2005;434(7029):88-93.

140. Badovinac VP, Messingham KA, Griffith TS, Harty JT. TRAIL deficiency delays, but does not prevent, erosion in the quality of “helpless” memory CD8 T cells. *J Immunol*. 2006;177(2):999-1006.

141. Norvell A, Mandik L, Monroe JG. Engagement of the antigen-receptor on immature murine B lymphocytes results in death by apoptosis. *J Immunol*. 1995;154(9):4404-4413.

142. Akashi K, Kondo M, von Freeden-Jeffry U, Murray R, Weissman IL. Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. *Cell*. 1997;89(7):1033-1041.

143. Maraskovsky E, O'Reilly LA, Teepe M, Corcoran LM, Peschon JJ, Strasser A. Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant rag-1-/- mice. *Cell*. 1997;89(7):1011-1019.

144. Grabstein KH, Waldschmidt TJ, Finkelman FD, et al. Inhibition of murine B and T lymphopoiesis in vivo by an anti-interleukin 7 monoclonal antibody. *J Exp Med*. 1993;178(1):257-264.

145. Namen AE, Lupton S, Hjerrild K, et al. Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature*. 1988;333(6173):571-573.

146. Oliver PM, Wang M, Zhu Y, White J, Kappler J, Marrack P. Loss of Bim allows precursor B cell survival but not precursor B cell differentiation in the absence of interleukin 7. *J Exp Med*. 2004;200(9): 1179-1187.

147. Opferman JT, Letai A, Beard C, Sorcinelli MD, Ong CC, Korsmeyer SJ. Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. *Nature*. 2003;426(6967):671-676.

148. Sandel PC, Monroe JG. Negative selection of immature B cells by receptor editing or deletion is determined by site of antigen encounter. *Immunity*. 1999;10(3):289-299.

149. Wechsler-Reya RJ, Monroe JG. Lipopolysaccharide prevents apoptosis and induces responsiveness to antigen receptor cross-linking in immature B cells. *Immunology*. 1996;89(3):356-362.

150. Hardy RR, Hayakawa K. B cell development pathways. *Annu Rev Immunol*. 2001;19:595-621.

151. King LB, Norvell A, Monroe JG. Antigen receptor-induced signal transduction imbalances associated with the negative selection of immature B cells. *J Immunol*. 1999;162(5):2655-2662.

152. Rathmell JC, Cooke MP, Ho WY, et al. CD95 (Fas)-dependent elimination of self-reactive B cells upon interaction with CD4+ T cells. *Nature*. 1995;376(6536):181-184.

153. Rathmell JC, Townsend SE, Xu JC, Flavell RA, Goodnow CC. Expansion or elimination of B cells in vivo: dual roles for CD40-and Fas (CD95)-ligands modulated by the B cell antigen receptor. *Cell*. 1996;87(2):319-329.

154. Choi MS, Boise LH, Gottschalk AR, Quintans J, Thompson CB, Klaus GG. The role of bcl-XL in CD40-mediated rescue from anti-muinduced apoptosis in WEHI-231 B

lymphoma cells. *Eur J Immunol* . 1995;25(5):1352-1357.

155. Foote LC, Marshak-Rothstein A, Rothstein TL. Tolerant B lymphocytes acquire resistance to Fas-mediated apoptosis after treatment with interleukin 4 but not after treatment with specific antigen unless a surface immunoglobulin threshold is exceeded. *J Exp Med*. 1998;187(6):847-853.

156. Rothstein TL. Inducible resistance to Fas-mediated apoptosis in B cells. *Cell Res*. 2000;10(4):245-266.

157. Stranges PB, Watson J, Cooper CJ, et al. Elimination of antigen-presenting cells and autoreactive T cells by Fas contributes to prevention of autoimmunity. *Immunity*. 2007;26(5):629-641.

158. Vikstrom I, Carotta S, Luthje K, et al. Mcl-1 is essential for germinal center formation and B cell memory. *Science*. 2010;330(6007): 1095-1099.

159. Clybouw C, Fischer S, Auffredou MT, et al. Regulation of memory B-cell survival by the BH3-only protein Puma. *Blood*. 2011;118(15):4120-4128.

160. Ekland EH, Forster R, Lipp M, Cyster JG. Requirements for follicular exclusion and competitive elimination of autoantigen-binding B cells. *J Immunol* . 2004;172(8):4700-4708.

161. Enders A, Bouillet P, Puthalakath H, Xu Y, Tarlinton DM, Strasser A. Loss of the pro-apoptotic BH3-only Bcl-2 family member Bim inhibits BCR stimulation-induced apoptosis and deletion of autoreactive B cells. *J Exp Med*. 2003;198(7):1119-1126.

162. Oliver PM, Vass T, Kappler J, Marrack P. Loss of the proapoptotic protein, Bim, breaks B cell anergy. *J Exp Med*. 2006;203(3):731-741.

163. Khan WN. B cell receptor and BAFF receptor signaling regulation of B cell homeostasis. *J Immunol* . 2009;183(6):3561-3567.

164. Craxton A, Draves KE, Gruppi A, Clark EA. BAFF regulates B cell survival by downregulating the BH3-only family member Bim via the ERK pathway. *J Exp Med*. 2005;202(10):1363-1374.

165. Graninger WB, Steiner CW, Graninger MT, Aringer M, Smolen JS. Cytokine regulation of apoptosis and Bcl-2 expression in lymphocytes of patients with systemic lupus erythematosus. *Cell Death Differ* . 2000;7(10):966-972.

166. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J*. 1992;11(11):3887-3895.

167. Devireddy LR, Teodoro JG, Richard FA, Green MR. Induction of apoptosis by a secreted lipocalin that is transcriptionally regulated by IL-3 deprivation. *Science*. 2001;293(5531):829-834.

168. Devireddy LR, Gazin C, Zhu X, Green MR. A cell-surface receptor for lipocalin 24p3 selectively mediates apoptosis and iron uptake. *Cell*. 2005;123(7):1293-1305.

169. Cleary ML, Smith SD, Sklar J. Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell*. 1986;47(1):19-28.

170. Ingulli E, Mondino A, Khoruts A, Jenkins MK. In vivo detection of dendritic cell antigen presentation to CD4(+) T cells. *J Exp Med*. 1997;185(12):2133-2141.

171. Wang J, Zheng L, Lobito A, et al. Inherited human caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. *Cell*. 1999;98(1): 47-58.

172. Chen M, Wang YH, Wang Y, et al. Dendritic cell apoptosis in the maintenance of immune tolerance. *Science*. 2006;311(5764):1160-1164.

173. Miao EA, Rajan JV, Aderem A. Caspase-1-induced pyroptotic cell death. *Immunol Rev*. 2011;243(1):206-214.

174. Hughes PD, Belz GT, Fortner KA, Budd RC, Strasser A, Bouillet P. Apoptosis regulators Fas and Bim cooperate in shutdown of chronic immune responses and prevention of autoimmunity. *Immunity*. 2008;28(2):197-205.

175. Hutcheson J, Scatizzi JC, Siddiqui AM, et al. Combined deficiency of proapoptotic regulators Bim and Fas results in the early onset of systemic autoimmunity. *Immunity*. 2008;28(2):206-217.

176. Weant AE, Michalek RD, Khan IU, Holbrook BC, Willingham MC, Grayson JM. Apoptosis regulators Bim and Fas function concurrently to control autoimmunity and CD8(+) T cell contraction. *Immunity*. 2008;28(2):218-230.

177. Oliveira JB, Bleesing JJ, Dianzani U, et al. Revised diagnostic criteria and classification for the autoimmune lymphoproliferative syndrome (ALPS): report from the 2009 NIH International Workshop. *Blood*. 2010;116(14):e35-e40.

178. Su H, Bidere N, Zheng L, et al. Requirement for caspase-8 in NF-kappaB activation by antigen receptor. *Science*. 2005;307(5714):1465-1468.

179. Bolze A, Byun M, McDonald D, et al. Whole-exome-sequencingbased discovery of

human FADD deficiency. *Am J Hum Genet*. 2010;87(6):873-881.

180. Lenardo MJ, Oliveira JB, Zheng L, Rao VK. ALPS-ten lessons from an international workshop on a genetic disease of apoptosis. *Immunity*. 2010;32(3):291-295.

181. Evan G, Littlewood T. A matter of life and cell death. *Science*. 1998; 281(5381):1317-1322.

182. Straus SE, Jaffe ES, Puck JM, et al. The development of lymphomas in families with autoimmune lymphoproliferative syndrome with germline Fas mutations and defective lymphocyte apoptosis. *Blood*. 2001;98(1):194-200.

183. Legembre P, Barnhart BC, Zheng L, et al. Induction of apoptosis and activation of NF-kappaB by CD95 require different signalling thresholds. *EMBO Rep*. 2004;5(11):1084-1089.

184. Cannons JL, Tangye SG, Schwartzberg PL. SLAM family receptors and SAP adaptors in immunity. *Annu Rev Immunol*. 2011;29:665-705.

185. Atkinson EA, Barry M, Darmon AJ, et al. Cytotoxic T lymphocyte-assisted suicide. Caspase 3 activation is primarily the result of the direct action of granzyme B. *J Biol Chem*. 1998;273(33): 21261-21266.

186. Harvey NL, Trapani JA, Fernandes-Alnemri T, Litwack G, Alnemri ES, Kumar S. Processing of the Nedd2 precursor by ICE-like proteases and granzyme B. *Genes Cells*. 1996;1(7):673-685.

187. Martin SJ, Amarante-Mendes GP, Shi L, et al. The cytotoxic cell protease granzyme B initiates apoptosis in a cell-free system by proteolytic processing and activation of the ICE/CED-3 family protease, CPP32, via a novel two-step mechanism. *EMBO J*. 1996;15(10):2407-2416.

188. Yang X, Stennicke HR, Wang B, et al. Granzyme B mimics apical caspases. Description of a unified pathway for trans-activation of executioner caspase-3 and -7. *J Biol Chem*. 1998;273(51):34278-34283.

189. Zapata JM, Takahashi R, Salvesen GS, Reed JC. Granzyme release and caspase activation in activated human T-lymphocytes. *J Biol Chem*. 1998;273(12):6916-6920.

190. Thiery J, Keefe D, Boulant S, et al. Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. *Nat Immunol*. 2011;12(8):770-777.

191. Gross A, Yin XM, Wang K, et al. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor

necrosis factor-R1/Fas death. *J Biol Chem*. 1999;274(2):1156-1163.

192. Heibein JA, Goping IS, Barry M, et al. Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members bid and Bax. *J Exp Med*. 2000;192(10):1391-1402.

193. Scaffidi C, Fulda S, Srinivasan A, et al. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J*. 1998;17(6):1675-1687.

194. Sutton VR, Davis JE, Cancilla M, et al. Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation. *J Exp Med*. 2000;192(10):1403-1414.

195. Yin XM, Wang K, Gross A, et al. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature*. 1999;400(6747): 886-891.

196. Ortaldo JR, Mason AT, O'Shea JJ. Receptor-induced death in human natural killer cells: involvement of CD16. *J Exp Med*. 1995;181(1): 339-344.

197. Ortaldo JR, Winkler-Pickett RT, Nagata S, Ware CF. Fas involvement in human NK cell apoptosis: lack of a requirement for CD16-mediated events. *J Leukoc Biol*. 1997;61(2):209-215.

198. Harrison LC, Hafler DA. Antigen-specific therapy for autoimmune disease. *Curr Opin Immunol*. 2000;12(6):704-711.

199. Waldmann TA, Dubois S, Tagaya Y. Contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for immunotherapy. *Immunity*. 2001;14(2):105-110.

200. Ogasawara J, Watanabe-Fukunaga R, Adachi M, et al. Lethal effect of the anti-Fas antibody in mice. *Nature*. 1993;364(6440):806-809.

201. Tracey KJ, Beutler B, Lowry SF, et al. Shock and tissue injury induced by recombinant human cachectin. *Science*. 1986;234(4775):470-474.

202. Ashkenazi A, Dixit VM. Apoptosis control by death and decoy receptors. *Curr Opin Cell Biol*. 1999;11(2):255-260.

203. Walczak H, Miller RE, Ariail K, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med*. 1999;5(2):157-163.

204. Fischer U, Schulze-Osthoff K. Apoptosis-based therapies and drug targets. *Cell Death Differ*. 2005;12(suppl 1):942-961.

205. Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X, Harran PG. A small

molecule Smac mimic potentiates TRAIL- and TNFalphamediated cell death. *Science*. 2004;305(5689):1471-1474.

206. Oltersdorf T, Elmore SW, Shoemaker AR, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature*. 2005;435(7042):677-681.

207. Walensky LD, Kung AL, Escher I, et al. Activation of apoptosis in vivo by a hydrocarbon-stapled BH3 helix. *Science*. 2004;305(5689): 1466-1470.

208. Eberstadt M, Huang B, Chen Z, et al. NMR structure and mutagenesis of the FADD (Mort1) death-effector domain. *Nature*. 1998; 392(6679):941-945.

209. Fesik SW. Insights into programmed cell death through structural biology. *Cell*. 2000;103(2):273-282.

210. Boatright KM, Salvesen GS. Mechanisms of caspase activation. *Curr Opin Cell Biol*. 2003;15(6):725-731.

211. Tu S, McStay GP, Boucher LM, Mak T, Beere HM, Green DR. In situ trapping of activated initiator caspases reveals a role for caspase-2 in heat shock-induced apoptosis. *Nat Cell Biol* . 2006;8(1):72-77.

212. Keller N, Mares J, Zerbe O, Grutter MG. Structural and biochemical studies on procaspase-8: new insights on initiator caspase activation. *Structure*. 2009;17(3):438-448.

213. Itoh N, Nagata S. A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen. *J Biol Chem*. 1993;268(15): 10932-10937.

214. Kischkel FC, Hellbardt S, Behrmann I, et al. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J*. 1995;14(22): 5579-5588.

215. Wang J, Chun HJ, Wong W, Spencer DM, Lenardo MJ. Caspase-10 is an initiator caspase in death receptor signaling. *Proc Natl Acad Sci U S A* . 2001;98(24):13884-13888.

216. Lin SC, Lo YC, Wu H. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. *Nature*. 2010;465(7300):885-890.

217. Wang L, Yang JK, Kabaleeswaran V, et al. The Fas-FADD death domain complex structure reveals the basis of DISC assembly and disease mutations. *Nat Struct Mol Biol*. 2010;17(11):1324-1329.

218. Spencer SL, Sorger PK. Measuring and modeling apoptosis in single cells. *Cell*.

2011;144(6):926-939.

219. Siegel RM, Muppidi JR, Sarker M, et al. SPOTS: signaling protein oligomeric transduction structures are early mediators of death receptorinduced apoptosis at the plasma membrane. *J Cell Biol* . 2004;167(4): 735-744.

220. Lee KH, Feig C, Tchikov V, et al. The role of receptor internalization in CD95 signaling. *EMBO J*. 2006;25(5):1009-1023.

221. Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ, Ashkenazi A. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity*. 2000;12(6): 611-620.

222. Kischkel FC, Lawrence DA, Tinel A, et al. Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. *J Biol Chem*. 2001;276(49):46639-46646.

223. Sprick MR, Weigand MA, Rieser E, et al. FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. *Immunity*. 2000;12(6): 599-609.

224. Wang EC, Thern A, Denzel A, Kitson J, Farrow SN, Owen MJ. DR3 regulates negative selection during thymocyte development. *Mol Cell Biol* . 2001;21(10):3451-3461.

225. Bayry J. Immunology: TL1A in the inflammatory network in autoimmune diseases. *Nat Rev Rheumatol* . 2010;6(2):67-68.

226. Liu J, Na S, Glasebrook A, et al. Enhanced CD4+ T cell proliferation and Th2 cytokine production in DR6-deficient mice. *Immunity*. 2001;15(1):23-34.

227. Zhao H, Yan M, Wang H, Erickson S, Grewal IS, Dixit VM. Impaired c-Jun amino terminal kinase activity and T cell differentiation in death receptor 6-deficient mice. *J Exp Med*. 2001;194(10):1441-1448.

228. Martinou JC, Youle RJ. Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. *Dev Cell* . 2011;21(1):92-101.

229. Golstein P. Controlling cell death. *Science*. 1997;275(5303):1081-1082.

230. Zou H, Li Y, Liu X, Wang X. An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem*. 1999;274(17):11549-11556.

231. Schafer ZT, Kornbluth S. The apoptosome: physiological, developmental, and

pathological modes of regulation. *Dev Cell*. 2006;10(5): 549-561.

232. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol* . 1999;15:269-290.

233. Shi Y. A structural view of mitochondria-mediated apoptosis. *Nat Struct Biol* . 2001;8(5):394-401.

234. Srinivasula SM, Hegde R, Saleh A, et al. A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. *Nature*. 2001;410(6824):112-116.

235. Wu G, Chai J, Suber TL, et al. Structural basis of IAP recognition by Smac/DIABLO. *Nature*. 2000;408(6815):1008-1012.

236. Hao Z, Duncan GS, Chang CC, et al. Specific ablation of the apoptotic functions of cytochrome C reveals a differential requirement for cytochrome C and Apaf-1 in apoptosis. *Cell*. 2005;121(4):579-591.

237. Scorrano L, Ashiya M, Buttle K, et al. A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. *Dev Cell*. 2002;2(1):55-67.

238. Cipolat S, Rudka T, Hartmann D, et al. Mitochondrial rhomboid PARL regulates cytochrome c release during apoptosis via OPA1-dependent cristae remodeling. *Cell*. 2006;126(1):163-175.

239. Frezza C, Cipolat S, Martins de Brito O, et al. OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. *Cell*. 2006;126(1):177-189.

240. Green DR. Apoptotic pathways: ten minutes to dead. *Cell*. 2005;121(5): 671-674.

241. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science*. 2004;305(5684):626-629.

242. Baines CP, Kaiser RA, Purcell NH, et al. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature*. 2005;434(7033):658-662.

243. Nakagawa T, Shimizu S, Watanabe T, et al. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature*. 2005;434(7033):652-658.

244. Susin SA, Lorenzo HK, Zamzami N, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature*. 1999;397(6718): 441-446.

245. Daugas E, Susin SA, Zamzami N, et al. Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. *FASEB J*. 2000;14(5):729-739.

246. Joza N, Susin SA, Daugas E, et al. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature*. 2001;410(6828):549-554.

247. Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature*. 2001;412(6842):95-99.

248. Vercammen D, Beyaert R, Denecker G, et al. Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. *J Exp Med*. 1998;187(9):1477-1485.

249. Vercammen D, Brouckaert G, Denecker G, et al. Dual signaling of the Fas receptor: initiation of both apoptotic and necrotic cell death pathways. *J Exp Med*. 1998;188(5):919-930.

250. Arnoult D, Gaume B, Karbowski M, Sharpe JC, Cecconi F, Youle RJ. Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. *EMBO J*. 2003;22(17):4385-4399.

251. Munoz-Pinedo C, Guio-Carrion A, Goldstein JC, Fitzgerald P, Newmeyer DD, Green DR. Different mitochondrial intermembrane space proteins are released during apoptosis in a manner that is coordinately initiated but can vary in duration. *Proc Natl Acad Sci U S A*. 2006;103(31):11573-11578.

252. Lakhani SA, Masud A, Kuida K, et al. Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. *Science*. 2006;311(5762):847-851.

253. Vercammen D, Vandenabeele P, Beyaert R, Declercq W, Fiers W. Tumour necrosis factor-induced necrosis versus anti-Fas-induced apoptosis in L929 cells. *Cytokine*. 1997;9(11):801-808.

254. Wyllie AH, Golstein P. More than one way to go. *Proc Natl Acad Sci U S A* . 2001;98(1):11-13.

255. Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nat Rev Mol Cell Biol* . 2010;11(10):700-714.

256. Stanger BZ, Leder P, Lee TH, Kim E, Seed B. RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell*. 1995;81(4):513-523.

257. Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity*.

1996;4(4):387-396.

258. Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P. The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. *Immunity*. 1998;8(3):297-303.

259. Ting AT, Pimentel-Muinos FX, Seed B. RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. *EMBO J*. 1996;15(22):6189-6196.

260. Challa S, Chan FK. Going up in flames: necrotic cell injury and inflammatory diseases. *Cell Mol Life Sci* . 2010;67(19):3241-3253.

261. Adachi M, Suematsu S, Suda T, et al. Enhanced and accelerated lymphoproliferation in Fas-null mice. *Proc Natl Acad Sci U S A* . 1996;93(5):2131-2136.

262. Zheng L, Bidere N, Staudt D, et al. Competitive control of independent programs of tumor necrosis factor receptor-induced cell death by TRADD and RIP1. *Mol Cell Biol* . 2006;26(9):3505-3513.

263. Oberst A, Dillon CP, Weinlich R, et al. Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature*. 2011;471(7338):363-367.

264. Lin Y, Devin A, Cook A, et al. The death domain kinase RIP is essential for TRAIL (Apo2L)-induced activation of IkappaB kinase and c-Jun N-terminal kinase. *Mol Cell Biol*. 2000;20(18):6638-6645.

265. Cho YS, Challa S, Moquin D, et al. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virusinduced inflammation. *Cell*. 2009;137(6):1112-1123.

266. He S, Wang L, Miao L, et al. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. *Cell*. 2009;137(6): 1100-1111.

267. Zhang DW, Shao J, Lin J, et al. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. *Science*. 2009;325(5938):332-336.

268. Lin Y, Choksi S, Shen HM, et al. Tumor necrosis factor-induced nonapoptotic cell death requires receptor-interacting protein-mediated cellular reactive oxygen species accumulation. *J Biol Chem*. 2004;279(11): 10822-10828.

269. Shisler JL, Moss B. Immunology 102 at poxvirus U: avoiding apoptosis. *Semin Immunol*. 2001;13(1):67-72.

270. Li M, Beg AA. Induction of necrotic-like cell death by tumor necrosis factor alpha and caspase inhibitors: novel mechanism for killing virusinfected cells. *J Virol*.

2000;74(16):7470-7477.

271. Basu S, Binder RJ, Suto R, Anderson KM, Srivastava PK. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. *Int Immunol*. 2000;12(11):1539-1546.

272. Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. *Nat Med*. 1999;5(11):1249-1255.

273. Upton JW, Kaiser WJ, Mocarski ES. Cytomegalovirus M45 cell death suppression requires receptor-interacting protein (RIP) homotypic interaction motif (RHIM)-dependent interaction with RIP1. *J Biol Chem*. 2008;283(25):16966-16970.

274. Barnitz RA, Chaigne-Delalande B, Bolton DL, Lenardo MJ. Exposed hydrophobic residues in human immunodeficiency virus type 1 vpr helix-1 are important for cell cycle arrest and cell death. *PLoS One*. 2011;6(9):e24924.

275. Bolton DL, Hahn BI, Park EA, Lehnhoff LL, Hornung F, Lenardo MJ. Death of CD4(+) T-cell lines caused by human immunodeficiency virus type 1 does not depend on caspases or apoptosis. *J Virol*. 2002;76(10):5094-5107.

276. Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR. The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell*. 1993;75(4):641-652.

277. Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem*. 1999;68:383-424.

278. Eckhart L, Ballaun C, Uthman A, et al. Identification and characterization of a novel mammalian caspase with proapoptotic activity. *J Biol Chem*. 2005;280(42):35077-35080.

279. Hitomi J, Katayama T, Taniguchi M, Honda A, Imaizumi K, Tohyama M. Apoptosis induced by endoplasmic reticulum stress depends on activation of caspase-3 via caspase-12. *Neurosci Lett*. 2004;357(2):127-130.

280. Saleh M, Mathison JC, Wolinski MK, et al. Enhanced bacterial clearance and sepsis resistance in caspase-12-deficient mice. *Nature*. 2006;440(7087):1064-1068.

281. Saleh M, Vaillancourt JP, Graham RK, et al. Differential modulation of endotoxin responsiveness by human caspase-12 polymorphisms. *Nature*. 2004;429(6987):75-79.

282. Boatright KM, Renatus M, Scott FL, et al. A unified model for apical caspase activation. *Mol Cell*. 2003;11(2):529-541.

283. Donepudi M, Mac Sweeney A, Briand C, Grutter MG. Insights into the regulatory mechanism for caspase-8 activation. *Mol Cell*. 2003; 11(2):543-549.

284. Read SH, Baliga BC, Ekert PG, Vaux DL, Kumar S. A novel Apaf-1-independent putative caspase-2 activation complex. *J Cell Biol*. 2002; 159(5):739-745.

285. Stennicke HR, Deveraux QL, Humke EW, Reed JC, Dixit VM, Salvesen GS. Caspase-9 can be activated without proteolytic processing. *J Biol Chem*. 1999;274(13):8359-8362.

286. Bidere N, Snow AL, Sakai K, Zheng L, Lenardo MJ. Caspase-8 regulation by direct interaction with TRAF6 in T cell receptor-induced NFkappaB activation. *Curr Biol*. 2006;16(16):1666-1671.

287. Keller N, Grutter MG, Zerbe O. Studies of the molecular mechanism of caspase-8 activation by solution NMR. *Cell Death Differ*. 2010;17(4):710-718.

288. Chao Y, Shiozaki EN, Srinivasula SM, Rigotti DJ, Fairman R, Shi Y. Engineering a dimeric caspase-9: a re-evaluation of the induced proximity model for caspase activation. *PLoS Biol*. 2005;3(6):e183.

289. Blanchard H, Kodandapani L, Mittl PR, et al. The three-dimensional structure of caspase-8: an initiator enzyme in apoptosis. *Structure Fold Des*. 1999;7(9):1125-1133.

290. Riedl SJ, Renatus M, Schwarzenbacher R, et al. Structural basis for the inhibition of caspase-3 by XIAP. *Cell*. 2001;104(5):791-800.

291. Varadhachary AS, Peter ME, Perdow SN, Krammer PH, Salgame P. Selective up-regulation of phosphatidylinositol 3′-kinase activity in Th2 cells inhibits caspase-8 cleavage at the death-inducing complex: a mechanism for Th2 resistance from Fas-mediated apoptosis. *J Immunol*. 1999;163(9):4772-4779.

292. Watt W, Koeplinger KA, Mildner AM, Heinrikson RL, Tomasselli AG, Watenpaugh KD. The atomic-resolution structure of human caspase-8, a key activator of apoptosis. *Structure Fold Des*. 1999;7(9): 1135-1143.

293. Wei Y, Fox T, Chambers SP, et al. The structures of caspases-1, -3, -7 and -8 reveal the basis for substrate and inhibitor selectivity. *Chem Biol*. 2000;7(6):423-432.

294. Chai J, Wu Q, Shiozaki E, Srinivasula SM, Alnemri ES, Shi Y. Crystal structure of a procaspase-7 zymogen. Mechanisms of activation and substrate binding. *Cell*. 2001;107(3):399-407.

295. Beidler DR, Tewari M, Friesen PD, Poirier G, Dixit VM. The baculovirus p35 protein

inhibits Fas- and tumor necrosis factor-induced apoptosis. *J Biol Chem*. 1995;270(28):16526-16528.

296. Bertin J, Armstrong RC, Ottilie S, et al. Death effector domain-containing herpesvirus and poxvirus proteins inhibit both Fas- and TNFR1-induced apoptosis. *Proc Natl Acad Sci U S A*. 1997;94(4):1172-1176.

297. Yang JK, Wang L, Zheng L, et al. Crystal structure of MC159 reveals molecular mechanism of DISC assembly and FLIP inhibition. *Mol Cell*. 2005;20(6):939-949.

298. Budd RC, Yeh WC, Tschopp J. cFLIP regulation of lymphocyte activation and development. *Nat Rev Immunol*. 2006;6(3):196-204.

299. Boatright KM, Deis C, Denault JB, Sutherlin DP, Salvesen GS. Activation of caspases-8 and -10 by FLIP(L). *Biochem J*. 2004;382(pt 2):651-657.

300. Pop C, Oberst A, Drag M, et al. FLIP(L) induces caspase 8 activity in the absence of interdomain caspase 8 cleavage and alters substrate specificity. *Biochem J*. 2011;433(3):447-457.

301. Cha SS, Sung BJ, Kim YA, et al. Crystal structure of TRAIL-DR5 complex identifies a critical role of the unique frame insertion in conferring recognition specificity. *J Biol Chem*. 2000;275(40):31171-31177.

302. Chai J, Shiozaki E, Srinivasula SM, et al. Structural basis of caspase-7 inhibition by XIAP. *Cell*. 2001;104(5):769-780.

303. Huang Y, Park YC, Rich RL, Segal D, Myszka DG, Wu H. Structural basis of caspase inhibition by XIAP: differential roles of the linker versus the BIR domain. *Cell*. 2001;104(5):781-790.

304. Rano TA, Timkey T, Peterson EP, et al. A combinatorial approach for determining protease specificities: application to interleukin-1beta converting enzyme (ICE). *Chem Biol*. 1997;4(2):149-155.

305. Thornberry NA, Rano TA, Peterson EP, et al. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J Biol Chem*. 1997;272(29):17907-17911.

306. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature*. 1998;391(6662):43-50.

307. Sakahira H, Enari M, Nagata S. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature*. 1998;391(6662): 96-99.

308. Rao L, Perez D, White E. Lamin proteolysis facilitates nuclear events during apoptosis. *J Cell Biol* . 1996;135(6, pt 1):1441-1455.

309. Wang ZQ, Stingl L, Morrison C, et al. PARP is important for genomic stability but dispensable in apoptosis. *Genes Dev*. 1997;11(18): 2347-2358.

310. Zhu S, Hsu AP, Vacek MM, et al. Genetic alterations in caspase-10 may be causative or protective in autoimmune lymphoproliferative syndrome. *Hum Genet*. 2006;119(3):284-294.

311. Chun HJ, Zheng L, Ahmad M, et al. Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency. *Nature*. 2002;419(6905):395-399.

312. Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, Boldin MP. Tumor necrosis factor receptor and Fas signaling mechanisms. *Annu Rev Immunol* . 1999;17:331-367.

313. Shu HB, Johnson H. B cell maturation protein is a receptor for the tumor necrosis factor family member TALL-1. *Proc Natl Acad Sci U S A* . 2000;97(16):9156-9161.

314. Wiley SR, Cassiano L, Lofton T, et al. A novel TNF receptor family member binds TWEAK and is implicated in angiogenesis. *Immunity*. 2001;15(5):837-846.

315. Tartaglia LA, Ayres TM, Wong GH, Goeddel DV. A novel domain within the 55 kd TNF receptor signals cell death. *Cell* . 1993;74(5):845-853.

316. Arch RH, Gedrich RW, Thompson CB. Tumor necrosis factor receptor-associated factors (TRAFs)—a family of adapter proteins that regulates life and death. *Genes Dev*. 1998;12(18):2821-2830.

317. Chan FK, Lenardo MJ. A crucial role for p80 TNF-R2 in amplifying p60 TNF-R1 apoptosis signals in T lymphocytes. *Eur J Immunol* . 2000;30(2):652-660.

318. Rothe M, Wong SC, Henzel WJ, Goeddel DV. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell* . 1994;78(4):681-692.

319. Weiss T, Grell M, Siemienski K, et al. TNFR80-dependent enhancement of TNFR60-induced cell death is mediated by TNFR-associated factor 2 and is specific for TNFR60. *J Immunol* . 1998;161(6):3136-3142.

320. Siegel RM, Chan FK, Chun HJ, Lenardo MJ. The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. *Nat Immunol* . 2000;1(6):469-474.

321. Hsu H, Xiong J, Goeddel DV. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell*. 1995;81(4):495-504.

322. Yeh WC, Shahinian A, Speiser D, et al. Early lethality, functional NFkappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. *Immunity*. 1997;7(5):715-725.

323. Li ZW, Chu W, Hu Y, et al. The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. *J Exp Med*. 1999;189(11):1839-1845.

324. Makris C, Godfrey VL, Krahn-Senftleben G, et al. Female mice heterozygous for IKK-gamma/NEMO deficiencies develope a dermatopathy similar to the human X-linked disorder incontinentia pigmenti. *Mol Cell*. 2000;5(6):969-979.

325. Rudolph D, Yeh WC, Wakeham A, et al. Severe liver degeneration and lack of NF-kappaB activation in NEMO/IKKgamma-deficient mice. *Genes Dev*. 2000;14(7):854-862.

326. Smahi A, Courtois G, Vabres P, et al. Genomic rearrangement in NEMO impairs NF-kappaB activation and is a cause of incontinentia pigmenti. The International Incontinentia Pigmenti (IP) Consortium. *Nature*. 2000;405(6785):466-472.

327. Tanaka M, Fuentes ME, Yamaguchi K, et al. Embryonic lethality, liver degeneration, and impaired NF-kappa B activation in IKK-betadeficient mice. *Immunity*. 1999;10(4):421-429.

328. Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death [see comments]. *Science*. 1996;274(5288):782-784.

329. Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. Suppression of TNF-alpha-induced apoptosis by NF-kappaB [see comments]. *Science*. 1996;274(5288):787-789.

330. Wang CY, Mayo MW, Baldwin AS Jr. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB [see comments]. *Science*. 1996;274(5288):784-787.

331. Lee SY, Kaufman DR, Mora AL, Santana A, Boothby M, Choi Y. Stimulus-dependent synergism of the antiapoptotic tumor necrosis factor receptor-associated factor 2 (TRAF2) and nuclear factor kappaB pathways. *J Exp Med*. 1998;188(7):1381-1384.

332. Chao DT, Korsmeyer SJ. BCL-2 family: regulators of cell death. *Annu Rev Immunol*. 1998;16:395-419.

333. Hawkins CJ, Vaux DL. The role of the Bcl-2 family of apoptosis regulatory proteins in the immune system. *Semin Immunol*. 1997;9(1): 25-33.

334. Raffeld M, Wright JJ, Lipford E, et al. Clonal evolution of t(14;18) follicular lymphomas demonstrated by immunoglobulin genes and the 18q21 major breakpoint region. *Cancer Res*. 1987;47(10): 2537-2542.

335. Vaux DL, Cory S, Adams JM. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature*. 1988;335(6189):440-442.

336. Fesik SW. Promoting apoptosis as a strategy for cancer drug discovery. *Nat Rev Cancer*. 2005;5(11):876-885.

337. Opferman JT, Korsmeyer SJ. Apoptosis in the development and maintenance of the immune system. *Nat Immunol*. 2003;4(5):410-415.

338. de Jong D, Prins FA, Mason DY, Reed JC, van Ommen GB, Kluin PM. Subcellular localization of the bcl-2 protein in malignant and normal lymphoid cells. *Cancer Res*. 1994;54(1):256-260.

339. Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature*. 1990;348(6299):334-336.

340. Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, Reed JC. Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res*. 1993;53(19):4701-4714.

341. Zamzami N, Brenner C, Marzo I, Susin SA, Kroemer G. Subcellular and submitochondrial mode of action of Bcl-2-like oncoproteins. *Oncogene*. 1998;16(17):2265-2282.

342. Wei MC, Zong WX, Cheng EH, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science*. 2001;292(5517):727-730.

343. Korsmeyer SJ, Shutter JR, Veis DJ, Merry DE, Oltvai ZN. Bcl-2/Bax: a rheostat that regulates an anti-oxidant pathway and cell death. *Semin Cancer Biol*. 1993;4(6):327-332.

344. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*. 1993;74(4):609-619.

345. Sedlak TW, Oltvai ZN, Yang E, et al. Multiple Bcl-2 family members demonstrate

selective dimerizations with Bax. *Proc Natl Acad Sci U S A* . 1995;92(17):7834-7838.

346. Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell*. 1995;80(2):285-291.

347. Yin XM, Oltvai ZN, Korsmeyer SJ. Heterodimerization with Bax is required for Bcl-2 to repress cell death. *Curr Top Microbiol Immunol* . 1995;194:331-338.

348. Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell*. 2004;116(2):205-219.

349. Westphal D, Dewson G, Czabotar PE, Kluck RM. Molecular biology of Bax and Bak activation and action. *Biochim Biophys Acta*. 2011; 1813(4):521-531.

350. Boise LH, Minn AJ, Noel PJ, et al. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity*. 1995;3(1):87-98.

351. Motoyama N, Wang F, Roth KA, et al. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science*. 1995;267(5203):1506-1510.

352. Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell*. 1993;75(2):229-240.

353. Rinkenberger JL, Horning S, Klocke B, Roth K, Korsmeyer SJ. Mcl-1 deficiency results in peri-implantation embryonic lethality. *Genes Dev*. 2000;14(1):23-27.

354. Linette GP, Hess JL, Sentman CL, Korsmeyer SJ. Peripheral T-cell lymphoma in lckpr-bcl-2 transgenic mice. *Blood*. 1995;86(4): 1255-1260.

355. Strasser A, Harris AW, Cory S. E mu-bcl-2 transgene facilitates spontaneous transformation of early pre-B and immunoglobulin-secreting cells but not T cells. *Oncogene*. 1993;8(1):1-9.

356. Marsden VS, O'Connor L, O'Reilly LA, et al. Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome. *Nature*. 2002;419(6907):634637.

357. Pattingre S, Tassa A, Qu X, et al. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell*. 2005;122(6):927-939.

358. Knudson CM, Tung KS, Tourtellotte WG, Brown GA, Korsmeyer SJ. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science*. 1995;270(5233):96-99.

359. Lindsten T, Ross AJ, King A, et al. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell*. 2000;6(6):1389-1399.

360. Sattler M, Liang H, Nettesheim D, et al. Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. *Science*. 1997;275(5302):983-986.

361. Cheng EH, Wei MC, Weiler S, et al. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell*. 2001;8(3):705-711.

362. Zong WX, Lindsten T, Ross AJ, MacGregor GR, Thompson CB. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. *Genes Dev*. 2001;15(12):1481-1486.

363. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell*. 1998;94(4):481-490.

364. Zha J, Weiler S, Oh KJ, Wei MC, Korsmeyer SJ. Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. *Science*. 2000;290(5497):1761-1765.

365. Eskes R, Desagher S, Antonsson B, Martinou JC. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol Cell Biol*. 2000;20(3):929-935.

366. Zhu Y, Swanson BJ, Wang M, et al. Constitutive association of the proapoptotic protein Bim with Bcl-2-related proteins on mitochondria in T cells. *Proc Natl Acad Sci U S A*. 2004;101(20):7681-7686.

367. Alves NL, Derks IA, Berk E, Spijker R, van Lier RA, Eldering E. The Noxa/Mcl-1 axis regulates susceptibility to apoptosis under glucose limitation in dividing T cells. *Immunity*. 2006;24(6):703-716.

368. Cartron PF, Gallenne T, Bougras G, et al. The first alpha helix of Bax plays a necessary role in its ligand-induced activation by the BH3-only proteins Bid and PUMA. *Mol Cell*. 2004;16(5):807-818.

369. Kuwana T, Bouchier-Hayes L, Chipuk JE, et al. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell*. 2005;17(4):525-535.

370. Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ. Distinct

BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* . 2002;2(3):183-192.

371. Tait SW, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. *Nat Rev Mol Cell Biol* . 2010;11(9):621-632.

372. Montessuit S, Somasekharan SP, Terrones O, et al. Membrane remodeling induced by the dynamin-related protein Drp1 stimulates Bax oligomerization. *Cell*. 2010;142(6):889-901.

373. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell*. 1997;90(3):405-413.

374. Inohara N, Nunez G. The NOD: a signaling module that regulates apoptosis and host defense against pathogens. *Oncogene*. 2001;20(44):6473-6481.

375. Li P, Nijhawan D, Budihardjo I, et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*. 1997;91(4):479-489.

376. Fortin A, Cregan SP, MacLaurin JG, et al. APAF1 is a key transcriptional target for p53 in the regulation of neuronal cell death. *J Cell Biol* . 2001;155(2):207-216.

377. Moroni MC, Hickman ES, Denchi EL, et al. Apaf-1 is a transcriptional target for E2F and p53. *Nat Cell Biol* . 2001;3(6):552-558.

378. Soengas MS, Alarcon RM, Yoshida H, et al. Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. *Science*. 1999; 284(5411):156-159.

379. Inohara N, Nunez G. NODs: intracellular proteins involved in inflammation and apoptosis. *Nat Rev Immunol* . 2003;3(5):371-382.

380. Hugot JP, Chamaillard M, Zouali H, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature*. 2001;411(6837):599-603.

381. Ogura Y, Bonen DK, Inohara N, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature*. 2001;411(6837):603-606.

382. Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, Lenardo MJ. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science*. 2000;288(5475):2351-2354.

383. Siegel RM, Frederiksen JK, Zacharias DA, et al. Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. *Science*.

2000;288(5475):2354-2357.

384. Jones EY, Stuart DI, Walker NP. Structure of tumour necrosis factor. *Nature*. 1989;338(6212):225-228.

385. McGeehan GM, Becherer JD, Bast RC Jr, et al. Regulation of tumour necrosis factor-alpha processing by a metalloproteinase inhibitor. *Nature*. 1994;370(6490):558-561.

386. Banner DW, D'Arcy A, Janes W, et al. Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell*. 1993;73(3):431-445.

387. Cha SS, Kim MS, Choi YH, et al. 2.8 A resolution crystal structure of human TRAIL, a cytokine with selective antitumor activity. *Immunity*. 1999;11(2):253-261.

388. Hymowitz SG, Christinger HW, Fuh G, et al. Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5. *Mol Cell*. 1999;4(4):563-571.

389. Mongkolsapaya J, Grimes JM, Chen N, et al. Structure of the TRAIL-DR5 complex reveals mechanisms conferring specificity in apoptotic initiation. *Nat Struct Biol*. 1999;6(11):1048-1053.

390. Naismith JH, Brandhuber BJ, Devine TQ, Sprang SR. Seeing double: crystal structures of the type I TNF receptor. *J Mol Recognit*. 1996;9(2):113-117.

391. Naismith JH, Devine TQ, Brandhuber BJ, Sprang SR. Crystallographic evidence for dimerization of unliganded tumor necrosis factor receptor. *J Biol Chem*. 1995;270(22):13303-13307.

392. Naismith JH, Devine TQ, Kohno T, Sprang SR. Structures of the extracellular domain of the type I tumor necrosis factor receptor. *Structure*. 1996;4(11):1251-1262.

393. Naismith JH, Sprang SR. Modularity in the TNF-receptor family. *Trends Biochem Sci*. 1998;23(2):74-79.

394. Park YC, Burkitt V, Villa AR, Tong L, Wu H. Structural basis for self-association and receptor recognition of human TRAF2. *Nature*. 1999;398(6727):533-538.

395. Ye H, Park YC, Kreishman M, Kieff E, Wu H. The structural basis for the recognition of diverse receptor sequences by TRAF2. *Mol Cell*. 1999;4(3):321-330.

396. Park YC, Ye H, Hsia C, et al. A novel mechanism of TRAF signaling revealed by

structural and functional analyses of the TRADD-TRAF2 interaction. *Cell*. 2000;101(7):777-787.

397. Tsao DH, McDonagh T, Telliez JB, et al. Solution structure of N-TRADD and characterization of the interaction of N-TRADD and C-TRAF2, a key step in the TNFR1 signaling pathway. *Mol Cell*. 2000;5(6): 1051-1057.

398. Fisher GH, Rosenberg FJ, Straus SE, et al. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell*. 1995;81(6):935-946.

399. Kimura M, Matsuzawa A. Autoimmunity in mice bearing Iprcg: a novel mutant gene. *Int Rev Immunol* . 1994;11(3):193-210.

400. Martin DA, Zheng L, Siegel RM, et al. Defective CD95/APO-1/Fas signal complex formation in the human autoimmune lymphoproliferative syndrome, type Ia. *Proc Natl Acad Sci U S A*. 1999;96(8):4552-4557.

401. Park HH, Logette E, Raunser S, et al. Death domain assembly mechanism revealed by crystal structure of the oligomeric PIDDosome core complex. *Cell*. 2007;128(3):533-546.

402. Holler N, Tardivel A, Kovacsovics-Bankowski M, et al. Two adjacent trimeric Fas ligands are required for Fas signaling and formation of a death-inducing signaling complex. *Mol Cell Biol* . 2003;23(4): 1428-1440.

403. O'Reilly LA, Tai L, Lee L, et al. Membrane-bound Fas ligand only is essential for Fas-induced apoptosis. *Nature*. 2009;461(7264): 659-663.

404. Minn AJ, Velez P, Schendel SL, et al. Bcl-x(L) forms an ion channel in synthetic lipid membranes. *Nature*. 1997;385(6614):353-357.

405. Schendel SL, Montal M, Reed JC. Bcl-2 family proteins as ion-channels. *Cell Death Differ*. 1998;5(5):372-380.

406. Huang B, Eberstadt M, Olejniczak ET, Meadows RP, Fesik SW. NMR structure and mutagenesis of the Fas (APO-1/CD95) death domain. *Nature*. 1996;384(6610):638-641.

407. Chou JJ, Matsuo H, Duan H, Wagner G. Solution structure of the RAIDD CARD and model for CARD/CARD interaction in caspase-2 and caspase-9 recruitment. *Cell*. 1998;94(2):171-180.

408. Qin H, Srinivasula SM, Wu G, Fernandes-Alnemri T, Alnemri ES, Shi Y. Structural basis of procaspase-9 recruitment by the apoptotic protease-activating factor 1. *Nature*. 1999;399(6736):549-557.

409. Zhou P, Chou J, Olea RS, Yuan J, Wagner G. Solution structure of Apaf-1 CARD and its interaction with caspase-9 CARD: a structural basis for specific adaptor/caspase interaction. *Proc Natl Acad Sci U S A* . 1999;96(20):11265-11270.

410. Bertin J, DiStefano PS. The PYRIN domain: a novel motif found in apoptosis and inflammation proteins. *Cell Death Differ* . 2000;7(12):1273-1274.

411. Fairbrother WJ, Gordon NC, Humke EW, et al. The PYRIN domain: a member of the death domain-fold superfamily. *Protein Sci*. 2001;10(9):1911-1918.

412. Martinon F, Hofmann K, Tschopp J. The pyrin domain: a possible member of the death domain-fold family implicated in apoptosis and inflammation. *Curr Biol* . 2001;11(4):R118-R120.

413. Xu G, Cirilli M, Huang Y, Rich RL, Myszka DG, Wu H. Covalent inhibition revealed by the crystal structure of the caspase-8/p35 complex. *Nature*. 2001;410(6827):494-497.

414. Lu M, Min T, Eliezer D, Wu H. Native chemical ligation in covalent caspase inhibition by p35. *Chem Biol*. 2006;13(2):117-122.

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Chapter 31

Immunologic Memory

Shane Crotty

Susan M. Kaech

Stephen P. Schoenberger

MEMORY AS A BIOLOGIC CONCEPT

The Protected State

> *Yet it was with those who had recovered from the disease that the sick and the dying found most compassion. These knew what it was from experience, and had now no fear for themselves; for the same man was never attacked twice- never at least fatally. And such persons not only received the congratulations of others, but themselves also, in the elation of the moment, half entertained the vain hope that they were for the future safe from any disease whatsoever.*[1]

This quote comes from the Greek historian Thucydides (ca. 460 BC to ca. 395 BC) and describes his firsthand account of an ancient plague that struck Athens during a protracted war with Sparta. Although the identity of the causative agent continues to be a matter of speculation,[2] these words transcend the ages to vividly illustrate the concept of *immune memory*—the phenomenon in which prior exposure to an infectious pathogen endows an individual with *immunity*, a durable state of protection against reinfection with the same organism. Unknown to Thucydides and his fellow Greeks, the immunity observed in the survivors of this plague reflected changes in the operational status of their immune systems as a result of the first (primary) response to the infecting pathogen. In the act of responding to the initial infection, the immune system expands a diverse population of antigen-specific B- and T-lymphocyte clones possessing a range of affinities and effector capacities. Through a process that is just now beginning to be understood at the molecular level, a portion of this repertoire is retained within the memory pool in the form of cells that are able to persist for long periods of time at relatively stable numbers by maintaining a slow but steady rate of division that is roughly equivalent to their rate of loss (death). When faced with a renewed challenge from the same (or antigenically related) infectious agents, memory cells mount a strong and rapid effector response that is capable of stopping reinfection at its earliest stages. The functional units of immune memory, therefore, are the long-lived B and T cells that mount rapid secondary (recall) responses upon reencounter with their cognate antigen.

In the broader teleologic concept of memory, however, it is the antigen receptors that were shown to be “useful” in combating the initial infection that are selected and preserved at an increased frequency on clonal progeny that possess enhanced response kinetics and specialized functions. Whether generated through infection or vaccination, the value of the protected state they confer to the individual is self-evident, as preexisting immunity can prevent or limit the potential damage of an otherwise unrestrained infection. This can be of benefit from the earliest days of life, as seen in the example of maternal antibodies transmitted to the neonate in milk and serum that may serve to limit infections and transform them into “natural vaccinations” and thereby diminishing the severity of childhood infections that will be encountered during the next 1 to 2 years.[3]

This chapter will examine the phenomena and mechanisms of long-lasting protection that can develop following infection or vaccination, and will focus on the factors that govern the establishment and maintenance of specialized subsets of lymphocytes generated from the naïve repertoire during the primary response to a given antigen. This concept stands in contrast to the “memory” associated with immune tolerance, through which the adaptive arm of the immune system can prevent inappropriate responses against ubiquitous self-antigens. Immune memory involves a stable increase in the number of antigen-experienced B- and T-lymphocytes that have acquired specialized functional properties, allowing them to generate secondary responses that are more rapid and effective than those made by their clonal antecedents during the primary response. It is in the establishment, maintenance, and execution of memory responses that the adaptive immune system finds its greatest purpose for the preservation of both the individual and population. As will be discussed in this chapter, immune memory depends on a remarkable degree of interaction and cooperation between many different cell types in an elegant division of labor aimed at preserving those T- and B-cell specificities that have proved useful in the battles fought and won against previously encountered pathogens. It is important in considering the concept of immune memory, however, that distinctions be drawn between the protected state, an operational definition that can vary with the nature and magnitude of the antigenic challenge used to test its integrity, and memory cells, the clonal elements of memory whose contribution to the protected state can only be inferred from their phenotypic and functional properties.

Lifelong Memory can be Induced through Infection or Vaccination

Observations of acquired resistance to recurring diseases are recorded throughout the history of human epidemics. The careful observations of the Danish physician Ludwig Panum were among the first to clearly illustrate just how durable the protected state can be in an individual. Working on the

remote Faroe Islands in the North Sea, Panum studied two separate measles epidemics that took place 65 years apart between the 18th and 19th centuries. In the apparent absence of outbreaks in the intervening period, he found that people who had contracted measles in the first epidemic were not affected during the second, while those of appropriate age that were infected during the second outbreak had not been affected during the first.[4] Thus, Panum observed, a single infection was capable of endowing an individual with lifelong immunity through a process that did not require reexposure to the pathogen. Although immune reactivity can be boosted by repeated exposure to a given organism, the extent to which this

determines the maintenance of immune memory remains a source of some controversy.[5] A source of uncertainty in applying this concept to human immunology is the difficulty in determining whether a single or multiple exposures have taken place before the memory state is assessed. With infrequently encountered life-threatening infections such measles, yellow fever, or polio, it is relatively straightforward to infer that a single exposure can establish a state of protection for up to 75 years that appears to be mediated by antibodies.[4,6,7] With less threatening ubiquitous agents such as those causing the childhood infections chicken pox (*Varicella zoster*) and whooping cough (*Bordetella pertussis*), it is unclear whether immune memory may depend on occasional subclinical reinfection.[8]

Given the long history of immunity in survivors of infectious disease, it is notable that even the most rudimentary understanding of how the protected state could be achieved was realized long after methods for its induction had been devised. Among the earliest practices was that of *variolation*, which had long been in practice in China and India as a means for enhancing protection against the deadly smallpox virus, which had been a major source of mortality in humans since recorded history.[9] Variolation involves the intentional inoculation with desiccated material obtained from the open pustules of smallpox victims, in hopes that exposed individual would develop a milder form of the disease and, upon recovery, would be immune to smallpox (variola virus). Although effective in many cases, the procedure was not without risk to the recipient, who risked contracting a more serious life-threatening form of the disease that could be spread to others. In 1796, Edward Jenner famously (and dramatically) overcame these obstacles by using material obtained from the pustules of milkmaids infected with cowpox (vaccinia virus), an antigenically related but less virulent type of poxvirus, to inoculate young James Phipps who subsequently survived his exposure to infectious smallpox patients.[10] In the 19th century, Louis Pasteur further developed the concept of using less-virulent or intentionally disabled forms of infectious organisms to confer immunity to bacterial diseases such as chicken cholera and anthrax, or viral diseases such as rabies. Pasteur used the word *vaccine* as a generic term to describe antigenic preparations administered to produce immunity, in reference to Jenner's earlier work with cowpox (vaccinia) virus (*vacca* is Latin for cow). As described in more detail in the chapter on vaccines (Chapter 43), current vaccines can be generated from organisms that are antigenically related to the one against which immunity is desired, from inactivated forms of the entire disease-causing organism, or from specific constituent portions of the infectious organism such as proteins, polysaccharides, or nucleic acids.

Despite their various compositions, all effective vaccines recapitulate several important aspects of the immune response leading to memory formation: they mimic the threat of an infectious pathogen through stimulation of innate immunity, and they contain distinct antigens that can become recognizable by cells of the adaptive immune system.[11,12] The power of vaccination to induce long-lasting immune memory is best illustrated by the example of the smallpox vaccine (vaccinia). This once-devastating organism has been virtually eradicated since the late 1970s through a worldwide mass vaccination program that still stands as one of the crowning achievements of the 20th century.[13] The immunity induced by the smallpox vaccine is of remarkable durability, with both antibody and cluster of differentiation (CD)4+ and CD8+ T cell responses detectable 50 years after initial priming,[14,15,16] and effectiveness, with a lifelong protection rate of 90% to 95% in vaccinated individuals, and a

fatality rate of only 2% in those vaccinated within a decade before exposure.[17,18] With the advent of modern immunologic tools such as enzyme-linked immunosorbent assay, intracellular cytokine staining, and enzyme-linked immunospot assays for the detection of rare vaccinia-specific T and B cells, a more detailed longitudinal picture of the response to the smallpox vaccine has emerged. These studies suggest that humoral immunity is more durable than cellular immunity, as increased numbers of vaccinia-specific memory B (Bmem) cells remain relatively stable for more than 50 years after vaccination, whereas CD4+ and CD8+ T-cell responses continue to decline over time, with a half-life of 8 to 15 years.[14,16,19] This indicates that Bmem-cell responses, and to a lesser extent memory T-cell responses, are maintained by robust mechanisms.

Given the remarkable longevity of the immunity induced through either infection or vaccination, it is clear that the cells mediating the protected state must possess several key properties in order to preserve useful specificities generated in previous antigenic experiences, with the study of memory T cells revealing insights into a number of these features. First among these is that antigen-specific memory cells must be present in the immune repertoire in greater numbers than in the naïve repertoire as a result of their initial proliferation.[20,21,22,23,24,25] Secondly, they must have undergone a program of differentiation involving alterations in chromatin structure and activation of specific transcription factors to allow the expression of key effector molecules such as cytokines, chemokines or cytotoxic proteins, in the case of cytotoxic T lymphocytes (CTLs), immediately upon recognition of antigen-expressing target cells.[26,27,28,29,30,31,32,33] Memory cells must be able to survive as a clonal population over time; that is, they must be able to replace cells lost to normal homeostatic turnover.[34,35] Memory cells also must perform a sentinel function by occupying specific physiologic niches where naïve cells are not usually found and where antigen exposure will either occur or be detected such as the mucosa of the lung or gastrointestinal tract.[36,37] Consistent with this

sentinel function, memory cells are found to be capable of responding to lower concentrations of antigen than naïve cells through the selective expansion of high-affinity clones and the upregulation of adhesion molecules and reorganization of their T-cell receptor (TCR).[38,39,40,41] Lastly, memory cells should be able to respond quickly to reencounter with their cognate antigen with the induction of rapid effector functions and renewed rounds of clonal expansion.[42] Memory cells have developed a variety of mechanisms to achieve each of these specialized properties. In the following section, we will examine how memory cells are generated during immune responses and how these key features are imparted during priming, maintained over long periods of time, and expressed when needed to confer rapid protection from reinfection (Fig. 31.1).

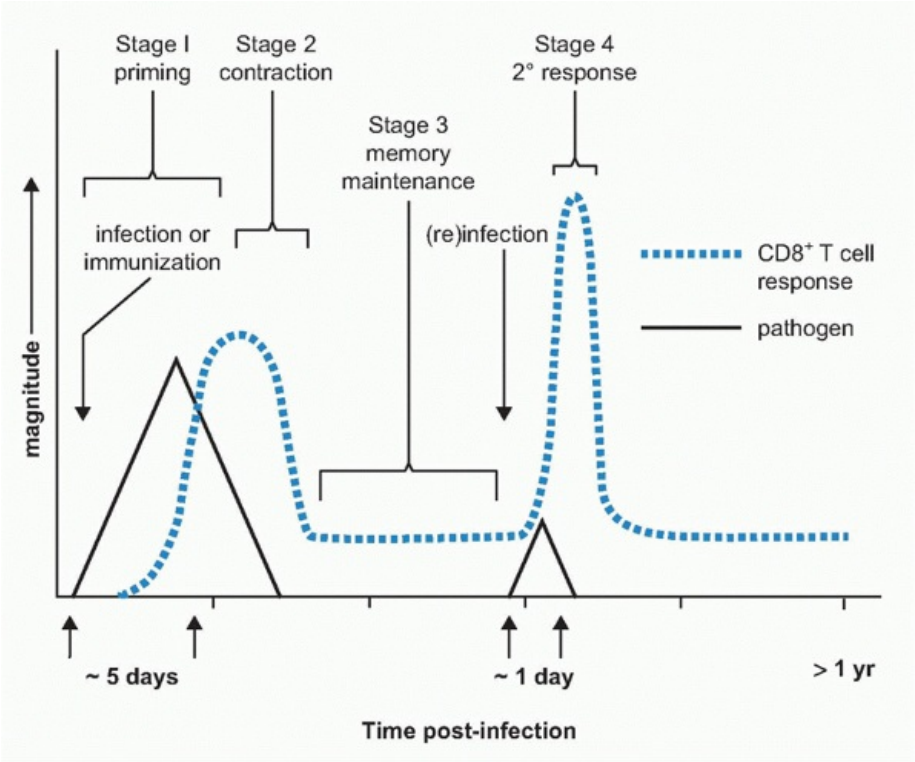

FIG. 31.1. Stages of the T-Cell Response to Infection. Antigen-specific T cells clonally expand during stage 1 and differentiate into effector T cells. After viral clearance, the majority of the cells die over the next 2 to 4 weeks (ie, stage 2, the bulk contraction phase). Soon thereafter, the number of virus-specific T cells stabilizes and the cells enter the memory phase and are maintained for long periods of time (stage 3). The majority of the memory T cells undergo slow, periodic basal homeostatic proliferation in response to interleukin-15 and interleukin-7. Upon reinfection, the memory T cells rapidly produce effector cytokines and other antimicrobial molecules and proliferate robustly to thwart infection.

THE GENERATION OF MEMORY T CELLS

Introduction

Antigen-driven T-cell responses can be broadly categorized as having two main components: a comparatively shortlived effector arm that comprises large numbers of cells that mediate direct functional response against target cells, and a smaller but longer-lived memory population that insures the potential of rapid and potent recall responses against the inducing antigen. The memory component can itself be distinguished into two separate activities: one that resides mainly within lymph nodes where it can respond to antigenic challenge by renewed clonal expansion and production of secondary effectors ("central memory" phenotype T cells [T_{CM}]) and one that is largely disseminated throughout peripheral tissues where the first contact with infectious pathogens is likely to occur ("effector memory" [T_{EM}] or "resident memory" [T_{RM}] phenotype T cells), which are capable of mediating immediate effector functions upon antigen recognition.[36,42,43,44] Memory T cells disseminate widely throughout the body and circulate passing from blood to tissues and lymphoid organs, but

more tissue resident T_{RM}, noncirculating memory T cells, can also be found in the brain and mucosal tissues such as the gut and skin that reside long-term in the tissues following infection.[45] The specific roles in immunity and the signals that may govern the development of these subsets will be discussed in subsequent sections in this chapter.

Models of Effector and Memory T-Cell Development

There are several models for how both effector and memory populations could be concomitantly generated, either in parallel or sequentially (Fig. 31.2). 1) The first model suggests that memory cells are the clonal progeny of a distinct precursor population that follows a separate differentiation fate from that of the primary effectors, and instead survive to execute these effector functions during the recall phase (separate precursor model). However, little support for this model exists currently because elegant lineage tracing experiments have demonstrated that the progeny of a single, naïve antigen-specific CD8 T cell can have diverse fates.[46,47] 2) The second, the "decreasing potential model," postulates that increasing amounts or prolonged periods of TCR stimulation causes the effector T cells to progressively lose memory cell potential and, ultimately, reach a terminally differentiated state.[48] This may occur in a linear or step-wise manner, driving the T cells through several distinct differentiation steps. Although this model was originally based mostly on antigenic signaling (signal 1), it is likely the collective history of signaling through costimulatory receptors (signal 2) and

inflammatory cytokines (signal 3) that determine the differentiation set-points of the T cells during infection. 3) The third model also allows for formation of heterogenous effector cell populations dependent on the overall "strength" of the signals 1, 2, and 3 encountered early during T-cell priming as first described by Lanzavecchia and Sallusto.[49] High or excessively strong signals drive greater clonal expansion, but might also cause terminal effector T-cell differentiation. This model differs conceptually from the decreasing potential hypothesis in that different cell fates can be specified early according to the intensity of the signals received, but do not necessarily require multiple rounds of stimulation to create heterogeneous cell fates. 4) This model is also conceptually similar to an alternative "asymmetric cell fate model"

that supports the notion that T_{EM} and T_{RM} fates can arise from a single precursor T cell via asymmetric cell division that occur as the activated T cells clonally expand, and may even begin as early as the first cell division.[50] This fourth model envisions memory as a cellular fate governed by the same common biologic strategy used during ontogeny and development to specify distinct functional properties in selected cells and their clonal progeny.[51] The latter three models are not mutually exclusive, and elements of each of these models most likely occur simultaneously during an immune response. Moreover, although there is some experimental evidence for early cell fate commitment, a more likely scenario is that signals encountered early during T-cell priming setup or specify T-cell fates, but that some plasticity remains in the dividing cells and their differentiation states and fates become determined through the subsequent contact with antigen-presenting cells (APCs) or other signals over the next several days of infection.

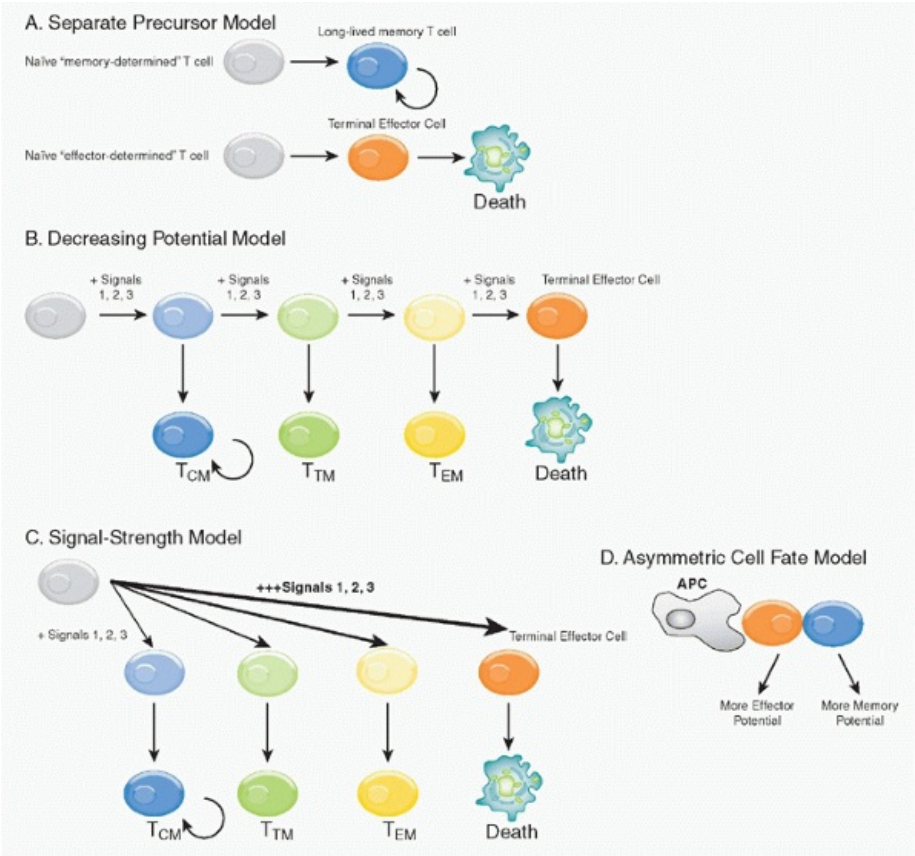

FIG. 31.2. Models of Memory T-Cell Generation. Four models are proposed to describe the formation the memory T cells. Please see text for detailed descriptions of each model. **A:** Separate precursor model. This model suggests that naïve T cells can be preprogrammed following thymic development to develop into memory T cells or terminal effector cells that die following infection. **B:** Decreasing potential model. This model states that a short duration of antigenic stimulation favors development of effector cells that will give rise to greater numbers of central memory phenotype T cells (T_{CM}; *blue*), whereas longer durations of stimulation promotes terminal effector cell differentiation (*red*) and death. According to the number of interactions with antigen-presenting cells and possibly cell divisions, intermediate stages of effector differentiation can also be acquired, giving rise to memory T cells with more effector memory phenotype T cell (TEM)-like (*orange*) or transitional memory (T_{TM}; *yellow*) phenotypes that can convert from $T_{EM} \rightarrow T_{CM}$ phenotypes overtime. *Curved arrow* denotes basal homeostatic proliferation. **C:** Signal strength model. This model proposes that the effector cells differentiate according to the "strength of signal" they receive at the time of priming (or possibly later). Larger amounts of antigen, costimulation, and inflammation (*thicker arrow*) drive cells toward a more terminal differentiation state, whereas weaker signals (*thinner arrows*) promote formation of T_{CM}, T_{TM}, or T_{EM} cells in a progressive manner. **D:** Asymmetric cell fate model. This model proposes that T-cell fates are specified (or possibly even determined) as early as the first cell division by the asymmetric portioning of cell fate determinants.

In each of these models, the initiating event leading to memory cell development is the

primary activation of a naïve T cell in response to immunization or infection. We shall therefore begin our consideration of memory T-cell generation within the different phases of the immune response: 1) the first phase of naïve T-cell priming, clonal expansion, and effector cell differentiation (the naïve to effector T-cell transition); 2) the second phase of effector cell contraction and formation of a surviving pool of memory T cells (the effector to memory T cell transition); 3) the third phase of memory T-cell maintenance and homeostasis; and 4) the reactivation of memory T cells during reinfection. Within each phase, we will discuss the similarities and differences between CD4 and CD8 T cells in effector and memory T-cell development.

Methods of Detecting Effector and Memory T Cells

Before discussing the factors responsible for the development of effector and memory T cells, it is important to understand the types of experimental approaches that have been used in their study and how these influence the various conclusions that can be drawn from the experimental data. In general, there have been two main experimental settings through which memory T-cells have been studied in vivo. One involves enumeration of cells that express markers associated with antigen-experience such as $CD44^{hi}$ or $CD62L^{lo}$ for activated cells, in either immunized or nonmanipulated normal mice. In an immunized host, the antigen-specific T cells can be generated from the endogenous polyclonal repertoire or adoptively transferred monoclonal transgenic T cells. In the latter case (a naïve host), the cells in question are the spontaneously arising “memory phenotype” (MP) T cells of unknown derivation and specificity, which increase in frequency with age.[52,53] In contrast, antigenspecific effector and memory T cells can be distinguished and tracked longitudinally in mice and humans using major histocompatibility complex (MHC) class I or II tetramers, which are soluble forms of the ligand structure recognized by T cells and allow the detection of cells recognizing a specific antigenic determinant.[54,55] Alternatively, antigen-specific T cells can also be identified functionally using intracellular cytokine staining, which allows enumeration of cells secreting cytokines in response to their cognate peptide antigen. Finally, the use of TCR-transgenic T cells has allowed the fate of a monoclonal population of antigen-specific T cells to be followed throughout their development from naive to memory cells during immune responses in mice. This typically involves the adoptive transfer of relatively small numbers of naïve TCR-transgenic T cells into a wild-type mouse that is subsequently immunized or infected to activate the T cells.

Phase I. Naïve to Effector T-Cell Transition: T-Cell Priming, Clonal Expansion, and Effector Differentiation

T-Cell Priming

The process of generating T-cell memory begins at priming, which is the initial activation of mature postthymic precursors (ie, naïve T cells) by foreign antigens presented by professional APCs. This is not a simple task, as a T cell recognizing any one single antigen is rare in the naïve repertoire, occurring with a frequency of perhaps 1 in 10^5 to 10^6 among the total population.[56,57] The antigen-bearing APCs are also likely to be infrequent among the (tens of) millions of cells within a given lymph node or in the spleen, as in most cases they must first obtain their antigenic cargo at peripheral sites of infection and then migrate via the

lymphatics to lymph node or via the bloodstream to the spleen.[58] As will be described, the complex interaction of rare and migrating cell types necessary to the generation of immune responses is facilitated by highly regulated patterns of migration and by the structural features of the secondary lymphoid organs themselves.

Naïve T cells are produced at a modest rate by the thymus, with approximately 1 to 2 × 10^6 cells per day emerging into the bloodstream in young mice.[59,60] The survival of naïve cells/recent thymic emigrants requires at least two types of extrinsic signals: 1) MHC molecules that are presumably occupied with low-affinity ligands (self- or environmental peptides) and provide tonic TCR stimulation, and 2) a critical cytokine, interleukin (IL)-7, which has many roles in B- and T-cell lymphopoeisis.[61,62,63,64,65,66,67,68] Mice or humans deficient in either IL-7 or IL-7Rα have considerably reduced numbers of T cells.[69,70,71,72,73,74] IL-7 is a member of the common γ-chain (γ_C) cytokine family that signals through a heterodimer comprised of γ_C and IL-7Rα.[75] Neither of these components are unique to IL-7, as γ_C is shared with IL-2, IL-4, IL9, IL-15, and IL-21, and IL7Rα is shared with thymic stromal lymphopoietin (TSLP).

To become stimulated (primed), naïve T cells must recognize antigens in the form of peptide fragments bound to MHC molecules on professional APCs, such as dendritic cells (DCs), in lymphoid organs. In response to microbial pathogens, immature DC migrate to sites of infection and inflammation in response to a range of CC and CXC chemokines and acquire soluble and particulate antigens. The immature DCs become activated by recognition of pathogen-associated molecular patterns or host molecules released by tissue damage and transition into an APC by

increasing expression of MHC:peptide complexes, the chemokine receptor CCR7 to facilitate migration toward T-cell areas of secondary lymphoid organs, and costimulatory ligands and cytokines to aide in T-cell activation and effector cell differentiation.[76] Once a naïve T cell recognizes its cognate peptide/MHC ligand on an activated DC within the secondary lymphoid organs, the interactions of priming (ie, the “antigenic signal”) can take place. The molecular details of this process involve the formation of immune synapses at the T/APC contact site that are composed of rapidly-clustering of TCR molecules binding peptide/MHC complexes on APC plus the local accumulation of intracellular signaling molecules such PKC-θ, LAT, and LCK.

Activation of T Cells by Antigen (Signal 1) and Costimulatory Receptors (Signal 2)

Another critical consequence of DC activation is the upregulated expression of numerous costimulatory molecules and cytokines that contribute to T-cell priming by promoting cell division, survival, and differentiation.[58,77,78,79] The primary activation of naïve T cells has traditionally been believed to require at least two signals: an antigen-specific “signal 1” that is transmitted upon TCR-mediated recognition of its cognate peptide/MHC complex, and an antigen nonspecific “signal 2” costimulus.[80,81] This “two-signal” model predicted that engagement of the TCR in the absence of costimulation is insufficient for sustained clonal expansion and cytokine production, and cells instead become anergic and unresponsive to

further stimulation.[82] While this outcome has been demonstrated in a number of studies, the number of ligand/receptor pairs capable of functioning as “signal(s) two” has grown considerably in the last decade. CD80 and CD86 are considered to be the prototypical costimulatory molecules expressed on APC capable of transmitting “signal 2” through their interaction with CD28 expressed on T cells. CD80/86-mediated ligation of CD28 produces signals in T cells that are distinct from those of the TCR and enhances clonal expansion, effector functions, and memory development.[83,84,85,86] Although many CD4+ and CD8+ T-cell responses depend on CD28, the requirement is not absolute, as many types of T-cell responses can occur in its absence in vivo, including allograft rejection, induction of allogeneic graft-versus-host and delayed-type hypersensitivity responses, and the generation of cytotoxic CD8+ T cells.[87,88,89,90,91] These observations suggest that additional pathways exist for costimulation of T cells other than CD28.[92] A number of these have emerged from the tumor necrosis factor receptor (TNFR) family (CD27/CD70, 4-1BB (CD137)/4-1BBL, OX40 (CD134)/OX40-L, HVEM-LIGHT, CD30/CD30-L, and GITR), each of which can provide costimulatory stimuli that synergize with TCR signals to sustain T-cell activation after priming, and that have been shown to be important for the magnitude and longevity of CD8+ T-cell responses in vivo in studies of knockout mice lacking either ligand or receptor.[92,93,94] Interestingly, the costimulatory requirements for CD4 and CD8 T cells are not completely overlapping. For example, CD8+ T-cell activation and clonal expansion occurs efficiently in mice lacking CD28, CD40L, or OX40, whereas CD4+ T-cell responses are substantially reduced. The opposite situation is seen in mice lacking 4-1BB-/-, which mount normal CD4+, but diminished CD8+, T-cell responses to lymphocytic choriomeningitis virus (LCMV) infection.[95] It should be pointed out that the influence of costimulatory interactions on the regulation of CD8+ T-cell responses is by no means limited to the priming event, as several receptor-ligand pairs, notably 4-1BB/4-1BBL, OX4O/OX40L, BTLA/HVEM, and PD-1/PDL1/PDL1, can act on the expanded population, with either positive or negative effects on T-cell function and survival.[94,96,97]

Inflammatory Cytokines (Signal 3) and Effector T-Cell Specification

In addition to contact-dependent costimulation, a number of inflammatory cytokines including interferon (IFN)α, IFNβ, IFNγ, IL-1, IL-4, IL-6, IL-12, and IL-21 have been implicated in the transmission of a “signal 3” that is required for optimal proliferation and differentiation of effector T cells.[98,99] Signal 3 cytokines are produced mostly by innate immune cells in response to pattern recognition receptor activation, but the types of cytokines produced will vary according to the pathogen, its tropism, and the types of pattern recognition receptors and sentinel cells activated during infection or vaccination. This selectivity of cytokine production ensures development of the appropriate types of effector T cells to defend against the given infecting pathogen.[99] For instance, intracellular pathogens, such as viruses and certain bacteria, elicit production of IFNα/β, IL-12, and IFNγ, which drive development of type I immune responses that mainly consist of cell-mediated defenses aimed at direct killing of infected cells via CD8+ CTLs or suppression of viral replication and activation of macrophages through T_H1-polarized CD4+ responses.[100] Infection by extracellular pathogens including parasites and helminthes produces TSLP and IL-4, which induces development of more humoral-based type 2 responses that generate Th2-polarized CD4+

effector T cells and recruitment of eosinophils, basophils, and mast cells. Other extracellular pathogens, including gram-negative bacteria, fungi, and some protozoa, induce IL-6 and IL-1; in the presence of basal transforming growth factor (TGF)-β, this instructs development of Th17 CD4+ T cells that recruit neutrophils and macrophages to sites of infection.[101,102,103,104] Additionally, as discussed in greater detail subsequently, CD4 T cells also form follicular helper T cells,which are specialized for B-cell help and control of germinal center (GC) responses and antibody production.[105] Lastly, regulatory T (T_{reg}) cell populations that develop in the thymus or in the periphery prevent autoimmunity and, during infection, keep effector T cells in check to reduce the amount of collateral damage (ie, tissue damage) associated with immune responses. Additional varieties of CD4 T cells have been proposed that differ based on cytokine production or localization.[106,107]

Much progress has been made in elucidating the genetic programs that govern development of CD8+ CTLs and CD4+ T_H1, T_H2, and Th17 effector T cells. During type I responses (eg, antiviral responses) TCR and IL-12, IFNαβ, and IFNγ signaling act together to activate transcription factors such as signal transducer and activator of transcription factor (STAT) 4 and T-bet in CD4+ T cells to induce formation of IFNγ-producing T_H1 cells. Likewise, in CD8+

T cells STAT4, T-bet and eomesodermin expression are needed to generate CTLs to directly kill infected cells.[107,108,109,110] Similarly, during type 2 responses, IL-4-dependent activation of STAT6 and GATA-3 creates T_H2 effector cells that produce IL-4, IL-5, and IL-13 as “signature cytokines” to trigger immunoglobulin (Ig)E production by B cells and activate eosinophils, basophils, and mast cells.[107] This, for example, creates a “weep and sweep” mode of defense to combat infection of helminths.[111,112,113] Although the exact details of T_H17 development remain to be elucidated, the cytokines IL-6, IL-1, and TGF-β play an instructive role in their differentiation through engagement of STAT-3 and the nuclear orphan receptor RORγ.[114,115,116,117,118] The development of T_H17 or T_{reg} cells appears to be reciprocal and dependent on the ratio of IL6:TGF-β as well the abundance of vitamin A metabolite retinoic acid.[119,120,121] For example, steady-state production of TGF-β may favor T_{reg} formation, but upon bacterial infection, the combination of newly synthesized IL-1 and IL-6 will favor Th17 differentiation.[122] Signals for Tfh cell differentiation are incompletely characterized, but IL-6 and ICOS are important factors in most cases.[105,123,124,125,126] In addition, Tfh cells depend on multiple stages of APC interactions, first requiring signals from DCs and then subsequent interactions and signals from B cells.[125,127,128,129]

Clonal Expansion of Effector T cells

The kinetics of T-cell clonal expansion can be remarkable. In murine model systems of *Listeria* and LCMV infection, it has been documented that CD8 T cells undergo between 15 to 20 sequential rounds of division within the first 7 days of infection, at rates as fast as ˜4 to 8 hours, and increase the frequency of antigen-specific T cells more than 10,000-fold over their initial frequency in the naïve.[32,130,131,132,133,134,135,136,137] CD4 T cells also undergo profound clonal expansion, but their burst sizes are typically smaller than that of

CD8 T cells. The factors that govern the magnitude of T-cell proliferation in vivo are numerous, but several studies have demonstrated a direct relationship between antigen load and the number of effector cells generated.[135,136,138,139,140,141,142] However, it is important to point out antigen-driven T-cell proliferation cannot be sustained indefinitely, as in the case of chronic infections (such as human immunodeficiency virus [HIV] and hepatitis C virus infection in humans and LCMV infection in mice), where antigen persists for prolonged periods. That is, despite the presence of antigen, the antigen-specific T cells still undergo contraction 1 to 2 weeks post infection. In these situations, it has been postulated that this occurs due to "programmed" contraction of activated CD8 T cells, but another critical element is that these cells differentiate into senescent, less functional "exhausted" T cells that may be sensitive to apoptosis.[143,144,145,146] Another difference observed between CD4+ and CD8+ T cells is that CD8+ T cells require a comparatively shorter period of stimulation (2 to 4 hours) for commitment to clonal expansion and effector development than do CD4+ T cells (~20 hours).[135,137,147,148] CD8+ T cells also begin dividing earlier after priming than CD4+ T cells and have a faster rate of division.[148,149,150,151] Additionally, and in contrast to CD8+ T cells, it has been observed that CD4+ T cells require continued antigenic stimulation throughout their period of expansion in order to achieve full proliferative and effector potential.[152,153] Based on these and other findings, Lanzavecchia and Sallusto have suggested that the optimal development of memory T cells will occur in cells that receive a specific level of antigenic stimulation during their priming and subsequent expansion that enables them to utilize and access survival signals in a process termed progressive differentiation.[49,147,154,155]

In addition to the effects of the inflammatory cytokines on effector T-cell specification, some of these cytokines also play a critical role in effector cell survival and clonal expansion. For instance, studies on CD8+ T cells primed in vitro with peptide/MHC and CD80 showed that optimal expansion and development of lytic functions required addition of IL-12 or IFNα.[156] IFNαβR-deficient LCMV GPspecific CD8+ T cells transferred to wild-type mice showed dramatic reductions in their ability to undergo clonal expansion and to generate a memory pool following infection with LCMV.[157,158] When the LCMV GP was expressed from a recombinant vaccinia virus, however, only a modest defect in CD8+ T-cell expansion and survival was observed, suggesting that the IFNαβ-dependence of CD8+ T-cell memory is influenced not by the target antigen, but rather by the immunogen.[157,159] This idea has found support in a recent study showing that the response of IFNαβR-deficient transgenic CD8+ T cells to the same peptide-pulsed DC vaccine offered in the context of four different infections (LCMV, vaccinia virus, vesicular stomatitis virus, and *Listeria monocytogenes*) were most severely inhibited for LCMV infection.[158,160] IFNγ has also been shown to support optimal CD8+ T-cell expansion after LCMV infection or peptide vaccination, and transgenic CD8+ T cells lacking the IFNγR1 display reduced expansion compared to wildtype cells in the same host.[161,162,163] Thus, multiple types of innate-immune induced cytokines can boost expansion of activated T cells.

CD4+ T Cells Help CD8+ T-Cell Priming

Although DCs increase their immunogenicity in response to inflammatory stimuli delivered at

peripheral sites of antigen uptake, their stimulatory capacity can be further enhanced within the lymph node through the action of CD4+ "helper" cells (T_H) that recognize peptide antigens bound to MHC class II molecules at the surface of the same APC presenting to the CD8+ T cell[164,165,166] (Fig. 31.3). The contribution of T_H to CD8+ priming had initially been thought to be conditional (ie, required for response to some immunogens but not others) and limited in mechanism to the production of paracrine IL-2 for the benefit of CD8+ T cells present in a "three-cell cluster" together with APCs.[167,168,169] More recent evidence, however, has shown that CD4+ T cells make important contributions to the generation and maintenance of CD8+ T cell responses at a number of discrete steps.[165,170] CD4 T cells can produce IL-21 and IL-4, in addition to IL-2, to support CTL expansion.[171,172,173,174,175] Several studies have shown that a key function of T_H is that it involves the engagement of CD40 on DCs that leads to a number of immunostimulatory alterations, including high-level expression of

costimulatory and adhesion molecules, among them CD80, CD86, 4-1BB, OX40, and CD70, as well as high-level production of IL-12.[94,176,177,178,179,180] CD4+ T cells can also help guide naïve CCR5-expressing CD8+ T cell precursors to relevant APCs within secondary lymph nodes by enhancing production of the chemokines CCL3 and CCL4 at the CD4+ T-cell/DC interface.[181,182]

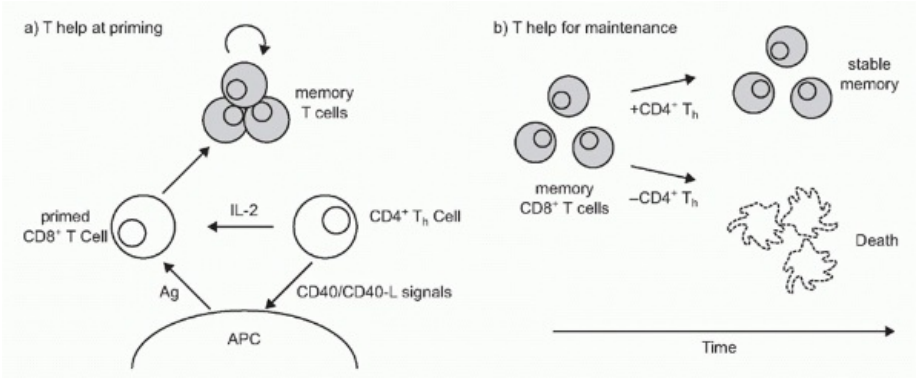

FIG. 31.3. Models of CD4+ T-Cell Help for CD8+ Memory Formation. CD4 T cells can help at two different stages of effector and memory CD8 T-cell development. **A:** CD4 T cells can help CD8 T-cell activation via multiple putative mechanisms including CD40:CD40L interactions between CD4 T cells and antigen-presenting cells (APCs), interleukin-2 secretion, or enhancing chemokine production by causing APCs. **B:** CD4 T cells also help generate functional memory CD8 T cells that are maintained long-term.

The absence of T_H can have different effects on the CD8+ T-cell response depending on the immunogen and the functional parameter measured. In certain settings of noninfectious immunizations using heat-killed or fixed preparations of pathogens or cocktails of foreign protein plus adjuvants, the extent of antigenic stimulation and inflammation can be relatively weak compared to immunization with live, replicating pathogens. In these cases, the antigens will often be cross-presented to CD8 T cells and their response will be CD4 T-cell dependent.

In contrast, the primary CD8 T-cell response to many, but not all, acute viral or bacteria responses are only modestly decreased in the absence of CD4 T cells. It is thought that the overt stimulation of the innate immune response, and perhaps, more prolonged and widespread inflammation and antigen presentation delivers a stronger signal to the CD8 T cells to overcome the need of CD4 T cells for activation during infection. However, in chronic viral infections, where antigen persists, CD4 T-cell help becomes essential to sustain virus-specific CD8 T-cell survival and function overtime.[183,184,185,186,187,188,189]

Effector T-Cell Migration

Following commitment to clonal expansion and acquisition of effector functions, CD8+ T cells begin to express new surface patterns of chemokine, homing, and adhesion receptors that mediate their egress from lymph node and facilitate their migration to peripheral sites. An initial event in this process is the downregulation of CD62L and CCR7, the receptors used by naïve precursors to enter the lymph node, and restoration of the chemotactic response to the lysophospholipid sphingosine-1-phosphate through reexpression of its receptor (sphingosine-1-phosphate-receptor-1), which promotes migration to blood and lymph by distinct sources of its ligand.[36,190,191,192] Following this, the migration of effector CD8+ T cells to nonlymphoid tissues will be largely determined by homing receptors and chemokines.[193] These include chemokine receptors such as CCR2 and CCR5 that guide cells to inflammatory sites through recognition of a variety of chemokines induced by inflammatory cytokines. In some cases, and for reasons that are poorly understood, a subset of these cells will reexpress CD62L and CCR7, thereby allowing their reentry into the lymph node where they can function as T_{CM}, the functional properties of that will be described in the following section.[194] Others will migrate to the spleen via the blood and take up residence in the red pulp and are largely excluded from the white pulp.[195] The ultimate “address” to which the cell will migrate, however, can be influenced by the conditions under which it was activated. For instance, in the intestine, DC from Peyer patches—specialized secondary lymphoid organs within the intestine—have been shown to induce the selective expression of the integrin α4β7and CCR9 chemokine receptor, both of which are essential for gut homing, in CD8+ T cells.[196,197,198,199] Likewise, epicutaneous priming of T cells enhances expression of E-and P-selectin ligands and their subsequent homing to the skin via chemokine receptors CCR4 and CCR10, which bind CCL17 expressed by skin vascular epithelium and TACK expressed on keratinocytes.[200,201,202] Thus, DCs not only “alert” naïve T cells to the presence of an infectious pathogen, but also instruct T cells to express the appropriate homing

receptors that enable their migration to the specific anatomic site where they are needed.[37]

Phase II: Effector to Memory Transition: Resolution of Immune Response and Formation of Memory T Cells

Contraction of Effector T-Cell Populations

Coincident with pathogen clearance at the end of the primary response, the majority of effector T cells are eliminated through a complex and poorly understood process that

involves their orderly apoptosis (programmed cell death) via interactions between growth factors, various member of the TNFR superfamily and elements of the mitochondrial pathway of apoptosis.[203,204] The contraction phase leaves a small population of cells, ~5% to 10% of the numbers present at the peak of the response, to survive as memory cells, which in some cases remain stable for the life of the animal.[205,206] The removal of effector cells achieves two important goals for the immune system: it prevents the T-cell repertoire from being overly populated or "focused" with T cells specific to a particular pathogen, and it restores homeostasis to the available environmental niches for support of T cells so that new antigenic challenges can be met. The magnitude of expansion of different epitope-specific T-cell populations can vary during an infection, creating what is referred to as "immunodominant" or "subdominant" T-cell populations based on their relative cell numbers. Interestingly, for CD8 T cells, the magnitude of contraction remains fairly constant across both immunodominant and subdominant clones such that a similar frequency of cells (~90% to 95%) die during the contraction phase.[21,131,132] These data suggest that the process of contraction occurs fairly equally across various populations of effector T-cell clones, irrespective of differences in precursor frequency and/or the number of times the cells may have divided during clonal expansion.

The loss of antigen-specific T cells at the end of immune responses is largely attributable to apoptotic death, a process traditionally characterized by the activation of a caspase cascade that results in the death of the host cell by cleavage of critical cellular proteins and creation of deoxyribonucleic acid double-stranded breaks.[207] There are two main pathways by which caspases can be activated in T cells: the death receptor pathway and the mitochondrial pathway.[206,208,209] Although the regulation of these pathways in activated T cells in vivo is not entirely clear, several chief factors are thought to be involved: 1) The abundance and duration of antigen is a major determinant of clonal burst size of activated T cells, and stemming from the original observation that restimulation of activated T cells with antigen in vitro can induce cell death, a process known as activation-induced cell death (AICD), antigenic stimulation likely also modulates T-cell death during infection.[210] For example, in certain cases of chronic viral infection, such as LCMV infection in mice, it is thought that persistent antigenic stimulation can lead to the physical deletion of certain epitope-specific CD8 T-cell populations, leaving holes in the repertoire of pathogen-specific T cells.[187,189] However, this does not happen to all virus-specific CD8 T cells during chronic LCMV infection, as virus-specific CD8 T cells directed against other viral epitopes are spared but develop into less functional CTLs, referred to as "exhausted" T cells that express inhibitory receptors that reduce TCR signal transduction.[146,189] In contrast, during other latent infections, such as the murine cytomegalovirus (a herpes virus), some epitope-specific populations contract like normal, but others recognizing latently produced viral proteins may actually "inflate" after the acute phase of infection due to low-grade antigen persistence from viral reactivation.[211,212] 2) Likewise, subsequent encounter with death receptor ligands during or near the end of their clonal expansion phase can also affect effector cell contraction.[213,214,215] 3) The third is the decline in T-cell growth factors and survival signals, such as IL-2, following resolution of infection. In this scenario, it is postulated that the effector cells compete for the dwindling supply of resources. 4) The fourth is the intrinsic heterogeneity of the effector cell population and the balance of proapoptotic (Bim, FADD,

FLICE, etc.) and antiapoptotic (c-FLIP, Bcl-2, Bcl-X_L, etc.) proteins they express.[207,216] 5) Lastly, the anatomic location of the T cells within tissues may also regulate the exposure of effector and memory T cells to signals that promote survival or apoptosis.[34,35,36,217,218,219] In the following, we will outline the major molecules that have been found to control effector T-cell apoptosis following infection.

Extrinsic Control of Effector T-Cell Apoptosis. The death receptor pathway activates apoptosis via the interaction of TNFR family receptors with protein activators such as TNFα and effector caspases 3 and 7.[208,220] With regard to the death receptor pathways, fewer effector cells died following viral infection in mice lacking both p55 and p75 TNFRs, but surprisingly, there was little effect of deleting Fas or Trail.[213,214,221,222,223,224] This result was unexpected given the critical role of Fas in T-cell homeostasis and the in vitro process of AICD.[210,225] However, Fas was found to play an important role in contraction of antigen-specific T cells during chronic infections or autoimmune situations where prolonged interaction with antigen occurs.[215,226,227] Moreover, Trail also plays a more prominent role in controlling the survival of memory CD8 T cells that are formed without robust CD4 T-cell help.[224,228,229] Thus, control of effector and memory T-cell survival operates differently according to the type of situation encountered.

In addition to the death-inducing signals delivered by some TNFR family members, others also serve a role in costimulating T cells during priming, as discussed previously, and inducing survival signals for activated CD8 T cells during the contraction phase. In particular, interference with or deletion of 4-1BB or OX40 on T cells impairs effector cell expansion and augments their contraction following viral infection. Consequently, fewer memory T cells develop in the absence of these receptors.[230,231,232,233] Interestingly, 41BB preferentially functions in CD8+ T cells and OX40 preferentially functions in CD4+ T cells, albeit OX40 also has a role in promoting effector CD8 T-cell differentiation and survival.[234,235] Additionally, the cytokines IL-7 and IL-15 upregulate the costimulatory TNFR family members, OX40 and 4-1BB, respectively, and this may provide additional survival signals to activated T cells.[236,237] Thus, distinct

niches may be created between DCs and antigen-specific T cells during the contraction phase whereby the interactions between cytokines, cytokine receptors, and TNFRs sustain T-cell survival.[236]

Intrinsic Control of Effector T-Cell Apoptosis . Another form of T-cell apoptosis referred to as activated T cell autonomous death (ACAD) differs from the death receptor pathway in that it does not initiate from a specific receptor, but rather from disruption of the mitochondrial or lysosomal membranes, leading to activation of proteases such as caspases or cathepsins, respectively.[238] The mitochondrial-regulated pathway of apoptosis is well characterized and initiates upon disruption of the mitochondrial membrane and release of cytochrome c into the cytoplasm. This integrates into a multisubunit complex (the apoptosome) to activate the downstream effector caspases 3 and 7,[205,208] an endpoint common to the death receptor pathways mentioned previously.[206,239] ACAD also involves the proapoptotic activities of the Bcl-2-related protein Bim and PUMA (p53-upregulated modulator of apoptosis) that are

induced by a variety of cellular stressors including the withdrawal of key survival cytokines such as IL-2, IL-7, or IL-15.[204,221,240,241,242,243,244,245] BIM can bind Bcl-2, BCL-X_L, and MCL-1 on the mitochondrial membrane, and promote the release of cytochrome *c* and the induction of cell death.[246,247] During their expansion phase, T cells also decrease their expression levels of Bcl-2 and Bcl-X_L and thereby allow for activation of the intrinsic ACAD pathway.[248] Indeed, ACAD, rather than AICD, is thought to be responsible for the contraction of CD8+ T cells that follows the expansion phase because a single deficiency in Bcl-2 results in greater death of effector T cells, and conversely, single deficiency in Bim or double deficiency in Bax and Bak profoundly enhances effector T-cell survival following infection.[215,227,242,249,250,251,252,253,254] Interestingly though, Bim-/- T cells do gradually decay, approaching near wild-type numbers several months after infection.[215,227,251,252] This finding suggests that alternative homeostatic control mechanisms and/or possibly competition for survival niches or factors, ultimately control T-cell numbers in the face of Bim deficiency.

The actions of effector T cells themselves, especially cytotoxic T cells, can cause self-inflicted injury and promote cell death. For instance, effector molecules such as perforin, granzymes, and IFNγ play a role in contraction, as mice deficient in either of these have increased numbers of CD8+ T cells in both the expansion and contraction phases.[161] A set of proteins involved in protecting CTLs from their own cytotoxic machinery are the serine protease inhibitors Spi2a and Spi6.[255,256,257] Spi2a inhibits the activation of cathepsin B in the lysosomal cell death pathway, a lesser-characterized cell death program that may involve both caspase-independent and -dependent mechanisms.[257] When overexpressed, Spi2a was cytoprotective and reduced effector CD8 T-cell contraction following LCMV infection. In contrast, “knockdown” of Spi2a increased the magnitude of effector cell death and fewer memory CD8 T cells formed. Another serine protease inhibitor, Spi6, that targets granzyme B, maintains the integrity of cytotoxic granules by inhibiting the release of granzymes into the cytosol and preventing CTL “suicide.”[256,258] Consequently, Spi6-deficient CD8 T cells have substantially reduced clonal expansion during viral infection.[256]

Heterogeneity of Effector T Cells and Memory Cell Potential

The previous section described numerous mechanisms that determine *how* effector T cells die following clonal expansion, but the factors that actually determine *which* of the effector cells die or live to populate the memory T-cell pool remains to be better resolved. Traditionally, it was proposed that effector T-cell death was more or less random and dependent on the competition between cells for limiting T-cell survival factors, such as common-gamma chain cytokines (IL-7, IL-2, IL-15). This permissive model implied that all the effector cells were equipotent in their ability to persist and give rise to memory T cells, and it was availability of extrinsic signals that ultimately determined their fate. Although competition for survival signals in cellular niches does impact effector and memory T-cell homeostasis, it is also evident that the effector cell population is heterogenous and contains effector cells that are intrinsically more “fit” to survive long-term and develop into memory T cells. These cells have been referred to as memory precursor cells (MPCs) and have best been characterized in CD8 T-cell populations based on IL-7Rα expression following acute viral and

bacterial infections. Both naïve and memory CD8+ T cells express high levels of IL-7Rα, but this receptor is downregulated in the majority of effector CTLs during clonal expansion.[64,70,259,260,261] However, a small percentage of the effector cells express relatively higher amounts of IL-7Rα and separation of virus-specific IL-7Rα^{hi} and IL-7Rα^{lo} CD8 T cells from LCMV-infected animals demonstrated that IL-7Rα^{hi} effector cells had the greatest capacity to give rise to long-lived memory CD8 T cells that could proliferate and protect against secondary infection.[260,262,263] Subsequent experiments have indicated that this is more likely due to a survival advantage mediated by IL-7Rα expression in conferring enhanced sensitivity to ambient IL-7 and increased expression of bcl-2, and that CD8+ memory T cells are not selected solely on the basis of IL-7/IL-7Rα interactions, but result from a more complex series of differentiation events, as described subsequently.[264,265,266,267,268] Although the IL-7Rα^{hi} effector CD8 T cells preferentially survive following infection compared to IL-7Rα^{lo} cells, there is some death in this population as the memory CD8 T cells form, and therefore this does not represent an absolute determinant of memory T-cell potential.[142,249,269] Moreover, not all types of immunizations lead to distinguishable populations of IL-7Rα^{hi} and IL-7Rα^{lo} effector cells.

Memory CD4+ T-cell development may be considered more complicated than that of CD8 T cells because of the diversity in the types of effector T cells formed (eg, Th1, Th2, Th17, Tfh, T_{reg} cells) during immune responses.[107] It is unclear if all types of CD4 T cells can generate memory and if so, whether the pathways involved are similar or

distinct. Like CD8 T cells, IL-7Rα is also critical for formation of memory CD4 T-cell populations, and it is similarly repressed in effector CD4 T cells and then reexpressed on memory CD4 T cells during infection.[270,271,272] It remains to be determined if IL-7Rα^{hi} CD4+ effector T cells have greater memory cell potential following infection. It is likely that other IL-7Rα-dependent cytokines (such as TSLP) are involved as blockade of IL-7 or STAT5 had little effect on short-term effector CD4 T-cell survival.[273,274] Other factors known to regulate activated CD4 T-cell survival long-term are Akt and CD44, one of the most widely used markers for memory T cells.[275,276]

How are heterogeneous populations of effector and memory CD8+ T cells established? It is clear that the duration of infection, level of inflammatory signals, time point at which naïve cells are recruited into the response, and tissue localization can impact both the type of effector T cell generated as well as the memory potential of the activated T cell.[142,262,277,278,279,280,281,282] The vast phenotypic and functional heterogeneity observed in antigen-specific CD8+ T-cell populations (as described previously) indicates that this differentiation process is not based on a simple binary cell fate decision, for instance to become a terminal effector cell versus becoming a memory precursor cell. Rather, a more appropriate model for T-cell differentiation may be one in which activated T cells can occupy not just two differentiation states, but a whole spectrum of differentiation states, and these cells can transition from one state to another according to changes in signal input. Indeed, recent in vivo lineage-tracing studies using transgenic mice that express reporter genes indicate that different types of effector CD4+ T cells are likely not fixed lineages as previously

thought, but contain cells with greater plasticity that can acquire certain features and functions of other types of CD4+ effector T cells.[106,283] For instance, IL-4-producing Th2 cells and IL-17-producing Th17 cells can acquire IFNγ production when restimulated under Th1-conditions, respectively,[284,285] and FoxP3+ T_{reg}s can acquire IFNγ and IL-17 expression.[286,287,288]

Transcriptional Control of Effector and Memory Cell Fates

Although memory T cells share many features with shortlived effectors, their capacity for self-renewal and recall response suggests that they are guided by a distinct program of gene expression. Moreover, the distinction between MPCs and other effector cells that are less fit to populate the memory T-cell pool indicate that variations in effector T-cell differentiation govern these cell fate choices early during the immune response. Investigations into the molecular events that guide the generation memory T cells and their precursors are at a relatively early stage in comparison to the phenotypic studies through which these phenomena have been defined. Nonetheless, a framework for understanding the genetic program of memory is beginning to emerge through the identification of genes and regulatory elements that control key functional parameters of T-cell memory.[30,289] Initial studies focused on performing genomescale gene expression analysis in T cells using gene array hybridization techniques, first to resting versus activated T cells, and subsequently to T cells undergoing homeostatic proliferation.[31,138,290,291] These studies revealed that the gene expression in T cells is a dynamic process that undergoes substantial alterations in response to TCR-mediated and environmental stimuli during the transition from resting to activated status. The phenotypic features of this process (eg, proliferation, effector differentiation, survival) are matched in the global patterns of genes expressed (eg, cyclins, effector molecules, antiapoptotic factors, etc.).

A key study on the transcriptional profile of memory T cells comes from Kaech et al., who performed genomewide analyses of gene expression on naïve, effector, and memory transgenic CD8+ T cells activated in vivo by LCMV.[30] Among the findings emerging from a comparative analysis was that each phase of development was characterized by specific patterns of gene expression directly related to the development or function of the cells at that stage of differentiation, with roughly 30% of the genes expressed by effector cells were also maintained in the memory cells, supporting the direct lineage relationship between these cell types.[30] In some cases, the expression level of the genes was set at the peak of the effector response (d8), but in others the level of expression decreased as the gradual conversion to memory occurred, perhaps reflecting a global reduction in transcriptional activity in these cells. Whereas the distinction of T_{EM} and T_{CM} subsets have traditionally been defined by phenotypic markers, Willinger et al. used transcriptional profiling to analyze the molecular signature of these subsets in human CD8+ T cells and found that the T_{CM} subset had a pattern of gene expression that was intermediate between naïve and T_{EM} and effector/T_{EM} phenotype cells.[292] It has long been appreciated that memory T cells share several notable features related to self-renewal with long-term hematopoietic stem cells. Consistent with this, Luckey et al. have found that memory T cells appear to share many elements of their transcriptional program with long-term hematopoietic stem cells, with notable increases in the

expression of prosurvival and antiapoptosis genes.[293]

In parallel with the genomic-scale studies of gene expression at the population level in T cells, efforts have been made to define the genetic program of memory in single cells or with respect to specific transcription factors. The recent study of Peixoto et al. is notable in its study of gene expression patterns at the level of single cells at various stages during an immune response.[294] This study revealed surprisingly heterogeneous and dynamic patterns of gene expression from the earliest point in the response, with both proinflammatory and effector functionality revealed in discrete cells, and was followed by a more uniform pattern in secondary responders. It is unclear if the reduction in heterogeneity at later time points reflects a selection process or progressive development of the entire memory populations. A number of studies have analyzed the contribution of specific transcription factors to the enhanced longevity and function of memory T cells. These include T-bet and eomesodermin, the transcription factors that coordinately regulate a number of genes related to lineage commitment and effector differentiation in both CD4+ and CD8+ T cells.[108,109,295,296,297] T-bet-/-

eomesodermin+/- mice have a near-total loss of effector and memory T cells through a mechanism that is believed to involve their role in regulating the expression of CD122 and thereby responsiveness to IL-15.[298] The roles of T-bet and eomesodermin in determining whether effector cells are selected for memory development may involve their reciprocal regulation in response to inflammatory signals via IL-12. In response to pathogen-induced IL-12, eomesoderminis repressed and T-bet levels increase which can lead to inhibition of IL-7Rα and MPCs, but increased clonal expansion and formation of terminal effector cells.[50,299,300] When IL-12 signals are reduced, eomesodermin levels rise while T-bet levels decrease, favoring the development of long-lived MPCs.[142,299] Another pair of transcription factors that are relevant for memory formation is inhibitor of deoxyribonucleic acid binding 2 (Id2), and Id3, antagonists of E protein transcription factors that are upregulated in CD8+ T cells during infection and appear to have somewhat reciprocal expression patterns and temporal modes of actions.[301,302] Id2 plays in important early during clonal expansion in sustaining viability of proliferating CD8+ T cells, and it is expressed to a higher level in terminal effector CD8 T cells, whereas Id3 is expressed to a higher extent in memory precursor cells and is required for their survival following infection during the effector to memory transition.[301,302,303] Id2- and Id3-deficient CD8+ T cells have altered patterns of expression in genes that influence cell survival, but they appear to operate on distinct sets of genes.[301,302,303]

In addition to the transcription factors described previously, several transcriptional repressors have emerged as regulators of T-cell memory. Bcl-6, first described as a transcriptional repressor with pleiotropic functions in B-cell differentiation,[304] appears to play a role the generation of both CD4+ and CD8+ T-cell memory, as the survival of Bcl-6-deficient CD4+ effector T cells was greatly diminished compared to wild-type controls after priming, and Bcl-6-deficient mice were found to lack T_{CM} memory phenotype CD8+ T cells following immunization.[305,306,307] The Bcl-6 homologue Bcl-6b, known to repress IL-2-induced B-cell differentiation, is expressed by a small subset of CD8+ T cells and regulates their capacity

for secondary expansion in response to antigenic challenge.[308] Another transcriptional repressor first described in B cells that influences T cell homeostasis is Blimp-1, which is required for terminal differentiation of B cells through coordinate regulation of hundreds of genes.[309,310] Mice lacking Blimp-1 suffer from a variety of immune-mediated pathologies including colitis and multiorgan inflammatory diseases that are characterized by accumulation of effector and memory T cells.[311,312] Moreover, Blimp-1 represses Id3 expression and promotes the formation of highly functional, yet terminal, IL-7Rα^{lo} CTLs during viral infection.[301,313,314] In the absence of Blimp-1, many more IL-7Rα^{hi} memory CD8 T cells and their precursors form; however, these cells are less efficient in migrating out of the lymphoid organs and defending against respiratory infection. Therefore, the Blimp-1:Bcl-6 axis is an important regulator of effector and memory CD8 T-cell fates. Interestingly, the balance between Blimp-1 and Bcl-6 also regulates T_H1:follicular helper T cell fates during viral infection.[128]

Phase III. Maintenance and Function of T-Cell Memory

The small fraction of effector T cells that survives contraction will initiate the memory pool that confers, in many cases, lifelong protection to the individual. Despite their extensive culling from peak numbers, antigen-specific cells in the postcontraction population are found at a far higher precursor frequency than in the naïve repertoire, a fact that undoubtedly impacts the increased speed and intensity that is characteristic of secondary responses. Although they possess some features associated with memory, having arisen in response to an antigenic challenge and being able to persist after the majority of their fellow effectors have been eliminated, the postcontraction T cells nonetheless comprise a heterogeneous population whose maintenance in the memory pool is a dynamic process involving phenotypic changes that will determine their function, distribution, and protective capacity over time.[36,42,315] A central theme governing this process involves adaptation to the homeostatic control mechanisms that regulate the equilibrium of naïve versus memory T cells. These pathways appear to be distinct and under independent control, as the homeostatic mechanisms that regulate the naïve and memory T-cell pools differ in their requirement for extrinsic factors such as TCR stimulation by self-MHC molecules (presumably occupied with peptide derived from self- or environmental antigens) and signals provided by the γ_c cytokines IL-7 and IL-15.

T cells sense each other and maintain homeostasis by competing with one another for survival signals, likely in the confines of cellular niches within tissues. When the T-cell compartment is “full,” as normally found in lymphoreplete states, naïve T cells are fairly quiescent and memory T cells divide periodically, approximately once every 2 to 4 weeks. This slow turnover of memory T cells is referred to as “basal proliferation” or “homeostatic turnover,” and is thought to be important for maintaining the pool of memory T cells.[259] In contrast, in lymphopenic states, such as following irradiation or transfer into immunocompromised mice lacking T cells (ie, recombination activating gene-deficient mice) both naïve and memory T cells will divide more rapidly to fill the available “space.” This “homeostatic expansion” more commonly referred to as “homeostatic proliferation” is polyclonal and occurs independent of foreign antigen recognition. For the purposes of this section, we shall refer to the slow turnover of T cells in a normal immunocompetent host as

"basal homeostatic proliferation" and the faster, more extensive division of T cells within lymphopenic hosts as "homeostatic expansion."

The Role of Major Histocompatibility Complex in Memory T-Cell Homeostasis

As discussed previously in this chapter, adoptive transfer studies have shown that the long-term survival of naïve T cells in lymphopenic hosts requires contact with self-MHC.[62,63,64,65,66,67] Whereas initial studies in the 1990s suggested that both types of memory cells were maintained by occasional TCR contact with undetectable amounts of sequestered foreign or cross-reactive environmental antigens,

more recent studies have shown that antigen-specific CD8+ memory T cells can survive and remain functionally competent in the absence of either antigen, TCR signaling adaptors, or MHC molecules, arguing against any *obligatory* role for TCR signaling in their maintenance.[32,67,316,317,318,319,320,321] However, the same may not be true during cases of chronic antigen exposure, such as during chronic infections. One study has demonstrated that virus-specific CD8 T cells isolated from mice with a chronic LMCV infection require antigen for their persistence,[145] and a similar situation may be evidenced after control of viremia by highly active antiretroviral therapy in patients with HIV.[322,323] Moreover, in another study, inducible ablation of the TCR on mature MP T cells showed that MP CD8+ T cells declined slowly over time, possibly indicating differences between antigen-derived versus MP T cells. The situation for CD4+ memory T cells is less clear, because while the antigen-specific CD4+ T cells do not strictly depend on MHC for their maintenance, the rate of both basal homeostasis and homeostatic expansion of MP CD4+ T cells does appear to depend on the TCR-MHC interactions, and declines significantly when these signals are extinguished, leaving the cells in one study critically dependent on IL-7 or less responsive to antigen reencounter in vitro and in vivo.[62,324,325,326,327,328,329] Thus, it is likely that the homeostasis of memory CD4 and CD8 T cells depends on some common, but also distinct, signals.

The Role of Common γ-Chain Cytokines in Memory T-Cell Homeostasis: Interleukin-7 and Interleukin-15

These findings of MHC-independent survival of memory T cells have brought cytokines into focus as the main extrinsic factors responsible for their maintenance, with particular emphasis on the common γ_C family members IL-7 and IL-15. One of the important initial observations linking cytokines with memory T-cell survival came from studies in which agents capable of stimulating IFN production by cells of the innate immune system, such as lipopolysaccharide and Poly I:C caused a transient burst of TCR-independent proliferation from the major $CD122^{hi}$ $CD44^{hi}$ subset of antigen-experienced CD8+ T cells through the IFNαβ-induced production of IL-15.[330,331,332,333,334,335] Importantly, the IFN-induced proliferative burst of $CD122^{hi}$ $CD44^{hi}$ CD8+ T cells was not observed in IL-15-/- mice, and the in vivo administration of exogenous IL-15 was found to mimic the effect of IFNs in stimulating the bystander proliferation of the MP CD8+ T-cell subset.[331,336,337]

The receptor for IL-15 is composed of three subunits: a unique α chain, a β chain shared with the IL-2 receptor and γc, the latter sheared with the receptors for with IL-2, IL-4, IL-7, IL9, and

IL-21.[338,339,340] Although a soluble cytokine under in vivo conditions, IL-15 is presented in trans via cell-associated complexes bound to IL-15Rα that are preassembled in the cytoplasm of the (non-T) cells that make both molecules.[341,342,343] Paradoxically, CD8+ T cells also express IL15Rα, and although this is not essential for them to respond to IL-15 (which requires expression of only the β and γ chains), it may enable them to activate other cell types such as T cells or APC via transpresentation.[341,342,344,345] The expression of CD122 is selectively elevated on the MP ($CD44^{hi}$) subset of CD8+ T cells that undergo bystander proliferation in response to IL-15, and both IL-15-/- and IL-15Rα-/- mice contain significantly reduced numbers of $CD44^{hi}$ $CD122^{hi}$ MP CD8+ T cells.[331,346,347,348] The effect of IL-15 on MP CD8+ T cells may reflect its role in cell survival rather than development, as these fail to proliferate and disappear rapidly upon transfer to IL-15-/- recipients.[331] Consistent with this view, IL-15 transgenic mice were found to contain elevated numbers of MP CD8+ T cells.[337,349] Taken together, these findings have led to the current view that the generation, survival, and basal homeostatic proliferation of MP CD8+ T cells is mediated through contact with ambient IL-15. At steady state, DCs and macrophages are the most important cell types that need to express IL-15:IL-15Rα complexes to sustain memory CD8 T cells in lymphoid organs, and intestinal epithelial cells are critical in the gut.[217] However, this does not exclude the production of IL-15 by a variety of other cell types including skeletal muscle, kidney, placenta, and hematopoietic stromal cells under conditions of innate immune activation via the IFN pathway.[334,350,351]

Studies of Ag-specific CD8+ T-cell responses following viral infection (LCMV or vesicular stomatitis Indiana virus) in IL-15-/- or IL-15Ra-/- mice have shown that, similar to MP CD8+ T cells, IL-15 is also important for the maintenance of Ag-specific memory CD8+ T cells.[259,352,353] These studies found that although Ag-specific CD8+ T cells were generated in the absence of IL-15 signals, they did not proliferate and their numbers declined over time.[352,353] Taken together, these observations show that IL-15 mediates the homeostatic turnover of both MP and Ag-specific memory CD8+ T cells. Although important for memory CD8+ T-cell homeostasis, IL-15 does not appear to be required for their function, as LCMV-specific memory CD8+ T cells are able to produce IFNγ and TNFα and respond to secondary infection.[352,353] The main effect of IL-15 on the homeostatic survival of CD8+ T cells is likely mediated through upregulation of antiapoptotic factors such as BCL-2 and BCL-X_L, and avoidance of death via the mitochondrial pathway of apoptosis, a common mechanism of γ_C cytokines described previously.[68,354,355,356] With regard to CD4 T cells, IL-15 was initially considered irrelevant for the development and homeostasis of MP CD4+ T cells, as these express low levels of CD122 and can be found in normal numbers in IL-15-/- mice.[335,347,357] However, recent studies using transgenic and endogenous LCMV-specific CD4+ memory T cells have shown that memory CD4+ T cells, are dependent on IL-15 for their basal homeostatic proliferation and long-term survival.[270] Consistent with this view, it has been shown that human and mouse CD4+ T cells respond to exogenous IL-15 and that IL-15- transgenic mice display enhanced CD4+ T cells responses to infectious pathogens.[349,358] Taken together, these data indicate that both CD4+ and CD8+ memory T cells are dependent on IL-15 for their homeostatic survival.

The other cytokine known to affect the homeostatic survival of memory T cells is IL-7. As discussed previously, IL-7 is constitutively produced by nonhematopoietic cells and is recognized by a heterodimeric receptor consisting of the IL-7Rα chain and γ_c.[359,360] IL-7 protects T cells from death by maintaining glucose uptake and inducing the antiapoptotic

factors Bcl-2 and Mcl-1, and inhibiting the proapoptotic factors Bax, Bad, and Bim.[69,361,362,363,364,365,366,367] Furthermore, the T-cell developmental defect in IL-7- or IL-7Rα-deficient mice can be rescued by a Bcl-2 transgene or deletion of Bax, and addition of anti-IL-7/anti-IL-7Rα to memory cell cultures was found to reduce Bcl-2 expression.[271,361,368,369] IL-7:IL-7Rα signaling is critical for the survival of antigen-specific memory CD8 T cells in most but not all cases.[64,259,260,265,370] However, in lymphoreplete animals, unlike IL-15, IL-7 does not seem essential for basal proliferation, but increasing the level of IL-7 in vivo through transgenic expression can overcome the requirement for IL-15 in promoting survival and homeostatic expansion of $CD122^{hi}$ memory CD8+ T cells.[259,371] Within a lymphopenic host, IL-7 and IL-15 appear to have redundant roles in promoting memory CD8+ T-cell homeostatic expansion because single deficiency of either gene alone does not impair memory T-cell proliferation; however, when both IL-7 and IL-15 are removed through gene knockouts and use of antireceptor antibodies, homeostatic static expansion ceases and memory CD8+ T cells rapidly die.[259,260,273] Together, these results indicate that IL-7 plays a more dominant role in memory CD8+ T-cell survival and IL-15 plays a more dominant role in basal proliferation, but these roles are not entirely separable and alterations in the levels of these cytokines or the numbers of lymphocytes competing for these cytokines can modulate the effects these cytokines have on memory T-cell homeostasis.

Similarly, multiple studies have shown the requirement for IL-7 in the long-term maintenance of Ag-specific memory CD4 T cells. For instance, the basal proliferation Agspecific memory CD4 T cells was heavily reliant on IL-7, in addition to IL-15,[248,270,357] and homeostatic expansion of such memory T cells in irradiated, lymphopenic hosts was acutely dependent on IL-7.[270] Interestingly, homeostatic expansion of polyclonal MP CD4+ T cells was initially found to occur independent of IL-7 or IL-15.[372] However, subsequent work revealed that the proliferation of MP CD4+ T cells in these circumstances was not due solely to lymphopenia, but likely also involved antigen-dependent proliferation occurring in response to commensal-related antigens.[373] In addition to its effects on basal proliferation of Ag-specific memory CD4+ T cells, IL-7 is also important for the longevity of memory CD4 T cells. Transfer of Ag-specific memory CD4 T cells into mice lacking IL-7 or blocking IL-7 with antibodies showed that IL-7 was needed for survival of the donor memory T-cell population.[270,271,272] However, the role for IL-7 in memory CD4+ T-cell survival may be temporally regulated because in contrast to the previously discussed experiments depriving Ag-specific effector CD4 T cells of both IL-7 and IL-15—or deletion of STAT5, a critical molecule downstream of γ_c cytokines—during the contraction phase following viral infection had little effect on effector CD4+ T cell apoptosis.[273,274] In contrast, the effector CD8 T cells were acutely sensitive to these cytokines during the contraction phase and the magnitude of contraction was increased by IL-7, IL-15, or STAT5 deficiency.[273] These results suggest that there may be

other factors that regulate effector CD4 T-cell survival early after infection, but IL-7 and IL-15 are essential to sustain Ag-specific memory CD4+ T cells long term.

Related to this point, compared to memory CD8 T cells, the pool of memory CD4+ T cells may be less stable, in general, because gradual erosion of Ag-specific memory CD4 T cells has been observed in several longitudinal studies following acute infection in mice. For instance, LCMV- and *Listeria*-specific CD4+ T cells initially contract rapidly, but then show a continual slow rate of disappearance whereas the CD8+ T response in these studies achieved a stable "set-point" of memory after approximately 30 to 40 days.[374,375] Similarly, the numbers of CD4+ Sendai virus-specific T cells in the lung declined during the first 3 months after pulmonary infection, while the CD8+ T-cell response remained relatively stable during this period.[376] There are several potential reasons for this difference between CD4 and CD8 T cells, such as 1) the rate of basal proliferation of memory CD4 T cells is slower than that of CD8 T cells and 2) although not essential for memory T-cell maintenance, CD4 T cells may compete more for MHC interactions because MHC class II has more limited array of expression compared to MHC class I. Additionally, several reports have implicated IFNγ as a regulator of T_H1 effector cell contraction. For example, IFNγ-deficient mice generate 30% to 50% more activated CD4+ T cells in murine models of bacterial, mycobacterial infection, and experimental autoimmune encephalitis.[377,378] Moreover, activated CD4 T-cell longevity can be extended when IFNγ signaling is blocked during priming in vitro or *Listeria* infection in vivo.[379,380,381] The mechanism underlying the selective susceptibility of IFNγ-producing T_H1 cells appears to involve the induction of apoptosis via a caspase 8-dependent pathway and is selective for this CD4+ T-cell subset, as T_H2 cells display a greater propensity for memory formation.[382,383] However, it is important to mention that elegant lineagetracing studies have clearly shown that T_H1 cells that expressed IFNγ can populate the memory pool and, therefore, IFNγ may influence, but it is not the sole determinant of effector CD4+ T-cell apoptosis.[384] Lastly, there is currently little information on the ability of the T_{reg} and T_H17 subsets to form memory. Functional Th1, Th2, Th17, and Tfh cells can be found in the memory T-cell compartment in humans, suggesting they can form memory cells.[385,386,387] However, the functional polarization of human T_{CM} Th1, Th2, or Tfh cells is modest compared to that of effector cells, suggesting functional heterogeneity in different types of human memory T cells.[385,388] It is less clear if memory T_{reg}s exist, and if so, a number of observations suggest that their differentiation state is unstable.[389,390]

Lastly, there is currently little information on the ability of the T_{reg} and T_H17 subsets to form memory, although this will certainly change in the near future due to active experimentation in these fields.

CD4+ T-Cell Help for Memory CD8+ T-Cell Development

CD4+ T cells have also been shown to be important for the function and maintenance of memory CD8+ T cells whether they were primed in the presence or absence of

CD4+ T_H cells.[391] The presence of CD4+ T cells during the priming of CD8+ T-cell precursors endows their clonal progeny with the ability to undergo secondary proliferative

responses upon reencounter with antigen.[392] That is, in the absence of CD4+ T_H cells, memory CD8 T cells can, in some circumstances, display a “helpless” or “lethargic” phenotype in which they proliferate less and produce fewer cytokines, especially IL-2, during primary responses, and are defective in their ability to mount secondary proliferative responses, a hallmark of immune memory.[392,393,394,395,396] The “help” under these conditions apparently need not be cognate (antigen-specific) as it is in the case of help for priming, suggesting that its role may involve the direct or indirect provision of homeostatic survival factors (discussed in the next section).[35]

How do CD4 T cells actually help memory CD8 T cells persist and function? In the case of memory CD8 T cells formed after a cellular vaccine, one mechanism involves the T_H-mediated modification of the CD8+ T-cell developmental program to avoid apoptosis upon secondary stimulation by TNF-related apoptosis-inducing ligand.[229] The role of TNF-related apoptosis-inducing ligand in this process may be limited in its duration, because although TNF-related apoptosis-inducing ligand appears to regulate the secondary expansion defect of “helpless” CD8+ T cells for at least 4 weeks after priming in the absence of T_H, additional factors supplied by CD4+ T cells are required for their homeostatic survival in the months afterwards.[224] Cytokines like IL-2 and IL-21 also help memory CD8 T cells. For example, CD8+ T cells deprived of IL-2 signaling due to CD25 deficiency have reduced secondary expansion after some, but not all, types of immunizations.[397,398,399] Although CD4 T cells are a likely source of IL-2, a more recent study showed that autocrine-production of IL-2 by memory CD8 T cells themselves, rather than CD4+ T_H cells, was necessary for optimal responses to secondary infection. 398 However, this does not rule out the possible role of CD4 T cells or other IL-2-producing cell types in other situations.[400,401,402,403] IL-21 also helps memory CD8 T cells develop and form protective CD8 T cells, and CD4+ T cells are the predominant producers of IL-21, but it is not known which types of CD4 T cells may be the physiologically relevant source CD4 T cells.[307,404,405]

TABLE 31.1 Phenotypic and Functional Properties of Memory T Cells

	Naive	1° Effector	T_{EM}	T_{CM}	T_{RM}
Surface	Human: CD45RA+	Human: CD45RA-	Human: CD45RA+/-*	Human: CD45RA-	Human: CD45RA-
Markers (a)	Mouse: $CD44^{lo}$	Mouse: $CD44^{hi}$	Mouse: $CD44^{hi}$	Mouse: $CD44^{hi}$	Mouse: $CD44^{hi}$
	CD62L+	CD62L-	CD62L-	CD62L+	CD103+
	CCR7+	CCR7-	CCR7-	CCR7+	CD62L-,

					CCR7-
	CD127+	CD127-	CD127+/-	CD127+	CD69+
			CD28+/- CD27+/-	CD28+ CD27+/-	Skin: CXCR3-, CLA+, PSGL1+
					Lung: CXCR3+, PSGL1+, VLA+(α1β1)
			CD57+/-	CD57-	Gut: CCR9+, α4β7+†
			KLRG1+/-	KLRG1-	
			PD1+/-‡	PD1-	
Functional					
Potential					
Proliferation	+++	-	+	+++	?
Effector Functions :					
IFNγ	-	+++	++	+ (CD4+), +++ (CD8+)	++
IL-2	+	+/-	+/-	++	
cytotoxicity	-	+++	++	+	++
Homing	2° L.O.	2° L.O., P.T.	2° L.O., P.T.	2° L.O.	Mucosal tissues/brain

CD, cluster of differentiation; IL, interleukin; T_{CM}, central memory phenotype T cells;

T_{EM}, effector memory phenotype T cells; T_{RM}, resident memory phenotype T cells. Data in table summarized from Sallusto et al.,42 Joshi et al.,142 Gebhardt et al.,202 Romero et al.,427 van Leeuwen et al.,428 Teijaro et al.,445 Masopust et al.,446 Clark et al.,647 Ravkov et al.,648 Purwar et al.,649 Joshi et al.650 Ibegbu et al.,651 and Sallusto et al.652

*Despite being a marker of naïve T cells, CD45RA is reexpressed on some memory T cells. Initial evidence suggested these cells were terminally differentiated effector cells, but more recent data suggests that CD45RA may also become reexpressed on resting memory CD8 T cells.653,654

†α4β7needed for entrance into intestine, but may not be maintained on resident memory T cells.

‡PD-1 is also a marker on "exhausted" CD8 T cells and follicular helper T cell CD4+ T cells, but recent work also shows it is prevalent on functional T_{EM} cells in humans.

The Phenotypic Heterogeneity of Memory T Cells

There is substantial heterogeneity within the memory population with regard to phenotype, function, and anatomic location, both in CD4+ and CD8+ compartments (Table 31.1). Moreover, the phenotypes of memory T cells, and likely also their differentiation states, are not completely stable and can change over time in response to various environmental stimuli. It has long been appreciated that memory T cells express surface markers that distinguish them from naïve cells.[406,407] Much early work was focused on the high- and

low-molecular-weight isoforms of the surface-expressed tyrosine phosphatase CD45, which can be generated through differential splicing of three extracellular exons, called A, B, and C, which are differentially expressed on naïve versus memory T cells.[408] In the human system, naïve T cells express the high-molecular-weight form called CD45RA that contains all three of the spliced exons, while memory cells tend to more often express the low-molecular-weight form called CD45RO, with the different phenotypes assigned based on reactivity with isoform-specific monoclonal antibodies.[409,410] There is significant heterogeneity in CD45 expression, however, with variation in surface levels of the low-molecular weight isoforms on memory T cells as a result of reexpression of CD45RA after its initial downregulation, which itself occurs at different rates on CD4+ versus CD8+ T cells.[411] Furthermore, although murine T cells also express variant isoforms of CD45, this has also proven to be a less reliable marker of naïve versus memory cells.[412,413,414] A number of studies have attempted to correlate the upregulation of adhesion molecules on memory cells, including members of the β1 (CD49d, CD40e, CD29) and β2 (CD11a, CD11b, CD18) family of integrins, as well as CD2, CD44, CD54, and CD58.[53] Among these, however, most appear to reflect recent activation status of the T cells, and only CD44 appears to be a stably expressed marker of memory cells that is expressed at high levels when T cells are transferred to antigen-free recipient mice.[317,321,415,416,417] However, high-level CD44

expression is also found on the MP T cells discussed in a previous section that may have developed from lymphopenia or exuberant cytokine production, so that CD44 expression alone cannot serve as an informative marker for antigen-specific memory cells. When used in conjunction with other markers, however, CD44 can be used to more specifically distinguish memory from naïve T cells. A number of other molecules can also differentiate between naïve and antigen-experienced cells. On CD8 T cells, these include both CD122, a component of both the IL-2 and IL-15 receptors, CD49d (a4 integrin), and Ly-6c, a small GPI-linked surface protein.[335,418,419,420,421] CD8+ T cells have also been shown to undergo changes in their cell-surface glycosylation that can help distinguish effectors from resting memory cells,[422,423] which may influence their reduced TCR signaling threshold.[424,425,426]

In addition to these phenotypic markers of memory T cells, efforts have been made to identify these cells based on their function, usually defined by the parameters of protection, proliferation, and persistence. The heterogeneity is broad and there are no absolute sets of defining characteristics, but generalizations or trends are emerging from the numerous investigations on different subsets of memory T cells identified. For example, increased expression of CD57 or KLRG1 or decreased expression of CD27 and CD28 identify subsets of T cells with heightened effector functions that typically express higher amounts of perforin, but appear to be terminally differentiated effector cells with reduced proliferative capacity and shortened telomeres.[42,427,428,429,430,431] Increased expression of inhibitory receptors such as PD-1, lag3, or TIM3 also identify CD8 T cells that have encountered high amounts of or prolonged periods of antigenic stimulation, such as during chronic viral infection or in tumors.[146,432,433,434,435] Memory T cells that produce the most IL-2 and have the greatest proliferative responses to antigen or rates of basal homeostatic proliferation express higher amounts of CD27, IL-7R, CXCR3, and CD62L.[278,436,437,438,439,440] These cells also tend to have longer telomeres compared to the $CD27^{lo}$ $CD28^{lo}$ $CD62L^{lo}$ cells.[42,427,441] The physiologic location of memory cells has been thought to represent a defining feature, as naïve T cells are not normally thought to occupy extralymphoid environments.[442] Recent studies using sphingosine-1-phosphate-receptor antagonists, however, have revealed that naïve cells may pass through peripheral tissues as part of their normal recirculation pattern.[442,443,444] Furthermore, T_{RM} cells that reside in the brain and mucosal tissues (gut, skin, and lung) also display a specialized phenotype characterized by increased expression of CD103, CD69, and granzyme B, and protect against pathogen reentry at these sites.[45,445,446,447,448,449]

Chemokine receptors represent another class of molecules used to functionally define different subsets of memory T cells, in this case based on their specific homing behavior.[450,451,452] Skin-homing memory T cells, for example, do not express lymph node-homing receptors CCR7 and CD62L, but do express high levels of β1 and β2 integrins and CLA, a molecule involved in lymphocyte homing to the dermis, as well as a number of inflammatory chemokine receptors including CCR1, CCR3, and CCR5.[42] CCR7+ memory T cells, in contrast, tend to express chemokine receptors that will guide them to lymphoid tissues such as CCR4, CCR6, and CXCR3, as well as CD62L. Based on the distinct homing potential of these two subsets (eg, CCR7+ CD62L+ and CCR7- CD62L-), it was proposed

that they represent two specialized types of memory T cells, each charged with a specific task in host defense.[453] The CCR7-CD62L-T_{EM} resides in peripheral tissues where pathogens are likely to first gain access to the body and can mediate direct effector functions against infected cells. The CCR7+ CD62L+ T_{CM} is found mostly within lymph nodes where it responds to antigenic stimulation by renewed proliferation and the generation of secondary effectors. It should be pointed out that expression of both CD62L and CCR7 also define naïve T cells, so that additional markers are required to distinguish T_{CM} such as high-level expression of CD44 in the mouse or the absence of CD45RA in the human. Extending from this work, differential expression of CXCR3, CCR4, CXCR5, and CCR6 can help to crudely distinguish polyclonal populations of human Th1, Th2, Tfh, and Th17 central memory cells, respectively.[385,387]

Although CD62L and CCR7 do not represent a perfect set of phenotypic markers with which to define memory subsets, the concept of a functional division of labor between T_{EM} versus T_{CM} has been remarkably influential idea with numerous studies dedicated to elucidating their lineage relationship and relative role in maintaining the protected state. Some of these have concluded that T_{EM} and T_{CM} arise sequentially through a linear pathway of differentiation, while others support the idea that they are separate populations that arise independently.[282,436,454,455,456,457,458,459] More recently, it has been suggested that the initial precursor frequency of antigen-specific T cells may influence the commitment

of T cells to one or the other lineage, or that changing the anatomic location (eg, from lymphoid to peripheral tissues) can cause the conversion from T_{CM} to T_{EM} phenotype.[277,456] Numerous laboratories are working on the elucidation of the origins and lineage relationships of T_{EM} and T_{CM} phenotypes, their relative roles in protective immunity, and their potential for interconversion.

Function of T-Cell Memory: The Secondary Response

From an operational perspective, it can be argued that the ultimate purpose for the long-term maintenance of memory T cells is found in the secondary response to previously encountered antigens, which comprises both direct effector functions and renewed proliferation. The structures and signals required to elaborate the secondary responses differ somewhat from those involved in the priming of naïve T cells. For many infections, the secondary response to antigenic challenge can proceed in the absence of encapsulated lymph nodes or spleen,[460,461] but nonetheless appear to require DCs and, depending on the model, can require costimulation by molecules such as CD28, OX40, and 4-1BB.[462,463,464] As described previously, the types of memory T cells present in mucosal tissues or other peripheral organs differ from each other and those predominantly found in lymph nodes, and it is likely that these different subsets will play, to a varying degree, different roles in protection against secondary infections. For example, T_{EM} cells (including the more restricted T_{RM} cells in mucosal tissues) can be found in the blood and in a variety of peripheral tissues such as bone marrow, liver, gastrointestinal tract, lung parenchyma, and fat pads where they can mediate immediate effector functions, and T_{CM} cells are found mostly within the lymph nodes where they are able to produce IL-2 (in addition to IFNγ in

some cases) and expand to a greater degree than T_{EM} cells.[436,465,466,467] Although initial studies supported a clear division of labor between these subsets, it has become clear that some degree of overlap exists in their location, function, and phenotype. In the CD8+ lineage, T_{EM} can undergo substantial secondary expansion, and conversely, T_{CM} can display cytotoxic potential and production of effector cytokines equivalent to that of T_{EM}.[436,468,469,470] The relative contribution of T_{EM} versus T_{CM} subsets to recall responses and effective immunity can depend on the nature of the antigenic challenge. For example, in some cases T_{CM} cells are better able to protect from challenge with a virulent forms of LCMV (eg, Cl.13) or vesicular stomatitis Indiana virus, predominantly lymphoid-trophic pathogens, while T_{EM} are superior in mediating protection from a less virulent form of LCMV (eg, Armstrong), vaccinia virus, Sendai virus, and *Listeria*, which predominantly replicate in the marginal zones of the spleen or peripheral organs.[440,471,472,473,474,475,476] The timing of the challenge can also play a role, as T_{EM} in the lung have been shown to be more effective at mediating protection against Sendai virus at early time points, while T_{CM} are superior at later time points.[477]

In addition to the increased frequencies of antigen-specific cells, the T cells that comprise the secondary response display a higher functional avidity and lower signaling thresholds than primary responders, and consequently can mount faster and more effective responses to infection that the naïve repertoire.[27,478,479,480] This “faster and better” response of memory T cells involves alterations in TCR signal transduction components that results in enhanced sensitivity to antigen, as well as epigenetic modification of key cytokine gene targets for TCR-mediated transcriptional activation, such as IFNγ and IL-2, resulting in more efficient and higher-level production.[23,478,479] In the case of the some effector molecules including IFNγ and RANTES, memory T cells contained stored messenger ribonucleic acid that is rapidly translated upon TCR stimulation.[30,481,482,483,484,485] The enhanced functionality of the memory cells at the population level may reflect a mechanism in which antigen-driven clonal selection is controlled by affinity-based thresholds.[41,480,486] Additionally, a number of studies have shown that the secondary T-cell response is prolonged compared to the primary, with emerging evidence suggesting that renewed exposure to antigen can further improve the function of the available repertoire, as secondary memory CD8+ T cells have been shown to provide better per-cell protective immunity after some reinfections.[32,143,279,465,487,488,489,490,491] Thus, the vast heterogeneity of memory T cells formed after an infection creates a multiple layered backup scheme to maximize containment. And given that pathogens differ in their tropism and pathogenesis, it will be important to identify which types of memory T cells offer the greatest protection for each.

THE GENERATION OF MEMORY B CELLS AND LONG-LIVED PLASMA CELLS

Introduction

B cells are the source of antibodies, and antibodies are efficient at protecting the body from reinfection by a previously encountered pathogen. Vaccines are predicated on the existence of immunologic memory, and 25 of the 27 licensed human vaccines protect primarily or exclusively on the basis of protective antibody responses.[492,493,494] B cells are also

important effectors of many human autoimmune diseases, and B-cell memory drives the maintenance or relapsing-remitting cycles of those diseases. Many allergies and allergic diseases are also defined by the antibody responses against the allergens, and B-cell memory is central to the recurring cycles of allergic responses. Hence, B-cell memory is central to a wide range of protective immunologic responses and pathologic immunologic responses.

Immunologic memory in the B-cell compartment consists of two very different cell types: Bmem cells and long-lived memory plasma cells (PCs). These two cell types perform two different functions. Long-lived PCs can be considered “active memory” as they continuously secrete voluminous quantities of antibodies that circulate in the blood. Bmem cells can be considered “quiescent memory” as these cells are resting, waiting for a second infection or reexposure to antigen to reactivate them. The proposed basic lineage relationship between naïve mature B cells, Bmem cells, and plasma cells is shown in Figure 31.4. Different stages of B-cell differentiation occur in histologically defined locations, as illustrated in Figure 31.5. In this basic model, shown in Figures 31.4 and 31.5, naïve mature B cells are activated by antigen (protein

in adjuvant, viral infection, or autoantigen) and CD4 T-cell help. These initial interactions occur at the border between the T- and B-cell areas of the lymphoid organ (spleen or lymph node).[495,496] Both Bmem and memory PCs are primarily generated in GCs. This chapter section will describe the generation of B-cell memory in GCs, the capabilities of B-cell memory, and the maintenance of B-cell memory.

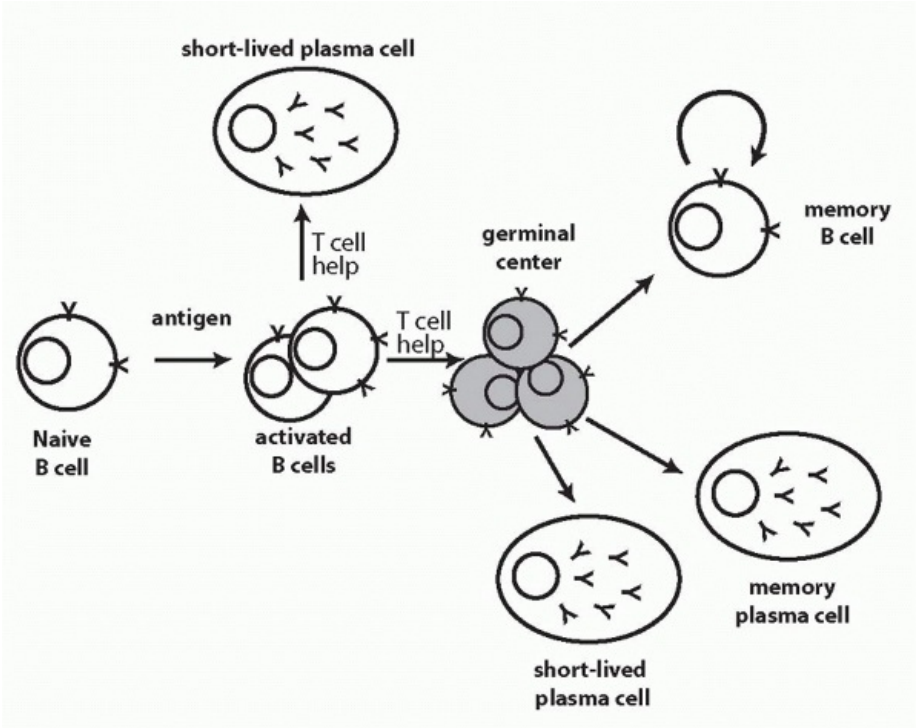

FIG. 31.4. Development of B Lineage Immune Response and Memory. Following antigenic stimulation, naïve B cells undergo clonal expansion and may differentiate into multiple different cell types, including the two cell types of B cell memory: memory B cells and plasma cells.

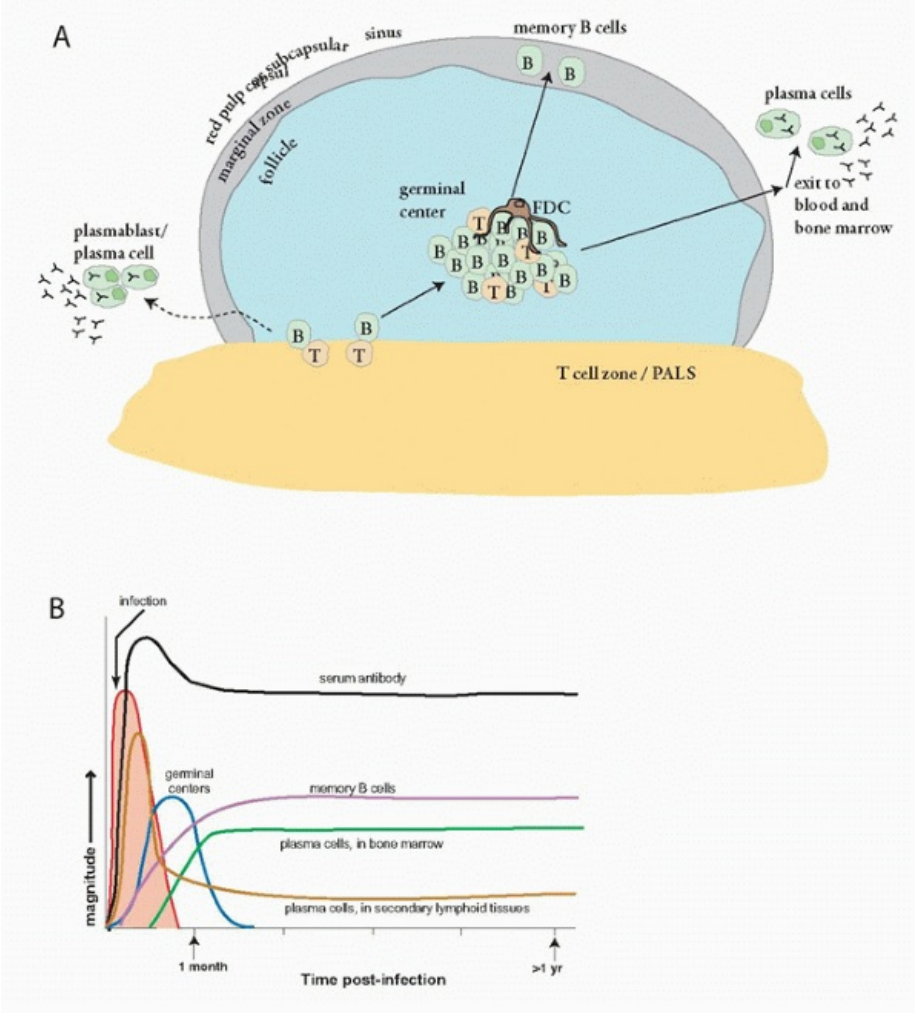

FIG. 31.5. Anatomic Features of Effector and Memory B-Cell Differentiation. A: Locations of stages of B-cell memory development in spleen or lymph node. See text for details. **B:** Kinetics of a conventional T cell-dependent B-cell response to an acute viral infection or protein immunization.

Germinal Centers: The Foundry of B-Cell Memory

B-cell memory is primarily generated in GCs. This is most evident in humans by the severe reductions in total Bmem cells as well as loss of vaccine and pathogen-specific longterm circulating antibody responses seen in humans with genetic immunodeficiencies that cause a loss of GCs, such as CD40L, CD40, SAP, and ICOS.[497,498,499,500,501,502] GCs are distinct microanatomic sites of B-cell selection in response to antigens (see Fig. 31.5).[503,504] B cells undergo clonal expansion, Ig gene hypermutation, and affinity maturation within GCs.[505] The purpose of a GC is two-fold. First, it is the site of rapid evolution of high affinity B-cell clones. The second job of the GC is to produce Bmem cells and plasma cells for long-term humoral immunity.

During an immune response, B-cell clones within the mature B-cell repertoire respond to an antigen, a pathogen component, on the basis of their affinity for the antigen. Those activated B-cell clones inherently have suboptimal affinity for the pathogen component they are responding against, given the universe of possible pathogen structures. It is important to improve that affinity to then generate antibodies with maximal capacity to neutralize the infecting pathogen and give the host the highest chance of survival. GCs are the site of the affinity maturation process. B-cell clones that randomly mutate an Ig gene that encodes a B-cell receptor (BCR) (and hence secreted antibody) with higher affinity for the pathogen are positively selected for survival and further rounds of proliferation in the GC. Cycles of affinity maturation can occur approximately every 6 hours (nonsynchronously),

allowing for > 20 generations of mutation and affinity maturation within a week. This powerful GC process of generation of genetic variation and selection of the most fit clones has been termed “evolution in miniature.”[506]

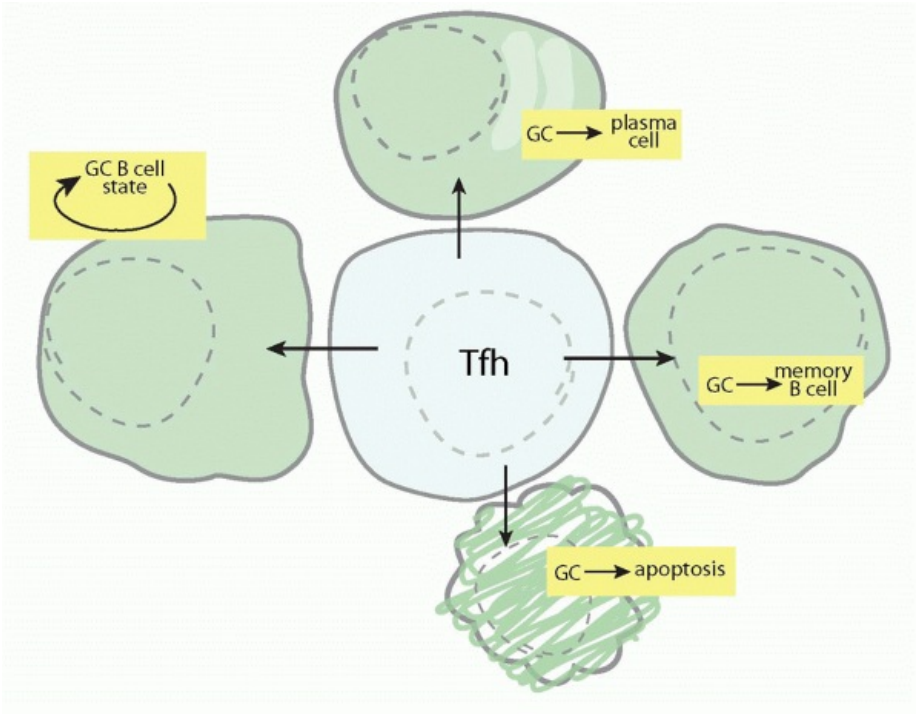

FIG. 31.6. Regulation of Germinal Center (GC) B-Cell Fate Decisions by T Follicular Helper (Tfh) Cells. Tfh cells regulate multiple different B-cell differentiation processes within the GC, via numerous molecular signals. Tfh cells are required for maintenance and proliferation of GC B cells (*left*). Tfh cells are also required for induction of differentiation of GC B cells into plasma cells (*top*). Tfh kill GC B cells that fail to present cognate antigen (*bottom*). Tfh may also signal GC B cells to differentiate into memory B cells (*right*). See text for details.

What is the mechanism of selection in GCs? In the absence of T-cell help, GC B cells rapidly die by neglect within hours.[507] Tfh cells are the uniquely specialized CD4 T cells for B-cell help.[105,505] Tfh cell help is the primary limiting factor for the magnitude of GC responses in

most settings.[105,128,508,509] The function of Tfh cells is to provide B-cell help. However, that is a vast oversimplification of the complex tasks Tfh cells perform. B-cell help is not a singular molecular signal or even a single process. Tfh cells are required for the formation of GCs, the maintenance of GCs, and for the generation of virtually all Bmem and PCs.[105] The control of these processes hinges on Tfh cells regulation of four B-cell fate decisions (Fig. 31.6) and the bidirectional communication between the Tfh cell and GC B cell.[105]

As stated previously, GC B cells rapidly die by neglect within hours without T-cell help, highlighting their critical dependence on continuous extrinsic survival signals.[507,510] To survive, GC B cells must bind antigen, process the antigen, present the antigen in MHCII, and obtain Tfh-cell help. Within a population of GC B cells, all specific for the same antigen, competition for Tfh cell help occurs. Tfh cells distinguish which GCB cells have the highest affinity BCRs by the amount of peptide-MHC complex each GC B cell presents. GC B cells presenting more cognate peptide to Tfh cells receive more survival signals from Tfh cells. The Bcl2 family member Mcl1 is required for GC B-cell survival.[511] The primary role of GC B cells in this selection process is to present antigen.[505,508] The observation that BCR-mediated antigen endocytosis or mitogenic signaling are mutually exclusive events supports this interpretation.[512] The role of Tfh cells in the process of B cell evolution in a GC center is to sample the population of GC B cells, interpret the environmental signals, rescue only the highest affinity GC B cells for survival, and potentiate further affinity maturation. Tfh cells physically bind cognate B cells in interactions that are sustained for long durations due to TCR and SLAM-associated protein (SAP, SH2D1A) dependent adhesion and development of immunologic synapse.[105,499,513] This allows for extensive signaling between Tfh and GC B cells in each round of interaction.

It is at this point that a critical decision occurs: simultaneously with the Tfh survival signals, each surviving GC B cell is provided an additional set of instructions by a Tfh cell to either undergo an additional round of somatic hypermutation and proliferation (maintain the GC B cell program), or differentiate into a PC or Bmem cell (see Fig. 31.6).[105,506,514,515] This is an iterative process constantly performed by the Tfh cells for the duration of a GC. (Tfh regulation of the B-cell response starts even before the GC,[105,516] but the discussion here focuses on the GC stage, as both high- and low-affinity BCR B cells enter GCs.[517,518]) For further information on the molecular processes of somatic hypermutation and class switch recombination, please see chapters 5, 7 and 9. Here, we focus on the GC as the foundry of B-cell memory.

The signal strength of “help” provided by the Tfh cells to the B cell will increase in proportion to the amount of antigen being presented by the GC B cell in the T-B conjugate. There is likely a threshold of antigen affinity at which the B cell presents sufficient amounts of peptide-MHC to the Tfh cells to elicit stronger Tfh signals that preferentially induce differentiation to plasma cell (or Bmem cell) and exit from the GC cycle.[514] As BCR affinity evolves, it becomes intrinsically more and more difficult to retain the B cell in the GC. Signals from the Tfh cells influence Bmem cell versus plasma

cell differentiation by GC B cells. Signals from follicular DCs (FDCs) may also be involved.

Tfh cells are capable of producing large amounts of IL-21 during interactions with B cells. GC B cells express high levels of IL-21R. IL-21 acts directly on GC B cells and is important for maintenance of GC B-cell proliferation and Bcl6 expression.[519,520] However, IL-21 is also the most potent known cytokine for induction of plasma cell differentiation, in a STAT3-dependent manner.[521,522,523,524,525,526] Therefore, combinatorial signaling must be involved in the cell fate decision of a GC B cell to maintain the GC B-cell program or initiate plasma cell differentiation. CD40 signaling inhibits plasma cell differentiation and potently enhances B-cell proliferation.[527] CD40L and IL-21 are both expressed at high levels by Tfh cells, and surface expression of CD40L and secretion of IL-21 are both induced by TCR signaling during Tfh-GC B cell interactions.

Numerous signals involved in T:B interactions have been proposed to modulate the likelihood of generation of PCs versus Bmem. These signals include CD70,[528] OX40,[529] PD-1,[530] and others. When comparing Bmem and bone marrow PCs specific for the same antigen, evidence of affinity maturation is strong for PCs but less so for Bmem.[531] In addition, experimental manipulation reducing the susceptibility of GC B cells to apoptosis results in larger GCs, more Bmem cells, and more plasma cells; however, affinity maturation is not equally affected.[532,533] An abundance of low-affinity Bmem are observed, but the PC compartment primarily contains high-affinity cells.[532,533] Based on these and other data, it has been postulated that a bias exists for PCs to primarily derive from GC B cells late in a GC reaction, due to development of high-affinity BCRs, whereas Bmem are generated throughout the GC reaction irrespective of increases in BCR affinity.[515] Nevertheless, high-affinity PCs can be generated early in GC responses.[531]

A parallel process occurring in the GC is class switch recombination (isotype switching). Loss of IgD expression occurs immediately after initial B-cell activation, before GC formation. Class switch recombination from IgM to any IgG isotype, or IgA, can occur before the GC reaction or during the GC reaction. Class switch recombination is regulated by signal strength and isotype specifying switch factors. All class switch recombination depends on activation-induced cytidine deaminase expression, as does somatic hypermutation. Multiple signals can induce activation-induced cytidine deaminase expression in B cells, including strong BCR crosslinking and CD40L-CD40 signaling from CD4 T cells. Activation-induced cytidine deaminase expression does not specific which isotype switch occurs. Cytokines, primarily from CD4 T cells, are potent switch factors, regulating which heavy chain constant region recombination event occurs. For example, in mice IFNγ enhances IgG2a class switch recombination, and IL4 enhances IgE and IgG1 class switch recombination. For further information on the molecular and cellular processes of class switch recombination, see chapters 5, 7 and 9..

Differentiation and Phenotype of Memory Plasma Cells: Active Memory

There are at least two populations of PCs: memory (longlived) and short-lived. Memory PCs are a central part of immune memory, as these cells are largely responsible for the long-term continuous secretion of antibody. In contrast to Bmem, PCs are terminally differentiated and cannot be stimulated by antigen to either divide or increase their rate of antibody production. Because preexisting antibody provides the first line of defense against infection by microbial pathogens, the importance of PCs in protective immunity cannot be overstated.

PC development starts in a GC when a GC B cell downregulates Bcl6 and begins to upregulate XBP1 and Blimp1.[534] The developing PC downregulates CXCR5 and other migration-associated molecules while increasing responsiveness to CXCR4, thereby allowing the cell to leave the GC environment and leave the B-cell follicle.[535,536] Blimp1 is considered the master regulator of PC differentiation.[534,537,538] Blimp1 orchestrates a program of PC gene expression, including maximal XBP1 expression,[536,539] which is required for synthesis and maintenance of the massive endoplasmic reticulum and Golgi apparatus necessary to power the extraordinary amounts Ig secretion accomplished by PCs.[534,540,541] Blimp1 is also required for survival of PCs.[542] There are steps of PC development that can occur before upregulation of Blimp-1, including partial upregulation of XBP1.[539] At this point of development, the PC is CD20^{low}CD3 8++CD27+++ Blimp-1+.[536] The PC is still MHCII+ (human leukocyte antigen-DR+) and is still likely CD138-.[536,543,544,545] Cells with this phenotype in vitro can still undergo some amount of proliferation and are therefore sometimes termed plasmablasts.[536,546] As PC development progresses further, with greater upregulation of Blimp-1, the PC stops dividing. The PC can further mature by downregulating MHCII and through additional gene expression changes.[536,547] During this period, the PC may migrate through the blood to the bone marrow.[531,535,536,546] Plasma cells are only in the blood for a brief period of time. Antigen-specific PCs are a vaccination or infection that can be found in human blood during brief windows of time representing the peak of PC production.[543,544,545,548] Once in bone marrow, human PCs upregulate CD138.[549,550] Memory PCs are identified as PCs in bone marrow, as CD138+ PCs, or functionally as PCs that have survived well past the time of the peak PC generation.[551,552,553,554] Memory PCs can also be readily observed in murine or human spleen,[550,555] as well as inflamed tissues.[556,557,558] First focusing on the bone marrow PCs, these cells can survive for prolonged periods in vivo, measured experimentally to have a 3- to 4-month half-life[554,559] or to live indefinitely.[547] However, it has been determined that PCs in bone marrow are not intrinsically long lived but are highly dependent on continuous provision of multiple extrinsic survival signals.[506,553] There is a PC survival niche in bone marrow consisting of a rich environment of survival cytokines and adhesions molecules,[553,560] such as IL-6,[549,550,557,560,561] CXCL12/SDF-1,[560] the ligand for CD138,[546] LFA-1, VLA-4,[562] APRIL,[557,563] and additional poorly defined cues that have been difficult to reproduce in vitro.[546,564] Efforts to maintain PC survival in vitro have met with limited success.[557,561] As long-lived PCs are not intrinsically long-lived, these cells may be referred to as bone marrow PCs or memory PCs.[565] Memory PCs of similar phenotype can be found in spleen[550]

and lamina propria of the small intestine.[557] Inflamed tissue can also possess memory PCs, particularly in the context of an autoimmune condition such as systemic lupus erythematosus. Such cells are identified as postmitotic PCs, having been in residence for a substantial period of time.[556,558]

PCs can come from other sources besides GC B cells. There are myriad pathways for B cells to flow into PC differentiation.[566] Most focus on memory PC development has fixed on the generation of memory PC from GC B cells or Bmem, but it has not been excluded that there can be other sources. For example, during an immune response, an activated B cell may either enter a GC or instead migrate outside the follicle and form extrafollicular foci, which rapidly develop into clusters of PCs (see Fig. 31.5).[567] These are predominantly short-lived PCs that survive for days[568] and are IgM in most cases; however, extrafollicular PCs can be IgG+, are localized adjacent to blood vessels, and could potentially migrate to bone marrow.

Many more GC-derived PCs are generated than become memory PCs.[547,554] What distinguishes a PC that will live long term versus a PC that will not? A prominent model is "competence and competition."[553] Bone marrow plasma cells predominantly possess high-affinity BCRs, even when selective pressure on the GC reaction is relaxed,[569] indicating that high affinity for antigen contributes to the programming of a PC to become a memory PC. Emigrating PCs from the GC are affinity matured, suggesting that affinity maturation is a prerequisite for exit. Short-lived PCs and memory PCs can coexist in the same environment during a chronic autoimmune response.[556] Those observations indicate there are development signals that mark a PC as "competent" to enter the memory PC population. The difference may involve differences in migration, responsiveness to survival signals, or both.[553] However, no cell surface or intracellular marker has been identified to distinguish a precursor shortlived PC from a precursor memory PC,[536,539,546,553] indicating either that the difference is subtle or a stochastic process is likely dominant for development of "competence" to enter the memory PC population. The stochastic process could be a probabilistic occurrence based on the location of the PC and the survival signals it receives at a specific, transient point in time.

The second challenge facing a PC that is competent to become a memory PC is competition. It appears there are a limited number of survival niches in the bone marrow and other tissues for memory PCs.[553] In that case, competition must occur between PCs of different specificities and affinities for those survival niches. This is discussed further in the following.

DIFFERENTIATION AND PHENOTYPE OF MEMORY B CELLS

Bmem cells are primarily generated in GCs.[515,570] The signals that induce a GC B cell to differentiate into a Bmem cell remain unclear.[105,515,570] Bmem cells can be found in spleen, blood, bone marrow, other lymphoid tissues such as tonsils, and mucosal sites.[571,572,573,574,575] Within spleen, Bmem cells largely localize to the marginal zone of the follicles.[573]

Human Bmem selectively express CD27,[573,576,577] a member of the TNFR superfamily.[578] Surface expression of CD27 is now widely used as a specific marker, in conjunction with lack of IgD expression, to discriminate human Bmem cells from naive B cells. CD27 was initially determined as a marker of human Bmem cells in both peripheral blood and spleen by demonstrating that CD27+ B cells express somatically mutated Ig V genes, while CD27- B cells do not.[573,577] This was also consistent with the observation that nearly all isotype

switched B cells in peripheral blood are CD27+, and the majority of IgD+ B cells are not.[576,577] Bmem cells in tonsillar tissue can be identified as IgD- CD38- B cells as well as IgD- CD38- CD27+.[571,572] Vaccine induced antigen-specific Bmem cells have also been shown to be predominantly CD27+.[548,579] Antigen-specific Bmem cells in humans can be identified by fluorescent antigen binding and flow cytometry,[545,580] or functional identification by in vitro reactivation.[548,579] Antigen-specific Bmem cells are present at low frequencies (approximately 0.005% to 0.2% of total peripheral blood B cells[14,581]). Using either approach, antigen-specific human Bmem cells have been identified to many antigens, including the smallpox vaccine,[14] the anthrax vaccine,[582] the tetanus vaccine,[19,548,581,583] measles,[19,548] rubella,[19] malaria,[584] influenza,[585,586] rotavirus,[545,587] severe acute respiratory syndrome,[588] and wasp venom allergen.[581] Substantially higher percentages of human B cells in spleen are Bmem cells,[574] and it has been calculated there are 10 to 100 times more antigen-specific Bmem in spleen than in blood.[570]

Human Bmem exhibit extensive heterogeneity. Bmem can be unswitched IgM[570] or switched to any IgG isotype, or IgA, and therefore have different effector and activation properties. Bmem can have no evidence of somatic hypermutation and affinity maturation, or evidence of sequential accumulation of a massive series of mutations.[589,590] CD27+ Bmem have extensive diversity in their surface phenotype, expressing many different surface receptors and homing receptors in a highly heterogeneous fashion.[591] Furthermore, not all human Bmem are CD27+. There are substantial populations of CD27- Bmem that themselves exhibit significant heterogeneity in surface phenotypes.[570,592] FCRL4+ Bmem are an additional population of Bmem predominantly localized to mucosal tissue.[575] In the context of chronic viral infections (eg, HIV and hepatitis C virus) and chronic autoimmune diseases (eg, lupus) additional populations of B cells have been observed that have been categorized as “exhausted” or “aberrent” Bmem.[593,594,595] These cells have evidence of recent activation and poor responsiveness to restimulation, and therefore appear related to exhausted CD8 T cells found in chronic viral infections due to continuous antigenic stimulation.

Similar observations of extensive heterogeneity have now been made for murine Bmem. There is no single cell surface marker that can identify murine Bmem. Murine Bmem are not selectively CD27+. Indeed, there is no set of surface markers that can identify all murine Bmem. Murine Bmem exhibit a wide range of polymorphic cell surface phenotypes,[596,597] and gene expression analysis has not found a consistent gene expression profile that defines B-cell

memory.[598,599] Murine Bmem may be IgM or IgG.[597,600,601] Generation of murine Bmem does not depend on affinity maturation.[306,596] While the majority of Bmem are generated in GCs, it is possible to generate GC-independent memory[306] and even T cell-independent memory in some cases,[602] which is further evidence of the immense heterogeneity possible in the Bmem population. Altogether, human and murine B cell data show that Bmem are not a

singular cell type with a singular phenotype, but are instead an extraordinarily varied population of cells, united only by their property of having previously recognized antigen. The various phenotypes of Bmem may or may not be stable. Bmem cells generated at different times during an immune response can have different phenotypes.[597] This observed diversity suggests that diversity of Bmem phenotypes and functions are biologically valuable for protection against infectious disease.[515] By having Bmem specific to the same antigen with different effector functions (isotypes), epitope specificities and affinities (divergent selection and affinity maturation), homing properties (tissue localization and recruitment to sites of inflammation), and responsiveness to restimulation (modulated by the multitudinous surface receptors with various functions in cell-cell interactions), the Bmem may be better poised to more effectively respond to rechallenge and unpredictable changes in the pathogen.[597,600]

Function of Memory B Cells: Quiescent Memory

What is the value of long-term maintenance of Bmem cells? Bmem cells have several features that indicate they are valuable for protection against infections. First, Bmem cells are present in much greater numbers than naive mature B cells of a given antigen specificity.[548,581,603,604,605] Second, Bmem cells respond to reactivation faster than naive B cells,[548,550] differentiate into PCs faster,[550] and have a larger burst size, indicative of resistance to apoptosis.[606] Third, Bmem cells have undergone affinity maturation and therefore produce antibodies after reactivation that have substantially higher affinity and/or avidity than antibody produced from naive B cells. Each of these properties likely make Bmem cells highly valuable for protection against reinfection, as the Bmem cells are able to make a rapid recall response and produce high levels of high-affinity antibodies quickly to limit the spread of the infecting microbe and quell the infection.

In situations where antibodies are known to be protective but are not present at high enough levels for sterilizing immunity (sterilizing immunity defined as a complete prevention of infection of any cells or tissue), Bmem cells are likely to contribute to the observed protection against disease. The hepatitis B virus (HBV) vaccine is a well-characterized example of a known situation where antibody titers that drop within 5 years.[607,608,609] Antibodies against HBsAg are the defined correlate of protection for the HBV vaccine, and the minimum protective level has been established as 10 mIU/mL.[492,494] When serum anti-HBsAg antibody levels drop below that level, booster immunization was recommended for many years. However, it is now recognized that booster immunizations are not required, because previous vaccination is protective even if anti-HBsAg titers have fallen to undetectable levels.[494,610] Why? Bmem cells provide protection.[610,611,612,613,614] HBVsAg antibodies are the correlate of protection for the vaccines, and high-affinity HBsAg-specific Bmem will rapidly differentiate into anti-HBsAg antibody secreting cells within 3 to 5 days of virus exposure.[611,612,613] This is sufficient for protection against disease because the kinetics of the HBV infection are slow enough so that the anamnestic antibody response generated by the Bmem occurs rapidly enough to quell the spread of the virus and prevent clinical disease. In any situation where antibodies contribute to protective immunity, reactivation of Bmem cells is likely to contribute to protection as long as the infectious agent is not so virulent as to cause disease before the Bmem cells have time to proliferate and differentiate into PCs.

Longevity of Humoral Immunity

There are several classic examples that clearly documented long-term protective immunity lasting up to 75 years in humans in the absence of reexposure to the pathogen (Table 31.2).[6,615,616] Those observations were crucial in shaping our ideas about immunologic memory because they showed that the immune system could remember an encounter that occurred many years ago. As antibodies are the main protective mechanism for measles, sustained measles immunity on the Faroe Islands suggested that B-cell memory to measles lasts at least 65 years.[615] A similar situation was observed with rare polio outbreaks in a remote Eskimo village. Three serotypes of poliovirus exist, and antibodies against one serotype were observed only in Eskimos more than 40 years old and antibodies against a second serotype were observed only in individuals older than 20. This epidemiologic observation indicated that production of antipoliovirus antibodies is long-lived even in the absence of reexposure to the virus.[6] Similar epidemiologic data of long-lasting antibody responses to specific influenza strains has been used extensively in more recent studies to predict immunity to new influenza strains.[617,618,619] Numerous human serologic studies of antigen-specific IgG have directly established that antibody responses against a variety of agents can be sustained for decades.[14,16,19,617,619,620,621,622] Astonishingly, substantial antibody levels can be sustained for 90 years in numerous instances.[619,621] At the cellular level, studies have

established that Bmem survive long term in mice and for many decades in humans.[14,548,623] Cellular studies have also shown that PCs can survive for the life of a mouse.[547,554,559] The mechanisms for maintenance of these cell types are described in the following.

TABLE 31.2 Long-Term Human Immunity in the Absence of Reexposure to the Pathogen

Infection	Duration of Immunity
Measles on the Faroe Islands615	65 years
Yellow fever virus in Norfolk, VA616	75 years
Polio in remote Eskimo villages6	40 years
Inactivated poliovirus vaccine in Sweden620	30+ years
Smallpox vaccine14,16,621,645	30 to 60+ years

Longevity of Memory B Cells

Bmem have been demonstrated to survive in the absence of antigen in mice.[623] In humans, Bmem are found in peripheral blood and spleen for decades after smallpox vaccination.[14,574] Bmem specific for numerous other immunizations or infections have been detected decades after exposure.[19,548,614,619] For the antigen-specific responses tested, circulating Bmem numbers decline slowly over a period of decades.[19]

There are four models for how human Bmem cells are maintained: 1) antigen-dependent, 2) intermittent stimulation by cross-reactive antigens, 3) bystander activation, and 4) programmed homeostatic maintenance. These models are not mutually exclusive, and all are topics of active research in the field to determine their importance in memory maintenance. The first model is antigen-dependent maintenance. Reinfection or reimmunization is one clear mechanism to maintain memory via recall. Microbial persistence is another clear mechanism to maintain memory. A number of microbes (herpesviruses being the most wellknown examples) can persist at low levels in healthy individuals, and this chronicity can provide either a continuous or intermittent antigenic stimulus to the immune system. Antigen depots are an antigen-dependent mechanism for memory maintenance that does not depend on reinfection or reexposure or microbial replication. The antigen depot model of memory maintenance relies on the ability of FDCs to trap antigen-antibody complexes on their cell surface and retain them for extended periods of time.[624,625] FDCs express Fc receptors on their cell surface, and antigen-antibody complexes can bind to these Fc receptors. It appears that FDCs do not internalize these antigen-antibody complexes but instead display them on their cell surface, thus promoting maintenance of GCs.[504,510,626,627] Some studies have suggested that antigen can persist on FDCs for greater than 1 year, and it has been proposed that after the generation of Bmem, the cells are maintained by periodic reencounter with antigen on FDCs.[624] However, data from the only study directly tracking antigen in GCs after a viral infection, using a mouse model, indicated that GCs are sustained by antigen on FDCs for ~30 days after clearance of the virus.[625] This was corroborated by a second study with a nonreplicating antigen,[559] indicating that FDCs were critical for maintenance of GCs but not Bmem. Furthermore, a genetic study demonstrated that murine Bmem are maintained in the absence of antigen.[623] This is analogous to murine CD8 T cells.[321]

A second model proposes that memory is maintained by stimulation of Bmem cells by cross-reactive environmental or self-antigens. B cells are stimulated by direct interaction of the BCR with antigen, and the specificity of a BCR is not absolute; cross-reactivity is observed. Therefore, it is possible that Bmem cells are maintained long term by intermittent interaction with environmental antigens (ie, allergens, food products, unrelated pathogens) or self-antigens. This hypothesis is exceptionally difficult to test. Rajewsky and colleagues have shown that mature naïve B cells must constantly maintain BCR expression for survival,[628] presumably due to a need for tonic signaling through the BCR. Tonic signaling may or may not be dependent on low-affinity binding of the BCR to self-antigens. If the signaling is dependent on self-reactivity, Bmem BCRs may also have a requirement for low-grade self-cross-reactivity. On the other hand, Bmem cells may have different requirements for BCR stimulation and may no longer require antigen for their survival or homeostatic proliferation. This would be similar to the differential requirements observed between naïve and memory T cells. A third potential mechanism for the maintenance of Bmem cells is bystander polyclonal

activation. This model is based on the observation that Bmem cells stimulated in vitro with toll-like receptor ligands or select polyclonal activators will undergo several rounds of proliferation and differentiate into plasma cells, whereas naïve B cells will not.[548,629] If this same process occurs in vivo, Bmem cells would be likely to proliferate in response to irrelevant microbes containing toll-like receptor ligands or proliferate due to production of cytokines secreted by responding cells of the immune system.[548,630] Intermittent bystander activation in this manner would result in small-scale expansion of Bmem cells, thereby maintaining the population by replacing any Bmem cells dying over time. This model is mechanistically plausible but currently seems unlikely. While one study observed bystander activation of human Bmem cells in vivo, as measured by increased antigen-specific serum antibody titers,[548] three others human studies have observed no bystander activation in vivo.[583,631,632] Bystander activation was not observed in a mouse model system.[633,634]

A fourth potential mechanism for the maintenance of Bmem cells is programmed homeostatic proliferation. This hypothesis draws from the known mechanisms of CD8 T-cell memory maintenance in mice.[352,635] As discussed earlier in the chapter, murine memory CD8 T cells accomplish prolonged maintenance by proliferation primarily via IL-15 signals. Memory CD8 T cells are thought to undergo homeostatic proliferation when they migrate through the bone marrow, where there are high concentrations of IL-15 and other proliferation and survival signals.[219,636] Therefore, antigen-specific Bmem cells may maintain themselves by a similar programmed homeostatic maintenance involving intermittent proliferation triggered by certain paracrine cytokines or other as yet unknown factors. Data from both mice and man indicates that Bmem cells undergo a slow rate of proliferation in the absence of rechallenge.[559,592,603,637,638] This is consistent with slow homeostatic proliferation and does not require CD4 T cells.[639]

Longevity of Memory Plasma Cells

Serum antibody titers have long been used as a surrogate measure for the presence of PCs, but that measurement is not sufficient for assessing the longevity of PCs, because the population of PCs may consist of a stable number of

long-lived cells or may be partially or fully replenished by Bmem differentiating to PCs. Therefore, it is necessary to directly quantify PCs and their longevity. The acute PC response to an immunization or acute infection peaks within 1 to 2 weeks and then declines within 2 to 4 weeks after infection.[531,536,555] After the GC reaction subsides, bone marrow becomes the predominant site of long-term antibody production, with bone marrow PCs responsible for 80% to 95% of the host's antibody production[547,555,640] (see Fig. 31.5). A seminal study on PC longevity determined that antigen-specific plasma cells can survive in bone marrow for the lifespan of a mouse (1.5 to 2 years) with a half-life of 3 to 4 months, even when Bmem were ablated.[554] A separate study concluded that murine bone marrow PCs have an indefinite lifespan.[547,552]

Due to obvious technical constraints, there is limited data on the longevity of PCs in humans. PCs that phenotypically match murine memory PCs, including evidence of somatic mutation,

are present in human spleen and bone marrow.[550,641] Regarding longevity of the cells, this can be inferred by anti-CD20 therapy (rituximab) studies. Anti-CD20 is used in human to deplete all mature B cells and Bmem in order to treat autoimmune diseases or malignant B-cell tumors, but anti-CD20 therapy does not deplete PCs. Serum antibody titers to some vaccine antigens remain unchanged in rituximab-treated individuals for at least 1 year.[642,643] These results indicate that human memory PCs survive for substantial periods. Murine studies utilizing B-cell depletion techniques found similar results of PC maintenance for months in the absence of mature B cells or Bmem.[562,644]

A difficulty in interpreting the longevity of PC in humans comes from serologic studies of different vaccines and infectious diseases. Serum antibody titers against the smallpox vaccine,[14,16,621,645] polio vaccine,[620] rubella vaccine,[19] and measles vaccine[19] are sustained indefinitely, consistent with indefinite survival of PCs (see Table 32.2). However, serum antibody titers against tetanus and diphtheria decline.[19,620] Serum antibody titers against HBV regularly decline to undetectable levels within 5 years.[492,494,607,608,609] Therefore, not all PC memory is of equivalent duration. This may be due to differing capacities of PC to compete with newly generated PC over time.[553] After an infection or other event inducing inflammation, some PCs may become dislodged from their survival niche, opening up availability of the niche to new PCs.[553]

Interplay between Memory B and Plasma Cell Compartments

Bmem and PCs appear to be independently maintained cellular compartments in the absence of antigen. While in many situations there is an overall correlation between Bmem and PC numbers (or serum antibody levels, as a surrogate for quantitating PCs),[14,19,548,646] the correlation is often modest,[19] and sometimes no relationship is observed between the abundance of Bmem and PCs specific for an antigen.[492,611,612,613,614] Data from humans having undergone splenectomy or B-cell depletion therapy has convincingly shown that serum antibody titers can be maintained for many years even with very reduced levels of Bmem.[574,642,643] Data from mice have shown that depletion of Bmem does not have a major impact on the memory PCs,[554,644] and depletion of PCs but not Bmem does not result in a rapid replenishment of PC by the Bmem in the absence of antigen.[562] Therefore, once Bmem and memory PCs are generated, they are maintained by independent processes in the absence of antigen.

CONCLUSION

Immunologic memory represents a remodeling of the immune system that allows the immune system to be “imprinted” with a memory of exposure to infectious pathogens as well as noninfectious foreign molecules. Much progress has been made identifying the signals and genes that induce T- and B-cell activation and differentiation during an immune response that ultimately results in the formation of memory T and B cells. However, less is understood about the permanence or plasticity of memory cells and how their differentiation states are achieved and maintained over time, or how phenotypically and functionally distinct memory T and B cells contribute to protection. These are important areas of ongoing research.

With the knowledge of immunologic memory that had been developed over the past decade, a move toward more rationale forms of vaccine design has begun. Nevertheless, the field is left with many hurdles for generating optimal numbers of memory cells in the right locations that form the most effective response, while avoiding excessive immunopathology. Formulating vaccines that balance these objectives remains a challenge, and having a "universal" vaccine design for different types of pathogens is likely unrealistic as the approaches taken will be varied and tailored toward each pathogen of interest. Moreover, we are finding a more intricate relationship between our immune system and the metabolic fitness and function of organs and tissues. The tissue environment can affect the phenotype, function, and longevity of memory T and B cells, but much remains to be learned about how organ function is, conversely, affected by the memory T cells that dwell within. This may have important implications on tissue homeostasis, inflammatory disease, obesity, and aging. The next advances in our understanding of immune memory will come from the application of more integrated molecular approaches to the study of physiologic immune responses in vivo, which will allow for the study of the activities of the whole *immune system*, as opposed to a specific *immune cell*. Although this might seem a daunting task in terms of its complexity and sheer volume of information to be obtained, the results will be transformative in terms of our fundamental understanding of the immune system and our ability to manipulate it for the prevention of disease.

REFERENCES

1. Thucydides RC. *History of the Peloponnesian War* : Courier Dover Publications. Book 2, Chapter VII.

2. Cunha BA. The cause of the plague of Athens: plague, typhoid, typhus, smallpox, or measles? *Infect Dis Clin North Am*. 2004;18(1):29-43.

3. Zinkernagel RM. On natural and artificial vaccinations. *Annu Rev Immunol*. 2003;21:515-546.

4. Panum PL. Beobachtungenuber das maserncontagium. *Virchows Arch*. 1847;1:492-512.

5. Zinkernagel RM. On differences between immunity and immunological memory. *Curr Opin Immunol*. 2002;14(4):523-536.

6. Paul JR, Riordan JT, Melnick JL. Antibodies to three different antigenic types of poliomyelitis virus in sera from north Alaskan eskimos. *Am J Hygiene*. 1951;54:275-285.

7. Sawyer WA. The persistence of yellow fever immunity. *J Prevent Med*. 1931;5:413-428.

8. Zinkernagel RM, Hengartner H. Protective "immunity" by pre-existent neutralizing antibody titers and preactivated T cells but not by socalled "immunological memory." *Immunol Rev*. 2006;211:310-319.

9. Barquet N, Domingo P. Smallpox: the triumph over the most terrible of the ministers of death. *Ann Intern Med*. 1997;127(8, pt 1):635-642.

10. Jenner E. *An Inquiry into the Causes and Effects of the Variolae Vaccinae* . London, United Kingdom: Samson Low; 1798.

11. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol*. 2004;5(10):987-995.

12. Pulendran B, Ahmed R. Translating innate immunity into immunological memory: implications for vaccine development. *Cell*. 2006;124(4):849-863.

13. Henderson DA. The eradication of smallpox. *Sci Am*. 1976;235(4):25-33.

14. Crotty S, et al. Cutting edge: long-term B cell memory in humans after smallpox vaccination. *J Immunol*. 2003;171(10):4969-4973.

15. Demkowicz WE Jr, et al. Human cytotoxic T-cell memory: long-lived responses to vaccinia virus. *J Virol*. 1996;70(4):2627-2631.

16. Hammarlund E, et al. Duration of antiviral immunity after smallpox vaccination. *Nat Med*. 2003;9(9):1131-1137.

17. Gayton W. *The Value of Vaccination as Shown by an Analysis of 10,403 Cases of Smallpox*. London, United Kingdom: Gillet & Henty; 1885.

18. Mack TM. Smallpox in Europe, 1950-1971. *J Infect Dis*. 1972;125(2): 161-169.

19. Amanna IJ, Carlson NE, Slifka MK. Duration of humoral immunity to common viral and vaccine antigens. *New Engl J Med* . 2007;357(19):1903-1915.

20. Bousso P, et al. The composition of a primary T cell response is largely determined by the timing of recruitment of individual T cell clones. *J Exp Med*. 1999;189(10):1591-1600.

21. Busch DH, et al. Coordinate regulation of complex T cell populations responding to bacterial infection. *Immunity*. 1998;8(3):353-362.

22. Hou S, et al. Virus-specific CD8+ T-cell memory determined by clonal burst size. *Nature*. 1994;369(6482):652-654.

23. Northrop JK, et al. Epigenetic remodeling of the IL-2 and IFN-gamma loci in memory CD8 T cells is influenced by CD4 T cells. *J Immunol*. 2006;177(2):1062-1069.

24. Varga SM, Welsh RM. High frequency of virus-specific interleukin-2-producing CD4(+) T cells and Th1 dominance during lymphocytic choriomeningitis virus infection. *J Virol*. 2000;74(9):4429-4432.

25. Whitmire JK, et al. Long-term CD4 Th1 and Th2 memory following acute lymphocytic choriomeningitis virus infection. *J Virol*. 1998;72(10):8281-8288.

26. Agarwal S, Rao A. Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity*. 1998;9(6):765-775.

27. Bachmann MF, et al. Distinct kinetics of cytokine production and cytolysis in effector and memory T cells after viral infection. *Eur J Immunol* . 1999;29(1):291-299.

28. Grayson JM, et al. Gene expression in antigen-specific CD8+ T cells during viral infection. *J Immunol*. 2001;166(2):795-799.

29. Hathcock KS, et al. Induction of telomerase activity and maintenance of telomere length in virus-specific effector and memory CD8+ T cells. *J Immunol*. 2003;170(1):147-152.

30. Kaech SM, et al. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell*. 2002;111(6):837-851.

31. Teague TK, et al. Activation changes the spectrum but not the diversity of genes expressed by T cells. *Proc Natl Acad Sci U S A* . 1999;96(22):12691-12696.

32. Veiga-Fernandes H, et al. Response of naive and memory CD8+ T cells to antigen stimulation in vivo. *Nat Immunol*. 2000;1(1):47-53.

33. Strutt TM, et al. Memory CD4+ T cells induce innate responses independently of pathogen. *Nat Med*. 2010;16(5):558-564.

34. Schluns KS, Lefrancois L. Cytokine control of memory T-cell development and survival. *Nat Rev Immunol* . 2003;3(4):269-279.

35. Surh CD, et al. Homeostasis of memory T cells. *Immunol Rev*. 2006;211:154-163.

36. Lefrancois L. Development, trafficking, and function of memory T-cell subsets. *Immunol Rev*. 2006;211:93-103.

37. Rodrigo Mora J, Von Andrian UH. Specificity and plasticity of memory lymphocyte migration. *Curr Top Microbiol Immunol* . 2006;308:83-116.

38. Busch DH, Pamer EG. T cell affinity maturation by selective expansion during infection. *J Exp Med*. 1999;189(4):701-710.

39. Fahmy TM, et al. Increased TCR avidity after T cell activation: a mechanism for sensing low-density antigen. *Immunity*. 2001;14(2):135-143.

40. Rees W, et al. An inverse relationship between T cell receptor affinity and antigen dose during CD4(+) T cell responses in vivo and in vitro. *Proc Natl Acad Sci U S A* . 1999;96(17):9781-9786.

41. Savage PA, Boniface JJ, Davis MM. A kinetic basis for T cell receptor repertoire selection during an immune response. *Immunity*. 1999;10(4):485-492.

42. Sallusto F, et al. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*. 1999;401(6754):708-712.

43. Lanzavecchia A, Sallusto F. Understanding the generation and function of memory T cell subsets. *Curr Opin Immunol* . 2005;17(3):326-332.

44. Hamann D, et al. Phenotypic and functional separation of memory and effector human CD8+ T cells. *J Exp Med*. 1997;186(9):1407-1418.

45. Sheridan BS, Lefrancois L. Regional and mucosal memory T cells. *Nat Immunol* . 2011;12(6):485-491.

46. Stemberger C, et al. A single naive CD8+ T cell precursor can develop into diverse effector and memory subsets. *Immunity*. 2007;27(6):985-997.

47. Schepers K, et al. Dissecting T cell lineage relationships by cellular barcoding. *J Exp Med*. 2008;205(10):2309-2318.

48. Ahmed R, Gray D. Immunological memory and protective immunity: understanding their relation. *Science*. 1996;272(5258):54-60.

49. Lanzavecchia A, Sallusto F. Progressive differentiation and selection of the fittest in the immune response. *Nat Rev Immunol* . 2002;2(12): 982-987.

50. Chang JT, et al. Asymmetric T lymphocyte division in the initiation of adaptive immune responses. *Science*. 2007;315(5819):1687-1691.

51. Roegiers F, Jan YN. Asymmetric cell division. *Curr Opin Cell Biol* . 2004;16(2):195-205.

52. Linton PJ, Dorshkind K. Age-related changes in lymphocyte development and function. *Nat Immunol* . 2004;5(2):133-139.

53. Sprent J, Surh CD. T cell memory. *Annu Rev Immunol*. 2002;20:551-579.

54. Altman JD, et al. Phenotypic analysis of antigen-specific T lymphocytes. *Science*. 1996;274(5284):94-96.

55. Labalette-Houache M, et al. Improved permeabilization procedure for flow cytometric detection of internal antigens. Analysis of interleukin-2 production. *J Immunol Methods*. 1991;138(2):143-153.

56. Blattman JN, et al. Estimating the precursor frequency of naive antigen-specific CD8 T cells. *J Exp Med*. 2002;195(5):657-664.

57. Moon JJ, et al. Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity*. 2007;27(2):203-213.

58. Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol*. 1991;9:271-296.

59. Scollay RG, Butcher EC, Weissman IL. Thymus cell migration. Quantitative aspects of cellular traffic from the thymus to the periphery in mice. *Eur J Immunol* . 1980;10(3):210-218.

60. Tough DF, Sprent J. Turnover of naive- and memory-phenotype T cells. *J Exp Med*. 1994;179(4):1127-1135.

61. Hofmeister R, et al. Interleukin-7: physiological roles and mechanisms of action. *Cytokine Growth Factor Rev* . 1999;10(1):41-60.

62. Polic B, et al. How alpha beta T cells deal with induced TCR alpha ablation. *Proc Natl Acad Sci U S A*. 2001;98(15):8744-8749.

63. Rooke R, et al. Targeted complementation of MHC class II deficiency by intrathymic delivery of recombinant adenoviruses. *Immunity*. 1997;7(1):123-134.

64. Schluns KS, et al. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat Immunol* . 2000;1(5):426-432.

65. Takeda S, et al. MHC class II molecules are not required for survival of newly generated CD4+ T cells, but affect their long-term life span. *Immunity*. 1996;5(3):217-228.

66. Tan JT, et al. IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc Natl Acad Sci U S A* . 2001;98(15):8732-8737.

67. Tanchot C, et al. Differential requirements for survival and proliferation of CD8 naive

or memory T cells. *Science*. 1997;276(5321): 2057-2062.

68. Marrack P, Kappler J. Control of T cell viability. *Annu Rev Immunol*. 2004;22:765-787.

69. Rathmell JC, et al. IL-7 enhances the survival and maintains the size of naive T cells. *J Immunol*. 2001;167(12):6869-6876.

70. Vella A, et al. Interleukin 4 (IL-4) or IL-7 prevents the death of resting T cells: stat6 is probably not required for the effect of IL-4. *J Exp Med*. 1997;186(2):325-330.

71. Boise LH, et al. Growth factors can enhance lymphocyte survival without committing the cell to undergo cell division. *Proc Natl Acad Sci U S A* . 1995;92(12):5491-5495.

72. von Freeden-Jeffry U, et al. Lymphopenia in interleukin (IL)-7 genedeleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med*. 1995;181(4):1519-1526.

73. Peschon JJ, et al. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med*. 1994;180(5):1955-1960.

74. Puel A, et al. Defective IL7R expression in T(-)B(+)NK(+) severe combined immunodeficiency. *Nat Gen*. 1998;20(4):394-397.

75. Boyman O, et al. Cytokines and T-cell homeostasis. *Curr Opin Immunol* . 2007;19(3):320-326.

76. Sallusto F, Mackay CR, Lanzavecchia A. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu Rev Immunol*. 2000;18:593-620.

77. Guermonprez P, et al. Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol*. 2002;20:621-667.

78. Lanzavecchia A, Sallusto F. Regulation of T cell immunity by dendritic cells. *Cell*. 2001;106(3):263-266.

79. Banchereau J, et al. Immunobiology of dendritic cells. *Annu Rev Immunol*. 2000;18:767-811.

80. Fuchs E. Two signal model of lymphocyte activation. *Immunol Today*. 1992;13(11):462.

81. Lafferty KJ, et al. Immunological induction of T lymphocytes: role of antigen and the lymphocyte costimulator. *Blood Cells*. 1978;4(3):395-406.

82. Chambers CA, Allison JP. Costimulatory regulation of T cell function. *Curr Opin Cell*

Biol. 1999;11(2):203-210.

83. Slavik JM, Hutchcroft JE, Bierer VE. CD28/CTLA-4 and CD80/CD86 families: signaling and function. *Immunol Res*. 1999;19(1):1-24.

84. Chambers CA. The expanding world of co-stimulation: the two-signal model revisited. *Trends Immunol*. 2001;22(4):217-223.

85. Greenfield EA, Nguyen KA, Kuchroo VK. CD28/B7 costimulation: a review. *Crit Rev Immunol*. 1998;18(5):389-418.

86. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. *Annu Rev Immunol*. 1996;14:233-258.

87. Shahinian A, et al. Differential T cell costimulatory requirements in CD28-deficient mice. *Science*. 1993;261(5121):609-612.

88. Kawai K, et al. Skin allograft rejection in CD28-deficient mice. *Transplantation*. 1996;61(3):352-355.

89. Speiser DE, et al. Acute graft-versus-host disease without costimulation via CD28. *Transplantation*. 1997;63(7):1042-1044.

90. Wen T, et al. CD28 is not required for rejection of unmanipulated syngeneic and autologous tumors. *Eur J Immunol* . 1997;27(8):1988-1993.

91. Suresh M, et al. Role of CD28-B7 interactions in generation and maintenance of CD8 T cell memory. *J Immunol*. 2001;167(10):5565-5573.

92. Watts TH, DeBenedette MA. T cell co-stimulatory molecules other than CD28. *Curr Opin Immunol*. 1999;11(3):286-293.

93. Croft M. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? *Nat Rev Immunol* . 2003;3(8):609-620.

94. Watts TH. TNF/TNFR family members in costimulation of T cell responses. *Annu Rev Immunol*. 2005;23:23-68.

95. Whitmire JK, Ahmed R. Costimulation in antiviral immunity: differential requirements for CD4(+) and CD8(+) T cell responses. *Curr Opin Immunol* . 2000;12(4):448-455.

96. Keir ME, Francisco LM, Sharpe AH. PD-1 and its ligands in T-cell immunity. *Curr Opin Immunol*. 2007;19(3):309-314.

97. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. *Nat Rev Immunol*. 2002;2(2):116-126.

98. Curtsinger JM, et al. Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J Immunol*. 1999;162(6):3256-3262.

99. Haring JS, Badovinac VP, Harty JT. Inflaming the CD8+ T cell response. *Immunity*. 2006;25(1):19-29.

100. Murphy KM, et al. Signaling and transcription in T helper development. *Annu Rev Immunol*. 2000;18:451-494.

101. Harrington LE, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol*. 2005;6(11):1123-1132.

102. Kennedy J, et al. Mouse IL-17: a cytokine preferentially expressed by alpha beta TCR+ CD4-CD8-T cells. *J Interferon Cytokine Res*. 1996;16(8):611-617.

103. Weaver CT, et al. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol*. 2007;25:821-852.

104. Yao Z, et al. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity*. 1995;3(6):811-821.

105. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol*. 2011;29:621-663.

106. Murphy KM, Stockinger B. Effector T cell plasticity: flexibility in the face of changing circumstances. *Nat Immunol*. 2010;11(8):674-680.

107. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Ann Rev Immunol*. 2010;28:445-489.

108. Szabo SJ, et al. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*. 2000;100(6):655-669.

109. Szabo SJ, et al. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science*. 2002;295(5553):338-342.

110. Intlekofer AM, et al. Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin. *Science*. 2008;321(5887):408-411.

111. Lee GR, Fields PE, Flavell RA. Regulation of IL-4 gene expression by distal regulatory elements and GATA-3 at the chromatin level. *Immunity*. 2001;14(4):447-459.

112. Nelms K, et al. The IL-4 receptor: signaling mechanisms and biologic functions. *Annu Rev Immunol*. 1999;17:701-738.

113. Seder RA, Paul WE. Acquisition of lymphokine-producing phenotype by CD4+ T cells. *Annu Rev Immunol*. 1994;12:635-673.

114. Mangan PR, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature*. 2006;441(7090):231-234.

115. Nurieva R, et al. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature*. 2007;448(7152):480-483.

116. Veldhoen M, et al. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity*. 2006;24(2):179-189.

117. Zhou L, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol*. 2007;8(9):967-974.

118. Ma CS, et al. Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J Exp Med*. 2008;205(7):1551-1557.

119. Bettelli E, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*. 2006;441(7090):235-238.

120. Mucida D, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science*. 2007;317(5835):256-260.

121. Xu L, et al. Cutting edge: regulatory T cells induce CD4 +CD25-Foxp3-T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. *J Immunol*. 2007;178(11):6725-6729.

122. Zhou L, et al. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature*. 2008;453(7192): 236-240.

123. Nurieva RI, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity*. 2008;29(1):138-149.

124. Nurieva RI, et al. Bcl6 mediates the development of T follicular helper cells. *Science*. 2009;325(5943):1001-1005.

125. Choi YS, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity*. 2011;34(6):932-946.

126. Harker JA, et al. Late interleukin-6 escalates T follicular helper cell responses and controls a chronic viral infection. *Science*. 2011;334(6057):825-829.

127. Haynes NM, et al. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. *J Immunol*. 2007;179(8):5099-5108.

128. Johnston RJ, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science*. 2009;325(5943):1006-1010.

129. Goenka R, et al. Cutting edge: dendritic cell-restricted antigen presentation initiates the follicular helper T cell program but cannot complete ultimate effector differentiation. *J Immunol*. 2011;187(3):1091-1095.

130. Whitmire JK, et al. Antiviral CD4 and CD8 T-cell memory: differences in the size of the response and activation requirements. *Philos Trans R Soc Lond B Biol Sci*. 2000;355(1395):373-379.

131. Butz EA, Bevan MJ. Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity*. 1998;8(2):167-175.

132. Murali-Krishna K, et al. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity*. 1998;8(2):177-187.

133. Masopust D, Murali-Krishna K, Ahmed R. Quantitating the magnitude of the lymphocytic choriomeningitis virus-specific CD8 T-cell response: it is even bigger than we thought. *J Virol*. 2007;81(4):2002-2011.

134. Foulds KE, et al. Cutting edge: CD4 and CD8 T cells are intrinsically different in their proliferative responses. *J Immunol*. 2002;168(4): 1528-1532.

135. Kaech SM, Ahmed R. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nat Immunol*. 2001;2(5):415-422.

136. Mercado R, et al. Early programming of T cell populations responding to bacterial infection. *J Immunol*. 2000;165(12):6833-6839.

137. van Stipdonk MJ, Lemmens EE, Schoenberger SP. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat Immunol*. 2001;2(5):423-429.

138. Kedl RM, et al. T cells compete for access to antigen-bearing antigenpresenting cells. *J Exp Med*. 2000;192(8):1105-1113.

139. Ludewig B, et al. Dendritic cells efficiently induce protective antiviral immunity. *J Virol*. 1998;72(5):3812-3818.

140. Wherry EJ, et al. The induction of virus-specific CTL as a function of increasing epitope expression: responses rise steadily until excessively high levels of epitope are attained. *J Immunol*. 1999;163(7):3735-3745.

141. Wherry EJ, McElhaugh MJ, Eisenlohr LC. Generation of CD8(+) T cell memory in response to low, high, and excessive levels of epitope. *J Immunol*. 2002;168(9):4455-4461.

142. Joshi NS, et al. Inflammation directs memory precursor and shortlived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity*. 2007;27(2):281-295.

143. Badovinac VP, Porter BB, Harty JT. Programmed contraction of CD8(+) T cells after infection. *Nat Immunol*. 2002;3(7):619-626.

144. Wherry EJ, et al. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol*. 2003;77(8):4911-4927.

145. Shin H, et al. Viral antigen and extensive division maintain virusspecific CD8 T cells during chronic infection. *J Exp Med*. 2007;204(4): 941-949.

146. Barber DL, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*. 2006;439(7077):682-687.

147. Iezzi G, Karjalainen K, Lanzavecchia A. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity*. 1998;8(1):89-95.

148. Wong P, Pamer EG. Cutting edge: antigen-independent CD8 T cell proliferation. *J Immunol*. 2001;166(10):5864-5868.

149. Gudmundsdottir H, Wells AD, Turka LA. Dynamics and requirements of T cell clonal expansion in vivo at the single-cell level: effector function is linked to proliferative capacity. *J Immunol*. 1999;162(9): 5212-5223.

150. Jelley-Gibbs DM, et al. Two distinct stages in the transition from naive CD4 T cells to effectors, early antigen-dependent and late cytokine-driven expansion and differentiation. *J Immunol*. 2000;165(9): 5017-5026.

151. Lee WT, et al. Continued antigen stimulation is not required during CD4(+) T cell clonal expansion. *J Immunol*. 2002;168(4):1682-1689.

152. Obst R, et al. Antigen persistence is required throughout the expansion phase of a CD4(+) T cell response. *J Exp Med*. 2005;201(10):1555-1565.

153. Obst R, et al. Sustained antigen presentation can promote an immunogenic T cell response, like dendritic cell activation. *Proc Natl Acad Sci U S A* . 2007;104(39):15460-15465.

154. Gett AV, et al. T cell fitness determined by signal strength. *Nat Immunol*. 2003;4(4):355-360.

155. van Stipdonk MJ, et al. Dynamic programming of CD8+ T lymphocyte responses. *Nat Immunol*. 2003;4(4):361-365.

156. Curtsinger JM, et al. Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. *J Immunol*. 2005;174(8):4465-4469.

157. Aichele P, et al. CD8 T cells specific for lymphocytic choriomeningitis virus require type I IFN receptor for clonal expansion. *J Immunol*. 2006;176(8):4525-4529.

158. Kolumam GA, et al. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *J Exp Med*. 2005;202(5):637-650.

159. Xiao Z, et al. Programming for CD8 T cell memory development requires IL-12 or type I IFN. *J Immunol*. 2009;182(5):2786-2794.

160. Thompson LJ, et al. Innate inflammatory signals induced by various pathogens differentially dictate the IFN-I dependence of CD8 T cells for clonal expansion and memory formation. *J Immunol*. 2006;177(3):1746-1754.

161. Badovinac VP, Tvinnereim AR, Harty JT. Regulation of antigenspecific CD8+ T cell homeostasis by perforin and interferon-gamma. *Science*. 2000;290(5495):1354-1358.

162. Sercan O, et al. Innate immune cells contribute to the IFN-gammadependent regulation of antigen-specific CD8+ T cell homeostasis. *J Immunol*. 2006;176(2):735-739.

163. Whitmire JK, Benning N, Whitton JL. Cutting edge: early IFN-gamma signaling directly enhances primary antiviral CD4+ T cell responses. *J Immunol*. 2005;175(9):5624-5628.

164. Bennett SR, et al. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. *J Exp Med*. 1997;186(1):65-70.

165. Castellino F, Germain RN. Cooperation between CD4+ and CD8+ T cells: when,

where, and how. *Annu Rev Immunol*. 2006;24:519-540.

166. Smith CM, et al. Cognate CD4(+) T cell licensing of dendritic cells in CD8(+) T cell immunity. *Nat Immunol*. 2004;5(11):1143-1148.

167. Bevan MJ. Immunology. Stimulating killer cells. *Nature*. 1989;342(6249): 478-479.

168. Keene JA, Forman J. Helper activity is required for the in vivo generation of cytotoxic T lymphocytes. *J Exp Med*. 1982;155(3):768-782.

169. Mitchison NA, O'Malley C. Three-cell-type clusters of T cells with antigen-presenting cells best explain the epitope linkage and noncognate requirements of the in vivo cytolytic response. *Eur J Immunol* . 1987;17(11):1579-1583.

170. Bevan MJ. Helping the CD8(+) T-cell response. *Nat Rev Immunol*. 2004;4(8):595-602.

171. Tham EL, Shrikant P, Mescher MF. Activation-induced nonresponsiveness: a Th-dependent regulatory checkpoint in the CTL response. *J Immunol*. 2002;168(3):1190-1197.

172. Elsaesser H, Sauer K, Brooks DG. IL-21 is required to control chronic viral infection. *Science*. 2009;324(5934):1569-1572.

173. Yi JS, Du M, Zajac AJ. A vital role for interleukin-21 in the control of a chronic viral infection. *Science*. 2009;324(5934):1572-1576.

174. Zeng R, et al. Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function. *J Exp Med*. 2005;201(1):139-148.

175. Carvalho LH, et al. IL-4-secreting CD4+ T cells are crucial to the development of CD8+ T-cell responses against malaria liver stages. *Nat Med*. 2002;8(2):166-170.

176. Bennett SR, et al. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature*. 1998;393(6684):478-480.

177. Grewal IS, Flavell RA. The role of CD40 ligand in costimulation and T-cell activation. *Immunol Rev*. 1996;153:85-106.

178. Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature*. 1998;393(6684):474-478.

179. Schoenberger SP, et al. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature*. 1998;393(6684):480-483.

180. Cella M, et al. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med*. 1996;184(2):747-752.

181. Beuneu H, Garcia Z, Bousso P. Cutting edge: cognate CD4 help promotes recruitment of antigen-specific CD8 T cells around dendritic cells. *J Immunol*. 2006;177(3):1406-1410.

182. Castellino F, et al. Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. *Nature*. 2006;440(7086):890-895.

183. Battegay M, et al. Enhanced establishment of a virus carrier state in adult CD4+ T-cell-deficient mice. *J Virol*. 1994;68(7):4700-4704.

184. Belz GT, et al. Compromised influenza virus-specific CD8(+)-T-cell memory in CD4(+)-T-cell-deficient mice. *J Virol*. 2002;76(23):12388-12393.

185. Cardin RD, et al. Progressive loss of CD8+ T cell-mediated control of a gamma-herpesvirus in the absence of CD4+ T cells. *J Exp Med*. 1996;184(3):863-871.

186. Matloubian M, Concepcion RJ, Ahmed R. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J Virol*. 1994;68(12):8056-8063.

187. Moskophidis D, et al. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature*. 1993;362(6422):758-761.

188. von Herrath MG, et al. CD4-deficient mice have reduced levels of memory cytotoxic T lymphocytes after immunization and show diminished resistance to subsequent virus challenge. *J Virol*. 1996;70(2):1072-1079.

189. Zajac AJ, et al. Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med*. 1998;188(12):2205-2213.

190. Matloubian M, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature*. 2004;427(6972):355-360.

191. Pappu R, et al. Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. *Science*. 2007;316(5822):295-298.

192. Schaerli P, Moser B. Chemokines: control of primary and memory T-cell traffic. *Immunol Res*. 2005;31(1):57-74.

193. Campbell DJ, Kim CH, Butcher EC. Chemokines in the systemic organization of

immunity. *Immunol Rev*. 2003;195:58-71.

194. Lanzavecchia A, Sallusto F. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science*. 2000;290(5489):92-97.

195. Potsch C, Vohringer D, Pircher H. Distinct migration patterns of naive and effector CD8 T cells in the spleen: correlation with CCR7 receptor expression and chemokine reactivity. *Eur J Immunol* . 1999;29(11): 3562-3570.

196. Hamann A, et al. Role of alpha 4-integrins in lymphocyte homing to mucosal tissues in vivo. *J Immunol*. 1994;152(7):3282-3293.

197. Kantele A, et al. Differential homing commitments of antigen-specific T cells after oral or parenteral immunization in humans. *J Immunol*. 1999;162(9):5173-5177.

198. Mora JR, et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature*. 2003;424(6944):88-93.

199. Svensson M, et al. CCL25 mediates the localization of recently activated CD8alphabeta(+) lymphocytes to the small-intestinal mucosa. *J Clin Invest*. 2002;110(8):1113-1121.

200. Campbell JJ, et al. The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. *Nature*. 1999;400(6746):776-780.

201. Morales J, et al. CTACK, a skin-associated chemokine that preferentially attracts skin-homing memory T cells. *Proc Natl Acad Sci U S A* . 1999;96(25):14470-14475.

202. Gebhardt T, et al. Different patterns of peripheral migration by memory CD4+ and CD8+ T cells. *Nature*. 2011;477(7363):216-219.

203. Harty JT, Badovinac VP. Influence of effector molecules on the CD8(+) T cell response to infection. *Curr Opin Immunol* . 2002;14(3):360-365.

204. Hildeman DA, et al. Molecular mechanisms of activated T cell death in vivo. *Curr Opin Immunol*. 2002;14(3):354-359.

205. Lakhani S, Flavell RA. Caspases and T lymphocytes: a flip of the coin? *Immunol Rev*. 2003;193:22-30.

206. Siegel RM. Caspases at the crossroads of immune-cell life and death. *Nat Rev Immunol*. 2006;6(4):308-317.

207. Marsden VS, Strasser A. Control of apoptosis in the immune system: Bcl-2, BH3-only proteins and more. *Annu Rev Immunol*. 2003;21:71-105.

208. Strasser A. The role of BH3-only proteins in the immune system. *Nat Rev Immunol* . 2005;5(3):189-200.

209. Green DR, Droin N, Pinkoski M. Activation-induced cell death in T cells. *Immunol Rev*. 2003;193:70-81.

210. Van Parijs L, Biuckians A, Abbas AK. Functional roles of Fas and Bcl-2-regulated apoptosis of T lymphocytes. *J Immunol* . 1998;160(5): 2065-2071.

211. Karrer U, et al. Memory inflation: continuous accumulation of antiviral CD8+ T cells over time. *J Immunol* . 2003;170(4):2022-2029.

212. Sierro S, Rothkopf R, Klenerman P. Evolution of diverse antiviral CD8+ T cell populations after murine cytomegalovirus infection. *Eur J Immunol* . 2005;35(4):1113-1123.

213. Suresh M, Singh A, Fischer C. Role of tumor necrosis factor receptors in regulating CD8 T-cell responses during acute lymphocytic choriomeningitis virus infection. *J Virol* . 2005;79(1):202-213.

214. Singh A, Suresh M. A role for TNF in limiting the duration of CTL effector phase and magnitude of CD8 T cell memory. *J Leukoc Biol* . 2007;82(5):1201-1211.

215. Weant AE, et al. Apoptosis regulators Bim and Fas function concurrently to control autoimmunity and CD8+ T cell contraction. *Immunity*. 2008;28(2):218-230.

216. Budd RC, Yeh WC, Tschopp J. cFLIP regulation of lymphocyte activation and development. *Nat Rev Immunol* . 2006;6(3):196-204.

217. Mortier E, et al. Macrophage- and dendritic-cell-derived interleukin-15 receptor alpha supports homeostasis of distinct CD8+ T cell subsets. *Immunity*. 2009;31(5):811-822.

218. Wang XZ, et al. Virus-specific CD8 T cells in peripheral tissues are more resistant to apoptosis than those in lymphoid organs. *Immunity*. 2003;18(5):631-642.

219. Becker TC, et al. Bone marrow is a preferred site for homeostatic proliferation of memory CD8 T cells. *J Immunol* . 2005;174(3):1269-1273.

220. Lakhani SA, et al. Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. *Science*. 2006;311(5762):847-851.

221. Lohman BL, Razvi ES, Welsh RM. T-lymphocyte downregulation after acute viral infection is not dependent on CD95 (Fas) receptor-ligand interactions. *J Virol* .

1996;70(11):8199-8203.

222. Nguyen LT, et al. TNF receptor 1 (TNFR1) and CD95 are not required for T cell deletion after virus infection but contribute to peptide-induced deletion under limited conditions. *Eur J Immunol* . 2000;30(2):683-688.

223. Sacks JA, Bevan MJ. TRAIL deficiency does not rescue impaired CD8+ T cell memory generated in the absence of CD4+ T cell help. *J Immunol* . 2008;180(7):4570-4576.

224. Badovinac VP, et al. TRAIL deficiency delays, but does not prevent, erosion in the quality of “helpless” memory CD8 T cells. *J Immunol* . 2006;177(2):999-1006.

225. Singer GG, Abbas AK. The fas antigen is involved in peripheral but not thymic deletion of T lymphocytes in T cell receptor transgenic mice. *Immunity*. 1994;1(5):365-371.

226. Zhou S, et al. Critical role for perforin-, Fas/FasL-, and TNFR1-mediated cytotoxic pathways in down-regulation of antigen-specific T cells during persistent viral infection. *J Virol* . 2002;76(2):829-840.

227. Hughes PD, et al. Apoptosis regulators Fas and Bim cooperate in shutdown of chronic immune responses and prevention of autoimmunity. *Immunity*. 2008;28(2):197-205.

228. Hamilton SE, et al. The generation of protective memory-like CD8+ T cells during homeostatic proliferation requires CD4+ T cells. *Nat Immunol* . 2006;7(5):475-481.

229. Janssen EM, et al. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature*. 2005;434(7029):88-93.

230. Rogers PR, et al. OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. *Immunity*. 2001;15(3):445-455.

231. Dawicki W, et al.4-1BB and OX40 act independently to facilitate robust CD8 and CD4 recall responses. *J Immunol* . 2004;173(10):5944-5951.

232. Tan JT, et al. 4-1BB ligand, a member of the TNF family, is important for the generation of antiviral CD8 T cell responses. *J Immunol* . 1999;163(9):4859-4868.

233. Gramaglia I, et al. The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. *J Immunol* . 2000;165(6):3043-3050.

234. Mousavi SF, et al. OX40 costimulatory signals potentiate the memory commitment of

effector CD8+ T cells. *J Immunol*. 2008;181(9): 5990-6001.

235. Lee SW, et al. Functional dichotomy between OX40 and 4-1BB in modulating effector CD8 T cell responses. *J Immunol*. 2006;177(7):4464-4472.

236. Sabbagh L, Snell LM, Watts TH. TNF family ligands define niches for T cell memory. *Trends Immunol*. 2007;28(8):333-339.

237. Pulle G, Vidric M, Watts TH. IL-15-dependent induction of 4-1BB promotes antigen-independent CD8 memory T cell survival. *J Immunol*. 2006;176(5):2739-2748.

238. Ashton-Rickardt PG. The granule pathway of programmed cell death. *Crit Rev Immunol*. 2005;25(3):161-182.

239. Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science*. 1998;281(5381):1312-1316.

240. Erlacher M, et al. BH3-only proteins Puma and Bim are rate-limiting for gamma-radiation- and glucocorticoid-induced apoptosis of lymphoid cells in vivo. *Blood*. 2005;106(13):4131-4138.

241. Hieronymus T, et al. CD 95-independent mechanisms of IL-2 deprivation-induced apoptosis in activated human lymphocytes. *Cell Death Differ*. 2000;7(6):538-547.

242. Hildeman DA, et al. Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. *Immunity*. 2002;16(6):759-767.

243. Lohman BL, Welsh RM. Apoptotic regulation of T cells and absence of immune deficiency in virus-infected gamma interferon receptor knockout mice. *J Virol*. 1998;72(10):7815-7821.

244. Razvi ES, et al. Lymphocyte apoptosis during the silencing of the immune response to acute viral infections in normal, lpr, and Bcl-2-transgenic mice. *Am J Pathol*. 1995;147(1):79-91.

245. Reich A, et al. Immune down-regulation and peripheral deletion of CD8 T cells does not require TNF receptor-ligand interactions nor CD95 (Fas, APO-1). *Eur J Immunol*. 2000;30(2):678-682.

246. Sandalova E, et al. Regulation of expression of Bcl-2 protein family member Bim by T cell receptor triggering. *Proc Natl Acad Sci U S A*. 2004;101(9):3011-3016.

247. Willis SN, et al. Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science*. 2007;315(5813):856-859.

248. Schmitz I, et al. An IL-2-dependent switch between CD95 signaling pathways sensitizes primary human T cells toward CD95-mediated activation-induced cell death. *J Immunol*. 2003;171(6):2930-2936.

249. Wojciechowski S, et al. Bim/Bcl-2 balance is critical for maintaining naive and memory T cell homeostasis. *J Exp Med*. 2007;204(7): 1665-1675.

250. Bouillet P, et al. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science*. 1999;286(5445):1735-1738.

251. Wojciechowski S, et al. Bim mediates apoptosis of CD127(lo) effector T cells and limits T cell memory. *Eur J Immunol* . 2006;36(7):1694-1706.

252. Pellegrini M, et al. Shutdown of an acute T cell immune response to viral infection is mediated by the proapoptotic Bcl-2 homology 3-only protein Bim. *Proc Natl Acad Sci U S A*. 2003;100(24):14175-14180.

253. Zong WX, et al. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. *Genes Dev*. 2001;15(12):1481-1486.

254. Rathmell JC, et al. Deficiency in Bak and Bax perturbs thymic selection and lymphoid homeostasis. *Nat Immunol*. 2002;3(10):932-939.

255. Zhang M, et al. Differential survival of cytotoxic T cells and memory cell precursors. *J Immunol*. 2007;178(6):3483-3491.

256. Zhang M, et al. Serine protease inhibitor 6 protects cytotoxic T cells from self-inflicted injury by ensuring the integrity of cytotoxic granules. *Immunity*. 2006;24(4):451-461.

257. Liu N, et al. Serine protease inhibitor 2A is a protective factor for memory T cell development. *Nat Immunol*. 2004;5(9):919-926.

258. Opferman JT, et al. Suicide induced by cytolytic activity controls the differentiation of memory CD8(+) T lymphocytes. *Int Immunol*. 2001;13(4):411-419.

259. Goldrath AW, et al. Cytokine requirements for acute and Basal homeostatic proliferation of naive and memory CD8+ T cells. *J Exp Med*. 2002;195(12):1515-1522.

260. Kaech SM, et al. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol*. 2003;4(12):1191-1198.

261. Vella AT, et al. Cytokine-induced survival of activated T cells in vitro and in vivo. *Proc Natl Acad Sci U S A*. 1998;95(7):3810-3815.

262. Sarkar S, et al. Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *J Exp Med*. 2008;205(3):625-640.

263. Huster KM, et al. Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. *Proc Natl Acad Sci U S A*. 2004;101(15):5610-5615.

264. Hand TW, Morre M, Kaech SM. Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. *Proc Natl Acad Sci U S A*. 2007;104(28):11730-11735.

265. Klonowski KD, et al. Cutting edge: IL-7-independent regulation of IL-7 receptor alpha expression and memory CD8 T cell development. *J Immunol*. 2006;177(7):4247-4251.

266. Lacombe MH, et al. IL-7 receptor expression levels do not identify CD8+ memory T lymphocyte precursors following peptide immunization. *J Immunol*. 2005;175(7):4400-4407.

267. Sun JC, Lehar SM, Bevan MJ. Augmented IL-7 signaling during viral infection drives greater expansion of effector T cells but does not enhance memory. *J Immunol*. 2006;177(7):4458-4463.

268. Kurtulus S, et al. Bcl-2 allows effector and memory CD8+ T cells to tolerate higher expression of Bim. *J Immunol*. 2011;186(10):5729-5737.

269. Kaech SM, Wherry EJ. Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. *Immunity*. 2007;27:393-405.

270. Purton JF, et al. Antiviral CD4+ memory T cells are IL-15 dependent. *J Exp Med*. 2007;204(4):951-961.

271. Kondrack RM, et al. Interleukin 7 regulates the survival and generation of memory CD4 cells. *J Exp Med*. 2003;198(12):1797-1806.

272. Li J, Huston G, Swain SL. IL-7 promotes the transition of CD4 effectors to persistent memory cells. *J Exp Med*. 2003;198(12):1807-1815.

273. Tripathi P, et al. STAT5 is critical to maintain effector CD8+ T cell responses. *J Immunol*. 2010;185(4):2116-2124.

274. Tripathi P, et al. Cutting edge: limiting amounts of IL-7 do not control contraction of CD4+ T cell responses. *J Immunol*. 2007;178(7):4027-4031.

275. Baaten BJ, et al. CD44 regulates survival and memory development in Th1 cells. *Immunity*. 2010;32(1):104-115.

276. Song J, et al. The costimulation-regulated duration of PKB activation controls T cell longevity. *Nat Immunol*. 2004;5(2):150-158.

277. Marzo AL, Yagita H, Lefrancois L. Cutting edge: migration to nonlymphoid tissues results in functional conversion of central to effector memory CD8 T cells. *J Immunol*. 2007;179(1):36-40.

278. Hikono H, et al. Activation phenotype, rather than central- or effectormemory phenotype, predicts the recall efficacy of memory CD8+ T cells. *J Exp Med*. 2007;204(7):1625-1636.

279. Badovinac VP, Porter BB, Harty JT. CD8+ T cell contraction is controlled by early inflammation. *Nat Immunol*. 2004;5(8):809-817.

280. Cui W, et al. Effects of Signal 3 during CD8 T cell priming: bystander production of IL-12 enhances effector T cell expansion but promotes terminal differentiation. *Vaccine*. 2009;27(15):2177-2187.

281. D'Souza WN, Hedrick SM Cutting edge: latecomer CD8 T cells are imprinted with a unique differentiation program. *J Immunol*. 2006;177(2):777-781.

282. Badovinac VP, Haring JS, Harty JT. Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8(+) T cell response to infection. *Immunity*. 2007;26(6):827-841.

283. O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science*. 2010;327(5969):1098-1102.

284. Hegazy AN, et al. Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions. *Immunity*. 2010;32(1):116-128.

285. Krawczyk CM, Shen H, Pearce EJ. Functional plasticity in memory T helper cell responses. *J Immunol*. 2007;178(7):4080-4088.

286. Lee YK, et al. Developmental plasticity of Th17 and Treg cells. *Curr Opin Immunol*. 2009;21(3):274-280.

287. Lee YK, et al. Late developmental plasticity in the T helper 17 lineage. *Immunity*. 2009;30(1):92-107.

288. Yang XO, et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity*. 2008;29(1):44-56.

289. Hyatt G, et al. Gene expression microarrays: glimpses of the immunological genome. *Nat Immunol*. 2006;7(7):686-691.

290. Alizadeh A, et al. Probing lymphocyte biology by genomic-scale gene expression analysis. *J Clin Immunol*. 1998;18(6):373-379.

291. Goldrath AW, et al. The molecular program induced in T cells undergoing homeostatic proliferation. *Proc Natl Acad Sci U S A* . 2004;101(48): 16885-16890.

292. Willinger T, et al. Molecular signatures distinguish human central memory from effector memory CD8 T cell subsets. *J Immunol*. 2005;175(9):5895-5903.

293. Luckey CJ, et al. Memory T and memory B cells share a transcriptional program of self-renewal with long-term hematopoietic stem cells. *Proc Natl Acad Sci U S A* . 2006;103(9):3304-3309.

294. Peixoto A, et al. CD8 single-cell gene coexpression reveals three different effector types present at distinct phases of the immune response. *J Exp Med*. 2007;204(5):1193-1205.

295. Juedes AE, et al. T-bet controls autoaggressive CD8 lymphocyte responses in type 1 diabetes. *J Exp Med*. 2004;199(8):1153-1162.

296. Pearce EL, et al. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. *Science*. 2003;302(5647):1041-1043.

297. Sullivan BM, et al. Antigen-driven effector CD8 T cell function regulated by T-bet. *Proc Natl Acad Sci U S A* . 2003;100(26):15818-15823.

298. Intlekofer AM, et al. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat Immunol*. 2005;6(12):1236-1244.

299. Takemoto N, et al. Cutting Edge: IL-12 inversely regulates T-bet and eomesodermin expression during pathogen-induced CD8+ T cell differentiation. *J Immunol*. 2006;177(11):7515-7519.

300. Intlekofer AM, et al. Requirement for T-bet in the aberrant differentiation of unhelped memory CD8+ T cells. *J Exp Med*. 2007;204(9):2015-2021.

301. Ji Y, et al. Repression of the DNA-binding inhibitor Id3 by Blimp-1 limits the formation of memory CD8(+) T cells. *Nat Immunol*. 2011;12(12):1230-1237.

302. Yang CY, et al. The transcriptional regulators Id2 and Id3 control the formation of distinct memory CD8(+) T cell subsets. *Nat Immunol*. 2011;12(12):1221-1229.

303. Cannarile MA, et al. Transcriptional regulator Id2 mediates CD8+ T cell immunity. *Nat Immunol*. 2006;7(12):1317-1325.

304. Klein U, Dalla-Favera R. Germinal centres: role in B-cell physiology and malignancy. *Nat Rev Immunol*. 2008;8(1):22-33.

305. Ichii H, et al. Bcl6 is essential for the generation of long-term memory CD4+ T cells. *Int Immunol*. 2007;19(4):427-433.

306. Toyama H, et al. Memory B cells without somatic hypermutation are generated from Bcl6-deficient B cells. *Immunity*. 2002;17(3):329-339.

307. Cui W, et al. An IL-21-IL-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells. *Immunity*. 2011;35(5):792-805.

308. Manders PM, et al. BCL6b mediates the enhanced magnitude of the secondary response of memory CD8+ T lymphocytes. *Proc Natl Acad Sci U S A*. 2005;102(21):7418-7425.

309. Davis MM. Blimp-1 over Budapest. *Nat Immunol*. 2007;8(5):445-447.

310. Kallies A, Nutt SL. Terminal differentiation of lymphocytes depends on Blimp-1. *Curr Opin Immunol*. 2007;19(2):156-162.

311. Kallies A, et al. Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance. *Nat Immunol*. 2006;7(5):466-474.

312. Martins GA, et al. Transcriptional repressor Blimp-1 regulates T cell homeostasis and function. *Nat Immunol*. 2006;7(5):457-465.

313. Rutishauser RL, et al. Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity*. 2009;31(2):296-308.

314. Kallies A, et al. Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses. *Immunity*. 2009;31(2):283-295.

315. Weninger W, Manjunath N, von Andrian UH. Migration and differentiation of CD8+ T cells. *Immunol Rev*. 2002;186:221-233.

316. Beverley PC. Is T-cell memory maintained by crossreactive stimulation? *Immunol*

Today. 1990;11(6):203-205.

317. Lau LL, et al. Cytotoxic T-cell memory without antigen. *Nature*. 1994;369(6482):648-652.

318. Mullbacher A. The long-term maintenance of cytotoxic T cell memory does not require persistence of antigen. *J Exp Med*. 1994;179(1):317-321.

319. Zinkernagel RM, et al. On immunological memory. *Annu Rev Immunol*. 1996;14:333-367.

320. Wiehagen KR, et al. Loss of tonic T-cell receptor signals alters the generation but not the persistence of CD8+ memory T cells. *Blood*. 2010;116(25):5560-5570.

321. Murali-Krishna K, et al. Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science*. 1999;286(5443):1377-1381.

322. Alter G, et al. Longitudinal assessment of changes in HIV-specific effector activity in HIV-infected patients starting highly active antiretroviral therapy in primary infection. *J Immunol*. 2003;171(1):477-488.

323. Casazza JP, et al. Decay kinetics of human immunodeficiency virusspecific CD8+ T cells in peripheral blood after initiation of highly active antiretroviral therapy. *J Virol*. 2001;75(14):6508-6516.

324. Garcia S, DiSanto J, Stockinger B. Following the development of a CD4 T cell response in vivo: from activation to memory formation. *Immunity*. 1999;11(2):163-171.

325. Hu H, et al. CD4(+) T cell effectors can become memory cells with high efficiency and without further division. *Nat Immunol*. 2001;2(8):705-710.

326. Seddon B, Tomlinson P, Zamoyska R. Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells. *Nat Immunol*. 2003;4(7):680-686.

327. Swain SL, Hu H, Huston G. Class II-independent generation of CD4 memory T cells from effectors. *Science*. 1999;286(5443):1381-1383.

328. Bushar ND, et al. Ablation of SLP-76 signaling after T cell priming generates memory CD4 T cells impaired in steady-state and cytokinedriven homeostasis. *Proc Natl Acad Sci U S A*. 2010;107(2):827-831.

329. Kassiotis G, et al. Impairment of immunological memory in the absence of MHC despite survival of memory T cells. *Nat Immunol*. 2002;3(3):244-250.

330. Belardelli F, et al. The induction of in vivo proliferation of long-lived CD44hi CD8+ T

cells after the injection of tumor cells expressing IFNalpha1 into syngeneic mice. *Cancer Res*. 1998;58(24):5795-5802.

331. Judge AD, et al. Interleukin 15 controls both proliferation and survival of a subset of memory-phenotype CD8(+) T cells. *J Exp Med*. 2002;196(7):935-946.

332. Tough DF, Borrow P, Sprent J. Induction of bystander T cell proliferation by viruses and type I interferon in vivo. *Science*. 1996;272(5270):1947-1950.

333. Tough DF, Sun S, Sprent J. T cell stimulation in vivo by lipopolysaccharide (LPS). *J Exp Med*. 1997;185(12):2089-2094.

334. Waldmann TA, Tagaya Y. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Annu Rev Immunol*. 1999;17:19-49.

335. Zhang X, et al. Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15. *Immunity*. 1998;8(5):591-599.

336. Fehniger TA, et al. Fatal leukemia in interleukin 15 transgenic mice follows early expansions in natural killer and memory phenotype CD8+ T cells. *J Exp Med*. 2001;193(2):219-231.

337. Marks-Konczalik J, et al. IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice. *Proc Natl Acad Sci U S A* . 2000;97(21):11445-11450.

338. Di Santo JP, Colucci F, Guy-Grand D. Natural killer and T cells of innate and adaptive immunity: lymphoid compartments with different requirements for common gamma chain-dependent cytokines. *Immunol Rev*. 1998;165:29-38.

339. Knoops L, Renauld JC. IL-9 and its receptor: from signal transduction to tumorigenesis. *Growth Factors*. 2004;22(4):207-215.

340. Nutt SL, et al. Interleukin 21: a key player in lymphocyte maturation. *Crit Rev Immunol*. 2004;24(4):239-250.

341. Burkett PR, et al. Coordinate expression and trans presentation of interleukin (IL)-15Ralpha and IL-15 supports natural killer cell and memory CD8+ T cell homeostasis. *J Exp Med*. 2004;200(7):825-834.

342. Dubois S, et al. IL-15Ralpha recycles and presents IL-15 In trans to neighboring cells. *Immunity*. 2002;17(5):537-547.

343. Sandau MM, et al. Cutting edge: transpresentation of IL-15 by bone marrow-derived cells necessitates expression of IL-15 and IL-15R alpha by the same cells. *J Immunol*.

2004;173(11):6537-6541.

344. Budagian V, et al. Reverse signaling through membrane-bound interleukin-15. *J Biol Chem*. 2004;279(40):42192-42201.

345. Lodolce JP, et al. T cell-independent interleukin 15R alpha signals are required for bystander proliferation. *J Exp Med*. 2001;194(8):1187-1194.

346. Berard M, et al. IL-15 promotes the survival of naive and memory phenotype CD8+ T cells. *J Immunol*. 2003;170(10):5018-5026.

347. Kennedy MK, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J Exp Med*. 2000;191(5):771-780.

348. Lodolce JP, et al. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity*. 1998;9(5):669-676.

349. Nishimura H, et al. Differential roles of interleukin 15 mRNA isoforms generated by alternative splicing in immune responses in vivo. *J Exp Med*. 2000;191(1):157-170.

350. Grabstein KH, et al. Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. *Science*. 1994;264(5161):965-968.

351. Tagaya Y, et al. IL-15: a pleiotropic cytokine with diverse receptor/signaling pathways whose expression is controlled at multiple levels. *Immunity*. 1996;4(4):329-336.

352. Becker TC, et al. Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. *J Exp Med*. 2002;195(12):1541-1548.

353. Schluns KS, et al. Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells. *J Immunol*. 2002;168(10):4827-4831.

354. Ilangumaran S, et al. Suppressor of cytokine signaling 1 regulates IL-15 receptor signaling in CD8+CD44high memory T lymphocytes. *J Immunol*. 2003;171(5):2435-2445.

355. Wu TS, et al. Reduced expression of Bcl-2 in CD8+ T cells deficient in the IL-15 receptor alpha-chain. *J Immunol*. 2002;168(2):705-712.

356. Yajima T, et al. IL-15 regulates CD8+ T cell contraction during primary infection. *J Immunol*. 2006;176(1):507-515.

357. Lenz DC, et al. IL-7 regulates basal homeostatic proliferation of antiviral CD4+ T cell memory. *Proc Natl Acad Sci U S A* . 2004;101(25):9357-9362.

358. Geginat J, Sallusto F, Lanzavecchia A. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. *J Exp Med*. 2001;194(12):1711-1719.

359. de Saint-Vis B, et al. The cytokine profile expressed by human dendritic cells is dependent on cell subtype and mode of activation. *J Immunol*. 1998;160(4):1666-1676.

360. Fry TJ, Mackall CL. The many faces of IL-7: from lymphopoiesis to peripheral T cell maintenance. *J Immunol*. 2005;174(11):6571-6576.

361. Khaled AR, et al. Bax deficiency partially corrects interleukin-7 receptor alpha deficiency. *Immunity*. 2002;17(5):561-573.

362. Kim K, et al. The trophic action of IL-7 on pro-T cells: inhibition of apoptosis of pro-T1, -T2, and -T3 cells correlates with Bcl-2 and Bax levels and is independent of Fas and p53 pathways. *J Immunol*. 1998;160(12):5735-5741.

363. Li WQ, et al. IL-7 promotes T cell proliferation through destabilization of p27Kip1. *J Exp Med*. 2006;203(3):573-582.

364. Li WQ, et al. Interleukin-7 inactivates the pro-apoptotic protein Bad promoting T cell survival. *J Biol Chem*. 2004;279(28):29160-29166.

365. Opferman JT, et al. Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. *Nature*. 2003;426(6967):671-676.

366. Pellegrini M, et al. Loss of Bim increases T cell production and function in interleukin 7 receptor-deficient mice. *J Exp Med*. 2004;200(9): 1189-1195.

367. Jacobs SR, Michalek RD, Rathmell JC. IL-7 is essential for homeostatic control of T cell metabolism in vivo. *J Immunol*. 2010;184(7):3461-3469.

368. Akashi K, et al. Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. *Cell*. 1997;89(7):1033-1041.

369. Maraskovsky E, et al. Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant rag-1-/-mice. *Cell*. 1997;89(7):1011-1019.

370. Osborne LC, et al. Impaired CD8 T cell memory and CD4 T cell primary responses in IL-7R alpha mutant mice. *J Exp Med*. 2007;204(3):619-631.

371. Kieper WC, et al. Overexpression of interleukin (IL)-7 leads to IL-15-independent generation of memory phenotype CD8+ T cells. *J Exp Med*. 2002;195(12):1533-1539.

372. Tan JT, et al. Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of

memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells. *J Exp Med*. 2002;195(12):1523-1532.

373. Kieper WC, et al. Recent immune status determines the source of antigens that drive homeostatic T cell expansion. *J Immunol*. 2005;174(6):3158-3163.

374. Homann D, Teyton L, Oldstone MB. Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. *Nat Med*. 2001;7(8):913-919.

375. Pepper M, et al. Different routes of bacterial infection induce longlived TH1 memory cells and short-lived TH17 cells. *Nat Immunol*. 2010;11(1):83-89.

376. Cauley LS, et al. Cutting edge: virus-specific CD4+ memory T cells in nonlymphoid tissues express a highly activated phenotype. *J Immunol*. 2002;169(12):6655-6658.

377. Chu CQ, Wittmer S, Dalton DK. Failure to suppress the expansion of the activated CD4 T cell population in interferon gamma-deficient mice leads to exacerbation of experimental autoimmune encephalomyelitis. *J Exp Med*. 2000;192(1):123-128.

378. Dalton DK, et al. Interferon gamma eliminates responding CD4 T cells during mycobacterial infection by inducing apoptosis of activated CD4 T cells. *J Exp Med*. 2000;192(1):117-122.

379. Wu CY, et al. Distinct lineages of T(H)1 cells have differential capacities for memory cell generation in vivo. *Nat Immunol*. 2002;3(9):852-858.

380. Xu H, et al. The mechanism and significance of deletion of parasite-specific CD4(+) T cells in malaria infection. *J Exp Med*. 2002;195(7):881-892.

381. Haring JS, Harty JT. Aberrant contraction of antigen-specific CD4 T cells after infection in the absence of gamma interferon or its receptor. *Infect Immun*. 2006;74(11):6252-6263.

382. Refaeli Y, et al. Interferon gamma is required for activation-induced death of T lymphocytes. *J Exp Med*. 2002;196(7):999-1005.

383. Zhang X, et al. Unequal death in T helper cell (Th)1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid Fas/FasL-mediated apoptosis. *J Exp Med*. 1997;185(10):1837-1849.

384. Harrington LE, et al. Memory CD4 T cells emerge from effector T-cell progenitors. *Nature*. 2008;452(7185):356-360.

385. Rivino L, et al. Chemokine receptor expression identifies Pre-T helper (Th)1, Pre-Th2, and nonpolarized cells among human CD4+ central memory T cells. *J Exp Med*.

2004;200(6):725-735.

386. Zielinski CE, et al. Dissecting the human immunologic memory for pathogens. *Immunol Rev*. 2011;240(1):40-51.

387. Morita R, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity*. 2011;34(1):108-121.

388. Chevalier N, et al. CXCR5 expressing human central memory CD4 T cells and their relevance for humoral immune responses. *J Immunol* . 2011;186(10):5556-5568.

389. Zhou X, et al. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol* . 2009;10(9):1000-1007.

390. McClymont SA, et al. Plasticity of human regulatory T cells in healthy subjects and patients with type 1 diabetes. *J Immunol* . 2011;186(7): 3918-3926.

391. Sun JC, Williams MA, Bevan MJ. CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nat Immunol* . 2004;5(9):927-933.

392. Janssen EM, et al. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature*. 2003;421(6925):852-856.

393. Bourgeois C, Rocha B, Tanchot C. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. *Science*. 2002;297(5589):2060-2063.

394. Bourgeois C, et al. CD8 lethargy in the absence of CD4 help. *Eur J Immunol* . 2002;32(8):2199-2207.

395. Shedlock DJ, Shen H. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science*. 2003;300(5617):337-339.

396. Sun JC, Bevan MJ. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science*. 2003;300(5617):339-342.

397. Obar JJ, et al. CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses. *Proc Natl Acad Sci U S A*. 2010;107(1):193-198.

398. Feau S, et al. Autocrine IL-2 is required for secondary population expansion of CD8(+) memory T cells. *Nat Immunol* . 2011;12(9):908-913.

399. Williams MA, Tyznik AJ, Bevan MJ. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature*. 2006;441(7095):890-893.

400. Feau S, et al. Dendritic cell-derived IL-2 production is regulated by IL-15 in humans and in mice. *Blood*. 2005;105(2):697-702.

401. Granucci F, et al. Early IL-2 production by mouse dendritic cells is the result of microbial-induced priming. *J Immunol*. 2003;170(10):5075-5081.

402. Granucci F, et al. Inducible IL-2 production by dendritic cells revealed by global gene expression analysis. *Nat Immunol*. 2001;2(9):882-888.

403. Rogers NC, et al. Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity*. 2005;22(4):507-517.

404. Novy P, et al. Intrinsic IL-21 signaling is critical for CD8 T cell survival and memory formation in response to vaccinia viral infection. *J Immunol*. 2011;186(5):2729-2738.

405. Yi JS, Ingram JT, Zajac AJ. IL-21 deficiency influences CD8 T cell quality and recall responses following an acute viral infection. *J Immunol*. 2010;185(8):4835-4845.

406. Beverley PC. Human T cell subsets. *Immunol Lett*. 1987;14(4):263-267.

407. Dutton RW, Bradley LM, Swain SL. T cell memory. *Ann Rev Immunol*. 1998;16:201-223.

408. Thomas ML. The leukocyte common antigen family. *Ann Rev Immunol*. 1989;7:339-369.

409. Bell EB. Function of CD4 T cell subsets in vivo: expression of CD45R isoforms. *Sem Immunol*. 1992;4(1):43-50.

410. Clement LT. Isoforms of the CD45 common leukocyte antigen family: markers for human T-cell differentiation. *J Clin Immunol*. 1992;12(1):1-10.

411. Gray D. Immunological memory. *Ann Rev Immunol*. 1993;11:49-77.

412. Bell EB, Sparshott SM. Interconversion of CD45R subsets of CD4 T cells in vivo. *Nature*. 1990;348(6297):163-166.

413. Beverley PC. Functional analysis of human T cell subsets defined by CD45 isoform expression. *Sem Immunol*. 1992;4(1):35-41.

414. Boursalian TE, Bottomly K. Stability of naive and memory phenotypes on resting

CD4 T cells in vivo. *J Immunol*. 1999;162(1):9-16.

415. Griffin JP, Orme IM. Evolution of CD4 T-cell subsets following infection of naive and memory immune mice with Mycobacterium tuberculosis. *Infect Immun*. 1994;62(5):1683-1690.

416. Picker LJ, et al. Differential expression of homing-associated adhesion molecules by T cell subsets in man. *J Immunol*. 1990;145(10):3247-3255.

417. Swain SL, Bradley LM. Helper T cell memory: more questions than answers. *Sem Immunol*. 1992;4(1):59-68.

418. Cho BK, et al. Functional differences between memory and naive CD8 T cells. *Proc Natl Acad Sci U S A*. 1999;96(6):2976-2981.

419. Curtsinger JM, Lins DC, Mescher MF. CD8+ memory T cells (CD44high, Ly-6C+) are more sensitive than naive cells to (CD44low, Ly-6C-) to TCR/CD8 signaling in response to antigen. *J Immunol.* 1998;160(7):3236-3243.

420. Walunas TL, et al. Ly-6C is a marker of memory CD8+ T cells. *J Immunol*. 1995;155(4):1873-1883.

421. Haluszczak C, et al. The antigen-specific CD8+ T cell repertoire in unimmunized mice includes memory phenotype cells bearing markers of homeostatic expansion. *J Exp Med*. 2009;206(2):435-448.

422. Daniels MA, Hogquist KA, Jameson SC. Sweet 'n' sour: the impact of differential glycosylation on T cell responses. *Nat Immunol*. 2002;3(10):903-910.

423. Tsuboi S, Fukuda M. Roles of O-linked oligosaccharides in immune responses. *BioEssays*. 2001;23(1):46-53.

424. Galvan M, et al. Alterations in cell surface carbohydrates on T cells from virally infected mice can distinguish effector/memory CD8+ T cells from naive cells. *J Immunol*. 1998;161(2):641-648.

425. Harrington LE, et al. Differentiating between memory and effector CD8 T cells by altered expression of cell surface O-glycans. *J Exp Med*. 2000;191(7):1241-1246.

426. Priatel JJ, et al. The ST3Gal-I sialyltransferase controls CD8+ T lymphocyte homeostasis by modulating O-glycan biosynthesis. *Immunity*. 2000;12(3):273-283.

427. Romero P, et al. Four functionally distinct populations of human effectormemory CD8+ T lymphocytes. *J Immunol*. 2007;178(7):4112-4119.

428. van Leeuwen EM, et al. Human virus-specific CD8+ T cells: diversity specialists. *Immunol Rev*. 2006;211:225-235.

429. Henson SM, Akbar AN. KLRG1—more than a marker for T cell senescence. *Age*. 2009;31(4):285-291.

430. Henson SM, et al. KLRG1 signaling induces defective Akt (ser473) phosphorylation and proliferative dysfunction of highly differentiated CD8+ T cells. *Blood*. 2009;113(26):6619-6628.

431. Cellerai C, et al. Proliferation capacity and cytotoxic activity are mediated by functionally and phenotypically distinct virus-specific CD8 T cells defined by interleukin-7R{alpha} (CD127) and perforin expression. *J Virol*. 2010;84(8):3868-3878.

432. Blackburn SD, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol*. 2009;10(1):29-37.

433. Blackburn SD, et al. Selective expansion of a subset of exhausted CD8 T cells by alphaPD-L1 blockade. *Proc Natl Acad Sci U S A* . 2008;105(39):15016-15021.

434. McMahan RH, et al. Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed in vitro cytotoxicity. *J Clin Invest*. 2010;120(12):4546-4557.

435. Fourcade J, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J Exp Med*. 2010;207(10):2175-2186.

436. Wherry EJ, et al. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol*. 2003;4(3):225-234.

437. Hand TW, Morre M, Kaech SM. Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. *Proc Natl Acad Sci U S A*. 2007;104(28):11730-11735.

438. Bachmann MF, et al. Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. *J Immunol*. 2005;175(7):4686-4696.

439. Bachmann MF, et al. Recall proliferation potential of memory CD8+ T cells and antiviral protection. *J Immunol*. 2005;175(7):4677-4685.

440. Nolz JC, Harty JT. Protective capacity of memory CD8+ T cells is dictated by antigen exposure history and nature of the infection. *Immunity*. 2011;34(5):781-793.

441. van Baarle D, et al. Significance of senescence for virus-specific memory T cell responses: rapid ageing during chronic stimulation of the immune system. *Immunol Lett*. 2005;97(1):19-29.

442. Mackay CR. Homing of naive, memory and effector lymphocytes. *Curr Opin Immunol*. 1993;5(3):423-427.

443. Cose S, et al. Evidence that a significant number of naive T cells enter non-lymphoid organs as part of a normal migratory pathway. *Eur J Immunol* . 2006;36(6):1423-1433.

444. Hofmann M, Brinkmann V, Zerwes HG. FTY720 preferentially depletes naive T cells from peripheral and lymphoid organs. *Int Immunopharmacol*. 2006;6(13-14):1902-1910.

445. Teijaro JR, et al. Cutting edge: tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection. *J Immunol*. 2011;187(11):5510-5514.

446. Masopust D, et al. Dynamic T cell migration program provides resident memory within intestinal epithelium. *J Exp Med*. 2010;207(3):553-564.

447. Masopust D, et al. Cutting edge: gut microenvironment promotes differentiation of a unique memory CD8 T cell population. *J Immunol*. 2006;176(4):2079-83.

448. Gebhardt T, et al. Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat Immunol*. 2009;10(5):524-530.

449. Wakim LM, Woodward-Davis A, Bevan MJ. Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence. *Proc Natl Acad Sci U S A*. 2010;107(42): 17872-17879.

450. Ley K, Kansas GS. Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation. *Nat Rev Immunol* . 2004;4(5):325-335.

451. Mohan K, et al. CXCR3 is required for migration to dermal inflammation by normal and in vivo activated T cells: differential requirements by CD4 and CD8 memory subsets. *Eur J Immunol* . 2005;35(6):1702-1711.

452. Tietz W, Hamann A. The migratory behavior of murine CD4+ cells of memory phenotype. *Eur J Immunol* . 1997;27(9):2225-2232.

453. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Ann Rev Immunol*. 2004;22:745-763.

454. Bouneaud C, et al. Lineage relationships, homeostasis, and recall capacities of central- and effector-memory CD8 T cells in vivo. *J Exp Med*. 2005;201(4):579-590.

455. Manjunath N, et al. Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes. *J Clin Invest*. 2001;108(6):871-878.

456. Marzo AL, et al. Initial T cell frequency dictates memory CD8+ T cell lineage commitment. *Nat Immunol*. 2005;6(8):793-799.

457. Opferman JT, Ober BT, Ashton-Rickardt PG. Linear differentiation of cytotoxic effectors into memory T lymphocytes. *Science*. 1999;283(5408):1745-1748.

458. Sallusto F, Lanzavecchia A. Exploring pathways for memory T cell generation. *J Clin Invest*. 2001;108(6):805-806.

459. Zaph C, et al. Persistence and function of central and effector memory CD4+ T cells following infection with a gastrointestinal helminth. *J Immunol*. 2006;177(1):511-518.

460. Moyron-Quiroz JE, et al. Persistence and responsiveness of immunologic memory in the absence of secondary lymphoid organs. *Immunity*. 2006;25(4):643-654.

461. Obhrai JS, et al. Effector T cell differentiation and memory T cell maintenance outside secondary lymphoid organs. *J Immunol*. 2006;176(7):4051-4058.

462. Bertram EM, et al. A switch in costimulation from CD28 to 4-1BB during primary versus secondary CD8 T cell response to influenza in vivo. *J Immunol*. 2004;172(2):981-988.

463. Borowski AB, et al. Memory CD8+ T cells require CD28 costimulation. *J Immunol*. 2007;179(10):6494-6503.

464. Zammit DJ, et al. Dendritic cells maximize the memory CD8 T cell response to infection. *Immunity*. 2005;22(5):561-570.

465. Marshall DR, et al. Measuring the diaspora for virus-specific CD8+ T cells. *Proc Natl Acad Sci U S A*. 2001;98(11):6313-6318.

466. Masopust D, et al. Preferential localization of effector memory cells in nonlymphoid tissue. *Science*. 2001;291(5512):2413-2417.

467. Reinhardt RL, et al. Visualizing the generation of memory CD4 T cells in the whole body. *Nature*. 2001;410(6824):101-105.

468. Barber DL, Wherry EJ, Ahmed R. Cutting edge: rapid in vivo killing by memory CD8 T cells. *J Immunol*. 2003;171(1):27-31.

469. Roberts AD, Woodland DL. Cutting edge: effector memory CD8+ T cells play a

prominent role in recall responses to secondary viral infection in the lung. *J Immunol*. 2004;172(11):6533-6537.

470. Unsoeld H, et al. Cutting edge: CCR7+ and CCR7- memory T cells do not differ in immediate effector cell function. *J Immunol*. 2002;169(2):638-641.

471. Bachmann MF, et al. Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. *J Immunol*. 2005;175(7):4686-4696.

472. Bachmann MF, et al. Recall proliferation potential of memory CD8+ T cells and antiviral protection. *J Immunol*. 2005;175(7):4677-4685.

473. Kagi D, et al. Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. *Annu Rev Immunol*. 1996;14:207-232.

474. Lowin B, Peitsch MC, Tschopp J. Perforin and granzymes: crucial effector molecules in cytolytic T lymphocyte and natural killer cell-mediated cytotoxicity. *Curr Top Microbiol Immunol*. 1995;198:1-24.

475. Ramshaw IA, et al. Cytokines and immunity to viral infections. *Immunol Rev*. 1997;159:119-135.

476. Klonowski KD, et al. CD8 T cell recall responses are regulated by the tissue tropism of the memory cell and pathogen. *J Immunol*. 2006;177(10):6738-6746.

477. Roberts AD, Ely KH, Woodland DL. Differential contributions of central and effector memory T cells to recall responses. *J Exp Med*. 2005;202(1):123-133.

478. Kersh EN, et al. Rapid demethylation of the IFN-gamma gene occurs in memory but not naive CD8 T cells. *J Immunol*. 2006;176(7):4083-4093.

479. Kersh EN, et al. TCR signal transduction in antigen-specific memory CD8 T cells. *J Immunol*. 2003;170(11):5455-5463.

480. Slifka MK, Whitton JL. Functional avidity maturation of CD8(+) T cells without selection of higher affinity TCR. *Nat Immunol*. 2001;2(8):711-717.

481. Mohrs K, et al. A two-step process for cytokine production revealed by IL-4 dual-reporter mice. *Immunity*. 2005;23(4):419-429.

482. Stetson DB, et al. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J Exp Med*. 2003;198(7):1069-1076.

483. Swanson BJ, et al. RANTES production by memory phenotype T cells is controlled by a posttranscriptional, TCR-dependent process. *Immunity*. 2002;17(5):605-615.

484. Walzer T, et al. Cutting edge: immediate RANTES secretion by resting memory CD8 T cells following antigenic stimulation. *J Immunol*. 2003;170(4):1615-1619.

485. Kersh EN, et al. Rapid demethylation of the IFN-gamma gene occurs in memory but not naive CD8 T cells. *J Immunol*. 2006;176(7):4083-4093.

486. Malherbe L, et al. Clonal selection of helper T cells is determined by an affinity threshold with no further skewing of TCR binding properties. *Immunity*. 2004;21(5):669-679.

487. Badovinac VP, et al. Regulation of CD8+ T cells undergoing primary and secondary responses to infection in the same host. *J Immunol*. 2003;170(10):4933-4942.

488. Choi EY, et al. Quantitative analysis of the immune response to mouse non-MHC transplantation antigens in vivo: the H60 histocompatibility antigen dominates over all others. *J Immunol*. 2001;166(7):4370-4379.

489. Jabbari A, Harty JT. Secondary memory CD8+ T cells are more protective but slower to acquire a central-memory phenotype. *J Exp Med*. 2006;203(4):919-932.

490. Masopust D, et al. Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination. *J Immunol*. 2006;177(2):831-839.

491. Grayson JM, et al. Differential sensitivity of naive and memory CD8+ T cells to apoptosis in vivo. *J Immunol*. 2002;169(7):3760-3770.

492. Plotkin SA. Vaccines: correlates of vaccine-induced immunity. *Clin Infect Dis*. 2008;47(3):401-409.

493. Siegrist CA. Vaccine immunology. In: Plotkin S, Orenstein W, Offit P, eds. *Vaccines*. Philadelphia: Elsevier; 2008:17-36.

494. Plotkin SA. Correlates of protection induced by vaccination. *CVI*. 2010;17(7):1055-1065.

495. Pape KA, et al. Visualization of the genesis and fate of isotypeswitched B cells during a primary immune response. *J Exp Med*. 2003;197(12):1677-1687.

496. Okada T, et al. Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS Biol*. 2005;3(6):e150.

497. Allen RC, et al. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science*. 1993;259(5097):990-993.

498. Conley ME, et al. Primary B cell immunodeficiencies: comparisons and contrasts. *Annu Rev Immunol*. 2009;27:199-227.

499. Cannons JL, Tangye SG, Schwartzberg PL. SLAM family receptors and SAP adaptors in immunity. *Annu Rev Immunol*. 2011;29:665-705.

500. Grimbacher B, et al. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. *Nat Immunol*. 2003;4(3):261-268.

501. Warnatz K, et al. Human ICOS deficiency abrogates the germinal center reaction and provides a monogenic model for common variable immunodeficiency. *Blood*. 2006;107(8):3045-3052.

502. Bossaller L, et al. ICOS deficiency is associated with a severe reduction of CXCR5+CD4 germinal center Th cells. *J Immunol*. 2006;177(7): 4927-4932.

503. Allen CD, et al. Imaging of germinal center selection events during affinity maturation. *Science*. 2007;315(5811):528-531.

504. Tsiagbe VK, Thorbecke GJ. Overview of germinal center function and structure in normal and genetically engineered mice. *Biol Germinal Centers*. 1998:1-103.

505. Allen CDC, Okada T, Cyster JG. Germinal-center organization and cellular dynamics. *Immunity*. 2007;27(2):190-202.

506. Tarlinton DM. Evolution in miniature: selection, survival and distribution of antigen reactive cells in the germinal centre. *Immunol Cell Biol*. 2008;86(2):133-138.

507. Liu YJ, et al. Mechanism of antigen-driven selection in germinal centres. *Nature*. 1989;342(6252):929-931.

508. Victora GD, et al. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell*. 2010;143(4):592-605.

509. Rolf J, et al. Phosphoinositide 3-kinase activity in T cells regulates the magnitude of the germinal center reaction. *J Immunol*. 2010;185(7):4042-4052.

510. MacLennan IC. Germinal centers. *Annu Rev Immunol*. 1994;12:117-139.

511. Vikstrom I, et al. Mcl-1 is essential for germinal center formation and B cell memory. *Science*. 2010;330(6007):1095-1099.

512. Hou P, et al. B cell antigen receptor signaling and internalization are mutually exclusive events. *PLoS Biol*. 2006;4(7):e200.

513. Qi H, et al. SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature*. 2008;455(7214):764-769.

514. Phan TG, et al. High affinity germinal center B cells are actively selected into the plasma cell compartment. *J Exp Med*. 2006;203(11):2419-2424.

515. Good-Jacobson KL, Shlomchik MJ. Plasticity and heterogeneity in the generation of memory B cells and long-lived plasma cells: the influence of germinal center interactions and dynamics. *J Immunol*. 2010;185(6):3117-3125.

516. Lee SK, et al. B cell priming for extrafollicular antibody responses requires Bcl-6 expression by T cells. *J Exp Med*. 2011;208(7):1377-1388.

517. Chan TD, et al. Antigen affinity controls rapid T-dependent antibody production by driving the expansion rather than the differentiation or extrafollicular migration of early plasmablasts. *J Immunol*. 2009;183(5):3139-3149.

518. Shih T-AY, et al. Role of BCR affinity in T cell dependent antibody responses in vivo. *Nat Immunol*. 2002;3(6):570-575.

519. Linterman MA, et al. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J Exp Med*. 2010;207(2):353-363.

520. Zotos D, et al. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J Exp Med*. 2010;207(2):365-378.

521. Eto D, et al. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. *PLoS One*. 2011;6(3):e17739.

522. Bryant VL, et al. Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. *J Immunol*. 2007;179(12):8180-8190.

523. Kuchen S, et al. Essential role of IL-21 in B cell activation, expansion, and plasma cell generation during CD4+ T cell-B cell collaboration. *J Immunol*. 2007;179(9):5886-5896.

524. Ozaki K, et al. A critical role for IL-21 in regulating immunoglobulin production. *Science*. 2002;298(5598):1630-1634.

525. Avery DT, et al. B cell-intrinsic signaling through IL-21 receptor and STAT3 is required for establishing long-lived antibody responses in humans. *J Exp Med*. 2010;207(1):155-171.

526. Fornek JL, et al. Critical role for Stat3 in T-dependent terminal differentiation of IgG B cells. *Blood*. 2006;107(3):1085-1091.

527. Randall TD, et al. Arrest of B lymphocyte terminal differentiation by CD40 signaling: mechanism for lack of antibody-secreting cells in germinal centers. *Immunity*. 1998;8(6):733-742.

528. Agematsu K, et al. B cell subpopulations separated by CD27 and crucial collaboration of CD27+ B cells and helper T cells in immunoglobulin production. *Eur J Immunol*. 1997;27(8):2073-2079.

529. Morimoto S, et al. CD134L engagement enhances human B cell Ig production: CD154/CD40, CD70/CD27, and CD134/CD134L interactions coordinately regulate T cell-dependent B cell responses. *J Immunol*. 2000;164(8):4097-4104.

530. Good-Jacobson KL, et al. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol*. 2010;11(6):535-542.

531. Blink EJ, et al. Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization. *J Exp Med*. 2005;201(4):545-554.

532. Smith KG, et al. bcl-2 transgene expression inhibits apoptosis in the germinal center and reveals differences in the selection of memory B cells and bone marrow antibody-forming cells. *J Exp Med*. 2000;191(3):475-484.

533. Takahashi Y, Ohta H, Takemori T. Fas is required for clonal selection in germinal centers and the subsequent establishment of the memory B cell repertoire. *Immunity*. 2001;14(2):181-192.

534. Shapiro-Shelef M, Calame K. Regulation of plasma-cell development. *Nat Rev Immunol*. 2005;5(3):230-242.

535. Hargreaves DC, et al. A coordinated change in chemokine responsiveness guides plasma cell movements. *J Exp Med*. 2001;194(1):45-56.

536. Kallies A, et al. Plasma cell ontogeny defined by quantitative changes in blimp-1 expression. *J Exp Med*. 2004;200(8):967-977.

537. Shapiro-Shelef M, et al. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity*. 2003;19(4):607-620.

538. Crotty S, Johnston RJ, Schoenberger SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat Immunol*. 2010;11(2):114-120.

539. Kallies A, et al. Initiation of plasma-cell differentiation is independent of the transcription factor Blimp-1. *Immunity*. 2007;26(5):555-566.

540. Reimold AM, et al. Plasma cell differentiation requires the transcription factor XBP-1. *Nature*. 2001;412(6844):300-307.

541. Shaffer AL, et al. XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. *Immunity*. 2004;21(1):81-93.

542. Shapiro-Shelef M, et al. Blimp-1 is required for maintenance of longlived plasma cells in the bone marrow. *J Exp Med*. 2005;202(11): 1471-1476.

543. Wrammert J, et al. Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. *Nature*. 2008;453(7195):667-671.

544. Odendahl M, et al. Generation of migratory antigen-specific plasma blasts and mobilization of resident plasma cells in a secondary immune response. *Blood*. 2005;105(4):1614-1621.

545. Gonzalez AM, et al. Rotavirus-specific B cells induced by recent infection in adults and children predominantly express the intestinal homing receptor alpha4beta7. *Virology*. 2003;305(1):93-105.

546. Tarlinton D, et al. Plasma cell differentiation and survival. *Curr Opin Immunol*. 2008;20(2):162-169.

547. Manz RA, et al. Survival of long-lived plasma cells is independent of antigen. *Int Immunol*. 1998;10(11):1703-1711.

548. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science*. 2002;298(5601):2199-2202.

549. González-García I, et al. Immunization-induced perturbation of human blood plasma cell pool: progressive maturation, IL-6 responsiveness, and high PRDI-BF1/BLIMP1 expression are critical distinctions between antigen-specific and nonspecific plasma cells. *J Immunol*. 2006;176(7):4042-4050.

550. Ellyard JI, et al. Ag-selected Ig-secreting cells persist in human spleen as well as bone marrow. *Blood*. 2004;103(10):3805-3812.

551. Manz RA, et al. Maintenance of serum antibody levels. *Annu Rev Immunol*.

2005;23:367-386.

552. Manz RA, Thiel A, Radbruch A. Lifetime of plasma cells in the bone marrow. *Nature*. 1997;388(6638):133-134.

553. Radbruch A, et al. Competence and competition: the challenge of becoming a long-lived plasma cell. *Nat Rev Immunol* . 2006;6(10): 741-750.

554. Slifka MK, et al. Humoral immunity due to long-lived plasma cells. *Immunity*. 1998;8(3):363-372.

555. Slifka MK, Matloubian M, Ahmed R. Bone marrow is a major site of long-term antibody production after acute viral infection. *J Virol* . 1995;69(3):1895-1902.

556. Hoyer BF, et al. Short-lived plasmablasts and long-lived plasma cells contribute to chronic humoral autoimmunity in NZB/W mice. *J Exp Med*. 2004;199(11):1577-1584.

557. Mesin L, et al. Long-lived plasma cells from human small intestine biopsies secrete immunoglobulins for many weeks in vitro. *J Immunol* . 2011;187(6):2867-2874.

558. Hiepe F, et al. Long-lived autoreactive plasma cells drive persistent autoimmune inflammation. *Nat Rev Rheumatol* . 2011;7(3):170-178.

559. Gatto D, et al. Regulation of memory antibody levels: the role of persisting antigen versus plasma cell life span. *J Immunol* . 2007;178(1):67-76.

560. Cassese G, et al. Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. *J Immunol* . 2003;171(4):1684-1690.

561. Corti D, et al. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science*. 2011;333(6044):850-856.

562. DiLillo DJ, et al. Maintenance of long-lived plasma cells and serological memory despite mature and memory B cell depletion during CD20 immunotherapy in mice. *J Immunol*. 2008;180(1):361-371.

563. Wolf AI, et al. Protective antiviral antibody responses in a mouse model of influenza virus infection require TACI. *J Clin Invest*. 2011;121(10):3954-3964.

564. Jourdan M, et al. An in vitro model of differentiation of memory B cells into plasmablasts and plasma cells including detailed phenotypic and molecular characterization. *Blood*. 2009;114(25):5173-5181.

565. Yoshida T, et al. Memory B and memory plasma cells. *Immunol Rev*. 2010;237(1):117-139.

566. Tarlinton DM, Hodgkin PD. Targeting plasma cells in autoimmune diseases. *J Exp Med*. 2004;199(11):1451-1454.

567. Maclennan ICM, et al. Extrafollicular antibody responses. *Immunol Rev*. 2003;194:8-18.

568. Ho F, et al. Distinct short-lived and long-lived antibody-producing cell populations. *Eur J Immunol* . 1986;16(10):1297-1301.

569. Smith KG, et al. bcl-2 transgene expression inhibits apoptosis in the germinal center and reveals differences in the selection of memory B cells and bone marrow antibody-forming cells. *J Exp Med*. 2000;191(3):475-484.

570. Tangye SG, Tarlinton DM. Memory B cells: effectors of long-lived immune responses. *Eur J Immunol* . 2009;39(8):2065-2075.

571. Liu YJ, et al. Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid up-regulation of B7-1 and B7-2. *Immunity*. 1995;2(3):239-248.

572. Basso K, et al. Tracking CD40 signaling during germinal center development. *Blood*. 2004;104(13):4088-4096.

573. Tangye SG, et al. Identification of functional human splenic memory B cells by expression of CD148 and CD27. *J Exp Med*. 1998;188(9):1691-1703.

574. Mamani-Matsuda M, et al. The human spleen is a major reservoir for long-lived vaccinia virus-specific memory B cells. *Blood*. 2008;111(9):4653-4659.

575. Ehrhardt GRA, et al. Expression of the immunoregulatory molecule FcRH4 defines a distinctive tissue-based population of memory B cells. *J Exp Med*. 2005;202(6):783-791.

576. Agematsu K, et al. Absence of IgD-CD27(+) memory B cell population in X-linked hyper-IgM syndrome. *J Clin Invest*. 1998;102(4):853-860.

577. Klein U, Rajewsky K, Kuppers R. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J Exp Med*. 1998;188(9):1679-1689.

578. Paul WE. *Fundamental Immunology*. Philadelphia: Lippincott Williams & Wilkins; 2003.

579. Crotty S, Ahmed R. Immunological memory in humans. *Semin Immunol*. 2004;16(3):197-203.

580. Leyendeckers H, et al. Frequent detection of thyroid peroxidase-specific IgG+ memory B cells in blood of patients with autoimmune thyroid disease. *Eur J Immunol* . 2002;32(11):3126-3132.

581. Leyendeckers H, et al. Correlation analysis between frequencies of circulating antigen-specific IgG-bearing memory B cells and serum titers of antigen-specific IgG. *Eur J Immunol*. 1999;29(4):1406-1417.

582. Crotty S, et al. Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system. *J Immunol Methods*. 2004;286(1-2):111-122.

583. Nanan R, et al. Acute and long-term effects of booster immunisation on frequencies of antigen-specific memory B-lymphocytes. *Vaccine*. 2001;20(3-4):498-504.

584. Dorfman JR, et al. B cell memory to 3 Plasmodium falciparum blood-stage antigens in a malaria-endemic area. *J Infect Dis*. 2005;191(10):1623-1630.

585. Sasaki S, et al. Comparison of the influenza virus-specific effector and memory B-cell responses to immunization of children and adults with live attenuated or inactivated influenza virus vaccines. *J Virol*. 2007;81(1):215-228.

586. Malaspina A, et al. Compromised B cell responses to influenza vaccination in HIV-infected individuals. *J Infect Dis*. 2005;191(9):1442-1450.

587. Weitkamp JH, et al. Generation of recombinant human monoclonal antibodies to rotavirus from single antigen-specific B cells selected with fluorescent virus-like particles. *J Immunol Methods*. 2003;275(1-2):223-237.

588. Traggiai E, et al. An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. *Nat Med*. 2004;10(8):871-875.

589. Walker LM, et al. Broad neutralization coverage of HIV by multiple highly potent antibodies. *Nature*. 2011;477(7.65):466-470.

590. Scheid JF, et al. Sequence and structural convergence of broad and potent HIV antibodies that mimic CD4 binding. *Science*. 2011;333(6049):1633-1637.

591. Sanz I, et al. Phenotypic and functional heterogeneity of human memory B cells. *Sem Immunol*. 2008;20(1):67-82.

592. Wirths S, Lanzavecchia A. ABCB1 transporter discriminates human resting naive B

cells from cycling transitional and memory B cells. *Eur J Immunol* . 2005;35(12):3433-3441.

593. Moir S, Fauci AS. B cells in HIV infection and disease. *Nat Rev Immunol* . 2009;9(4):235-245.

594. Moir S, et al. Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. *J Exp Med*. 2008;205(8):1797-1805.

595. Charles ED, et al. Clonal B cells in patients with hepatitis C virus-associated mixed cryoglobulinemia contain an expanded anergic CD21low B-cell subset. *Blood*. 2011;117(20):5425-5437.

596. Anderson SM, et al. New markers for murine memory B cells that define mutated and unmutated subsets. *J Exp Med*. 2007;204(9):2103-2114.

597. Tomayko MM, et al. Cutting edge: hierarchy of maturity of murine memory B cell subsets. *J Immunol* . 2010;185(12):7146-7150.

598. Tomayko MM, et al. Systematic comparison of gene expression between murine memory and naive B cells demonstrates that memory B cells have unique signaling capabilities. *J Immunol* . 2008;181(1):27-38.

599. Bhattacharya D, et al. Transcriptional profiling of antigen-dependent murine B cell differentiation and memory formation. *J Immunol* . 2007;179(10):6808-6819.

600. Dogan I, et al. Multiple layers of B cell memory with different effector functions. *Nat Immunol* . 2009;10(12):1292-1299.

601. Dell CL, Lu YX, Claflin JL. Molecular analysis of clonal stability and longevity in B cell memory. *J Immunol* . 1989;143(10):3364-3370.

602. Foote JB, Kearney JF. Generation of B cell memory to the bacterial polysaccharide alpha-1,3 dextran. *J Immunol* . 2009;183(10):6359-6368.

603. Schittek B, Rajewsky K. Maintenance of B-cell memory by long-lived cells generated from proliferating precursors. *Nature*. 1990;346(6286):749-751.

604. McHeyzer-Williams MG, Nossal GJ, Lalor PA. Molecular characterization of single memory B cells. *Nature*. 1991;350(6318):502-505.

605. Crotty S, et al. SAP is required for generating long-term humoral immunity. *Nature*. 2003;421(6920):282-287.

606. Martin SW, Goodnow CC. Burst-enhancing role of the IgG membrane tail as a molecular determinant of memory. *Nat Immunol*. 2002;3(2):182-188.

607. Jilg W, Schmidt M, Deinhardt F. Persistence of specific antibodies after hepatitis B vaccination. *J Hepatol*. 1988;6(2):201-207.

608. Nakao K, et al. Analysis of anti-HBs levels in healthcare workers over 10 years following booster vaccination for hepatitis B virus. *Vaccine*. 2003;21(25-26):3789-3794.

609. Floreani A, et al. Long-term persistence of anti-HBs after vaccination against HBV: an 18 year experience in health care workers. *Vaccine*. 2004;22(5-6):607-610.

610. Plotkin SA, Orenstein WA, Offit PA. *Vaccines*. London, UK: Saunders-Elsevier; 2008:1691.

611. West DJ, Calandra GB. Vaccine induced immunologic memory for hepatitis B surface antigen: implications for policy on booster vaccination. *Vaccine*. 1996;14(11):1019-1027.

612. Tuaillon E, et al. Detection of memory B lymphocytes specific to hepatitis B virus (HBV) surface antigen (HBsAg) from HBsAg-vaccinated or HBV-immunized subjects by ELISPOT assay. *J Immunol Methods*. 2006;315(1-2):144-152.

613. Jilg W, Schmidt M, Deinhardt F. Immune response to hepatitis B revaccination. *J Med Virol*. 1988;24(4):377-384.

614. Ward SM, et al. Direct ex vivo evaluation of long-lived protective antiviral memory B cell responses against hepatitis B virus. *J Infect Dis*. 2008;198(6):813-817.

615. Panum PL. *Beobachtungen uber das Maserncontagium* (Vichows Archives 1847; 1:492-503) Reprinted. Medical Classics. Baltimore, MD: The Williams & Wilkins Company; 1936:829-840.

616. Sawyer WA. Persistence of yellow fever immunity. *J Prevent Med*. 1930;5:413-428.

617. Hancock K, et al. Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. *N Engl J Med*. 2009;361(20):1945-1952.

618. Itoh Y, et al. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. *Nature*. 2009;460(7258):1021.

619. Yu X, et al. Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors. *Nature*. 2008;455(7212):532-536.

620. Bottiger M, Gustavsson O, Svensson A. Immunity to tetanus, diphtheria and

poliomyelitis in the adult population of Sweden in 1991. *Int J Epidemiol* . 1998;27(5):916-925.

621. Taub DD, et al. Immunity from smallpox vaccine persists for decades: a longitudinal study. *Am J Med*. 2008;121(12):1058-1064.

622. Pütz MM, et al. Prevalence of antibodies to Vaccinia virus after smallpox vaccination in Italy. *J Gen Virol*. 2005;86(pt 11):2955-2960.

623. Maruyama M, Lam KP, Rajewsky K. Memory B-cell persistence is independent of persisting immunizing antigen. *Nature*. 2000;407(6804):636-642.

624. Mandel TE, et al. The follicular dendritic cell: long term antigen retention during immunity. *Immunol Rev*. 1980;53:29-59.

625. Bachmann MF, et al. Induction of long-lived germinal centers associated with persisting antigen after viral infection. *J Exp Med*. 1996;183(5):2259-2269.

626. Tew JG, et al. Follicular dendritic cells as accessory cells. *Immunol Rev*. 1990;117:185-211.

627. Wu Y, et al. Immune complex-bearing follicular dendritic cells deliver a late antigenic signal that promotes somatic hypermutation. *J Immunol* . 2008;180(1):281-290.

628. Lam KP, Kuhn R, Rajewsky K. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell*. 1997;90(6):1073-1083.

629. Ruprecht CR, Lanzavecchia A. Toll-like receptor stimulation as a third signal required for activation of human naive B cells. *Eur J Immunol* . 2006;36(4):810-816.

630. Lanzavecchia A, et al. Understanding and making use of human memory B cells. *Immunol Rev*. 2006;211:303-309.

631. Di Genova G, et al. Vaccination of human subjects expands both specific and bystander memory T cells but antibody production remains vaccine specific. *Blood*. 2006;107(7):2806-2813.

632. Lee FE, et al. Circulating human antibody-secreting cells during vaccinations and respiratory viral infections are characterized by high specificity and lack of bystander effect. *J Immunol* . 2011;186(9):5514-5521.

633. Hebeis BJ, et al. Activation of virus-specific memory B cells in the absence of T cell help. *J Exp Med*. 2004;199(4):593-602.

634. Richard K, Pierce SK, Song W. The agonists of TLR4 and 9 are sufficient to activate memory B cells to differentiate into plasma cells in vitro but not in vivo. *J Immunol*. 2008;181(3):1746-1752.

635. Kaech SM, Wherry EJ, Ahmed R. Effector and memory T-cell differentiation: implications for vaccine development. *Nat Rev Immunol*. 2002;2(4):251-262.

636. Parretta E, et al. CD8 cell division maintaining cytotoxic memory occurs predominantly in the bone marrow. *J Immunol*. 2005;174(12): 7654-7664.

637. Macallan DC, et al. B-cell kinetics in humans: rapid turnover of peripheral blood memory cells. *Blood*. 2005;105(9):3633-3640.

638. Anderson SM, Tomayko MM, Shlomchik MJ. Intrinsic properties of human and murine memory B cells. *Immunol Rev*. 2006;211:280-294.

639. Vieira P, Rajewsky K. Persistence of memory B cells in mice deprived of T cell help. *Int Immunol*. 1990;2(6):487-494.

640. McMillan R, et al. Immunoglobulin synthesis by human lymphoid tissues: normal bone marrow as a major site of IgG production. *J Immunol*. 1972;109(6):1386-1394.

641. Jiménez-Gómez G, et al. Modulated selection of IGHV gene somatic hypermutation during systemic maturation of human plasma cells. *J Leukocyte Biol*. 2010;87(3):523-530.

642. Edwards JCW, et al. Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *N Engl J Med*. 2004;350(25): 2572-2581.

643. Vallerskog T, et al. Treatment with rituximab affects both the cellular and the humoral arm of the immune system in patients with SLE. *Clin Immunol*. 2007;122(1):62-74.

644. Ahuja A, et al. Maintenance of the plasma cell pool is independent of memory B cells. *Proc Natl Acad Sci U S A*. 2008;105(12):4802-4807.

645. Putz MM, et al. Prevalence of antibodies to Vaccinia virus after smallpox vaccination in Italy. *J Gen Virol*. 2005;86(pt 11):2955-2960.

646. Rohner GB, et al. The magnitude of the antibody and memory B cell responses during priming with a protein-polysaccharide conjugate vaccine in human infants is associated with the persistence of antibody and the intensity of booster response. *J Immunol*. 2008;180(4):2165-2173.

647. Clark RA, et al. The vast majority of CLA+ T cells are resident in normal skin. *J Immunol*. 2006;176(7):4431-4439.

648. Ravkov EV, Myrick CM, Altman JD. Immediate early effector functions of virus-specific CD8+CCR7+ memory cells in humans defined by HLA and CC chemokine ligand 19 tetramers. *J Immunol*. 2003;170(5): 2461-2468.

649. Purwar R, et al. Resident memory T cells (T(RM)) are abundant in human lung: diversity, function, and antigen specificity. *PLoS One*. 2011;6(1):e16245.

650. Joshi NS, et al. Increased numbers of preexisting memory CD8 T cells and decreased T-bet expression can restrain terminal differentiation of secondary effector and memory CD8 T cells. *J Immunol*. 2011;187(8):4068-4076.

651. Ibegbu CC, et al. Expression of killer cell lectin-like receptor G1 on antigen-specific human CD8+ T lymphocytes during active, latent, and resolved infection and its relation with CD57. *J Immunol*. 2005;174(10):6088-6094.

652. Sallusto F, Lanzavecchia A. Heterogeneity of CD4+ memory T cells: functional modules for tailored immunity. *Eur J Immunol* . 2009;39(8):2076-2082.

653. Miller JD, et al. Human effector and memory CD8+ T cell responses to smallpox and yellow fever vaccines. *Immunity*. 2008;28(5):710-722.

654. Carrasco J, et al. CD45RA on human CD8 T cells is sensitive to the time elapsed since the last antigenic stimulation. *Blood*. 2006;108(9):2897-2905.

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Chapter 32

Immunologic Tolerance

Christopher C. Goodnow

Pamela S. Ohashi

INTRODUCTION

How the immune system tolerates molecules that are part of our body yet mounts a destructive response against molecules that are foreign has been a major biologic and medical conundrum for decades. The idea that the repertoire of lymphocytes is made actively tolerant to each individual's unique set of self-molecules has posed many practical and conceptual challenges. If all lymphocytes that have the potential to respond to self-antigens are silenced or eliminated, will the residual repertoire be sufficient to respond to the enormous array of unknown pathogens? If all self-reactive lymphocytes are actively tolerized, why then do a bewildering array of autoimmune diseases collectively affect more than 5% of people? If we are programmed during development to eliminate tissue-specific lymphocytes, how does immune surveillance against tumors occur? Most importantly, how can we harness the physiologic processes of immunologic tolerance either to prevent transplant rejection and allergy or to promote rejection of tumor cells? In this chapter, we will cover our understanding of the basic mechanisms that are currently known to promote immunologic self-tolerance.

HISTORY

The history of immunologic tolerance illustrates superbly how scientific progress wobbles and sways between two pillars: what people can conceive and what they can test experimentally.

The Discovery of Actively Acquired Tolerance

“Horror autotoxicus” was the insightful term used by Paul Ehrlich early in the 20th century to describe the immune system's avoidance of producing autoantibodies.[1] There were nevertheless much more pressing issues at that time defining the nature of protective antibodies against microbes and the production of vaccines, and little consideration was given to the problem of avoiding autoantibodies.

A key turning point came in 1948 and 1949 in the form of a book and a genetics article synthesizing all that was known about *The Production of Antibodies* by Burnet and Fenner.[2,3] Owen had published in 1945 an extraordinary analysis of antibody production against blood cell alloantigens in cattle.[4] He discovered that nonidentical twins had become hemopoietic chimeras in utero and that as adults they avoided making antibodies against the foreign alloantigens that were continuously presented on their sibling's red blood cells. Traub had earlier observed that mice infected with lymphocytic choriomeningitis virus (LCMV) in utero became lifelong carriers of the virus without producing neutralizing antibodies.[5] Burnet and Fenner put these observations together[2]: “This finding … has the important implication that cells foreign to the host may be tolerated indefinitely provided they are implanted early in embryonic life.”

How the immune system could distinguish self- from foreign antigens was clearly a big problem for Burnet and Fenner's theory. They proposed that tolerance-inducing antigens would be recognized by antibody on lymphocytes alongside a second receptor for a “self-marker.” It is ironic that Burnet and Fenner, as microbiologists first and foremost, locked onto this idea and did not conceive the reciprocal mechanism: that microbial antigens provoke immunity because they are seen by antibody on lymphocytes together with a second receptor for a “microbe marker.”

The failure to conceive of specific receptors for microbes, other than antibody-like molecules, proved to be a prolonged conceptual handicap for the field of actively acquired tolerance and immunity. It stemmed from a turn-of-thecentury rivalry between the schools of thought spearheaded by 1908 Nobel laureates Ehrlich and Metchnikoff, which artificially divided immunologists into those focused on adaptive immunity, exemplified by Ehrlich's antibodies with their extraordinary antigen specificity, and students of Metchnikoff's innate immunity which was viewed by those that followed Ehrlich as “nonspecific.”

In 1963, Claman[6] and Talmage[7] published experiments and theory that bacterial lipopolysaccharide (LPS) or similar components of acid-fast bacilli used in adjuvants provided a second “nonspecific” signal needed to switch lymphocytes from tolerance to immunity. Citing Talmage's theoretical work, Claman concluded,

> These experiments may nevertheless be regarded in the framework of a theory which considers that the production of antibody requires two stimuli, one specific and (at least) one nonspecific. The specific stimulus (or antigenic determinant) designates which specific antibody will be produced. The nonspecific stimulus (or adjuvant factor) carries the ability to stimulate cell proliferation, and is concerned with the quantity of antibody produced.

In 1975, Moller published further experimental and theoretical evidence for this idea, naming it the "one nonspecific signal" model.[8] Their use of the term "nonspecific" to describe the effects of LPS exemplifies the erroneous assumption held by most adaptive immunologists, but not

by those that studied innate immunity, that the adjuvant effects of microbial products like LPS were not mediated by specific receptors. Janeway articulated the need for a collective *mea culpa* among adaptive immunologists in 1989, describing the attitude to microbial adjuvants as "the immunologist's dirty little secret,"[9] and went on to hypothesize a parallel set of pathogen-associated molecular pattern receptors.[10] The question of how LPS, and by extension many microbial adjuvants, were recognized by the vertebrate immune system was answered dramatically with the discovery of precise mutations in the signaling domain of a receptor that looked nothing like antibody, toll-like receptor (TLR) 4, in strains of mice that were specifically unable to respond to LPS.[11] Mouse molecular genetics subsequently illuminated specific sensing functions for a diverse family of vertebrate TLRs and other receptors for "microbe markers."[12]

Partly because of this conceptual gap, Burnet's experiments to test the "self-marker" theory that tolerance was specifically acquired in utero yielded negative results. Chickens[13] or mice[14] exposed to foreign antigens *in ovo* or *in utero* were still fully capable of making antibodies against those antigens. However, most of the experiments employed influenza virus and bacterial flagellin that, like LPS, are recognized by antibody-like receptors and by various members of the TLR family, and would have resulted in the induction of immunity rather than tolerance. Although nonmicrobial antigens were also employed, they were not of the type that persisted long-term to maintain tolerance, unlike the allogeneic blood stem cells that had been exchanged *in utero* between Owen's twin cattle. True to the Popperian tradition of attempting to falsify one's hypothesis, Burnet noted that these results "may mean that the whole conception of the development of selfmarker recognition during embryonic life is wrong."[13]

Burnet and Fenner's book had nevertheless triggered Medawar to approach the problem of immunologic tolerance from a different angle, as a transplant scientist seeking to solve the problem of human skin graft rejection in burn patients. He extended Owen's findings to show that nonidentical twin cattle also accepted skin grafts from each other, representing extraordinary evidence for immunologic tolerance as allogeneic skin grafts remain to this day the most difficult to block from rejection. Medawar's team then performed the famous experiment of injecting allogeneic blood cells from one inbred mouse strain into newborn mice of another.[15] The blood cells would have engrafted to establish lifelong chimerism like the twin cattle and would not have stimulated the TLR system, allowing Medawar to reveal profound tolerance to skin grafts that was extraordinarily specific for the foreign tissue antigens encountered neonatally. In their 1953 paper, Medawar crystallized the concept: "This phenomenon is the exact inverse of actively acquired immunity and we therefore propose to describe it as actively acquired tolerance."[15]

These concepts and experimental findings are now employed in clinics around the world, with greatest success in children with primary immune deficiency diseases where tolerance is acquired so completely to semiallogeneic bone marrow transplants that immunosuppressive drugs can be withdrawn several months after transplantation.

The Clonal Selection Theory

While Medawar's experiments put the phenomenon of actively acquired tolerance on firm foundations, it opened up an even more perplexing problem: how were both tolerance and immunity acquired at the cellular level? Talmage[16] and Burnet[17,18] independently arrived at the "one cell-one antibody" concept of clonal proliferation to explain acquisition of immunity. Burnet's PhD student at the time, Gus Nossal, soon obtained the first experimental evidence for that concept using single cell micromanipulation methods with the bacterial geneticist Lederberg.[19] Many experiments followed in the 1960s, 1970s, and beyond that affirmed the role of clonal proliferation in acquired immunity. Clonal proliferation itself made the experiments feasible, by increasing the frequency and the specificity of antigen-reactive cells so that they could be accurately enumerated, micromanipulated, and experimentally studied.

The reciprocal mechanism—clonal deletion to explain the acquisition of tolerance—was Burnet's elegant mirror image concept in *The Clonal Selection Theory*.[18] Lederberg[20] extended the concept by pointing out that the timing of antigen encounter, at an immature stage of lymphocyte development, could itself be the "self-marker" that triggered clonal deletion or maturation arrest to prevent the cells from making antibody: "The distinction between the function of an antigen as inhibitor (self-marker) or as inducer of antibody formation is … the time when the antigen is introduced into the potential antibody forming cell."

Talmage[7] and Claman[6] proposed a prescient alternative: that antigen alone would trigger exhaustive differentiation of specific lymphocytes without clonal expansion, depleting the pool of lymphocytes left to respond to a second challenge. In their view, antigen plus a second "nonspecific" signal from LPS, other adjuvants, or protein aggregation was necessary for clonal expansion of specific lymphocytes to prevent depletion of the pool of responders when some differentiated into effector cells.

Testing for the disappearance of self-reactive clones in tolerant animals was nevertheless like asking if a needle had been eliminated from a haystack. It was confounded for 30 years by the fundamental technical barrier that antigenbinding lymphocytes are rare and heterogeneous in the preimmune repertoire.

The Experimental and Conceptual Swing against Clonal Deletion in the 1960s and 1970s

Experiments were performed in the 1960s and 1970s to see if lymphocytes capable of binding self-antigens were absent from the circulating repertoire, either by direct antigenbinding measurements, by polyclonally activating cells into antibody secretion in culture, or by immunizing with foreign antigens that resemble self-antigens. Lymphocytes with self-antigen binding antibodies could be found,[21] leading many to question the idea of clonal deletion.

We know now that many B cells in the preimmune repertoire bear antibodies that are polyreactive, binding with low and variable affinity to many different antigens including self-antigens.[22,23,24,25,26,27,28] The same is true for most circulating

T cells, which are indeed biased to be weakly self-major histocompatibility complex (MHC) reactive. Many of these polyreactive receptors do not pose a risk of autoimmune disease unless made in extraordinary concentrations, and when expressed in transgenic mice some have an affinity for self that falls below that which invokes actively acquired tolerance, as described later. But the assays used at the time could not distinguish between lymphocytes bearing frequent but harmless polyreactive receptors and much rarer cells bearing potentially destructive receptors with higher affinity and specificity, which we know now are usually controlled by active tolerance mechanisms. In other cases, highaffinity receptors were isolated but the assays used confused genuine autoantibodies with antibodies that bound to misfolded, proteolytically cleaved, or posttranslationally modi-fied "altered self."[29]

Contemporaneous experiments in the 1970s supported clonal deletion or inactivation of immature lymphocytes, but the necessary experimental contrivances made it hard to shift the growing consensus that self-reactive lymphocytes were not deleted. Cooper neatly bypassed the problem of varying antigen affinity by showing that treatment of developing chicks or neonatal mice with antibodies to the constant region of immunoglobulin (Ig)M completely blocked B-cell development and antibody formation.[30] He noted: "Acting at an early stage of differentiation, anti-mu antibody might inactivate or eliminate…cells at the time that they begin to express IgM surface antibody. This concept is analogous to the elimination of 'forbidden clones' recognizing self-antigens."[30] Nossal[31] and Klinman[32] performed sophisticated tissue culture experiments that showed immature B cells exposed to haptenic antigens, but not their mature counterparts, were actively incapacitated from making antibody-secreting progeny, although they could not see the fate of the antigen-specific cells directly.

Set against these findings of clonal deletion or inactivation in immature lymphocytes, Claman,[6] Dresser,[33] Weigle,[34] and others showed that even mature antigenspecific lymphocytes were somehow incapacitated when chronically exposed to nonimmunogenic forms of antigenlike xenogeneic gammaglobulin, provided these persisted in the body, were deaggregated so that they did not provoke inflammatory responses, and were free of the bacterial ligand for TLR 4, LPS.

Miller's discovery of helper T cells in the 1960s provided an alternative way to reconcile the presence of self-antigen binding B cells with Burnet's concept of clonal deletion or inactivation. While B cells might not actively acquire tolerance, the repertoire of helper T cells could still be purged of self-reactive clones. This revised view was made poignantly by Miller when he received the inaugural Burnet Medal in 1971[35]: "Specific tolerance can be induced much more readily in T cells than in B cells and, in many cases, tolerance in B cells in vivo is just not demonstrable. It may in fact turn out that tolerance to self-components is a property

confined exclusively to the T cell population, tolerance in B cells being merely a laboratory artifice." Effectively, this allowed the goalposts of clonal deletion to be moved from B cells to T cells. But that solution soon came into doubt as well: the 1974 discovery of MHC restriction by Doherty and Zinkernagel[36] indicated that all circulating T cells were inherently self-reactive.

Tolerance by Cell-Cell Interaction Networks and Suppressor T Cells

The inherent self-MHC reactivity of T cells was probably the final trigger that drove many to discard clonal deletion altogether and conceive alternative mechanisms for actively acquired tolerance. By 1983 when Nossal reviewed the field of immunological tolerance,[37] he noted that most immunologists had discarded Burnet's concept of clonal deletion as well as Nossal's "clonal anergy." One camp pursued alternative ideas of Jerne, who hypothesized that tolerance was achieved by complex networks of consultation between lymphocytes through idiotype-anti-idiotype interactions.[38] It is fair to say that experimental studies have yet to establish the importance of idiotype networks for actively acquired tolerance.

An important alternative school of thought was fostered in the 1970s by Cohn's two-signal model.[39] Its inception hypothesized that antibody responses were only triggered when a newly introduced antigen was recognized simultaneously by two antigen-binding lymphocytes: the B cell and a helper T cell that delivered a second signal to the B cell. In this model, tolerance could still be actively acquired in the B cell by deletion, anergy, or some other mechanism that paralyzed antibody formation if it encountered antigen (signal 1) without receiving signal 2 from the helper T cell. Cohn's model extended to include T cells the two-signal concepts developed 10 years earlier by Dresser, Claman, and Talmage, that recognition of antigen alone promoted tolerance whereas immunity required a second signal coming directly from microbes through the mitogenic action of LPS and other microbial components.[6,7,8,40] This still left the question of how T cells might select between tolerating or rejecting an antigen. Based on transplantation tolerance experiments, Lafferty initiated the conceptual leap that T-cell proliferation and rejection of foreign tissue depended upon recognizing antigen and MHC alongside a second "costimulator" signal unrelated to antigen, on specialized antigenpresenting cells that lay as sentinels in each tissue of the body.[41] Steinman discovered dendritic cells as fulfilling this property in the 1970s.[42]

An intensely popular new idea was that self-reactive helper or cytotoxic T cells could be controlled by suppressor T cells that were actively induced by self-antigens or idiotypes, and were marked by CD8 and a distinctive MHC molecule, I-J.[43,44] Thousands of publications appeared in the 1970s and 1980s on suppressor T cells, including more than 300 referring to I-J. However, the experimental tools available at the time to test these ideas were not up to the task, and this period illustrates the potential for even the best minds to be engulfed in mass hallucination when our ideas are not grounded by falsifiable experiments. As molecular genetics revolutionized the field in the 1980s, "suppressor T cell" became a tainted term: Steinmetz and Hood sequenced the MHC and showed there was no

I-J molecule,[45,46] and antigen-specific suppressor T-cell clones were found to lack a T-cell receptor (TCR).[47] While molecular genetics swept I-J+ CD8+ suppressor T cells away in the 1980s, in 2001 these same powerful experimental methods gave back CD4+ Foxp3+ "regulatory T (T_{reg}) cells" and showed these were critical for tolerance, when Ramsdell and colleagues revealed that deficiency of the Foxp3 transcription factor unleashed lethal autoimmunity in the scurfy mutant mouse and in human immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome.[48,49,50]

Effectively, this turbulent history has laid out a conceptual landscape comprising three general ways to govern tolerance and immunity: 1) "individual choice" where tolerance is acquired by cell-autonomous processes of clonal deletion or inactivation; 2) "committee governance" where cell-cell interactions and additional signals are needed to launch immune reactions; and 3) "governance by police" where tolerance is imposed through the action of dedicated inhibitory cells. As detailed in the following, modern molecular and cellular methods have established important roles in actively acquired self-tolerance for each of these three fundamental concepts.

THYMIC TOLERANCE

T Cells are Positively Selected for Self-Reactivity

The finding that T cells were self-MHC restricted[51,52] was soon followed by transplantation studies demonstrating their self-MHC requirement was imprinted during immature T-cell development in the thymus[53,54,55]: a result that ran entirely contrary to the idea of clonal deletion. The transplantation experiments involved construction of bone marrow chimeras or thymus chimeric mice, where bone marrow of MHC type A × B gave rise to T cells within a mouse or a transplanted thymus of MHC type B or MHC type A. Although controversial, the

resulting T cells primarily recognized antigen when it was presented by cells that bore the same MHC type as the thymus in which they had differentiated.[53,54,55] These experiments showed T cells were somehow positively selected during their development to recognize self-MHC, triggering intense discussions about whether T cells employed two receptors—one for antigen and one for self-MHC—or if there was one receptor for "altered self." While we take "altered self" for granted now, it is important to bear in mind that these discoveries took place 7 years before biochemical experiments showed that antigen peptides bind MHC molecules[56] and 9 years before the peptidebinding groove of MHC was revealed crystallographically.[57]

The key event that opened up experiments on thymic tolerance was the identification and cloning of the TCR.[58,59,60,61,62,63,64] This led to key experiments that established that there was a single receptor for antigen and MHC,[65,66,67,68] and also resulted in the development of two key experimental approaches to analyze the cellular basis of T-cell self-tolerance: antibodies specific for TCR Vβ regions and the ability to generate TCR transgenic mice.[69,70,71,72,73] Both of these approaches allowed investigators to follow the fate of T cells with defined specificity during thymocyte development.

The series of experiments that conclusively demonstrated positive selection of self-MHC reactive T cells was the use of TCR transgenic mice. TCRs were cloned from T-cell lines of a defined specificity, and TCR transgenic mice were generated from these receptors. Here, the prediction would be that the rearranged transgenic TCRs would only mature in mice that had the same MHC type as the original donor T cell. Studies using a TCR from a female $H2^b$ CD8+ T cell, which was restricted to recognizing H-Y male-specific antigen associated with the H2-D^b MHC molecule, clearly demonstrated that CD8+ T cells bearing this TCR matured in female mice expressing $H2^b$ but not in mice with other MHC types.[74] These studies demonstrated that the transgenic TCR was positively selected and skewed the repertoire to the CD8 lineage during development, as the receptor was specific for the class I MHC molecule.

T Cells are Negatively Selected for Self-Reactivity

One reagent that turned out to be critical in defining T-cell clonal deletion as a mechanism of self-tolerance was the generation of TCR-specific antibodies. The majority of TCR antibodies were specific for Vβ regions. Kappler et al. evaluated the T-cell populations that react with a monoclonal antibody specific for Vβ17. They showed that Vβ17+ T cells react with the class II MHC molecule I-E and are not present in the peripheral lymphoid tissues of mice that express I-E. Importantly, in I-E+ mice, immature CD4+8+ thymocytes were present bearing Vβ17, but these were eliminated from the mature CD4+8- single-positive (SP) thymocyte subset, demonstrating that the self-MHC-specific T cells were deleted during development in the thymus.[70] Other studies showed that the infusion of tolerizing agents such as staphylococcal enterotoxin B (SEB) led to clonal deletion of specific cells in the thymus.[75] These experiments clearly supported the idea that self-reactive T cells are removed from the repertoire because they encountered their antigen when still immature in the thymus.

Similar studies were done using other Vβ-specific antibodies that correlated the absence of particular Vβ with the presence of self-antigens. One of the most popular models to evaluate tolerance in this way used different strains of mice that expressed minor histocompatibility antigens known as minor lymphocyte stimulatory (Mls) antigens. This group of molecules was defined by Festenstein in the early 1970s[76] and later shown to associate with endogenous retroviruses. Mice were characterized as expressing Mls-1^a, Mls-1^b, or Mls-1^c antigens, by the ability of T cells to generate a proliferative response against cells from different strains of mice. Early studies demonstrated that T cells expressing Vβ6 or Vβ8.1 were absent in mouse strains that express Mls-1^a.[77,78] Collectively, these studies demonstrated that clonal deletion of T cells occurred in the presence of a defined self-antigen.

Conceptually, it is surprising that antigen specificity could be associated with a particular Vβ segment independent of the Dβ, Jβ, and Vα segments of the TCR. Importantly, antigens such as Mls-1^a or SEB defined a unique class of antigens dubbed "superantigens" because they were able to stimulate a much higher proportion of T cells than conventional antigens and in a way that did not obey the rules of MHC restriction, although certain MHC types were shown to preferentially present superantigens.[79] Research has shown

that superantigens bind the sides of MHC II and TCR Vβ segments in an unconventional way that does not involve the conventional peptide-binding groove of MHC and complementarity determining regions of the TCR.

Although Vβ-specific monoclonal antibodies provided key evidence for clonal deletion, it was essential to demonstrate that this also occurred for self-reactive T cells recognizing conventional peptide antigens bound to self-MHC. The first study to demonstrate clonal

deletion of conventional selfreactive T cells employed a TCR transgenic mouse.[73] In this model, the TCR α and β transgenes were taken from a CD8+ T-cell clone that was specific for the H-Y male antigen and H-2D^{b}. When female transgenic mice were examined, many T cells with the H-Y specific TCR were positively selected to the CD8 lineage due to the class I H-2D^{b} restriction, as noted previously. In TCR transgenic male mice, however, no T cells developed with the characteristics of the original H-Y T-cell clone bearing high levels of CD8 and the H-Y TCR, although many unusual T cells developed bearing the H-Y TCR and low levels of CD8. In the thymus of male TCR-transgenic mice, there was a drastic reduction in the number of immature CD4+8+ double-positive (DP) thymocytes, implying that thymic deletion occurred at or before this stage. However, follow-up studies established that the loss of DP cells and accumulation of unusual H-Y T cells in male mice was due to premature expression of the TCR alpha chain at the CD4-8-double-negative stage, which caused lineage misdirection at this stage as opposed to clonal deletion.[80] When the H-Y TCR alpha chain transgene was controlled so that it was only activated at the DP stage, there was little decrease in the DP population in male mice and negative selection occurred at the CD8+CD4- SP stage of thymocyte development,[80] comparable to the findings with superantigens.[70] The H-Y studies were reinforced by parallel TCR transgenic experiments with other conventional TCR specificities, providing solid evidence that self-reactive T cells are eliminated from the T-cell repertoire during thymic development.[72,81]

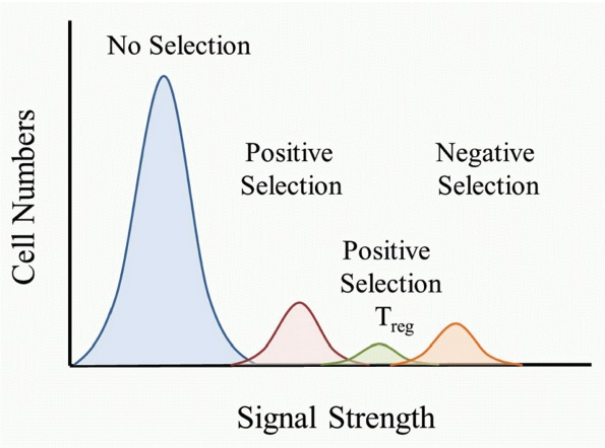

FIG 32.1. The Strength of T-Cell Receptor (TCR) Peptide/Major Histocompatibility Complex (MHC) Interactions Programs Tolerance Mechanisms. The random rearrangements of TCR gene segments lead to a wide range of specificities. Thymocyte fate will be determined by the strength of interaction between the TCR and peptide/MHC complexes that are found in the thymus. The majority of cells will not meet the threshold for positive selection. Stronger TCR interactions lead to the maturation of regulatory T cells or T-cell tolerance resulting in anergy or clonal deletion. The relative proportion of selected T cells is estimated.

Anergy as a Mechanism for Thymocyte Tolerance

Anergy is a term proposed by Nossal to describe lymphocytes that were present but rendered intrinsically unable to respond to stimulation with cognate antigen because of earlier exposure to the same antigen.[37] In T cells, evidence for anergy as an alternative mechanism of self-tolerance came from studies using parent into F1 bone marrow chimeras.[82] In these studies, Mls-1^{b} bone marrow was transplanted into irradiated Mls-1^{a} Mls-1^{b} hybrid recipients. In this case, where synthesis of the Mls-1^{a} self-superantigen was limited to radioresistant cells in the thymus, Vβ6+ T cells that would normally be deleted in Mls-1^{a} mice were able to mature and emigrate from the thymus but were unresponsive to Mls-1^{a}. This demonstrated that an unresponsive state to self-antigen could be induced in the thymus and suggested that anergy versus deletion were alternative fates for selfreactive thymocytes depending upon the amount of TCR stimulation or the cell type presenting the self-antigen.

T-Cell Affinity/Avidity Defines Thresholds for Positive and Negative Selection

The observation that T cells were positively selected for selfreactivity during thymic development and also negatively selected for self-reactivity drew into sharp focus the central paradox for actively acquired tolerance and immunity. How would a TCR be able to transmit signals for differentiation and survival (positive selection) versus death (negative selection)?

Lederberg's[20] and Nossal's[31] earlier concept of a developmental window for deletion could not be invoked here because the alternative fates were both acquired in immature T cells. Over the years, evidence accumulated to support an affinity/avidity model that suggested that T-cell fate was determined by the strength of the signal transmitted by the TCR (Fig. 32.1).[83,84,85] The absence of a detectable signal in thymocytes would result in a process called death by neglect. A T cell expressing a receptor that could not engage

self-peptide/MHC complexes (pMHC) would be destined to die because it would be a "useless" TCR to have in the repertoire. TCRs that bound only weakly to self-pMHC complexes in the thymus would send a signal sufficient for the T cell to survive and differentiate to either the CD4 or CD8 lineage. According to this model, a strong signal would be sent if the TCR bound strongly to self-pMHC complexes and would lead to the death of the T cell. It is important to keep in mind that as the thymocyte undergoes positive selection and matures, the TCR level increases, and therefore the T cell would have increased sensitivity to self-pMHC complexes as they progress through thymocyte selection.

Studies to evaluate the affinity/avidity models of thymocyte selection were again dependent upon the development of new technologies. In the 1980s, Smithies and Capecchi reported a way to generate knockout mice, where the expression of a defined gene was disrupted.[86,87] This led to a flurry of activity with several groups generating a variety of genedeficient mice. Amongst the early gene knockout mice were animals with a defect in MHC class I protein display due to the absence of the β2-microglobulin subunit of MHC I molecules or the absence of self-peptides available to bind and fold MHC I molecules because of disruption of the transporter associated with antigen processing.[88,89] These mice were the basis for a very unique set of experiments in fetal thymic organ cultures, where defined peptides could be added in culture to evaluate their consequences for thymic selection.[90,91] When a TCR transgenic model was combined with the class I mutant mice, this provided a novel way to evaluate the consequences of defined peptides on positive selection as well as negative selection.

A series of experiments were done with the OT-1 TCR transgenic mice, that are specific for the ovalbumin (OVA) peptide SIINFEKL and H-2D^{b}.[83] The OT-1 TCR transgene was bred onto the transporter associated with antigen processing-deficient background. Fetal thymic organ culture was done using OT-1 transporter associated with antigen processing-1-/- (Tap1-/-) thymic lobes. In this model, no mature OT-1 T cells were generated in the thymus due to the absence of class I expression. However, a series of variant peptides resembling the OVA SIINFEKL peptide could be added to the culture and these would restore class I expression, with the resulting MHC molecules homogeneously complexed with a defined peptide. In this model, the SIINFEKL peptide itself and related peptide sequences that were capable of activating mature OT-1 T cells (agonist peptides) triggered negative selection of immature thymocytes bearing the OT-1 TCR. By contrast, variant peptides that were unable to activate mature OT-1 cells but instead antagonized activation by SIINFEKL itself (antagonist peptide ligands) promoted positive selection of immature thymocytes to accumulate in the cultures as CD8 SP T cells.

At the same time, other studies were done using P14 TCR transgenic mice that express a TCR specific for the LCMVglycoprotein (gp). These studies bred the TCR transgene on a β2-microglobulin-deficient background. Two groups demonstrated that different concentrations of the same agonist peptide promoted positive selection at low concentrations and negative selection at high concentrations.[84,85,92] Notably, in the P14 model, antagonist peptide ligands could not support positive selection of the P14 TCR.[93] Collectively, these studies provided key evidence that supported an affinity/avidity model for thymocyte selection.

The affinity of TCR binding to MHC molecules bearing the various peptides was subsequently measured by surface plasmon resonance. Using this approach for the OT-1 TCR, Alam et al. demonstrated that the difference between positive selection and negative selection was a threefold difference in affinity for pMHC.[94] Interestingly, the agonist peptides (A4Y, L6F) that could support positive selection of the P14 TCR had a similar affinity to the antagonist peptides that supported positive selection of the OT-1 TCR.[95] Further studies have also shown that in some models, agonist ligands lead to the differentiation of T cells bearing a CD8αα homodimer and this population of cells have innate-like properties.[93,96,97] Collectively, these studies support the concept that a certain affinity/avidity is required to promote positive selection and that increasing the affinity/avidity leads to the induction of negative selection (see Fig. 32.1).

What are the biochemical differences in the signal transmitted by the TCR that leads to survival and differentiation of weak pMHC binding thymocytes but death of strongly self-reactive thymocytes? Several studies demonstrated a critical role for the ERK MAP-kinase signaling pathway for positive selection.[98,99] Further in-depth studies to follow TCR signaling events used the OT-1 and P14 models to evaluate differences in peptide-specific interactions that were known to result in positive or negative selection. Interestingly, positive

selection was shown to occur as a result of sustained low level ERK signaling, while negative selection resulted a stronger, yet transient peak of ERK activation.[100,101,102,103] Accordingly, studies have shown that there was impaired thymocyte positive selection in the absence of ERK1 and ERK2.[104,105] Evidence also suggests that calcineurin has an impact on Erk activation and positive selection,[106] but not negative selection. An essential upstream activator of ERK, RasGRP1, was also found to be essential for positive but not negative selection of thymocytes.[107] Further studies have identified downstream ERK-activated transcription factors that are also important for positive selection.[108,109]

Recent studies also provided evidence that the outcome of thymocyte positive or negative selection is correlated with a different TCR conformation.[110] Further in-depth analyses have observed that signals that induce negative selection lead to the membrane compartmentalization of molecules involved in signal transduction such as ZAP-70, LAT, and ERK1/2. Importantly, similar changes in localization of signaling components were not observed under positively selecting conditions.[111] Therefore, the different thymocyte fates of positive or negative selection involve detectable molecular changes on the cell surface and in intracellular compartments.

Regulatory T Cells are Selected by High Affinity/Avidity Interactions

T_{reg} cells were first identified as a critical population that prevented autoimmunity and arose during thymic development. Early studies demonstrated neonatal thymectomy of some

inbred rodent strains led to the development of autoimmunity targeting the gonads and thyroid gland.[112,113,114] Further studies using various markers identified a subset of cells, first in the rat and then in the mouse, that had the potential to block the induction of autoimmunity. Mason's team demonstrated that adoptive transfer of naïve T cells to immunodeficient rats resulted in autoimmune diabetes and inflammatory bowel disease that was blocked if a subset of CD4 T cells with markers of activated or memory T cells was cotransferred.[115,116] These findings were extended to mice by Powrie[117] and by Sakaguchi, whose group demonstrated that the transfer of CD4+CD25- cells into 6-week-old BALB/c nude mice resulted in the development of autoimmune oophoritis, gastritis, and thyroiditis that was suppressed by the cotransfer of CD4+ CD25+ cells.[118] While these experiments suggested the existence of a new type of suppressor T cell (dubbed a T_{reg} cell to avoid confusion with the CD8+ suppressor T cells of the 1980s), their interpretation was uncertain: suppression by cells with markers of activated or memory T cells could simply reflect better control of microbial flora in the immunodeficient recipients, so that there was less stimulus for inflammatory bowel disease and autoimmunity.

The critical turning point establishing the existence of T_{reg}s came from the identification of the transcription factor, FoxP3, as a specific marker of mouse T_{reg}s that was required for CD4+25+ T cell differentiation in the thymus. Foxp3 was first identified by genetic mapping of an autoimmune mutation in the scurfy mouse strain.[48] That led to the identification of *FOXP3* mutations as the cause of a human inflammatory disorder known as IPEX syndrome.[49,50,119] Foxp3 was necessary and sufficient for T_{reg} differentiation, and thymic-derived Foxp3+ "natural T_{regs}" were shown to be critical to suppress autoimmunity, allergy, and inflammation of mucosal and cutaneous barriers.[120,121,122,123,124] However, it should be noted that the marker FoxP3 is not exclusively linked to T_{reg} cells in humans.

Caton's group was the first to show that natural T_{reg} differentiation was an alternative fate to clonal deletion for CD4 thymocytes with a strongly self-reactive TCR, further complicating the question of how alternative cell fates are determined in developing thymocytes.[125] The strength of TCR signal has again been invoked as an instructive in-fluence, supported by additional studies that indicate that T_{reg}s bear TCRs that bind to self-pMHC more strongly than TCRs that promote positive selection of helper T cells 126-129 (see Fig. 32.1). However, other studies argue that Foxp3 expression may be stochastic and protect strongly self-reactive CD4 cells from deletion.[130]

MOLECULAR PATHWAYS INVOLVED IN THYMIC CLONAL DELETION

The precise molecular pathway that leads to the death of self-reactive T cells remains unknown. Evaluating the role of a given signaling molecule in positive and negative selection often points to a role for that molecule in both events, supporting the idea that TCR signals are critical for determining T-cell fate.[131,132,133,134,135] However, in general, the role of molecules involved in regulating negative selection has met with controversy, although interesting candidates are beginning to emerge. Some of the uncertainty is likely due to the models that were used to study negative selection. In the early literature, mice were often treated with anti-CD3 antibodies to induce death of T cells. However, because the majority of T cells are activated, this leads to a stress-induced death that is dependent upon

glucocorticoids,[136] and therefore is not a good model to study negative selection.

Controversial Role for Apoptotic Pathways in Negative Selection

Two major mechanisms that regulate apoptotic cell death are known as the intrinsic and extrinsic pathways and are regulated by Bcl2 family members and tumor necrosis factor receptor (TNFR) family members, respectively. Because many members of the TNFR family possess a “death domain” that can interact with other death domain-containing proteins, this family became the focus for studies investigating the mechanisms of death during thymocyte negative selection. TNFR, Fas, CD30, Trail, DR3, as well as the downstream effector molecules have all been investigated, and the results remain controversial.[137,138,139,140,141,142,143,144,145,146,147,148,149,150,151]

The Role for ERK and Nur77 in Negative Selection

Several approaches have been taken to address the question of whether the ERK pathway is essential for negative selection. Experiments using dominant negative forms of RAS or MEK, which limit Erk activation, supported the idea that ERK was important for positive selection but not negative selection.[98] Other studies using pharmacologic inhibitors for the Erk pathway demonstrated that Erk played a role in both positive and negative selection,[101,152,153] whereas yet other studies supported the idea that Erk did not influence negative selection.[154] It is possible that the discrepancies in these studies are related to the strength of the signal required for selection of the TCR in the different models that were being studied. For example, because positive selection requires sustained signaling that results from weak TCR interactions, these signals would be more likely to be perturbed by transgenic forms of dominant negative signaling molecules, because this system would still have the endogenous molecules that could provide some signals.

Studies using mice that were deficient in both ERK1 and ERK2 demonstrated that thymic clonal deletion could still occur by following deletion of the OT-1 TCR transgenic T cells using various models.[155] However, there are many ERK family members and it is therefore possible that only certain members of the ERK family are critical or that the various ERK family members can compensate for each other. Studies using dominant negative or constitutively activated MEK5, which activates the ERK5 pathway, was able to modulate clonal deletion of thymocytes, but had no role in positive selection.[156] Importantly, ERK5 activity correlated with the induction of *Nur77*, which has been previously implicated in thymocyte negative selection.[157,158,159] In several other models, *Nur77* is also upregulated during thymocyte

negative selection, thereby supporting the importance of this molecule.[160] Therefore, studies support a role for ERK5 and *Nur77* in thymocyte clonal deletion.

Bim Links Negative Selection and Autoimmunity

If a particular molecule is essential for the death of selfreactive T cells, one prediction would be that the absence of this molecule may lead to spontaneous autoimmune disease. This prediction would not hold true if that particular molecule was also essential for T-cell differentiation or activation. One such candidate that was considered is the BH3-only-containing protein Bim. Bim is a proapoptotic molecule that gives rise to three alternatively spliced forms: Bim_S, Bim_L, and Bim_{EL}. In general, Bim is thought to promote apoptosis by binding and sequestering survival molecules Bcl-2, Bcl-x_L, and other members of that family. Initial studies reported that Bim-deficient mice spontaneously develop autoimmunity, and that Bim was critical for thymocyte negative selection.[161,162]

Recent studies, however, question the role of Bim in negative selection and autoimmunity. Baldwin's group evaluated negative selection using the HYcd4 TCR transgenic model.[163] They demonstrated that Bim played a role in apoptosis of thymocytes but not in self-antigen-specific negative selection. Other studies used an elegant TCR transgenic model to accurately follow the fate of Bim-deficient thymocytes. Kovalovsky et al. demonstrated that self-reactive Bim-/-T cells undergoing negative selection were arrested at a $CD4^{lo}CD8^{lo}$ immature stage of development.[160] Death of these cells was dependent on an alternative pathway that required the thymic microenvironment. By contrast, evidence from unpublished work has shown that Bim deficiency almost completely abolished negative selection in 3A9 TCR transgenic mice crossed with hen egg lysozyme transgenic mice.[163a] In these animals, the negatively selecting antigen was expressed selectively in Aire+ thymic medullary epithelial cells by the insulin promoter, and in their Bim+/+ counterparts negative selection was induced at the early CD4 SP stage. Thus, the relative dependence on Bim may vary depending on the stage of thymocyte development when self-antigen is encountered, the amount of self-antigen presented in the thymus, or the nature of the antigen-presenting cells that induce negative selection. Further studies using Bim-/- mice have shown that these mice have impaired induction of experimental autoimmune encephalomyelitis. Unexpectedly, Ludwinski

et al. also showed that Bim-deficient CD4+ T cells have a strikingly reduced ability to produce cytokines, particularly interleukin (IL)-6 and interferon-γ.[164]. This adds further complexity as to the mechanism of spontaneous autoimmune disease in Bim-deficient mice.[161]

Many insights have been uncovered by evaluating both animal models for autoimmunity and clinical studies. Several groups have shown that the nonobese diabetic (NOD) mouse strain that spontaneously develop diabetes has impaired negative selection as one of several factors that may contribute to the onset of disease.[165,166] Interestingly, recent studies have evaluated the genes that are expressed in stimulated NOD thymocytes versus wild-type thymocytes. Interestingly, Bim and Nur77 are among the genes that are not upregulated in the NOD background, lending potential support for these molecules in tolerance induction.[167,168,169]

MINK Links Jnk and Bim in Negative Selection

There is compelling evidence published by Cantor's group that a molecule known as MINK plays a role in negative selection but not positive selection.[170] They showed that MINK expression is increased 20-fold in DP thymocytes compared to the double negative subset. Expression of MINK was also shown to be very low in SP thymocytes as well as peripheral T cells. McCarty et al. studied the role of MINK in thymocyte selection by generating chimeric mice with bone marrow that was infected with a lentivirus that expressed green fluorescent protein as well as a small hairpin ribonucleic acid that reduced MINK expression. Three models of thymocyte negative selection supported a role for MINK. These include the superantigen Mtv-9-induced Vβ5 deletion in the C57Bl/6 and BALB/c backgrounds, the male-specific H-Y TCR transgenic model, as well as peptide-induced deletion using OT-II transgenic mice. Interestingly, they provided evidence that MINK acts via c-jun N-terminal kinase activation and the upregulation of the proapoptotic molecules Bim and BimEL.

Several groups have also evaluated the role of the c-jun N-terminal kinase/SAPK or p38 MAPK pathways in negative selection. Using knockout animals or pharmacologic inhibitors, evidence suggests that both of these pathways play a role in the death of thymocytes.[154,171,172] However, further experiments are necessary to solidify a role for Jnk in thymocyte deletion. Importantly, Jnk activation has been linked with Bim expression in neuronal cell types, further strengthening the possibility that Jnk may be involved in thymocyte deletion.[173,174]

EXPRESSION OF SELF-ANTIGENS IN THE THYMUS

Negative selection in the thymus provides a key mechanism to eliminate self-reactive cells early in development. However, this mechanism was thought to be incapable of establishing self-tolerance to the numerous self-antigens that are expressed only in specific organs, such as insulin or elastase in the endocrine and exocrine pancreas, respectively. Pioneering work by Hanahan identified cells in the thymic medulla that he dubbed “peripheral antigen-expressing cells” that were able to express organ-specific genes like insulin and elastase, and induced T-cell tolerance to a transgenic self-antigen controlled by the insulin promoter.[175,176] Several years later, work by Derbinski et al. showed that medullary thymic epithelial cells were able to express a wide array of self-antigens.[177,178]

At the same time as Hanahan's definition of peripheral antigen-expressing cells, two human molecular genetics teams discovered that mutations in a novel gene, dubbed autoimmune regulator (*AIRE*), were responsible for a devastating recessive syndrome known as autoimmune polyendocrinopathy-candidiasis ectodermal dystrophy

(APECED) or autoimmune polyendocrinopathy syndrometype 1 (APS-1).[179,180] APS-1 is remarkable because of the progressive development of multiple organ-specific autoimmune diseases, most frequently autoimmune adrenocortical failure and hypoparathyroidism, but also autoimmune thyroid disease, diabetes, vitiligo, alopecia, ovarian failure, hypogonadism, liver disease, pernicious anemia, and exocrine pancreatic insuffi-ciency.[181] Almost all patients develop mucocutaneous *Candida albicans* infections because they produce neutralizing autoantibodies to IL-17, a cytokine that is essential for immune control of *Candida*.[182,183] While this experiment of nature established that *AIRE* underpinned a critical mechanism for self-tolerance to a wide range of discrete organs, the *AIRE* gene and protein provided few clues to its mechanism of action: it contained motifs that suggested a role in transcription regulation,[179,180] it was mostly expressed in the thymus and not in the organs that were affected by autoimmunity,[180] and in the thymus the protein was primarily expressed in medullary thymic epithelial cells where it was concentrated in nuclear dots.[184]

Aire-deficient mice were generated and were also found to develop spontaneous organ-specific autoimmune diseases, albeit not an identical spectrum to humans.[185,186] No overt abnormalities in T-cell development or T-cell subsets were apparent in *Aire*-knockout mice. Consistent with the predominant Aire expression in medullary thymic epithelial cells,

transplantation studies established that spontaneous autoimmunity resulted even when AIRE was only absent from thymic epithelium and not when it was absent from lymphoid cells.[186] The key turning point connecting Hanahan's peripheral antigen-expressing cells with the human autoimmune disease came when *Aire*-deficient mice were analyzed for expression of organ-specific genes in the thymus[186] and for thymic negative selection of organ-specific T cells,[187] which revealed these processes were selectively and severely disrupted. AIRE has been shown to be an unusual transcription factor that promotes ectopic expression of a variety of organ-specific genes in thymic medullary epithelial cells by relieving the tendency for ribonucleic acid polymerase to stall shortly after the transcription start site.[188]

These studies supported the idea that human APS-1 results from failure of thymic negative selection to organ-specific genes and the development of a T-cell repertoire that is poised to develop organ-specific autoimmunity. To further test this hypothesis, Anderson's group identified an autoantigen that was specifically expressed in the retina of the eye, interphotoreceptor retinoid-binding protein (IRBP), as a target of autoantibodies in AIRE-deficient mice that had developed autoimmune uveitis.[189] By crossing *Aire*-/- mice with IRBP gene knockout mice, they showed that IRBP expression in the eye was essential for spontaneous development of autoimmune eye disease in *Aire*-/- mice but not for other organ-specific autoimmune diseases in the same animals. IRBP messenger ribonucleic acid (mRNA) expression in the thymus was abolished in AIRE-deficient mice, and selective deficiency of IRBP in thymic stromal cells was sufficient to recapitulate the eye specific autoimmunity that occurs in AIRE-deficient mice.[189] With a similar rationale, Trucco's group generated mice where insulin was deleted from thymic epithelial cells while maintaining expression in the pancreas.[190] Importantly, diabetes spontaneously developed in 3-week-old animals. Anderson's group has gone on to identify other genes that are regulated by AIRE, that are also targets of autoimmunity in mice and humans.[191]

An important question concerns the possibility that organ-specific autoimmunity caused by AIRE deficiency might reflect failure to induce organ-specific T_{reg} cells, as opposed to the failure to delete organ-specific helper and cytotoxic T cells. Anderson et al.[192] addressed this question definitively by performing elegant double-thymus transplant experiments in *nude* mice, which lack normal thymic epithelium. When the mice were engrafted with one *Aire*-deficient thymus and one *Aire*-sufficient thymus, half of the circulating T-cell repertoire would have been selected in the thymus that lacked expression of organ-specific genes, while the other half was formed in the thymus that expressed organ-specific genes. If a deficiency of organ-specific T_{regs} was responsible for the organ-specific autoimmunity in *Aire*-deficient mice, this would have been complemented by the production of organ-specific T_{reg}s from the wild-type thymus in the doubly transplanted mice. By contrast, the wild-type thymus would have no impact if autoimmunity resulted from self-reactive effector T cells that escaped deletion in the *Aire*-deficient thymus. Consistent with the latter, autoimmunity developed with the same organ-specific pattern in the doubly transplanted mice. The same conclusion was reached when equal mixtures of T cells from *Aire*-deficient and wild-type mice were cotransferred into immunodeficient Rag1-knockout mice.[192] Further evidence that AIRE mediates self-tolerance through mechanisms that do not involve T_{reg} formation comes from comparing Foxp3-deficient mice with normal AIRE or lacking AIRE, because the latter have markedly accelerated autoimmunity.[193] Likewise, analysis of mice with a T-cell repertoire constrained to junctional diversity between a single TCR Vα and Jα found that the great majority of T_{reg}-specific TCRs were selected independently of AIRE.[194]

Collectively, these studies demonstrate that thymic clonal deletion is an important mechanism for actively acquired self-tolerance in man and mouse. This conclusion is reinforced by the finding that human susceptibility to type 1 diabetes correlates with genetic variants in the insulin promoter that selectively diminish insulin gene expression in the thymus.[195,196] Similarly, a genetic variant in the promoter of the *CHRNA* gene that diminishes its transcription in the thymus contributes to susceptibility to forming autoantibodies against the acetylcholine receptor chain encoded by this gene and development of myasthenia gravis.[197]

PERIPHERAL T-CELL TOLERANCE

Powerful mechanisms exist to regulate mature T-cell responses against self-antigens after they have emigrated from the thymus: a process known as peripheral tolerance. One line of evidence for this conclusion comes from an experiment of nature: most organs and antigens remain unaffected by autoimmunity in *AIRE*-deficient humans and mice despite the failure of thymic tolerance to antigens in almost every tissue. Experimental evidence supporting this conclusion traces back to the demonstration that adult

mice could be rendered tolerant to adjuvant-free, deaggregated gammaglobulin from other species,[6,33,34] and this tolerant state would persist for months provided the animals had

been thymectomized to prevent generation of fresh T cells.[198] However, unlike in the thymus, where the engagement of strong TCR-pMHC interactions leads to clonal deletion or anergy, strong pMHC signals in mature T cells normally leads to the development of an effective immune response. Therefore, with mature T cells we are left with the puzzle of how a strong TCR interaction results in tolerance versus activation. Solving this puzzle holds the most practical potential for overcoming the major clinical problems of transplant rejection and tumor immunology and for restoring tolerance in people with autoimmunity or allergy.

As summarized in the section "History" at the start of this chapter, early work began to lay out two-signal concepts for tolerance or immunity by demonstrating that bacterial LPS, adjuvants, or protein aggregation were required together with antigen for inducing immunity and not tolerance in animal models, although the adjuvant effect was not viewed as a specific sensing pathway.[6,40,199] In the early 1970s, Gery and Waksman discovered that LPS-stimulated macrophages produced a soluble activity that augmented T-cell proliferation, dubbed "lymphocyte activating factor."[200] "Monokines" with other names such as leukocyte pyrogen[201] and mitogenic protein[202] were subsequently observed to have similar T-cell-augmenting activity and shown to represent the same small protein, renamed IL-1.[203] Independently, experiments inducing tolerance to allografts led Lafferty to conclude that T cells need to receive not only an antigen stimulus but also a specific "costimulus" from a dedicated antigen-presenting leukocyte for induction of immunity and not tolerance.[41,204] It nevertheless became apparent that a cell-bound T-cell costimulator that was not IL-1 also existed,[205,206] leading to the discovery of the T-cell costimulatory receptor, CD28, and its membrane bound ligands CD80/B7-1 and CD86/B7-2.[12,207,208,209,210]

The study of T-cell costimulation by IL-1 languished after the discovery of CD28 and its ligands, and only recently has IL-1 been demonstrated to serve as a powerful additional signal that is necessary—in conjunction with CD80 and CD86—for survival and accumulation of large numbers of daughter cells when CD4 T cells are triggered into division by antigen in vivo.[211] IL-12 and interferon alpha and beta, made by antigen-presenting cells in response to bacteria and viruses, similarly provide critical signals for CD8 cells that have received TCR and CD28 stimulation, promoting the survival and accumulation of progeny over successive cell divisions and their acquisition of cytolytic effector functions including interferon gamma and granzyme B expression.[212,213,214,215,216,217,218,219,220] Interferon alpha and beta have also been shown to promote survival of activated CD4 cells.[221]

Much of the early work on T-cell costimulators focused on macrophages as a principal source,[206] but parallel studies by Steinman led to the recognition that another myeloid cell, the dendritic cell (DC), was the key antigen-presenting cell required to induce CD4 and CD8 immunity.[222,223] Furthermore, if the DC had not been stimulated by adjuvants to "mature," then antigens encountered on the "immature" DC induced T-cell tolerance, while antigens encountered on mature DCs induced immunity.[224,225,226] DC maturation was shown to be triggered by pathogen-derived signals, such as TLR signals, and that maturation included the upregulation of a variety of key costimulatory molecules on the DC, including CD80/B7-1 and CD86/B7-2 ligands for CD28,[12,207,208,209,210] as well as the production of IL-1, IL-12, or other cytokines. The theory governing peripheral T-cell tolerance versus immunity in its simplest form often reports signal 1 as the TCR-derived antigen-specific signal, and signal 2 that stems from interactions with mature DCs has been typically thought of as CD28/B7 interactions. However, it is clear that multiple receptor ligand interactions as well as cytokine signals are critical to promote T cell-mediated immunity.

In this section, we will introduce the basic mechanisms of peripheral T-cell tolerance, which include clonal deletion and anergy. Tolerance is also maintained by two other mechanisms. One is known as T-cell ignorance, which is the inability of the T cells to detect sequestered self-antigens. In addition, potentially autoreactive cell function is held in check by T_{reg} cells that arise either in the thymus or may also be induced from the mature CD4 T cell population. It is also clear that CD8 T_{reg} cells exist; however, much less work has been done with this population.

Evidence for T Cell Anergy

The first direct evidence for T-cell clonal anergy came from tissue culture experiments with human T-cell clones that recognized specific influenza virus peptides and MHC II molecules.[227] When these T cells were cultured for more than 3 hours (optimally 18 hours) with high concentrations of the relevant peptide in the absence of stimulatory antigen-presenting cells (an unfractionated mixture that included monocytes, DCs, and B cells), the T cells became profoundly refractory to restimulation of proliferation with antigen presented by stimulatory antigen-presenting cells yet retained a normal proliferative response to IL-2. This selective tolerance to antigen persisted despite culturing the anergic T cells for more than 168 hours in the presence of IL-2. Human T-cell clones express MHC II molecules, so that in the presence of high concentrations of peptide the T cells could present p/MHC II complexes

to each other[228] in such a way that a high proportion of surface CD3 molecules were initially modulated from the cell surface.[229] Whether the long-term unresponsive cells regained normal CD3 expression was not examined in this study.

The concept of T cell anergy resulting from antigen recognition in the absence of a specific costimulatory signal from specialized antigen-presenting cells was extended in experiments where cloned mouse T cells were cultured with the relevant peptide antigen presented on chemically fixed antigen-presenting cells[230,231] or on synthetic lipid bilayers containing only MHC II molecules.[232] Like the human T-cell clones, this also resulted in a persistent state of antigen tolerance that blocked the proliferation response of T cells when subsequently exposed to antigen on unfixed antigen-presenting cells, yet left untouched the proliferative response to IL-2.

This antigen-tolerant state, dubbed T cell clonal anergy by Schwartz and coworkers, was not accompanied by long-lasting downregulation of surface CD3 and TCR. The missing costimulatory signal from the antigen-presenting cells could not be replaced by exogenous IL-1 or IL-2,[232,233,234] but was later shown could be replaced by stimulatory antibodies against CD28.[235] The tolerance-inducing signal from antigen on fixed antigen-presenting cells could be mimicked by selectively elevating intracellular calcium and required calcium-activation of calcineurin and induction of protein synthesis,[233,234] leading to the concept that TCR-induced activation of calcium, calcineurin, and the transcription factor NFAT induced the expression of a lasting tolerance program in mature T cells if it was not accompanied by costimulatory signals.[205,236]

To extend these tissue culture experiments to a more physiologic milieu, an essential requirement for studying peripheral T-cell tolerance was the ability to follow a T cell with a defined TCR specificity in vivo. Early studies using monoclonal antibodies specific for Vβ regions allowed one to evaluate peripheral T-cell tolerance induction to superantigens such as Mls-1^a and others. Pioneering studies performed by Rammensee et al. provided evidence for the induction of T-cell anergy in vivo. Mls-1^a cells were given to $Mls\text{-}1_b$ mice and by following Vβ6+ Mls-1^a-specific T cells, it was shown that the remaining Vβ6+ T cells were unresponsive to the $Mls\text{-}1_a$ antigen.[237] A multitude of studies using superantigens or conventional antigens supported these findings that mature T cells could be present in a functionally tolerant state after encountering a tolerogenic antigen in vivo.[212,213,214,215,238,239,240,241,242,243,244,245,246,247,248,249,250,251,252,253,254,255,256,257,258,259,260,261,262,263,264,265,266,267,268] Examples of these studies are summarized in Table 32.1.

Tolerance by Anergy, Abortive Proliferation, and Clonal Deletion of Peripheral T Cells

Almost all the in vivo examples of peripherally induced T-cell clonal anergy cited previously are embedded in a more dynamic process that includes T-cell division, death, and differentiation. A large body of evidence, examples of which are summarized in Table 32.1, demonstrates that TCR stimulation of mature T cells without CD28 or microbial adjuvant costimulation in vivo does not immediately induce anergy but frequently induces several rounds of cell division. Tolerance nevertheless follows because the burst of T-cell proliferation is aborted by apoptosis, and because the daughter cells fail to differentiate into fully fledged helper or cytotoxic effector cells.

Peripheral Tolerance to Exogenous Superantigens

In a pivotal set of studies analyzing peripheral T-cell tolerance in vivo, mice were thymectomized to prevent new T-cell production and then infused with Mls-1^a+ spleen cells. Vβ6+ Mls-1^a reactive T cells proliferated in response to the antigen, but subsequently were eliminated from the T-cell repertoire presumably by exhaustive differentiation into short-lived effector cells[269] —a fate analogous to an original two-signal model for acquisition of tolerance articulated by Talmage nearly three decades earlier.[7]

These findings of abortive proliferation were reinforced and extended by analyses of tolerance in mice that had been injected with a bacterial superantigen, SEB.[242,243,244,245,246,247,248,249] SEB engages TCRs bearing the Vβ8 element. Twenty-four hours after SEB injection into BALB/c mice, flow cytometric analysis of deoxyribonucleic acid (DNA) content in Vβ8+ CD4 T cells revealed that 30% were in S, G2, or M phase of cell cycle.[245] The same fraction were induced into cell division in CD28-knockout mice, establishing that CD28 costimulation is unnecessary to initiate T-cell proliferation at least in the context of an unconventionally potent superantigen stimulus.[249] CD28 deficiency, nevertheless, had a large effect: it prevented normal levels of CD25 from being induced and caused a rapid drop in the fraction of dividing Vβ8+ CD4 T cells and in their accumulation 48 hours and 72 hours after SEB injection. This effect of CD28 in

promoting the survival of dividing daughter cells can at least partly be explained through induction of the prosurvival protein, Bcl-X_L.[270]

Despite evidence for CD28 costimulation during acquisition of tolerance to SEB, when carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled Vβ8+ CD4 cells were tracked, the fraction that had divided peaked on day 3 and remained steady on day 4, with most of the cells dividing only three to five times and many displaying the apoptosis marker, annexin V, after their fourth or fifth division.[248] Continuous labeling with BrdU from the time of SEB injection revealed that almost all of the Vβ8+ CD4 T cells that were induced to divide had disappeared by 14 days.[246] Approximately 45% of Vβ8+ CD4 T cells never entered division, despite almost all responding by CD69 induction, and it was these “nondividers” that persisted at later times in an anergic state characterized by diminished proliferation or IL-2 production to restimulation with SEB in vitro. It is conceivable that the “nondividers” to SEB either received a weaker TCR stimulus by chance, by the nature of the antigen-presenting cell they encountered, or because of their particular TCRβ junction sequence or TCRα chain sequence.

Deletion of most of the divided T cells between days 4 and 7 during the response to SEB appears to reflect combined actions of the intrinsic (*Bcl2*-regulated) and extrinsic (*Fas*- or *Tnfr1* -regulated) pathways for apoptosis. Thus, several studies found the decrease in Vβ8+ CD4 T cells was either slower or less marked in mice homozygous for the Fas^{lpr} mutation.[247,271,272] Hildeman et al.,[273] however, found deletion was unaffected in mice with defective Fas-ligand or with a triple deficiency of *Fas*, *Tnfr1*, and *Tnfr2* but was inhibited in *Bcl2*-transgenic mice or in mice lacking Bim, the proapoptotic inhibitor of Bcl-2. Strasser et al.[274] found that complete blockade of peripheral deletion occurred only when both pathways were blocked in $Fas^{lpr/lpr}$ *Bcl2*-transgenic mice, resulting in a dramatic increase in Vβ8+ CD4 T cells 7 days after SEB treatment.

Peripheral Tolerance to Exogenous Peptide Antigens in T-Cell Receptor-Transgenic Mice

Abortive T-cell proliferation and anergy in response to conventional protein antigens has been demonstrated by tracking CD8 or CD4 cells with defined TCR specificities in TCR-transgenic mice (see Table 32.1). One of the first of

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these studies was by Kyburz et al.,[254] who compared the fate of TCR-transgenic CD8 cells recognizing a dominant peptide from LCMV presented by the MHC class I molecule, $H2D^b$, after injecting thymectomized TCR-transgenic mice with LCMV peptide in incomplete Freund's adjuvant (which provides no microbial costimulus but creates a reservoir of peptide). The specific CD8 T cells became large, increased in number, and developed cytotoxic T-lymphocyte (CTL) activity transiently, peaking on day 2 when many appeared apoptotic, with a rapid loss of blast cells and ex vivo CTL activity by day 3 and a drop in overall numbers below starting levels on day 5 and decreasing further on days 10 and 20. This abortive activation was independent of CD28, although CD28 deficiency made the response even more transient.[256] The cells present after day 2 appeared functionally tolerant, as they could not be induced to proliferate or form CTLs in vitro from this time onwards, even when their subsequent deletion was abolished by transgenic expression of either Bcl-2 or Bcl-X_L.[255] Deletion of the activated T cells in this model thus resulted from activation of the intrinsic pathway of apoptosis, implying that the peptide-activated T-cell blasts received insufficient external signals for cell growth and metabolism, whereas the extrinsic apoptosis pathways triggered by Fas or TNFR1 had no measurable role.[257,275]

TABLE 32.1 Examples of Experimental Studies Demonstrating Abortive Proliferation, With or Without Anergy, Induced in Mature T Cells by Tolerogenic Antigens in Vivo

Tolerizing Antigen	TCR Population Followed in Vivo	Evidence of Abortive Proliferation	Evidence of Functionally Tolerant Cells	Pathways Involved	Citation
Exogenous Mls-a spleen cells	Vb6+ CD4 cells in thymectomized mice	Increased frequency day 4, 30% of starting	Poor thymidine incorporation		269

		frequency by day 22			
Exogenous SEB	Vb8+ CD4 cells in Balb/c mice	Increased frequency day 4, 75% of starting frequency by day 7	Poor thymidine incorporation and IL-2 on day 7		242,243
Exogenous SEB	Vb8+ CD4 cells in Balb/c or C57BL/6 mice	Increased DNA content day 1, increased frequency day 4, CFSE dilution, BrDU incorporation, frequency below starting by day 7, many CFSE low cells divided four to five times are Annexin V+ day, many subdiploid DNA content day 7	Poor thymidine incorporation and IL-2 protein and mRNA for IL-2, IL-3, IFNg starting day 3	Deletion partly inhibited by Fas-lpr	244,245,246,247,248
Exogenous SEB	Vb8+ CD4 cells in Balb/c mice	Increased DNA content: 30% in cycle day 1 regardless of CD28-/-, sustained day 2 and increased frequency day 4 in CD28+/+ but aborted by day 2 in CD28-/- and frequencies dropped, decline in frequency of CD28-/- on day 3 and 4	Poor thymidine incorporation	CD28-/- diminished survival and sustained division	249
Exogenous SEB	Vb8+ CD4 cells in C3H/HeJ mice	Decreased numbers in wild-type controls on day 7		Deletion fully inhibited in Fas-lpr + Bcl2-transgenic mice resulting in 300% increased numbers day 7. Partial effect of single mutants.	274
Exogenous SEB	Vb8+ CD4 cells in MRL mice	Decline in frequency days 4 to 6		Deletion partly inhibited by Fas-lpr	272

Exogenous SEB	Vb8+ CD4 cells in MRL mice	Decline in frequency days 7 to 14		Deletion partly inhibited by Fas-lpr	271
Exogenous SEB	Vb8+ CD4 cells in C57BL/6 mice	Decline in frequency days 7 to 15		Deletion blocked in Bim-/- and Bcl2-transgenic mice, but not in B6-gld or B6-lpr/lpr Tnfr1-/- Tnfr2-/- mice	273
Exogenous SEB	Vb8+ CD4 cells in C57BL/10 or 129/Sv mice	Increased death of cells isolated 2 days after SEB and placed in culture		In vitro apoptosis suppressed by IFN alpha or beta	221
Exogenous SEA	Vb3+ CD4 cells in Balb/c mice	Increased frequency relative to starting on day 4, decline in frequency relative to day 4 on days 5 to 7		Deletion prevented by LPS independently of CD80-86 blockade but partly reversed by TNFa blockade	280
Exogenous cytochrome c peptide in PBS (I-Ek)	2B4 TCR-Tg CD4 cells in TCR-transgenic mice	Increased numbers 200% to 300% of starting in MRL-lpr/lpr day 7; decreased numbers 30% of starting in MRL+/+ controls day 7		Deletion blocked by Fas-lpr mutation	137
Exogenous OVA peptide in PBS IV (I-Ed)	DO11.10 TCR-Tg CD4 cells transferred to Balb/c	DNA content, BrdU incorporation, and increased numbers days 2 to 3, numbers dropped by day 5 and below untreated controls by day 17	Poor proliferation in vitro or after rechallenge in vivo with peptide/CFA or peptide/IFA; failure to form follicular T cells in vivo, lower surface TCR, diminished IL-2 mRNA, and protein	Complete Freund adjuvant sustains numbers day 5, requiring CD28: reversed by CD80 and CD86 blockade and enhanced by CTLA4 blockade	250,251,252
Exogenous OVA in PBS SC (I-Ed)	DO11.10 TCR-Tg CD4 cells transferred to Balb/c	Increased numbers peaking day 3, declining days 5 to 12, LPS enhanced numbers at all times and		CD28-/- diminished IL-2 production and the increase in numbers day 3, and abolished the effect of LPS. Accumulation of	281

		increased IL2 production per cell 8 to 12 hours after OVA		T cells not decreased by IL-2 deficiency but enhanced.	
Exogenous OVA in IFA SC	DO11.10 TCR-Tg CD4 cells transferred to Balb/c	CFSE dilution on day 3, number of cells day 6		CD80 and CD86 blockade diminished induction of CD25, fraction dividing, and number of divisions, but had little effect on IL-2 production as a function of cell division. BclXL transgene prevented the diminution of cell numbers by CTLA4-Ig on day 6.	282,283
Exogenous OVA or cytochrome c in PBS IV (I-Ad, I-Ek)	OT-II or 5CC7 TCR-Tg CD4 cells transferred to B6 or B10.A	CFSE dilution on day 7 but low accumulation of divided cells		Concurrent IL-1a or IL-1b increased accumulation of divided cells massively, 10- to 50-fold more than LPS alone. Blocked cell autonomously by IL1R deficiency in half the OT-II cells.	211
Endogenous or exogenous lysozyme presented by anergic or activated B cells or via other APCs (I-Ak)	3A9 TCR-Tg CD4 cells transferred to B10. Br mice	Induction of CD69 and in vivo B cell killing via Fas, CFSE dilution, peak numbers of divided cells day 3 declined by day 5		Activation and CD86 induction on B cells did not affect proliferation or loss of divided cells on day 5, whereas lysozyme in CFA promoted large accumulation of divided cells	284
Exogenous lysozyme peptide coupled to anti-DEC205 antibody (I-Ak)	3A9 TCR-Tg CD4 cells transferred to B10. Br mice	Increased numbers of CFSE diluted cells on day 3, decline in numbers of divided CFSE-low cells by days 7 and 20		Agonistic antibody to CD40 prevents deletion of CFSE low cells on day 7	226
Exogenous LCMV peptide in IFA (Db)	P14 TCR-Tg CD8 cells in thymectomized TCR-transgenic mice	Transient cell enlargement, increased numbers, ex vivo CTL activity days	Loss of in vitro proliferation and CTL activity from day 2, loss of CD69 induction	Deletion blocked by Bcl2 and BclX transgenes but not by Fas&TNFR1 double	254,255,256,257

		1 and 2 with peptide, most with subdiploid DNA content day 2 after peptide, decrease frequency relative to starting by day 5 completely blocked by Bcl2 or BclXl transgenes. Slower but sustained and greater increase after LCMV infection.	after in vivo peptide rechallenge, inability to control subsequent challenge with LCMV	deficiency. Persisting cells anergic. Initial proliferation unaffected by CD28-/- but unable to sustain ex vivo CTL activity unless peptide administered every 12 hours.	
Exogenous OVA peptide in PBS IV (Kb)	OT-1 TCR-Tg CD8 cells transferred to C57BL/6	CFSE dilution on day 2 or 3 but little accumulation of divided cells with lower peptide dose and no CTL activity unless ive IL-12 or LPS concurrently.	Little increase in numbers and no CTL activity on rechallenge with peptide+LPS day 40, unless given IL-12 initially	IL-12 as “signal 3” for survival of daughter cells and acquisition of effector function. Sustained exposure to antigen, b7 and IL-12 needed for 1 to 2 days. IFN alpha had similar activity in vitro.	212,213,214,215
Exogenous heart graft expressing ovalbumin (Kb and I-Ab)	OT-1 TCR-Tg CD8 cells and OT-II TCR-Tg CD4 cells transferred to C57BL/6	In absence of helper cells, OT-1 CFSE dilution on day 2 or 5 not accompanied by increase in numbers or acquisition of ex vivo or in vivo CTL activity due to low granzyme B and IFN gamma		Helper cells increased OT-1 CD25 induction, numbers accumulating on day 5, and CTL activity, IFN gamma and granzyme B. Helper effect blocked by IL-12Rb-/- in OT-1 cells, or CD40L-/- in OT-II cells, and mimicked by IL-12 treatment.	216
Exogenous influenza peptide in PBS IP (Dd)	F5 TCR-Tg CD8 cells in thymectomized TCRtransgenic mice	Increased frequency and CTL activity day 4, low frequency after 5 weeks of peptide injections		IL2-/- did not block proliferation in vivo days 2 to 4, but prevented acquisition of ex vivo CTL activity	515,516
Exogenous influenza HA peptide in PBS IP (Kd)	Clone 4 TCR-Tg CD8 cells transferred to Balb/c mice	Higher fraction diluting CFSE on day	CD8 cells on day 4 after highdose peptide have	Propose that low, repeated stimulation with antigen triggers	258,259

		4 with high-dose peptide, and more divisions but no CTL activity or IFN gamma expression	poor ERK phosphorylation in response to peptide in vitro	proliferation followed by deletion, whereas high repeated stimulation desensitizes dividing cells and diminishes deletion	
Endogenous H-Y antigen ubiquitously expressed (Db)	T1.70 TCR-Tg CD8 cells transferred to C57BL/6 nude	Increased number day 5, decline in numbers day 9 and 60	Poor proliferation in vitro days 5 onwards, unable to increase frequency upon secondary transfer to fresh male nude mice	Deletion blocked by Bcl2 transgene but not by Fas-lpr mutation	240,253,286
Endogenous H2-Kb from metallothionein or albumin promoters	DES TCR-Tg CD8 cells in TCR-Tg mice or transferred to B10.A	CD69 induction, BrdU incorporation, CFSE dilution, increased numbers peaking 7 days if LN activation, transient hepatitis, and ALT elevation. Almost complete loss of cells by 30 days after transfer, failure to accumulate cells after emigration from thymus in thymus grafted mice	No evidence for anergy: normal CFSE measured in vitro proliferation of Bim-deficient cells from LN of Alb-Kb on day 15	When antigen presented only by hepatocytes induced almost normal initial CFSE dilution on day 2.5, but poor CD25 or ICAM1 induction, high Bim, and Bim-dependent failure to accumulate in large numbers in LNs or acquire CTL activity ex vivo or based on liver ALT	287,288,289,290
Endogenous LCMV gp33 from H2Kb promoter (Db)	P14 TCR-Tg CD8 cells transferred to C57BL/6	CFSE dilution on day 2 but no detectable expansion or CTL activity unless concurrently give LCMV, VSV, *Listeria*, LPS or poly I:C within 1 to 3 days of T-cell transfer	LCMV-expanded cells day 35 had lower surface TCR, could not be stimulated to proliferate or form CTLs in vitro		268
Endogenous OVA from insulin promoter presented by	OT-1 TCR-Tg CD8 cells transferred to C57BL6.H2-bm1 chimeric	CD69 induction, CFSE dilution, diabetes	No. Persistent cells when deletion blocked by Bim or Bcl2	Deletion not blocked by Fas-lpr but completely blocked by Bim-	292,293,294

DCs (Kb)	mice with H2b marrow	induction if antigen also presented directly by beta cells, decline in numbers after 8 weeks or after thymectomy	proliferated normally in vitro.	/- or Bcl2 transgene resulting in accumulation. Deletion also blocked by OT-II helper T cells.	
Endogenous OVA from keratin5 promoter presented by mature Langerhans and DCs (Kb)	OT-1 TCR-Tg CD8 cells transferred to C57BL/6 mice.	CFSE dilution on day 3, decline in numbers after 6 weeks		High CD86 on mature Langerhans and DCs insufficient to prevent deletion	517
Endogenous OVA from keratin14 promoter presented by mature Langerhans cells, or from actin or H2k promoter systemically	OT-1 TCR-Tg CD8 cells transferred to C57BL/6 mice.	CFSE dilution but little accumulation of OT-1 cells in systemic expressors or in K14-OVA × actin-OVA double transgenics. CTL activity acquired and 100- to 1000-fold expansion of OT-1 cells days 4 to 6 in K14-OVA mice or B6 mice infected with OVA-vaccinia virus, declining to very low numbers by day 21	Normal expansion upon in vivo rechallenge with OVA peptide + LPS on day 21 in K14-OVA animals, but suppressed in Actin-OVA recipients	High CD86 (+ signal 3 cytokines?) on mature Langerhans sufficient to promote accumulation and acquisition of CTL activity	260
Endogenous HSV peptide from insulin promoter presented by CD8+ DCs (Kb)	gBT1 TCR-Tg CD8 cells transferred to C57BL/6 mice	CFSE dilution on day 3, elimination by 7 weeks		Same CD8 + DCs induce tolerance and priming of HSV T cells	518
Endogenous influenza HA peptide from insulin promoter (Kd)	Clone 4 TCR-Tg CD8 cells transferred to Balb/c mice	CFSE dilution day 3 in fraction of cells (transgene dose-dependent) but only slight increase in numbers day 4 and no CTL activity	Ten to fourteen days after transfer of low numbers of T cells, influenza infection no longer precipitated diabetes. Divided cells sorted on day 4 and parked in non-transgenic	Bim-deficiency or Bcl2-transgene increased numbers of divided cells day 4, whereas Faslpr or TNFR1-/- did not. Concurrent influenza infection at time of transfer	259, 296,297,298

		or IFN gamma expression, unaffected by CD80 and CD86 blockade. Numbers fell by day 8.	recipient for 4 weeks expand normally following infection.	precipitated diabetes, greatly increased Cl4 number, CD25 and IFN gamma expression and CTL activity day 4, inhibited by CD80 and CD86 blockade.	
Endogenous influenza HA peptide from insulin promoter (Kd and I-Ad)	Clone 4 TCR-Tg CD8 cells plus HNT TCR-Tg CD4 cells transferred to Balb/c mice	CFSE dilution in fraction of CD4 and CD8 cells, no enhancement of CD8 cell division or accumulation or CTL activity by naïve helper cells. In vitro activated CD4 cells increased accumulation of CD8 cells and IFNg and CTL activity, precipitating diabetes.		CD80 and CD86 blockade negated effects of activated CD4 cells. Agonistic antibody to CD40 did not mimic helper cells despite inducing CD86 on DCs unless accompanied by exogenous IL-12 as “signal 3.”	217
Endogenous influenza HA peptide at low levels from H2K promoter in hemopoietic cells (Dd)	F5 TCR-Tg CD8 cells in RAG1-/- TCR-Tg mice	Induction of CD44 and in vivo CTL activity, but normal numbers	Increased CD5, some with lower TCR, poor in vitro proliferation, IL-2 or IFN gamma production but normal CTL activity	Evidence for elimination of antigen-presenting DCs and macrophages	261
Endogenous SV40 T-antigen expressed by insulin promoter (I-Ak)	TCR1 TCR-Tg CD4 cells in TCR-Tg mice	CD69 and CD44 induction in draining lymph nodes, BrdU incorporation in vivo over 12 days, insulitis at 1 to 3 weeks of age. Decline in frequency and numbers 4 to 12 weeks of age, rapid decline after thymectomy.	Poor proliferation to peptide in vitro		262
Endogenous influenza HA peptide from C3(I) systemic promoter at low or high	6.5 TCR-Tg CD4 cells transferred to Balb/c mice	CFSE dilution on day 5, increased numbers on day 9 in HA-	Poor proliferation and IL-2 secretion in vitro		263,264

levels (I-Ed)		high strain but not in HA-low strain			
Endogenous lysozyme from insulin promoter (I-Ak)	3A9 TCR-Tg CD4 cells transferred to MRL+/+ mice	CFSE dilution in ~20% cells in pancreatic node, one to three divisions after 10 days, increased to ~70% by CTLA4-deficiency but no increase in numbers and no diabetes.	Six days after transfer cells have poor proliferation to HELpeptide in vitro	Coadministration of lysozyme in PBS or alum increases CTLA4-/- numbers in pancreatic node sixfold, and precipitates diabetes. Lysozyme in CFA has similar effect for CTLA4+/+ cells.	266,267

ALT, alanine transaminase; APC, antigen-presenting cell; CD, cluster of differentiation; CFA, complete Freund's adjuvant; CFSE, carboxyfluorescein diacetate succinimidyl ester; CTL, cytotoxic T-lymphocyte; DC, dendritic cell; DNA, deoxyribonucleic acid; HA, hemagglutinin; HEL, hen egg lysozyme; HSV, IFA, incomplete Freund's adjuvant; IFN, interferon; IL, interleukin; LCMV, lymphocytic choriomeningitis virus; LN, lymph node; LPS, lipopolysaccharide; MHC, major histocompatibility complex; mRNA, messenger ribonucleic acid; OVA, ovalbumin; PBS, Phosphate Buffered Saline; SEB, staphylococcal enterotoxin B; TCR, T-cell receptor; Tg, transgenic; TNF, tumor necrosis factor; VSV, vesicular stomatitis virus.

Color codes denote studies involving the following: yellow, exogenous superantigens; orange, exogenous conventional protein antigens; purple, endogenous conventional protein antigens; blue, MHC II-restricted CD4 T cells; green, MHC I-restricted CD8 T cells; red, in vivo evidence of functionally tolerant (“anergic”) T cells.

The *Fas*^{lpr} mutation nevertheless interfered with peripheral deletion of CD4 T cells in a parallel study of peripheral tolerance induced by injecting a peptide from cytochrome c without adjuvant into transgenic mice expressing an MHC II-restricted TCR.[137] Similar studies in TCR-transgenic mice expressing an influenza hemagglutinin (HA)-specific MHC II-restricted TCR found that deletion could only be blocked by injecting an antibody to TNFα combined with the *Fas*^{lpr} mutation.[139] Likewise, other studies also indicate a role for Fas in peripheral tolerance in certain settings.[276,277] Thus, like the studies previously mentioned with superantigens, the relative role of the intrinsic and extrinsic pathways for deleting activated T cells in response to conventional peptide antigens may vary depending upon the TCR specificity and/or the duration and strength of TCR stimulation.

Peripheral Tolerance to Exogenous Peptide Antigens in Adoptively Transferred T-Cell Receptor-Transgenic T Cells

The studies previously mentioned analyzed peripheral T-cell tolerance in situ either in TCR-transgenic mice with very high initial frequencies of antigen-specific T cells or in superantigen treated mice where the starting frequency of Vβ8+ CD4 T cells was also very high. A large number of experiments have nevertheless reinforced and extended these results by tracking acquisition of peripheral tolerance in smaller numbers of antigen-specific T cells that have been adoptively transferred from TCR-transgenic mice into wildtype mice with a normal T-cell repertoire (see Table 32.1).

In one of the first studies of this type, Kearney et al.[250] harvested lymph node cells from DO11.10 TCR-transgenic mice, where most of the CD4 T cells expressed a TCR that could be detected by a clonotypic antibody (KJ1-26) and recognized OVA peptide presented by MHC II I-E^{d} molecules. A total of 2.5 million KJ1-26+ CD4+ cells were injected into the circulation of normal BALB/c mice so that the antigen-specific T cells could just be detected by flow cytometry of lymph nodes, at a frequency of 1 cell in 300. The recipients were then challenged with a tolerizing regime comprising OVA peptide given intravenously in saline or intraperitoneally in incomplete Freund's adjuvant so that the antigen was encountered without a bacterial costimulus. For comparison, other recipients were given antigen as an immunizing regime comprising the same amount of peptide given subcutaneously emulsified in complete Freund's adjuvant containing an extract of *Mycobacterium tuberculosis*.

With either regime, a fraction of the T cells were initially induced into S, G2, and M phase of cell cycle, and increased in frequency and absolute number in brachial and axillary lymph node on day 2 and 3. However, the tolerizing treatment caused the T-cell number to peak at

day 3 and drop precipitously by day 5, so that the frequency of antigen-specific CD4 cells fell below untreated control recipients by day 17. By contrast, in response to the immunizing regime the T cells accumulated to higher numbers at day 3 and sustained these numbers at day 5, then falling gradually by day 17 although still very elevated over untreated controls. In the immunized animals, most of the KJ1-26+ T cells were in B-cell follicles on day 5, reflecting what is now known to be differentiation into T-follicular helper cells that are critical for antibody responses,[278,279] whereas they remained outside the follicles in the T-cell zone in the tolerized recipients. On day 17, compared to untreated control recipients, the T cells in the immunized recipients were hyperresponsive to antigen as measured by recall proliferation in vitro, whereas those remaining in the tolerized animals were hyporesponsive as measured by proliferation or IL-2 production even adjusted for their lower frequency. The latter anergic T cells had diminished surface TCR expression, yet had been induced to proliferate initially based on labeling with BrdU during the tolerizing regime.[252] Profound hyporesponsiveness of the latter was also observed in vivo, upon immunization of the tolerized mice with peptide in complete Freund's adjuvant. Thus, peripheral tolerance to a foreign peptide reflected abortive proliferation of CD4 T cells, failure to differentiate into fully fledged helper cells, and deletion of most of the activated T cells and anergy in the residuum.

Further studies in the DO11.10 adoptive transfer model analyzed the effect of adjuvant and role of CD28 costimulation in the decision between peripheral tolerance and immunization. The effect of complete Freund's adjuvant in increasing the numbers of OVA-specific T cells that accumulated and their persistence was reversed by blocking CD80 and CD86 with monoclonal antibodies or a CTLA4-Ig fusion protein, whereas neutralizing the competing receptor for CD28 ligands, CTLA-4, further enhanced T-cell accumulation.[251] These results are consistent with at least one of the effects of bacterial adjuvant being the induction of CD80 and CD86 to higher levels on DCs, although they contrast with studies of the response to bacterial superantigen where CTLA4-Ig treatment did not block the ability of LPS to sustain the numbers of activated CD4 cells, and instead the bacterially induced cytokine tumor necrosis factor-α had a partial role.[280] More recent studies using adoptive transfer

of TCR-transgenic, OT-I, CD8 or OT-II, CD4 cells bearing other OVA-specific TCRs have demonstrated that two other bacterially induced cytokines, IL-12 and IL-1, have a direct impact on the induction of T-cell proliferation and effector function, in addition to induction of CD28 ligands.[211,212,213,214,215,216]

Along similar lines, peripheral tolerance induced by peptide without adjuvant could not be explained simply by absence of a CD28 costimulus, because when CD28-deficient DO11.10 T cells were adoptively transferred and the recipients treated with the tolerizing regime, the specific T cells exhibited an even more truncated proliferative response.[281] By adoptively transferring CFSE-labeled DO11.10-transgenic Rag1-/- T cells and treating the recipients with the tolerizing peptide regime, Turka and colleagues[282] showed that blocking CD80 and CD86 with CTLA4-Ig had no effect on the percentage of specific T cells induced by the peptide to express CD69, diminished the fraction of T cells that were induced into division on day 3 by only 30%, and had little effect on induction of IL-2 in these cells provided it was measured as a function of cell division. Like CD28 deficiency,[281] CTLA4-Ig treatment nevertheless greatly decreased the number of accumulating daughter cells that had made multiple divisions, and this effect was blocked by transgenic expression of Bcl-X_L.[283] Thus, peripheral tolerance in this setting involves receipt of a limiting CD28 costimulus that sustains the survival of divided progeny through several divisions but is insufficient to sustain them after more than four divisions and for more than a few days. This abortion of proliferation after several cell divisions parallels the results from analyzing peripheral tolerance to SEB.[248]

Complementary studies using a similar adoptive transfer design with other T-cell specificities reinforce this conclusion (see Table 32.1). For example, when CFSE-labeled CD4 cells bearing a lysozyme-specific TCR were adoptively transferred from TCR-transgenic mice into normal B10.BR mice, they underwent abortive proliferation for several divisions but most of the divided progeny disappeared by day 5, even when the lysozyme peptide was presented by activated B cells bearing high levels of CD86[284] or by DCs.[226]

These studies collectively demonstrate that when T cells encounter exogenous antigen in the absence of microbial or adjuvant costimuli, various stages of T-cell induction occur but the response is limited or truncated, leading to the outcome of tolerance. Although CD28 ligands play an important role as costimulators favoring T-cell immunity over tolerance, they are not necessarily sufficient on their own nor are they the only costimuli that favor immunity. Accordingly, the presence or absence of a CD28 signal is not the sole determining factor for whether or not T-cell tolerance is induced.

Peripheral Tolerance to Endogenous Peptide Antigens

These studies have the caveat that they studied acquisition of tolerance in animals exposed

to an exogenous, foreign antigen. Parallel studies nevertheless reinforce these conclusions by analyzing specific T cells during acquisition of tolerance to antigens that are made endogenously by the animal, either as natural self-antigens or in most cases "neo-self-antigens" encoded by transgenes that are present in the test animals but absent from controls and from the TCR-transgenic donor mouse (see Table 32.1). In this way self-antigens could be chosen where the peptide that was presented in a certain MHC type was known and where it was possible to follow the fate of initially naïve peripheral T cells specific for a particular endogenous antigen in vivo. Although TCR-transgenic models have their own caveats,[285] in general this was a tremendous step forward as researchers could now ask the question what happens to self-reactive T cells in mice expressing an "endogenous" self-antigen, as opposed to studying what happens when a foreign antigen is administered to mice in a tolerogenic way.

Rocha and von Boehmer[240,253] were among the first to follow this approach to peripheral T-cell tolerance, taking advantage of a natural self-antigen, HY, that is present in males but absent in females because it is encoded by the *Smcy* gene on the Y chromosome. They harvested HY+D^b-specific CD8 T cells from the spleen of female T1.70 TCR-transgenic mice, where they had matured without any exposure to HY antigen. These were injected into male mice, where they would encounter HY expressed ubiquitously, or into female controls that lacked HY. To facilitate tracking of the transferred T cells, nude mice that lack T cells were used as the recipients. The T-cell lymphopenia in these recipients also introduces a complication, however, because it profoundly alters signals for T-cell proliferation and survival. Nevertheless, the numbers of T1.70 TCR+ CD8 cells remained steady in female recipients but in the male recipients these dramatically increased by day 5 and then declined to half this number by day 9 and 25% of the peak number by day 60. On day 5, and especially days 9 and 20, both CD8 and TCR were decreased on the surface of the cells in male recipients, and the day 9 and day 20 T cells were completely unresponsive to restimulation with male antigen presenting cells in vitro. Secondary transfers to male or female recipients showed that maintenance of the unresponsive state required continued exposure to HY antigen. The authors concluded that peripheral T cells become tolerant through a sequence of activation, proliferation, anergy, and death. Subsequent analysis showed that the deletion of activated HY-specific T cells in these experiments was blocked if they expressed a *Bcl2* transgene but was unaffected by the Fas^{lpr} mutation, indicating it was due primarily to the intrinsic pathway of apoptosis.[286]

Bertolino and Miller[287] were among the first to extend these studies to trace peripheral T-cell tolerance to endogenous antigens after adoptive transfer of TCR-transgenic T cells into animals with a "full" complement of otherwise normal T cells. These studies tracked CD8 T cells bearing the DES TCR, which could be detected with a clonotypic antibody and recognized $H2K^b$ with an unknown self-peptide. While $H2K^b$ is normally expressed ubiquitously and would induce thymic deletion of DES TCR+ cells, to study peripheral tolerance they engineered B10.BR mice expressing $H2K^b$ as a transgene controlled by the metallothionein (MT) promoter, which is predominantly expressed in liver hepatocytes although also at low levels in other cells in thymus and lymph nodes. Two complementary approaches were followed to ensure that they studied only peripheral T-cell tolerance. First, they surgically removed

the *MT-*K^b transgenic thymus and replaced it with a nontransgenic thymus and traced the development of DES TCRtransgenic cells as they emigrated from the thymus and encountered self-peptide+K^b in the liver and lymph nodes. Second, they adoptively transferred naïve T cells from DES TCR-transgenic mice into *MT-*K^b transgenic B10.BR mice. These experiments revealed that the specific CD8 cells were stimulated to proliferate and accumulate transiently in the liver, resulting in hepatitis and release of the liver enzyme ALT into the circulation that was self-resolving and followed by apoptosis and deletion of the T cells in the liver and other tissues, establishing a state of tolerance.

Subsequent studies using this model[288,289,290] revealed that the tempo and transient phase of tissue damage preceding peripheral tolerance were dramatically affected by whether the CD8 T cells had encountered endogenous antigen on hepatocytes or on DCs. When they only encountered selfpeptide plus K^b on hepatocytes, proliferation of DES TCR+ CD8 cells was aborted quickly after a number of cell divisions and between days 2.5 and 6 as a result of Bim-dependent apoptosis, without acquisition of CTL activity or induction of liver damage. The divided CD8 cells were rescued from deletion by Bim-deficiency, but these did not acquire CTL activity or induce liver damage yet also did not appear to have become anergic as they could be stimulated to divide in response to K^b in vitro. By contrast, when transferred DES TCR+ cells also encountered self-peptide plus K^b on hemopoietic cells in lymph nodes (presumably DCs), this induced 100-fold greater accumulation of divided progeny and acquisition of CTL activity and hepatic damage, although that response was also ultimately self-limiting presumably due to exhaustive differentiation. The efficient

induction of peripheral tolerance by hepatocytes compared to other cells potentially explains how cotransplantation of liver with other organs improves acceptance of allografts.

Abortive proliferation as a mechanism for peripheral T-cell tolerance to tissue-specific endogenous antigens has been elegantly analyzed by Heath's group.[291,292] The rat insulin promoter (RIP) was used to express membrane bound OVA in the β-islet cells of the pancreas in C57BL/6 mice. Kurts et al. harvested naïve CD8 T cells from lymph nodes of TCR-transgenic mice bearing the OT-ITCR against an OVA peptide and $H2K^b$, labeled the cells with CFSE, and adoptively transferred them into *RIP-mOVA* transgenic recipients and nontransgenic controls. They made the surprising discovery that when mature OT-1 CD8 T cells reached the draining nodes of the pancreas, they encountered antigen that had been captured from pancreatic beta cells and cross-presented by cells that were later shown to be immature DCs. Cross-presentation by immature DCs did not cause anergy at any point but instead activated the OT-1 cells into more than eight rounds of cell division during the first 3 days, accompanied in this case by acquisition of cytotoxic function that destroyed the pancreatic islet beta cells within 9 days if 5 million OT-1 CD8 cells were transferred. If islet cell destruction was avoided by analyzing chimeric mice where a different $H2K^{bm1}$ molecule was expressed on the pancreatic islets, so that OVA could only be presented by DCs and not by the islet beta cells, proliferation of OT-1 cells was followed 8 weeks later by the loss of most of the T cells, demonstrating peripheral T-cell deletion as a mechanism of T-cell tolerance. Deletion of the activated OT-1 cells was blocked by a *Bcl2* transgene or Bim deficiency but unaffected by the Fas^{lpr} mutation,[293] implying that insufficient survival signals or cytokines or acute cytokine withdrawal was the cause of peripheral tolerance in this setting.

Like the DES-TCR into MT-K^b experiments where lymph node cells presented the self-antigen,[288,289,290] abortive proliferation was extensive in the transferred OT-1 cells and was accompanied by CTL activity, yet a key finding here was that this was only sufficient to cause diabetes if a very large bolus of CD8 cells was introduced synchronously into the animals. If 250,000 OT-1 cells were transferred instead of 5 million, their abortive activation was insufficient to cause diabetes. Physiologically, T cells that escape thymic deletion or anergy will emigrate to the periphery asynchronously in much lower steady-state frequencies, so that their transient proliferation and acquisition of effector activity before deletion may not create any measurable tissue dysfunction. In this regard, a key extension of the RIP-mOVA model was to cotransfer several million naïve CD4 T cells expressing the OT-II TCR, which recognized another OVA peptide presented by $I\text{-}A^b$. While the CD4 cells did not cause diabetes, nor did they appear to increase OT-I cell division, they nevertheless prevented deletion of the activated OT-1 cells and allowed 250,000 transferred OT-I cells to be sufficient to precipitate diabetes.[294] The decision between peripheral tolerance and immunity in this setting corresponds closely to Cohn's idea[39] that immunity requires coincident recognition of two epitopes on an antigen, one by a helper T cell and one—in this case—by a cytotoxic T cell.

Precisely how helper T cells protected cytotoxic T cells from peripheral deletion remains to be fully elucidated, as it could not be mimicked in the OT-1 RIP-OVA experiments simply by activating or “licensing” the DCs using an injection of agonistic antibody to CD40.[295] Parallel findings were also made in another TCR-transgenic adoptive transfer model developed by Sherman's group,[217,259,296,297,298] tracking CD8 T cells bearing the Clone 4 TCR that recognizes an influenza HA peptide presented by $H2K^d$. The Clone 4 T cells were transferred into normal BALB/c mice expressing a transgene encoding HA under the insulin promoter (InsHA transgenic mice), either alone or together with CD4 cells from another TCR-transgenic strain expressing the HNT TCR against an IA^d-presented HA peptide. In the absence of helper cells, a subset of the HA-specific CD8 cells were induced to divide several times in the pancreatic lymph node but the daughter cells failed to accumulate in increased numbers due to Bim-dependent apoptosis and failed to acquire CTL activity or interferon gamma expression. When the HA-specific CD4 cells were cotransferred, a subset of these also divided one to four times in the pancreatic node, but this abortive proliferation of the CD4 cells failed to help the CD8 cells and did not alter their abortive proliferation. However if the HA-specific CD4 cells were activated by influenza-infected spleen cells in vitro for 3 days before adoptive transfer, these fully activated helper T cells greatly increased the accumulation and persistence of divided HA-specific CD8 cells over

the following 4 to 8 days, inducing them to make interferon gamma and precipitating diabetes.[217] Treating the InsHA recipients of HA-specific CD8 cells with an agonistic antibody to CD40 was shown to increase the number of DCs in the draining node and their expression of CD86, but was insufficient to mimic the effect of activated helper cells unless IL-12 was also given. Taken together with other studies mentioned previously,[211,212,213,214,215,216] these results indicate that abortive T-cell proliferation leading to peripheral tolerance is only switched into sustained proliferation and effector

differentiation and tissue destruction when the T cells receive multiple costimulatory signals via CD28 together with other cytokines like IL-12, interferon alpha, or IL-1, and potentially other cytokines that are made directly by appropriately activated helper T cells.

The conclusion that peripheral T-cell tolerance is acquired through abortive proliferation and deletion because of insufficient costimulatory signals is nevertheless unable to explain the fate of peripheral T cells encountering endogenous antigens in all adoptive transfer models. A striking example comes from the experiments of Mayerova and Hogquist,[260] who transferred OT-1 TCR-transgenic CD8 cells into C57BL/6 mice that were either transgenic for a Keratin 14 promoter-OVA peptide minigene that was expressed in skin keratinocytes or were transgenic for an actin promoter-mOVA construct expressed systemically. Although CFSElabeled OT-1 cells divided in skin-draining lymph nodes in both recipients, there was little accumulation of the progeny in the systemic expressors, and the OT-1 cells underwent abortive proliferation, failed to acquire effector function, and the remaining cells after 21 days were anergic. By contrast in the K14-OVA animals, a 100- to 1000-fold increase in OT-1 cells was observed peaking on days 4 to 6 and accompanied by the acquisition of interferon gamma and CTL function. These cells induced autoimmune dermatitis and vitiligo that became lethal if more than half a million OT-1 cells were transferred. Remarkably, the OT-1 T-cell response to endogenous K14-OVA was comparable to that induced in B6 recipients infected with vaccinia virus expressing OVA.

A partial explanation for such strong induction of immunity and not peripheral tolerance in K14-OVA recipients was that the OT-1 T cells were activated by antigen presented by mature Langerhans cells, which had migrated from the skin to the draining node and expressed high levels of CD86 and MHC II. However, given the evidence discussed previously and in Table 32.1 that TCR and CD28 costimulatory signals are insufficient to drive accumulation of large numbers of divided T cells and acquisition of effector function, it is likely that the Langerhans cells were also expressing IL-12 or other cytokines because of their exposure to products of skin bacteria. One might expect the presence of pathogenic stimuli to exert a “dominant” effect, resulting in the induction of immunity when Langerhans cells and immature DCs were both presenting antigen in K14-OVA × actin-OVA double transgenic mice. However, the opposite was observed: OT-1 CD8 cells encountering endogenous antigen from the two sources underwent abortive proliferation, did not acquire CTL activity or cause dermatitis, and became anergic. Like other studies where dividing T cells become anergic when continuously stimulated by endogenous antigen distributed systemically,[240,253,261,263,264,268] the “dominance” of tolerance over immunity in K14-OVA × actin-OVA double transgenic mice may also reflect desensitization of TCR signaling to critical pathways for T-cell growth and differentiation like RAS-ERK-AP1 or NF-κB. When endogenous antigen is only available from rare DCs in particular sites, and especially if these antigen-bearing DCs are eliminated by abortively activated T cells,[299] the frequency of TCR engagement may in this setting not be sufficient to activate TCR desensitization and anergy, explaining the absence of anergy in other examples of abortive proliferation.[289,293,297]

Chronic TCR engagement during abortive T-cell proliferation to endogenous or exogenous antigens also induces inhibitory signaling molecules on the activated T cells, which play an important role in suppressing the accumulation of divided progeny and their acquisition of effector functions. This has been particularly well demonstrated by Probst et al.[300] in the case of PD-1, a negative signaling receptor displayed by chronically stimulated T cells.[301,302,303,304,305] They engineered an elegant transgenic mouse strain that endogenously expressed two dominant peptide epitopes of LCMV in a subset of DCs. Expression was controlled by a drug-inducible genetic switch, which allowed a burst of peptide-presenting DCs to be formed in adult mice that had a normal T-cell repertoire and had not been immunized or infected. In chimeric mice, where half of the T cells lacked the PD-1 inhibitory receptor and the other half were wild-type, switching on peptide display induced accumulation of high frequencies (˜2%) of LCMV peptide-specific CD8 T cells expressing interferon gamma, revealed by flow cytometric straining with p/MHC I tetramers. However, these were all derived selectively from the subset of T cells that were PD-1 deficient, with no measurable accumulation of corresponding T cells from the wild-type T cells in the same animals. If wild-type T cells were exposed to the burst of peptidedisplaying DCs for 12 days, they became actively tolerant as revealed indirectly by infecting the mice with LCMV and observing a 90% decrease in the accumulation of tetramer positive CD8 cells recognizing the two LCMV peptides that had been expressed by DCs endogenously but not a third LCMV peptide that had not been encountered as “self” in the 12 days before infection. By infecting animals with equal mixtures of tolerant and naïve T cells, they excluded T_{reg} induction as a mechanism. While the actual fate of antigenspecific T cells could not be visualized in this setting due to their very low frequency, these studies establish that presentation of antigen by immature DCs leads to actively acquired tolerance by peripheral T-cell deletion or anergy, and this requires induction of inhibitory receptors on the responding T cells to abort their proliferation.

While abortive T-cell activation generally leads to a cell autonomous process of peripheral

tolerance that is limited to the T cells that are deleted or anergic, in some settings partially activated self-reactive T cells accumulate and suppress other self-reactive T cells. This has been revealed by elegant analyses of a prevalent population of CD8 T cells in diabetes-prone NOD mice, which proliferate in response to a pancreatic autoantigen, islet-specific

glucose-6-phosphatase catalytic subunit-related protein (IGRP).[306,307] CD8 T cells bearing TCRs with intermediate affinity for an IGRP peptide are not rendered tolerant in the thymus but, in combination with CD4 cells, are stimulated to proliferate in the pancreatic lymph node and destroy the pancreatic islet beta cells. However, other CD8 T cells with very similar TCRs recognizing IGRP peptide with low affinity are stimulated to accumulate in the lymph nodes in a partially activated $CD44^{hi}$ CD62L+ state expressing interferon gamma and granzyme B but not IL-2. These low affinity self-reactive T cells suppress the islet antigen-induced proliferation of high-affinity T cells, in part by eliminating antigen-presenting DCs and B cells through perforin-mediated killing. This mechanism may explain observations in the 1970s and 1980s of CD8+ suppressor T cells. It also suggests that one of the functions of thymic positive selection is to populate the peripheral T-cell repertoire with low-affinity self-reactive T cells that actively suppress activation of any high-affinity self-reactive T cells that had escaped thymic deletion.

Collectively, the studies summarized in the previous sections demonstrate that peripheral T-cell tolerance induced by exogenous or endogenous antigens is a result of suboptimal T-cell stimulation, that generally leads to the tolerized T cell undergoing rounds of division, acquiring effector function in some cases, but following an abortive sequence that is generally self-limiting. One major distinction between T-cell proliferation to tolerogenic antigens and immunogenic foreign antigens is that the former does not normally leave a population of memory T cells with heightened responsiveness, but instead leaves either a "hole" in the repertoire or T cells with diminished responsiveness that may in some cases be actively suppressive. Costimulatory signals such as those through CD28, and other important cytokine signals that promote T-cell activation, contribute to the overall signals that drive T-cell immunity versus tolerance. However, the ability of specific costimuli to influence T-cell fate varies substantially for reasons that are imperfectly understood.

T Cell Ignorance Toward Undetected Self-Antigens

A number of transgenic models traced the fate of T cells expressing a well-defined transgenic TCR specific for a tissue-restricted antigen and found that tolerance mechanisms such as thymic or peripheral deletion or anergy were not observed.[308,309,310,311] In some cases, animals developed spontaneous autoimmunity and in other situations no spontaneous autoimmunity occurred. This situation has become known as T cell ignorance. In one model, the LCMV-gp was expressed using the RIP. This led to the expression of the LCMV-gp in the β-islet cells of the pancreas. These mice were bred with P14 TCR-transgenic mice, expressing the receptor specific for the LCMV-gp. Importantly, these double transgenic P14/RIP-gp mice and single transgenic RIP-gp mice clearly showed that the islet-specific T cells were not deleted in the thymus or tolerized in the periphery. LCMV infection of either RIP-gp mice or P14/RIP-gp mice resulted in the induction of diabetes, due to the cytotoxic activity of CD8+ gp-specific T cells. Infection with a control virus such as vaccinia or an LCMV strain that did not encode the major gp epitope did not induce diabetes. Studies have suggested that immunologic ignorance is a consequence of low levels of self-antigen presentation.[312,313]

Regulatory T Cell Populations Maintain Tolerance and Limit Autoimmunity

Many studies have shown that a population of T_{reg} cells can also be induced in the periphery. These cells have become known as induced T_{reg} (iT_{reg}) cells. These are also an important population of cells that inhibits autoimmunity. Evidence has shown that transforming growth factor β is an important factor that promotes the differentiation of this population, and that IL-10 production is important for their inhibitory function.[314,315] Further analysis has shown that iT_{reg}s arise upon presentation of low levels of antigen on DCs together with suboptimal maturation signals. Transforming growth factor β promotes the generation of iT_{regs} in this model.[316]

Both natural T_{regs} and iT_{regs} appear to use several molecules to suppress antigen presentation and costimulation of peripheral T cells, thereby contributing to actively acquired tolerance. One of these is IL-10,[317,318] which has long been known to downregulate the surface display of both MHC II and CD86 on DCs and macrophages.[319,320,321] Recently, this action of IL-10 has been shown to be through the induction of the ubiquitin ligase, March1, which attaches ubiquitin molecules to cytoplasmic lysines in MHC II and CD86 and tags these proteins for degradation.[322,323] When DCs mature in response to LPS or other bacterial or viral products, they dramatically increase expression of CD83, and this protein functions by blocking the effects of IL-10 and March1 to tip the balance in favor of T-cell

stimulation.[323] Another critical molecule for tolerance and T_{reg} function in vivo is CTLA-4, which has recently been established to function by stripping CD86 and CD80 from the surface of DCs and other activated antigen-presenting cells.[124,324,325,326]

CTLA-4 and IL-10 from T_{reg}s thus work in concert to suppress delivery of signal 1 and signal 2 to helper and cytotoxic T cells in the absence of microbial costimuli. However, when large numbers of MHC II^{high} $CD86_{high}$ $CD83_{high}$ mature DCs accumulate in a lymph node because of a microbial stimulus, these will exceed the inhibitory capacity of CTLA-4 and IL-10 from T_{reg}s and favor T-cell activation. CTLA-4 and IL-10 are also produced by activated Foxp3- T cells under various circumstances, providing a potential feedback loop whereby clonal expansion and differentiation of activated T cells may similarly dampen signal 1 and signal 2.

Fate of Autoreactive Mature T Cells

These different mechanisms of peripheral tolerance act in different situations (Fig. 32.2). Autoimmunity may be prevented when self-reactive T-cell activity is limited by the influence of T_{reg}s. Alternatively, if self-antigen is presented at a level that is detectable by the T cell, then tolerance may occur either by deletion or anergy. If there is no detectable self-antigen, then T cells remain ignorant of the antigen.

Studies have shown that the level of self-antigen determines the fate of the self-reactive T cell.[313] Further studies have also provided evidence that low-avidity tissue-specific T cells can escape central and peripheral tolerance and potentially cause autoimmune disease.[327,328,329]

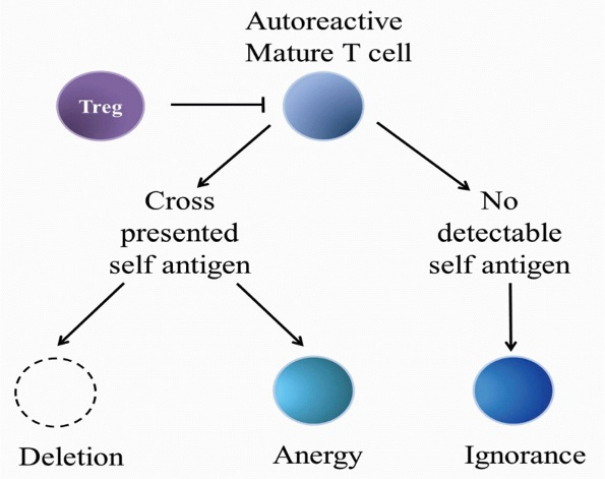

FIG. 32.2. Fate of Potentially Autoreactive Mature T Cells. If an autoreactive T cell does not encounter self-antigen in the thymus, then autoimmunity may be avoided if the T cell is silenced by a number of peripheral tolerance mechanisms. Regulatory T cells may inhibit the activity of the autoreactive T cell. Alternatively, if the T cell engages cross-presented self-antigen on a resting antigen-presenting cell, this would result in T-cell tolerance either by rendering the T cell anergic or by deleting the T cell. If the T cell does not encounter self-antigen, this would be known as T-cell ignorance and the T cell would persist as a naïve T cell in the repertoire.

Why do Anergic T Cells Exist?

Although T-cell anergy has been defined in vitro and in vivo, the idea that nonfunctional T cells remain in the T-cell repertoire is intuitively not attractive. How could the host benefit from having nonfunctional T cells in the repertoire? Several groups have proposed a model where T-cell activation thresholds could be modulated, such that the resting threshold is set by tonic signals in the environment, and TCR engagement with “stronger” antigens could lead to T-cell activation. 330 In this way, the “anergic” T cells would actually be responsive to other antigens, thereby diversifying the T-cell repertoire. Experimental evidence has been published that supports this model.[92,331,332] Others have suggested a model called adaptive tolerance.[333] However, the concept that T cells have tunable activation thresholds still remains unclear.

Importantly, T_{regs} have often been considered to be anergic cells. Consistent with the classic definition of anergy, T_{regs} can proliferate only in the presence of higher doses of IL-2 and

cannot themselves produce IL-2. Whether the mechanism of anergy exists solely for the capacity for T_{reg} function is currently unknown. However, studies have been reported that evaluate connections between anergy and suppression.[334,335]

Molecular Pathways Involved in T-Cell Anergy

Regardless of the functional reason why anergy exists, a tremendous number of studies have addressed the molecular pathway that programs the T-cell unresponsive state. Understanding the molecular basis for anergy may be important for manipulating this state in order to promote antitumor immunity or limit autoimmunity. The signaling events that lead to T-cell anergy have been studied from multiple perspectives. Studies have investigated pathways that fail to be activated in anergic cells. Others have evaluated various knockout animals to determine whether the induction of anergy or activation is impaired. Importantly, inconsistencies may stem from the model that is used to induce T-cell anergy. Experiments that rely on chemicals to induce anergy, such as treating T cells with ionomycin alone, are evaluating a distinct way to disable signaling events in T cells. Furthermore, it is possible that CD4 T-cell anergy is different from pathways that induce CD8 T-cell anergy.

Macian and colleagues have proposed that anergy occurs when NFAT is preferentially activated in the absence or reduced activation of AP-1 and NF-κB. Accordingly, NFAT deficient T cells cannot be anergized. By inhibiting the formation of NFAT-AP-1 complexes, the cells can be shifted from activation to anergy.[336,337] In anergic cells, NFAT induces genes that inhibit TCR signaling cascades as well as inhibit cytokine production. Importantly, NFAT-dependent genes include Itch, Cbl-b, Grail, and caspase-3. Studies using a CD4-dependent system demonstrate that caspase-3 is essential for the development of anergy in T cells.[338] However, other studies demonstrate that caspase-3 is not required for the induction of CD8 anergy in vivo.[151]

Because CD28 is a key costimulatory molecule that promotes full T-cell activation, signaling pathways downstream of CD28 should be critical in limiting anergy. Evidence from several studies has shown that PKCθ is triggered downstream of TCR and CD28 stimulation.[339,340,341] Importantly, anergy of CD8+ T cells can be readily induced in CD28- and PKCθ-deficient mice, and is regulated via the c-Rel

subunit of NF-κB but not NF-κB1 (p50).[342,343] Other models have also supported a role for PKCθ in preventing anergy and promoting T-cell activation.[344] Early studies using a variety of models have also shown that the activation of the transcription factor AP-1, which is downstream of PKCθ,[345,346] is important for regulating anergy.[347] Evidence also supports a role for the Erk pathway in the activation of AP-1 and accordingly has also been shown to play a role in T-cell anergy.[348,349] These and other studies support an important role for NF-κB and AP-1 in regulating CD4+ T-cell anergy.[350]

Early studies examining anergy suggested that this state is an actively maintained state and that continuous TCR derived signals are necessary.[232,253] Therefore, researchers have looked for molecules that are important in maintaining the anergic state. These molecules would be predicted to be upregulated in anergic cells. One candidate is known as Tob. Tob is a member of the APRO (antiproliferative) family and is expressed at low levels in quiescent cells.[351] Another example is diacylglycerol kinase-α. Diacylglycerol kinase-α is upregulated in anergic cells and has been shown to associate with RasGRP-Ras complexes and thereby inhibit signaling via the Ras-GRP/Ras complex and ERK activation.[352] Accordingly, the induction of anergy is impaired in diacylglycerol kinase-α-deficient T cells or in the presence of pharmacologic inhibitors.[353]

Several E3 ubiquitin ligases are involved in regulating anergy. As protein degradation is regulated by ubiquitination, it is possible that a functional T-cell response is regulated by the degradation of proteins involved in signaling and function of T cells. Gene related to anergy in lymphocytes (GRAIL) is an E3 ubiquitin ligase that was identified using an in vitro screen for anergy. A T-cell clone specific for sperm whale myoglobin was stimulated with a fibroblast line transduced with the appropriate MHC molecule and/or CD80 and with or without antigen. In this way, the authors evaluated the expression of genes when the T-cell clone was stimulated with antigen and costimulation (CD80).[354] Grail is expressed in resting CD4 mouse and human T cells and is upregulated in anergic cells. It is required for the induction of anergy in mouse CD4 T cells.[355,356,357]

Another E3 ubiquitin ligase that is important for regulating anergy is Cbl-b. Initial interest in this molecule was piqued when Cbl-b knockout mice were generated.[358,359] Studies showed that the absence of Cbl-b rendered T-cell activation to be independent of CD28. Therefore, it appeared that Cbl-b negatively regulated costimulatory signals. Although *Cblb*-null mice generally do not develop spontaneous autoimmunity, they were more susceptible to experimentally induced autoimmunity. Interestingly, *Cblb* was also identified as a key gene

that was mutated in the KDP rat strain that spontaneously develops type 1 diabetes.[360] Experiments have also shown that Cbl-b-deficient T cells are unable to be anergized in vivo.[344,361] While *Cblb*-deficient B10.BR mice exhibit no evidence of autoimmunity, when it was combined with Aire-deficiency (which also caused little autoimmunity on its own), the combination resulted in autoimmunity directed specifically at the exocrine pancreas and submandibular salivary gland that was lethal by 20 to 30 days of age.[362] These results underscore the importance of peripheral T-cell tolerance through Cbl-b as a failsafe when self-reactive T cells escape thymic deletion. Importantly, studies have also shown that the absence of Cbl-b also results in T cells that are resistant to the regulatory properties of T_{reg}.[363,364,365] However, if the Cbl-b-deficient T cells were resistant to T_{reg} activity, then one prediction would be that these mice develop autoimmunity in a similar way to T_{reg}-deficient mice. This, however, does not occur.

One of the hallmarks of anergy is the reduced proliferative capacity of anergic T cells. This has prompted many groups to evaluate the importance of cell cycle regulation in anergy. Several important studies have been done with anergic cells that demonstrate alterations in the molecules involved in cell cycle progression. For example, rapamycin inhibits the mammalian target of rapamycin pathway, which is important for cell division and can induce anergy in T cells.[366,367] Other studies have focused on a molecule known as p27kip that inhibits cell cycle progression. Forced upregulation of p27kip induces an anergy-like state, and accordingly anergy cannot be induced in p27kip-deficient cells.[368,369] Further studies of the p27kip pathway has identified a role for Smad3 in the induction of anergy.[370]

In general, T-cell anergy is a consequence of impaired signaling capacity in T cells, which has been shown to be regulated at many levels.[371,372,373,374] It is not yet clear how these pathways are interconnected in anergic cells or what internal programming is required to maintain anergy.

Breaking T-Cell Tolerance

Autoimmunity can arise by many different mechanisms, some of which include the failure of tolerance mechanisms described previously. There is much interest in uncovering strategies to break tolerance, either to promote antitumor immunity or limit autoimmunity. In order to reverse an anergic state, several approaches have been taken, including stimulation of T cells through the common γ chain of the IL-2R or triggering costimulatory molecules.[375,376,377,378] Many approaches have also been taken to appropriately activate ignorant T cells. These strategies generally involve activation of the immune system with viruses, the administration of antigen together with other costimulatory signals, or other stimuli that lead to DC maturation.[224,308,379,380,381]

Notably, tumor growth can generate sufficient amounts of antigen to activate ignorant cells and promote immune surveillance in animal models.[382,383] One can speculate that similar tissue-specific “ignorant” cells may be responsible for providing immune surveillance in patients. Correlative studies have been reported by many groups that demonstrate that patients with a defined T-cell signature have a better prognosis.[384,385,386,387] It is compelling to speculate that there are ignorant T cells that exist in the T-cell repertoire that are activated to tumor antigens and can prolong survival of patients by promoting an antitumor response.

Studies have clearly shown that the costimulatory/coinhibitory family of molecules play an important role in T-cell activation and also maintaining tolerance (Fig. 32.3). The absence of CTLA4, PD-1, or PD-L1 results in spontaneous autoimmunity and has been proposed to act largely by controlling autoreactive T-cell activity.[300,301,302,324,388,389] PD-1 has two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC). PD-L1 is expressed on T cells, B cells, DCs, macrophages, as well as parenchymal cells, whereas PD-L2 expression is induced on DCs and macrophages. Importantly, studies have shown that the expression of PD-L1 on parenchymal cells also plays a role in maintaining peripheral tolerance.[390] There may be many unexplored molecules expressed by tissues that are critical for maintaining tolerance.

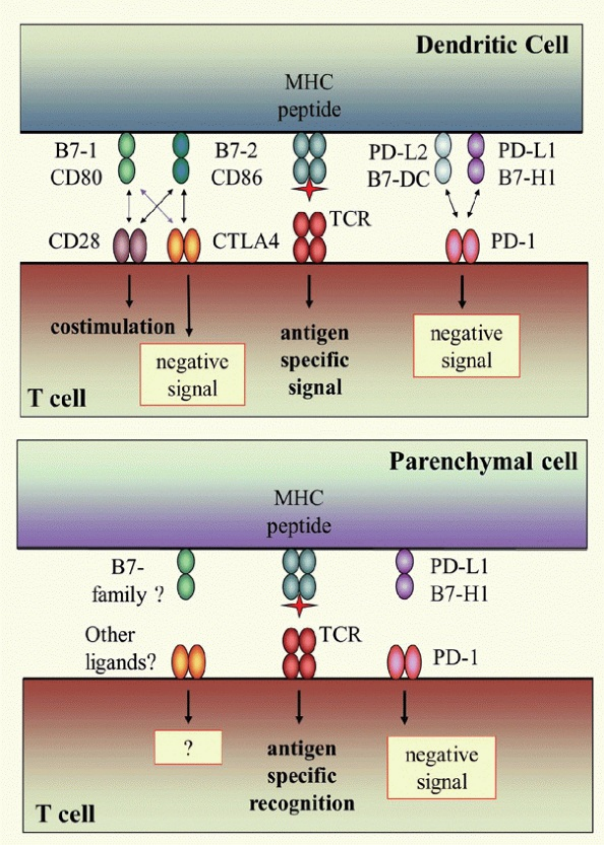

FIG. 32.3. Regulating Tolerance and Immunity by Costimulation and Coinhibition. **A:** Interactions between naïve T cells and mature dendritic cells are critical for inducing the adaptive immune response but are also critical for maintaining tolerance. The costimulatory molecule cluster of differentiation (CD)28, binds to its ligands B7-1 (CD80) and B7-2 (CD86). The coinhibitory molecule CTLA4 is induced after T-cell activation and competitively binds to the same ligands, with a higher affinity. Other members of the immunoglobulin superfamily also regulate these processes. PD-1 binds to PD-L1 (B7-H1) and PD-L2 (B7-DC) and sends an inhibitory signal to T cells. **B:** Members of the B7 family are expressed on a variety of cells other than the hematopoietic system, such as the islets cells in the pancreas. It is possible that the B7 family of molecules contribute to tissue homeostasis in a variety of settings.

B-CELL TOLERANCE: ACTIVELY ACQUIRED TOLERANCE IN THE PREIMMUNE B-CELL REPERTOIRE

Actively Acquired B-Cell Self-Tolerance Varies with Antibody Affinity, Antigen Abundance, and Antigen Form

Tracing the fate of self-reactive B cells accurately and controlling for the confounding effects of antibody heterogeneity, polyspecificity, and affinity, as discussed in the history section, has depended on engineering transgenic mice with rearranged Ig heavy (IgH) and light (IgL) chain genes that encode antibodies of defined specificity and affinity. Initially, these transgenes were introduced in multiple copies cointegrated at a random chromosomal locus. Subsequently, the transgenes were targeted as single copies integrated into the endogenous IgH and IgL chromosomal loci. Collectively, these models have revealed that actively acquired tolerance in B cells is extensive.

The first use of this approach by Goodnow and colleagues in 1988 employed an antibody against hen egg lysozyme, HyHEL10.[391] This antibody-antigen pair had been intensely characterized by Smith-Gill and Davies, including high-resolution crystal structures, and exhibited the monospecificity and affinity typical of highly selected, hypermutated antibodies that dominate long-term immunity against foreign protein antigens.[392,393] In transgenic mice inheriting both the rearranged IgH and IgL chain genes, most of the B cells that developed expressed cell surface IgM and IgD that only bound hen egg lysozyme. These foreign-specific B cells matured and recirculated amongst peripheral lymphoid tissue and, when mice were immunized with the foreign hen egg lysozyme antigen, these B cells were efficiently stimulated into clonal proliferation and antibody formation.

Parallel sets of transgenic mice engineered to express hen egg lysozyme as a “neo-self”

protein displayed actively acquired tolerance and were specifically unable to make antibody upon immunization with lysozyme.[391] In some lysozyme transgenic strains, only trace amounts of the antigen circulated in their blood (˜10^{-10} M), which resulted in tolerance induction only in the helper T cell repertoire and not in B cells themselves.[394,395] This greater sensitivity of T cells for acquiring tolerance, which was also observed in transgenic animals where lysozyme expression was limited to the pancreatic islets or thyroid gland, was later shown to be due to *AIRE*-independent and *AIRE*-dependent expression of the lysozyme gene in thymic medullary epithelial cells, which induced clonal deletion of lysozyme-specific CD4 T cells.[167,396] However, T-cell tolerance could be bypassed and high-affinity antibodies to lysozyme induced in these low-lysozyme animals by immunizing with lysozyme covalently coupled to horse red blood cells, establishing either absence or breakdown of B cell tolerance to low-abundance, low-valency autoantigens.[394] However, in mice with 10^{-9} M or higher levels of circulating lysozyme, the same treatment

elicited only low-affinity antibody-secreting cells, implying that B-cell tolerance was induced as a result of sufficient exposure and affinity to the self-antigen and could not be overcome by strong T cell help to a linked foreign “carrier.” The existence of actively acquired tolerance in individual B cells was clearly shown when lysozyme transgenic mice were crossed with the antilysozyme Ig-transgenic mice, yielding four outcomes depending on the concentration and form of lysozyme expressed as a self-antigen (Fig. 32.4). Self-reactive B cells remained “ignorant” and exhibited little functional or numerical change when they developed in mice with ˜10^{-10} M circulating antigen in their blood, which was only sufficient to engage a few percent of the lysozyme binding receptors on the B cells.[395] This circumstance included transgenic strains with tissue-specific lysozyme expression that was present at greater than 10^{-6} M on thyroid epithelium or the pancreatic islet beta cells, because in these animals the preimmune B cells only encountered trace amounts of lysozyme that had been cleaved and released into the circulation.[397]

When lysozyme circulated at higher concentrations of 10^{-9} M or greater, the monomeric antigen continuously engaged > 40% of the binding sites in the IgM and IgD on the surface of the B cells, starting as soon as IgM was displayed on the surface of immature B cells in the bone marrow.[391,395,398] This did not delete or arrest the maturation of the B cells into IgD_{high} recirculating follicular B cells but caused them to selectively downregulate their surface IgM as they matured and induced a lasting functional change that made them much less responsive to lysozyme immunization as measured by proliferation and antibody secretion. For simplicity, this state is referred to as B cell clonal anergy but, as with T cell clonal anergy discussed previously, this single term should not be mistaken to connote a single mechanism. In fact, there are multiple separate inhibitory processes responsible for actively acquired tolerance in “anergic” B cells that are imperfectly understood, and these appear to vary depending on the nature of the autoantigen and antibody.

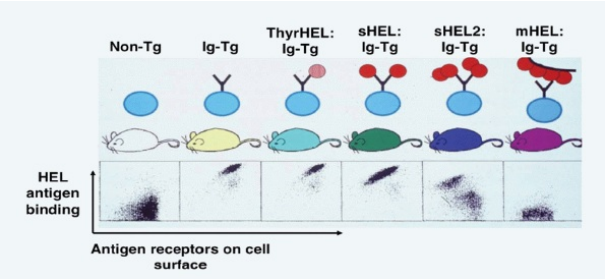

FIG. 32.4. Tracing the Fate of Self-Reactive B Cells in Transgenic Mice. Flow cytometric plots are gated on spleen B cells, with each *dot* representing one cell. These show on the x-axis the $\log_{10}$ relative abundance of surface immunoglobulin (Ig) on each B cell, stained with antibody to kappa light chain. On the y-axis is the $\log_{10}$ relative amount of hen egg lysozyme (HEL) bound to each B cell, detected by staining the cells first with a saturating concentration of lysozyme and then a fluorescent antibody to lysozyme. In normal C57BL/6 nontransgenic mice, the rare lysozyme-binding B cells detected are heterogeneous and probably represent poly-specific or nonspecific binding. In mice inheriting IgH and IgL transgenes, almost all the B cells bind lysozyme monospecifically, and cell-to-cell variation in binding is tightly correlated with variation in the amount of surface Ig on each cell. Clonal ignorance is observed in double-transgenic mice expressing the lysozyme gene selectively in the thyroid epithelium: the frequency of lysozyme binding cells is unaltered and the amount of surface Ig they display is decreased by less than twofold. In double transgenic mice where monomeric lysozyme accumulates to higher concentrations in the circulation and continuously engages ˜50%

of the antigen receptors on the developing B cells, actively acquired tolerance in the B cells is reflected by selective downregulation of their surface IgM receptors but the cells mature to the IgD^{high} recirculating stage. In a matched double transgenic strain where the same proportion of receptors is engaged by dimeric lysozyme, IgM and IgD surface antigen receptors are downregulated accompanied by developmental arrest at the T1-T2 stage, and edited B cells accumulate with normal densities of antigen receptors that do not bind lysozyme. Finally, in a double-transgenic strain where lysozyme is displayed in polyvalent form on the membrane of other blood and marrow stromal cells, lysozyme-binding B cells are almost completely eliminated and replaced by edited B cells.

Dimeric lysozyme, circulating at concentrations that engaged an identical percentage of B-cell antigen receptors, triggered a more extreme surface IgM downregulation and arrested the maturation of the B cells at the immature IgD^{low} CD21^{low} CD24^{hi} CD93+ CD62L- stage that characterizes their first arrival from the bone marrow to the spleen (see Fig. 32.4).[399] These cells were short-lived in the spleen, profoundly “anergic” not only to antigen but to LPS and, because of their lack of the CD62L lymph node homing receptors, very few reached peripheral lymph nodes. Anergic B cells encountering monomeric lysozyme were similarly arrested as a shortlived IgM^{low} IgD^{low} immature population in the spleen in mice that contained normal numbers of competing B cells.[400]

The extreme fate for self-reactive B cells was observed in transgenic mice displaying membrane-tethered lysozyme present on the surface of many bone marrow and blood cells, which resulted in almost complete absence of lysozymebinding B cells in the spleen and peripheral lymph nodes,[401] including cells with very low affinity (˜10^{-6}) for the antigen.[402] This fate was first demonstrated for B cells in Ig-transgenic mice expressing an allo-antibody against MHC I proteins that were ubiquitously displayed on bone marrow cells.[403,404]

The existence of this spectrum of actively tolerant states in B cells has been reinforced and extended through the analysis of Ig-transgenic mice expressing many clinically significant antibody specificities. These are summarized in Table 32.2 and specific mechanisms described in more detail subsequently.

TABLE 32.2 Summary of Fate of Self-Reactive B Cells in Different Immunoglobulin-Transgenic Mouse Models

Fate of B Cells	Antigen	Antibody	Reference
Ignorance characterized by maturation into IgMhi IgDhi follicular and IgMhi marginal zone B cells	Monomeric lysozyme in circulation ˜0.1 nM	HyHEL10 from repeatedly immunized mouse	394,395
	0.1 nM circulating lysozyme cleaved from thyroid or islets	HyHEL10 from repeatedly immunized mouse	397
	IgG2a (low affinity)	AM15, from MRL-lpr lupus mouse	509,510
	IgG, myoglobin, thyroglobulin, actin, ssDNA (low affinity)	SMI VH1-69 germline and Vk3 from human chronic lymphocytic leukemia	473,511
	Human La autoantigen, not crossreactive with mouse La	A3 Vh from mouse immunized with human La	512
	Single-stranded DNA	3H9 anti-DNA VH and Vk8 from MRL/lpr lupus mouse	469

B cells selectively form marginal zone B cells	Thy-1 glycoform at 10% of normal levels	VH3609 and Vk21 germline from spontaneous anti-thymocyte mouse B cell	508
Anergic cells mature to IgMlowIgDhigh CD62L+ CD24- CD93- follicular B cells, recirculate to lymph nodes, desensitized to BCR but retain response to CD40 and LPS	Single-stranded DNA, polyreactive?	HK165 anti-arsgermline VH and mutated Vk from hapten immunized mouse	441,442
	Single-stranded DNA, polyreactive?	ArsA1 anti-arsgermline VH and mutated Vk from hapten immunized mouse	440,453
	Single-stranded DNA, polyreactive?	3H9 anti-DNA VH from MRL/lpr lupus mouse and Vk8	415,438,439
	Sm Ribonucleoprotein	2-12H from lupus mouse with Vk8 or Vk4	443,444
	Insulin	125 H and L chain	445,446
	Monomeric lysozyme in circulation ~1 to 10 nM	HyHEL10 from repeatedly immunized mouse	391,417,452
Anergic B cells in spleen at IgMlowIgDlow CD62L- $CD24^{hi}$ CD93+ transitional stage, short-lived, poor activation to BCR and in some cases anergic to LPS, subset of edited cells preferentially accumulate over time	Erythrocyte membrane antigen	4C8 VH and VL from NZB mouse with autoimmune hemolyticanemia	476,477,478
	Thy-1 membrane antigen glycoform	VH3609 and Vk21 germline natural antibody	479
	MHC cell surface protein Kb expressed in liver or anti-kappa light chain “superantigen” in liver	3.83 H and L from allo-immunized mouse	482,483,484
	Dimeric lysozyme ~1 nM in circulation, or monomer lysozyme 1 nM with competing B cells	HyHEL10 from repeatedly immunized mouse	399,400
	Membrane lysozyme or MHC I Kb on 5% to 10% of blood cells	HyHEL10 or 3-83	412,481
	IgG high affinity rheumatoid factor	Les VH4-34 and Vk328 mutated from chronic lymphocytic	472,473,474

		leukemia	
	Glucose-6-phosphate isomerase (high affinity)	6.121 mutated H and L from TCR transgenic mouse w/ autoimmune arthritis	475
	Double-stranded DNA, nuclear antigens	3H9 VH with Vlambda1 from MRL/lpr lupus mouse	467,468
	Double-stranded DNA, nuclear antigens	3H9 VH with Vlambda2 from MRL/lpr lupus mouse	469
	Double-stranded DNA, nuclear antigens	HKlR anti-Ars Vh with Arg55 in CDR2 and mutated Vk from immunized mouse	441,470
	RNA, ssDNA, cytoplasmic and nucleolar antigens	564 H and L chain from SWRxNZB mouse with lupus	471
Profound downregulation of surface IgM and developmental arrest at small pre-B/immature B cell stage in bone marrow, loss of proliferative response to LPS, followed by editing or apoptosis within 24 to 48 hours	Laminin and single-stranded DNA	LAM nephrotoxic antibody from lupus mouse	435
	a3(IV)collagen NC1 domain	1G6 H and L from mouse hyperimmunized with a3(IV)collagen NC1 domain	434
	IgG2a of a-allotype (high affinity)	20.8.3 H and L chain from allo-immunized mouse	430
	Myelin oligodendrocyte glycoprotein and other	8.18C5 from MOG-immunized mouse with EAE	433
	MHC 1 Kb or Kk on blood or stromal cells	3.83 H&L from allo-immunized mouse	404,413,416
	Membrane-bound lysozyme on blood or stromal cells	HyHEL10 from repeatedly immunized mouse	401,412,417
	Alpha-Gal carbohydrate xenoantigen	M86 H and L from alphaGal-transferase KO mouse	428
	Cardiolipin, histones, cytoplasmic and nuclear antigens	2F5 H and L antibody to HIV gp41 from chronically infected patient	420
	Membrane	3H9 germline VH	432

phosphatidylserine		
Double-stranded DNA, phosphatidylserine	3H9 VH and Vk (Vk4) from MRL/lpr lupus mouse	414,415,438
Double-stranded DNA, nuclear antigens	3H9 VH with Vlambda2* from MRL/lpr lupus mouse	438,519
Double-stranded DNA higher affinity	3H9 VH with 56R CDR2 mutation from MRL/lpr lupus mouse	419,520
Double-stranded DNA	SP6 H and L chain to TNP hapten	431
Sialylated glycolipids, Sialyl-Lewis ×	GAS VH5-51 and Vk1 from Waldenstroms macroglobulinemia cryoglobulin	429

BCR, B-cell receptor; CD, cluster of differentiation; DNA, deoxyribonucleic acid; Ig, immunoglobulin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; RNA, ribonucleic acid.

Green means “go”; yellow means moderate warning (moderately self-reactive); orange means strong warning (strongly self-reactive); and red means “stop” (very self-reactive).

Monospecificity, Polyspecificity, and the Need to Balance Immunity with Self-Tolerance

An overwhelming lesson from the studies summarized in Table 32.2 is that many self-reactive B cells are not eliminated in the bone marrow but reach the spleen and in many cases circulate to lymph nodes. Why are so many self-reactive B cells allowed to reach the spleen and circulation? The likely explanation is that the immune system cannot afford simply to discard antibodies that have unwanted self-reactivity and has the opportunity to remodel these antibodies by hypermutation and purifying selection in germinal centers[405,406] (Fig. 32.5). There are compelling mathematical arguments that germline antibodies need to be polyspecific to provide adequate coverage of microbial epitopes with a finite pool of preimmune B cells. Increasing the crossreactivity and polyspecificity of each antibody minimizes the chance of having a gap in the repertoire due to limited B cells but this is offset by progressively larger holes in the antibody repertoire due to these antibodies also binding self-antigens.[407,408,409,410]

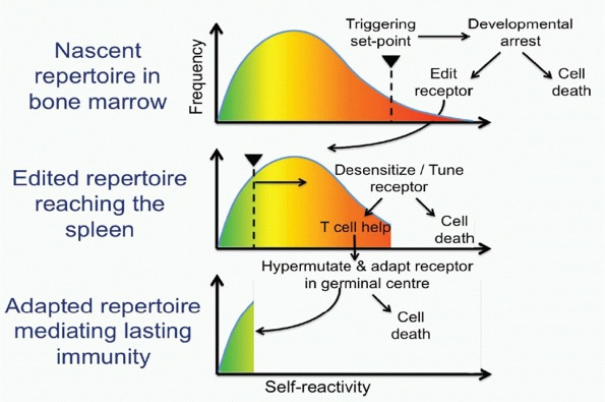

FIG. 32.5. Gradual Evolution of Antibodies with Minimal Self-Reactivity. Self-reactive B cells are eliminated in a series of steps that balances the need for self-tolerance against the need to maintain clones for immunity. (*Top*) Hypothetical distribution of clones are plotted by degree of autoreactivity (x axis) against clone frequency (y axis). The degree of autoreactivity is a function of the amount of autoantigen presented and the avidity with which it is bound by the clone. Most newly formed B-cell clones have low but appreciable self-reactivity, but only a subset appear to exceed an inherited set point and trigger either elimination in the bone marrow or editing to lower self-reactivity. Clones with less autoreactivity are exported to the periphery, illustrated by the hypothetical distribution of clones in the *middle*. By this time, repeated binding of autoantigens has tuned down surface immunoglobulin signaling in the more self-reactive end of the spectrum, illustrated by *orange shading*. Binding of autoantigen and competition for follicular niches also trigger exclusion and death of the more self-reactive clones in the T-cell zones, although these cells can potentially be rescued by T cells if they bind foreign antigens with much higher avidity than they bind to self. As a result of follicular competition, together with hypermutation and further selection in germinal centers, the B-cell repertoire that recirculates for weeks or months among lymphoid tissues is skewed toward a small subset of B cells with the least autoreactivity (*bottom*). A much larger range of newly produced clones is nevertheless available in the T-cell zones of the spleen (*middle*) to be tested for its fit against microbial antigens and potentially recruited for transient antibody responses or remodeling in germinal centers. (Adapted from Goodnow[405]).

Indeed, a large fraction of the preimmune antibody repertoire does exhibit polyspecificity that includes binding to a variety of self-antigens.[22,23,24,25,26,27,28] This may reflect conformational flexibility in the binding sites of germline antibodies, which then become locked into a monospecific shape by point mutations arising during germinal center selection.[411] Allowing many B cells bearing antibodies with self-reactivity to reach the peripheral lymphoid tissue may enable gradual refinement of these antibodies in germinal centers by hypermutation and the twin forces of purifying selection to “breed out” the negative trait of self-reactivity and positive selection for better binding to foreign microbes.[405,406]

B-Cell Receptor Editing in the Bone Marrow

Nemazee's analysis of Ig-transgenic mice expressing an antibody to the MHC I protein, $H2K^k$, initially suggested the B cells were deleted as soon as they expressed this antibody and bound the self-antigen on neighboring bone marrow cells, because no cells with surface IgM could be detected.[403,404] However, more sensitive staining and cell mixing experiments in membrane lysozyme transgenic mice revealed that the immature B cells did not initiate apoptosis in the first day or two after binding the self-antigen but instead they actively downregulated their surface IgM to very low levels and arrested their maturation at a stage that was intermediate between immature B cells and small pre-B cells.[412] This response was reversible if self-antigen ceased to bind to the arrested B cells before the onset of apoptosis: when removed from membrane lysozyme, the B cells regained high surface IgM and matured to the IgD positive stage. This seemed an oddly precarious way to eliminate forbidden clones of B cells.

Nemazee[413] and Weigert[414] independently recognized that the transgenic mice had illuminated an entirely new mechanism for actively acquired tolerance: instead of clonal selection as envisaged by Burnet, this was a system for *receptor selection*. Nemazee showed the B cells that had downregulated their surface IgM had also increased their expression of *Rag2* mRNA, encoding the Ig-gene recombinase, and contained abundant circular DNA excision products from rearrangements of the endogenous lambda light chain.[413] He proposed that strong binding of self-antigens actively induced “receptor editing”: by initiating another round of light-chain rearrangement, the self-reactive antibody could be replaced by one that was less self-reactive. Indeed, a subset of the B cells with the self-reactive antibody to H2K or to double-stranded DNA in Weigert's experiments were salvaged from clonal deletion and matured by expressing less self-reactive antibodies comprised of the transgenic heavy chain paired with an “editor” light chain derived from an endogenous light chain gene rearrangement. The latter were highly selected and did not bind MHC I or double-stranded DNA.

In the transgenic experiments, receptor editing was nevertheless inefficient because the transgenic light chain gene was located at a random chromosomal site where it was not flanked by upstream Vκ elements that could be joined to downstream Jκ elements. Second generation Ig-transgenic mice were then generated with the rearranged light chain VJκ exons integrated into the endogenous light chain locus flanked by upstream Vκ and downstream Jκ elements.[415,416,417] In these “knock-in” Ig-transgenic animals, self-antigen binding

triggered efficient replacement of the offending light chain by joining an upstream Vκ element to a downstream Jκ element. Indeed, when competitive bone marrow chimeras were established, bone marrow B cells that started out with prerearranged but self-reactive receptors nevertheless produced edited mature B cells as efficiently as competing B cells that needed to rearrange their H and L chain genes de novo.[418] The efficiency with which a self-reactive antibody can be edited is likely to vary depending on what fraction of the light chain repertoire can serve as "editors" and exhibit sufficiently lower binding to self. Some H-chains have such a strong propensity to bind self-antigens like DNA, (for example, binding to DNA due to long CDR3 loops and multiple charged arginines in their binding site) that a very small minority of oppositely charged light chains can salvage these H-chains.[419,420]

Analyses of mice with normal antibody genes indicate that 20% to 50% of productive light chain rearrangements are edited by a second light chain rearrangement.[421,422,423] This is likely to be driven primarily by editing of self-reactivity because 55% of the antibodies produced by immature B cells with undetectably low surface IgM are poly-specific for self-antigens whereas only 7% of the antibodies in surface IgM+ immature B cells are poly-specific. The downregulation of surface IgM that is induced by self-antigen binding[412] is comparable to the downregulation of non-self-reactive H-chain that occurs when they pair with surrogate light chains in pre-B cells that have yet to rearrange their light chain genes.[424] When a light chain displaces surrogate light chain and IgM accumulates on the B cell surface, the receptor complex spontaneously signals through the PI3-kinase pathway and inactivates the Foxo1 transcription factor, which in turn is required for *Rag1* and *Rag2* gene expression. Downregulation of surface IgM by self-antigen allows Foxo1 to accumulate, reactivate *Rag1* and *Rag2*, and induce a second round of light chain recombination.[425,426,427]

Dramatic downregulation of surface IgM, developmental arrest at the pre-B/immature B cell transition, followed by receptor editing or deletion, has been demonstrated in Igtransgenic mice expressing many different antibodies (see Table 32.2). These include damaging antibodies to the blood group surface antigens alpha-Gal[428] and sialyl-Lewis X,[429] antibody with high affinity for IgG2a,[430] a bispecific antibody that binds DNA and the foreign hapten TNP,[431] antibody against membrane phosphatidylserine with low affinity for DNA,[432] a demyelinating antibody to myelin oligodendrocyte glycoprotein that cross-reacts with an additional unknown selfantigen,[433] nephrotoxic antibodies to glomerular basement collagen or laminin—the latter bispecific for DNA[434,435]—and a broadly neutralizing antibody to human immunodeficiency virus (HIV) that is also polyspecific for cardiolipin, histones, cytoplasmic, and nuclear antigens.[420] The last example analyzing the 2F5 antibody to HIV provides a compelling clinical example of why the immune system cannot afford to discard antibodies with self-reactivity: in this case, it appears that cross-reactivity between a conserved epitope on the virus and multiple self-antigens stands in the way of developing broadly neutralizing immunity.

Anergy and Peripheral Deletion of Self-Reactive B Cells

Depending on their affinity and specificity, self-reactive B cells that escape or fall below the threshold required to induce developmental arrest and editing in the bone marrow follow two general fates in the spleen: 1) antigen/antibody pairs where the self-reactive B cells become mature follicular cells marked as IgD^{high} $CD24^{low}$ CD93- and L-selectin+, which recirculate to lymph nodes and may be relatively longlived; and 2) B cells that are very short-lived, mostly found in the spleen at the immature transitional stage marked as IgD^{low} $CD24^{high}$ CD93+ and L-selectin-, and few recirculate and reach the lymph nodes (see Table 32.2).

Anergy in Recirculating Immunoglobulin D^{high} B Cells

The longer-lived, recirculating self-reactive B cells are characterized by selective downregulation of surface IgM but high surface IgD. In Ig-transgenic mice with varying concentrations of soluble lysozyme, the extent of IgM downregulation is closely correlated with the fraction of antigen-engaged receptors and occurs after the cells have passed through an IgM^{high} IgD^{low} CD93+ $CD24^{hi}$ transitional stage.[395,398] This prompted the hypothesis that the broad range of surface IgM that exists from cell to cell among IgD^{high} L-selectin+ B cells in the normal repertoire reflects variable degrees of self-reactivity and that the 25% of IgD+ B cells with lowest surface IgM were anergic.[395] Recent analyses in humans confirm this is indeed the case.[436,437] The IgM^{low} IgD^{high} fate has been observed for B cells in Ig-transgenic mice with antibodies that have relatively low affinity to single-stranded DNA and potentially other autoantigens,[415,438,439] antibodies that are polyspecific for DNA and the foreign hapten arsonate,[440,441,442] a lupus antibody to the Smith ribonucleoprotein antigen,[443,444] and an antibody to insulin.[445,446] A similar fate

occurs in normal humans for the ~10% of naive B cells that use the VH4-34 heavy chain variable segment, which is almost invariably self-reactive to I/i glycolipids in the membrane of erythrocytes.[447]

Selective downregulation of IgM but not IgD on anergic cells is due to rapid recycling of internalized antigen-IgD complexes back to the cell surface[448] and to degradation of newly synthesized IgM in the endoplasmic reticulum.[449] The halt to IgM traffic probably results from being outcompeted for CD79 subunits with IgD, whose mRNA is increased in anergic cells,[450] and to changes in chaperone activity from depletion of endoplasmic reticulum calcium, due to constant release of calcium to the cytoplasm by inositol-3-phosphate. Selective downregulation of IgM occurs in the absence of antigen-binding when inositol-3-phosphate activity is not dampened by the enzyme inositol 1,4,5-trisphosphate 3-kinase B.[451]

The antigen receptors on the surface of anergic B cells are desensitized at a proximal point in their intracellular signaling cascade, activating less tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motifs in CD79alpha and beta and Syk kinase, with a concomitant decrease in the magnitude of intracellular calcium.[440,452,453,454] Desensitization of antigen receptors on anergic cells depends upon normal function of the SHP-1 (PTP1C) tyrosine phosphatase[455] and the antigen receptor-activated tyrosine kinase Lyn.[456] Lyn initiates antigen receptor signaling but at the same time it activates SHP-1 as a negative feedback, by phosphorylating immunoreceptor tyrosine-based inhibitory motifs in inhibitory cell surface receptors such as CD22, Siglec-G, and CD72 that recruit SHP-1 to antigenbound receptors.[456,457,458,459,460,461]

Desensitization of the intracellular response to antigen in anergic B cells is nevertheless pathway specific. It selectively dampens intracellular signals that activate the NF-κB transcription factor and the c-jun N-terminal kinase.[462] Low magnitude (~200 nM) spikes of intracellular calcium are repeatedly induced as long as anergic B cells continue to be exposed to self-antigen.[462] While these are too low for NF-κB activation, they are sufficient to promote repeated cycles of calcineurin-mediated dephosphorylation of the NFATc1 and NFATc2 transcription factors, triggering their nuclear translocation and inducing a subset of NFAT-target genes.[450,462,463] Likewise, continuous activation of the MEK-ERK kinase pathway in anergic B cells induces target genes like *Egr1* and opposes induction of the plasma cell transcription factor Blimp-1.[450,462,464,465] Rather than making no response to antigen, the anergic B cells induce a unique pattern of mRNAs when they encounter antigen compared to B cells that encounter the same antigen as a foreign protein.[450]

These early events of continuous calcium spiking and Egr1 induction cease if the B-cell antigen receptors stop being exposed to self-antigen.[453,462] Recovery of the intracellular calcium flux in response to a new round of antigen-engagement occurred within minutes of removal from self-antigen in polyspecific DNA/TNP B cells,[453] whereas it had not recovered after 36 hours in lysozyme-anergic B cells.[452] In the latter, full recovery of surface IgM takes between 4 and 10 days, and the capacity to make a T-cell-dependent plasma cell response does not recover even 10 days after removal from antigen, as long as the B cells remain in a resting, follicular state and are not activated into cycle through other receptors such as TLRs or by primed helper T cells.[466] Thus both short-lived and long-lived inhibitory feedbacks are at work in anergic B cells, and the latter are still poorly understood.

Short-lived, Immunoglobulin M^{low} Immunoglobulin D^{low} Anergic B Cells

The short-lived splenic fate has been observed for self-reactive B cells bearing antibodies with intermediate affinity to double-stranded and single-stranded DNA,[441,467,468,469,470] ribonucleic acid,[471] high-affinity human rheumatoid factor antibody,[472,473,474] high-affinity arthritogenic antibodies to glucose-6-phosphate isomerase,[475] hemolytic antibody to erythrocytes,[476,477,478] very-low-affinity autoantibody to a glyco-epitope on the T-cell membrane protein Thy1,[479] membrane lysozyme or membrane H2K^b limited to 5% to 10% of blood cells,[480,481] and membrane H2K^b MHC molecule or a kappa light chain crosslinking "superantigen" limited to liver hepatocytes.[482,483,484]

In each of these examples, the self-reactive B cells develop in the bone marrow with little downregulation of IgM and hence most cells are not edited at that point. The B cells are found in the spleen primarily as transitional immature cells

with downregulated surface IgM and low IgD, low complement receptor CD21, little CD62L lymph node homing receptor, and high expression of CD24 and CD93. Anergic, self-reactive B cells with this phenotype represent ~5% of the peripheral B cell pool in normal mice.[485] The IgM^{low} B cells persist in the spleen for only 1 to 3 days before being eliminated by the

intrinsic, Bcl2-regulated apoptosis pathway[400] that reflects antigen-induced increase in the proapoptotic Bcl-2 inhibitor Bim.[486,487] Anergic B cells in the spleen require higher concentrations of the anti-apoptotic cytokine BAFF for their survival compared to B cells that are not selfreactive,[487] and their elimination can be delayed or blocked in animals that have increased BAFF.[484,488] This heightened dependence upon BAFF provides a basis for the selective clinical benefits of BAFF antagonists in some people with autoantibody-mediated diseases.[489]

During the time that short-lived anergic B cells remain in the spleen, they are excluded from the B-cell follicles and concentrate at the junction with the T-cell zones.[400,467,471,475] Concentration at the T-B junction is a general effect observed in B cells that have bound antigen, either self or foreign, and is due to BCR signals that increase CCR7 expression and enhance responsiveness to the CCL19 and CCL21 chemokines produced by stroma in the T zone.[490] When B cells that have acutely bound foreign antigen are attracted to this site, they are also triggered to express high levels of the T-cell costimulatory molecule CD86 and to enter G1 of cell cycle. By contrast, self-reactive B cells have already been exposed to the same antigen in the bone marrow,[398,445] and as a result have become desensitized and do not induce CD86 or enter cell cycle when antigen signals them to migrate towards the T zone in the spleen.[452,491] Although they can present antigen to unprimed T cells, this leads to active killing of the anergic B cells through the sequential action of T cell-derived CD40L and FasL.[284,492,493]

Salvaging Self-Reactive Antibodies from Anergic B Cells

A key point is that desensitization to antigen is relative and not absolute. Anergic B cells can activate high intracellular calcium flux, induce CD86, and collaborate with naïve T cells to proliferate and make antibody if their antigen receptors are stimulated by a much more potent agonist than the one to which they have grown accustomed. In other words, they are not unresponsive but have tuned down their responsiveness to antigen, similar to that suggested for selfreactive T cells (see previous discussion). This is illustrated by the calcium flux and plasma cell reaction generated when B cells anergic to monomeric soluble lysozyme are acutely stimulated by polymeric membrane lysozyme and provided with helper T cells that recognize foreign MHC II molecules on the B cells.[452] Similarly, biotin-streptavidin polymerized lysozyme, but not monomeric lysozyme, was able to transduce signals that block Fas-mediated apoptosis in lysozyme anergic B cells, whereas both agonists delivered the protective signal in naive B cells.[494]

Primed T follicular helper cells[278,279] may also salvage anergic B cells that are multispecific and also bind foreign antigen. As antigen-presenting cells, anergic B cells are poor inducers of IL-4 and OX40 from unprimed helper T cells and this is a result of their lack of CD86, because anergic B cells that express CD86 constitutively recover the capacity to induce these helper T cell proteins, to evade Fas-killing, and to mount T-cell-dependent antibody responses.[495] IL-4 is well documented to override Fas-mediated apoptosis in B cells.[494,496,497] Moreover, while anergic B cells make a muted proliferative response to CD40 stimulation alone, the combination of CD40 and IL-4 stimuli elicits heightened proliferation compared to naïve B cells.[452,468,498] This switching capacity of IL-4 may enable primed follicular helper T cells to salvage B cells with antibodies that cross-react with foreign and self-antigens and induce their proliferation in germinal center reactions, where their antibodies can be remodeled to eliminate self-reactivity but preserve or enhance binding to foreign antigens.[405]

T cell-independent stimuli, in the form of foreign antigens linked to ligands for TLRs, can also divert short-lived anergic B cells from death to proliferation. This is best exemplified by the short-lived anergic B cells that result from an encounter with the H-2K^b protein in the liver while en route to the spleen.[483] Potent cross-linking of their antigen receptors by bacteriophages bearing a mimotope of the K^b molecule rescued these anergic B cells from peripheral deletion in the spleen and stimulated them into autoantibody formation. Reversal from anergy in this instance may result from the absence of liver-specific antigen in the spleen. Continuous exposure to self-antigen in lysozyme anergic B cells resulted in the sustained activation of the ERK signaling pathway, which blocked plasma cell differentiation and antibody secretion even when the B cells were induced to divide with TLR9 or TLR4 stimulation.[464,465] In addition, some self-antigen/antibody pairs result in a state of anergy in the short-lived splenic B cells that blocks the proliferative response to TLR4 ligands.[468,471] Collectively, these experiments indicate that abnormal processing of endogenous TLR ligands like DNA or ribonucleic acid,[499,500,501,502,503] or an overactive response of TLRs, may cause aberrant rescue of selfreactive B cells.[471,504,505,506,507]

In several antigen/antibody pairs analyzed in transgenic mice, B cells bearing self-reactive

antibodies are anergic and short-lived in the spleen, yet become expanded in the peritoneal cavity as B1 cells where they secrete autoantibodies. In Ig-transgenic mice expressing an antibody against erythrocytes, expansion of B1 cells depends upon their relative shielding from erythrocytes in this compartment[476,477] and requires concurrent infection with pathogenic or commensal microorganisms.[478] It is not known whether the infectious microorganisms present a cross-reactive foreign antigen that stimulates the self-reactive B cells or if they simply provide potent TLR stimulation of B cells. In either case, the IgM antibody that is secreted binds with high avidity to erythrocytes and causes severe autoimmune hemolytic anemia.

A similar situation occurs in Ig-transgenic mice expressing a low-affinity germline antibody against a carbohydrate epitope on the T-cell protein, Thy1, where the B cells are arrested as anergic immature cells in the spleen, few reach the lymph nodes, but they accumulate as B1 cells in the peritoneal cavity and secrete anti-Thy1 IgM autoantibody.[479]

In this case, the antibody binds with insufficient affinity to Thy1 to cause any depletion of T cells. Remarkably, the induction of B1 cells and autoantibodies requires the Thy1 autoantigen and is abolished in Thy1-deficient mice. It is not yet known if this also requires infection with commensal or pathogenic microbes, like the antierythrocyte B1 cells. When Thy1 is restored at 10% of normal levels in the mice, it changes the fate of the self-reactive B cells again by inducing their maturation into marginal zone B cells that do not secrete autoantibody.[508] These experiments support the view that some low-affinity polyspecific antibodies may be positively selected into the plasma cell, B1, or marginal zone pool by their binding to self-antigens, because these antibodies are not harmful to self-tissues but represent protective antibodies against common microbial antigens.[27]

Clonal Ignorance in Self-Reactive B Cells

The fate of "clonal ignorance," where self-reactive B cells are not tolerized because they do not bind sufficient autoantigen, has also been observed for a number of antibodies expressed in Ig-transgenic mice. These include a low affinity rheumatoid factor antibody against IgG,[509,510] a polyspecific VH1-69 germline antibody from a chronic lymphocytic leukemia,[473,511] an antibody against the lupus and Sjögren syndrome antigen SSB/La,[512] and a low-affinity antibody against single-stranded DNA,[469] although in this case the B cells displayed some level of anergy.[415,438,439] In the case of the low-affinity rheumatoid factor antibody, careful analysis revealed that the self-reactive B cells were not ignorant per se, but a small number were actively stimulated by selfantigen to form short-lived, nonproductive germinal center reactions.[510] This has some parallels with the stimulation of autoantibody production by low-affinity binding of Thy-1 self-antigen, although in that case the B cells were excluded from the follicular/germinal center pathway and formed plasma cells via the B1 B-cell pathway.[479]

TOLERANCE FROM BENCH TO BEDSIDE—A PLAY IN THREE ACTS

This chapter summarizes the enormous progress that has been made in understanding immunologic tolerance and the long and winding road involved. But it is fair to say that we are only in the early parts of a play of three acts.

The first act has been to define the physiologic mechanisms of actively acquired tolerance. This has proved much more complex than any human mind could have dreamt, and while many mechanisms have been defined, the recent discoveries of Foxp3+ T^{reg}s and *AIRE* indicate that even this first act is still in full swing. There are likely to be entirely new mechanisms revealed in the future, and answers are yet to come about fundamental questions like how lymphocytes choose alternative fates of death, survival, or growth at a given tolerance checkpoint.

The second act is to define the root cause of spontaneous autoimmune disease. Rapid progress is currently occurring in this area, particularly in simpler, monogenic forms of autoimmunity following on from autoimmune lymphoproliferative syndrome, APS-1, and IPEX. In the more common sporadic autoimmune diseases, MHC is the largest shared genetic factor, yet we still do not understand why particular MHC haplotypes are so strongly associated with particular autoimmune diseases and not with particular infectious diseases. With respect to the non-MHC heritable elements, unanswered questions remain as to what proportion are common versus rare genetic variants. Currently, this is an area of great activity, through genome-wide association studies of unparalleled size and the dawn of an exciting era of whole exome and genome sequencing in patient cohorts.

The third act will be to use the knowledge gained in the first and second acts to develop specific agents that induce or restore physiologic mechanisms of actively acquired tolerance in autoimmunity, allergy, or transplantation, or agents that break tolerance to allow immune clearance of cancer cells or chronic infections like HIV. The success of bone marrow transplantation in children with primary immunodeficiency, where immunosuppressive agents

can be withdrawn within months, represents progress directly from Medawar's bench to the modern bedside. The induction of tolerance to foreign RhD erythrocyte alloantigens in primiparous RhD-negative mothers is the best present-day example of the ability to induce antigen-specific tolerance reliably, although in this case clinical development was empirical and we do not really understand why it works. Analysis of B- and T-cell anergy has revealed the limitations of cyclosporin and tacrolimus as blocking both lymphocyte activation and actively acquired tolerance,[236,450] and helped promote a shift to "tolerance sparing" immunosuppressive drugs like rapamycin and mycophenolate.

Perhaps the most exciting evidence for what is to come is the clinical approval in 2011 of two human antibodies that really begin to use the amassed knowledge of tolerance for rational therapeutics. One is a blocking antibody to BAFF, belimumab (Benlysta), which is supported by the principle that self-reactive B cells require more BAFF than non-self-reactive B cells.[400,484,487,488] This heightened dependence upon BAFF provides a basis for the selective clinical benefits of BAFF antagonists in some people with autoantibody-mediated diseases.[489]

The other milestone is the clinical approval for a blocking antibody to CTLA-4, ipilimumab (Yervoy),[513,514] which works on the principle that T cells that recognize melanoma antigens are not all deleted but are unable to mount a beneficial autoimmune response. Neither Yervoy nor Benlysta represent a "penicillin moment" for clinical therapy. They are a small step for most patients, but the clinical response that occurs in a subset represents a giant leap for mankind. These agents mark the start of the age when we understand immunologic tolerance sufficiently to induce, restore, or suspend it in practical ways.

REFERENCES

1. Ehrlich P, Morgenroth J. *On Haemolysins*. London, United Kingdom: Pergamon Press; 1957.

2. Burnet FM, Fenner F. Genetics and immunology. *Heredity*. 1948;2: 289-324.

3. Burnet FM, Fenner F. *The Production of Antibodies. 2nd ed* . Melbourne, Australia: Macmillan & Co; 1949.

4. Owen RD. Immunogenetic consequences of vascular anastomoses between bovine twins. *Science*. 1945;102:400-401.

5. Traub E. Factors influencing the persistence of choriomeningitis virus in the blood after clinical recovery. *J Exp Med.* 1938;68:229-251.

6. Claman HN. Tolerance to a protein antigen in adult mice and the effect of nonspecific factors. *J Immunol*. 1963;91:833-839.

7. Talmage DW, Pearlman DS. The antibody response: a model based on antagonistic actions of antigen. *J Theoret Biol* . 1963;5:321-339.

8. Moller G. One non-specific signal triggers B lymphocytes. *Transplant Rev.* 1975;23:126-137.

9. Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harbor Symp Quant Biol*. 1989;54:1-13.

10. Janeway CA Jr, Goodnow CC, Medzhitov R. Danger - pathogen on the premises! Immunological tolerance. *Curr Biol* . 1996;6:519-522.

11. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*. 1998;282:2085-2088.

12. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell*. 2006;124:783-801.

13. Burnet FM, Stone JD, Edney M. The failure of antibody production in the chick embryo. *Aust J Exp Biol Med Sci*. 1950;28:291-297.

14. Nossal GJV. The immunological response of foetal mice to influenza virus. *Aust J Exp Biol*. 1957;35:549-558.

15. Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells.

Nature. 1953;172:603-606.

16. Talmage DW. Allergy and immunology. *Ann Rev Med*. 1957;8:239-256.

17. Burnet FM. A modification of Jerne's theory of antibody production using the concept of clonal selection. *Aust J Sci.* 1957;20:67-69.

18. BurnetFM. *The Clonal Selection Theory of Acquired Immunity* . Cambridge, MA: Cambridge University Press; 1959.

19. Nossal GJ, Lederberg J. Antibody production by single cells. *Nature*. 1958;181:1419-1420.

20. Lederberg J. Genes and antibodies. *Science*. 1959;129:1649-1653.

21. Moller G. The V gene repertoire as revealed by polyclonal B cell activators. *Annales d'immunologie*. 1976;127:449-465.

22. Prabhakar BS, Saegusa J, Onodera T, Notkins AL. Lymphocytes capable of making monoclonal autoantibodies that react with multiple organs are a common feature of the normal B cell repertoire. *J Immunol*. 1984;133:2815-2817.

23. Dighiero G, Lymberi P, Holmberg D, Lundquist I, Coutinho A, Avrameas S. High frequency of natural autoantibodies in normal newborn mice. *J Immunol*. 1985;134:765-771.

24. Ternynck T, Avrameas S. Murine natural monoclonal autoantibodies: a study of their polyspecificities and their affinities. *Immunol Rev*. 1986;94:99-112.

25. Seigneurin JM, Guilbert B, Bourgeat MJ, Avrameas S. Polyspecific natural antibodies and autoantibodies secreted by human lymphocytes immortalized with Epstein-Barr virus. *Blood*. 1988;71:581-585.

26. Casali P, Notkins AL. Probing the human B-cell repertoire with EBV: polyreactive antibodies and CD5+ B lymphocytes. *Ann Rev Immunol*. 1989;7:513-535

27. Avrameas S. Natural autoantibodies: from "horror autotoxicus" to "gnothi seauton." *Immunol Today*. 1991;12:154-159.

28. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. *Science*. 2003;301:1374-1377.

29. Goodnow CC, Basten A. Self-tolerance in B lymphocytes. *Sem Immunol*. 1989;1:125-135.

30. Lawton AR, Cooper MD. Modification of B lymphocyte differentiation by anti-immunoglobulins. *Comtemp Topics Immunobiol.* 1974;3: 193-225.

31. Nossal GJV, Pike BL. Evidence for the clonal abortion theory of B-lymphocyte tolerance. *J Exp Med.* 1975;141:904-917.

32. Metcalf ES, Klinman NR. In vitro tolerance induction of neonatal murine B cells. *J Exp Med.* 1976;143:1327-1340.

33. Dresser DW, Mitchison NA. The mechanism of immunological paralysis. *Adv Immunol*. 1968;8:129-181.

34. Chiller JM, Habicht GS, Weigle WO. Kinetic differences in unresponsiveness of thymus and bone marrow cells. *Science*. 1971;171:813-815.

35. Miller JFAP. The thymus in immunity. *Rec Austral Acad Sci*. 1971;2:82-98.

36. Zinkernagel RM, Doherty PC. Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis. *Nature*. 1974;251:547-548.

37. Nossal GJV. Cellular mechanisms of immunologic tolerance. *Ann Rev Immunol*. 1983;1:33-62.

38. Jerne NK. Towards a network theory of the immune system. *Ann Immunol (Inst Pasteur)*. 1971;125C:373-389.

39. Bretscher P, Cohn M. A theory of self-nonself discrimination: paralysis and induction involve the recognition of one and two determinants on an antigen, respectively. *Science*. 1970;169:1042-1049.

40. Dresser DW. Effectiveness of lipid and lipidophilic substances as adjuvants. *Nature*. 1961;191:1169-1171.

41. Lafferty KJ, Cunningham AJ. A new analysis of allogeneic interactions. *Aust J Exp Biol Med Sci*. 1975;53:27-42.

42. Steinman RM, Witmer MD. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc Natl Acad Sci U S A* . 1978;75:5132-5136.

43. Green DR, Flood PM, Gershon RK. Immunoregulatory T-cell pathways. *Ann Rev Immunol*. 1983;1:439-463.

44. Dorf ME, Benacerraf B. I-J as a restriction element in the suppressor T cell system. *Immunol Rev*. 1985;83:23-40.

45. Steinmetz M, Minard K, Horvath S, et al. A molecular map of the immune response region from the major histocompatibility complex of the mouse. *Nature*. 1982;300:35-42.

46. Kronenberg M, Steinmetz M, Kobori J, et al. RNA transcripts for I-J polypeptides are apparently not encoded between the I-A and I-E subregions of the murine major histocompatibility complex. *Proc Natl Acad Sci U S A* . 1983;80:5704-5708.

47. Blanckmeister CA, Yamamoto K, Davis MM, Hammerling GJ. Antigen-specific, I-A-restricted suppressor hybridomas with spontaneous cytolytic activity. Functional properties and lack of rearrangement of the T cell receptor beta chain genes. *J Exp Med*. 1985;162:851-863.

48. Brunkow ME, Jeffery EW, Hjerrild KA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Gen*. 2001;27:68-73.

49. Bennett CL, Christie J, Ramsdell F, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Gen*. 2001;27:20-21.

50. Wildin RS, Ramsdell F, Peake J, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Gen*. 2001;27:18-20.

51. Zinkernagel RM, Doherty PC. Restriction of in vitro T cell mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature*. 1974;248:701-702.

52. Shearer GM. Cell mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. *Eur J Immunol* . 1974;4:257.

53. Bevan MJ. In a radiation chimera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells. *Nature*. 1977;269: 417-418.

54. Zinkernagel RM, Callahan GN, Klein J, Dennert G. Cytotoxic T cells learn specificity for self H-2 during differentiation in the thymus. *Nature*. 1978;271:251-253.

55. Kappler JW, Marrack P. The role of H-2 linked genes in helper T cell function. IV. Importance of T cell genotype and host environment in I-region and Ir gene expression. *J Exp Med*. 1978;148:1510-1522.

56. Babbitt BP, Allen PM, Matsueda G, Haber E, Unanue ER. Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature*. 1985;317:359-361.

57. Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature*. 1987;329:512-518.

58. Allison JP, McIntyre BW, Bloch D. Tumor-specific antigen of murine T-lymphoma defined with monoclonal antibody. *J Immunol*. 1982;129:2293-2300.

59. McIntyre BW, Allison JP. The mouse T cell receptor: structural heterogeneity of molecules of normal T cells defined by xenoantiserum. *Cell*. 1983;34:739-746.

60. Kappler J, Kubo R, Haskins K, et al. The major histocompatibility complex-restricted antigen receptor on T cells in mouse and man: identification of constant and variable peptides. *Cell*. 1983;35:295-302.

61. Meuer SC, Acuto O, Hussey RE, et al. Evidence for the T3-associated 90K heterodimer as the T-cell antigen receptor. *Nature*. 1983;303:808-810.

62. Hedrick SM, Nielsen EA, Kavaler J, Cohen DI, Davis MM. Sequence relationships between putative T-cell receptor polypeptides and immunoglobulins. *Nature*. 1984;308:153-158.

63. Yanagi Y, Yoshikai Y, Leggett K, Clark SP, Aleksander I, Mak TW. A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. *Nature*. 1984;308:145-149.

64. Chien Y, Becker DM, Lindsten T, Okamura M, Cohen DI, Davis MM. A third type of murine T-cell receptor gene. *Nature*. 1984;312:31-35.

65. Kappler J, Skidmore B, White J, Marrack P. Antigen-inducible H-2 restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *J Exp Med*. 1981;153:1198-1214.

66. HÅnig T, Bevan MJ. Specificity of T-cell clones illustrates altered self hypothesis. *Nature*. 1981;294:460-462.

67. Saito T, Weiss A, Miller J, Norcross MA, Germain RN. Specific antigen-Ia activation of transfected human T cells expressing murine Ti alpha beta-human T3 receptor complexes. *Nature*. 1987;325:125-130.

68. Dembic Z, Haas W, Weiss S, et al. Transfer of specificity by murine alpha and beta T-cell receptor genes. *Nature*. 1986;320:232-238.

69. Haskins K, Hannum C, White J, et al. The antigen-specific, major histocompatibility complex-restricted receptor on T cells. VI. An antibody to a receptor allotype. *J Exp Med*. 1984;160:452-471.

70. Kappler JW, Roehm N, Marrack P. T cell tolerance by clonal elimination in the thymus. *Cell*. 1987;49:273-280.

71. Sha WC, Nelson CA, Newberry RD, Kranz DM, Russel JH, Loh DY. Selective expression of an antigen receptor on CD8-bearing T lymphocytes in transgenic mice. *Nature*. 1988;335:271-274.

72. Pircher H, BÅrki K, Lang R, Hengartner H, Zinkernagel R. Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. *Nature*. 1989;342:559-561.

73. Kisielow P, BlÅthmann H, Staerz UD, Steinmetz M, von Boehmer H. Tolerance in T cell receptor transgenic mice involves deletion of nonmature CD4 + 8 + thymocytes. *Nature*. 1988;333:742-746.

74. Teh HS, Kisielow P, Scott B, et al. Thymic major histocompatibility complex antigens and the αβ T cell receptor determine the CD4/CD8 phenotype of T cells. *Nature*. 1988;335:229-233.

75. White J, Herman A, Pullen AM, Kubo R, Kappler JW, Marrack P. The V beta-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal

deletion in neonatal mice. *Cell*. 1989;56:27-35.

76. Festenstein H. The Mls system. *Transplant Proc*. 1976;8:339-342.

77. Kappler JW, Staerz UD, White J, Marrack P. Self tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. *Nature*. 1988;332:35-40.

78. MacDonald HR, Schneider R, Lees RK, et al. T cell receptor V beta use predicts reactivity and tolerance to Mls a - encoded antigens. *Nature*. 1988;332:40-45.

79. Scherer MT, Ignatowicz L, Winslow GM, Kappler JW, Marrack P. Superantigens: bacterial and viral proteins that manipulate the immune system. *Ann Rev Cell Biol*. 1993;9:101-128.

80. Baldwin TA, Sandau MM, Jameson SC, Hogquist KA. The timing of TCR alpha expression critically influences T cell development and selection. *J Exp Med*. 2005;202:111-121.

81. Sha WC, Nelson CA, Newberry RD, Kranz DM, Russell JH, Loh DY. Positive and negative selection of an antigen receptor on T cells in transgenic mice. *Nature*. 1988;336:73-76.

82. Ramsdell F, Lantz T, Fowlkes BJ. A nondeletional mechanism of thymic self tolerance. *Science*. 1989;246:1038-1041.

83. Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR. T cell receptor antagonist peptides induce positive selection. *Cell*. 1994;76:17-27.

84. Ashton-Rickardt PG, Bandeira A, Delaney JR, et al. Evidence for a differential avidity model of T cell selection in the thymus. *Cell*. 1994;76:651-663.

85. Sebzda E, Wallace VA, Mayer J, Yeung RSM, Mak TW, Ohashi PS. Positive and negative thymocyte selection induced by different concentrations of a single peptide. *Science*. 1994;263:1615-1618.

86. Thomas RK, Folger KR, Capecchi MR. High frequency targeting of genes to specific sites in the mammalian genome. *Cell*. 1986;44: 419-428.

87. Koller BH, Smithies O. Inactivating the β-microglobulin locus in mouse embryonic stem cells by homologous recombination. *Proc Natl Acad Sci U S A* . 1989;86:8932-8935.

88. Koller BH, Marrack P, Kappler JW, Smithies O. Normal development of mice deficient in beta 2M, MHC class I proteins, and CD8+ T cells. *Science*. 1990;248:1227-1230.

89. Van Kaer L, Ashton-Rickardt PG, Ploegh HL, Tonegawa S. TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4-CD8+ T cells. *Cell*. 1992;71:1205-1214.

90. Hogquist KA, Gavin MA, Bevan MJ. Positive selection of CD8+ T cells induced by major histocompatibility complex binding peptides in fetal thymic organ culture. *J Exp Med*. 1993;177:1469-1473.

91. Ashton-Rickardt PG, Van Kaer L, Schumacher TNM, Ploegh HL, Tonegawa S. Peptide contributes to the specificity of positive selection of CD8+ T cells in the thymus. *Cell*. 1993;73:1041-1049.

92. Sebzda E, KÅndig TM, Thomson CT, et al. Mature T cell reactivity altered by a peptide agonist that induces positive selection. *J Exp Med*. 1996;183:1093-1104.

93. Chidgey AP, Boyd RL. Positive selection of low responsive potentially autoreactive T cells induced by high avidity, non-deleting interactions. *Int Immunol*. 1998;10:999-1008.

94. Alam SM, Travers PJ, Wung JL, et al. T-cell-receptor affinity and thymocyte positive selection. *Nature*. 1996;381:616-620.

95. Gronski MA, Boulter JM, Moskophidis D, et al. TCR affinity and negative regulation limit autoimmunity. *Nat Med.* 2004;10:1234-1239.

96. Leishman AJ, Gapin L, Capone M, et al. Precursors of functional MHC class 1- or class II-restricted CD8alphaalpha + T cells are positively selected in the thymus by agonist self-peptides. *Immunity*. 2002;16:355-364.

97. Yamagata T, Mathis D, Benoist C. Self-reactivity in thymic doublepositive cells commits cells to a CD8 alpha alpha lineage with characteristics of innate immune cells. *Nat Immunol*. 2004;5:597-605.

98. Alberola-Ila J, Forbush KA, Seger R, Krebs EG, Perlmutter RM. Selective requirement for MAP kinase activation in thymocyte differentiation. *Nature*. 1996;373:620-623.

99. Delgado P, Fernandez E, Dave V, Kappes D, Alarcon B. CD3Î couples T-cell receptor signalling to ERK activation and thymocyte positive selection. *Nature*. 2000;406:426-430.

100. Werlen G, Hausmann B, Palmer E. A motif in the alpha beta T-cell receptor controls positive selection by modulating ERK activity. *Nature*. 2000;406:422-426.

101. Mariathasan S, Zakarian A, Bouchard D, Ohashi PS. Duration and strength of extracellular signal-regulated kinase signals are altered during positive versus negative thymocyte selection. *J Immunol*. 2001;167:4966-4973.

102. McNeil LK, Starr TK, Hogquist KA. A requirement for sustained ERK signaling during thymocyte positive selection in vivo. *Proc Natl Acad Sci U S A* . 2005;102:13574-13579.

103. Hailman E, Burack R, Shaw AS, Dustin ML, Allen PM. Immature CD4 + CD8 + thymocytes form a multifocal immunological synapse with sustained tyrosine phosphorylation. *Immunity*. 2002;16:839-848.

104. Pages G, Guerin S, Grall D, et al. Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. *Science*. 1999;286:1374-1377.

105. Fischer AM, Katayama CD, Pages G, Pouyssegur J, Hedrick SM. The role of erk1 and erk2 in multiple stages of T cell development. *Immunity*. 2005;23:431-443.

106. Neilson JR, Winslow MM, Hur EM, Crabtree GR. Calcineurin B1 is essential for positive but not negative selection during thymocyte development. *Immunity*. 2004;20:255-266.

107. Priatel JJ, Teh SJ, Dower NA, Stone JC, Teh HS. RasGRP1 transduces low-grade TCR signals which are critical for T cell development, homeostasis, and differentiation. *Immunity*. 2002;17:617-627.

108. Costello PS, Nicolas RH, Watanabe Y, Rosewell I, Treisman R. Ternary complex factor SAP-1 is required for Erk-mediated thymocyte positive selection. *Nat Immunol*. 2004;5:289-298.

109. Mylona A, Nicolas R, Maurice D, et al. The essential function for serum response factor in T-cell development reflects its specific coupling to extracellular signal-regulated kinase signaling. *Mol Cell Biol*. 2011;31:267-276.

110. Risueno RM, van Santen HM, Alarcon B. A conformational change senses the strength of T cell receptor-ligand interaction during thymic selection. *Proc Natl Acad Sci U S A*. 2006;103:9625-9630.

111. Daniels MA, Teixeiro E, Gill J, et al. Thymic selection threshold defined by compartmentalization of Ras/MAPK signalling. *Nature*. 2006;444:724-729.

112. Nishizuka Y, Sakakura T. Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. *Science*. 1969;166:753-755.

113. Penhale WJ, Farmer A, McKenna RP, Irvine WJ. Spontaneous thyroiditis in thymectomized and irradiated Wistar rats. *Clin Exp Immunol*. 1973;15:225-236.

114. Sakaguchi S, Fukuma K, Kuribayashi K, Masuda T. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. *J Exp Med*. 1985;161:72-87.

115. Fowell D, Mason D. Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. Characterization of the CD4+ T cell subset that inhibits this autoimmune potential. *J Exp Med*. 1993;177:627-636.

116. Powrie F, Mason D. OX-22high CD4+ T cells induce wasting disease with multiple organ pathology: prevention by the OX-22low subset. *J Exp Med*. 1990;172:1701-1708.

117. Powrie F, Leach MW, Mauze S, Menon S, Caddle LB, Coffman RL. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RB hi CD4 + T cells. *Immunity*. 1994;1:553.

118. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). *J Immunol*. 1995;155:1151-1164.

119. Chatila TA, Blaeser F, Ho N, et al. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. *J Clin Invest*. 2000;106:R75-81.

120. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003;299:1057-1061.

121. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*. 2003;4:330-336.

122. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol*. 2003;4:337-342.

123. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol*. 2007;8:191-197.

124. Wing K, Onishi Y, Prieto-Martin P, et al. CTLA-4 control over Foxp3+ regulatory T cell function. *Science*. 2008;322:271-275.

125. Jordan MS, Boesteanu A, Reed AJ, et al. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol*. 2001;2:301-306.

126. Hsieh CS, Liang Y, Tyznik AJ, Self SG, Liggitt D, Rudensky AY. Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. *Immunity*. 2004;21:267-277.

127. Itoh M, Takahashi T, Sakaguchi N, et al. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol*. 1999;162:5317-5326.

128. Sakaguchi S, Sakaguchi N. Thymus and autoimmunity: capacity of the normal thymus to produce pathogenic self-reactive T cells and conditions required for their induction of autoimmune disease. *J Exp Med*. 1990;172:537-545.

129. Moran AE, Holzapfel KL, Xing Y, et al. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J Exp Med*. 2011;208:1279-1289.

130. van Santen HM, Benoist C, Mathis D. Number of T reg cells that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells. *J Exp Med*. 2004;200:1221-1230.

131. Negishi I, Motoyama N, Nakayama K, et al. Essential role for ZAP-70 in both positive and negative selection of thymocytes. *Nature*. 2005;376:435-438.

132. Turner M, Mee PJ, Walters AE, et al. A requirement for the Rho-family GTP exchange factor Vav in positive and negative selection of thymocytes. *Immunity*. 1997;7:451-460.

133. Sommers CL, Lee J, Steiner KL, et al. Mutation of the phospholipase C-γ1-binding site of LAT affects both positive and negative thymocyte selection. *J Exp Med*. 2005;201:1125-1134.

134. Rivera RR, Johns CP, Quan J, Johnson RS, Murre C. Thymocyte selection is regulated by the helix-loop-helix inhibitor protein, Id3. *Immunity*. 2000;12:17-26.

135. Jang IK, Zhang J, Chiang YJ, et al. Grb2 functions at the top of the T-cell antigen receptor-induced tyrosine kinase cascade to control thymic selection. *Proc Natl Acad Sci U S A*. 2010;107:10620-10625.

136. Brewer JA, Kanagawa O, Sleckman BP, Muglia LJ. Thymocyte apoptosis induced by T cell activation is mediated by glucocorticoids in vivo. *J Immunol*. 2002;169:1837-1843.

137. Singer GG, Abbas AK. The Fas antigen is involved in peripheral but not thymic deletion of T lymphocytes in T cell receptor transgenic mice. *Immunity*. 1994;1:365-371.

138. Amakawa R, Hakem A, Kundig TM, et al. Impaired negative selection of T cells in Hodgkin's disease antigen CD30-deficient mice. *Cell*. 1996;84:551-562.

139. Sytwu HK, Liblau RL, McDevitt HO. The roles of Fas/APO-1 (CD95) and TNF in antigen-induced programmed cell death in T cell receptor transgenic mice. *Immunity*. 1996;5:17-30.

140. Alam A, Braun MY, Hartgers F, et al. Specific activation of the cysteine protease CPP32 during the negative selection of T cells in the thymus. *J Exp Med*. 1997;186:1503-1512.

141. Kishimoto H, Surh CD, Sprent J. A role for Fas in negative selection of thymocytes in vivo. *J Exp Med*. 1998;187:1427-1438.

142. Page DM, Roberts EM, Peschon JJ, Hedrick SM. TNF receptor-deficient mice reveal striking differences between several models of thymocyte negative selection. *J Immunol*. 1998;160:120-133.

143. Izquierdo M, Grandien A, Criado LM, et al. Blocked negative selection of developing T cells in mice expressing the baculovirus p35 caspase inhibitor. *EMBO J*. 1999;18:156-166.

144. Doerfler P, Forbush KA, Perlmutter RM. Caspase enzyme activity is not essential for apoptosis during thymocyte development. *J Immunol*. 2000;164:4071-4079.

145. DeYoung AL, Duramad.O, Winoto A. The TNF receptor family member CD30 is not essential for negative selection. *J Immunol*. 2000;165:6170-6173.

146. Wang ECY, Thern A, Denzel A, Kitson J, Farrow SN, Owen MJ. DR3 regulates negative selection during thymocyte development. *Mol Cell Biol*. 2001;21:3451-3461.

147. Hara H, Takeda A, Takeuchi M, et al. The apoptotic protease-activating factor 1-mediated pathway of apoptosis is dispensable for negative selection of thymocytes. *J Immunol*. 2002;168:2288-2295.

148. Wang J, Fu YX. LIGHT (a cellular ligand for herpes virus entry mediator and lymphotoxin receptor)-mediated thymocyte deletion is dependent on the interaction between TCR and MHC/self-peptide. *J Immunol*. 2003;170:3986-3993.

149. Villunger A, Marsden VS, Strasser A. Efficient T cell receptor-mediated apoptosis in nonobese diabetic mouse thymocytes. *Nat Immunol*. 2003;4:717.

150. Lamhamedi-Cherradi SE, Zheng SJ, Maguschak KA, Peschon J, Chen YH. Defective thymocyte apoptosis and accelerated autoimmune diseases in TRAIL -/- mice. *Nat Immunol*. 2003;4:255-260.

151. Murakami K, Liadis N, Sarmiento J, et al. Caspase 3 is not essential for the induction of anergy or multiple pathways of CD8+ T-cell death. *Eur J Immunol*. 2010;40:3372-3377.

152. Mariathasan S, Ho SSN, Zakarian A, Ohashi PS. Degree of ERK activation influences both positive and negative thymocyte selection. *Eur J Immunol*. 2000;30:1060-1068.

153. Bommhardt U, Scheuring Y, Bickel C, Zamoyska R, HÅnig T. MEK activity regulates negative selection of immature CD4+ CD8+ thymocytes. *J Immunol*. 2000;164:2326-2337.

154. Sugawara T, Moriguchi T, Nishida E, Takahama Y. Differential roles of ERK and p38 MAP kinase pathways in positive and negative selection of T lymphocytes. *Immunity*. 1998;9:565-574.

155. McGargill MA, Ch'en IL, Katayama CD, Pages G, Pouyssegur J, Hedrick SM. Cutting edge: extracellular signal-related kinase is not required for negative selection of developing T cells. *J Immunol*. 2009;183: 4838-4842.

156. Sohn SJ, Lewis GM, Winoto A. Non-redundant function of the MEK5-ERK5 pathway in thymocyte apoptosis. *EMBO J*. 2008;27:1896-1906.

157. Calnan BJ, Szychowski S, Chan FKM, Cado D, Winoto A. A role for the orphan steroid receptor Nur77 in apoptosis accompanying antigeninduced negative selection. *Immunity*. 1995;3:273-282.

158. Zhou T, Cheng J, Yang Z, et al. Inhibition of Nur77/Nurr1 leads to inefficient clonal deletion of self-reactive T cells. *J Exp Med*. 1996;183:1879-1892.

159. Cheng LE, Chan FK, Cado D, Winoto A. Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T-cell apoptosis. *EMBO J*. 1997;16:1865-1875.

160. Kovalovsky D, Pezzano M, Ortiz BD, Sant'Angelo DB. A novel TCR transgenic model reveals that negative selection involves an immediate, Bim-dependent pathway and a delayed, Bim-independent pathway. *PLoS One*. 2010;5:e8675.

161. Bouillet P, Metcalf D, Huang DCS, et al. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science*. 1999;286:1735-1738.

162. Bouillet P, Purton JF, Godfrey DI, et al. BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature*. 2002;415:922-926.

163. Hu Q, Sader A, Parkman JC, Baldwin TA. Bim-mediated apoptosis is not necessary for thymic negative selection to ubiquitous self-antigens. *J Immunol*. 2009;183:7761-7767.

163a. Daley S and CCG, submitted

164. Ludwinski MW, Sun J, Hilliard B, et al. Critical roles of Bim in T cell activation and T cell-mediated autoimmune inflammation in mice. *J Clin Invest*. 2009;119:1706-1713.

165. Kishimoto H, Sprent J. A defect in central tolerance in NOD mice. *Nat Immunol*. 2001;2:1025-1031.

166. Lesage S, Hartley SB, Akkaraju S, Wilson J, Townsend M, Goodnow CC. Failure to censor forbidden clones of CD4 T cells in autoimmune diabetes. *J Exp Med*. 2002;196:1175-1188.

167. Liston A, Lesage S, Gray DH, et al. Generalized resistance to thymic deletion in the NOD mouse; a polygenic trait characterized by defective induction of Bim. *Immunity*. 2004;21:817-830.

168. Zucchelli S, Holler P, Yamagata T, Roy M, Benoist C, Mathis D. Defective central tolerance induction in NOD mice: genomics and genetics. *Immunity.* 2005;22:385-396.

169. Liston A, Hardy K, Pittelkow Y, et al. Impairment of organ-specific T cell negative selection by diabetes susceptibility genes: genomic analysis by mRNA profiling. *Genome Biol*. 2007;8:R12.

170. McCarty N, Paust S, Ikizawa K, Dan I, Li X, Cantor H. Signaling by the kinase MINK is essential in the negative selection of autoreactive thymocytes. *Nat Immunol*. 2005;6:65-72.

171. Rincç¢n M, Whitmarsh A, Yang DD, et al. The JNK pathway regulates the in vivo

deletion of immature CD4 + CD8 + thymocytes. *J Exp Med*. 1998;188:1817-1830.

172. Sabapathy K, Kallunki T, David JP, Graef I, Karin M, Wagner EF. c-Jun NH2-terminal kinase (JNK)1 and JNK2 have similar and stage-dependent roles in regulating T cell apoptosis and proliferation. *J Exp Med*. 2001;193:317-328.

173. Harris CA, Johnson EM Jr. BH3-only Bcl-2 family members are coordinately regulated by the JNK pathway and require Bax to induce apoptosis in neurons. *J Biol Chem.* 2001;276:37754-37760.

174. Whitfield J, Neame SJ, Paquet L, Bernard O, Ham J. Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. *Neuron.* 2001;29:629-643.

175. Jolicoeur C, Hanahan D, Smith KM. T-cell tolerance toward a transgenic beta-cell antigen and transcription of endogenous pancreatic genes in thymus. *Proc Natl Acad Sci U S A*. 1994;91:6707-6711.

176. Smith KM, Olson DC, Hirose R, Hanahan D. Pancreatic gene expression in rare cells of thymic medulla: evidence for functional contribution to T cell tolerance. *Int Immunol*. 1997;9:1355-1365.

177. Derbinski J, Schulte A, Kyewski B, Klein L. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat Immunol.* 2001;2:1032-1039.

178. Derbinski J, Gabler J, Brors B, et al. Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J Exp Med*. 2005;202:33-45.

179. Aaltonen J, Consortium TF-GA. An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zincfinger domains. *Nat Genet*. 1997;17:399-403.

180. Nagamine K, Peterson P, Scott HS, et al. Positional cloning of the APECED gene. *Nat Genet*. 1997;17:393-398.

181. Perheentupa J. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. *J Clin Endocrinol Metab*. 2006;91:2843-2850.

182. Kisand K, Boe Wolff AS, Podkrajsek KT, et al. Chronic mucocutaneous candidiasis in APECED or thymoma patients correlates with autoimmunity to Th17-associated cytokines. *J Exp Med*. 2010;207:299-308.

183. Puel A, Doffinger R, Natividad A, et al. Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I. *J Exp Med*. 2010;207:291-297.

184. Heino M, Peterson P, Kudoh J, et al. Autoimmune regulator is expressed in the cells regulating immune tolerance in thymus medulla. *Biochem Biophysic Res Comm.* 1999;257:821-825.

185. Ramsey C, Winqvist O, Puhakka L, et al. Aire deficient mice develop multiple features of APECED phenotype and show altered immune response. *Hum Mol Genet.* 2002;11:397-409.

186. Anderson MS, Venanzi ES, Klein L, et al. Projection of an immunological self-shadow within the thymus by the Aire protein. *Science.* 2002;298:1395-1403.

187. Liston A, Lesage S, Wilson J, Peltonen L, Goodnow CC. Aire regulates negative selection of organ-specific T cells. *Nat Immunol*. 2003;4: 350-354.

188. Giraud M, Yoshida H, Abramson J, et al. Aire unleashes stalled RNA polymerase to induce ectopic gene expression in thymic epithelial cells. *Proc Natl Acad Sci U S A* . 2012;109(2):535-540.

189. DeVoss J, Hou Y, Johannes K, et al. Spontaneous autoimmunity prevented by thymic expression of a single self-antigen. *J Exp Med.* 2006;203:2727-2735.

190. Fan Y, Rudert WA, Grupillo M, He J, Sisino G, Trucco M. Thymusspecific deletion of insulin induces autoimmune diabetes. *EMBO J*. 2009;28:2812-2824.

191. Shum AK, DeVoss J, Tan CL, et al. Identification of an autoantigen demonstrates a link between interstitial lung disease and a defect in central tolerance. *Sci Transl Med*. 2009;1:9ra20.

192. Anderson MS, Venanzi ES, Chen Z, Berzins SP, Benoist C, Mathis D. The cellular mechanism of Aire control of T cell tolerance. *Immunity*. 2005;23:227-239.

193. Chen Z, Benoist C, Mathis D. How defects in central tolerance impinge on a deficiency in regulatory T cells. *Proc Natl Acad Sci U S A* . 2005;102:14735-14740.

194. Daniely D, Kern J, Cebula A, Ignatowicz L. Diversity of TCRs on natural Foxp3+ T cells in mice lacking Aire expression. *J Immunol* . 2010;184:6865-6873.

195. Vafiadus P, Bennett ST, Todd JA, et al. Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus. *Nat Genet.* 1997;15:289-292.

196. Pugliese A, Zeller M, Fernandez A Jr, et al. The insulin gene is transcribed in the human thymus and transcription levels correlate with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type I diabetes. *Nat Genet.* 1997;15:293-297.

197. Giraud M, Taubert R, Vandiedonck C, et al. An IRF8-binding promoter variant and AIRE control CHRNA1 promiscuous expression in thymus. *Nature*. 2007;448:934-937.

198. Claman HN, Talmage DW. Thymectomy: prolongation of immunological tolerance in the adult mouse. *Science*. 1963;141:1193-1194.

199. Brooke MS. Conversion of immunological paralysis to immunity by endotoxin. *Nature*. 1965;206:635-636.

200. Gery I, Waksman BH. Potentiation of the T-lymphocyte response to mitogens. II. The cellular source of potentiating mediator(s). *J Exp Med*. 1972;136:143-155.

201. Rosenwasser LJ, Dinarello CA, Rosenthal AS. Adherent cell function in murine T-lymphocyte antigen recognition. IV. Enhancement of murine T-cell antigen recognition by human leukocytic pyrogen. *J Exp Med*. 1979;150:709-714.

202. Calderon J, Kiely JM, Lefko JL, Unanue ER. The modulation of lymphocyte functions by molecules secreted by macrophages. I. Description and partial biochemical analysis. *J Exp Med*. 1975;142: 151-164.

203. Aarden LA. Revised nomenclature for antigen-nonspecific T cell proliferation and helper factors. *J Immunol* . 1979;123:2928-2929.

204. Talmage DW, Dart G, Radovich J, Lafferty KJ. Activation of transplant immunity: effect of donor leukocytes on thyroid allograft rejection. *Science*. 1976;191:385-388.

205. Mueller DL, Jenkins MK, Schwartz RH. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Ann Rev Immunol* . 1989;7:445-480.

206. Weaver CT, Unanue ER. The costimulatory function of antigen-presenting cells. *Immunol Today*. 1990;11:49-55.

207. Linsley PS, Clark EA, Ledbetter JA. T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. *Proc Natl Acad Sci U S A* . 1990;87:5031-5035.

208. Azuma M, Ito D, Yagita H, et al. B70 antigen is a second ligand for CTLA-4 and CD28. *Nature*. 1993;366:76-79.

209. Freeman GJ, Gribben JG, Boussiotis VA, et al. Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. *Science*. 1993;262:909-911.

210. Steinman RM, Nussenzweig MC. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc Natl Acad Sci U S A* . 2002;99:351-358.

211. Ben-Sasson SZ, Hu-Li J, Quiel J, et al. IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. *Proc Natl Acad Sci U S A* . 2009;106:7119-7124.

212. Curtsinger JM, Lins DC, Mescher MF. Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. *J Exp Med*. 2003;197: 1141-1151.

213. Curtsinger JM, Johnson CM, Mescher MF. CD8 T cell clonal expansion and development of effector function require prolonged exposure to antigen, costimulation, and signal 3 cytokine. *J Immunol*. 2003;171:5165-5171.

214. Curtsinger JM, Valenzuela JO, Agarwal P, Lins D, Mescher MF. Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. *J Immunol*. 2005;174:4465-4469.

215. Curtsinger JM, Lins DC, Johnson CM, Mescher MF. Signal 3 tolerant CD8 T cells degranulate in response to antigen but lack granzyme B to mediate cytolysis. *J Immunol*. 2005;175:4392-4399.

216. Filatenkov AA, Jacovetty EL, Fischer UB, Curtsinger JM, Mescher MF, Ingulli E. CD4 T cell-dependent conditioning of dendritic cells to produce IL-12 results in CD8-mediated graft rejection and avoidance of tolerance. *J Immunol*. 2005;174:6909-6917.

217. Hernandez J, Aung S, Marquardt K, Sherman LA. Uncoupling of proliferative potential and gain of effector function by CD8(+) T cells responding to self-antigens. *J Exp Med*. 2002;196:323-333.

218. Kolumam GA, Thomas S, Thompson LJ, Sprent J, Murali-Krishna K. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *J Exp Med*. 2005;202:637-650.

219. Lang KS, Recher M, Junt T, et al. Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. *Nat Med.* 2005;11:138-145.

220. Aichele P, Unsoeld H, Koschella M, Schweier O, Kalinke U, Vucikuja S. CD8 T cells specific for lymphocytic choriomeningitis virus require type I IFN receptor for clonal expansion. *J Immunol*. 2006;176: 4525-4529.

221. Marrack P, Kappler J, Mitchell T. Type I interferons keep activated T cells alive. *J Exp Med*. 1999;189:521-530.

222. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice: I. Morphology, quantitation, tissue distribution. *J Exp Med*. 1973;137:1142-1162.

223. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice: II. Functional properties in vitro. *J Exp Med*. 1974;139:380-397.

224. Garza KM, Chan SM, Suri R, et al. Role of antigen presenting cells in mediating tolerance and autoimmunity. *J Exp Med*. 2000;191: 2021-2027.

225. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med*. 2001;193:233-238.

226. Hawiger D, Inaba K, Dorsett Y, et al. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med*. 2001;194:769-779.

227. Lamb JR, Skidmore BJ, Green N, Chiller JM, Feldmann M. Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza hemagglutinin. *J Exp Med*. 1983;157: 1434-1447.

228. Lamb JR, Feldmann M. Essential requirement for major histocompatibility complex recognition in T-cell tolerance induction. *Nature*. 1984;308:72-74.

229. Zanders ED, Lamb JR, Feldmann M, Green N, Beverley PC. Tolerance of T-cell

clones is associated with membrane antigen changes. *Nature*. 1983;303:625-627.

230. Jenkins MK, Schwartz RH. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J Exp Med*. 1987;165:302-319.

231. Schwartz RH. A cell culture model for T lymphocyte clonal anergy. *Science*. 1990;248:1349-1356.

232. Quill H, Schwartz RH. Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. *J Immunol*. 1987;138:3704-3712.

233. Jenkins MK, Pardoll DM, Mizuguchi J, Chused TM, Schwartz RH. Molecular events in the induction of a nonresponsive state in interleukin 2-producing helper T-lymphocyte clones. *Proc Natl Acad Sci U S A* . 1987;84:5409-5413.

234. Jenkins MK, Ashwell JD, Schwartz RH. Allogeneic non-T spleen cells restore the responsiveness of normal T cell clones stimulated with antigen and chemically modified antigen-presenting cells. *J Immunol*. 1988;140:3324-3330.

235. Harding FA, McArthur JG, Gross JA, Raulet DH, Allison JP. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature*. 1992;356:607-609.

236. Macian F, Garcia-Cozar F, Im SH, Horton HF, Byrne MC, Rao A. Transcriptional mechanisms underlying lymphocyte tolerance. *Cell*. 2002;109:719-731.

237. Rammensee HG, Kroschewski R, Frangoulis B. Clonal anergy induced in mature V beta 6 T lymphocytes on immunizing Mls-1 b mice with Mls-1 a expressing cells. *Nature*. 1989;339:541-544.

238. Burkly LC, Lo D, Kanagawa O, Brinster RL, Flavell RA. T-cell tolerance by clonal anergy in transgenic mice with nonlymphoid expression of MHC class II I-E. *Nature*. 1989;342:564-566.

239. Rellahan BL, Jones LA, Kruisbeek AM, Fry AM, Matis LA. In vivo induction of anergy in peripheral V beta 8+ T cells by staphylococcal enterotoxin B. *J Exp Med*. 1990;172:1091-1100.

240. Rocha B, von Boehmer H. Peripheral selection of the T cell repertoire. *Science*. 1991;251:1225-1228.

241. Sloan-Lancaster J, Evavold B, Allen PM. Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen presenting cells. *Nature*. 1993;363:156-159.

242. Kawabe Y, Ochi A. Selective anergy of V beta 8+,CD4+ T cells in Staphylococcus enterotoxin B-primed mice. *J Exp Med*. 1990;172: 1065-1070.

243. Kawabe Y, Ochi A. Programmed cell death and extrathymic reduction of V·8+ CD4+ T cells in mice tolerant to Staphylococcus aureus enterotoxin B. *Nature*. 1991;349:245-248.

244. MacDonald HR, Baschieri S, Lees RK. Clonal expansion precedes anergy and death of V beta 8+ peripheral T cells responding to staphylococcal enterotoxin B in vivo. *Eur J Immunol* . 1991;21:1963-1966.

245. Baschieri S, Lees RK, Lussow AR, MacDonald HR. Clonal anergy to staphylococcal enterotoxin B in vivo: selective effects on T cell subsets and lymphokines. *Eur J Immunol*. 1993;23:2661-2666.

246. Renno T, Hahne M, MacDonald HR. Proliferation is a prerequisite for bacterial superantigen-induced T cell apoptosis in vivo. *J Exp Med*. 1995;181:2283-2287.

247. Renno T, Hahne M, Tschopp J, MacDonald HR. Peripheral T cells undergoing superantigen-induced apoptosis in vivo express B220 and upregulate Fas and Fas ligand. *J Exp Med*. 1996;183:431-437.

248. Renno T, Attinger A, Locatelli S, Bakker T, Vacheron S, MacDonald HR. Cutting edge: apoptosis of superantigen-activated T cells occurs preferentially after a discrete number of cell divisions in vivo. *J Immunol*. 1999;162:6312-6315.

249. Mittrucker HW, Shahinian A, Bouchard D, Kundig TM, Mak TW. Induction of unresponsiveness and impaired T cell expansion by staphylococcal enterotoxin B in CD28-deficient mice. *J Exp Med*. 1996;183:2481-2488.

250. Kearney ER, Pape KA, Loh DY, Jenkins MK. Visualization of peptidespecific T cell immunity and peripheral tolerance induction in vivo. *Immunity*. 1994;1:327-339.

251. Kearney ER, Walunas TL, Karr RW, et al. Antigen-dependent clonal expansion of a trace population of antigen-specific CD4+ T cells in vivo is dependent on CD28 costimulation and inhibited by CTLA-4. *J Immunol*. 1995;155:1032-1036.

252. Pape KA, Merica R, Mondino A, Khoruts A, Jenkins MK. Direct evidence that functionally impaired CD4+ T cells persist in vivo following induction of peripheral tolerance. *J Immunol*. 1998;160:4719-4729.

253. Rocha B, Tanchot C, von Boehmer H. Clonal anergy blocks in vivo growth of mature T cells and can be reversed in the absence of antigen. *J Exp Med*. 1993;177:1517-1521.

254. Kyburz D, Aichele P, Speiser DE, Hengartner H, Zinkernagel RM, Pircher H. T cell immunity after a viral infection versus T cell tolerance induced by soluble viral peptides. *Eur J Immunol* . 1993;23: 1956-1962.

255. Petschner F, Zimmerman C, Strasser A, Grillot D, Nunez G, Pircher H. Constitutive expression of Bcl-xL or Bcl-2 prevents peptide antigeninduced T cell deletion but does not influence T cell homeostasis after a viral infection. *Eur J Immunol* . 1998;28:560-569.

256. Kundig TM, Shahinian A, Kawai K, et al. Duration of TCR stimulation determines costimulatory requirement of T cells. *Immunity*. 1996;5: 41-52.

257. Nguyen LT, McKall-Faienza K, Zakarian A, Speiser DE, Mak TW, Ohashi PS. TNF receptor 1 (TNFR1) and CD95 are not required for T cell deletion after virus infection but contribute to peptideinduced deletion under limited conditions. *Eur J Immunol* . 2000;30: 683-688.

258. Redmond WL, Marincek BC, Sherman LA. Distinct requirements for deletion versus anergy during CD8 T cell peripheral tolerance in vivo. *J Immunol*. 2005;174:2046-2053.

259. Hernandez J, Aung S, Redmond WL, Sherman LA. Phenotypic and functional analysis of CD8(+) T cells undergoing peripheral deletion in response to cross-presentation of self-antigen. *J Exp Med*. 2001;194:707-717.

260. Mayerova D, Parke EA, Bursch LS, Odumade OA, Hogquist KA. Langerhans cells activate naive self-antigen-specific CD8 T cells in the steady state. *Immunity*. 2004;21:391-400.

261. Stamou P, de Jersey J, Carmignac D, Mamalaki C, Kioussis D, Stockinger B. Chronic exposure to low levels of antigen in the periphery causes reversible functional impairment correlating with changes in CD5 levels in monoclonal CD8 T cells. *J Immunol*. 2003;171:1278-1284.

262. Forster I, Lieberam I. Peripheral tolerance of CD4 T cells following local activation in adolescent mice. *Eur J Immunol* . 1996;26:3194-3202.

263. Adler AJ, Marsh DW, Yochum GS, et al. CD4+ T cell tolerance to parenchymal self-antigens requires presentation by bone marrow-derived antigen-presenting cells. *J Exp Med*. 1998;187:1555-1564.

264. Adler AJ, Huang CT, Yochum GS, Marsh DW, Pardoll DM. In vivo CD4+ T cell tolerance induction versus priming is independent of the rate and number of cell divisions. *J Immunol*. 2000;164:649-655.

265. Akkaraju S, Ho WY, Leong D, Canaan K, Davis MM, Goodnow CC. A range of CD4 T cell tolerance: partial inactivation to organ-specific antigen allows nondestructive thyroiditis or insulitis. *Immunity*. 1997;7:255-271.

266. Walker LS, Ausubel LJ, Chodos A, Bekarian N, Abbas AK. CTLA-4 differentially regulates T cell responses to endogenous tissue protein versus exogenous immunogen. *J Immunol*. 2002;169:6202-6209.

267. Ausubel LJ, Chodos A, Bekarian N, Abbas AK, Walker LS. Functional tolerance is maintained despite proliferation of CD4 T cells after encounter with tissue-derived antigen. *Dev Immunol*. 2002;9:173-176.

268. Ehl S, Hombach J, Aichele P, et al. Viral and bacterial infections interfere with peripheral tolerance induction and activate CD8+ T cells to cause immunopathology. *J Exp Med*. 1998;187:763-774.

269. Webb S, Morris C, Sprent J. Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. *Cell*. 1990;63: 1249-1256.

270. Boise LH, Minn AJ, Noel PJ, et al. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity*. 1995;3:87-98.

271. Scott DE, Kisch WJ, Steinberg AD. Studies of T cell deletion and T cell anergy following in vivo administration of SEB to normal and lupusprone mice. *J Immunol*. 1993;150:664-672.

272. Mogil RJ, Radvanyi L, Gonzalez-Quintial R, et al. Fas (CD95) participates in peripheral T cell deletion and associated apoptosis in vivo. *Int Immunol*. 1995;7:1451-1458.

273. Hildeman DA, Zhu Y, Mitchell TC, et al. Activated T cell death in vivo mediated by proapoptotic Bcl-2 family member bim. *Immunity*. 2002;16:759-767.

274. Strasser A, Harris AW, Huang DC, Krammer PH, Cory S. Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. *EMBO J*. 1995;14:6136-6147.

275. Reich A, Korner H, Sedgwick JD, Pircher H. Immune down-regulation and peripheral deletion of CD8 T cells does not require TNF receptorligand interactions nor CD95. *Eur J Immunol*. 2000;30:678-682.

276. Bellgrau D, Gold D, Selawry H, Moore J, Franzusoff A, Duke RC. A role for CD95 ligand in preventing graft rejection. *Nature*. 1995;377: 630-632.

277. Griffith TS, Yu X, Herndon JM, Green DR, Ferguson TA. CD95-induced apoptosis of lymphocytes in an immune privileged stite induces immunological tolerance. *Immunity*. 1996;5:7-16.

278. Vinuesa CG, Tangye SG, Moser B, Mackay CR. Follicular B helper T cells in antibody responses and autoimmunity. *Nat Rev Immunol*. 2005;5:853-865.

279. Vinuesa CG, Cyster JG. How T cells earn the follicular rite of passage. *Immunity*. 2011;35:671-680.

280. Vella AT, McCormack JE, Linsley PS, Kappler JW, Marrack P. Lipopolysaccharide interferes with the induction of peripheral T cell death. *Immunity*. 1995;2:261-270.

281. Khoruts A, Mondino A, Pape KA, Reiner SL, Jenkins MK. A natural immunological adjuvant enhances T cell clonal expansion through a CD28-dependent, interleukin (IL)-2-independent mechanism. *J Exp Med*. 1998;187:225-236.

282. Gudmundsdottir H, Wells AD, Turka LA. Dynamics and requirements of T cell clonal expansion in vivo at the single-cell level: effector function is linked to proliferative capacity. *J Immunol*. 1999;162: 5212-5223.

283. Wells AD, Li XC, Li Y, et al. Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat Med*. 1999;5: 1303-1307.

284. Townsend SE, Goodnow CC. Abortive proliferation of rare T cells induced by direct or indirect antigen presentation by rare B cells in vivo. *J Exp Med*. 1998;187:1611-1621.

285. Fîrster I, Hirose R, Arbeit JM, Clausen BE, Hanahan D. Limited capacity for

tolerization of CD4+ T cells specific for a pancreatic beta cell neo-antigen. *Immunity*. 1995;2:573-585.

286. Teh SJ, Dutz JP, Motyka B, Teh HS. Fas (CD95)-independent regulation of immune responses by antigen-specific CD4-CD8+ T cells. *Int Immunol*. 1996;8:675-681.

287. Bertolino P, Heath WR, Hardy CL, Morahan G, Miller JFAP. Peripheral deletion of autoreactive CD8+ T cells in transgenic mice expressing H-2K b in the liver. *Eur J Immunol*. 1995;25:1932-1942.

288. Bertolino P, Bowen DG, McCaughan GW, Fazekas de St Groth B. Antigen-specific primary activation of CD8+ T cells within the liver. *J Immunol*. 2001;166:5430-5438.

289. Holz LE, Benseler V, Bowen DG, et al. Intrahepatic murine CD8 T-cell activation associates with a distinct phenotype leading to Bimdependent death. *Gastroenterology*. 2008;135:989-997.

290. Bowen DG, Zen M, Holz L, Davis T, McCaughan GW, Bertolino P. The site of primary T cell activation is a determinant of the balance between intrahepatic tolerance and immunity. *J Clin Invest*. 2004;114:701-712.

291. Kurts C, Heath WR, Carbone FR, Allison J, Miller JFAP, Kosaka H. Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J Exp Med*. 1996;184:923-930.

292. Kurts C, Kosaka H, Carbone FR, Miller JFAP, Heath WR. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8+ T cells. *J Exp Med*. 1997;186:239-245.

293. Davey GM, Kurts C, Miller JFAP, et al. Peripheral deletion of autoreactive CD8 T cells by cross presentation of self-antigen occurs by a Bcl-2-inhibitable pathway mediated by Bim. *J Exp Med*. 2002;196:947-955.

294. Kurts C, Carbone FR, Barnden M, et al. CD4+ T cell help impairs CD8+ T cell deletion induced by cross-presentation of self-antigens and favors autoimmunity. *J Exp Med*. 1997;186:2057-2062.

295. Behrens GM, Li M, Davey GM, et al. Helper requirements for generation of effector CTL to islet beta cell antigens. *J Immunol*. 2004;172: 5420-5426.

296. Morgan DJ, Kreuwel HT, Sherman LA. Antigen concentration and precursor frequency determine the rate of CD8+ T cell tolerance to peripherally expressed antigens. *J Immunol*. 1999;163:723-727.

297. Redmond WL, Hernandez J, Sherman LA. Deletion of naive CD8 T cells requires persistent antigen and is not programmed by an initial signal from the tolerogenic APC. *J Immunol*. 2003;171:6349-6354.

298. Redmond WL, Wei CH, Kreuwel HT, Sherman LA. The apoptotic pathway contributing to the deletion of naive CD8 T cells during the induction of peripheral tolerance to a cross-presented self-antigen. *J Immunol*. 2008;180:5275-5282.

299. Belz GT, Zhang L, Lay MD, Kupresanin F, Davenport MP. Killer T cells regulate antigen presentation for early expansion of memory, but not naive, CD8+ T cell. *Proc Natl Acad Sci U S A*. 2007;104:6341-6346.

300. Probst HC, McCoy K, Okazaki T, Honjo T, van den BM. Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. *Nat Immunol*. 2005;6:280-286.

301. Nishimura H, Nose M, Hiai H, Minator N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity*. 1999;11: 141-151.

302. Nishimura H, Okazaki T, Tanaka Y, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor deficient mice. *Science*. 2001;291:319-322.

303. Okazaki T, Honjo T. The PD-1-PD-L pathway in immunological tolerance. *Trends*

Immunol. 2006;27:195-201.

304. Barber DL, Wherry EJ, Masopust D, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*. 2006;439:682-687.

305. Day CL, Kaufmann DE, Kiepiela P, et al. PD-1 expression on HIVspecific T cells is associated with T-cell exhaustion and disease progression. *Nature*. 2006;443:350-354.

306. Han B, Serra P, Amrani A, et al. Prevention of diabetes by manipulation of anti-IGRP autoimmunity: high efficiency of a low-affinity peptide. *Nat Med*. 2005;11:645-652.

307. Tsai S, Shameli A, Yamanouchi J, et al. Reversal of autoimmunity by boosting memory-like autoregulatory T cells. *Immunity*. 2010;32: 568-580.

308. Ohashi PS, Oehen S, Buerki K, et al. Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell*. 1991;65:305-317.

309. Goverman J, Woods A, Larson L, Weiner LP, Hood L, Zaller DM. Transgenic mice that express a myelin basic protein specific T cell receptor develop spontaneous autoimmunity. *Cell*. 1993;72:551-560.

310. Katz JD, Wang B, Haskins K, Benoist C, Mathis D. Following a diabetogenic T cell from genesis through pathogenesis. *Cell*. 1993;74: 1089-1100.

311. Lafaille JJ, Nagashima K, Katsuki M, Tonegawa S. High incidence of spontaneous autoimmune encephalomyelitis in immunodeficient antimyelin basic protein T cell receptor transgenic mice. *Cell*. 1994;78: 399-408.

312. Schild HJ, Rîtzschke O, Kalbacher H, Rammensee HG. Limit of T cell tolerance to self proteins by peptide presentation. *Science*. 1990;247:1587-1589

313. Kurts C, Sutherland RM, Davey G, et al. CD8 T cell ignorance or tolerance to islet antigens depends on antigen dose. *Proc Natl Acad Sci U S A* . 1999;96:12703-12707.

314. Chen W, Jin W, Hardegen N, et al. Conversion of peripheral CD4+ CD25-naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med*. 2003;198:1875-1886.

315. Groux H, O'Garra A, Bigler M, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature*. 1997;389:737-742.

316. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol*. 2005;6:1219-1227.

317. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med*. 1999;190:995-1004.

318. Hara M, Kingsley CI, Niimi M, et al. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J Immunol*. 2001;166:3789-3796.

319. de Waal Malefyt R, Haanen J, Spits H, et al. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J Exp Med*. 1991;174:915-924.

320. Buelens C, Willems F, Delvaux A, et al. Interleukin-10 differentially regulates B7-1 (CD80) and B7-2 (CD86) expression on human peripheral blood dendritic cells. *Eur J Immunol*. 1995;25:2668-2672.

321. Redpath S, Angulo A, Gascoigne NR, Ghazal P. Murine cytomegalovirus infection down-regulates MHC class II expression on macrophages by induction of IL-10. *J Immunol*. 1999;162:6701-6707.

322. Thibodeau J, Bourgeois-Daigneault MC, Huppe G, et al. Interleukin-10-induced MARCH1 mediates intracellular sequestration of MHC class II in monocytes. *Eur J Immunol*. 2008;38:1225-1230.

323. Tze LE, Horikawa K, Domaschenz H, et al. CD83 increases MHC II and CD86 on dendritic cells by opposing IL-10-driven MARCH1-mediated ubiquitination and degradation. *J Exp Med*. 2011;208:149-165.

324. Schmidt EM, Wang CJ, Ryan GA, et al. Ctla-4 controls regulatory T cell peripheral homeostasis and is required for suppression of pancreatic islet autoimmunity. *J Immunol*. 2009;182:274-282.

325. Qureshi OS, Zheng Y, Nakamura K, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science*. 2011;332:600-603.

326. Ise W, Kohyama M, Nutsch KM, et al. CTLA-4 suppresses the pathogenicity of self antigen-specific T cells by cell-intrinsic and cell-extrinsic mechanisms. *Nat Immunol*. 2010;11:129-135.

327. Liu GY, Fairchild PJ, Smith RM, Prowle JR, Kioussis D, Wraith DC. Low avidity recognition of self-antigen by T cells permits escape from central tolerance. *Immunity*. 1995;3:407-415.

328. Anderton SM, Viner NJ, Matharu P, Lowrey PA, Wraith DC. Influence of a dominant cryptic epitope on autoimmune T cell tolerance. *Nat Immunol*. 2002;3:175-181.

329. Zehn D, Bevan MJ. T cells with low avidity for a tissue-restricted antigen routinely evade central and peripheral tolerance and cause autoimmunity. *Immunity.* 2006;25:261-270.

330. Grossman Z, Paul WE. Adaptive cellular interactions in the immune system: the tunable activation threshold and the significance of subthreshold responses. *Proc Natl Acad Sci U S A*. 1992;89:10365-10369.

331. Kawai K, Ohashi PS. Immunological function of a defined T-cell population tolerized to low-affinity self antigens. *Nature*. 1995;374: 68-69.

332. Sandberg JK, Franksson L, Sundback J, et al. T cell tolerance based on avidity thresholds rather than complete deletion allows maintenance of maximal repertoire diversity. *J Immunol*. 2000;165:25-33.

333. Choi S, Schwartz RH. Molecular mechanisms for adaptive tolerance and other T cell anergy models. *Sem Immunol*. 2007;19:140-152.

334. Lombardi G, Sidhu S, Batchelor R, Lechler R. Anergic T cells as suppressor cells in vitro. *Science*. 1994;264:1587-1589.

335. Knoechel B, Lohr J, Zhu S, et al. Functional and molecular comparison of anergic and regulatory T lymphocytes. *J Immunol*. 2006;176:6473-6483.

336. Macin F, Garca-C¢zar F, Im SH, Horton HF, Byrne MC, Rao A. Transcriptional mechanisms underlying lymphocyte tolerance. *Cell*. 2002;109:719-731.

337. Soto-Nieves N, Puga I, Abe BT, et al. Transcriptional complexes formed by NFAT dimers regulate the induction of T cell tolerance. *J Exp Med*. 2009;206:867-876.

338. Puga I, Rao A, Macian F. Targeted cleavage of signaling proteins by caspase 3 inhibits T cell receptor signaling in anergic T cells. *Immunity.* 2008;29:193-204.

339. Coudronniere N, Villalba M, Englund N, Altman A. NF- k B activation induced by T cell receptor / CD28 costimulation is mediated by protein kinase C-È. *Proc Natl Acad Sci U S A*. 2000;97:3394-3399.

340. Bi K, Tanaka Y, Coudronniere N, et al. Antigen-induced translocation of PKC-È to membrane rafts is required for T cell activation. *Nat Immunol*. 2001;2:556-563.

341. Huang J, Lo PF, Zal T, et al. CD28 plays a critical role in the segregation of PKCÈ within the immunologic synapse. *Proc Natl Acad Sci U S A* . 2002;99:9369-9373.

342. Berg-Brown NN, Gronski MA, Jones RG, et al. PKCÈ signals activation versus tolerance in vivo. *J Exp Med*. 2004;199:743-752.

343. Deenick EK, Po L, Chapatte L, et al. c-Rel phenocopies PKCtheta but not Bcl-10 in regulating CD8(+) T-cell activation versus tolerance. *Eur J Immunol* . 2009;40(3):867-877.

344. Heissmeyer V, Macian F, Im SH, et al. Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. *Nat Immunol*. 2004;5:255-265.

345. Baier-Bitterlich G, Uberall F, Bauer B, et al. Protein kinase C-È isoenzyme selective stimulation of the transcription factor complex AP-1 in T lymphocytes. *Mol Cell Biol* . 1996;6:1842-1850.

346. Sun Z, Arendt CW, Ellmeier W, et al. PKC-È is required for TCRinduced NF- k B activation in mature but not immature T lymphocytes. *Nature*. 2000;404:402-407.

347. Kang SM, Beverly B, Tran AC, Brorson K, Schwartz RH, Lenardo MJ. Transactivation by AP-1 is a molecular target of T cell clonal anergy. *Science*. 1992;257:1134-1138.

348. Li W, Whaley CD, Mondino A, Mueller DL. Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4+ T cells. *Science*. 1996;271:1272-1276.

349. Fields PE, Gajewski TF, Fitch FW. Blocked Ras activation in anergic CD4+ T cells. *Science*. 1996;271:1276-1278.

350. Sundstedt A, Sigvardsson M, Leanderson T, Hedlund G, Kalland T, Dohlsten M. In vivo anergized CD4+ T cells express perturbed AP-1 and NF- k B transcription factors. *Proc Natl Acad Sci U S A* . 1996;93:979-984.

351. Tzachanis D, Freeman GJ, Hirano N, et al. Tob is a negative regulator of activation that is expressed in anergic and quiescent T cells. *Nat Immunol*. 2001;2:1174-1182.

352. Zha Y, Marks R, Ho AW, et al. T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-alpha. *Nat Immunol*. 2006;7:1166-1173.

353. Olenchock BA, Guo R, Carpenter JH, et al. Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. *Nat Immunol*. 2006;7:1174-1181.

354. Anandasabapathy N, Ford GS, Bloom D, et al. GRAIL: an E3 ubiquitin ligase that inhibits cytokine gene transcription is expressed in anergic CD4+ T cells. *Immunity.* 2003;18:535-547.

355. Seroogy CM, Soares L, Ranheim EA, et al. The gene related to anergy in lymphocytes, an E3 ubiquitin ligase, is necessary for anergy induction in CD4 T cells. *J Immunol*. 2004;173:79-85.

356. Soares L, Seroogy C, Skrenta H, et al. Two isoforms of otubain 1 regulate T cell anergy via GRAIL. *Nat Immunol*. 2004;5:45-54.

357. Lin JT, Lineberry NB, Kattah MG, et al. Naive CD4 t cell proliferation is controlled by mammalian target of rapamycin regulation of GRAIL expression. *J Immunol* . 2009;182:5919-5928.

358. Chiang YJ, Kole HK, Brown K, et al. Cbl-b regulates the CD28 dependence of T-cell activation. *Nature*. 2000;403:216-220.

359. Bachmaier K, Krawczyk C, Kozieradzki I, et al. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature*. 2000;403:211-216.

360. Yokoi N, Komeda K, Wang HY, et al. Cblb is a major susceptibility gene for rat type 1 diabetes mellitus. *Nat Genet.* 2002;31:391-394.

361. Jeon MS, Atfield A, Venuprasad K, et al. Essential role of the E3 ubiquitin ligase Cbl-b in T cell anergy induction. *Immunity*. 2004;21:167-177.

362. Teh CE, Daley SR, Enders A, Goodnow CC. T-cell regulation by casitas B-lineage lymphoma (Cblb) is a critical failsafe against autoimmune disease due to autoimmune regulator (Aire) deficiency. *Proc Natl Acad Sci U S A* . 2010;107:14709-14714.

363. Wohlfert EA, Callahan MK, Clark RB. Resistance to CD4+CD25+ regulatory T cells and TGF-beta in Cbl-b-/- mice. *J Immunol*. 2004;173:1059-1065.

364. Wohlfert EA, Gorelik L, Mittler R, Flavell RA, Clark RB. Cutting edge: deficiency in the E3 ubiquitin ligase Cbl-b results in a multifunctional defect in T cell TGF-beta sensitivity in vitro and in vivo. *J Immunol*. 2006;176:1316-1320.

365. Adams CO, Housley WJ, Bhowmick S, et al. Cbl-b(-/-) T cells demonstrate in vivo resistance to regulatory T cells but a context-dependent resistance to TGF-beta. *J Immunol*. 2010;185:2051-2058.

366. Powell JD, Lerner CG, Schwartz RH. Inhibition of cell cycle progression by rapamycin induces T cell clonal anergy even in the presence of costimulation. *J Immunol*. 1999;162:2775-2784.

367. Zheng Y, Collins SL, Lutz MA, et al. A role for mammalian target of rapamycin in regulating T cell activation versus anergy. *J Immunol*. 2007;178:2163-2170.

368. Boussiotis VA, Freeman GJ, Taylor PA, et al. p27kip1 functions as an anergy factor inhibiting interleukin 2 transcription and clonal expansion of alloreactive human and mouse helper T lymphocytes. *Nat Med.* 2000;6:290-297.

369. Rowell EA, Walsh MC, Wells AD. Opposing roles for the cyclin-dependent kinase inhibitor p27kip1 in the control of CD4+ T cell proliferation and effector function. *J Immunol*. 2005;174:3359-3368.

370. Li L, Iwamoto Y, Berezovskaya A, Boussiotis VA. A pathway regulated by cell cycle inhibitor p27Kip1 and checkpoint inhibitor Smad3 is involved in the induction of T cell tolerance. *Nat Immunol*. 2006;7: 1157-1165.

371. Boussiotis VA, Barber DL, Lee BJ, Gribben JG, Freeman GJ, Nadler LM. Differential association of protein tyrosine kinases with the T cell receptor is linked to the induction of anergy and its prevention by B7 family-mediated costimulation. *J Exp Med*. 1996;184:365-376.

372. Hundt M, Tabata H, Jeon MS, et al. Impaired activation and localization of LAT in anergic T cells as a consequence of a selective palmitoylation defect. *Immunity.* 2006;24:513-522.

373. Demotte N, Stroobant V, Courtoy PJ, et al. Restoring the association of the T cell receptor with CD8 reverses anergy in human tumor-infiltrating lymphocytes. *Immunity.* 2008;28:414-424.

374. Villarino AV, Katzman SD, Gallo E, et al. Posttranscriptional silencing of effector cytokine mRNA underlies the anergic phenotype of selfreactive T cells. *Immunity.* 2011;34:50-60.

375. Boussiotis VA, Barber DL, Nakarai T, et al. Prevention of T cell anergy by signaling through the g c chain of the IL-2 receptor. *Science*. 1994;266:1039-1042.

376. Teague RM, Sather BD, Sacks JA, et al. Interleukin-15 rescues tolerant CD8+ T cells for use in adoptive immunotherapy of established tumors. *Nat Med.* 2006;12:335-341.

377. Wilcox RA, Tamada K, Flies DB, et al. Ligation of CD137 receptor prevents and reverses established anergy of CD8+ cytolytic T lymphocytes in vivo. *Blood.* 2004;103:177-184.

378. Bansal-Pakala P, Jember AGH, Croft M. Signaling through OX40 (CD134) breaks peripheral T-cell tolerance. *Nat Med.* 2001;7:907-912.

379. Oldstone MBA, Nerenberg M, Southern P, Price J, Lewicki H. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. *Cell*. 1991;65: 319-331.

380. Wilcox RA, Flies DB, Zhu G, et al. Provision of antigen and CD137 signaling breaks immunological ignorance, promoting regression of poorly immunogenic tumors. *J Clin*

Invest. 2002;109:651-659.

381. Waldner H, Collins M, Kuchroo VK. Activation of antigen-presenting cells by microbial products breaks self tolerance and induces autoimmune disease. *J Clin Invest*. 2004;113:990-997.

382. Nguyen LT, Elford AR, Murakami K, et al. Tumor growth enhances cross-presentation leading to limited T cell activation without tolerance. *J Exp Med* 2002;195:423-435.

383. Spiotto MT, Yu P, Rowley DA, et al. Increasing tumor antigen expression overcomes "ignorance" to solid tumors via crosspresentation by bone marrow-derived stromal cells. *Immunity*. 2002;17:737-747.

384. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* . 2003;348:203-213.

385. Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science.* 2006;313:1960-1964.

386. Finak G, Bertos N, Pepin F, et al. Stromal gene expression predicts clinical outcome in breast cancer. *Nat Med.* 2008;14:518-527.

387. Tosolini M, Kirilovsky A, Mlecnik B, et al. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res*. 2011;71:1263-1271.

388. Waterhouse P, Penninger JM, Timms E, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science*. 1995;270:985-988.

389. Tivol EA, Boriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction. *Immunity*. 1995;3:541-547.

390. Keir ME, Liang SC, Guleria I, et al. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med*. 2006;203:883-895.

391. Goodnow CC, Crosbie J, Adelstein S, et al. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature*. 1988;334:676-682.

392. Padlan EA, Silverton EW, Sheriff S, Cohen GH, Smith-Gill SJ, Davies DR. Structure of an antibody-antigen complex: crystal structure of the HyHEL-10 Fab-lysozyme complex. *Proc Natl Acad Sci U S A* . 1989;86:5938-5942.

393. Smith-Gill SJ, Wilson AC, Potter M, Feldmann RJ, Mainhart CR. Mapping the antigenic epitope for a monoclonal antibody against avian lysozyme. *J Immunol*. 1982;128:314-322.

394. Adelstein S, Pritchard-Briscoe H, Anderson TA, et al. Induction of selftolerance in T cells but not B cells of transgenic mice expressing little self antigen. *Science*. 1991;251:1223-1225.

395. Goodnow CC, Crosbie J, Jorgensen H, Brink RA, Basten A. Induction of self-tolerance in mature peripheral B lymphocytes. *Nature*. 1989;342:385-391.

396. Liston A, Gray DH, Lesage S, et al. Gene dosage—limiting role of Aire in thymic expression, clonal deletion, and organ-specific autoimmunity. *J Exp Med*. 2004;200:1015-1026.

397. Akkaraju S, Canaan K, Goodnow CC. Self-reactive B cells are not eliminated or inactivated by autoantigen expressed on thyroid epithelial cells. *J Exp Med*. 1997;186:2005-2012.

398. Mason DY, Jones M, Goodnow CC. Development and follicular localization of tolerant B lymphocytes in lysozyme/anti-lysozyme IgM/IgD transgenic mice. *Int Immunol*. 1992;4:163-175.

399. Rayner J. *Characterisation of a Novel Transgenic Model of B Cell Tolerance* . Canberra, Australia: Australian National University; 2002.

400. Cyster JG, Hartley SB, Goodnow CC. Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire. *Nature*. 1994;371:389-395.

401. Hartley SB, Crosbie J, Brink R, Kantor AB, Basten A, Goodnow CC. Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. *Nature*. 1991;353:765-769.

402. Hartley SB, Goodnow CC. Censoring of self-reactive B cells with a range of receptor affinities in transgenic mice expressing heavy chains for a lysozyme-specific antibody. *Int Immunol*. 1994;6:1417-1425.

403. Nemazee D, Buerki K. Clonal deletion of autoreactive B lymphocytes in bone marrow chimeras. *Proc Natl Acad Sci U S A* . 1989;86:8039-8043.

404. Nemazee DA, Burki K. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature*. 1989;337: 562-566.

405. Goodnow CC. Balancing immunity and tolerance: deleting and tuning lymphocyte repertoires. *Proc Natl Acad Sci U S A* . 1996;93:2264-2271.

406. Goodnow CC, Adelstein S, Basten A. The need for central and peripheral tolerance in the B cell repertoire. *Science*. 1990;248:1373-1379.

407. Perelson AS, Oster GF. Theoretical studies of clonal selection: minimal antibody repertoire size and reliability of self-non-self discrimination. *J Theoret Biol* . 1979;81:645-670.

408. De Boer RJ, Perelson AS. How diverse should the immune system be? *Proc Biol Sci Royal Soc*. 1993;252:171-175.

409. Nemazee D. Antigen receptor “capacity” and the sensitivity of self-tolerance. *Immunol Today*. 1996;17:25-29.

410. Wucherpfennig KW, Allen PM, Celada F, et al. Polyspecificity of T cell and B cell receptor recognition. *Sem Immunol*. 2007;19:216-224.

411. Wedemayer GJ, Patten PA, Wang LH, Schultz PG, Stevens RC. Structural insights into the evolution of an antibody combining site. *Science*. 1997;276:1665-1669.

412. Hartley SB, Cooke MP, Fulcher DA, et al. Elimination of self-reactive B lymphocytes proceeds in two stages: arrested development and cell death. *Cell*. 1993;72:325-335.

413. Tiegs SL, Russell DM, Nemazee D. Receptor editing in self-reactive bone marrow B cells. *J Exp Med*. 1993;177:1009-1020.

414. Gay D, Saunders T, Camper S, Weigert M. Receptor editing: an approach by autoreactive B cells to escape tolerance. *J Exp Med*. 1993;177: 999-1008.

415. Chen C, Prak EL, Weigert M. Editing disease-associated autoantibodies. *Immunity*. 1997;6:97-105.

416. Pelanda R, Schwers S, Sonoda E, Torres RM, Nemazee D, Rajewsky K. Receptor editing in a transgenic mouse model: site, efficiency, and role in B cell tolerance and antibody diversification. *Immunity*. 1997;7:765-775.

417. Hippen KL, Schram BR, Tze LE, Pape KA, Jenkins MK, Behrens TW. In vivo assessment of the relative contributions of deletion, anergy, and editing to B cell self-tolerance. *J Immunol* . 2005;175:909-916.

418. Halverson R, Torres RM, Pelanda R. Receptor editing is the main mechanism of B cell tolerance toward membrane antigens. *Nat Immunol* . 2004;5:645-650.

419. Li H, Jiang Y, Prak EL, Radic M, Weigert M. Editors and editing of anti-DNA receptors. *Immunity*. 2001;15:947-957.

420. Verkoczy L, Chen Y, Bouton-Verville H, et al. Rescue of HIV-1 broad neutralizing antibody-expressing B cells in 2F5 VH × VL knockin mice reveals multiple tolerance controls. *J Immunol*. 2011;187:3785-3797.

421. Harada K, Yamagishi H. Lack of feedback inhibition of V kappa gene rearrangement by productively rearranged alleles. *J Exp Med*. 1991;173:409-415.

422. Retter MW, Nemazee D. Receptor editing occurs frequently during normal B cell development. *J Exp Med*. 1998;188:1231-1238.

423. Casellas R, Shih TA, Kleinewietfeld M, et al. Contribution of receptor editing to the antibody repertoire. *Science*. 2001;291:1541-1544.

424. Kohler F, Hug E, Eschbach C, et al. Autoreactive B cell receptors mimic autonomous pre-B cell receptor signaling and induce proliferation of early B cells. *Immunity*. 2008;29:912-921.

425. Tze LE, Schram BR, Lam KP, et al. Basal immunoglobulin signaling actively maintains developmental stage in immature B cells. *PLoS Biol*. 2005;3:e82.

426. Verkoczy L, Duong B, Skog P, et al. Basal B cell receptor-directed phosphatidylinositol 3-kinase signaling turns off RAGs and promotes B cellpositive selection. *J Immunol*. 2007;178:6332-6341.

427. Amin RH, Schlissel MS. Foxo1 directly regulates the transcription of recombination-activating genes during B cell development. *Nat Immunol*. 2008;9:613-622.

428. Benatuil L, Kaye J, Cretin N, et al. Ig knock-in mice producing anticarbohydrate antibodies: breakthrough of B cells producing low affinity anti-self antibodies. *J Immunol*. 2008;180:3839-3848.

429. Havouis S, Dumas G, Ave P, et al. Negative regulation of autoreactive B cells in transgenic mice expressing a human pathogenic cold agglutinin. *Eur J Immunol* . 2000;30:2290-2299.

430. Wang H, Shlomchik MJ. High affinity rheumatoid factor transgenic B cells are eliminated in normal mice. *J Immunol*. 1997;159:1125-1134.

431. Andersson J, Melchers F, Rolink A. Stimulation by T cell independent antigens can relieve the arrest of differentiation of immature auto-reactive B cells in the bone marrow. *Scand J Immunol*. 1995;42:21-33.

432. Li H, Jiang Y, Cao H, Radic M, Prak EL, Weigert M. Regulation of antiphosphatidylserine antibodies. *Immunity*. 2003;18:185-192.

433. Litzenburger T, Bluthmann H, Morales P, et al. Development of myelin oligodendrocyte glycoprotein autoreactive transgenic B lymphocytes: receptor editing in vivo after encounter of a self-antigen distinct from myelin oligodendrocyte glycoprotein. *J Immunol*. 2000;165:5360-5366.

434. Zhang Y, Su SC, Hecox DB, et al. Central tolerance regulates B cells reactive with Goodpasture antigen alpha3(IV)NC1 collagen. *J Immunol*. 2008;181:6092-6100.

435. Brady GF, Congdon KL, Clark AG, et al. Kappa editing rescues autoreactive B cells destined for deletion in mice transgenic for a dual specific anti-laminin Ig. *J Immunol*. 2004;172:5313-5321.

436. Duty JA, Szodoray P, Zheng NY, et al. Functional anergy in a subpopulation of naive B cells from healthy humans that express autoreactive immunoglobulin receptors. *J Exp Med*. 2009;206:139-151.

437. Quach TD, Manjarrez-Orduno N, Adlowitz DG, et al. Anergic responses characterize a large fraction of human autoreactive naive B cells expressing low levels of surface IgM. *J Immunol*. 2011;186:4640-4648.

438. Erikson J, Radic MZ, Camper SA, Hardy RR, Carmack C, Weigert M. Expression of anti-DNA immunoglobulin transgenes in non-autoimmune mice. *Nature*. 1991;349:331-

334.

439. Nguyen KA, Mandik L, Bui A, et al. Characterization of anti-singlestranded DNA B cells in a non-autoimmune background. *J Immunol*. 1997;159:2633-2644.

440. Benschop RJ, Aviszus K, Zhang X, Manser T, Cambier JC, Wysocki LJ. Activation and anergy in bone marrow B cells of a novel immunoglobulin transgenic mouse that is both hapten specific and autoreactive. *Immunity*. 2001;14:33-43.

441. Alabyev B, Rahman ZS, Manser T. Quantitatively reduced participation of anti-nuclear antigen B cells that down-regulate B cell receptor during primary development in the germinal center/memory B cell response to foreign antigen. *J Immunol*. 2007;178:5623-5634.

442. Heltemes-Harris L, Liu X, Manser T. Progressive surface B cell antigen receptor down-regulation accompanies efficient development of antinuclear antigen B cells to mature, follicular phenotype. *J Immunol*. 2004;172:823-833.

443. Borrero M, Clarke SH. Low-affinity anti-Smith antigen B cells are regulated by anergy as opposed to developmental arrest or differentiation to B-1. *J Immunol*. 2002;168:13-21.

444. Diz R, McCray SK, Clarke SH. B cell receptor affinity and B cell subset identity integrate to define the effectiveness, affinity threshold, and mechanism of anergy. *J Immunol*. 2008;181:3834-3840.

445. Henry RA, Acevedo-Suarez CA, Thomas JW. Functional silencing is initiated and maintained in immature anti-insulin B cells. *J Immunol*. 2009;182:3432-3439.

446. Rojas M, Hulbert C, Thomas JW. Anergy and not clonal ignorance determines the fate of B cells that recognize a physiological autoantigen. *J Immunol*. 2001;166:3194-3200.

447. Pugh-Bernard AE, Silverman GJ, Cappione AJ, et al. Regulation of inherently autoreactive VH4-34 B cells in the maintenance of human B cell tolerance. *J Clin Invest*. 2001;108:1061-1070.

448. Blery M, Tze L, Miosge LA, Jun JE, Goodnow CC. Essential role of membrane cholesterol in accelerated BCR internalization and uncoupling from NF-kappa B in B cell clonal anergy. *J Exp Med*. 2006;203:1773-1783.

449. Bell SE, Goodnow CC. A selective defect in IgM antigen receptor synthesis and transport causes loss of cell surface IgM expression on tolerant B lymphocytes. *EMBO J.* 1994;13:816-826.

450. Glynne R, Akkaraju S, Healy JI, Rayner J, Goodnow CC, Mack DH. How self-tolerance and the immunosuppressive drug FK506 prevent B-cell mitogenesis. *Nature*. 2000;403:672-676.

451. Miller AT, Beisner DR, Liu D, Cooke MP. Inositol 1,4,5-trisphosphate 3-kinase B is a negative regulator of BCR signaling that controls B cell selection and tolerance induction. *J Immunol*. 2009;182:4696-4704.

452. Cooke MP, Heath AW, Shokat KM, et al. Immunoglobulin signal transduction guides the specificity of B cell-T cell interactions and is blocked in tolerant self-reactive B cells. *J Exp Med.* 1994;179:425-438.

453. Gauld SB, Benschop RJ, Merrell KT, Cambier JC. Maintenance of B cell anergy requires constant antigen receptor occupancy and signaling. *Nat Immunol*. 2005;6:1160-1167.

454. Weintraub BC, Jun JE, Bishop AC, Shokat KM, Thomas ML, Goodnow CC. Entry of B cell receptor into signaling domains is inhibited in tolerant B cells. *J Exp Med*. 2000;191:1443-1448.

455. Cyster JG, Goodnow CC. Protein tyrosine phosphatase 1C negatively regulates antigen receptor signaling in B lymphocytes and determines thresholds for negative selection. *Immunity*. 1995;2:13-24.

456. Cornall RJ, Cyster JG, Hibbs ML, et al. Polygenic autoimmune traits: Lyn, CD22, and SHP-1 are limiting elements of a biochemical pathway regulating BCR signaling and selection. *Immunity*. 1998;8:497-508.

457. Smith KG, Tarlinton DM, Doody GM, Hibbs ML, Fearon DT. Inhibition of the B cell by CD22: a requirement for Lyn. *J Exp Med*. 1998;187:807-811.

458. Nishizumi H, Horikawa K, Mlinaric-Rascan I, Yamamoto T. A doubleedged kinase Lyn: a positive and negative regulator for antigen receptor-mediated signals. *J Exp Med*. 1998;187:1343-1348.

459. Chan VW, Lowell CA, DeFranco AL. Defective negative regulation of antigen receptor signaling in Lyn-deficient B lymphocytes. *Curr Biol* . 1998;8:545-553.

460. Adachi T, Flaswinkel H, Yakura H, Reth M, Tsubata T. The B cell surface protein CD72 recruits the tyrosine phosphatase SHP-1 upon tyrosine phosphorylation. *J Immunol*. 1998;160:4662-4665.

461. Jellusova J, Wellmann U, Amann K, Winkler TH, Nitschke L. CD22 × Siglec-G double-deficient mice have massively increased B1 cell numbers and develop systemic autoimmunity. *J Immunol*. 2010;184: 3618-3627.

462. Healy JI, Dolmetsch RE, Timmerman LA, et al. Different nuclear signals are activated by the B cell receptor during positive versus negative signaling. *Immunity*. 1997;6:419-428.

463. Healy JI, Dolmetsch RE, Lewis RS, Goodnow CC. Quantitative and qualitative control of antigen receptor signalling in tolerant B lymphocytes. *Novartis Found Symp*. 1998;215:137-144.

464. Rui L, Vinuesa CG, Blasioli J, Goodnow CC. Resistance to CpG DNAinduced autoimmunity through tolerogenic B cell antigen receptor ERK signaling. *Nat Immunol*. 2003;4:594-600.

465. Rui L, Healy JI, Blasioli J, Goodnow CC. ERK signaling is a molecular switch integrating opposing inputs from B cell receptor and T cell cytokines to control TLR4-driven plasma cell differentiation. *J Immunol*. 2006;177:5337-5346.

466. Goodnow CC, Brink R, Adams E. Breakdown of self-tolerance in anergic B lymphocytes. *Nature*. 1991;352:532-533.

467. Mandik-Nayak L, Bui A, Noorchashm H, Eaton A, Erikson J. Regulation of anti-double-stranded DNA B cells in nonautoimmune mice: localization to the T-B interface of the splenic follicle. *J Exp Med*. 1997;186:1257-1267.

468. Mandik-Nayak L, Seo S, Eaton-Bassiri A, Allman D, Hardy RR, Erikson J. Functional consequences of the developmental arrest and follicular exclusion of anti-double-stranded DNA B cells. *J Immunol*. 2000;164:1161-1168.

469. Noorchashm H, Bui A, Li HL, et al. Characterization of anergic anti-DNA B cells: B cell anergy is a T cell-independent and potentially reversible process. *Int Immunol*. 1999;11:765-776.

470. Notidis E, Heltemes L, Manser T. Dominant, hierarchical induction of peripheral tolerance during foreign antigen-driven B cell development. *Immunity*. 2002;17:317-327.

471. Berland R, Fernandez L, Kari E, et al. Toll-like receptor 7-dependent loss of B cell tolerance in pathogenic autoantibody knockin mice. *Immunity*. 2006;25:429-440.

472. Tighe H, Chen PP, Tucker R, et al. Function of B cells expressing a human immunoglobulin M rheumatoid factor autoantibody in transgenic mice. *J Exp Med*. 1993;177:109-118.

473. Widhopf GF 2nd, Brinson DC, Kipps TJ, Tighe H. Transgenic expression of a human polyreactive Ig expressed in chronic lymphocytic leukemia generates memory-type B cells that respond to nonspecific immune activation. *J Immunol*. 2004;172:2092-2099.

474. Tighe H, Heaphy P, Baird S, Weigle WO, Carson DA. Human immunoglobulin (IgG) induced deletion of IgM rheumatoid factor B cells in transgenic mice. *J Exp Med*. 1995;181:599-606.

475. Huang H, Kearney JF, Grusby MJ, Benoist C, Mathis D. Induction of tolerance in arthritogenic B cells with receptors of differing affinity for self-antigen. *Proc Natl Acad Sci U S A*. 2006;103:3734-3739.

476. Okamoto M, Murakami M, Shimizu A, et al. A transgenic model of autoimmune hemolytic anemia. *J Exp Med*. 1992;175:71-79.

477. Murakami M, Tsubata T, Okamoto M, et al. Antigen-induced apoptotic death of Ly-1 B cells responsible for autoimmune disease in transgenic mice. *Nature*. 1992;357:77-80.

478. Murakami M, Nakajima K, Yamazaki K, Muraguchi T, Serikawa T, Honjo T. Effects of breeding environments on generation and activation of autoreactive B-1 cells in anti-red blood cell autoantibody transgenic mice. *J Exp Med*. 1997;185:791-794.

479. Hayakawa K, Asano M, Shinton SA, et al. Positive selection of antithy-1 autoreactive B-1 cells and natural serum autoantibody production independent from bone marrow B cell development. *J Exp Med*. 2003;197:87-99.

480. Hartley SB. *B Cell Tolerance in Transgenic Mice*. Sydney, Australia: University of Sydney; 1993.

481. Lang J, Nemazee D. B cell clonal elimination induced by membranebound self-antigen may require repeated antigen encounter or cell competition. *Eur J Immunol* . 2000;30:689-696.

482. Russell DM, Dembic Z, Morahan G, Miller JF, Burki K, Nemazee D. Peripheral deletion of self-reactive B cells. *Nature*. 1991;354:308-311.

483. Kouskoff V, Lacaud G, Nemazee D. T cell-independent rescue of B lymphocytes from peripheral immune tolerance. *Science*. 2000;287:2501-2503.

484. Ota T, Ota M, Duong BH, Gavin AL, Nemazee D. Liver-expressed Igkappa superantigen induces tolerance of polyclonal B cells by clonal deletion not kappa to lambda receptor editing. *J Exp Med*. 2011;208:617-629.

485. Merrell KT, Benschop RJ, Gauld SB, et al. Identification of anergic B cells within a wild-type repertoire. *Immunity*. 2006;25:953-962.

486. Enders A, Bouillet P, Puthalakath H, Xu Y, Tarlinton DM, Strasser A. of the pro-apoptotic BH3-only Bcl-2 family member Bim inhibits BCR stimulation-induced apoptosis and deletion of autoreactive B cells. *J Exp Med*. 2003;198:1119-1126.

487. Lesley R, Xu Y, Kalled SL, et al. Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF. *Immunity*. 2004;20:441-453.

488. Thien M, Phan TG, Gardam S, et al. Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches. *Immunity*. 2004;20: 785-798.

489. Navarra SV, Guzman RM, Gallacher AE, et al. Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial. *Lancet*. 2011;377: 721-731.

490. Reif K, Ekland EH, Ohl L, et al. Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature*. 2002;416:94-99.

491. Cyster JG, Goodnow CC. Antigen-induced exclusion from follicles and anergy are separate and complementary processes that influence peripheral B cell fate. *Immunity*. 1995;3:691-701.

492. Rathmell JC, Cooke MP, Ho WY, et al. CD95 (Fas)-dependent elimination of self-reactive B cells upon interaction with CD4+ T cells. *Nature*. 1995;376:181-184.

493. Rathmell JC, Townsend SE, Xu JC, Flavell RA, Goodnow CC. Expansion or

elimination of B cells in vivo: dual roles for CD40- and Fas (CD95)-ligands modulated by the B cell antigen receptor. *Cell*. 1996;87: 319-329.

494. Foote LC, Marshak-Rothstein A, Rothstein TL. Tolerant B lymphocytes acquire resistance to Fas-mediated apoptosis after treatment with interleukin 4 but not after treatment with specific antigen unless a surface immunoglobulin threshold is exceeded. *J Exp Med*. 1998;187: 847-853.

495. Rathmell JC, Fournier S, Weintraub BC, Allison JP, Goodnow CC. Repression of B7.2 on self-reactive B cells is essential to prevent proliferation and allow Fas-mediated deletion by CD4(+) T cells. *J Exp Med*. 1998;188:651-659.

496. Nakanishi K, Matsui K, Kashiwamura S, et al. IL-4 and anti-CD40 protect against Fas-mediated B cell apoptosis and induce B cell growth and differentiation. *Int Immunol*. 1996;8:791-798.

497. Foote LC, Howard RG, Marshak-Rothstein A, Rothstein TL. IL-4 induces Fas resistance in B cells. *J Immunol*. 1996;157:2749-2753.

498. Eris JM, Basten A, Brink R, Doherty K, Kehry MR, Hodgkin PD. Anergic self-reactive B cells present self antigen and respond normally to CD40-dependent T-cell signals but are defective in antigen-receptor-mediated functions. *Proc Natl Acad Sci U S A*. 1994;91:4392-4396.

499. Nagata S. Autoimmune diseases caused by defects in clearing dead cells and nuclei expelled from erythroid precursors. *Immunol Rev*. 2007;220:237-250.

500. Napirei M, Karsunky H, Zevnik B, Stephan H, Mannherz HG, Moroy T. Features of systemic lupus erythematosus in Dnase1-deficient mice. *Nat Gen*. 2000;25:177-181.

501. Yasutomo K, Horiuchi T, Kagami S, et al. Mutation of DNASE1 in people with systemic lupus erythematosus. *Nat Gen*. 2001;28:313-314.

502. Lee-Kirsch MA, Gong M, Chowdhury D, et al. Mutations in the gene encoding the 3′-5′ DNA exonuclease TREX1 are associated with systemic lupus erythematosus. *Nat Gen*. 2007;39:1065-1067.

503. Rice G, Newman WG, Dean J, et al. Heterozygous mutations in TREX1 cause familial chilblain lupus and dominant Aicardi-Goutieres syndrome. *Am J Hum Gen*. 2007;80:811-815.

504. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature*. 2002;416: 603-607.

505. Pisitkun P, Deane JA, Difilippantonio MJ, Tarasenko T, Satterthwaite AB, Bolland S. Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. *Science*. 2006;312:1669-1672.

506. Deane JA, Pisitkun P, Barrett RS, et al. Control of toll-like receptor 7 expression is essential to restrict autoimmunity and dendritic cell proliferation. *Immunity*. 2007;27:801-810.

507. Subramanian S, Tus K, Li QZ, et al. A Tlr7 translocation accelerates systemic autoimmunity in murine lupus. *Proc Natl Acad Sci U S A*. 2006;103:9970-9975.

508. Wen L, Brill-Dashoff J, Shinton SA, Asano M, Hardy RR, Hayakawa K. Evidence of marginal-zone B cell-positive selection in spleen. *Immunity*. 2005;23:297-308.

509. Hannum LG, Ni D, Haberman AM, Weigert MG, Shlomchik MJ. A disease-related rheumatoid factor autoantibody is not tolerized in a normal mouse: implications for the origins of autoantibodies in autoimmune disease. *J Exp Med*. 1996;184:1269-1278.

510. William J, Euler C, Primarolo N, Shlomchik MJ. B cell tolerance checkpoints that restrict pathways of antigen-driven differentiation. *J Immunol*. 2006;176:2142-2151.

511. Koenig-Marrony S, Soulas P, Julien S, et al. Natural autoreactive B cells in transgenic mice reproduce an apparent paradox to the clonal tolerance theory. *J*

Immunol. 2001;166:1463-1470.

512. Aplin BD, Keech CL, de Kauwe AL, Gordon TP, Cavill D, McCluskey J. Tolerance through indifference: autoreactive B cells to the nuclear antigen La show no evidence of tolerance in a transgenic model. *J Immunol*. 2003;171:5890-5900.

513. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363:711-723.

514. Robert C, Thomas L, Bondarenko I, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med*. 2011;364:2517-2526.

515. Mamalaki C, Tanaka Y, Corbella P, Chandler P, Simpson E, Kioussis D. T cell deletion follows chronic antigen specific T cell activation in vivo. *Int Iimmunol*. 1993;5:1285-1292.

516. Kramer S, Mamalaki C, Horak I, Schimpl A, Kioussis D, Hung T. Thymic selection and peptide-induced activation of T cell receptor-transgenic CD8 T cells in interleukin-2-deficient mice. *Eur J Immunol*. 1994;24:2317-2322.

517. Waithman J, Allan RS, Kosaka H, et al. Skin-derived dendritic cells can mediate deletional tolerance of class I-restricted self-reactive T cells. *J Immunol*. 2007;179:4535-4541.

518. Belz GT, Behrens GM, Smith CM, et al. The CD8alpha(+) dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens. *J Exp Med*. 2002;196:1099-1104.

519. Roark JH, Bui A, Nguyen KA, Mandik L, Erikson J. Persistence of functionally compromised anti-double-stranded DNA B cells in the periphery of non-autoimmune mice. *Int Immunol*. 1997;9:1615-1626.

520. Chen C, Radic MZ, Erikson J, et al. Deletion and editing of B cells that express antibodies to DNA. *J Immunol*. 2004;152:1970-1982.

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Chapter 33

Regulatory/Suppressor T Cells

Ethan M. Shevach

INTRODUCTION

T cells are crucial in the immune response because they can function as both effector cells in cell-mediated responses and as helper cells in both humoral and cell-mediated responses. Most biologic systems are subject to complex regulatory controls, and the immune system is not an exception. In addition to T cells that upregulate (help), other populations that downregulate (suppress), the immune response must exist. Once a normal immune response is initiated by antigenic stimulation, mechanisms must be in place to control the magnitude of that response and to terminate it over time. Downregulation should contribute to the homeostatic control of all immune responses serving to limit clonal expansion and effector cell activity in response to any antigenic stimulus. An active mechanism of T-cell suppression is also needed to control potentially pathogenic autoreactive T cells. The primary mechanism that leads to tolerance to self-antigens is thymic deletion of autoreactive T cells, but some autoreactive T cells may escape thymic deletion or recognize antigens that are expressed only extra-thymically. T-cell anergy[1] and T-cell ignorance/indifference[2] have been proposed as the primary mechanisms used to control autoreactive T cells in the periphery, although these “passive” mechanisms for self-tolerance may not be sufficient to control potentially pathogenic T cells.

It was proposed more than 40 years ago that a distinct subset of T cells is responsible for immune suppression.[3] A suppressor T cell is functionally defined as a T cell that inhibits an immune response by influencing the activity of another cell type. Although a strong theoretical basis exists for T cell-mediated suppression, this area of immunologic research has been plagued by controversy. The past decade has seen a resurgence of interest in the concept of T-cell suppression mediated by a distinct subset of T cells that are uniquely equipped to mediate suppressor activity.

HISTORICAL PERSPECTIVE

Gershon and Kondo[3,4] first identified suppressor T cells during studies designed to understand the process of “highzone” tolerance. Injection of supraoptimal doses of an antigen including sheep red blood cells (SRBCs) resulted in specific tolerance or nonresponsiveness to subsequent challenge with that antigen. It was believed at that time that the antibody-producing B cell was rendered nonresponsive by exposure to the high concentration of antigen. To investigate whether B-cell tolerance was dependent on the

presence of T cells, Gershon and Kondo injected high doses (2.5×10^{10}) of SRBCs into thymectomized (Tx), irradiated, bone marrow-reconstituted mice and then assayed the functional status of B cells from these mice by a secondary challenge with SRBCs in the presence of added thymocytes as a source of T-cell help. Surprisingly, nonresponsiveness as measured by deficient antibody production was only induced in the B cells of animals that had received thymocytes as well as bone marrow cells during the initial exposure to high dose antigen, but not in mice that received bone marrow alone (Fig. 33.1). This result fulfilled Gershon's prediction that under certain conditions, antigen seen by T cells can induce not only helper and effector cells, but also cells that are able to suppress immune responses. Furthermore, when spleen cells from tolerized animals were transferred into secondary recipients together with normal thymocytes and bone marrow cells, they were capable of suppressing the otherwise competent response of these animals to SRBCs. This suppression, or “infectious tolerance” as it was originally termed, was antigen-specific, as the immune response to an unrelated antigen, horse red blood cells, was not inhibited.[4] T cells were necessary for the induction of B-cell tolerance, and these T suppressor cells were assumed to be a distinct cell population with a fully differentiated gene program that allowed them to perform a very specialized function. Other studies[5,6,7] during the 1970s supported the existence of T cell-mediated suppression.

Most of the early studies in these models demonstrated that the T cells mediating suppression were distinct from T cells mediating help because the former were cluster of differentiation (CD)8+, while the latter were CD8-. The finding that T suppressor effector cells were CD8+ distinguished them from helper cells, but did not allow them to be distinguished from cytotoxic cells, which are also CD8+. It remained possible that suppressor T cells were actually cytotoxic cells that killed the helper or effector T cells. A cell surface marker that seemed to identify a suppressor cell-specific antigen was discovered in 1976. It was found that an antiserum raised by immunizing the congenic strains B10. A(3R) with cells from B10. A(5R) mice or vice versa gave rise to an antiserum that seemed to react exclusively with suppressor cells.[8] CD8+ suppressor cells were also shown to bind antigen in the absence of major histocompatibility complex (MHC) molecules. These experiments suggested that the suppressor effector cells differed from other T cells in that they were capable of binding antigen directly and did not recognize processed antigenic peptides in association with

products of the MHC on the surface of antigen-presenting cells (APCs). Soon after the existence of T cell-mediated suppression was appreciated, some studies suggested that interactions among multiple distinct T-cell subpopulations might be involved. A CD4+ cell was described that appeared to induce CD8+ suppressor cells and was called the suppressor inducer cell. Contrasuppressor T cells had no independent helper, suppressor, or cytotoxic activity on an immune response, but enhanced immune responses by preventing the downregulation mediated by suppressor cells.

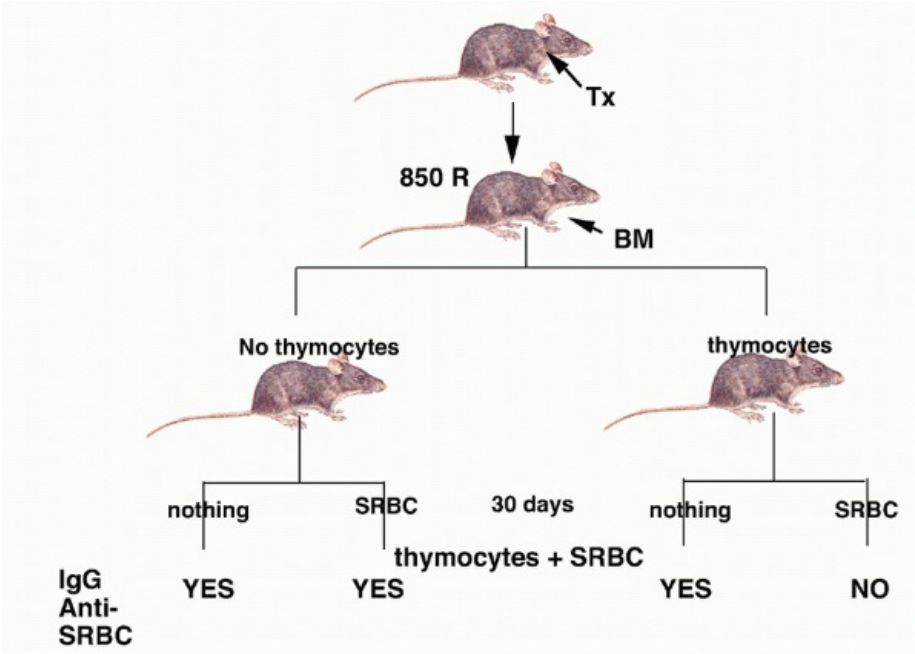

FIG. 33.1. First Demonstration of Suppressor T Cells. Adapted from Gershon and Kondo.[3]

Research in this area rapidly shifted from studies of the function of intact T cells to studies of their soluble products.[9] By the late 1970s, soluble factors produced by T suppressor cells were described by several groups, and cloned T-cell hybridomas that produced such factors were generated. T-cell suppression was regarded as being mediated by numerous soluble antigen-specific and nonspecific factors that comprised a functionally unique network.[10,11,12] The cascade involved antigen-specific, I-J-restricted CD4+ suppressor inducer cells (Ts1 cells), CD8+ anti-idiotype-specific cells (Ts2 cells), and CD8+ antigen-specific effector cells (Ts3 cells), whose suppressor function was not restricted by the MHC. Some of these cells were capable of binding directly to immobilized antigen in the absence of MHC molecules. Connectivity in this cellular cascade was mediated by a series of soluble factors: TsF1 was idiotypic and antigen-specific and immunoglobulin (Ig) heavy chain variable (V_H) region restricted. TsF2 was anti-idiotypic and required delivery by a macrophage. TsF3 acted totally nonspecifically. TsF1 and TsF2 required APCs, but TsF3 did not. Most of these factors were composed of two polypeptide chains, one of which was capable of binding native antigen and the other which bore a determinant recognized by anti-I-J antibodies.

These elaborate highly convoluted suppressor cell pathways and circuits fell out of favor in the mid-1980s for a number of important reasons. The existence of I-J was called into question by the finding that the region of the MHC complex to which I-J mapped did not contain a gene that could encode a unique I-J polypeptide.[13] When the genes encoding the T-cell receptor (TCR) were isolated, they were completely unrelated to the genes encoding Ig heavy chains, thereby calling into question the existence of T-cell factors that expressed Ig V region products. Many of the suppressor T cell hybridomas that produced antigen-specific suppressor factors were found either to have unrearranged genes for the α- or β- chains of the TCR or to have deleted genes for TCR β-chain.[14] No studies were ever performed that

convincingly characterized at the molecular level any of the suppressor

T cell factors. These studies, together with the inability to identify a marker specific for suppressor T cells and the inability to purify suppressor T cells, raised considerable doubts about the existence of a distinct functional lineage of suppressor T cells.

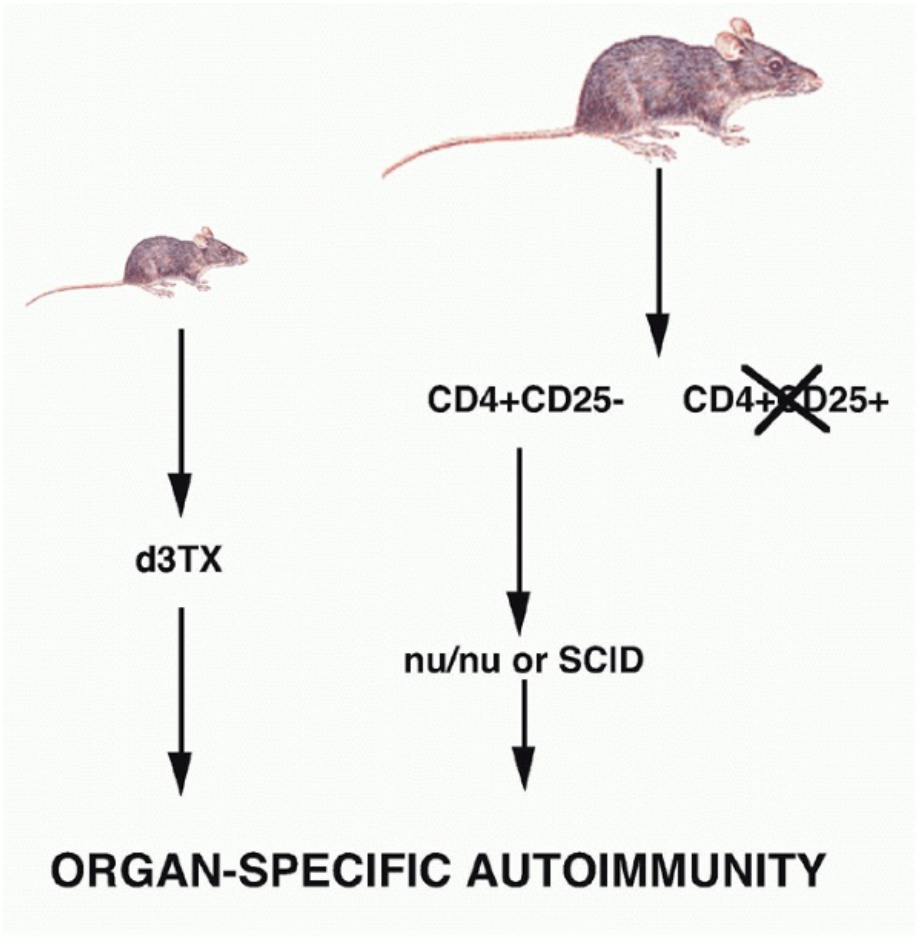

FIG. 33.2. Depletion of CD4+ CD25+ T Cells Results in Organ-Specific Autoimmunity. Adapted from Nishizuka and Sakakura[15] and Sakaguchi et al.[21]

Two completely different approaches to the demonstration of the importance of regulatory or suppressor T cells in the prevention of organ-specific autoimmunity were also developed in the 1970s. In one, mice that were Tx on the third day of life (d3Tx) were shown to develop organ-specific autoimmune diseases (Fig. 33.2). The specific disease that developed varied with the strain of mouse under study; more than one organ could be involved in a given mouse. Most importantly, autoimmunity was not seen if the mouse was Tx on day 1 or day 7 of life, and disease could be completely prevented if the d3Tx mouse received a thymus transplant between days 10 and 15 of life.[15] These observations led to the hypothesis that autoreactive T cells were exported from the thymus during the first 3 days of life and that somewhat later in ontogeny a population of suppressor cells emigrated from the thymus that controlled the autoreactive T cells. Removal of the thymus before the suppressor cells reached the periphery resulted in autoimmune disease. A number of other protocols (Table

33.1) that induced a lymphopenic state also resulted in the development of organ-specific autoimmunity. It was believed that these procedures resulted in a selective depletion of suppressor T cells, while leaving the autoreactive effector populations intact. Subsequent studies demonstrated that the effector cells in this model were CD4+ T cells. The suppressor T cells were also CD4+ cells, and the development of autoimmune disease could be prevented by reconstitution of the d3Tx animals with peripheral CD4+ T cells from normal adult mice.[16]

A second approach to define the role of regulatory T (T_{reg}) cells in the control of autoimmunity was described by Penhale and coworkers in the 1970s.[17,18] They devised a procedure to deplete Tregs from adult animals, while leaving

the helper population responsible for autoantibody production intact. The disease model was autoimmune thyroiditis because circulating antibody to thyroglobulin was believed to play an important pathogenic role. Spontaneous thyroiditis and circulating IgG autoantibodies developed in 60% of rats following the selective depletion of T cells by adult Tx followed by irradiation. Tx was performed between 3 and 5 weeks of age, and the rats were then given four to five repeated doses of 200 rad at 14-day intervals (Fig. 33.3). No evidence of thyroiditis was seen in rats that received only local irradiation to the thyroid region, indicating that irradiation itself did not induce pathologic changes. The conclusion drawn from these studies was that in the normal animal, B cells that recognized thyroid antigens were prohibited from differentiating into autoantibody-producing cells by an active controlling T cell mechanism. It was assumed that the suppressor T-cell population was mediating its functions by acting directly on the B cell and not by regulating other T cells. The active role of T cells in preventing the development of autoimmunity in this model was confirmed by reconstituting, shortly after the final dose of irradiation, the Tx-irradiated mice with lymphoid cells from normal donors. Penhale and colleagues[19] also demonstrated that autoimmune diabetes would develop following the Txirradiation protocol in a strain of rats that was normally not susceptible to this disease. Taken together, the d3Tx model in the mouse and the Tx-irradiation model in the rat demonstrated that normally autoreactive helper and suppressor cells may coexist and that certain autoimmune responses are held in check by the equilibrium favoring suppressor activity.

TABLE 33.1 Organ-Specific Autoimmune Disease and Lymphopenia

- d3Tx
- Neonatal administration of cyclosporine
- Thymectomy + repeated low-dose irradiation
- High-dose fractionated total lymphoid irradiation
- Adult thymectomy + cyclophosphamide
- Single TCR a-chain mice
- Transfer of T cells to T cell-deficient mice

d3Tx, Tx on the third day of life; TCR, T-cell receptor. (Adapted from Sakaguchi and Sakaguchi.16)

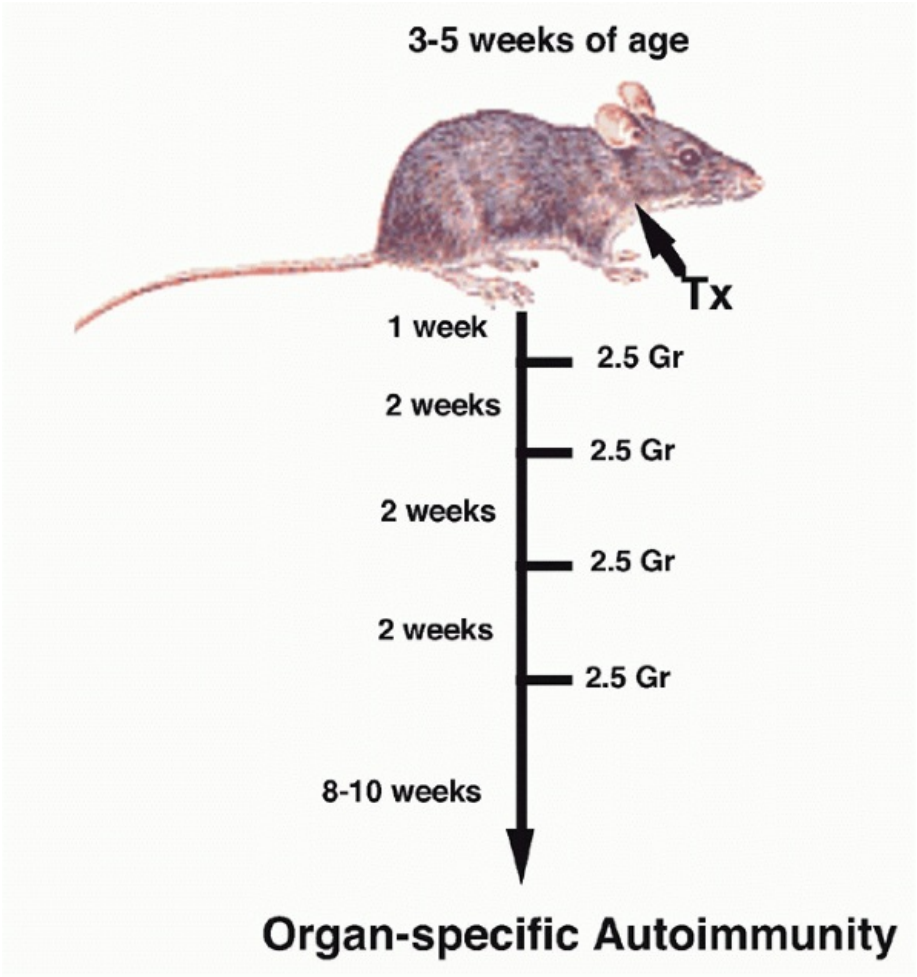

FIG. 33.3. Induction of Organ-Specific Autoimmunity in Rats by Adult Thymectomy and Irradiation. Adapted from Penhale et al.[17]

IDENTIFICATION OF CD4+ CD25+ FOXP3+ REGULATORY T CELLS

An important extension of this hypothesis was that inhibition of autoreactive T cells by suppressor T cells was not a phenomenon unique to the neonate, but that in the normal adult animal, autoreactive T cells are also under the constant control of the suppressor T cells. If the suppressor lineage was deleted, damaged, or compromised in the adult animal, autoimmune disease might develop. Although a number of studies suggested that the Treg cells in the normal adult animal might be identified by the expression of certain membrane antigens (eg, high levels of CD5[20]), a major advance in our understanding of

the role of Treg cells was the demonstration by Sakaguchi et al.,[21,22] that a minor population of CD4+ T cells (10%) that coexpressed the CD25 antigen (the interleukin [IL]-2R α-chain) appeared to function as Treg cells in the normal adult. When CD25+ T cells were depleted from a population of normal adult CD4+ T cells and the remaining CD4+CD25- T

cells transferred to an immunocompromised recipient such as a *nu/nu* mouse, the recipients developed a spectrum of autoimmune diseases that closely resembled those seen following d3Tx (see Fig. 33.2). Cotransfer of the CD25+ cells prevented the development of autoimmunity. Similarly, the induction of disease post-d3Tx could also be prevented by reconstitution of the animals with CD4+CD25+, but not CD4+CD25-, normal adult T cells before day 14 of life.[23] CD8+CD25-T cells alone were not capable of inducing autoimmunity and enhanced the induction of disease induced by CD4+CD25- T cells. These studies solidified the role of the CD4+CD25+ T cells as a major subset of cells that plays a unique role in the regulation of the immune response. The autoimmune diseases induced by depletion of CD4+CD25+ T cells are uniformly accompanied by the development of organ-specific autoantibodies, suggesting that this mode of loss of T-cell tolerance also results in the breakdown of B-cell tolerance as well. It is likely that the activated self-reactive T helper cells provide signals to self-reactive B cells, rescue them from apoptosis, and stimulate autoantibody production.

Powrie and Mason[24] were the first to identify cell surface markers that distinguished between regulatory and effector T-cell populations in the rat. When athymic rats were reconstituted with small numbers of CD4+CD45RC^{high} T cells, they developed a severe wasting disease characterized by extensive mononuclear cell infiltration in the lungs, liver, thyroid, stomach, and pancreas 6 to 10 weeks later. No pathology developed in rats that received unseparated CD4+ T cells or CD45RC^{low} cells. It seemed likely from these studies that the CD45RC^{low} subset controlled the capacity of the RC^{high} subset to mediate the wasting disease. Fowell and Mason[25] directly demonstrated the suppressive effect of the RC^{low} subset in the Tx-irradiation model develped by Penhale. Transfer of RC^{low} CD4+ T cells completely inhibited the development of diabetes and insulitis. RC^{low} T cells from long-term Tx donors could protect as efficiently as cells from normal donors, demonstrating that the Treg cell is long-lived in the periphery. Subsequently, CD4+CD45RB^{low} subset in the mouse was shown to have regulatory properties similar to the CD4+CD45RC^{low} subset of the rat.[26] Taken together, these studies in mouse and rat model systems demonstrated for the first time that two well characterized cell surface antigens (CD25 and CD45RC^{low}/CD45RB^{low}) could be used to identify suppressor CD4+ T-cell subpopulations present in normal animals. In later studies,[27] it was shown that the CD25+ T within the CD4+CD45RB^{low} population mediated their suppressor activity.

CD4+CD25+ T cells typically represent 5% to 8% of the total population of T cells in the normal mouse lymph node (LN), or 10% to 15% of mouse CD4+ T cells (Fig. 33.4). CD4+CD25+ T cells can also be found in the thymus where they also represent about 5% to 10% of the mature CD4+CD8- population, or 0.5% of mouse thymocytes.[21,28] A population with an identical phenotype has been identified in the rat, in human peripheral blood, and in human thymus.[29,30,31,32,33,34,35,36] When compared directly to CD4+CD25- T cells, CD4+CD25+ T cells express slightly higher levels of CD5, have a slightly higher proportion of CD62L^{low} cells, and have a higher proportion of CD69+ cells. They express both intermediate and low levels of CD45RB, and are completely absent from the CD45RB^{high} population.[37] All of the CD4+CD25+ T cells express the TCR αβreceptor and are NK 1.1

negative. CD25+ and CD25- T cells have a similar distribution of TCR Vα or Vβ specificities.[28] One other unique property of CD25+ T cells in both mouse and man is that they are the only nonactivated T-cell population that expresses high levels of the cytotoxic T-lymphocyte antigen (CTLA)-4 antigen intracellularly.[27,38,39] The glucocorticoidinduced tumor necrosis factor (TNF)-like receptor (GITR, TNFRSF18) has also been shown to be expressed on the majority of resting CD4+CD25+ T cells and to be expressed at very low levels on CD4+CD25- T cells.[40,41] The GITR is upregulated on CD4+CD25- T cells following TCRmediated activation.

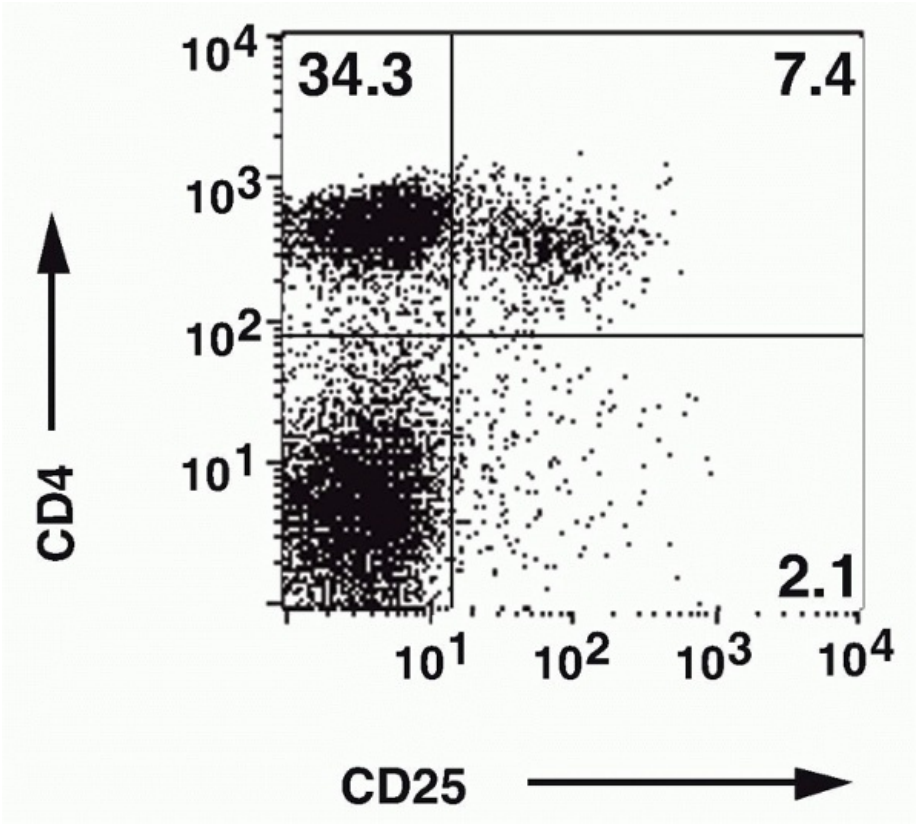

FIG. 33.4. Identification of CD4+ CD25+ T Cells in Normal Mouse Lymph Node. Adapted from Thornton and Shevach.[37]

A major advance in the study of T_{reg}s was derived from two related experiments of nature. Although CD4+CD25+ T cells were shown to display suppressive properties in multiple disease models, the value of CD25 has a marker is limited as CD25 is highly expressed on both activated CD4 and CD8 T cells, compromising its usefulness in studying Treg cells in settings of immune activation. The X-chromosome encoded forkhead transcription factor,

Foxp3, was identified as the key player in the biology of CD4+CD25+ T_{reg}s.[42,43,44,45] Young males with the immune dysregulation polyendocrinopathy enteropathy X-(IPEX) linked syndrome or a mutant mouse strain, scurfy, succumb to similar autoimmune and inflammatory diseases as a result of uncontrolled activation and expansion of CD4+ T cells. Both IPEX and

scurfy mice have mutations in a common gene, *Foxp3*, which encodes a forkheadwinged-helix transcription factor. There is an excellent correlation between expression of Foxp3 and CD25, but a minor population (˜10%) of Foxp3+ cells is CD25- and about 10% of CD25+ cells are Foxp3- (Fig. 33.5); the latter represent activated effector cells. Expression of Foxp3 only in the thymus using a proximal lck-driven transgene does not prevent disease in scurfy mice. Foxp3 expression in peripheral T cells is thus required for maintenance of Treg function. Retroviral-mediated transduction of Foxp3 expression in naïve T cells can convert these cells to a regulatory phenotype functioning in vitro in a manner similar to CD4+CD25+ Tregs. Furthermore, Foxp3-transduced naïve CD25- T cells also manifest suppressor activity in vivo and can inhibit the weight loss, diarrhea, and histologic development of colitis induced by transfer of CD25-CD45RB^{high} cells as effectively as Tregs.

Fontenot et al.[46] generated Foxp3 deficient (-/-) mice and demonstrated that Foxp3 is specifically required for the thymic development of Tregs. Mixed bone marrow chimeras using wild-type (WT) and Foxp3-/- bone marrow demonstrated that Foxp3-/- T cells behaved normally in the presence of WT Foxp3+ Tregs. Thus, the lethal autoimmune syndrome in Foxp3-/- mice results from a deficiency of Tregs and not from a cell intrinsic defect of Foxp3- T cells. Further study of the role of Foxp3 in Treg function has been greatly facilitated by the development of strains of mice in which a fluorescent protein such as green fluorescent protein (GFP) has been “knocked in” to the Foxp3 locus, permitting ready identification and isolation of Foxp3+ Tregs by cell sorting.[47] Studies of Treg development in the thymus of the Foxp3^{gfp} mice demonstrated that less than 0.1% of the CD4+CD8- thymocytes expressed Foxp3 within 12 hours after birth, and that the percentage of Foxp3+ CD4 single positive (SP) thymocytes increased slowly over the following days and reached a plateau of ˜4% at ˜21 days after birth. The largest single day change in the percentage of Foxp3 expressing SP occurred between days 3 and 4. Eighty percent of the Foxp3+ T cells on day 1 were CD4+CD8- SP cells. This result correlates with the early studies on the d3Tx mice that suggested that the generation of Treg cells is delayed relative to generation of nonregulatory CD4+ T cells. Foxp3 was not expressed in nonhematopoietic tissues. Foxp3 expression in peripheral TCR αβ+ T cells irrespective of CD25 expression correlates with suppressor activity.

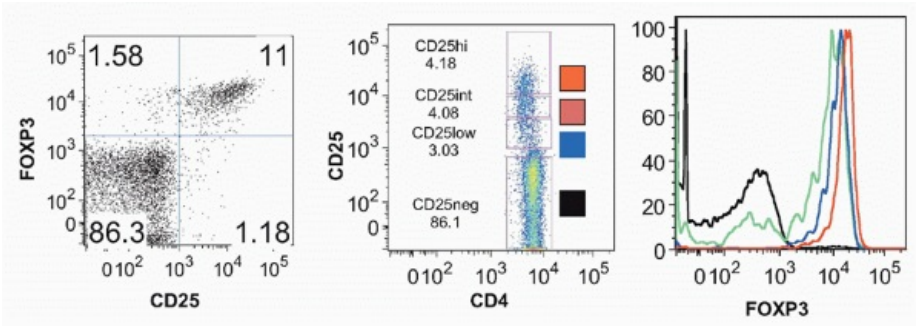

FIG. 33.5. Correlation between CD25 Expression and Foxp3 Expression in Mouse CD4+ Cells.

Direct proof that Foxp3+ T cells maintain control of autoreactive T cells in the periphery has been shown by using a targeting construct encoding the human diptheria toxin receptor fused to sequences encoding GFP and equipped with an internal ribosome entry site into the 3′ untranslated region of Foxp3.[48] Complete elimination of Foxp3+ cells was achieved after 7 days of treatment with diptheria toxin. Foxp3 elimination at birth led to a syndrome very similar to that seen in Foxp3-/- mice. Treg cell elimination in adult nonlymphopenic mice resulted in an even more rapid development of terminal autoimmune disease than in neonates. These studies demonstrate that T cells, probably expressing self-reactive TCRs, are targets of continuous Treg-mediated suppression. After Treg-cell ablation, those T cells become activated, produce secreted and membrane bound cytokines, and facilitate dendritic cell (DC) maturation. At day 7 after ablation, there are increased numbers of B cells, macrophages, Gr-1+ granulocytes, natural killer (NK) cells, and a 10-fold increase in the absolute numbers of DCs. Thus, DC dysregulation is a general phenomenon resulting from the absence of Treg cells. Furthermore, Foxp3-independent recessive and dominant tolerance mechanisms established in adult mice are not sufficient to protect mice from fatal autoimmunity after elimination of Treg. Even a partial

decreased level of Foxp3 protein in Treg cells as a result of induced dysregulation of Foxp3 gene transcription results in marked attenuation of Treg suppressor function.[49]

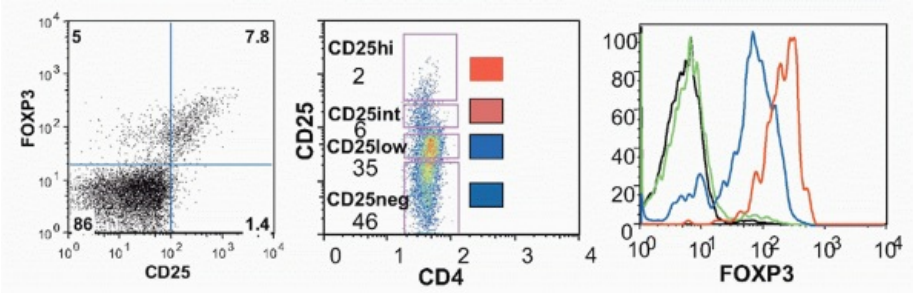

FIG. 33.6. Correlation between CD25 Expression and Foxp3 Expression in Human CD4+ Cells.

Isolation of human Tregs continues to be problematic as one must rely on expression of cell surface antigens. It was reported that among T cells, only the highest expressors of CD25 (˜2% of CD4+ T cells) exerted significant suppressive effects in vitro.[29] This observation was substantiated by a comparison of Foxp3 expression with CD25 expression on human CD4+ T cells. Almost all human CD4+ $CD25^{high}$ cells are Foxp3+, but a variable percentage of $CD25^{int}$ cells express lower, but substantial amounts of Foxp3 (Fig. 33.6). Flow cytometric isolation of the $CD25^{high}$ cells does allow one to obtain a population that is almost uniformly Foxp3+, but a substantial subset of Foxp3+ cells present in the $CD25^{int}$ population will be lost. It has been proposed that almost all of human Foxp3+ T cells can be identified as expressing low levels of the IL-7 receptor (CD127) and that $CD127^{low}$ expression can be

used to isolate human Treg.[50] However, CD127 is downregulated early in the course of T-cell activation, so the $CD127^{low}$ phenotype is also unlikely to be Treg-specific during an ongoing immune or inflammatory response. In addition, only about 40% of the $CD127^{low}$ population is Foxp3+ and even purified $CD4+CD127^{low}CD25+$ cells were only 85% to 90% Foxp3+. Nevertheless, the differential expression of CD127 has proven useful for the isolation of human Treg.

Because the number of Foxp3+ T cells is remarkably constant in normal animals in the absence of perturbation of the immune system, they have been frequently referred to as thymic-derived, "naturally occurring" Tregs,[51] whereas Foxp3+ T cells that are generated extrathymically are termed "adaptive or induced" Tregs. As both populations of Tregs can exert potent biologic functions in vivo and both are generated during the course of normal T-cell development and differentiation, this nomenclature is not accurate. This chapter will primarily describe the biologic functions of Foxp3+ T cells that will be referred to as Tregs irrespective of their site of generation. In the discussion of some of the papers published prior to the availability of reagents specific for the detection of Foxp3, they are referred to a CD4+CD25+ T cells. More precise definitions of the site of origin will be used where appropriate. The potential regulatory functions of Foxp3- T-cell populations will be summarized in section on Foxp3- Regulatory T cells.

BIOLOGIC PROPERTIES OF FOXP3+ REGULATORY T CELLS

Development of Regulatory T Cells in the Thymus

The potential role of the thymus in the generation of Treg cells was first described several years ago.[52] Most studies strongly support the view that the Foxp3+ Treg population is produced in the thymus as a functionally mature distinct T-cell subpopulation. Thymic Foxp3+ Tregs are not derived from peripheral cells that have recirculated from the periphery to the thymus because Tregs are developed in vitro in organ cultures of fetal thymus. Foxp3+ thymocytes are nonresponsive and suppress T-cell activation in vitro in a manner similar to Tregs derived from the periphery.[28] The capacity of Foxp3+ T cells to migrate from the thymus to the periphery was documented by injection of fluorescein isothiocyanate intrathymically. The percentage of Foxp3+ T cells within migrants and resident T cells was identical, suggesting that Foxp3+ T cells in the periphery can originate in the thymus.[53] Thymectomy at 4 to 5 weeks of age did not modify the number of Foxp3+ T cells in the periphery even when tested 19 months later.

It is widely accepted that conventional CD4+ and CD8+ T cells develop in the thymus by a process of positive and negative selection. Positive selection is mediated by interaction of developing T cells with MHC antigens on thymic cortical epithelium. Negative selection is mediated both by DCs and thymic medullary epithelium. As Treg cells have an antigen-experienced phenotype in the absence of exposure to foreign antigens, it seems logical that their TCRs would be biased toward recognition of self-antigens. A number of studies have suggested that Tregs undergo a unique developmental process during their generation in the thymus. When TCR-transgenic mice bearing a TCR specific for a determinant (S1) derived from influenza hemagglutinin (HA) in association with $I\text{-}E^{d}$ were crossed to mice expressing the HA transgene, the transgenic T cells were not deleted and a

large proportion expressed CD25 and functioned as Tregs.[54] Radioresistant elements of the thymus were shown to be both necessary and sufficient for the selection of Foxp3+ T cells in these doubly transgenic mice. Similar results were obtained when TCR transgenic mice on a RAG-/- background were mated to the HA transgenic mice, clearly indicating that thymocytes that can only express a single transgenic TCR can undergo selection to become Tregs. A second TCR transgenic mouse was generated that expressed a variant determinant of HA, but that recognized the S1 determinant with 100-fold less affinity. When these mice were bred to the HA transgenic mouse (S1), they did not have an increased frequency of Foxp3+ T cells. Thus, thymocytes with a low intrinsic affinity for the S1 peptide did not develop into Foxp3+ thymocytes in response to HA. These data are consistent with a model in which selection of Tregs that express a transgenic TCR depends on a high-affinity interaction of the TCR with its ligand. Treg differentiation results from interactions that lie in between the signaling strength required for conventional positive selection on one side and clonal deletion on the other side. It could not be determined from these studies whether this selection process occurs on cortical or medullary epithelial cells.

A number of other models have been proposed[55] for Treg differentiation in the thymus. Recent studies favor TCR specificity as playing a dominant role in thymic Treg cell development.[56,57] Transgenic mice expressing TCRs derived from Treg cells bore very few thymic Foxp3+ T cells in contrast to TCR-transgenic mice using foreign antigen-specific TCRs. The failure of these mice to develop significant numbers of Foxp3+ T cells suggested that developing T cells with the same antigenic specificity compete for a limited niche for Treg-cell development. In mixed bone marrow chimeras with varying proportions of polyclonal progenitors and Treg cell-derived TCRs, a striking inverse correlation was observed between the percentage of TCR-transgenic cells and the propensity of these cells to give rise to Foxp3+ T cells. When TCR-transgenic cells accounted for 10% of total cells, 1% expressed Foxp3, but when TCR-transgenic cells were only 0.1% of the total cells, 30% expressed Foxp3. This result suggests the existence of a niche that limits Treg cell development. Other studies have demonstrated efficient clonal deletion of CD4+Foxp3- T cells and the complete absence of Foxp3+ T cells in a transgenic mouse expressing a different Treg-derived TCR transgene consistent with the view that Treg-derived TCRs are self-reactive.[58]

Some studies have raised the possibility that Tregs are generated in the thymic cortex,[59] whereas others favor the view that the thymic medulla is the site where Treg precursors mature after contacts with tissue-specific peptides presented by thymic medullary epithelial cells or DCs.[60] It is likely that both both DCs and thymic medullary epithelial cells can facilitate Treg development.[61] It is still possible that different TCR specificities are selected by each APC subset. AIRE expression in thymic medullary epithelial cells might favor tissue specific antigens in the thymus, but Treg maturation is normal in AIRE-/- mice that have impaired presentation of tissue-specific antigens and in CCR7-/- mice that have defects in thymocyte trafficking. Some peripheral DCs can migrate into the thymus and present peripheral antigens to developing thymocytes. The ability of the different types of APCs to mediate negative selection versus Treg development may also differ.

Events in addition to TCR signaling are also required for the induction of Foxp3+ T cells. CD28 signaling also plays an important cell intrinsic role in the development of Foxp3+ T

cells in the thymus.[62] CD28 primarily improves the efficiency of Treg cell development rather than enhancing the number of TCRs that can facilitate Treg cell development. CD28 signaling is important for the generation of cytokine responsive Treg cell precursors, as the TCR repertoires of WT and CD28-/- mice were identical.[63] The ability of CD28 to support Treg generation does not require an intact phosphoinositide 3-kinase (PI3K)-binding motif as PI3K signaling through Akt and mammalian target of rapamycin (mTOR) will antagonize Treg differentiation.[64,65]

Cytokines also play a critical role in Treg development. The thymic population of Tregs is only mildly reduced in IL-2-/- or IL-2Rα-/- mice.[66] A more dramatic decrease is seen in mice with targeted mutations of IL-2Rγ, JAK3, or signal transducer and activator of transcription (STAT)5, signaling molecules shared by IL-7 and IL-15. Because neither IL-7 or IL-15 deficiency by itself affects the thymic production of Treg cells, it is likely that IL-2 is the principal common γ-chain cytokine required for intrathymic Treg cell development, but that in its absence IL-7 and IL-15 can compensate to a certain extent.[67] The development of Foxp3+ thymocytes occurs in two stages. A relatively small CD25+Foxp3- subset of CD4+ SP thymocytes is enriched in precursors of Foxp3+ Treg cells. These Foxp3- Treg precursors require common γ-chain cytokines, but no further TCR stimulation for acquisition of the mature Foxp3+ Treg phenotype. In this “two step model,” the first step is a TCR- and CD28-driven instructive phase, while the second step is the cytokine-driven consolidation phase.[68,69] Although multiple cytokines can induce Foxp3+ Treg cell differentiation, they all do so via STAT5 activation. Activated STAT5 is likely to directly bind and regulate the Foxp3 gene at the promoter and at an intronic enhancer region.[69] Transgenic mice expressing a constitutively active form of STAT5 have a marked increase in the percentage and total numbers of Foxp3+ Tregs.

While other cytokines that use the γ common chain can substitute for IL-2 for the development of Tregs in the thymus, IL-2 is required for the maintenance and survival of Foxp3+ T cells in the periphery. The numbers of Foxp3+ Treg cells in the periphery of IL-2-/-, IL-2Rα, and IL-2Rβ-/-[70] mice are substantially decreased or almost absent.[71] Partial or more profound defects both in the number and function of Foxp3+ T cells have been reported in mice deficient for CD80/CD86, CD40,[72] CD40L,[73] and Stat5a.[74] The one common factor that characterizes these mice is that the products of all the deficient genes have important roles in the production or responsiveness to IL-2. Treatment of mice with anti-IL-2 or with CTLA-4Ig to inhibit costimulatory signals also leads to a rapid decline in the number of Foxp3+ T cells.[38] As Foxp3+ T cells do not produce IL-2, this deficiency may

be secondary to the capacity of Foxp3- T cells to produce IL-2,[75] or to some intrinsic defect in the Foxp3+ T cells in their capacity to respond to IL-2. IL-2Rα-/- T cells in mixed bone marrow chimeras had a substantially decreased competitive fitness compared to WT Foxp3+ Tregs. The conclusion drawn from these studies was that IL-2 is an essential Treg cell growth factor in vivo and that in the absence of IL-2, Treg cells exhibit an impaired metabolic fitness. It appears that Foxp3 expression in Tregs establishes a gene expression program that renders Tregs critically dependent on paracrine IL-2 signalling resulting in repression of IL-2 production and the induction of IL-2Rα. IL-2 is not only required for the survival of Foxp3+

Tregs in the periphery, but also appears to be critical for maintenance of their suppressive functions.[76,77,78] Deletion of the proapoptotic factor, Bim, results in the restoration of normal numbers of Foxp3+ T cells in IL-2-/- mice, but Bim-deleted mice still exhibited lethal autoimmunity.[78]

The potential role of transforming growth factor (TGF)-β in the induction of Foxp3+ T cells during thymic development remains controversial. Liu et al.[79] demonstrated that very few Foxp3+ cells could be detected in the thymus of TGF-βRI-/- mice between 3 and 5 days of age. Later in life, the number of Foxp3+ T cells in the thymus increased and this expansion was driven by the production of IL-2. Very similar results were observed in TGF-βRII-/- mice, with a reduced number of Foxp3+ thymocytes at 3 to 5 days of age and increased numbers at 2 to 3 weeks of age.[80] The few Foxp3+ T cells in the TGF-βRII-/- mice expressed high levels of caspase and enhanced apoptosis associated with high amounts of the proapoptotic Bcl-2 family members, Bim, Bak, and Bax. Bim deficiency enhanced the number of peripheral Tregs. Thus, TGF-β is probably not essential for the induction of Foxp3 expression during Treg cell development, but the antiapoptotic effects of TGF-β are required for Treg survival.

Transcriptional Regulation of Regulatory T Cell Development

Recent studies of regulatory elements within the Foxp3 locus offer insights into the molecular mechanisms of Foxp3+ T-cell differentiation and maintenance.[81] Conserved noncoding deoxyribonucleic acid (DNA) sequence (CNS) elements at the Foxp3 locus encode information defining the size, composition, and stability of the Treg population (Fig. 33.7). CNS3 contains a DNase I hypersensitive site, and the NF-κB family member c-Rel binds to this element and likely facilitates the activity of the Foxp3 promoter. Gene targeting studies reveal an essential role for CNS3 in the induction of Foxp3 expression in the thymus and in the periphery. CNS3-/- mice remain healthy despite an approximately fivefold decrease in Treg cells compared to WT mice. Consistent with the documented binding of c-Rel to CNS3, c-Rel deficiency in thymic precursor cells results in a profound impairment in Treg differentiation.[82] In contrast to other CNS regions, CNS3 contains permissive chromatin in double positive (DP) and CD4SP cells that can facilitate binding of c-Rel as a homodimer. This result suggests that c-Rel may be a pioneer transcription factor that opens the Foxp3 locus to other transcription factors. It is likely that that c-Rel binding to CNS3 in cooperation with other transcription factors, including NFAT, CREB, p65, and Smad, facilitates formation of a c-Rel containing enhanceosome at the Foxp3 promoter. Other members of the NF-κB pathway are important for Treg differentiation, as mutations of PKCθ, CARMA1, Bcl-10, TAK1, and IKKβ greatly reduce thymic Treg numbers.[83]

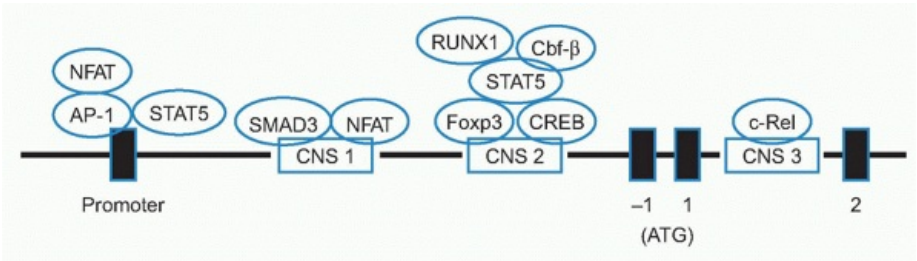

FIG. 33.7. Transcription Factors Controlling Foxp3 Expression. Adapted from Zheng et al.[81] and Ruan et al.[82]

CNS2 also known as the Treg-specific demethylated region contains a cytosin-phosphatidyl-guanosin (CpG) island whose methylation status is correlated with the stability of Foxp3 expression.[84] CNS2 CpGs are demethylated in Foxp3+ T cells, but fully methylated in Foxp3- T cells. While Foxp3 induction in the thymus and periphery was unimpaired in CNS2-/- mice, Treg cells lacking CNS2 progressively lost Foxp3 expression upon division. Thus, CNS2 has a nonredundant function for the maintenance of Foxp3 in the progeny of dividing Treg cells. Foxp3 is also recruited to CNS2 with the assistance of Runx-1 and its co-factor Cbf-β. The binding of the Foxp3-Runx-1-Cbf-β complex confers stability to Foxp3 expression and is also involved in facilitating Foxp3 promoter activity. The recruitment of Foxp3 to CNS2 and the resulting maintenance of Foxp3 expression represent the first description of a feed-forward mechanism enforcing heritable cell lineage destiny. Foxp3 binding to CNS2 occurs after and is dependent on demthylation of CNS2.

CNS1 contains NFAT, Smad3, and STAT5 binding sites. Analysis of mice deficient in CNS1 revealed that thymic differentiation was unaffected, whereas peripheral Foxp3 induction was severely impaired by CNS1 deficiency.[81] Deficiency in peripherally generated Treg cells did not result

in detectable tissue specific autoimmunity in CNS1-/- mice until 1 year of age. These results suggest that mechanistic requirements for thymic and peripherally induced Tregs are distinct from Tregs differentiated in the thymus.

T-Cell Receptor Repertoire Analysis of Foxp3+ Regulatory T Cells

As noted previously, several lines of evidence suggest that Treg development hinges upon a particular TCR specificity. Foxp3+ Tregs in general do not develop in TCR-transgenic mice lacking RAG genes, suggesting that only cells with certain TCR specificities can develop into Tregs. Several studies have addressed in detail the TCR repertoire of Foxp3+ Tregs and their antigen specificity.[85,86] Hsieh et al.[85] directly compared the sequences of the CDR3 region on Treg and conventional T cells and found that they are only partially overlapping. When immunodeficient mice were reconstituted with T cells expressing diverse Vα2 TCR α-chains from Treg or conventional T cells paired with a single Vβ chain, some of the Treg TCR reconstituted T cells were selfreactive, as they induced wasting disease in lymphopenic hosts, but not in conventional recipients. Although 40% of the TCR were strongly self-reactive in vivo, others were only intermittently and moderately self-reactive or nonreactive as measured by T-cell expansion in lymphopenic hosts. Other studies[86] have demonstrated a large degree of overlap in TCR usage between Foxp3+ and Foxp3- T cells. The recent experiments[56,57,58] demonstrating that TCR self-specificity is required for the intrathymic induction of Foxp3 expression strongly support the view that the TCRs of the Foxp3+ population are predominantly self-specific.

TCR repertoire analysis has also been used to determine the relationship between thymic Foxp3+ Treg cells and Foxp3+ Tregs in the periphery. A comparison of the TCR repertoire of thymic and peripheral Foxp3- and Foxp3+ T cells revealed that the TCRs from Foxp3+CD4+ peripheral T cells resembled those of thymic precursors and differed from TCRs expressed by Foxp3-CD4+ T cells; in addition, the diversity of Foxp3+ TCRs was greater than TCRs on Foxp3-CD4+ naïve T cells.[87] These studies suggested that the great majority of peripheral Foxp3+ Tregs derive from thymic precursors and that peripheral conversion from Foxp3- to Foxp3+ is rare. TCR repertoire analysis has also been applied to autoreactive T cells in mice deficient in Foxp3.[88] These mice did not exhibit a defect in negative selection. Activated, but not naïve, T cells in Foxp3-/- mice often used TCRs found in the Foxp3+ Treg TCR repertoire of normal mice, suggesting that T cells expressing these selfreactive TCR are not eliminated and might contribute to the pathology associated with Foxp3 deficiency.

Development of Foxp3+ Regulatory T Cells Extrathymically

One important question that must be addressed is whether the thymus is the only site for the generation of Foxp3+ Tregs. A number of studies performed both in vivo and in vitro have demonstrated that under certain conditions Foxp3+ Tregs can be generated extrathymically and that TGF-β is a key component in this process. Administration of peptide antigen to TCR transgenic mice bred onto the RAG-/- background, that lack Tregs, resulted in prolonged expression of CD25 only when the antigen was administered under tolerogenic conditions.[89,90] These CD25+ cells had some Treg properties. The mechanism of suppression used by these induced Tregs was not explored. One highly effective method for the induction of Tregs was to expose TCR transgenic T cells on a RAG-/- background in vivo to a continuous supply of subimmunogenic doses of agonist peptides delivered via a mini-osmotic pump. Many of the T cells became Foxp3+ and resembled thymic-derived Tregs in all their phenotypic and functional properties.[91] It was postulated from these studies that low doses of peptide presented over relatively long periods of time on nonactivated DCs represented a important mechanism by which Tregs could be generated in the periphery particularly to self-components that do not lead to tolerance in the thymus. Foxp3+ T cells were also induced when mice were treated with peptide agonist ligands targeted to DCs in minute doses by coupling them to antibodies directed to DCs under conditions of suboptimal DC activation.[92] Tregs induced with subimmunogenic conditions could subsequently be expanded by delivery of antigen in immunogenic conditions. DC-targeted de novo generation in vivo results in efficient demethylation of conserved CpG motifs within the Treg-specific demethylated region (CNS2) region of the Foxp3 locus, a predictive parameter for long-term stability of induced Foxp3 expression.[93]

One interpretation of the studies described previously is that weak TCR stimulation is favorable for the peripheral induction of Foxp3 and that robust TCR signaling during Treg cell generation stimulated the Akt-PI3K-mTOR pathway that antagonizes the induction of Foxp3[64] and results in cellcycle dependent maintenance of a silenced state of the Foxp3 locus.[94] More recent studies have examined the role of the strength of the antigenic signal required for the induction of Foxp3+ T cells in the periphery.[95] Foxp3+ cells induced by weak agonist peptides did not persist compared to cells generated by a higher affinity ligand. There appear to be qualitative differences between weak and strong signals whereby a few strong

signals may be different from many weaker signals. The few strong signals may specifically induce survival signals, while the many weaker signals may induce proapoptotic signals. There also appears to be a unique subpopulation of recent thymic emigrants that are $CD25^{hi}CD62L^{int}$ CD69+ that are capable of becoming Foxp3+ upon exposure to IL-2 alone in the absence of TGF-β.[96]

A number of groups[97,98,99,100] have presented studies that demonstrate a pivotal role for TGF-β in the generation of Foxp3+ Tregs in vitro. TCR activation in the presence of TGF-β converted naïve T cells into Foxp3+ (Fig. 33.8) cells with an anergic/suppressor phenotype. IL-2 plays a nonredundant role in TGF-β-mediated induction of Foxp3 expression in mouse CD4+ T cells. Once induced, Foxp3 expression was maintained both in vitro and in vivo in the absence of IL-2. Other cytokines utilizing the common γ-chain as part of their receptors were unable to induce Foxp3 expression in IL-2-/- cells. When T cells are activated through the TCR

in the presence of TGF-β, both RORγT and Foxp3 are transcribed and interact with each other. In the presence of IL-2, Foxp3 expression dominates leading to suppression of RORγT transcription, promoting Treg development. In contrast, in the presence of IL-6, and low levels of IL-2 the transcription of RORγT is favored which in turn represses Foxp3 transcription with resultant Th17 devlopment.[101]

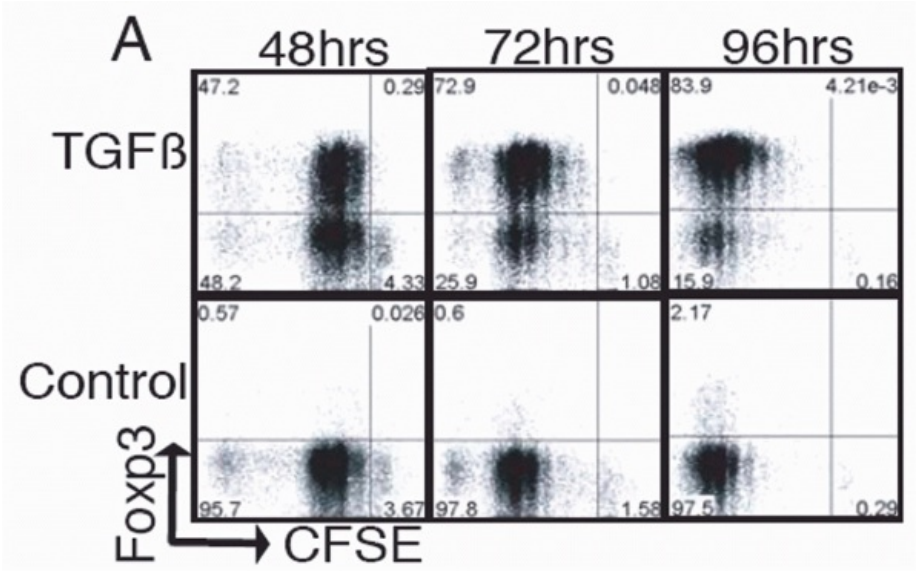

FIG. 33.8. Naïve Foxp3- T Cells were Cultured and Stimulated in the Presence of Transforming Growth Factor-χ. Foxp3 expression was determined by intracellular staining at the indicated times. Adapted from Davidson et al.[100]

TGF-β-induced Foxp3+ T cells not only exhibit unresponsiveness to TCR stimulation, but also suppress normal CD4+ T-cell activation and Th1 and Th2 cytokine production in vitro. The induction of Foxp3 results in downregulation of Smad7 and renders T cells highly susceptible to the regulatory effects of TGF-β signaling via Smad3/4.[98] Foxp3+ T cells

induced in vitro can suppress antigen-specific proliferation of CD4+ T cells in vivo and prevent house dust mite-induced mouse asthma.[97] One of the major factors preventing induction of Foxp3 expression in vitro is the presence of interferon (IFN)γ with subsequent STAT1 signaling.[102] Some studies suggest that TGF-β may also be capable of inducing Foxp3 expression in vivo. Peng et al.[103] used an inducible system for the transient induction of TGF-β in the pancreatic islets during the priming phase of diabetes. Approximately 40% to 50% of intraislet CD4+ T cells expressed CD25 and other characteristics of Tregs. However, it is not clear if high levels of TGF-β resulted in the in situ expansion of the small numbers of Tregs already present in the islets or if TGF-β induced the conversion of CD25- T cells to CD25+ suppressors.

The gut appears to be one of the major sites for the generation of Tregs in the periphery, and the gut contains a large number of Foxp3+ Tregs. Mucida et al.[104] first demonstrated that repeated antigen feeding induced antigenspecific Foxp3+ Treg cells in the absence of thymic-derived Tregs. Converted cells were rapidly found in the mesenteric LN, Peyer patches, and primarily in the small intestine lamina propria. Gut-associated lymphoid tissue has a number of unique properties that facilitate the generation of Foxp3+ T cells. A vitamin A metabolite, retinoic acid (RA), produced by gut DCs, can selectively induce molecules such as CCR9 and the integrin, α4β7, on both conventional T cells and Treg cells, and these cell surface antigens are involved in directing homing of cells to the gut. DCs from the lamina propria of the small intestine have the unique ability to generate Treg cells in vitro via a mechanism that involves the synergistic action of TGF-β and RA. A subpopulaton (25%) of lamina propria DCs express CD103, and this CD103+ subpopulation was responsible for the induction of Foxp3 in the absence of exogenously added TGF-β. Endogenous TGF-β was responsible for Treg conversion, as anti-TGF-β largely abrogated Treg induction. Addition of RA to splenic DC cultures enhanced Treg conversion, and blockade of nuclear RA signaling decreased converted Tregs, but RA alone is not sufficient for Foxp3 induction. Thus, the capacity of the intestinal immune system to produce TGF-β and RA explains the unique capacity of the gut to induce

tolerance.[105,106] In addition to the TGF-β induction pathway, Foxp3+ Tregs can also be induced via PD-L1-PD-1 ligand receptor pathway[107] and by environmental factors such as some aryl hydrocarbon receptor agonists.[108]

One major issue that remains to be addressed is the relative contribution of thymic-derived versus peripherally induced Tregs to the establishment of immune tolerance. Although Tregs are induced in the colitis model from Foxp3- T cells, the induced Tregs are not sufficient to prevent disease.[109] Some studies suggest that only the combination of thymic-derived Tregs and in vitro generated Treg cells could fully protect mice from developing inflammatory bowel disease (IBD) induced by naïve T cells from Foxp3-/- donors. When scurfy mice were reconstituted with thymic-derived Tregs alone, a marked inhibition of lethality was observed, but the treated mice still displayed signs of inflammation.[110] Surprisingly, the combined treatment with thymic-derived Tregs and conventional Foxp3- T cells, that could differentiate into Foxp3+ T cells, decreased inflammation. In vitro generated Treg cells could substitute for Tregs generated in vivo from Foxp3- conventional cells. One explanation for this result was that the TCR repertoires of the thymic-Tregs and the Tregs induced in the periphery were

largely nonoverlapping and that each Treg cell subset is responsible for recognizing different antigens. The major conclusions drawn from this study is that peripherally induced Treg cells are an essential, nonredundant regulatory subset that acts synergistically with thymic-Treg cells to enforce peripheral tolerance.

If a significant percentage of Foxp3+ T cells are generated at peripheral sites, one should expect that the TCR repertoires of peripheral- and thymus-derived Tregs to be different as peripheral conversion would allow for the generation of Treg cells specific for antigens not presented in the thymus such as those derived from commensal bacteria. Lathrop et al.[111] found a significant rate of conversion when CD4+Foxp3- T cells were transferred into lymphopenic hosts. There was considerable disparity in TCR usage between converted Foxp3+ and expanded Foxp3- T-cell subsets within the same host. The converted TCRs were found in the normal non-Treg cell subsets because the donor cells for these studies were normal Foxp3- T cells. However, some of these TCRs were also found in the normal Treg subset and made up 5% of the sequences in the normal Treg pool. It appears that some TCRs are able to facilitate both thymic and peripheral Treg cell development. It is likely that tissue-specific antigens influence the local Treg TCR repertoires as several studies suggest that the presence of an organ facilitates Treg-mediated tolerance to that organ.[112,113,114,115,116,117,118]

Peripherally induced Tregs may differ markedly from thymic-Tregs in terms of TCR repertoire expression, stability, suppressor mechanisms, and potential suitability for in vitro expansion and cellular therapy. Thus far, no cell surface antigen has been identified that allows one to reliably distinguish thymic-derived Tregs from Tregs induced in peripheral sites. Some studies have suggested that expression of Helios, a member of the Ikaros transcription factor family, distinguishes thymic Tregs from peripheral Tregs.[119] Helios was expressed intracellulary in 100% of Foxp3+ mouse thymocytes, but is only expressed by ~70% of Foxp3+ T cells in peripheral lymphoid tissues in both mouse and man (Fig. 33.9). Foxp3+ T cells induced in vivo by oral tolerance, following transfer of Foxp3- T cells to RAG-/- mice, or following induction by reconstitution of germ-free mice with bacteria,[120,121] were primarily Helios-. These studies suggest that a significant proportion of Foxp3+ Tregs are generared extrathymically. Further studies are needed to validate the use of Helios expression as a marker for thymic-derived Tregs. Major differences in the composition of the Treg pool may also exist between man and mouse. A subpopulation of human Tregs was highly proliferative in vivo with a doubling time of 8 days.[122] However, these cells were also susceptible to apoptosis and had critically short telomeres and low telomerase activity raising the possibility that they must be produced from another population source. Heteroduplex analysis of TCR from memory T cells and Tregs suggested that they are closely clonally related raising the possibility that a significant proportion of human Tregs are not thymus derived, but are generated from rapidly dividing highly differentiated memory CD4+ T cells.

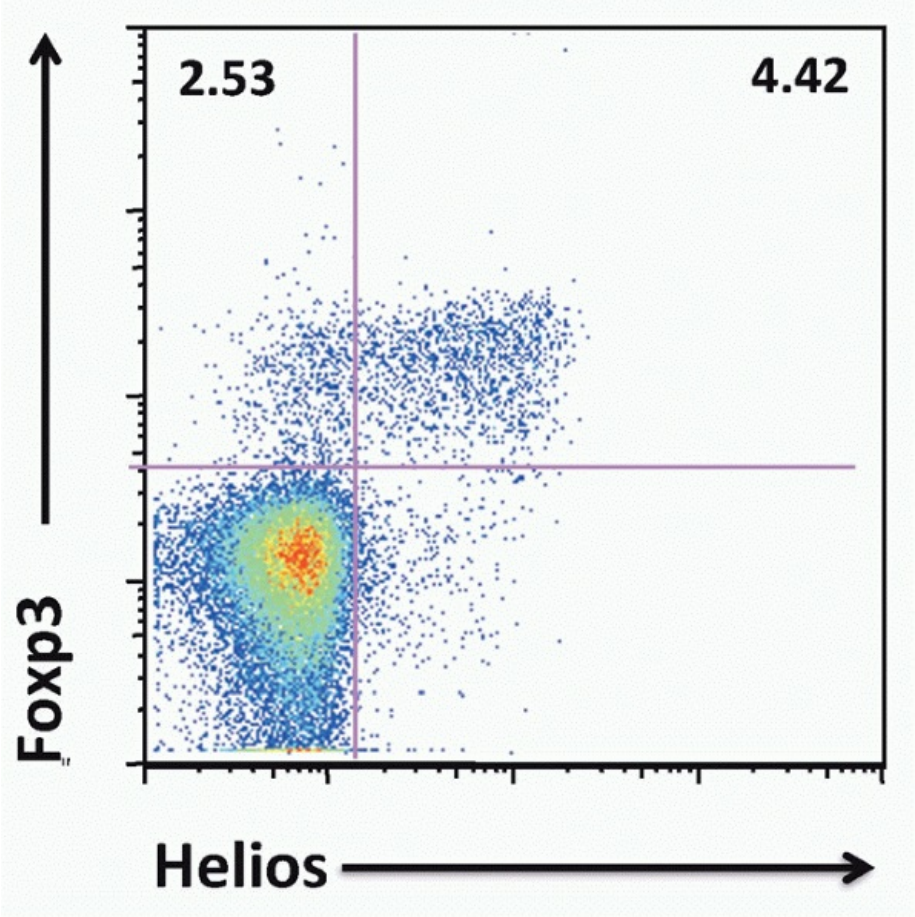

FIG. 33.9. Approximately 70% of Mouse Foxp3+ T Cells Express Helios. Adapted from Thornton et al.[119]

Analysis of Gene Expression by Foxp3+ Regulatory T Cells

Both DNA microarray technology and serial analysis of gene expression technology have been used to compare patterns of gene expression between different cell types.[40,123,124] These technologies have also been applied to compare the patterns of gene expression in Tregs with Foxp3- T cells and other cell types with regulatory functions. One major goal of this approach is to determine whether Tregs simply represent a population of previously activated T cells or whether they display a unique pattern of gene expression that is correlated

with their functional properties. Most of the results are consistent with the latter possibility. Some genes are differentially expressed between the resting Foxp3- T cells and Tregs, while others are differentially expressed following activation.

TABLE 33.2 Genes Selectively Activated in CD4+CD25+ T Cells

- Signaling: COS, SOCS-1, SOCS-2, SLAP-130

- Secreted molecules: IL-10, IL-17, Enkephalin, ETA-1, ECM-1, MIP-1a, MIP-1b
- Cell surface molecules: CD2, OX40, CD25, CD122, GPCR83, GITR, Ly-6, Galectin-1, Thy-1

(Adapted from Hill et al.125)

A comparison of a number of these studies has permitted the identification of groups of genes that are relatively Treg specific and allow one to begin to define a distinct Treg molecular signature (Table 33.2). The “signature” includes both genes encoding certain cell-surface receptors, and genes encoding a wider array of transcription factors and other intracellular proteins.[125] Using a Treg to T conventional fold ratio of 1.5/1 or 1/1.5, 603 genes were differentially expressed, with 407 overexpressed and 196 underexpressed in Tregs. The vast majority of the signature's differential elements were already present in the thymus. Thus, most of the Treg cell signature is already specified at an early stage of lineage commitment, independent of peripheral influences. Many of the Treg signature genes also identify activation antigens expressed on activated conventional T cells including CTLA-4, GITR, and other members of the TNF-receptor superfamily that are expressed by activated effector cells. Some, including neuropilin, GITR, CD38, and CD5, appear to be shared with Foxp3-anergic T cells that do not exert suppressive functions.[126] On balance, the studies on global gene expression indicate that Tregs express a unique pattern of gene expression that may contribute to their survival and anergic state.

A Foxp3 chromatin immunoprecipitation method has been used to analyze the direct targets of Foxp3.[127,128] About 700 to 1000 genes contain Foxp3 binding regions, and many of these genes are up- or downregulated in Foxp3+ T cells, confirming that Foxp3 can function as both a transcriptional activator and repressor. However, Foxp3 target genes comprise only a small portion of Foxp3-dependent gene expression, suggesting that Foxp3 regulates a large part of its transcriptional program by acting on other transcription factors (Table 33.3). It is likely that some of genes encoding molecules that could potentially mediate suppressor function (eg, IL-10, granzyme B) are secondary Foxp3 targets, as they are not found in the group of genes that are direct Foxp3 targets.

TABLE 33.3 Transcription Factors Bound and Regulated by Foxp3

Prdm1

Irf6

CREM

Helios

(Adapted from Zheng et al.127 and Marson et al.128)

One novel approach to understand the role of Foxp3 in directing Treg function is to engineer a mouse expressing the coding sequence of GFP knocked into the *Foxp3* locus together with disruption of Foxp3 protein expression.[129,130] Surprisingly, the GFP+Foxp3- T cells in these mice shared many phenotypic features with their GFP+Foxp3+ counterparts. The former are what might be termed Treg "wannabees," as they develop in normal mice and respond to signals required to induce Foxp3 expression. The GFP+Foxp3- cells were unable to produce IL-2 and effector cytokines, expressed elevated levels of CD25, were relatively nonresponsive to TCR stimulation, but lacked suppressor activity. Treg "wannabees" expressed some (including transcriptional activity at the Foxp3 locus), but not all, of the genes of the Treg signature. Thus, several of the characteristics of the Treg cell lineage can be selected and persist in the absence of Foxp3. Foxp3 does not function as the "master regulator" of the Treg lineage, but stabilizes or enhances certain Treg characteristics, particularly its immunoregulatory activities including suppression. Foxp3 is also not sufficient to elicit the full Treg signature after transfection or induction by TGF-β. Treg "wannabees" do not become conventional effector cells and transfer of these cells into RAG-/- recipients did not induce the scurfy phenotype.[88,131] It appears that WT mice have a population of self-reactive non-Tregs that express the Treg self-reactive TCR repertoire, which are not subject to negative selection but can potently induce autoimmune disease in the scurfy mouse.

Studies of the Treg "wannabee" mice indicate that certain Treg cell phenotypic properties including transcription of the Foxp3 locus and expression of some the Treg signature genes can be independent of Foxp3. One candidate family of genes that can regulate these Foxp3-independent Treg properties are the Foxo (Forkhead-box-O) family of transcription factors that play critical functions in the control of diverse cellular responses. Mice with a T cell-specific deletion of Foxo1 have defects in T-cell survival as well as defects in expression of the IL-7 receptor α-chain and CD62L.[132] Foxo1 is required for the inhibition of T-cell activation and Foxo1-/- mice develop a mild autoimmune disease. Foxo3 plays a role in DC function.[132] Mice with a deletion of both Foxo1 and Foxo3 develop a lethal inflammatory disease associated with a compromised Treg-cell differentiation. Foxo1 and Foxo3 function in a cell intrinsic manner to suppress IFNγ and IL-17 production. Foxo1/Foxo3-/- Treg cells fail to protect lymphopenic mice from autoimmunity induced by scurfy T cells or from colitis induced by Foxp3- T cells.[131] Mixed bone marrow chimeras containing bone marrow from Scurfy mice and Foxo1/Foxo3-/- developed a disease as severe as that observed with scurfy bone marrow alone; this was associated with fewer thymic and splenic Treg cells. Thus, it appears that Foxo proteins control the expression of Foxp3 and a subset of Treg cell-associated genes needed for suppressing inflammatory cytokine production in Treg cells. In chromatin-immunoprecipitation assays using antibody

to Foxo1 and Foxo3, Treg cells were selectively enriched in genomic fragments containing the CNS1 and CNS3 regions, but not the CNS2 region of the Foxp3 promoter. Foxo transcription factors constitute one element of higher order control[132,133] of Treg development and function.

SUPPRESSOR MECHANISMS UTILIZED BY FOXP3+ REGULATORY T CELLS

In Vitro Studies

An in vitro model system has been established for the analysis of Treg-cell function that allows rapid assays and that may offer some insights to the mechanism of action of Treg function in vivo.[37,134,135,136] This approach also allows a comparison of the requirements for activation (costimulation, antigen concentration) of Foxp3+ T cells compared to Foxp3- T cells. Purified Foxp3+ T cells were completely unresponsive to high concentrations of IL-2 alone, to stimulation with plate-bound or soluble anti-CD3, or to the combination of anti-CD3 and anti-CD28. They could be induced to proliferate when stimulated with the combination of anti-CD3 and IL-2. The most striking property of the Foxp3+ T cells is their ability to suppress proliferative responses of both CD4+Foxp3- and CD8+ Foxp3- T cells (Fig. 33.10). The Foxp3+ T cells must be activated via their TCR to suppress. No suppression was seen when Foxp3+ T cells were separated by semipermeable membrane from the CD25- T cells. This demonstrates that cell contact between CD25+ and CD25- T cells is required. Neutralization of the suppressor cytokines IL-4, IL-10, and TGF-β individually or in combination also had no effect on the Treg-mediated suppression. Similarly, Foxp3+ T cells from mice deficient in IL-4, IL-10, or TGF-β were fully competent suppressors. Indo-1-loaded Foxp3+ T cells did not flux calcium in response to TCR stimulation,[123] suggesting that they have a block in proximal signaling similar to that seen in T cells rendered anergic in vitro.

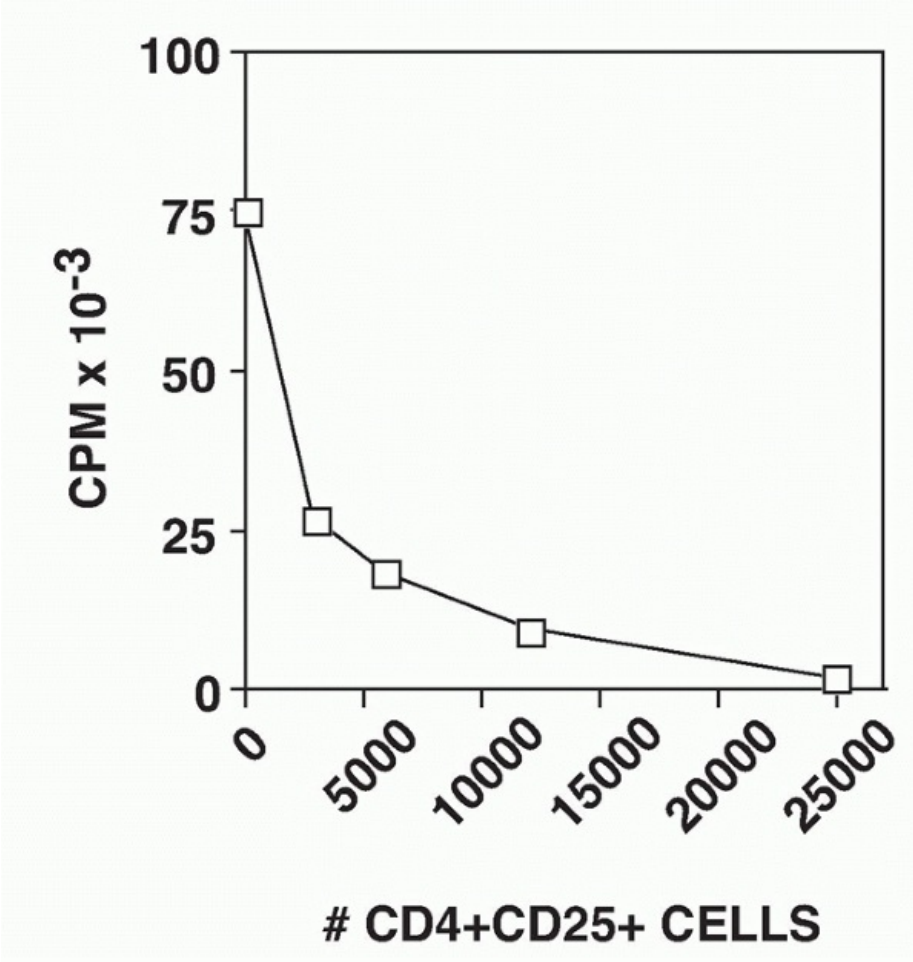

FIG. 33.10. CD4+ CD25+ T Cells Suppress the Proliferative Response of CD4+CD25-T cells. Graded numbers of CD4+CD25+ T cells were mixed with 5×10^4 CD4+CD25- T cells, T-depleted spleen cells, and soluble anti-CD3. Proliferation was measured after 72 hours. Adapted from Thornton and Shevach EM.[37]

Most studies have demonstrated that Foxp3+ T cells mediate suppression by inhibiting the induction of IL-2 messenger ribonucleic acid (mRNA) and mRNA for other effector cytokines in the responder Foxp3- T cells. Suppression can be abrogated by the addition of IL-2, thereby circumventing the block. Although IL-2 gene transcription was inhibited in the presence or absence of exogenous IL-2, the addition of anti-CD28 overcomes suppression by potently stimulating the production of endogenous IL-2 and overriding the suppressive effects of the Foxp3+ cells. Surprisingly, transcription of IL-2 mRNA was also restored in the cocultures in the presence of anti-IL-2. These results suggest that Tregs do not suppress the initial activation of Foxp3- T cells, but mediate their suppressive effects following production of IL-2 by the responder cells resulting in both the expansion of the Treg and induction/enhancement of their suppressor functions.[77] Foxp3+ T cells do not directly mediate the death of the responders, but induce a cell-cycle arrest at the G_1-S phase of the cell cycle. Such a cell-cycle arrest is often followed by cell death; it is difficult to recover significant numbers of viable cells when the suppressors and responders are cocultured for periods longer than 48 hours. Suppression of T-cell proliferation is the exclusive property of Foxp3+ T cells isolated from normal animals. The induction of CD25 expression by stimulating CD25- T cells via the TCR, in the absence of TGF-β, does not render the stimulated cells suppressive.

The role of IL-2 consumption in the suppressive function of Foxp3+ T cells is controversial. Treg cells express all three components of the high affinity IL-2R complex (CD25, CD122, and CD132), and one study[137] has suggested that Tregs may compete with Foxp3- T cells for IL-2 and thereby inhibit the proliferation of Foxp3- T cells secondary to the induction of a form of apoptosis dependent on the proapoptotic factor Bim. In a hybrid system in which human Treg cells were shown to efficiently suppress mouse Foxp3- T cells, the addition of an antihuman CD25 that blocks IL-2 binding had no effect on the function of the human Treg.[138] Taken together with the data that Foxp3+ Tregs inhibit the production of IL-2, it is highly unlikely that Tregs function as IL-2 “sinks,”[139] at least in vitro. IL-2 consumption might play a role in Treg suppression in vivo. However, WT Tregs suppress autoimmunity in IL-2Rβ-/- mice where all other cells in the recipient are nonresponsive to IL-2 directly demonstrating that effective suppression is not simply due to depriving IL-2 growth promoting signals to autoreactive T cells by Treg consumption of this cytokine.[140]

Foxp3+ T cells can be easily propagated in vitro for 3 to 14 days by stimulation initially with anti-CD3 and IL-2 and then expansion in IL-2 alone.[136] It has also been possible to clone murine Foxp3+ T cells by repeated stimulation with anti-CD3 and IL-2.[41] Following 7 to 14 days of activation and

culture in IL-2, activated Foxp3+ T cells remain nonresponsive and cannot be induced to proliferate when restimulated via their TCR in the absence of IL-2. The activated Foxp3+ T

cells have more potent suppressor activity on a per cell basis (three- to fourfold) than freshly explanted Foxp3+ T cells. Foxp3+ T cells that appear to be antigen-specific can be identified in mice that express a transgenic TCR. In general, the percentage of Foxp3+ cells is reduced in TCR transgenic mice (3% to 5% compared to 10% in normal animals). The expression of the transgenic TCR α-chain is a convenient tool that allows activation of the Foxp3+ T cells with peptide/MHC rather than with anti-CD3.[136] When Foxp3+ T cells from TCR-transgenic mice are activated with their peptide/MHC ligand and then expanded in vitro in IL-2, the activated suppressors are subsequently capable of suppressing the proliferative responses of fresh Foxp3- T cells from mice that express a different transgenic TCR. There is no MHC restriction in the interaction of the activated suppressors and the Foxp3- responders. Therefore, the suppressor effector function of activated Foxp3+ T cells in vitro is completely nonspecific (Table 33.4).

Treg-mediated suppression is highly sensitive to antigenic stimulation. For example, when Foxp3+ and Foxp3- T cells are prepared from the same TCR-transgenic mouse and stimulated with a specific peptide, the antigen concentration required to stimulate the Foxp3+ T cells to suppress is 10- to 100-fold lower than that required for triggering the proliferation of the Foxp3- T-cell population.[134] The partially activated phenotype of Foxp3+ T cells combined with their enhanced sensitivity to antigen stimulation suggests that they are highly differentiated in their function and are ready to mediate their suppressive functions immediately upon encounter with their target antigens. Their capacity to rapidly suppress responses in vitro suggests that they have been continuously stimulated by self-antigens in the normal physiologic state and continuously exert some degree of suppression in vivo.

Foxp3+ T cells suppress the proliferative responses of CD8+ T cells in a manner similar to that seen with CD4+ responders.[141] Marked suppression of the effector cytokine, IFNγ is also seen in the presence of Foxp3+ T cells. While suppression of the proliferation of CD4+ responders by Foxp3+ T cells can be completely reversed by the addition of IL-2 or anti-CD28, the suppression of CD8+ T-cell responses is not reversed by the addition of IL-2 or anti-CD28. The failure of IL-2 to abrogate suppression is secondary to a failure of full upregulation of the expression of CD25 on the responder CD8+ T cells. Foxp3+ T cells thereby prevent responses mediated by CD8+ T cells both by inhibiting their ability to produce IL-2 and by inhibiting their ability to respond to IL-2, thus disrupting CD4+ T cell help for CD8+ T cells.

TABLE 33.4 The Suppressor Effector Function of Activated CD4+ CD25+ T Cells is Completely Nonspecific

First Culture	Second Culture (TCR Tg CD4+CD25- T cells Specific For)	% Suppression
CD4+CD35+ from HA TCR Tg stimulated with $HA_{126\text{-}138}$ + IL-2 3 to 7 days	$I\text{-}A^d$ + $HA_{126\text{-}138}$	99

$I\text{-}E^d + HA_{110\text{-}119}$	99
$I\text{-}E^k + PCC_{88\text{-}104}$	95
$I\text{-}A^u + MBP_{Ac1\text{-}11}$	91

HA, hemagglutinin; TCR, T-cell receptor; Tg, transgenic.

CD4+CD25+ T cells from mice expressing a TCR transgene specific for $HA_{126\text{-}138}$ were cultured with peptide, T-depleted spleen cells, and IL-2. They were then washed and mixed with CD4+CD25-T cells from mice expressing the same or different TCR transgenes and then stimulated with the appropriate peptide. (Adapted from Thornton and Shevach.136)

In general, murine Tregs failed to inhibit responses induced by plate-bound anti-CD3, but readily inhibited responses induced by soluble anti-CD3.[37] This finding raised the possibility that the cellular target of the Treg was the APC rather than the responder T cell, as responses to platebound anti-CD3 are relatively APC-independent, but other studies demonstrated that Foxp3+ T cells can mediate suppression in vitro via a T-T cell interaction and that the APC is not directly required for delivery of the suppressive signal.[141] Similar conclusions were drawn in other studies in which Foxp3+ T cells were shown to suppress responses of either mouse[142] or human.[33] Foxp3- T cells induced by anti-CD3 coupled to beads in the absence of APCs and can efficiently directly target responder T cells. It remains unclear whether the mechanisms utilized by Tregs to suppress the proliferation of Foxp3- T cells in the presence or absence of APCs are the same or whether assays of Treg function under these distinct conditions measure distinct components of Treg-mediated suppressor function.

Molecular Analysis of Regulatory T Cell Suppression and Anergy

Recent studies have attempted to define the biochemical and molecular pathways that mediate the anergic or nonresponsive state of Foxp3+ Tregs.[143,144] A number of studies have shown that Tregs exhibit diminished responses of both proximal and distal signalling pathways both in absolute amplitude and in duration when compared to the responses of CD25- T cells. Tregs have reduced phosphorylation of the TCR ζ-chain, reduced recruitment of ZAP-70, and reduced phosphorylation of SLP-76. Defects were seen in the activity of PLC-γ1 and in signals downstream of this enzyme including calcium mobilization, NFAT, NF-κB, and Ras-ERK-AP-1

activation. The diminished activity of PLC-γ1 in Tregs is likely to contribute, not only to the delay and reduced appearance of NFAT in the nucleus, but also to the attenuated activation of the PKC- and Ras-ERK-directed pathways as well. Other studies[145] have suggested that the proximal promoter of the IL-2 gene fails to undergo chromatin remodeling and remains in

a closed chromatin configuration.

Despite the widely recognized importance of IL-2 in Treg homeostasis, very little is known about the intracellular mechanisms that regulate IL-2R signaling in Tregs. Indeed, a defining characteristic of Tregs is their inability to expand in vitro upon stimulation with IL-2 alone despite expression of all three chains of the IL-2 receptor. It has been shown that JAK/STAT-dependent signaling is intact in Tregs stimulated with IL-2, but that downstream mediators of PI3K are not activated.[146] The nonresponsiveness of Tregs is associated with elevated levels of PTEN, a phosphoinositol 3,4,5-triphosphatase that catalyzes the reverse reaction of PI3K, negatively regulating the activation of downstream signaling pathways. In conventional T cells, the level of PTEN is downregulated after T-cell activation, but PTEN remains highly expressed in Tregs. A critical role of PTEN, in the nonresponsiveness of Tregs to IL-2, was established by studying PTEN-/- mice.[147] PTEN-/- Tregs readily proliferated after stimulation with IL-2 alone in vitro and exhibited enhanced peripheral turnover in vivo. They retained their ability to suppress effector T-cell responses both in vitro and in vivo. Forced expression of PTEN in recently activated non-Tregs inhibited their ability to expand in response to IL-2 confirming the ability of this lipid phosphatase to negatively regulate IL-2-dependent proliferation.

As anticipated from their heightened expression of PTEN, Tregs have a defect in the activation of the PI3K-AKT pathway indicated by a reduction in AKT phosphorylation.[64] Thymic-derived Tregs with established and stable Foxp3 levels were resistant to the effects of constitutively activated AKT, while activated AKT inhibits the induction of Foxp3 expression in Foxp3- T cells both in vitro and in vivo. IL-6 may block the differentiation of Tregs by activating an AKTdependent pathway. The repression of Foxp3 expression by active AKT required its kinase activity and was partly counteracted by rapamycin treatment placing mTOR as a downstream target of AKT. mTORC1 is more sensitive to rapamycin treatment, so it is likely that AKT is exerting part of its effect via mTORC1. Conversely, inhibition of the PI3K-AKT-mTOR pathway results in de novo expression of Foxp3 in a TGF-β-independent manner.[148]

The role of Foxp3 in directly controlling the activation of Tregs has been extensively studied. Many of the genes regulated by Foxp3 are also target genes for NFAT. The IL-2 and IL-4 genes are activated by NFAT and repressed by Foxp3, while the CD25 and CTLA-4 genes are upregulated by both NFAT and Foxp3. It is likely that Foxp3 represses IL-2 expression by competing with NFAT for binding to DNA. Foxp3 may also repress NFAT-driven cytokine transcription by sequestering NFAT away from DNA. Examination of the crystal structure of an NFAT:FOXP2:DNA complex revealed an extensive protein-protein interaction interface between NFAT and Foxp2.[149] Structure guided mutations of Foxp3, predicted to disrupt its interaction with NFAT, interfere in a graded manner with the ability of Foxp3 to repress expression of IL-2 and upregulate Treg markers CTLA-4 and CD25. Thus, NFAT can switch its transcription partner from AP-1 that drives T-cell activation to Foxp3 that programs Treg function. Foxp3 also has been shown to interact with the transcription factor AML1/Runx1 that is required for normal hematopoiesis, as well as activation of IL-2 and IFNγ gene expression.[150] The physical interaction of Foxp3 with RUNX1 suppresses IL-2 and IFNγ production and induces Treg cell-associated molecules such as CD25, CTLA-4, and GITR. Treg-cell deficiency of Cbf-β, a cofactor for all RUNX proteins, attenuated expression of

Foxp3 and resulted in high expression of IL-4. The RUNX1-Cbf-β heterodimer is indispensable for optimal expression of Foxp3, maintenance of suppressive function, and suppression of T effector responses.[151] Foxp3 can also directly interact with c-JUN/AP-1 in Treg cells, sequestering activated AP-1 in the nucleus, altering the subnuclear distribution of activated AP-1, and disrupting chromatin binding of activated AP-1.[152] Foxp3 can form a transcriptional repressor complex with several posttranslational modification enzymes such as the histone deacetylases HDAC1, HDAC7, and HDAC9, and histone acetyltransferase TIP60.[153]

Other transcription factors including Eos, a member of the Ikaros family, have been shown to play critical roles in Foxp3 dependent gene silencing in Tregs.[154] Silencing of Eos in Tregs abrogates their ability to suppress in vitro immune reponses and endows them with partial effector function, as they are capable of inducing IBD in vivo. Knockdown of Eos reversed Foxp3-mediated suppression of IL-2 production. Ninety percent of the genes suppressed by Foxp3 were no longer downregulated when Eos was knocked down in Tregs, while ninety-five percent of the Foxp3 upregulated genes were unaltered by Eos knockdown. One other gene that appears to be important in the regulation of Foxp3 suppressor function is the genome organizer SATB1, which is one of the genes most repressed in human and mouse Treg cells.[155] Foxp3 binds directly to the SATB1 locus in Treg cells and prevents SATB1 transcription. Foxp3+ T cells overexpressing SATB1, despite expressing normal levels of Foxp3, lost their suppressive activity and produced T effector cytokines, suggesting that downregulation of SATB1 in Treg cells was necessary for a stable suppressive phenotype. Thus, Treg-cell function not only depends on the induction of Foxp3-dependent genes that mediate suppressor function, but also on the specific repression of molecules such as SATB1 that prevent T effector function

Microribonucleic acids (miRNAs) also act as posttranslational regulators of Treg-cell phenotype, as mice with a deletion of either DICER or DROSHA, RNAaseIII enzymes necessary for miRNA maturation, convert Treg cells to Th1 or Th2 cells.[156] Conditional deletion of DICER in Treg cells confirmed that miRNA are critical for the homeostatic potential of Treg cells, as well as Treg-cell function in inflammation.[157] miRNA-155 is essential for the competitive fitness of Treg cells by modulating SOCS-1 expression thus regulating the response of STAT5 to limiting amounts of IL-2.[158] In contrast, miRNA-146a is important in the suppression

pathogenic Th1 responses by downregulating STAT-1 expression in Treg cells.[159]

Much less is known about the molecular/biochemical signals generated in the responder cell following interaction with Foxp3+ Tregs. Detailed time course studies in vitro have shown that suppression is established after 6 to 12 hours of contact with Tregs, and that responders are refractory to suppression after 12 hours of activation. As suggested by the requirements for IL-2 to activate suppressor function,[77] the IL-2 response of the responders is normal for the first 6 hours of the coculture and then abruptly terminates. Costimulation with anti-CD28 generated resistance to suppression by stabilizing IL-2 transcripts enabling the responder T cells to counterbalance transcriptional down regulation by Tregs.[160] Tregs do not inhibit the early induction of CD69 or CD25 and viability of responder cells was excellent at 36 hours.

Gene expression analysis in responder T cells using a microarray approach has shown that a set of genes expressed after culture with Tregs was distinct from genes induced in anergized T cells, T cells deprived of IL-2, or T cells treated with TGF-β.[161] Hopefully, further detailed studies of these genes will elucidate the specific pathways involved in Treg-mediated inhibition.

Production of Suppressor Cytokines by Regulatory T Cells

Most of the studies with either human or mouse Tregs that have studied suppression of T-cell activation in vitro have failed to identify a soluble suppressor cytokine. It is difficult to rule out the involvement of a cytokine that acts over short distances or a cell-bound cytokine. Nakamura et al.[162] raised the possibility that TGF-β produced by Tregs and then bound to their cell surface by an as yet uncharacterized receptor might mediate suppression in a cell contact-dependent fashion. In their studies, TGF-β was detected on the surface of resting and activated CD25+ T cells, and suppression could be reversed by high concentrations of anti-TGF-β monocloncal antibodies (mAbs). They postulated that latent (inactive) TGF-β bound to the cell surface of activated Tregs is delivered directly to responder CD25- T cells and is then locally converted to its active form. In contrast to these studies, Piccirillo et al.[163] were unable to show a requirement for either the production of TGF-β or responsiveness to TGF-β in Treg-mediated suppression. CD25- T cells from *Smad3*-/- and from mice expressing a dominant negative form of the TGF-β receptor II (TGFβRII), that are completely resistant to the immunosuppressive effects of TGF-β were readily suppressed by Treg cells from WT mice (Table 33.5). Tregs from TGF-β-/- mice were as efficient as Tregs from WT mice in mediating suppression of WT CD25- T cells. High concentrations of anti-TGF-β did not reverse suppression, nor did anti-TGF-β or a soluble form of the TGF-βRII inhibit suppression mediated by activated CD25+ T cells.

An alternative possibility is that TGF-β plays a role in the enhancement or maintenance of Foxp3+ Treg suppressor activity. Indeed, Marie et al.[164] have demonstrated that peripheral TGFβ1-/- Tregs expressed diminished levels of Foxp3. These studies suggested that TGF-β is required for maintenance of Foxp3 expression in peripheral Tregs, that the source of the TGF-β is not the Treg, and that paracrine production of TGF-β by APCs is needed for optimal Treg survival and suppressor function. Further evidence for the role of TGF-β in the maintenance of Tregs was derived from studies of mice with T cell-specific deletion of TGF-βRII.[165] In these mice, TGF-β was not required for induction of Foxp3 expression in the thymus and for the thymic development of Treg cells. In fact, TGF-βRII-/- mice have an increase in the percentage and number of Foxp3+ T cells in the thymus and increased BrdU uptake, suggesting that TGF-β normally inhibits the proliferation of thymic Tregs. Increased Treg proliferation was also seen in the periphery of TGF-βRII-/- mice, but these mice have a decrease in the percentage of Foxp3+ Tregs in the periphery, consistent with the possibility that TGF-β is essential for their survival in the periphery. When mixed bone marrow chimeras between TGF-βRII-/- and WT mice were generated, the knock out Foxp3- T cells demonstrated complete resistance to suppression by WT Tregs. This result suggests that there is a defect in a cell intrinsic mechanism of TGF-β-mediated control of T-cell reactivity in the TGF-βRII-/- T cells.

TABLE 33.5 CD4+ CD25+ Suppressor Function Occurs Independently of Transforming Growth Factor-β

CD4+CD25+	CD4+CD25-	% Suppression
Wild-type	Wild-type	Yes
Wild-type	Wild-type + anti-TGF-β	Yes
Wild-type	SMAD3-/-	Yes
SMAD3-/-	Wild-type	Yes
Wild-type	DNTGF$_{\beta}$RII	Yes

TGF, transforming growth factor.

CD4+CD25+ T cells were mixed with CD4+CD25- T cells in the presence of T-depleted spleen cells and soluble anti-CD3. Proliferation was measured at 72 hours. (Adapted from Piccirillo et al.163)

The TGF-β gene has been deleted in activated T cells and Treg cells or in Treg cells alone to determine the exact source of TGF-β for regulating T-cell tolerance and differentiation. Abrogation of TGF-β1 in activated T cells and Treg cells, but not in Treg cells alone, protected mice from experimental autoimmune encephalomyelitis (EAE) and was associated with compromised Th17 differentiation.[166] Th17 cells were the main producers of TGF-β1 in vitro and in vivo. TGF-β1 produced by Treg cells is not essential for Th17 differentiation in the EAE model.[167] Mice with a specific deletion of TGF-β1 in Tregs showed no signs of inflammation even at the age of 9 months. These mice had increased frequencies of Foxp3+ T cells in peripheral and mesenteric nodes, but not in the spleen, with no increase in conventional CD4+ or CD8+ T cells. These results demonstrate that TGF-β produced by Treg cells alone is specifically required for inhibiting Treg-cell proliferation.

The potential role of TGF-β as a mediator of Treg suppression must be reevaluated, as recent studies[168,169,170] have

identified GARP (LRRC32) as the cell surface receptor for latent TGF-β. Although the GARP/Latent TGF-β complex is also expressed by platelets, within the immune system, GARP expression is most often observed on activated Foxp3+ Tregs (Fig. 33.11). However, the function of the GARP/latent TGF-β complex on Tregs has remained elusive. Tregs that lacked the surface expression of LAP following treatment with TGF-β siRNA or the expression of GARP and LAP following treatment with GARP siRNA were somewhat less suppressive than Tregs treated with the control siRNA in the in vitro suppression assay. The roles for secreted or cell surface associated TGF-β as major mediators of the in vitro or in

vivo suppressive functions or other functions of Tregs remains to be further explored.

TGF-β does appear to play a major role in the in Treg-NK cell interactions.[171] Tregs were shown to inhibit NKG2D-mediated cytolysis in vitro largely by a TGF-β-dependent, IL-10-independent mechanism. Similarly, Tregs directly inhibited NKG2D-mediated NK cell cytotoxicity in vivo, resulting in suppression of NK cell-mediated tumor rejection.[172] Other studies have shown that human Tregs could also directly inhibit NK cell effector functions by a TGF-β-dependent mechanism with a concomitant downregulation of NKG2D receptors on the NK cell surface.[173] NK cell cytotoxicity was restored in the presence of Tregs when the cocultures were performed in the presence of cytokines known to activate NK cells such as IL-2, IL-4, IL-7, and IL-12. Curiously, in both of these studies, Treg cells did not induce a global inhibition of NK-activating receptors, but only an inhibition of NKG2D. In other experiments, depletion of Tregs resulted in enhanced proliferation of NK cells as well as their cytotoxic potential. The role of Tregs in the regulation of NKcell function has also been studied in a bone marrow graft rejection model.[174] One explanation for the susceptibility of NK cells to Treg control is that uninhibited NK cells might be a danger to the host in autoimmune diseases.

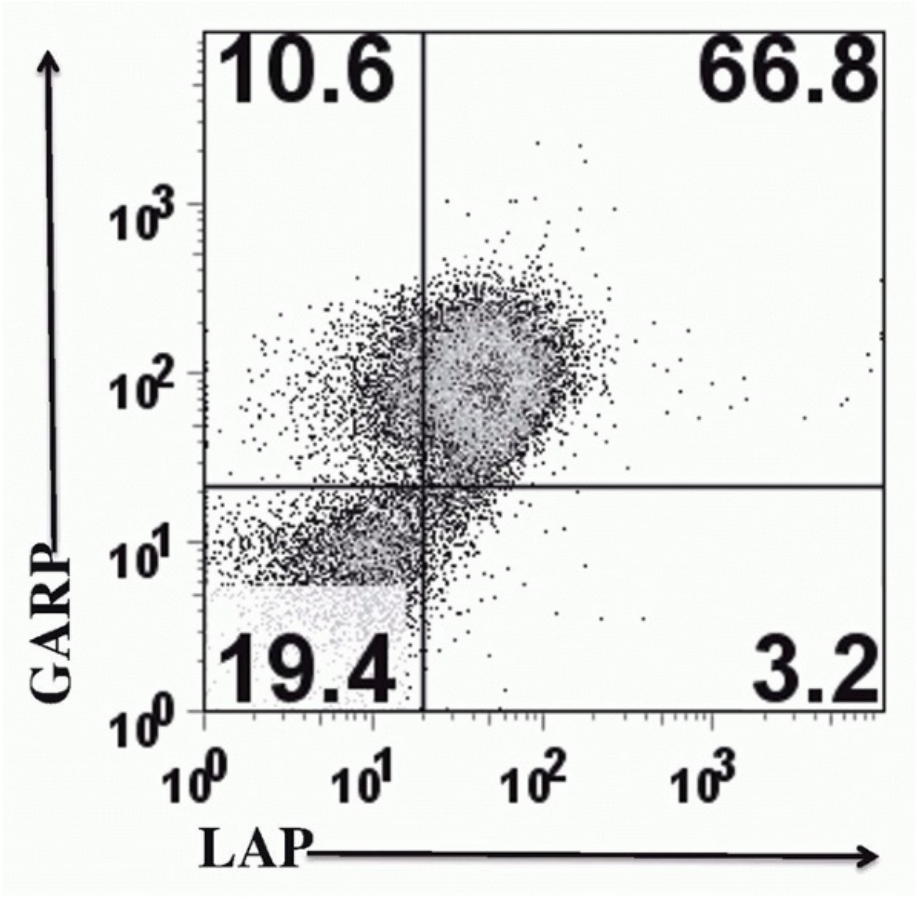

FIG. 33.11. A High Percentage of Activated Human Foxp3+ T Cells Express GARP and LAP. Adapted from Tran et al.[168]

The failure to observe suppression when Tregs are separated from responders does not rule

out the possibility that Tregs secrete an as yet uncharacterized factor that functions in gradient fashion and requires proximity between suppressor and responder. The newly described cytokine, IL-35, a heterodimeric member of the IL-12 family, composed of Epstein-Barr virus-induced gene 3 (Ebi3 which normally pairs with p28 to form IL-27) and p35 (which normally pairs with p40 to form IL-12) may subserve this function.[175] IL-35 is produced by activated Tregs and has been shown to act directly on responder T cells and suppress their proliferative responses. In addition, treatment of naïve mouse or human T cells with IL-35 induced a Foxp3- regulatory population (termed iTr35) that mediated suppression by secreting IL-35, but not IL-10 or TGF-β.[176] iTr35 were as effective as Foxp3+ T cells in preventing lethal autoimmunity in scurfy mice and completely protected mice from induction of EAE. Studies on the role of IL-35 have been hampered by the lack of a recombinant form of the cytokine, neutralizing antibodies, and characterization of its receptor.

Other secreted molecules may also play a role in Treg-mediated suppression including galectin-1 a member of a highly conserved family of β-galactoside binding proteins.[177] Galectin-1 is secreted as a homodimer and binds to many glycoproteins including CD45, CD43, and CD7 resulting in cell cycle arrest, apoptosis, and the inhibition of proinflammatory cytokine production on target cells. Galectin-1 is preferentially expressed in Treg cells and is upregulated upon TCR activation. Blocking galectin-1 markedly reduced Treg suppressor function in vitro and Treg cells from galectin-1-/- mice had reduced suppressor function. Galectin-10, another member of the Galectin family, is selectively expressed by Tregs.[178] However, Galectin-10 is primarily expressed intracellularly and is unlikely to be involved in suppression by Tregs.

Cell Contact-Dependent Mechanisms of Suppression

Cytolysis of target cells must also be considered as another potential mechanism for Treg-mediated suppression. Human Foxp3+ T cells that are activated with a combination of antibodies to CD3 and CD46 express granzyme A and kill activated CD4+ and CD8+ T cells and other cell types in a perforin-dependent, Fas/Fas-L-independent manner.[179] Activation of mouse Tregs also results in upregulation of granzyme B expression, and one report[180] has claimed that activated Treg kill responder cells by a granzyme B-dependent, perforin-independent mechanism. Other studies[181] demonstrated that activated mouse Treg cells inhibited the proliferative responses of B cells to polyclonal B-cell activators. Suppression of proliferation was due to increased cell death caused by the Treg cells in a cell contact-dependent manner. The induction of B-cell death was not mediated by Fas/Fas-L pathway, but depended on the upregulation of perforin and granzymes in the Treg.

It was originally proposed that Tregs target APCs by inhibiting the induction of the expression of costimulatory

molecules, or perhaps by competition for costimulatory signals.[182] In short-term cultures, Treg cells downregulated the expression of markers on immature bone marrow-derived DCs and inhibited their ability to elicit antigen-specific CD8+ T-cell responses in vitro. Activation of immature DCs with CpG DNA or lipopolysaccharide (LPS) abrogated the ability of the Treg to downregulate DC activation markers or to inhibit T-cell activation. Similarly, systemic activation of DC with CpG DNA or an agonistic anti-CD40 antibody completely inhibited the

antidiabetogenic activity of Treg cells in nonobese diabetic (NOD) mice.[183] Although these results suggest that Tregs exert their suppressive effects by keeping DCs in an immature state and that mature DCs are resistant to the effects of Tregs, other studies have shown that Tregs are capable of inactivating CD40L-prestimulated human DCs[184] and that Tregs can dampen immune responses induced by fully mature DCs in vivo.[185] Some of the differences in these studies may be secondary to Treg modulation of cytokine production by the DCs. For example, although Tregs were unable to prevent DC maturation or induction of IL-6 transcription in the presence of strong inflammatory stimuli, they were still able to modulate mature DC responses by enhancing transcription of the anti-inflammatory cytokine IL-10.[186] Treg-mediated enhancement of IL-10 production by fully mature DCs may result in inhibition of T-cell activation and is one mechanism by which Tregs can modulate the function of mature DCs.

Considerable controversy exists regarding the significance of the expression of CTLA-4 on Tregs and its potential involvement in their suppressor function. Takahashi et al.[39] have shown that the addition of the Fab fragment of anti-CTLA reverses suppression in cocultures of Treg and CD4+CD25- T cells. Read et al.[27] demonstrated that treatment of mice with anti-CTLA-4 abrogates suppression of IBD mediated by Treg cells. Reversal of suppression by anti-CTLA-4 or its Fab fragment in vitro has not been seen in all studies.[31,32,33,34,35,36,37] In studies[142] where Tregs suppress the activation of CD25- T cells in the absence of APCs, the addition of anti-CTLA-4 or anti-CD80/CD86 did not reverse suppression, suggesting engagement of CTLA-4 is not required for suppressor function. Treg from CTLA-4-/- mice are as efficient as WT Tregs in mediating suppressor function in vitro, but in contrast to WT Tregs, their suppressive effects were more dependent on TGF-β.[187] One possible functional role for CTLA-4 on Tregs would be to ligate CD80/CD86 on DCs or other cells. Some,[188] but not all,[189] studies have claimed that CD80/CD86-/- CD25- T cells are refractory to Treg suppression in vivo and in vitro, and that interaction of CD80/CD86 on CD25- T cells with a molecule on Tregs, most likely CTLA-4, is needed for suppression to be manifest.

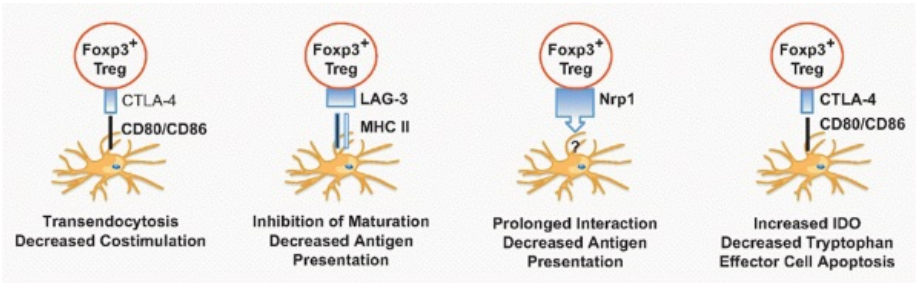

FIG. 33.12. Potential Mechanisms for Regulatory T Cell-Mediated Downregulation of Dendritic Cell Function.

The potential unique role of CTLA-4 as a mediator of Treg suppression has been

strengthened by the demonstration that animals with a selective deletion of the expression of CTLA-4 on Treg develop systemic autoimmunity by 7 weeks of age.[190] Thus, CTLA-4 deficiency in Treg cells alone is sufficient to cause fatal disease and maintenance of its expression in activated effector T cells is insufficient to prevent this outcome. Selective CTLA-4 deficiency does not alter the development or homeostasis of Treg cells or render them pathogenic. Foxp3+ T cells with a selective loss of CTLA-4 expression remain anergic, but are less suppressive in vitro. It has been proposed that the interaction of CTLA-4 on Tregs with its ligands, CD80 and CD86 on DCs blocks the subsequent increase of CD80 and CD86 expression or even downregulates CD80 and CD86 expression induced by CD40/CD40L interactions between T effector cells and DCs. Treg cells from CTLA-4-/- mice are defective when compared with Treg cells from WT mice in preventing the upregulation of CD80/CD86 expression. The major conclusions from these studies are that inhibition of CD80/CD86 expression limits the delivery of costimulatory signals to naïve T cells via CD28 resulting in defective T effector cell activation.

A molecular basis for this cell extrinsic function of CTLA-4 is needed (Fig. 33.12). Quershi et al.[191] postulated that because of its highly endocytic behavior, CTLA-4 can mediate the intercellular transfer of a ligand from one cell to its receptor on a different cell by a process termed transendocytosis. Studies by this group have demonstrated that following Treg activation, CTLA-4 interacts with CD80/CD86 and removes the costimulatory molecules from the cell surface, with subsequent degradation of these ligands inside the CTLA-4-expressing cells, thereby limiting CD28-driven costimulation. In contrast to CTLA-4, CD28 was not capable

of transendocytosis. Although this model can potentially account for Treg-mediated suppression and the phenotype of the Treg conditional CTLA-4-/- mouse, it should also be emphazsized that CTLA-4 expressed by activated T effector cells can also mediate transendocytosis of CD80/CD86.

An additional pathway (see Fig. 33.12) by which the interaction of CTLA-4 on Tregs with CD80/CD86 on DCs can result in suppression is the activation of indoleamine 2, 3-dioxygenase (IDO). IDO is responsible for the metabolism of the essential amino acid tryptophan. Reduced amounts of tryptophan after induction of IDO are associated with decreased activation of T cells.[192] Other studies have demonstrated that in addition to IDO, Tregs may induce multiple other enzymes that consume essential amino acids.[193] The reduction in essential amino acid levels resulted in reduced mTOR signaling that can potentially lead to the de novo induction of Foxp3+ T cells in a manner that is synergistic with TGF-β. This process may be primarily operative at sites of Treg-mediated suppression (eg, protection against skin graft rejection).

A number of other mechanisms have been proposed by which Treg cells abrogate the antigen-presenting activity of DCs or promote the secretion of suppressive factors by the target DC population. One cell surface antigen that may play in Treg-cell suppression of DC function is LAG-3 (CD223), a CD4 homolog that binds MHC class II molecules with very high affinity. Binding of LAG-3 to MHC class II molecules expressed by immature DCs induces an immunoreceptor tyrosine-based activation motif-mediated inhibitory signal that suppresses DC maturation and immunostimulatory capacity.[194] Because activated human, but not

mouse, T cells can express MHC class II, Treg cell-mediated ligation of LAG-3 on activated human T effector cells might also induce suppression of T-cell function. A second molecule that has been proposed to play a role in the interaction of Treg cells with DC is neuropilin-1 (Nrp-1). Nrp-1 is a receptor for class II semaphorins and a coreceptor for vascular endothelial growth factor. Nrp-1 is preferentially expressed on Tregs and can be induced by ectopic expression of Foxp3 in Foxp3- T cells.[195] Nrp-1 promotes long interactions between Treg cells and immature DCs. Anti-Nrp-1 abrogates suppression of proliferation mediated by Treg cells when the responder T cells are stimulated with low concentrations of antigen. These data suggest that the role of Nrp-1 is to give Treg cells a headstart over naïve responder T cells under conditions in which antigen is limiting.

Extracellular adenosine triphosphate (ATP) functions as an indicator of tissue destruction and may exert its effects on DCs. Intracellular ATP concentration is so high that substantial amounts of the nucleotide are released upon cell damage. CD39 is the dominant ectoenzyme in the immune system that hydrolyzes ATP or ADP to AMP and is expressed by a number of different cell types including Tregs. ATP can activate DCs and lead to the upregulation of CD86. The catalytic inactivation of extracellular ATP by CD39 is a potential anti-inflammatory mechanism used by Tregs.[196] The immodulatory effects of removal of ATP by CD39 can be amplified by the generation of adenosine. Adenosine can be generated by CD39 in concert with the 5′-ectonucleoside CD73, which dephosphorylates the CD39 product, AMP.[197] Adenosine signaling via the A2A receptor can inhibit the function of DCs and activated T cells. The documented ability of Tregs to convert ATP to adenosine provides direct evidence of the functional capacity of these enzymes and supports the notion that adenosine is a mediator of Treg function. Adenosine regulates T-cell function indirectly by reducing the secretion of proinflammatory cytokines including IL-12 and TNF-α from stimulated APC by 80% to 90%. The immunosuppressive function of adenosine may be mediated by increasing intracellular cyclic AMP.

Tregs can transfer cAMP to Teff via gap junctions, and elevating cAMP in Tregs renders them more suppressive.[198] Upon entrance to the target cell, cAMP inhibits IL-2 production and proliferation. Tregs also indirectly boost cAMP levels in target cells by augmenting adenosine-mediated signaling from extracellular nucleotides. One other metabolic pathway that can modulate Treg function targets the P2X7 receptor that is expressed at high levels on Foxp3+ T cells.[199] Nicotinomide adenine dinucleotide (NAD+) released by cell lysis or nonlytic mechanisms can activate the P2X7 receptor and induce Treg-cell death. The systemic administration of NAD+ has resulted in selective depletion of Treg cells and enhancement of an antitumor response.

Mechanisms of Suppression in Vivo

There are a number of major differences between the properties of Treg cells in vivo compared to in vitro. Although Treg cells are anergic or nonresponsive in vitro due to their failure to produce IL-2, Treg cells expand in vivo after engagement of their TCR by its cognate antigen in a manner indistinguishable from conventional T cells.[200,201] While most in vitro studies of Treg suppressor function do not implicate suppressor cytokines such as IL-10 as a mediator of suppression, studies in several in vivo models have shown that suppressor cytokines can be major contributors to the suppressive effects of Tregs.[202,203]

While the major effect of Tregs in vitro is to suppress the production of IL-2 by T effector cells, the role of IL-2 in the antigen drive expansion of CD4+ T cells has not been elucidated and antigen-specific cells from IL-2-/- mice expand normally in vivo when triggered via their TCR.[204] Taken together, it is difficult to translate from in vitro studies of Treg function to the in vivo situation. As a major goal of research in this area is the manipulation of Treg function in vivo in disease, a clear understanding of Treg biology in vivo is required. Specifically, which mechanisms from the long list of mechanisms proposed for Treg suppressor function in vitro are used in vivo?

The production of IL-10 by Foxp3+ Tregs plays an important role in many disease models including EAE, IBD, and the immune response to parasites. However, none of the mechanisms proposed for suppression have been proven to operate in unmanipulated animals. Surprisingly, selective disruption of IL-10 in Treg cells led to spontaneous colitis[205] and augmented immune response-associated pathology in skin and lungs. Thus, in unmanipulated mice, IL-10 produced by Tregs plays an important role in suppressing immune inflammation at environmental interfaces. While

the production of TGF-β by Foxp3+ Tregs has been implicated in the suppressive function of Tregs in vivo, a number of experiments have also demonstrated that autocrine TGF-β1 expression is not essential for Treg suppression in vivo. Transfer of TGF-β1-/- splenocytes to RAG-2-/- mice induced disease with features similar to those of the TGF-β1-/- mice and disease transfer was accelerated by the depletion of TGF-β-/- CD25+ T cells. Importantly, cotransfer of TGF-β1-/- Tregs clearly attenuated disease in RAG2-/- recipients of CD25-depleted spleen and lymph nodes, but suppression was incomplete when compared to WT Tregs.[206] Although TGF-β appears to play a nonredundant role in control of intestinal inflammation, Tregs from TGF-β1-/- mice were capable of inhibiting IBD induced by $CD45RB^{high}$[207] or CD4+CD25- T cells[208] in vivo. Furthermore, Tregs from mice expressing a dominant negative form of the TGF-βRII are also capable of inhibiting colitis, suggesting that TGF-β is not required for the function of Tregs. Importantly, the suppressive function of TGF-β-/- Treg cells was abrogated by anti-TGF-β indicating that TGF-β is absolutely required for protection from IBD in this model, but can be provided by a non-Treg cell source.

The in vitro studies demonstrating the cytotoxic activity of Tregs suggested that one should reevaluate the concept of “cytotoxic/suppressor cell” because it appears at least in vitro that Tregs can mediate both of these functions. It remains to be determined whether Tregs can develop into cytotoxic cells in vivo. It has been difficult to demonstrate granzyme B expression by Treg cells in vivo, but Cao et al.[209] have demonstrated that 5% to 30% of Treg cells in the tumor microenvironment express granzyme B and are lytic for NK cells and cytotoxic T-lymphocytes in a granzyme B- and perforin-dependent manner. Foxp3+ T cells also reduce granule exocytosis in cytotoxic T-lymphocytes. This inhibitory effect did not require long-lasting contacts between Foxp3+ T cells and cytotoxic T-lymphocytes, but was dependent on the secretion of TGF-β. It has also been shown that polyclonal Foxp3+ T cells limit DC survival in tumor draining LN through an antigen- and perforin-dependent mechanism involving direct contacts between Foxp3+ Tregs and tumor antigen presenting DCs.[210] Depletion of Foxp3+ Tregs resulted in an increase in the the number of DCs in tumor draining LN, a marked arrest in antitumor CD8+ T cell migration, increased T-cell priming, and

subsequent tumor rejection. This study concluded that the Foxp3+ T cells kill the DC directly, but did not rule out indirect effects of Tregs on the survival of the DC.

Mice that lack Foxp3+ T cells develop spontaneous germinal centers and have high titers of autoantibodies. In many models of organ-specific autoimmune disease, Treg cells suppressed the activation of autoantigen-specific T cells and inhibited the production of autoantibodies to the target organ. As B cells and autoantibody production are prominent mediators of pathology in many autoimmune diseases, it is critical to understand the potential for Tregs to control autoantibody production. While it is clear that Tregs can suppress antibody production by blocking T helper functions, a number of other studies have suggested that Tregs can exert a direct effect on certain B-cell functions. Bystry et al.[211] were the first to suggest that Treg cells could inhibit LPS-mediated B-cell activation. Lim et al.[212] demonstrated that human Tregs can suppress B cell-dependent Ig production and class switch recombination by a cell contact-dependent mechanism in the absence of T helper cells. Foxp3+ Treg cells were identified in T-B area borders and within germinal centers of human lymphoid tissues.[213] Treg cells were also able to suppress the expression of AID, a key enzyme involved in class switch recombination and affinity maturation. The biochemical mechanisms for the suppression of B-cell function in these studies remain unclear.

Treg cells can also control the magnitude and the quality of the germinal center response.[214,215] To complete maturation and exit the germinal center, a B cell must receive help in the form of prosurvival signals from a specialized subset of CD4+ T cells termed T follicular helper (Tfh) cells. Tfh cells mediate selection of only those B cells that express highaffinity variants of their original B-cell receptor. However, cross-reactive affinity-matured B cells that also recognize self-antigens could still be positively selected and allowed to exit the germinal center contributing to the development of autoimmunity. Together with results demonstrating that Treg cells can directly inhibit B-cell activation in vitro, these data suggest that Treg cells may have important function in regulating germinal center responses. Antigen-induced germinal centers contain a substantial population of Foxp3+ Treg cells that have been termed TfTreg. They phenotypically mirror Tfh cells in their expressison of CXCR5, a chemokine receptor that promotes T-cell migration into germinal centers and shares a common developmental pathway that requires contact with B cells and expression of both the signaling adapter SAP and the transcription factor Bcl-6. TfTreg cells likely play an important role in preventing autoantibody production and enforcing self-tolerance after somatic hypermutation. The molecular pathways by which they mediate these complex effects remain to be elucidated.

The potential pathways of Treg suppression in vivo are more complex than those used in vitro. The Treg must able to localize to various parts of the body such that close contact with most effector T cells with a given antigen specificity either directly or through an APC intermediate becomes possible. Unlike the culture situation, there is ample space for T cells to evade suppression in vivo unless Tregs can localize with them in response to antigenic stimulation that begins in antigen-draining lymph nodes. A number of possible mechanisms for the suppressive function of Tregs in vivo have been proposed including whether they inhibit the priming and expansion of T effector cells, block the migration of T effector cells from the site of immunization (eg, the draining LN) to the target organ, or inhibit the differentiation of naïve precursors into pathogenic T effector (Th1, Th2, or Th17) cells.

The first studies to directly examine the effects of Tregs on priming immune responses in vivo used two-photon laserscanning microscopy.[216,217] After entering the LN through high endothelial venules, T cells travel through a region near the paracortical T cell zone and B cell follicles where tissue emigrant DCs are found. In the absence of antigen, T cells move freely within the T cell zones of the LN. In contrast,

in the presence of antigen, T cells interact with the antigenloaded DCs first by a process termed swarming that ultimately results in arrest of their locomotion for a period of hours before they again become motile. Taken together, these studies demonstrated that both Treg cells and T effector cells formed similar clusters with DCs. In the absence of Tregs, cluster formation by T effector cells was more sustained. Cluster formation by Tregs preceded the inhibition of T effector activation. Treg cells appeared to block T effector cell priming by decreasing the contact time between DCs and the naïve CD4+ T cells. Surprisingly, no direct interactions between Treg and T effector cells were observed in these studies, indicating that the major suppressive effects of Tregs were directed to the DCs. The results of these studies are consistent with models that propose that Treg cells can preferentially interact with DCs and either prevent the subsequent interaction of DCs with T effector cells or ultimately lead to inactivation of the DCs. It remains unclear why Tregs that express the same TCR as the T effector cells would preferentially interact with DCs. One explanation is the expression of higher levels of molecules mediating cell adhesion such as LFA-1[218] or Nrp-1.[195]

Another approach to analyze the effects of Tregs on T effector expansion and differentiation involved the transfer of congenically marked carboxyfluorescein diacetate succinimidyl ester-labeled TCR-transgenic naïve T cells to normal recipients with or without congenically distinct pre-activated polyclonal Tregs.[219] Following immunization with antigen in complete Freund's adjuvant (CFA), the numbers and activation status of the transferred transgenic T cells were analyzed at various time points. Surprisingly, cotransfer of Tregs did not inhibit T effector proliferation and resulted in a twofold increase in the absolute number of T effector cells present in the draining LN. There was no difference in the expression of activation markers on the T effectors immunized in the presence or absence of Tregs, and most importantly, there was no difference in the percentages of T effector cells producing IFNγ and IL-17. The total number of T cells in the LN is determined not only by in situ proliferation and expansion, but also by the contribution of entry and exit from the LN. In mice that received T effector cells only, the ratio of T effector cells in the LN to T effector cells in the blood was approximately 2:1. In contrast, in the mice that received T effector cells and polyclonal Tregs, the ratio was 10:1. Mice that received Tregs had fewer antigenspecific T cells when challenged at a peripheral site 1 week after priming. Thus, in the presence of polyclonal Tregs, fewer cells leave the lymph node to enter the circulation, and few cells are therefore available to respond to antigen at a distant site. The retention of antigen-specific cells at the site of immunization was associated with a decreased level of expression of the sphingosine 1-phosphate receptor 1 (S1P1). S1P1 mediates the response of primed T cells to high levels of S1P in the circulation and promotes their exit from the LN. It is unclear if this is the only mechanism operative in this model or if the Tregs target the T effector cells directly or mediate their effects indirectly by acting on DCs.

Klein et al.[200] were the first to initiate a detailed in vivo analysis of the properties of antigen-

specific Tregs. They obtained Tregs from TCR/antigen doubly transgenic mice and transferred them alone or in combination with naïve T cells of the same antigen specificity to normal immunocompetent mice. They observed that both Tregs and naïve antigen-specific T cells expanded in similar fashion 8 days after immunization. In cotransfer studies, Tregs did not inhibit the proliferation of naïve T cells in vivo in the draining LN when analyzed 90 hours after immunization.[200] After this time point, in the absence of the Tregs, the T effectors continued to expand, while in the presence of the Tregs their numbers remained stable. The percent of cytokine producing cells 8 days after priming was identical in the groups that did or did not receive Tregs. The conclusions drawn from this study were that Tregs had a late effect in vivo on the expansion of T effector, but had no effect into their differentiation into cytokine producing T effector cells. Other studies used an identical cotransfer model but observed marked suppression of the expansion of the antigen-specific T effector cells, as well as marked inhibition of cytokine production (IL-2 and IFNγ) 96 hours after cell transfer.[220,221,222] A correlation between Treg-mediated suppression and Treg division was observed in these studies; this is consistent with the view that Treg activation is a key requirement for effective immunosuppression in vivo. The differences between these studies and the study of Klein et al.[200] are not clear but may reflect the type of immunization and the ratio of Tregs to naïve T cells in the transfer inoculum.

The failure of T effector cell expansion in the presence of Tregs suggests that the major target of the suppressive functions of the Tregs is APCs. Hanig and Lutz[221] cotransferred peptide-pulsed bone marrow DCs, that had been matured with LPS, with antigen-specific T effector cells with or without antigen-specific Tregs to normal recipients. Clustering of DCs with both T effector and Tregs was observed in the spleen at 24 hours after cell transfer. Similarly, when all populations were injected simultaneously, no effects of Tregs on total spleen cell size and T effector numbers were observed for the first 48 hours. Maximum expansion of spleen size and numbers of T effector cells were detected at 72 hours after injection and modest (25%) suppression was seen at this time point when Tregs were cotransferred. Lower numbers of T effector cells in the memory phase of the response were seen when Tregs were coinjected. In contrast, when Tregs were transferred 10 to 24 hours before the T effector cells, cluster formation was completely inhibited and marked suppression of T effector cell expansion was seen. Thus, in this model using antigen-pulsed bone marrow DCs that had been prematured in vitro by treatment with LPS, Treg-mediated suppression required that the Tregs be preactivated in vivo. When Tregs and DCs were injected 24 hours before the T effector cells, marked downregulation of the expression of CD80/CD86, PDL-1, and PD-L2 was observed 48 hours later on DCs matured with TNF or LPS. No changes of ICOS-L, CD70, CD40, or MHC class II expression were observed. The conclusions drawn were that the Treg-mediated block in T effector cell expansion was mediated by downregulation of specific costimulatory molecules on the DC surface that in turn indirectly control the T effector cell expansion. However, Tregs could not inhibit T effector cell expansion and modulation of DC cell surface antigen expression, when

the DCs were matured with the combination of LPS and anti-CD40. Thus, certain modes of DC activation may result in a stimulatory DC that is completely resistant to Treg-mediated suppression.

Similar results were obtained in cotransfer studies using preactivated antigen-specific Tregs

that had been induced in vitro by exposure to TGF-β and IL-2. Cotransfer of antigenspecific Tregs resulted in a > 90% inhibition of the expansion of the naïve T effector cells (Fig. 33.13) and complete inhibition of the induction of effector cytokine production.[222] While the expansion of the T effector cells was inhibited, the Tregs in these mice readily proliferated, consistent with the earlier studies.[200] Again, it is likely that the Tregs in this model were inhibiting antigen presentation by acting on DCs. No evidence for Treg killing of DCs was observed, and the Tregs did not produce IL-10 that could potentially suppress the capacity of DCs to present antigen or deliver costimulatory signals. An alternative possibility is that the Tregs may inhibit chemokine production by the DCs resulting in inhibition of further recruitment of the transferred DCs to the site of immunization and less T effector cell activation.[223] It is still difficult to rule out direct effects of the Tregs on the responder T cells in vivo. It is possible that Tregs and T effector cells could interact with the same antigen-presenting DCs and complex interactions between all three cells would occur in this milieu. T effector production of IL-2 is required for the maintenance of Foxp3 expression in the transferred TGF-β-induced Tregs.[224] The molecular pathways that might play a role in Treg-mediated inhibition of APC function in vivo remain to be elucidated. The interacton of CTLA-4 on the Treg with its ligands CD80/CD86 represents the leading candidate.[225,226]

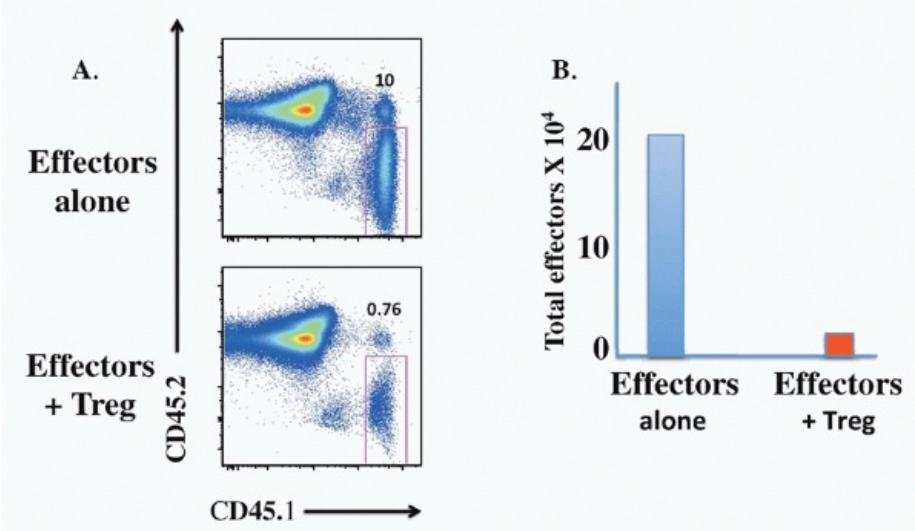

FIG. 33.13. Antigen-Specific Regulatory T (Treg) Cells Inhibit the Expansion of Antigen-Specific T Effector Cells in Response to Immunization. CD45.1 antigen-specific T cells were cotransferred to CD45.2 mice with Tregs specific for the same antigen. Following immunizations the **(A)** percentage and **(B)** absolute number of the T effectors were quarantined.

Reversal of and Resistance to Suppression

One approach to determining potential cell surface antigens involved in the process of Treg-mediated suppression has been to reverse suppression with antibodies to candidate antigens. One member of the TNFRSF, the GITR (TNFRSF18), has been shown to play an important role in regulation of T-cell suppressor activity. Both a polyclonal antiserum and an

mAb to the GITR appeared to be able to reverse suppression mediated by freshly isolated CD25+ T cells.[40,41] However, the GITR is expressed on two populations of cells (resting Tregs and activated CD4+CD25- cells) that do not manifest suppressor activity. It is therefore very unlikely that the GITR is the molecule responsible for mediating suppressor effector function. Surprisingly, culturing CD25+ T cells with the anti-GITR in the presence of IL-2, but in the absence of anti-CD3, resulted in a vigorous proliferative response and breaking the non-responsive state of the Tregs to stimulation with IL-2.

These studies were originally interpreted as indicating that anti-GITR functioned as an agonist for the GITR resulting in a signal that instructs the Tregs not to mediate their suppressive functions. As both CD25- and Tregs can

express the GITR, it was impossible to conclude that the anti-GITR mediated its effects by acting solely on the Tregs. When combinations of WT and GITR-/- CD4+CD25- and Tregs were used in coculture experiments, ligation of the GITR on the CD25- responders, not the Tregs, was required to abrogate suppression.[227] These results suggest that interactions of the GITR with its ligand, the GITR-L, provided a signal that renders effector T cells resistant to the inhibitory effects of Tregs. Similar conclusions were drawn from studies in which the costimulatory effects of anti-GITR in vivo were maintained following depletion of Tregs.[228] The GITR-L was shown to be expressed on a number of different resting APC populations including DCs, macrophages, and B cells, and to be downregulated on activation. Thus, engagement of the GITR on T effector cells by its ligand on APCs early during the course of an immune response may render the responder cells resistant to suppression by Tregs.

Other studies have implicated additional members of the TNFRSF as potential targets for reversal of Treg-mediated suppression. The capacity of Tregs to inhibit graft-versushost disease (GVHD) was reversed when Tregs were treated with an anti-OX40 mAb.[229] Injection of tumor-bearing mice with an agonistic anti-OX40 also appeared to inactivate intratumor Tregs and resulted in tumor rejection.[230] Stimulation of human Tregs in vitro in the presence of TNF or in the presence of an agonistic mAb to TNFRII reversed their in vitro suppressive activity and appeared to downregulate their expression of Foxp3.[231] One problem with the interpretation of these studies is that activated conventional T cells also express high levels of the GITR, OX40, and TNFRII, and it is difficult to determine the precise site of action of the agonistic mAbs. As shown with the anti-GITR, the majority of the “reversal” of suppression may actually be the result of enhanced resistance of the activated T effector cells to the suppressive effects of the Tregs. This explanation is also compatible with the observations of Pasare and Medzhitov[232] that soluble factors released by activated DCs can act directly on T effector cells, but not Tregs, to render them resistant to suppression. Other members of the TNFRSF appear to actually enhance Treg function. Stimulation of Tregs with an agonistic mAb to TNFRSF25 (also known as DR3) resulted in marked expansion of Tregs in vivo, and these expanded Tregs protected mice against the induction of allergic lung inflammation.[233]

In addition to the role of APC-derived signals in rendering responder cells resistant to suppression, a number of mutations in TCR signaling and costimulatory pathways also appear to render responder cells completely resistant to the inhibitory signals delivered by Tregs. One of the best examples of a defect in resistance to regulation is the cblb-/- mouse.

Cbl-b is an E3 ubiquitin ligase that negatively regulates the CD28 costimulatory pathway and downregulates the PI3K/Akt pathway. In the absence of cbl-b, mice develop CD28-independent hyperactive responses and autoimmunity. Treg from cbl-b-/- mice function normally in vitro, but CD25- T cells from cbl-b-/- mice are resistant to suppression in vitro and in vivo by WT Tregs.[234] Effector T cells from a number of other mutant mouse strains exhibit a resistance to suppression similar to that seen with cbl-b-/- effectors (Table 33.6). Many of these strains have in common a hyperactivated PI3K/Akt pathway that may be a direct or indirect target for Treg-mediated suppression.

TABLE 33.6 Genetically Deficient Mice That Are Resistant to Regulatory T Cell Suppression

Cbl-b-/-

TGF-β-RII-/-

TRAF6-/- T cell specific

NFATc2-/- / NFATc3-/-

TGF, transforming growth factor.

(Adapted from Wohlfert et al.234)

REGULATORY T CELL SPECIALIZATION

The functional specialization of T effector cells is due to the expression of master transcription factors, namely T-bet, GATA-3, and RORγt, which control the differentiation of Th1, Th2, and Th17 subsets, respectively. In general, these subsets mediate proinflammatory and potentially responses that are harmful. Treg cells also use the Th cell-associated transcription factors to maintain or restore immune homeostasis (Fig. 33.14). Treg cells expression of IFN regulatory factor 4 (IRF4), a transcription factor involved in the control of IL-4 production and and Th2 cell differentiation, is required for Treg-mediated control of Th2-type inflammatory responses. Mice in which IRF4 is specifically deleted in Foxp3+ Tregs develop a lymphoproliferative disease that is associated with a selective expansion of IL-4 and IL-5 producing Th2 cells and have elevated serum levels of of IgG1 and IgE, and develop splenic germinal centers.[235] IRF4 is also crucial for the differentiation of Tfh cells. The increase in germinal centers in mice with a Treg cell-specific deletion of IRF4 in the absence of an increase in IL-13-producing cells or eosinophil numbers suggested that Treg cell-specific expression of IRF4 may be needed to control a specific component of Th2 cell-associated inflammation, Tfh-cell activation, and high-affinity antibody production.

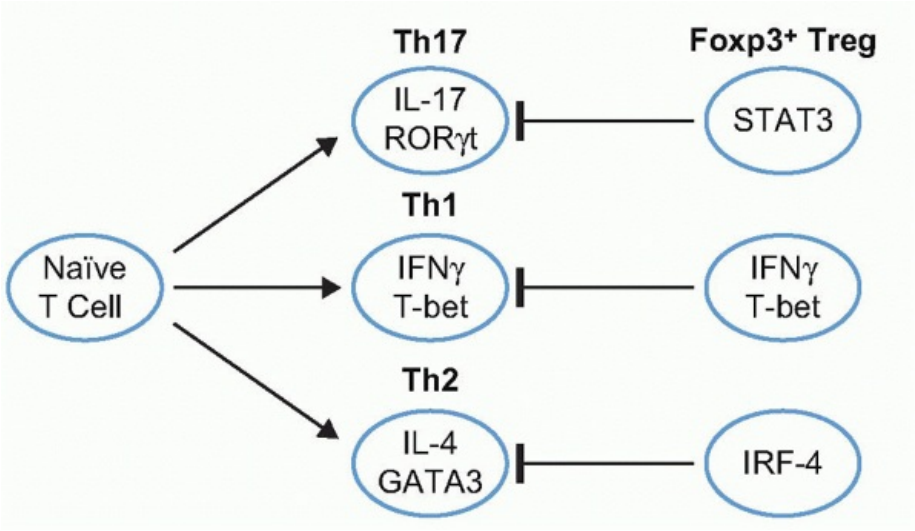

FIG. 33.14. Specialization of Regulatory T Cell Function. Adapted from Zheng et al.,[236] Koch et al.,[235] and Chaudry et al.[237]

While T-bet is the master transcription factor controlling Th1 responses, it is also expressed by a subset of Treg cells and is required for Treg cell homeostasis and function during polarized Th1 responses. While IFNγ can inhibits the peripheral generation of Foxp3+ Treg cells from naïve CD4+ T cells,[102] IFNγ signaling through STAT1 activation drives T-bet expression in Treg cells resulting in control of Th1-type immune responses. IFNγ may thus limit the magnitude and duration of Th1-type inflammatory responses. T-bet+ Treg accumulate at sites of Th1 inflammation and T-bet-/- Treg cells display impaired proliferation during Th1-type inflammation and do not control the expansion of IFNγ-producing Th1 cells when transferred into scurfy mice.[236] The failure of T-bet-/- Treg cells to control Th1-driven inflammatory responses may result from their inability to survive and proliferate in a highly polarized Th1-type environment.

Lastly, conditional deletion of STAT3 in Treg cells results in the selective dysregulation of Th17 responses accompanied by development of a spontaneous fatal intestinal inflammation with elevated levels of IL-17 production.[237] The mechanisms by which T-bet, IRF4, and STAT3 control Treg cell activity during Th1, 2, and 17 responses are still unclear but likely involve control of Treg-cell migration, function, and homeostasis. Selective expression of transcription factors associated with a given Th responses may induce molecular changes needed to restain a particular response. This hypothesis has important implications for the therapeutic uses of Treg cells as it implies that specific subsets of Treg cells may be needed in treating Th1-, Th2-, and Th17- mediated inflammatory diseases.

REGULATORY T CELL PLASTICITY

The studies on Treg specialization have demonstrated the potential benefical effects of T helper cell produced inflammatory cytokines on the ability of Tregs to control pathologic immune responses mediated by those cytokines. Other studies have challenged the notion of a committed Foxp3+ Treg cell lineage and raised the possibility that Treg cells can lose

Foxp3 expression when present in an inflammatory milieu or following transfer to an immunodeficient environment and be “reprogrammed” to become pathogenic T effector cells. For example, during infection with *Toxoplasma gondii*, Treg cells lose Foxp3 expression and become IL-12 responsive. They can then express high levels of T-bet, acquire Th1 effector characteristics, and produce IFNγ contributing to lethality.[238] Furthermore, when Treg cells were cultured under Th17 or Th1 polarizing conditions, a substantial fraction of IL-17+Foxp3+ and INFγ+Foxp3+ T cells were observed.[239] Thus, IL-6 may potentially convert Treg cells into proinflammatory Th17 cells in a proinflammatory environment.[240,241] No evidence of reciprocal conversion of Th17 cells to Treg was observed.

The Treg and Th17 programs of differentiation are clearly linked,[101] and Foxp3 and RORγt transcription factors can interact with one another.[101] Whereas transduction of naïve CD4+ T cells with RORγt induced IL-17 expression, cotransduction with Foxp3 abrogated IL-17-producing capacity when Foxp3 contains its exon 2-encoded domain, which is required for binding to to RORγt. After in vitro induction of RORγt and Foxp3 by TGF-β, cells do not express IL-17 and have the potential to differentiate into either Th17 or Treg depending on the cytokine environment. In the presence of proinflammatory cytokines (IL-6, IL-21, or IL-23) and low concentrations of TGF-β, RORγt expression is further elevated and Foxp3 expression is reduced resulting in Th17 differentiation. Conversely, in the absence of proinflammatory cytokines and high concentrations of TGF-β, Treg induction is promoted. IL-2 suppresses RORγt expression and promotes the induction of Tregs.[242] Runx1 can also form a complex with RORγt to promote Th17 differentiation, but can also interact with Foxp3 to suppress IL-2 and IFNγ production, resulting in enhancement of Treg suppressor function. Functional plasticity in cells that coexpress Runx1 and RORγt may be governed by the ability of Runx1 to cooperate with either transcription factor.[243] Foxp3+ Treg cells in Peyer patches differentiate into Tfh cells that can then participate in germinal center formation and IgA synthesis in the gut.[244]

The potential plasticity of Tregs is supported by epigenetic studies of cytokime gene expression. Wei et al.[245] have performed genome-wide examination of histone modifications and DNA methylation that accompany changes in gene expression in CD4+ T cell subsets. Trimethylation of histone H3 lysine 4 (H3K4me3) is a permissive mark found in the promoters and enhancers of active genes, whereas trimethylation of histone H3 lysine 27 (H3K27me3) is present in broad domains that encompass inactive genes. The epigenetic marks found at the IFNγ, IL-4, and IL-17 genes correlate precisely with Th1, Th2, and Th17 lineages. However, these genes in Tregs were bivalently modified, and this result is consistent with their potential plasticity. These bivalent modifications may allow specific lineage regulator gene loci to be activated under different polarizing conditions thus allowing reprogramming of Tregs into other lineages.

In addition to having permissive epigenetic modification of T effector cytokine genes, Tregs may express GATA3, the canonical Th2 transcription factor. GATA3 is highly expressed in Tregs at barrier sites such as the skin and the gut and is readily expressed in Tregs following TCR activation. Treg intrinsic expression of GATA3 is required for maintenance of high level of Foxp3 expression and promotes Treg accumulation at inflamed sites.[246] Expression of GATA3 was not seen in Tregs expressing T-bet or RORγt. In one study, GATA3 expression by

Tregs was required for Treg accumulation in inflammatory conditions such as GI infection or EAE and GATA3-/-. Tregs failed to control colitis due to impaired accumulation. Another study demonstrated a more profound defect in Treg function in Treg conditional GATA-/- mice, including the development of a generalized inflammatory disorder, decreased expression of Foxp3 and Foxp3 signature genes, and decreased suppressor function in vitro.[247]

Two groups recently generated mice that were engineered to track the fate of Foxp3+ cells in vivo. In these systems, mice expressing the Cre recombinase in Treg cells under control of regulatory elements from the Foxp3 gene, were crossed with mice in which the Cre-mediated recombination removes a stop codon from within a fluorescent protein

encoding a reporter gene that was knocked into the ubiquitously expressed Rosa 26 locus. Thus, in these animals, cells even transiently expressing Foxp3 are permanently marked, and their phenotypic properties can be spatially and temporarily examined. Using this system, Zhou et al.[248] reported that a portion of CD4+Foxp3+ T cells downregulate Foxp3 and acquire the ability to produce effector cytokines such as IFNγ even in the absence of any experimental manipulation. The frequency of these ex-Treg cells was increased in the pancreatic islets of NOD mice, indicating that they may contribute to immune pathology. These studies suggest that highly polarized inflammatory environments can subvert Treg-cell function by converting them to Foxp3- effector T cells. The other study demonstrated that Foxp3 expression by Treg cells is remarkably stable, even in highly inflammatory settings.[249] The discrepancies in these studies may be due to differences in the inflammatory sytems used to examine Treg stability in vivo, or to differences in the way the reporter mice were constructed. Alternatively, Tregs that have lost Foxp3 may not be the product of reprogramming of committed Treg cells, but may represent the products of a minor population of uncommitted cells that transiently express Foxp3.[250] These cells can accumulate by selective expansion and under the appropriate environmental conditons differentiate into Th1, Th2, or Th17 T effector cells.[251]

FOXP3- REGULATORY T CELLS

Thymic-derived Foxp3+ Tregs or Foxp3+ Tregs induced in peripheral sites represent the major populations of Tregs that have been characterized in both normal physiologic studies and in disease models. A number of other Treg populations have been described in both mouse and man, and many of these also appear to exert potent Treg functions in certain defined situations. One important distinction between these induced populations of Tregs and the thymicderived Foxp3+ Tregs is that the TCR of the former are very frequently specific for known antigens, while the antigenspecificity of the Foxp3+ T cells are likely preferentially specific for self-peptide MHC class II.

Th3 Cells

One of the first approaches used for the induction of Tregs was the administration of antigen via the oral route. Oral tolerance takes advantage of the normal physiologic process that is needed to prevent systemic immune responses to ingested proteins. Oral administration of antigen at low doses induces populations of Tregs that secrete suppressor cytokines, while higher antigen doses result in deletion or clonal anergy of autoreactive precursors. Pretreatment with orally administered antigen-induced suppressor populations that inhibited

pathology in a number of different animal models of autoimmunity including EAE, collagen-induced arthritis, and uveitis.[252] Bulk T cells from orally tolerized animals can suppress active immune responses to other antigens in the microenvironment, a phenomenon called antigen-driven bystander suppression. The suppressor cells from orally tolerant mice have been termed Th3 cells and mediate their suppressive effects primarily by secreting TGF-β. A major advance in our understanding of the function of regulatory cells following oral tolerance was the study by Chen et al.,[253] which successfully isolated T-cell clones from the mesenteric lymph nodes of SJL strain mice that had been orally tolerized to myelin basic protein. These clones produced large amounts of TGF-β and varying amounts of IL-4 and IL-10. Most importantly, upon adoptive transfer to normal recipients, they suppressed EAE induced with either myelin basic protein or proteolipid protein. Their in vivo suppressive activity could be neutralized with anti-TGF-β. The selective induction of Tregs via the oral route is thought to be secondary to certain poorly characterized properties of the gut mucosal microenvironment, most likely the type of resident APCs.[254,255]

Although the oral administration of antigen represents a potentially easy way to induce Tregs, progress in this area has been slow because it has been difficult to determine the antigen concentration that is capable of inducing Tregs, but which does not induce deletion. It has also been very difficult to isolate the types of clones described previously in the EAE model in other systems or to identify Treg cells that exclusively produce TGF-β in other models. The therapeutic utility of orally administered antigens in autoimmunity has primarily been demonstrated in pretreatment protocols, and oral administration of antigen was ineffective in treating animals once disease has been initiated. Nevertheless, in animal models, the oral administration of antigen can result in the induction of Foxp3+ antigen-specific Tregs that can modulate immune responses. These Foxp3+ Treg populations can produce TGF-β, but the relationship of the Foxp3+ Tregs to what was formally described as Th3 cells remains unclear.

T Regulatory 1 (Tr1) Cells

One important lesson that can be derived from the experiments on oral tolerance is that the milieu in which T cells are primed is critically important in determining whether regulatory rather than effector T cells will be generated. Decreased expression of costimulatory molecules on APCs or the presence of suppressor cytokines such as IL-10 and TGF-β may generate suppressor T cells rather than effector T cells. The production of these suppressor cytokines by Tregs may lead to the generation or expansion of additional regulatory cells via a positive feedback loop.

One of the first studies demonstrating the potential importance of IL-10 in the generation of Tregs was derived from an analysis[256] of patients with severe combined immunodeficiency (SCID) who received transplants of human leukocyte antigen (HLA)-mismatched hematopoietic stem cells. Complete immunologic reconstitution was achieved in the absence of graft versus host disease. CD4+ T-cell clones reactive with host MHC antigens from these patients produced IL-10, but not IL-2, after antigen-specific stimulation in vitro. It therefore seemed likely that endogenous IL-10 production in the transplanted patients was responsible for maintaining tolerance in vivo. The IL-10 may prevent the activation of host reactive T cells or suppress APC function and cytokine production by host APCs. The high IL-10

production in vivo may reflect a chronic activation of donor T cells and host monocytes.

IL-10 is a cytokine produced by numerous cell types including activated T cells, B cells, mast cells, and macrophages, and primarily acts by inhibiting the maturation and function of APCs. The activation of CD4+ human T cells in the presence of IL-10 renders them nonresponsive or anergic.[257] Activation of human CD8+ T cells with allogeneic APC and IL-10 also results in reduced proliferation and cytotoxicity. T cells rendered anergic by the addition of exogenous IL-10 in an mixed leukocyte reaction (MLR) become unable to respond to a rechallenge with the same antigen. IL-10-iinduced anergy is strictly antigen-specific as treated T cells retain normal proliferative and cytotoxic responses toward other protein antigens and third-party alloantigens.

Collectively, these studies suggest that IL-10 itself is a major factor for the induction of suppressive IL-10-producing T cells. Culture of mouse or human CD4+ T cells with antigen or alloantigen in the presence of IL-10 results in the generation of IL-10-producing T-cell clones. Most of these T-cell clones produce high levels of IL-10 and TGF-β, moderate amounts of IFNγ and IL-5, but no IL-2 or IL-4. CD4+ T cells generated in this manner have been termed T regulatory 1 (Tr1) cells.[258] Tr1 cells proliferate poorly following polyclonal TCR-mediated or antigen-specific activation and do not expand significantly under standard T-cell culture conditions. This low proliferative capacity is due in part to autocrine production of IL-10, as anti-IL-10 mAbs partially restore proliferative responses. The intrinsic low proliferative capacity of Tr1 cells has been a major limitation and has hindered their detailed characterization. The ability to generate human Tr1 cells is enhanced by the addition of IFNα .[259] Tr1 cells can be generated from human cord blood with IFNα alone, as cord blood T cells have the intrinsic ability to produce IL-10. IFNα and IL-10 do not act as general antiproliferative agents, but rather as factors that induce the differentiation of Tr1 cells and inhibit the growth of non-Tr1 cells in the culture.

Tr1 cells do not express Foxp3 and can be generated in the absence of Foxp3+ Tregs. Both human and mouse Tr1 clones suppress immune responses in vitro. Antigeninduced proliferation of naïve CD4+ T cells was dramatically reduced following coculture with activated Tr1 clones that were separated from the responding cells by a trans-well insert. The capacity of either human or mouse Tr1 clones to suppress CD4+ T cell proliferation was reversed by the addition of anti-TGF-β and IL-10 mAbs.[258] Some IL-10-producing Tr1 cells suppress the proliferation of naïve CD4+ T cells by an IL-10-independent, cell contact-dependent mechanism.[260,261] Human Tr1 cell clones also suppress the production of Ig by B cells, as well as the antigen-presenting capacity of monocytes and DCs. Most importantly, it could be shown[258] that mouse Tr1 clones have regulatory effects in vivo and suppress Th1-mediated colitis induced by transfer of $CD45RB^{hi}$ cells into SCID mice. Suppression was only seen if the Tr1 clones were activated by antigen-specific stimulation via their TCR. Because the function of Tr1 cells is mediated by IL-10 and TGF-β, these studies imply that Tr1 clones can suppress active immune responses to unknown antigens in the microenvironment by an antigen-driven bystander suppression mechanism similar to the mechanism proposed for Th3 cells in oral tolerance. Although IL-10 was originally described as a product of mouse Th2 cells, Tr1 clones are also capable of regulating Th2 responses including antigenspecific IgE production.[261] Anti-IL-10R antibodies reversed this inhibitory effect.

An alternate approach to the generation of Tr1 cells in vitro has involved pharmacologic manipulation of the microenvironment during T-cell priming.[262] The immunosuppressive drug 1, 25(OH)2-vitamin D3 (VitD3) acts on APCs and activated T cells. VitD3 inhibits antigen-induced T-cell proliferation, cytokine production, and the maturation of human DCs, leading to inhibition of the expression of CD40/CD80/CD86 expression. Similarly, the glucocorticoid dexamethasone inhibits key transcription factors involved in the regulation of a number of inflammatory cytokine genes. When naïve CD4+ T cells were stimulated through their TCR in the presence of the combination of VitD3 and dexamethasone, the primed T cells produced IL-10, but not IFNγ, IL-4, or IL-5. The IL-10-producing cells developed independently of Th1 (IL-12, IFNγ) and Th2 (IL-4) polarizing cytokines, and addition of these cytokines inhibited the development of the IL-10-producing cells. Although the induction of these Tregs could not be induced by IL-10, endogenous IL-10 production was required because addition of anti-IL-10R antibodies substantially reduced the number of IL-10-producing T cells. The development of the IL-10-producing T cells occurred under conditions where the expression and/or activation of key transcription factors involved in Th1 (T-bet) and Th2 (GATA3) differentiation was minimal, suggesting that the IL-10 producers were completely unrelated to conventional Th1 or Th2 cells. VitD3/dexamethasone-induced Tr1 cells are also capable of inhibiting the induction of Th1-mediated autoimmune disease in vivo. One common theme to emerge from the studies on the in vitro induction of Tr1 cells, either by the addition of IL-10 or of pharmacologic agents, is that both of these modalities are likely to inhibit the maturation and activation of DCs and to generate what has been termed “tolerogenic DCs.”[263] Clinical trials are ongoing to evaluate the potential therapeutic effects of Tr1 cells in the prevention and treatment of GVHD after bone marrow transplantation. The clinical protocol involves the transfer of ex vivo generated Tr1 cells to patients with hematologic cancers treated with hematopoietic stem cell transplantation. Treatment of patients with IL-10-anergized donor T cells has potential to reconstitute immunity, prevent GVHD, while guarding against infection and recurrence of cancer.

The inability to expand Tr1 cells in large quantity has hampered progress in understanding the biology of these cells. The relationships between Tr1 cells that produce IL-10 and Foxp3+ Tregs that also produce IL-10 remain unclear. IL-10 reporter mice have been generated[264] in which expression of IL-10 was replaced with expression of a Thy-1.1 reporter, facilitating identification of cells producing IL-10 spontaneously in vivo. This mouse stably identifies all cells in which IL-10 alleles have been previously activated as well as those

cells actively transcribing IL-10. Foxp3+IL-10- Treg cells were more frequenct in LN and spleen, while Foxp3+IL-10+ Treg cells were more frequent in the lymphoid tissues of the large intestine. In the small intestine and Peyer patches, Foxp3-IL-10+ Treg cells were the most predominant population and had a cytokine profile, proliferative response, and suppressive function typical of Tr1 cells. The Foxp3-IL-10+ population developed from Foxp3- precursors in all tissues and its development was dependent on TGF-β but not IL-10. The presence of these cells in the gut may explain why IL-10-/- mice and mice treated with an anti-IL-10R succumb to intestinal inflammation despite having normal numbers of Foxp3+ cells in the intestines. Thus, IL-10 has a nonredundant function in maintaining immune homeostasis to the diverse intestinal microbiota. The relative contributions of the Foxp3+IL-10+ and Foxp3-IL-10+ producing subsets is still unclear. As the two populations are located in different parts of the intestinal tract, it remains possible that a distinct class of commensal

organisms would preferentially induce both of the subsets.

Recent studies have demonstrated that IL-27, a member of the IL-12 cytokine family consisting of two subunits, p28 and EBI3, is a differentiation factor for the generation of IL-10-producing Tr1 cells.[265,266] IL-27 is secreted by tolerogenic DCs that were conditioned in vitro or in vivo by Foxp3+ Treg cells. These Treg-modified DCs express plasmacytoid-like markers. The addition of IL-27 to naïve T cells induced expansion and differentiation of CD4+ T cells that secreted high levels of IL-10.[267] IL-27-driven Tr1 cells proliferate poorly following TCR-mediated activation and suppress T effector responses through IL-10 production. Granzyme B is also induced by IL-27 during Tr1 differentiation and may mediate suppression of T effector responses by cytolysis. IL-27 also can induce IL-10-producing human Tr1 cells.[268] Signaling via the arylhydrocarbon receptor may modulate Tr1 differentiation. Naïve T cells differentiated in vitro with IL-27 in the presence of the arylhydrocarbon receptor ligand, TCDD, had enhanced secretion of both IL-10 and IL-21.

CD8+ Regulatory T Cells

Most of the early studies on T suppressor cells in the mouse demonstrated that they were confined to the CD8+ subset. Almost all of the recent studies on suppressor/regulatory T cells in mouse or man have focused on CD4+ T cells. Several studies have suggested that potent CD8+ suppressor cells may also exist. Repeated stimulation of human T cells in the MLR resulted in a progressive decrease of the capacity of CD4+ T cells to proliferate when rechallenged with the APCs used for priming. The relative nonresponsiveness of the stimulated CD4+ T cells could be restored by depletion of CD8+CD28-, but not CD8+CD28+, T cells from these cultures. The stimulated CD8+CD28- T-cell population was devoid of cytotoxic activity for either CD4+ T cells or the APCs used for priming. When the CD8+CD28- T cells were added to mixtures of CD4 cells and APCs, they inhibited proliferation,[269] but suppression was only observed when the stimulatory APCs shared at least one HLA class I allele with the original stimulator population. The regulatory effect of the CD8+ suppressors was not restricted either by class I or class II MHC antigens expressed by the responder CD4+ T cells. Suppression mediated by CD8+CD28- T cells required cell-cell contact and was not reversed by antisuppressor cytokine antibodies. Coincubation of the CD8+ suppressors with CD4+ responders had no effect, while coincubation of the CD8+ T cells with the APCs rendered the APCs unable to stimulate CD4+ proliferation. Phenotypic analysis indicated that the CD8+ suppressors blocked the upregulation of costimulatory molecules such as CD80/CD86, CD54, and CD58 on the APCs.[270] The mechanism by which the CD8+ suppressors deactivate APC functions has been shown to involve upregulation of the genes encoding Ig-like transcript 3 (ILT3) and ILT4.[271] These inhibitory receptors are structurally and functionally related to killer cell inhibitory receptors. Thus far, the biologic activity of these CD8+CD28- suppressor populations has only been studied in vitro. Their potential roles in mediating immunosuppression in vivo as well as their relationship to CD4+ suppressor cells remain to be explored. It has been proposed that antigen-specific MHC class I restricted CD8+CD28- Tregs first induce ILT-expressing tolerogenic DC, which in turn generate CD4+ Tregs.[272]

Regulatory CD8+ T cells have also been generated in vitro by stimulation with unique

subpopulations of DCs.[273] When naïve CD8+ T cells were stimulated in vitro with CD40L-activated monocyte-derived DCs (DC1), the primed CD8+ T cells proliferated when restimulated with allogeneic target cells, secreted large amounts of IFNγ, and had potent cytotoxic activity. In contrast, when naïve CD8+ T cells were stimulated with CD40L-activated plasmacytoid DCs (DC2), the primed CD8+ T cells proliferated poorly, displayed weak cytotoxic activity, and secreted primarily IL-10. DC2-primed CD8+ T cells inhibited the ability of naïve CD8+ T cells to proliferate to allogeneic monocytes, immature DCs, or mature DCs. Both the generation of CD8+ suppressor cells and their suppressor function could be markedly inhibited by anti-IL-10, but not by anti-TGF-β. Dhodapkar et al.[274] have measured the ability of immature DC cells to modulate the immune response in vivo in humans. Injection of immature DC pulsed with influenza matrix peptide resulted in an expansion of antigen-specific tetramer binding CD8+ T cells. These CD8+ cells were capable of proliferating when stimulated with antigen in vitro, but were defective in IFNγ secretion and lacked cytotoxic function. These findings indicate that immature DC can dampen preexisting antigen-specific effector function in man. The potential suppressive function of the CD8+ T cells that responded to the iDCs was not studied. The relationship of these CD8+ T cells to those induced in vitro with DC2 cells is unknown. Wei et al.[275] have isolated plasmacytoid DCs from malignant ascites of patients with ovarian cancer. These DCs, upon CD40L activation, could induce antigen-specific CD8+ IL-10-producing Tregs that suppressed the responses of tumor antigen-specific CD4+ T cells. A unique subpopulation of mouse CD8+ T cells that express high levels of the IL-2R β-chain (CD122) has been shown to have immunoregulatory activity.[276] CD8+CD122- are Foxp3- and inhibit the activation of both CD8+ and CD4+ T cells in vitro by an IL-10-dependent mechanism as CD8+CD122+ T

cells from IL-10-/- could not suppress T-cell activation in vitro, but may exert some suppressive effects in vivo.[277]

Qa-1 Restricted CD8+ Regulatory T Cells

Mice genetically deficient or depleted of CD8+ T cells have demonstrated a clear role for CD8+ T cells in regulating EAE. CD8+ T cells from mice that had recovered from EAE downregulated or killed some CD4+ neuroantigen-specific T-cell clones. Inhibition was blocked by antibodies to the MHC class Ib molecule Qa-1, but not by anti-MHC class Ia molecules.[278] It has been proposed that Qa-1 self-peptide complexes expressed by activated CD4+ T cells trigger the TCR on CD8+ cells. These CD8+ T cells then differentiate into suppressor cells that in turn suppress the CD4+ T cells that express the same Qa-1 self-peptide complex. The CD8+ Treg population can target a peptide from the conserved region of the TCR.[279] The mechanism of suppression has not been fully defined, although it may involve cytotoxicity or the secretion of suppressor cytokines. Human CD8+ T cells restricted by HLA-E, the human homologue of mouse Qa-1, may have similar functions.[280]

Double Negative Regulatory T Cells

The majority of T cells in normal mice and humans that express the TCR-αβ chains also express either the CD4 or CD8 coreceptor molecules. However, 1% to 5% of the peripheral TCR-αβ population express CD3 but not CD4 or CD8. Double negative (DN) T cells can be

divided into NKT cells and NK-marker negative DN Tregs. DN Tregs do not develop from CD8+ precursors, nor is CD8 expression required for their development in vivo.[281] Functional DN Tregs develop outside of the thymus and may mature from chronically activated CD4+ T cells. DN T cells have been shown to have immunoregulatory activity both in vivo and in vitro in both mouse and man.[282,283] DN Tregs have the ability to acquire alloantigens from APCs and present them to alloreactive syngenic effector T cells in human in vitro studies. DN suppressors generated in this way kill alloreactive CD4+ and CD8+ T responders that express functional Fas by a mechanism involving Fas/Fas-L interactions. These activated DN cells were only able to kill syngeneic targets activated by the same alloantigenic stimulus. A significant difference was noted between the mean percentages of DN Tregs in patients who developed GVHD after stem cell transplantation when compared to those that did not. All patients in whom DN Tregs were expanded to > 1% did not develop GVHD, suggesting that the expansion of these cells may prevent the development of GVHD.[284]

Regulatory T Cell-Induced Regulatory T Cells—Infectious Tolerance

One possible mechanism to increase the efficiency of Foxp3+ Tregs is that Tregs induce anergy or suppressor activity in the Foxp3- responders as a form of infectious immunologic tolerance. It has been shown that human CD4+CD25+ T cells induce CD4+CD25- T cells to become Tregs that are capable suppressing by producing IL-10[285] or TGF-β,[286] thereby mediating suppression of naïve CD4+ T cells. Similar studies in the mouse demonstrated that coculture of Tregs with Foxp3- responder cells resulted in the induction of both TCR and IL-2 unresponsiveness in the responder population.[287] The responder cells remained Foxp3- and exerted suppressor activity in a cell contact-dependent manner that was partially abrogated by anti-TGF-β.

One problem with these early studies is that it was difficult to determine if the regulatory populations induced by coculture with presumably Foxp3+ T cells actually were induced to express Foxp3. Recent studies have demonstrated that activation of Foxp3- T cells in the presence of activated Foxp3+ T cells resulted in the induction of Foxp3 expression in the Foxp3- population.[288] The induction of Foxp3 expression was mediated by TGF-β produced by the Foxp3+ inducer population in a contact-dependent fashion. The newly induced Foxp3+ T cells mediated suppression both in vivo and in vitro. Foxp3+ T cells have also been shown to mediate infectious tolerance locally at the site of a transplanted skin allograft. The induced cells were suppressive and prevented potential efectors from rejecting grafts.[289]

REGULATORY T CELLS AND DISEASE

Animal Models of Autoimmune Disease

One of the most widely used experimental models for the study of Treg function is the animal model of IBD. Transfer of CD4+CD45RB^{high} cells to SCID mouse recipients resulted in the development of a wasting disease and colitis 6 to 8 weeks after T-cell transfer. This disease was characterized pathologically by epithelial cell hyperplasia, goblet cell depletion, and transmural inflammation.[290] There was a 20- to 30-fold accumulation of Th1 cells in the intestine compared to normal mice. Treatment of recipients with anti-IFNγ, anti-TNF-α, or anti-

IL-12 inhibited the induction of disease. Transfer of CD45RB^{low} cells did not induce colitis, and cotransfer of RB^{high} and RB^{low} cells prevented the development of colitis. A ratio of 1:8 RB^{low} to RB^{high} was able to prevent disease. When CD45RB^{low} cells were fractionated into CD25+ and CD25- fractions, control of intestinal inflammation was primarily mediated by the CD25+ (Foxp3+) fraction.[27] CD45RB^{low} CD25- cells did exert some suppressive function when transferred at high cell concentrations.

Treatment of recipients of RB^{high} cells with IL-10 inhibited the development of colitis (Table 33.7). This treatment inhibited the accumulation of Th1 cells in the intestine, but did not induce Tregs, as colitis developed when IL-10 administration ceased. Treg isolated from IL-10-/- mice are able to inhibit colitis induced by CD4+CD45RB^{hi} cells, indicating that IL-10 secretion by Tregs is not required. However, control of colitis by CD25-CD45RB^{low} cells was found to be highly dependent on IL-10.[291] In contrast, Treg-mediated suppression of autoimmune gastritis involves a cell contact-dependent mechanism of suppression, and IL-10 and TGF-β are not involved.[292] One reason for this difference is that the pathogenesis of autoimmune gastritis and IBD are quite distinct. Bacteria play a required role in IBD, whereas

autoimmune gastritis can be induced by d3Tx of germ-free mice. It remains possible that suppression of IBD involves both the cell contact-dependent and the cytokine-mediated pathways of suppression. IL-10 is required to first dampen the inflammatory response induced by intestinal bacteria. Once this response is reduced, cell contact-mediated suppression can become operative.

TABLE 33.7 Role of Cytokines in the Pathogenesis and Treatment of Irritable Bowel Disease

Treatment	Effect on Disease
Anti-IFNγ(days 1 + 4)	Substantial protection
Anti-IFNα(days 1 + 4)	No protection
Anti-TNF (weekly)	Protection during treatment only
IL-10	Protection during treatment only
IL-4	No protection
CD4+CD25+ T cells	Complete protection
CD4+CD25+ T cells + anti-IL-4	Complete protection

CD4+CD25+ T cells + anti-IL-10R	Protection with naïve CD45RB^{hi}
CD4+CD25+ T cells + anti-IL-10R	No protection with naïve CD45RB^{low}
CD4+CD25+ T cells + anti-TGF-β	No protection

IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

(Adapted from references 290 and 291)

One advantage of the IBD model is that CD4+CD25 + CD45RB^{low}(Foxp3+), but not CD4+CD25-CD45RB^{low}, T cells can cure ongoing colitis 4 weeks after transfer of CD4+CD45RB^{high} T cells.[293,294] Tregs proliferate and accumulate in the mesenteric LN and also in the colonic lamina propria. At both sites, the progeny of the Tregs are in direct contact with CD11c+ DCs as well as effector T cells. These findings suggest that regulation of an active immune response by Tregs occurs in the draining LN as well as at the site of inflammation. During the cure of intestinal inflammation, the majority of the Foxp3+ T cells are IL-10-secreting cells. Whereas Foxp3+ cells are present in similar frequencies in both the secondary lymphoid organs and lamina propria of colitic animals, the IL-10-producing cells are selectively enriched in the colonic lamina propria. The gut environment may condition the Tregs to differentiate into IL-10-producing Tregs in the colon. Alternatively, the accumulation of Tregs in the colon may be attributed to the preferential expansion of a minor subset of IL-10 producers contained in the transferred population.

The NOD mouse represents the best experimental model for autoimmune diabetes. It is widely accepted that Foxp3+ Tregs play a critical role in the regulation of disease in this animal model.[295] The islets of Langerhans become rapidly infiltrated with immune cells several weeks prior to the onset of diabetes. Diabetes progression may relate to a subsequent failure in the maintenance of Treg function. Chatenoud et al.[296] have examined whether CD4+CD25+ Tregs play a protective role in the NOD mouse. Young NOD/SCID recipients were injected intravenously with mixtures of splenocytes from NOD mice and Tregs. Tregs from young (6-week-old) prediabetic mice significantly protected against diabetes in this model. Protection could be reversed by treatment of the recipient mice with anti-TGF-β, but not anti-IL-4 or anti-IL-10. Interestingly, anti-TGF-β did not reverse protection mediated by Tregs derived from the thymus. Analysis of Treg function in the NOD mouse is difficult because not all animals will develop disease and it takes a long time for disease to develop. Green et al.[297] have developed a model of insulin-dependent diabetes mellitus (IDDM) in which a regulatable TNF gene is expressed in the pancreatic islets. When these mice were crossed to mice expressing CD80 in their islets and TNF is expressed for 25 days from birth, diabetes is delayed. In contrast, if TNF expression is allowed to continue for 28 days, the regulatory mechanisms are overcome and the animals rapidly develop diabetes. The autoreactive effector cells in this model are exclusively CD8+ T cells. Tregs could be isolated from the islets and draining pancreatic node, but not from other nodes or spleen; they could suppress the development of disease in animals in which TNF was expressed for

28 days. These regulatory cells were extremely potent; protection could be transferred with as few as 2 × 10^3 Tregs. This sitespecific accumulation of regulatory cells may be the result of localized production of chemokines that specifically recruit Tregs to the site of inflammation. Alternatively, the Tregs in the draining node may be antigen-specific and generated in situ by stimulation by islet-derived antigens.

Antigen-specific Tregs from the TCR-transgenic BDC2.5 mice that recognize an islet-derived antigen have been expanded with coimmobilized anti-CD3/anti-CD28 in the presence of IL-2.[298] These expanded Tregs prevented the development of diabetes in NOD mice, survived long-term in vivo, but required antigen exposure for functional activity. Islet autoantigen-specific BDC2.5 Tregs were significantly more effective than polyclonal Tregs in regulating autoimmune responses in vivo. Importantly, expanded BDC2.5 Tregs were able to reverse diabetes, in conjunction with 500 syngeneic NOD islets, when transferred into NOD mice that had been diabetic for 2 weeks. The expanded Tregs were also capable of inhibiting diabetes in newly diagnosed NOD mice.

These results raise the possibility that Tregs might be isolated from patients during remission or soon after disease onset, expanded, and used to treat patients at the time of maximal disease activity to moderate the inflammatory response. Indeed, Masteller et al.[299] were able to expand Tregs with the BDC2.5 specificity by stimulating NOD Tregs with beads coated with a mimitope peptide complexed to MHC class II (I-Ag^7) that is recognized by the BDC2.5 TCR. The expanded Tregs were more efficient than polyclonal Tregs in suppressing autoimmune diabetes. Thus, small numbers of autoantigenspecific Tregs may be clinically efficacious because they can suppress polyclonal pathogenic T-cell responses either by bystander cytokine production or by recruitment of endogenous Tregs while avoiding the pan-immune suppression that might be induced by polyclonal Tregs.

The site of action and mechanisms of action of Tregs have been explored in autoimmune diabetes models.[300] Tregs had

little or no effect on the expansion of effector cells, but suppressed IFNγ production by the effectors in the pancreatic LN and seemed to slow the migration of effector cells into the islets perhaps through a reduction in their expression of CXCR3 in the pancreatic LN. One problem with studies of Treg-mediated protection from autoimmune disease is that they frequently involve transfer into lymphopenic hosts where interpretation of the results may be difficult secondary to stimulation of the effector population that is seen in response to lymphopenia. Foxp3-/- NOD mice have been used to analyze where and how Treg cells exert their control on diabetes under experimental conditions not influenced by homeostatic expansion of effector populations.[301] Tregs did not delay or hinder the activation and expansion of anti-islet T cells in the pancreatic LN nor the timing of their infiltration into the islets, but primarily prevented locally damaging consequences of autoimmune infiltration in the islets. It appears the Tregs primarily impinge on autoimmune diabetes by suppressing destructive T cells inside the islets, more than during the initial activation in the draining LN. Further studies are clearly needed to resolve these conflicting findings as a detailed understanding of the site and mechanism of suppression of experimental autoimmune disease will facilitate translation of the therapeutic use of Tregs for treatment of autoimmune disease in man.

In studies of the relationships between Treg cells and T effector cells in the pancreatic lymph nodes of NOD mice, it was noted that progression to diabetes was associated with an increase in Treg cells in the pancreatic LN at the time of diabetes onset.[302] However, disease progression was associated with a decrease in the percentage of Treg cells in the pancreatic islets with a concomitant loss of CD25 expression and of expression of the antiapoptotic factor, Bcl-2 in the intraislet Tregs. CD4+ T cells from islets produced less IL-2 following stimulation in vitro than did CD4+ T cells from spleen, suggesting that a deficiency in IL-2 production is responsible for the loss of Tregs in the islets. Administration of IL-2 promoted Treg survival and and protected the mice from diabetes. In the NOD mouse model of type I diabetes, the idd3 region on mouse chromosome 3 is associated with a lower production of IL-2, a decreased proportion of Treg cells, and increased susceptibility to disease. The agonistic activity of an IL-2/anti-IL-2 complex was exploited as a therapeutic agent for type 1 diabetes in NOD mice. This complex extends the half-life of IL-2 from 30 months for free IL-2 to 48 to 72 hours for complex IL-2. A low dose of complex increased expression of IL-2Rα and prosurvival factor, Bcl-2, by Treg cells and prevented the onset of diabetes. A high dose of the complex accelerated the progression to type 1 diabetes secondary to the enhancing autoreactive effector T cells that express the high affinity IL-2Rα .[303] Similar results were observed in the animal model of multiple sclerosis and EAE. Pretreatment of mice with the complex modulated disease induction, whereas administration of the complex after disease initiation resulted in a more severe disease course.[304]

EAE is the major animal model for the study of multiple sclerosis; Tregs have been shown to play a role in this model. When the Treg population of naïve C57BL/6 mice was supplemented with CD4+CD25+ Tregs from a normal donor before the active induction of EAE by immunization with an encephalitogenic peptide from myelin oligodendrocyte glycoprotein (MOG), the Tregs conferred significant protection. Protection was associated with the presence of Th2 cytokines and a markedly decreased CD4+ T cell and APC infiltrates in the CNS.[305] These studies suggested that Tregs might inhibit the homing of pathogenic T cells to the CNS. McGeachy et al.[306] demonstrated that that the accumulation of CD25+Foxp3+ cells in the CNS correlated with the recovery phase of EAE. As few as 104 CNS-derived CD25+ cells significantly reduced disease severity in recipients, whereas the same number of naïve CD25+ T cells had no effect. Although the mechanism of action of the Tregs in protection from EAE was not completely elucidated, CD25+ T cells were the major source of IL-10 in the CNS and IL-10 production was crucial for full recovery from EAE. Using tetramers, it was shown that MOG-specific Tregs expanded alongside MOG-reactive T effector cells in the draining LN before migration to the spleen and accumulation in the CNS. MOG-reactive Tregs in peripheral sites showed suppressive activity against naïve MOG-reactive T cells, whereas Tregs derived from the CNS failed to suppress the in vitro proliferation of CNS-derived T effector cells due to the high levels of inflammatory cytokinies coming from the T effector population. During recovery, there was a dramatic change in the Treg:T effector ratio in the CNS in favor of Tregs.[307]

Tregs suppress the differentiation and function of Th1 and Th2 cells in vitro and in vivo.[308,309] Other studies[310] have shown that freshly isolated Tregs inhibit the IL-4-induced development of Th2 cells, but had no influence on established Th2 cells. In contrast, preactivated Tregs inhibited cytokine production and proliferation of established Th2 cells.

CD4+Foxp3+ Tregs modulate the development of experimentally induced asthma in mice genetically predisposed or comparatively resistant to the development of airway resistance. In vivo depletion of Tregs in resistant animals before initial antigen contact increased airway hypersensitivity reaction, airway eosinophilia, and IgE synthesis, and was associated with Th2 cytokine production, while Treg-cell depletion had no effect on allergen-driven airway hypersensitivity reaction in susceptible mice.[311] Enhancement of the allergic phenotype in resistant mice was associated with stimulation of DC function, suggesting that the Tregs do not act on effector T cells, but act indirectly via the DC. Th2 polarized CD4+ T cells depleted of CD25+ cells induce increased airway eosinophilia after adoptive transfer.[312] Allergen-specific CD4+CD25+ T cells from TCR transgenic mice inhibited the classical pathology associated with allergic asthma including airway hypersensitivity reaction, lung eosinophilia, and Th2 cytokine production in immunocompetent mice. Their effects were dependent on IL-10, but Treg cells exerted their suppressive effects in vivo independently of their capacity to produce IL-10, by inducing IL-10 production from recipient CD4+ T cells.

Mice with an established helminth infection are less prone to allergic airway inflammation as measured by the inflammatory infiltrate in bronchial lavage fluid or peribronchial and perivascular inflammation. Helminth infection with *Heligmosomoides polygyrus* protects from allergic airway

responses by acting downstream from allergen sensitization and can be transferred from infected mice to uninfected presensitized animals by CD25+ T cells. Th2 effector cytokines (IL-5 and IL-13) were diminished, but allergen-specific IgE was not changed. Thus, chronic parasite infection may maintain a high level of activation in Tregs so that they primarily target the effector mechanisms resulting in decreased inflammation in a previously sensitized allergy-prone host.[313]

Immune Homeostasis

Lymphocyte numbers in a normal adult animal remain stable throughout life. A homeostatic equilibrium exists between the numbers of newly produced cells, self-renewal of peripheral T cells, and the numbers of dying cells. The mechanism that controls the number of peripheral lymphocytes is unknown. The regulation of the numbers of naïve and memory CD4+ and CD8+ T cells is independently controlled. Most studies of the effects of Tregs in vivo in the suppression of autoimmune disease involve the transfer of the effector populations to a T cell-deficient mouse. T-lymphocytes undergo a rapid, vigorous proliferative response when transferred to an immunodeficient recipient. We assume that some of the inhibitory effects of Tregs in autoimmunity are related to their ability to be specifically activated by autoantigens. An alternative possibility is that a major component of Treg function is to nonspecifically inhibit lymphopenia-induced proliferation[314] and thereby prevent the development of autoimmune disease. CD4+Foxp3+ T cells might be potent inhibitors of lymphopenia-induced proliferation, but any T-cell population with an activated effector/memory phenotype might also be capable of mediating inhibition.[315]

Surprisingly, both CD4+CD25- (including RBhigh cells) T cells and CD4+CD25+ T cells were capable of proliferating in a lymphopenic host with proliferation beginning on days 3 to 4 posttransfer and reaching as many as eight divisions by days 21 to 28 after transfer.

CD4+CD25+ T cells did not inhibit this early phase of “homeostatic proliferation” of CD4+CD25- T cells.[316] Although CD4+CD25+ T cells have been shown to inhibit the accumulation of CD25- T cells 2 to 6 months after transfer to a RAG-/- recipient by an IL-10-dependent mechanism, this result must be interpreted with caution.[317,318] The fact that CD25- T cells almost always induce some form of organ-specific autoimmune disease demonstrates that CD25- T cells have undergone some form of autoantigen-specific proliferation in addition to the lymphopenia-induced proliferation. As described previously, CD4+Foxp3+ T cells are highly efficient inhibitors of the induction of these autoimmune diseases. It should be noted that transfer of CD25+Foxp3+ T cells to immunodeficient mice results in loss of expression of CD25 by a majority of the transferred cells.[123] The significance of this finding is unknown as the recovered CD25- T cells retain the ability to suppress T-cell activation in vitro.

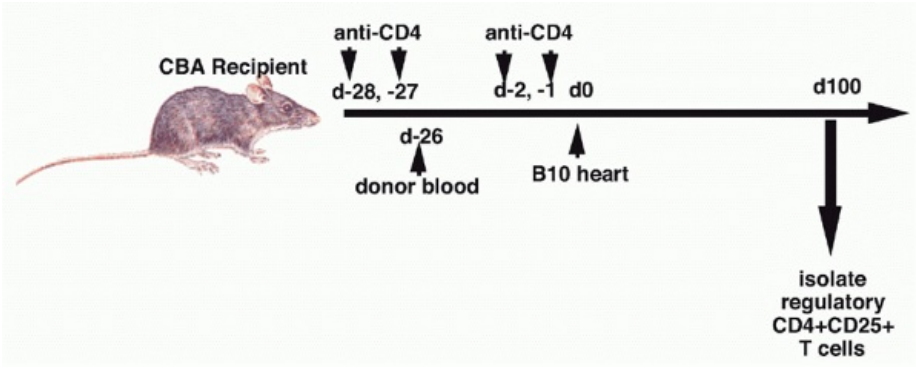

FIG. 33.15. Induction of Regulatory T Cells for Tolerance to Alloantigens. Adapted from Hara et al.[320]

Allograft Rejection and Graft-versus-Host Disease

A considerable body of data exists that regulatory/suppressor T cells exist in patients and animals with long-term surviving allografts.[319] Analysis of the properties of these cells in vitro has been hampered because bulk populations of T cells from tolerant recipients proliferate normally when cultured in vitro with donor alloantigens and frequently secrete a proinflammatory Th1 pattern of cytokines. It appears that the Tregs are masked by the presence of naïve T cells responding to alloantigens. In animal models, a number of protocols have been used to induce tolerance to allografts (Fig. 33.15). Tolerance to cardiac allografts in mice can be induced by pretreatment with a donor-specific blood transfusion combined with depleting or nondepleting anti-CD4 antibody.320 It is likely that the protective effects of this protocol were mediated by the induction of CD4+ Tregs, as complete depletion of CD4+ T cells fails to induce tolerance. Pretreated mice accepted cardiac grafts, and cells from these mice could transfer tolerance to naïve recipients. CD4+ T cells were responsible for both the induction and maintenance of tolerance.

In studies of allograft rejection across a full H-2 difference, the differential expression of CD45RB has facilitated separation of nontolerant naïve T cells from the induced Treg

cells.[319] RB^{high} cells from tolerant mice responded normally to challenge with alloantigen in vitro and were able to reject allogeneic skin grafts when transferred alone to T cell-deficient mice. In contrast, $CD45RB^{low}$ cells failed to mount a proliferative response, to secrete cytokines in response to alloantigen in vitro, and to induce allograft rejection in vivo. The addition of $CD45RB^{low}$ cells to an MLR with $CD45RB^{high}$ cells as responders resulted in inhibition of proliferation. $CD45RB^{low}$ T cells from long-term tolerant mice were able to suppress responses to alloantigen in vitro only when the donor alloantigens were presented by the indirect pathway of allorecognition by the APCs present in the culture. One explanation for this result is that it is potentially advantageous for the Tregs to respond to alloantigens via the indirect pathway because Tregs may require constant stimulation to maintain their function. Passenger leukocytes rapidly migrate out of the graft and the graft tissues lack costimulatory molecules. Donor-derived allopeptides must be presented by recipient APCs for stimulation of the Tregs in vivo and for the detection of functional activity of these cells in vitro. All of the suppressive activity of the $CD45RB^{low}$ cells was mediated by the CD4+CD25+ T-cell subset contained in that pool. The suppressive capacity of the Treg cells in this model in vitro and in vivo was reversed by anti-IL-10R but not by anti-IL-4. Tregs can also be isolated from mice pretreated with donor alloantigen in combination with depleting anti-CD4 but not transplanted with an allograft.[320]

In studies examining induction of tolerance to minor histocompatibility antigens on allografts following protocols similar to those used previously, both CD4+CD25+ and CD4+CD25- T cells from tolerant mice could mediate suppression but suppression required 10 times more CD4+CD25- T cells.[321] Both populations may have a significant role in maintaining transplantation tolerance as the number of CD25- T cells is 10 times higher than CD25+ T cells in the tolerant mouse. It is possible that some of the CD25- T cells have lost expression of CD25 during the tolerance induction protocol, that effector cell death in the CD25- T cell pool unmasked the presence of Tregs, or that some of the CD25- T cells express Foxp3. In contrast to the results with a major MHC difference, CD4+CD25+ T cells from naïve mice could also prevent naïve T cells from rejecting skin grafts from donors differing at minor loci, although five times more cells were required than from tolerant donors. The enhanced potency of CD25+ T cells from tolerant mice may be secondary to expansion of an alloantigen-specific population. The capacity of T cells from tolerant donors or CD25+ T cells from normal mice to mediate their suppressive function could not be neutralized by anti-CTLA-4, anti-IL-10, or anti-IL-4.

A short treatment of mice with saturating amounts of nondepleting anti-CD4 can induce tolerance to foreign protein antigens and enables long-term survival of minor antigen mismatched skin or MHC mismatched cardiac grafts. The addition of anti-CD40L can extend this effect to full donor-specific tolerance such that second donor grafts are accepted at any later time whereas third-party grafts are rapidly rejected. The maintenance of tolerance is dependent on CD4+ Tregs that can suppress both CD4+ and CD8+ naïve T cells after transfer into secondary recipients. Treg cells are found not only in recipient lymphoid tissue after transplantation but also at the graft site. The localization of Treg cells at more than one site in vivo is important if Tregs are to effectively control aggressive immune reactivity to the graft. In lymphoid tissues, Treg cells might be effective at blocking the initiation of an aggressive response against the graft whereas, at the graft site, they can inhibit the effector

activity of aggressive cells that have escaped regulation and migrated to the graft. Depletion of Tregs resulted in reversal of the tolerant state induced by anti-CD40L and allograft rejection.[322] Mast cells appear to play an important role in this mode of tolerance induction as tolerance induction by the combination of anti-CD40L and infusion of allogeneic cells could not be induced in mast cell-deficient animals.[323]

Regulation observed in therapeutic tolerance probably is maintained both by preexisting Tregs together with induced Tregs. In one model, antigen stimulation in the presence of a nondepleting anti-CD4 has been shown to convert CD4+Foxp3- T cells into Foxp3+ Tregs in a TGF-β dependent manner in vivo; this correlates with lifelong acceptance of the graft.[324] Treg cells that recognize donor MHC I molecules through the indirect pathway of allorecognition have been shown to be responsible for the phenomenon of linked unresponsiveness.[324] Linked suppression in vivo seems to correlate with the in vitro finding that once Tregs are activated, they can suppress the activation of other T cells responding to antigen presented by the same APCs in an antigen-nonspecific manner.

The inability of T-cell populations containing large numbers of CD25+ T cells to mediate GVHD strongly raised the possibility that CD25+ T cells might actually be capable of inhibiting CD25- effectors in cotransfer studies. Freshly isolated CD25+ T cells only modestly inhibited GVHD when mixed with CD25- T cells in equal numbers, while alloantigen-stimulated, cultured CD25+ T cells profoundly inhibited the capacity of CD25- T cells to inhibit rapidly lethal GVHD.[325] In murine models, transplantation of bone marrow into a lethally irradiated host does not cause GVHD; cotransfer of naïve donor T cells (CD4+, CD8+, or both) is required to induce GVHD. Depletion of Tregs from the donor graft accelerated the course of GVHD and increased lethality. Transfer of Tregs with naïve T cells from the donor protected lethally irradiated recipients from GVHD morbidity and decreased GVHD-related mortality[326] across minor and major MHC class I and/or class II barriers in various mouse strain combinations. The effect of Tregs on GVHD is likely to be early and to affect the maturation and expansion of effector cells. $CD62L^{hi}$ Tregs are more potent suppressors of GVHD presumably due to their ability to migrate to secondary LNs where they are primed and activated.[327] As host APCs are necessary to trigger acute GVHD, it is possible that the activated Treg function by downregulating costimulatory molecules and thereby prevent the activation of effector

T cells. Hoffman et al. showed decreased protection from GVHD with Tregs derived from IL-10-/- animals.[328]

The most analogous model to the murine models of GVHD induction is the administration of donor lymphocyte infusions to humans with relapsed leukemia after allogeneic hematopoietic cell transplantation so as to obtain a graftversus-tumor effect. The development of GVHD after donor lymphocyte infusion is associated with tumor clearance. However, Tregs controlling GVHD may also cause suppression of tumor immunity. Edinger and colleagues[329] found that adoptive transfer of donor Tregs effectively decreased the incidence and severity of GVHD without abrogating the graft-versus-tumor response. In this model, cotransfer of Tregs markedly decreased the proliferation of alloreactive effector cells in secondary lymphoid organs by > 90% at day 7 but did not inhibit the activation of the effectors as judged

by activation marker expression and cytokine production. One possibility is that the decrease in the proliferation and expansion of the alloreactive T effector cells was sufficient to control acute GVHD, but this decrease was not complete, and the remaining donor T effector cells were sufficient to effectively mount a graft-versus-tumor effect.

A model has been developed to demonstrate that human Treg cells can inhibit GVHD and graft rejection in vivo in the mouse. When human peripheral blood mononuclear cells are transferred to NOD/*scid*/IL2Rγc^{null} mice, the recipients develop a xeno-GVHD. When autologous expanded human Tregs are cotransferred with the peripheral blood mononuclear cells, marked amelioration of the GVHD is observed.[330] One marker of chronic allograft dysfunction is transplant arteriosclerosis with neointimal thickening of arteries in the allograft. A chimeric human mouse model that resembles transplant arteriosclerosis has been developed in which allogeneic human peripheral blood mononuclear cells elicit rejection of human arterial segments that have been transplanted into a mouse.[331] When human Treg cells that had been expanded in vitro were cotransferred with the peripheral blood mononuclear cells, abrogation of transplant arteriosclerosis was observed.

Tregs also play a facilitating role in bone marrow engraftment. Joffre et al.[332] found that Tregs provided allospecific protection of transplanted bone marrow from host rejection. Enhanced engraftment with Tregs also increased long-term donor chimerism in animals that received Tregs compared to those that received bone marrow transplants only. Recipients of Tregs demonstrated tolerance to host and donor antigens but mounted responses to third-party antigens.[333] Treg therapy has been evaluated for the prevention of GVHD following hematopoietic stem cell transplantation in man.[334,335] One trial was primarily a safety trial of human in vitro expanded Tregs isolated from umbilical cord blood. No serious side effects were observed and some reduction of GVHD was observed. The most comprehensive trial to date was performed by Di Ianni et al.[335] This study involved 28 patients with high-risk hematologic malignancies who underwent HLA-haploidentical stem cell transplantation. No posttransplantation immunosuppression was given, and freshly isolated Tregs were administered without in vitro expansion. The incidence of GVHD was markedly decreased, and the Treg treatment was not associated with an increased risk of leukemia relapse or with a increased incidence of infection with opportunistic pathogens. It is likely that multiple trials of of Treg therapy for the prevention of GVHD in man will be conducted in the future.

Tumor Immunity

Many tumor-associated antigens recognized by autologous tumor-reactive lymphocytes are antigenically normal selfconstituents. Tumor immunity is therefore autoimmunity, and the mechanisms for maintaining immunologic self-tolerance may hinder effective tumor immunity. Studies by North and Bursuker in the early 1980s[336] demonstrated the role of suppressor T cells in the prevention of tumor immunity. The suppressor T cells in these experiments of North and Awwad were shown to be CD4+CD8-, but were not characterized further.[337]. Onizuki et al.[338] were the first to suggest that Foxp3+ T cells played an important role in inhibiting tumor immunity. They first depleted CD4+CD25+ T cells by injecting a depleting anti-CD25 mAb and noted that this led to regression of a number of tumors that grew progressively in nondepleted mice. The depletion had to be performed not later than day 2

after injection of the tumor. Coadministration of anti-CD8 inhibited the tumor regression induced by anti-CD25 depletion, suggesting that CD8 T cells were responsible for tumor regression. Shimizu et al.[171] drew similar conclusions when they transferred CD25-depleted or CD25-containing spleen cells to *nu/nu* recipients and then challenged them with normally nonimmunogenic tumors. In the recipients of CD25-depleted cells, tumors grew but then regressed; in contrast, all recipients of spleen cells containing CD25+ T cells died from rapidly growing tumors. Long-lasting tumor immunity to rechallenge could also be demonstrated in the recipients of the CD25-depleted spleen cells.

Taken together, the studies on depletion of CD4+CD25+ T cells and the transfer of CD4+CD25- T cells strongly suggest that the effectiveness of a tumor vaccine would be greatly enhanced by removal of CD4+CD25+ T-cell suppressor activity. Indeed, Sutmuller et al.[339] were able to demonstrate that antibody-mediated depletion of CD25+ T cells followed by vaccination with a granulocyte macrophage-colony stimulating factor-transfected melanoma cell line resulted in enhanced tumor rejection. Tumor rejection was accompanied by skin depigmentation, suggesting that autoreactive immune responses are involved in this process. Depletion of CD25+ T cells followed by anti-CTLA-4 treatment and vaccination resulted in the most potent antitumor response (Table 33.8). Thus, CD25 depletion and anti-CTLA-4 treatment increase the immunogenicity of a tumor vaccine by distinct mechanisms involving nonredundant pathways. CTLA-4 blockade enhances the induction of antitumor effector cells by removing the normal inhibitory signals generated by CD80/CD86 interactions with CTLA-4.

Experiments with adoptively transferred mouse Treg cells provided a direct link between Treg cells and reduced tumor immunity. Tumor-specific CD8+ T cells were transferred with either Treg cells or conventional T cells into mice bearing B16 melanoma. In mice that received Treg cells, but not

in mice that received CD25- T cells, CD8+ T cell-mediated immunity was abolished. Treg cells may mediate their suppressive effect by inhibiting T-cell priming in lymphoid organs or by reducing the effector function of tumor antigen-specific cells. Antony et al.[340] have shown that transfer of Treg cells reduced the therapeutic efficiency of adoptively transferred tumor antigen-specific effector T cells in a mouse melanoma model. Depletion of intratumoral Treg cells induced potent T-cell tumor immunity and resulted in regression of large established tumors. Administration of anti-GITR antibody protected mice from B16 tumor challenge and induced tumor regression in mice bearing carcinogeninduced sarcomas and colon carcinomas.[341] The anti-GITR likely costimulated T effector functions to make the effector cells resistant to Treg-mediated suppression.

TABLE 33.8 Depletion of CD4+ CD25+ T Cells Augments the Immune Response to a Tumor Vaccine

Treatment	% Surviving d90

None	0
Anti-CD25 (day -4)	20
GM-CSF vaccine (day 0, 3, 6) + anti-CD25 (day -4)	50
GM-CSF vaccine (day 0, 3, 6) + anti-CTLA-4 (day 0, 3, 6)	50
GM-CSF vaccine (day 0, 3, 6) + anti-CD25 (day -4) + anti-CTLA-4 (day 0, 3, 6)	100

CTLA, cytotoxic T-lymphocyte antigen; GM-CSF, granulocyte macrophage-dolony stimulatinf factor.

(Adapted from Sutmuller et al.339)

Although these studies in well-characterized animals models demonstrate the role of CD4+CD25+ T cells in inhibiting tumor rejection, it is often difficult to extrapolate from tumor immunity studies in rodents to humans. Woo et al.[342] have shown that Foxp3+ T cells exist in high proportions (˜33%) in the tumor-infiltrating lymphocytes of patients with nonsmall-cell lung cancer. It is not known if these tumor-infiltrating Foxp3+ T cells are thymic-derived or are generated in the tumor microenvironment from Foxp3- T cells. Foxp3+ Tregs may also be induced at the site of the tumor because of the high concentrations of TGF-β secreted by tumor cells. Nevertheless, these findings suggest that one component of the immune response to tumors in humans is the generation of tumor-specific suppressor T cells. A high frequency of Treg cells has been noted in many other human cancers. Treg cells have been extensively studied in human ovarian cancer.[343] Treg cells within the ovarian tumor microenvironment expressed Foxp3, inhibited tumor antigen-specific CD8+ T-cell cytotoxicity, and contributed to tumor growth in vivo in a human-SCID mouse chimeric model. An accumulation of Treg cells in the tumor predicted a striking reduction in survival.

A number of mechanisms have been proposed for Treg-mediated suppression of tumor immunity. As Tregs express CTLA-4, one possible model is that engagement of CD80/CD86 on tumor APCs may induce production of the immunosuppressive molecule, IDO (see Fig. 33.12). IDO-expressing APCs are found in human tumors and within draining LNs. Alternatively, ovarian tumor-associated macrophages, but not normal macrophages, express B7-H4, a negative regulator of T-cell responses.[344] Treg, but not normal T, cells induced B7-H4 on monocytes, macrophages, and myeloid DCs, and rendered them immunosuppressive.

The spectrum of tumor-specific antigens recognized by Tregs has not yet been defined. In one case, cloned Tregs from a melanoma recognized LAGE-1, a cancer and testis-specific antigen, and suppressed LAGE-1-specific T-cell activation.[345] It is important to identify the ligands for Treg cells to compare them to the ligands for tumor-specific effector cells and to determine whether there are ligands that are exclusively recognized by Treg cells and whether Treg cells recognize mutated tumor antigens. Tregs are able to recognize some

tumor antigens identified serologically by recombinant expression cloning (SEREX). Adoptive transfer of Tregs from mice immunized with SEREX antigens enhanced pulmonary metastasis and accelerated tumor development indicating that acceleration of tumorigenesis and enhancement of metastasis may be secondary to the immunosuppressive effects of Tregs in the immunized hosts.[346] A major concern that remains to be addressed is whether vaccination with certain tumor antigens might promote Treg cell clonal expansion.

The concept of reversing immunosuppression in cancer has merit as a therapeutic approach. CTLA-4 blockade improves tumor immunity but also resulted in severe but treatable autoimmune responses in patients.[347] The mechanistic link between the effects of treatment with anti-CTLA and Treg function remain to be determined. Depletion of Tregs should be beneficial for cancer patients. Denileukin diftitox (Ontak [Eisai, Inc. Woodcliff Lek, NJ]), a ligandtoxin fusion protein, consists of full-length IL-2 fused to the enzymatically active and translocating domains of diptheria toxin. It has been hypothesized that this agent will lead to depletion of Tregs in vivo but it also might deplete CD25+ effector cells. In one trial, patients received a single does of Ontak followed by vaccination with DCs transfected with total tumor ribonucleic acid.[348] Treg cells were eliminated in a dose-dependent manner and Ontak-treated vaccinated patients had an improved tumor-specific effector T-cell response. In other studies, Ontak had variable effects on Foxp3 messenger ribonucleic acid expression in CD4+ T cells in patients with melanoma, and no significant clinical efficacy was observed.[349] Depletion of Tregs to improve tumor immunity should be a major goal in tumor immunotherapy. Thus far, attempts to deplete Tregs in patients with cancer using cyclophosphamide, the anti-CD25 mAb daclizimab, or Ontak have not been successful.[350]

Other models raise the possibility that Tregs may have a beneficial effect in preventing tumorigenesis. A widely used model for human colorectal carcinogenesis is the “multiple intestinal neoplasia” mouse, which has a germline mutation in the *Apc* tumor-suppressor gene.[351] Transfer of Treg cells reduced the multiplicity of epithelial adenomas in these mice by an IL-10-dependent mechanism. Recipients of Tregs showed increased apoptosis and downregulation of cyclooxygenase-2 within

tumors coinciding with tumor regression. In this model, aspirin and cyclooxygenase inhibitors also decreased the risk for colon cancer by inhibiting cyclooxygenase-2. As Tregs can suppress bacterially triggered inflammatory responses in the bowel of mice, the ability of Tregs to traffic and suppress inflammation likely explains their therapeutic efficacy in this model. As inflammatory mediators drive tumor development, Tregs and anti-inflammatory drugs exert their effect by modulating the levels of these molecules.

Immunity to Infectious Agents

Treg cells dampen immune responses in a wide variety of infections caused by bacteria, fungi, parasites, and viruses, especially those that persist in the host. One of the most important roles of Tregs may, in fact, involve modulation of the immune response to infectious agents to prevent the lethal consequences of an overwhelming inflammatory response during the course of a productive immune response to an invading microorganism. Treg and effector cells must maintain equilibrium between no immunity at all and immunopathology. This critical role of Tregs is well illustrated by the immune response of mice to infection with

Pneumocystis carinii (PC). When SCID mice chronically infected with PC are reconstituted with CD4+CD25- T cells, they develop a severe inflammatory response in their lungs that is ultimately fatal. Animals injected with CD25+ T cells alone did not become moribund and only manifested transient weight loss. Cotransfer of CD25+ T cells prevented the development of the PC-driven fatal pulmonary inflammation induced by CD25-T cells but also suppressed the elimination of PC mediated by the CD25- T cells. Protective CD25+ T cells are needed to inhibit the lethal immunopathologic response mediated by the PC-specific CD4+CD25- T cells but they also inhibited complete clearing of the organism.[352] Although Tregs may target CD4+ and CD8+ T cells responding to an infectious challenge, Tregs have also been shown to act on the innate immune system in the response to *Helicobacter hepaticus*.[353]

The role of Foxp3+ T cells in the immune response to infection includes more than suppression of inflammation.[354,355] Foxp3+ T cells also maintain persistence of infection and promote chronicity. The persistence of pathogens following clinical cure is a hallmark of certain viral, bacterial, and parasitic infections. In clinical and experimental forms of leishmaniasis, small numbers of viable organisms persist within lymphoid tissue and within the site of former skin lesions following self-cure or successful chemotherapy. As low numbers of parasites persisting in the dermis can be efficiently transmitted back to their vector sandflies, the expansion and/or recruitment of regulatory T cells to the site of *Leishmania major* infection might reflect a parasite adaptive strategy to maintain its transmission cycle in nature. Despite the absence of sterilizing immunity, these individuals maintain strong lifelong immunity to reinfection, a status similar to the concomitant immunity described in tumor models.[356]

In healed C57BL/6 mice, CD4+CD25+ Treg cells accumulate in sites of *L. major* infection in the skin.[357] These cells are exclusively derived from CD25+ T cells and not from activated CD25- T cells. They suppress the expansion of and killing mediated by *L. major*-specific effector cells. Although IL-10 produced by CD25+ T cells is essential to the establishment of persistent infection, early in the infectious process CD25+ T cells can promote parasite survival and growth in an IL-10-independent manner. Later in the course of infection, IL-10 is absolutely required for development of the chronic lesion as recipients of CD25+ T cells from IL-10-/- mice ultimately healed and completely cleared the parasite from the site. IL-10 produced by Tregs contributes directly to parasite persistence by either modulating APC function, inhibiting cytokine production by Th1 cells, or by rendering macrophages refractory to IFNγ that is needed for intracellular killing.

When rechallenge studies were performed in IL-10-/- mice or in WT mice that were treated during the chronic stage of their primary infection with anti-IL-10R, conditions that result in complete clearance of parasites from the skin and draining LN, reinfection at a site distant from the initial infection resulted in parasite loads that were comparable to those following primary infection in naïve mice. Because healed mice treated with control antibody maintained strong immunity to reinfection, the maintenance of a residual source of infection, secondary to IL-10 production by CD25+ T cells at the lesion site, is required for preservation of acquired immunity to *L. major*. Treg cell lines isolated from chronic *L. major* are able to respond to parasiteinfected DCs by proliferating and producing IL-10.[358] The cells that have undergone proliferation express and maintain Foxp3 expression. It appears that the parasite has specifically evolved to manipulate DCs in a manner that favors and sustains Treg

proliferation. Most importantly, the *L. major*-specific cell lines maintain their suppressive functions in vivo, as transfer of the lines to chronically infected mice results in massive disease reactivation and dissemination.

Th1 effector cells are induced during the course of infection with *Bordetella pertussis* and ultimately play a critical role in the clearance of bacteria from the respiratory tract.[359] Antigen-specific Th1 responses in the lung and local LNs are severely suppressed during the acute phase of infection. *B. pertussis* has evolved a number of strategies to circumvent protective immune responses. One bacterial component, filamentous HA, is capable of inhibiting LPS-driven IL-12 production by macrophages and DCs, and stimulating IL-10 production. Filamentous HA may contribute to the suppressed Th1 responses during acute infection with *B. pertussis* by the induction of T cells with regulatory activity as a result of its interactions with cells of the innate immune system. Repeated stimulation of T cells from the lungs of mice acutely infected with *B. pertussis* resulted in the generation of Tr1 clones specific for filamentous HA. Tr1 cells could only be generated from the lungs of infected animals and not from spleen. These Tr1 cells secreted high levels of IL-10 and inhibited protective immune responses against *B. pertussis* in vivo and in vitro. Suppression was substantially reversed by anti-IL-10 in vivo. The capacity to induce Tr1 cells is thereby exploited by a respiratory pathogen to evade protective immunity and suppress protective Th1 responses at local sites of infection.

Analysis of cytokine production by T cells from patients with chronic hepatitis C virus infection identified antigenspecific regulatory T cells that secreted IL-10 in addition to IFNγ-producing circulating Th1 cells[360]; no IL-4-producing T cells were identified. IL-10-producing cells were detected in a higher proportion of patients with chronic infection than in those who had cleared the virus. Taken together with the studies on *L. major* and *B. pertussis*, these studies on hepatitis C virus strongly support the general concept that many infectious agents have evolved mechanisms for selective activation of either naturally occurring CD25+ T cells or the generation of IL-10-producing Tr1 cells from CD25- T cells. The ultimate result is perpetuation of the chronic infectious state with incomplete clearing of the infection. Depending on the extent of suppression of effector T cells in the host, the consequences of the chronic state may be protective immunity (*L. major*) or continued pathogenmediated organ destruction (hepatitis C virus).

Many viruses such as herpesviruses, hepatitis viruses, and retroviruses evade immunologic destruction during acute infection and establish chronic, persistent infections that may culminate in life-threatening diseases. Friend virus (FV) infection in mice has been used as an experimental model to study retrovirus-induced immunosuppression and may offer insights into our understanding of immunosuppression associated with human immunodeficiency virus (HIV). Mice chronically infected with FV are unable to reject both FV-induced and unrelated immunogenic tumors. CD8+ T cells from acutely infected mice produced perforin, granzyme A, and granzyme B, and display recent evidence of degranulation and in vivo cytotoxicity. Activated T cells from chronically infected mice were deficient in cytolytic molecules and showed little evidence of recent degranulation and in vivo cytotoxicity. CD4+, but not CD8+, T cells from infected mice can transfer suppression to normal mice and can inhibit the generation of CTL in culture.[361] Suppression could be substantially reversed by the addition of anti-CTLA-4, but not anti-IL-10R, to the cultures. FV-induced Tregs suppress CD8+ T cells

in vitro regardless of the TCR specificity of the CD8+ T cells.[362] It is unknown whether the Tregs in chronically infected mice are specific for any viral proteins. Depletion of Foxp3+ T cells early after infection resulted in an increase in virus-specific CD8+ T cells on day 10 postinfection, enhanced production of IFNγ and TNFα, and a 10-fold reduction in viral load.[363]

As HIV infection in man in many respects mimics the animal models of chronic retroviral infections, several studies have begun to examine Treg function as different stages of HIV infection. Kinter et al.[364] found that in the majority of HIV-infected but still healthy individuals, $CD25^{hi}$ Treg cells significantly suppressed cellular proliferation and cytokine production by CD4+ and CD8+ T cells in response to HIV antigens in vitro. Suppression was cell contact-dependent and IL-10- and TGF-β-independent. Patients with strong HIVspecific suppression in vitro had lower levels of viremia and higher CD4/CD8 T-cell ratios than patients who did not have Treg activity. These data suggest that the suppression of CD4+ T-cell activation by Tregs may make HIV replication less favorable as the virus must replicate within the CD4+ T cell itself. Thus, in this chronic infection model, the suppressive functions of Tregs may actually be beneficial to the patient.

The contribution of Tregs has been most clearly shown in responses to chronic infections. Much less is known about the role of Tregs in modulating acute viral infections. In the murine model of herpetic stromal keratitis, depletion of Tregs before infection resulted in lesions of greater severity and permitted the induction of disease with lower infecting doses of virus.[365] Cotransfer of Tregs with CD25- T cells reduced lesion severity and diminished the Ag-specific cytokine response of splenic CD4+ T cells. The mechanism of action of Tregs in this model is not fully elucidated. Tregs inhibit the induction of virus specific CD4+ T cells, but may also modify the expression of homing molecules involved in T-cell migration to the ocular inflammatory site or of pathogenic T cells to extralymphoid inflammatory sites. Tregs can also control the intensity of secondary responses to herpes simplex virus and may also influence the magnitude of immunologic memory.

Pathogen-specific Treg cells have been detected in a few studies using whole pathogen preparations as targets without the identication of specific target epitopes. The role of Treg cells in facilitating the persistence of infections with *Mycobacterium tuberculosis* is unclear. Rigorous depletion of Treg cells during early infection results in enhanced bacterial clearance.[366] Early arrival in the lung of *M. tuberculosis*-specific T cells is associated with enhanced immune control and lower bacterial burdens; Treg cells slow this recruitment process. This result differs from studies of infection with herpesvirus concluded that Treg cells facilitate effector T-cell recruitment to tissue sites of infection.[367] Studies using TCR-transgenic cells specific for an epitope derived from *M. tuberculosis* attempted to identify Tregs specific for the same epitope recognized. Treg cells specific for this epitope were not found.[366] Virus-specific Treg cells have been detected in mice infected with a strain of mouse hepatitis virus. Polyclonal Treg cells from normal mice will decrease weight loss, clinical scores, and demyelination when adoptively transferred into mice infected with this viral strain. Treg cells for two viral epitopes were detected using peptide-MHC class II tetramers 6 to 7 days after infection, at the same time as CD4+ effector T cells. The virus-specific Foxp3+ cells expressed both IFNγ and IL-10 in the CNS after infection and

suppressed T-cell proliferation of cognate epitope-specific CD4+ T cells.[368] IFNγ expression by these cells was maintained during both acute and chronic phases of infection. The identification of Tregs specific for a pathogen-derived epitope is consistent with the view that exposure and immunization may expand not only protective T-cell subsets, but also T-cell subsets that impede protection. Overall protection likely depends on the relative ratio of protective versus suppressive T-cell subsets.

Autoimmune Disease in Man

A large body of data supports the existence of Foxp3+ T cells in humans,[29,30,31,32,33,34,35,36] and the in vitro characterization of human Foxp3+ cells suggests that they are identical to their murine counterparts. Tr1 cells have been readily induced in cultures of human T cells, and a number of studies have

supported the existence of cell populations with Tr1-like properties in humans. Danke et al.[369] have shown that deletion of Tregs in vitro allows marked clonal expansion of autoreactive T cells in vitro. In mice, Foxp3 seems to be a robust marker for thymic-derived and induced Treg cells. However, many human CD4+ T cells transiently express Foxp3 during activation.[370,371,372] It also remains controversial if human T cells that are induced to express Foxp3 by activation in the presence of TGF-β acquire regulatory function.[372] It has recently been shown that culture of naïve human T cells (CD4+CD25-CD45RA+) in the presence of TGF-β and rapamycin[373] or in the presence of TGF-β and RA[374] results in the generation of functional Foxp3+ human Tregs. Nevertheless, it is clear that Foxp3 alone is not a reliable marker for human Treg cells. The combined expression of Foxp3 and low levels of CD127 may be more reliable.

There is currently no clear evidence that a global deficiency in the number of Treg cells is the source of failed regulation in the more common forms of autoimmunity.[375] There may be differences in Treg phenotypes in disease including the numbers of CD4+CD45RA+Foxp3+ and CD4+CD45RA-Foxp3+ Tregs.[376] The peripheral induction of Treg might also be defective in IBD. In fact, increased numbers of Treg cells are frequently observed in the affected tissues of patients with rheumatoid arthritis, IBD, and psoriasis, suggesting that Treg function may be compromised by cell extrinsic factors such as the cytokine milieu or overactive APC function, resulting in resistance to suppression. Several studies in patients with different autoimmune diseases have demonstrated a defect in the function of Tregs, raising the issue as to whether this may be a common denominator in the cause of human autoimmune disease (Table 33.9). In many of these studies, it was definitively shown that the decrease in Treg function was due to defect in the Treg subset rather than secondary to responder T cells that were refractory to suppression. One possibility is that Tregs may have migrated into the target tissue so that blood Treg function is depressed, but Treg function from the target tissue may actually be enhanced. For example, the frequency of $CD25^{hi}$ T cells was much greater in the synovial fluid as compared to the peripheral blood in adult patients with rheumatoid arthritis and such synovial fluid-Treg demonstrated normal suppressive activity in vitro.[384] Functional defects in in the ability of Tregs from rheumatoid arthritis to suppress proliferation were not present but the Treg from rheumatoid arthritis patients appeared to have a selective defect in suppression of IFNγ secretion. This selective

suppression was associated with overexpression of TNFα in the synovial tissues of rheumatoid arthritis subjects, decreased Foxp3 expression, and impaired CTLA-4 expression.[385,386,387] Treatment of patients with rheumatoid arthritis with anti-TNFα restored the capacity of the Tregs to suppress cytokine production. It remains unclear whether the treatment acted directly on Tregs or whether it acted on the pathogenic T effector cells or modified the inflammatory milieu. Appropriate caution should be exercised in interpretation of these studies. As discussed previously, identification of human Foxp3+ Tregs is difficult; many of the studies published thus far have only utilized high levels of CD25 expression to identify human Tregs and did not validate the isolated populations for Foxp3 expression by intracellular staining.

TABLE 33.9 Human Diseases with Abnormal Regulatory T Cell Function

Disease	Reference
Multiple sclerosis	377
Myasthenia gravis	378
Rheumatoid arthritis	385
Autoimmune polyglandular II	379
Lupus erythematosis	380
Psoriasis	381
Type I diabetes	382
Human immunodeficiency virus/acquire immunodeficiency syndrome	383

Enhancement of either the numbers, function, or survival of Tregs represents a goal for the treatment of autoimmune and allergic diseases as well as for inhibition of allograft rejection.[334,335] One might expand either CD4+CD25+ T cells or Tr1 T cells to generate sufficient numbers of cells for infusion back into patients.[388,389] If Foxp3+ Tregs recognize organ-specific antigens and if these antigens can be identified, it may be possible to generate lines or clones of antigen-specific suppressor T cells in vitro that could be used therapeutically. Organ-specific Tregs would home to their target, be activated by their target autoantigen, but mediate bystander suppression, as their effector function would be nonspecific. Alternatively, as a number of factors (eg, IL-6 or the GITR-L) can render effector cells resistant to the suppressor function of Tregs, blocking or neutralizing these factors may enhance Treg function. Ultimately, further studies of the molecular basis of Treg-mediated

suppression should allow the development of mAbs or small molecules[390] that could enhance their suppressor effector function.

CONCLUSION

The hypothesis proposed[3] more than 40 years ago, that a distinct lineage of T cells mediated immune suppression, has been confirmed by major advances only in the last 15 years. The identification of the transcription factor, Foxp3, in 2003 solidified a large body of experimental data.[43,44,45] Yet, numerous critical issues remain to be studied. While Foxp3 is the major factor controlling Treg function, other, as yet uncharacterized, factors upstream of Foxp3 likely play a role in Treg development. Translation of a large body of experimental data on Tregs in animal models to the clinic represents a daunting task. Major questions remain as to the mechanisms used by Tregs to suppress distinct immune responses in different environmental conditions. Until we have a complete understanding of these mechanisms, manipulation of Treg function with small molecules or biologics will present a major challenge.

REFERENCES

1. Schwartz RH. A cell culture model for T lymphocyte clonal anergy. *Science*. 1990;248:1349-1356.

2. Miller JFAP, Heath WR. Self-ignorance in the peripheral T-cell pool. *Immunol Rev*. 1993;133:131-150.

3. Gershon RK, Kondo K. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology*. 1970;18:723-735.

4. Gershon RK, Kondo K. Infectious immunological tolerance. *Immunology*. 1971;21:903-914.

5. Baker PJ, Barth RF, Stashak PW, et al. Enhancement of the antibody response to type III pneumococcal polysaccharide in mice treated with anti-lymphocyte serum. *J Immunol*. 1970;104:1313-1315.

6. Tada T, Taniguchi M, Okumura K. Regulation of homocytotropic antibody response in the rat. II. Effect of X-irradiation. *J Immunol*. 1971;106:1012-1018.

7. Okumura K, Tada T. Regulation of homocytotrophic antibody in the rat. VI. Inhibitory effect of thymocyte on homocytotrophic antibody response. *J Immunol*. 1971;107:1682-1689.

8. Dorf ME, Benacerraf B. I-J as a restriction element in the suppressor cell system. *Immunol Rev*. 1985;83:23-40.

9. Tada T, Okumura O. The role of antigen-specific T cell factors in the immune

response. *Adv Immunol*. 1979;28:1-87.

10. Germain RN, Benacerraf B. A single major pathway of T-lymphocyte interactions in antigen-specific immune suppression. *Scand J Immunol*. 1981;13:1-10.

11. Green DR, Flood PM, Gershon RK. Immunoregulatory T-cell pathways. *Ann Rev Immunol*. 1983; 1;439-463.

12. Dorf ME, Benacerraf B. Suppressor cells and immunoregulation. *Ann Rev Immunol*. 1984;2:127-158.

13. Murphy DB. The I-J puzzle. *Ann Rev Immunol*. 1987;5:405-427.

14. Hedrick SM, Germain RN, Bevan MJ, et al. Rearrangement and transcription of a T-cell receptor β-chain gene in different T-cell subsets. *Proc Nat Acad Sci U S A*. 1985;82:531-535.

15. Nishizuka Y, Sakakura T. Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. *Science*. 1969;166:753-755.

16. Sakaguchi S, Sakaguchi N. Thymus, T cells, and autoimmunity: various causes but a common mechanism of autoimmune disease. In: Coutinho A, Kazatchkine MD, eds. *Autoimmunity: Physiology and Disease*. Wilmington, DE: Wiley-Liss, Inc.; 1994:203-227.

17. Penhale, WJ, Farmer, A, Irvine WJ. Thyroiditis in T cell-depleted rats: influence of strain, radiation dose, adjuvants and antilymphocyte serum. *Clin Exp Immunol*. 1975:21:362-375.

18. Penhale WJ, Irvine WJ, Inglis JR, et al. Thyroiditis in T cell-depleted rats: suppression of the autoallergic response by reconstitution with normal lymphoid cells. *Clin Exp Immunol*. 1976;25:6-16.

19. Penhale WJ, Stumbles PA, Huxtable CR, et al. Induction of diabetes in PVG/c strain rats by manipulation of the immune system. *Autoimmunity*.1990;7:169-179.

20. Sakaguchi S, Fukuma K, Kuribayashi K, et al. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. *J Exp Med*. 1985;161:72-87.

21. Sakaguchi S, Sakaguchi N, Asano M, et al. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). *J Immunol*. 1995;155:1151-1164.

22. Asano M, Toda M, Sakaguchi N, et al. Autoimmune disease as a consequence of

developmental abnormality of a T cell subpopulation. *J Exp Med*. 1996;184:387-396.

23. Suri-Payer E, Amar AZ, Thornton AM, et al. CD4+CD25+ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J Immunol*. 1998;160:1212-1218.

24. Powrie F, Mason D. OX-22^{high}CD4+ T cells induce wasting disease with multiple organ pathology: prevention by the OX-22^{low} subset. *J Exp Med*. 1990;172:1701-1708.

25. Fowell D, Mason D. Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. Characterization of the CD4+ T cell subset that inhibits this autoimmune potential. *J Exp Med*. 1993;177:627-636.

26. Powrie F, Leach MW, Mauze S, et al. Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C.B-17 scid mice. *Int Immunol*. 1993;5:1461-1471.

27. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25^{+}CD4^{+} regulatory cells that control intestinal inflammation. *J Exp Med*. 2000;192: 295-302.

28. Itoh, M, Takahashi T, Sakaguchi N, et al. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol*. 1999;162:5317-5326.

29. Baecher-Allan C, Brown JA, Freeman GJ, et al. CD4+CD25^{high} regulatory cells in human peripheral blood. *J Immunol*. 2001;167:1245-1253.

30. Stephens LA, Mottet C, Mason D, et al. Human CD4+CD25+ thymocytes and peripheral T cells have immune suppression activity. *Eur J Immunol* . 2001;31:1247-1254.

31. Levings MK, Sangregorio R, Roncarolo MG. Human CD25+CD4+ T regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med*. 2001;193:1295-1301.

32. Jonuleit H, Schmitt E, Stassen M, et al. Identification and functional characterization of human CD4+CD25+ T cells with regulatory properties isolated from peripheral blood. *J Exp Med*. 2001;193:1285-1294.

33. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4+CD25+ T cells with regulatory properties from human blood. *J Exp Med*. 2002;193:1303-1310.

34. Taams LA, Smith J, Rustin MH, et al. Human anergic/suppressive CD4+CD25+ T

cells: a highly differentiated and apoptosis-prone population. *Eur J Immunol* . 2001;31;1122-1131.

35. Ng WF, Duggan PJ, Ponchel F, et al. Human CD4+CD25+ cells: a naturally occurring population of regulatory T cells. *Blood*. 2001;98:2736-2744.

36. Stephens LA, Mason D. CD25 is a marker for CD4+ thymocytes that prevent autoimmune diabetes in rats, but peripheral T cells with this function are found in both CD25+ and CD25- subpopulations. *J Immunol* . 2001;165:3105-3110.

37. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med*. 1998;188:287-296.

38. Salomon B, Lenschow DJ, Rhee L, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity*. 2000;12:431-440.

39. Takahashi T, Tagami T, Yamazaki S, et al. Immunologic self-tolerance maintained by CD25+CD4+ regulatory T cells constitutively expressing cytotoxic T lymphcyte-associated antigen 4. *J Exp Med*. 2000;192:303-309.

40. McHugh RS, Whitters MJ, Piccirillo CA, et al. CD4+CD25+ immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity*. 2002;16:311-323.

41. Shimizu J, Yamazaki S, Takahashi T, et al. Stimulation of CD25+CD4+ regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* . 2002;3:135-142.

42. Brunkow ME, Jeffrey EW, Hjerrild KA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the surfy mouse. *Nat Genet*. 2001;27:68-73.

43. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* . 2003;4:330-336.

44. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor *Foxp3*. *Science*. 2003;299:1057-1061.

45. Khattri R, Cox T, Yasayko SA, et al. An essential role for scurfin in $CD4^{+}CD25^{+}$ T regulatory cells. *Nat Immunol* . 2003;4:337-342.

46. Fontenot JD, Rasmussen JP, Williams LM, et al. Regulatory T cell lineage specification by the Forkhead Transcription Factor Foxp3. *Immunity*. 2005; 22:329-341.

47. Fontenot JD, Dooley JL, Farr AG, et al. Development regulation of Foxp3 expression during ontogeny. *J Exp Med*. 2005;202:901-906.

48. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol*. 2007;8:191-197.

49. Wan YY, Flavell RA. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature*. 2007;445:766-770.

50. Liu WH, Putnam AL, Xu-Yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human $CD4^+$ T reg cells. *J Exp Med*. 2006;203:1701-1711.

51. Shevach EM. Certified professionals: CD4+CD25+ suppressor T cells. *J Exp Med*. 2001;193:F41-F45.

52. Modigliani Y, Coutinho A, Pereira P, et al. Establishment of tissue-specific tolerance is driven by regulatory T cells selected by thymic epithelium. *Eur J Immunol* . 1996;26:1807-1815.

53. Papiernik M, de Moraes ML, Pontoux C, et al. Regulatory CD4 T cells: expression of IL-2R chain, resistance to clonal deletion and IL-2 dependency. *Int Immunol*. 1998;10:371-378.

54. Jordan MS, Boesteanu A, Reed AJ, et al. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol*. 2001;2:301-306.

55. Van Santen HM, Benoist C, Mathis D. Number of T reg that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells. *J Exp Med*. 2004;200:1221-1230.

56. Bautista JL, Lio CW, Lathrop SK, et al. Intraclonal competition limits the fate determination of regulatory T cells in the thymus. *Nat Immunol*. 2009;6:610-617.

57. Leung MW, Shen SQ, Lafaille JJ. TCR-dependent differentiation of thymic $Foxp3^+$ cells is limited to small clonal sizes. *J Exp Med*. 2009;206:2121-2130.

58. DiPaolo RA, Shevach EM. CD4+ T cell development in a mouse expressing a transgenic TCR derived from a regulatory T cell. *Eur J Immunol* . 2009;39:234-240.

59. Bensinger SJ, Bandeira A, Jordan MS, et al. Major histocompatibility complex class II-positive cortical epithelium mediates the selection of CD4+25+immunoregulatory T cells. *J Exp Med*. 2001;194:427-438.

60. Aschenbrenner K, D'Cruz LM, Vollman EH, et al. Selection of Foxp3^{+} regulatory T cells specific for self-antigen expressed and presented by Aire^{+} medullary thymic epithelial cells. *Nat Immunol*. 2007;8:351-358.

61. Hinterberger, M, Aichinger M, da Costa OP, et al. Autonomous role of medullary thymic epithelial cells in central CD4^{+} T cell tolerance. *Nat Immunol*. 201;11:512-519.

62. Tai X, Cowan M, Feigenbaum L, et al. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol*. 2005;6:152-162.

63. Lio CW, Dodson LF, Deppong CM, et al. CD28 facilitates the generation of Foxp3-cytokine responsive regulatory T cell precursors. *J Immunol*. 2010;184(11):6007-6013.

64. Haxhinasto S, Mathis D, Benoist C. The AKT-mTOR axis regulates de novo differentiation of CD4^{+}Foxp3^{+} cells. *J Exp Med*. 2008;205:565-574.

65. Schmidt-Supprian M, Tian J, Grant EP, et al. Differential dependence of CD4^{+}CD25^{+} regulatory and natural killer-like cells on signals leading to NF-κB activation. *Proc Natl Acad Sci U S A*. 2004;101:4566-4571.

66. Fontenot JD, Rasmussen JP, Gavin MA, et al. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol*. 2005;6: 1142-1151.

67. Vang KB, Tang J, Mahmud SA, et al. IL-2-7 and -15, but not thymic stromal lymphopoietin, redundantly goven CD4+Foxp3+ regulatory T cell development. *J Immunol*. 2008;181:3285-3290.

68. Burchill MA, Yang J, Vang KB, et al. Linked T cell receptor and cytokine signaling govern the development of the regulatory T cell repertoire. *Immunity*. 2008;28:112-121.

69. Lio CW, Hsieh CS. A two-step process for thymic regulatory T cell development. *Immunity*. 2008;28:100-110.

70. Malek TR, Yu A, Vincek V, et al. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rβ-deficient mice: implications for the nonredundant function of IL-2. *Immunity*. 2002;17:167-178.

71. D'Cruz LM, Klein L. Development and function of agonist-induced CD24^{+}Foxp3^{+} regulatory T cells in the absence of interleukin 2 signaling. *Nat Immunol*. 2005;6:1152-1159.

72. Kumanogoh A, Wang X, Lee I, et al. Increased T cell autoreactivity in the absence of

CD40-CD40 ligand interactions: a role of CD40 in regulatory T cell development. *J Immunol*. 2001;166:353-360.

73. Singh B, Read S, Asseman C, et al. Control of inflammation by regulatory T cells. *Immunol Rev*. 2001;182:190-200.

74. Kagami SI, Nakajima H, Suto A, et al. Stat5a regulates T helper cell differentiation by several distinct mechanisms. *Blood*. 2001;97:2358-2365.

75. Setoguchi R, Hori S, Takahashi T, et al. Homeostatic maintenance of natural $Foxp3^{+}CD25^{+}CD4^{+}$ regulatory T cells by interleukin-2 and induction of autoimmune disease by IL-2 neutralization. *J Exp Med*. 2005;201:723-735.

76. Furtado GC, Curotto de Lafaille MA, Kutchukhidze N, et al. Interleukin 2 signaling is required for $CD4^{+}$ regulatory T cell function. *J Exp Med*. 2002;196:851-857.

77. Thornton AM, Donovan EE, Piccirillo CA, et al. Cutting edge: IL-2 is critically required for the in vitro activation of $CD4^{+}CD25^{+}$ T cell suppressor function. *J Immunol*. 2004;172:6519-6523.

78. Barron L, Dooms H, Hoyer KK, et al. Cutting edge: mechanisms of IL-2-dependent maintenance of functional regulatory T cells. *J Immunol*. 2010;185:6426-6430.

79. Liu Y, Zhang P, Li J, et al. A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. *Nat Immunol*. 2008;9:632-640.

80. Ouyang W, Beckett O, Ma Q, et al. Transforming growth factor-β signaling curbs thymic negative selection promoting regulatory T cell development. *Immunity*. 2010;32:642-653.

81. Zheng Y, Josefowicz, S, Chaudry A, et al. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature*. 2010;463:808-812.

82. Ruan Q, Kameswaran V, Tone Y, et al. Development of $Foxp3^{+}$ regulatory T cells is driven by the c-Rel enhanceosome. *Immunity*. 2009;31:932-940.

83. Molinero LL, Yand J, Gajewski T, et al. CARMA1 controls an early checkpoint in the thymic development of $FoxP3^{+}$ regulatory T cells. *J Immunol*. 2009;182:6736-6743.

84. Floess S, Freyer J, Siewert C, et al. Epigenetic control of the foxp3 locus in regulatory T cells. *PloS Biol*. 2007;5:169-178.

85. Hsieh CS, Liang, Y, Tyznik AJ, et al. Recognition of peripheral self by naturally arising

$CD25^+CD4^+$ T cell receptors. *Immunity*. 2004;21:267-277.

86. Pacholczyk R, Ignatowicz H, Kraj P, et al. Origin and T cell receptor diversity of $Foxp3^+CD4^+CD25^+$ T cells. *Immunity*. 2006;25:249-259.

87. Hsieh CS, Zheng, Y, Liang Y, et al. An intersection between self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nat Immunol*. 2006;7:401-410.

88. Kuczma M, Podolsky R, Garge N, et al. Foxp3-deficient regulatory T cells do not revert into conventional effector $CD4^+$ T cells but constitute a unique cell subset. *J Immunol*. 2009;183:3731-3741.

89. Thorstenson KM, Khoruts A. Generation of anergic and potentially immunoregulatory CD25+CD4+ T cells in vivo after induction of peripheral tolerance with intravenous or oral antigen. *J Immunol*. 2001;167:188-195.

90. Apostolou I, Sarukhan A, Klein L, et al. Origin of regulatory T cells with known specificity for antigen. *Nat Immunol*. 2002:3:756-763.

91. Apostoulou I, von Boehmer H. In vivo instruction of suppressor commitment in naive T cells. *J Exp Med*. 2004;199:1401-1408.

92. Kretschmer K, Apostolou I, Hawiger D, et al. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol*. 2005;6:1219-1227.

93. Polansky JK, Kretschmer K, Freyer J, et al. DNA methylation controls Foxp3 gene expression. *Eur J Immunol* . 2008;38:1654-1663.

94. Josefowicz SZ, Wilson CB, Rudensky AY. Cutting edge: TCR stimulation is sufficient for induction of Foxp3 expression in the absence of DNA methyltransferase I. *J Immunol*. 2009;182:6648-6652.

95. Gottschalk RA, Corse E, Allison JP. TCR liganddensity and affinity determine peripheral induction of Foxp in vivo. *J Exp Med*. 2010;207:1701-1711.

96. Schallenberg S, Tsai PY, Riewaldt JR, et al. Identification of an immediate $Foxp3^-$ precursor to $Foxp3^+$ regulatory T cells in peripheral lymphoid organs on nonmanipulated mice. *J Exp Med*. 2010;207:1393-1407.

97. Chen WJ, Jin W, Hardegen N, et al. Conversion of peripheral $CD4^+CD25^-$ naive T cells to $CD4^+CD25^+$ regulatory T cells by TGF-β induction of transcription factor *Foxp3*. *J Exp Med*. 2003;198: 1875-1886.

98. Fantini MC, Becker C, Monteleone G, et al. Cutting edge: TGF-β induces a regulatory phenotype in $CD4^+CD25^-$ T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol*. 2004;172:5149-5153.

99. Horwitz DA, Zheng SG, Gray JD, et al. Regulatory T cells generated ex vivo as an approach for the therapy of autoimmune disease. *Sem Immunol*. 2004;16:135-143.

100. Davidson T, DiPaolo RJ, Andersson J, et al. Cutting edge: IL-2 is essential for TGF-β -mediated induction of $Foxp3^+$ regulatory cells. *J Immunol*. 2007;178:4027-4032.

101. Zhou L, Lopes JE, Chong MM, et al. TGF-β-induced Foxp3 inhibits Th17 cell differentiation by antagonizing RORγt function. *Nature*. 2008;453:236-240.

102. Caretto D, Katzman SD, Villarino AV, et al. Cutting edge: The Th1 response inhibits the generation of peripheral regulatory T cells. *J Immunol*. 2010;184:30-34.

103. Peng Y, Laouar Y, Li MO, et al. TGF-β regulates in vivo expansion of Foxp3-expressing $CD4^+CD25^+$ regulatory T cells responsible for protection against diabetes. *Proc Nat Acad Sci U S A*. 2004;101:4572-4577.

104. Mucida D, Kutchukhidze N, Erazo A, et al. Oral tolerance in the absence of naturally occurring Tregs. *J Clin Invest*. 2005;115:1923-1933.

105. Mucida D, Park Y, Kim G, et al. Reciprocal Th17 and regulatory T cell differentiation mediated by retinoic acid. *Science*. 2007;317:256-260.

106. Coombes JL, Siddiqui KRR, Arancibia-Carcamo CV, et al. A functionally specialized population of mucosal $CD103^+$ DCs induces $Foxp3^+$ regulatory T cell via a TGF-beta- and retinoic acid-dependent mechanism. *J Exp Med*. 2007;204:1757-1764.

107. Francisco, LM, Salinas VH, Brown KE, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med*. 2009;206:3015-3029.

108. Quintana FJ, Basso AS, Iglesias AH, et al. Control of Treg and Th17 differentiation by the arylhydrocarbon receptor. *Nature*. 2008;453:65-71.

109. Haribhai D, Lin W, Edwards B, et al. A central role for induced regulatory T cells in tolerance induction in experimental colitis. *J Immunol*. 2009;182:3461-3468.

110. Haribhai D, Williams JB, Jia S, et al. A requisite role for induced regulatory T cells in tolerance based on expanding antigen receptor diversity. *Immunity*. 2011;35:109-122.

111. Lathrop SK, Santacruz, NA, Pham D, et al. Antigen-specific peripheral shaping of the

natural regulatory T cell population. *J Exp Med*. 2008;205:3105-3117.

112. McCullagh P. The significance of immune suppression in normal self-tolerance. *Immunol Rev*. 1996;149:127-153.

113. Seddon B. Mason D. Peripheral autoantigen induces regulatory T cells that prevent autoimmunity. *J Exp Med*. 1999;189:877-881.

114. Taguchi O, Nishizuka Y. Self-tolerance and localized autoimmunity. Mouse models of autoimmune disease that suggest tissue-specific suppressor T cells are involved in self-tolerance. *J Exp Med*. 1987;165: 146-156.

115. Taguchi O, Kontani K, Ikeda H, et al. Tissue-specific suppressor T cells involved in self-tolerance are activated extrathymically by self-antigens. *Immunology.* 1994;82:365-369.

116. Smith H, Lou YH, Lacy P, et al. Tolerance mechanism in experimental ovarian and gastric autoimmune diseases. *J Immunol*. 1992;149: 2212-2218.

117. Tung KSK, Agersborg SS, Alard P, et al. Regulatory T cell, endogenous antigen and neonatal environment in the prevention and induction of autoimmune disease. *Immunol Rev*. 2001;182:135-148.

118. Samy ET, Parker LA, Sharp CP, et al. Continuous control of autoimmune disease by antigen-dependent polyclonal $CD4^{+}CD25^{+}$ regulatory T cells in the regional lymph node. *J Exp Med*. 2005;202:771-782.

119. Thornton AM, Korty PE, Tran DQ, et al. Expression of Helios, and Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced $Foxp3^{+}$ T regulatory cells. *J Immunol*. 2010;184:3433-3441.

120. Atarashi K, Tanoue T, Shima T, et al. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science*. 2011;331:337-341.

121. Geuking MB, Cahenzli J, Lawson MAE, et al. Intestinal colonization induces mutualistic regulatory T cell responses. *Immunity*. 2011;34: 794-806.

122. Vukmanovic-Stejic M, Zhang Y, Cook JE, et al. Human $CD4^{+}CD25^{hi}$ $Foxp3^{+}$ regulatory T cells are derived by rapid turnover of memory populations in vivo. *J Clin Invest*. 2006;116:2423-2433.

123. Gavin MA, Clarke SR, Negrou E, et al. Homeostasis and anergy of CD4+CD25+ suppressor T cells in vivo. *Nat Immunol*. 2002;3:33-41.

124. Zelenika D, Adams E, Humm S, et al. Regulatory T cells overexpress a subset of Th2 gene transcripts. *J Immunol*. 2002;168:1069-1079.

125. Hill JA, Feuerer M, Tash K, et al. Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell signature. *Immunity*. 2007;27:786-800.

126. Knoechel B, Lohr J, Zhu S, et al. Functional and molecular comparison of anergic and regulatory T lymphocytes. *J Immunol*. 2006;176:6473-6483.

127. Zheng Y, Josefowicz SZ, Kas A, et al. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature*. 2007;445:936-940.

128. Marson A, Kretchmer K, Frampton GM, et al. Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature*. 2007;445:931-935.

129. Gavin MA, Rasmussen JP, Fontenot JD, et al. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature*. 2007;445:771-775.

130. Lin W, Haribhai D, Relland LM, et al. Regulatory T cell development in the absence of functional Foxp3. *Nat Immunol*. 2007;8:359-368.

131. Lahl K, Mayer CT, Bopp T, et al. Nonfunctional regulatory T cells and defective control of Th2 cytokine production in natural scurfy mutant mice. *J Immunol*. 2009;5662-5672.

132. Ouyang W, Beckett O, Ma Q, et al. Foxo proteins cooperatively control the differentiation of $Foxp3^{+}$ regulatory T cells. *Nat Immunol*. 2010;11:618-627.

133. Kerdiles YM, Stone EL, Beisner DL, et al. Foxo transcription factors control regulatory T cell development and function. *Immunity*. 2010;33:890-894.

134. Takahashi T, Kuniyasu Y, Toda M, et al. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and autoimmune disease by breaking their anergic/suppressive state. *Int Immunol*. 1998;10: 1969-1980.

135. Read S, Mauze S, Asseman C, et al. $CD38+CD45RB^{low}$ T cells: a population of T cells with immune regulatory activities in vitro. *Eur J Immunol* . 1998:28:3434-3447.

136. Thornton AM, Shevach EM. Suppressor effector function of CD4+ CD25+ immunoregulatory T cells is antigen nonspecific. *J Immunol*. 2000;164:183-190.

137. Padiyan P, Xheng L, Ishihara S, et al. $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector $CD4^{+}$ T cells. *Nat Immunol*. 2007;8:1353-1362.

138. Tran DQ, Glass DD, Uzel G, et al. Analysis of adhesion molecules, target cells, and role of IL-2 in human FOXP3+ regulatory T cell suppressor function. *J Immunol*. 2009;182:2929-2938.

139. Palacios R, Moller G. T-cell growth factor abrogates concanavalin A-induced suppressor cell-function. *J Exp Med*. 1981;153:1360-1365.

140. Malek TR, Yu A, Vincek V, et al. I. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rβ-deficient mice. Implications for the nonredundant function of IL-2. *Immunity*. 2002;17:167-178.

141. Piccirillo CA, Shevach EM. Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. *J Immunol*. 2000;167: 1137-1140.

142. Ermann, J, Szanya V, Ford GS, et al. CD4+CD25+ T cells facilitate the induction of T cell anergy. *J Immunol*. 2001;167:4271-4275.

143. Hickman SP, Yang J, Thomas RM, et al. Defective activation of protein kinase C and Ras-ERK pathways limits IL-2 production and proliferation by CD4+CD25+ regulatory T cells. *J Immunol*. 2006;177: 2186-2194.

144. Tsang JY, Camara NOS, Eren E, et al. Altered proximal T cell receptor signaling in human CD4+CD25+ regulatory T cells. *J Leuk Biol*. 2006;80:145-151.

145. Su L, Creusot RJ, Gallo EM, et al. Murine $CD4^{+}CD25^{+}$ regulatory T cells fail to undergo chromatin remodeling across the proximal promoter region of the IL-2 gene. *J Immunol*. 2004;173:4994-5001.

146. Bensinger SJ, Walsh PT, Zhang J, et al. Distinct IL-2 receptor signaling pattern in $CD4^{+}CD25^{+}$ regulatory T cells. *J Immunol*. 2004;172: 5287-5296.

147. Walsh PT, Buckler JL, Zhang J, et al. PTEN inhibits IL-2 receptormediated expansion of $CD4^{+}CD25^{+}$ Tregs. *J Clin Invest*. 2006;116: 2521-2531.

148. Sauer S, Bruno L, Hertweck A, et al. T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. *Proc Nat Acad Sci U S A*. 2008;105:7797-7802.

149. Wu Y, Borde M, Heissmeyer V, et al. FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell*. 2006;126:375-387.

150. Ono M, Yaguchi H, Ohkura N, et al. Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. *Nature*. 2007;446:685-689.

151. Kitoh A, Ono M, Naoe Y, et al. Indispensable role of the Runx1-Cbfβ transcription complex for in vivo-suppressive function of FoxP3 + regulatory T cells *Immunity*. 2009;31:609-620.

152. Lee SM, Gao B, Fang D. Foxp3 maintains Treg unresponsiveness by selectively inhibiting the promoter DNA-binding activity of AP-1. *Blood*. 2008;111:3599-3606.

153. Li B, Samanta A, Song X, et al. FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proc Nat Acad Sci U S A* . 2007;104:4571-4576.

154. Pam F, Yu H, Dang EV, et al. Eos mediates Foxp3-dependent gene silencing in $CD4^+$ regulatory T cells. *Science*. 2009;325:1142-1146.

155. Beyer M, Thabet Y, Muller RU, et al. Repression of the genome organizer SATB1 in regulatory T cells is required for suppressive function and inhibition of cellular differentiation. *Nat Immunol*. 2011;12: 898-908.

156. Zhou X, Jeker LT, Fife BT, et al. Selective miRNA disruption in Treg cells leads to uncontrolled autoimmunity. *J Exp Med*. 2008;205: 1983-1991.

157. Liston A, Lu LF, O'Caroll D, et al. Dicer-dependent micoRNA pathway safeguards regulatory T cell function. *J Exp Med*. 2008;205:1993-2004.

158. Lu LF, Thai TH, Calado DP, et al. Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. *Immunity*. 2009;30:80-91.

159. Lu, LF, Boldin MP Chaudry A, et al. Function of miRNA-146a in controlling Treg cell-mediated regulation of Th1 responses. *Cell*. 2010;142:914-929.

160. Sojka DK, Hughson A, Sukiennicki TL, et al. Early kinetic window of target T cell susceptibility to $CD25^+$ regulatory T cell activity. *J Immunol*. 2005;175:7274-7280.

161. Sukiennicki TL, Fowell DJ. Distinct molecular program imposed on $CD4^+$ T cell targets by $CD4^+CD25^+$ regulatory T cells. *J Immunol*. 2006;177:6952-6961.

162. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4+CD25+ regulatory T cells is mediated by cell surfacebound transforming growth factor β. *J Exp Med*. 2001;194:629-644.

163. Piccirillo CA, Letterio JJ, Thornton AM, et al. CD4+CD25+ regulatory T cells can mediate suppressor function in the absence of transforming growth factor β1 production and responsiveness. *J Exp Med*. 2002;196:1-10.

164. Marie JC, Letterio JJ, Gavin M, et al. TGF-β1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *J Exp Med*. 2005;201:1061-1067.

165. Marie JC, Liggitt D, Rudensky AY. Cellular mechanisms of fatal earlyonset autoimmunity in mice with the T cell-specifc targeting of transforming growth factor-β receptor. *Immunity*. 2006;25:441-454.

166. Li MO, Wan YY, Flavell RA. T cell-produced transforming growth factor-β1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. *Immunity*. 2007;26:579-591.

167. Gutcher I, Donkor MK, Ma Q, et al. Autocrine transforming growth factor-β1 promotes in vivo Th17 differentiation. *Immunity*. 2011;34: 396-408.

168. Tran DQ, Andersson J, Wang R, et al. GARP(LRRC32) is essential for the surface expression of latent TGF-β on platelets and activated FOXP3^{+} regulatory T cells. *Proc Natl Acad Sci U S A*. 2009;106: 13445-13450.

169. Wang R, Kozhaya L, Mercer F, et al. Expression of GARP selectively identifies activated human Foxp3^{+} regulatory T cells. *Proc Natl Acad Sci U S A* . 2009;106:13439-13444.

170. Stockis J, Colau D, Coulie PG, et al. Membrane protein GARP is a receptor for latent TGF-beta on the surface of activated Treg. *Eur J Immunol* . 2009; 12:3315-3322.

171. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing CD25^{+}CD4^{+} T cells: a common basis between tumor immunity and autoimmunity. *J Immunol*. 1999;163:5211-5218.

172. Smyth MJ, Teng MWL, Swann J, et al. CD4^{+}CD25^{+} T regulatory cells suppress NK cell-mediated immunotherapy of cancer. *J Immunol*. 2006;176:1582-1587.

173. Ghiringhelli, F, Menard C, Terme M, et al. CD4^{+}CD25^{+} regulatory T cells inhibit natural killer cell functions in a transforming growthfactor-β-dependent manner. *J Exp Med*. 2005;202:1075-1085.

174. Barao I, Hanash AM, Hallett W, et al. Suppression of natural killer cellmediated bone marrow cell rejection by CD4^{+}CD25^{+} regulatory T cells. *Proc Natl Acad Sci U S A* . 2006;103:5460-5465.

175. Collison LW, Workman CJ, Kuo TT, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature*. 2007;450:566-569.

176. Collison LW, Chaturvedi V, Henderson AL, et al. IL-35-mediated induction of a potent regulatory T cell population. *Nat Immunol*. 2010;11:1093-1101.

177. Garin MI, Chu CC, Golshayan D, et al. Galectin-1: a key effector of regulation mediated by $CD4^+CD25^+$ T cells. *Blood*. 2007;109:2058-2065.

178. Kubach J, Lutter P, Bopp T, et al. Human CD4+CD25+ regulatory T cells: proteome analysis identifies galectin-10 as a novel marker essential for their anergy and suppressive function. *Blood*. 2007;110:1550-1558.

179. Grossman, WJ, Verbsky JW, Barchet W, et al. Human regulatory T cells can use the perforin pathway to cause autologous target cell death. *Immunity*. 2004;21:589-601.

180. Gondek DC, Lu LF, Quezada SA, et al. Cutting edge: contact-mediated suppression by $CD4^+CD25^+$ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol*. 2005;174:1783-1786.

181. Zhao DM, Thornton AM, DiPaolo RJ, et al. Activated $CD4^+CD25^+$ regulatory T cells selectively kill B lymphocytes. *Blood*. 2006;107:3925-3932.

182. Cederbom L, Hall H, Ivars, F. CD4+CD25+ regulatory T cells downregulate co-stimulatory molecules on antigen-presenting cells. *Eur J Immunol* . 2000;30:1538-1543.

183. Serra P, Amrani A, Yamanouchi J, et al. CD40 ligation releases immature dendritic cells from the control of regulatory CD4+CD25+ T cells. *Immunity*. 2003;19:877-889.

184. Misra N, Bayry J, Lacroix-Desmazes S, et al. Cutting edge: human $CD4^+CD25^+$ T cells restrain the maturation and antigen-presenting function of dendritic cells. *J Immunol*. 2004;172:4676-4680.

185. Oldenhove G, de Heusch M, Urbain-Vansanten G, et al. $CD4^+CD25^+$ regulatory T cells control T helper 1 responses to foreign antigens induced by mature dendritic cells in vivo. *J Exp Med*. 2003;198:259-266.

186. Veldhoen M, Moncrieffe H, Hocking RJ, et al. Modulation of dendritic cell function by naive and regulatory $CD4^+$ T cells. *J Immunol*. 2006;176:6202-6210.

187. Tang Q, Boden EK, Henriksen KJ, et al. Distinct roles of CTLA-4 and TGF-β in CD4+CD25+ regulatory cell function. *Eur J Immunol* . 2004;34:2996-3005.

188. Paust S, Cantor H. Regulatory T cells and autoimmune disease. *Immunol Rev*. 2005;204:195-207.

189. May KF, Chang X, Zhang HM, et al. B7-deficient autoreactive T cells are highly susceptible to suppression by $CD4^+CD25^+$ regulatory T cells. *J Immunol*. 2007;178:1542-1552.

190. Wing K, Onishi Y, Prieto-Martin P, et al. CTLA-4 control over $Foxp3^+$ regulatory cell function. *Science*. 2008;322:271-275.

191. Qureshi OS, Zheng Y, Nakamura K, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell extrinsic function of CTLA-4. *Science*. 2011;332:600-603.

192. Grohmann U, Fallarino F, Puccetti P. Tolerance DCs and tryptophan: much ado about IDO. *Trends Immunol*. 2003;24:242-248.

193. Cobbold SP, Adams E, Farquhar CA, et al. Infectious tolerance via consumption of essential amino acids and mTOR signaling. *Proc Natl Acad Sci U S A* . 2009;106:12055-12060.

194. Liang BT, Workman C, Lee J, et al. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *J Immunol*. 2008;180:5916-5926.

195. Sarris M, Anderson KG, Randow F, et al. Neuropilin-1 expression on regulatory T cells enhances their interactions with dendritic cells during antigen recognition. *Immunity*. 2008;28:402-413.

196. Borsellino G, Kleinewietfeld M, Di Mitri, D, et al. Expression of ecto nucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood*. 2007;110:1225-1232.

197. Deaglio S, Dwyer KM, Gao W, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med*. 2007;204:1257-1265.

198. Bopp T, Becker C, Klein M, et al. Cyclic adenosine monophosphate is a key component of regulatory T cell mediated suppression. *J Exp Med*. 2007;204:1303-1310.

199. Hubert S, Rissiek B, Klages K, et al. Extracellular NAD^+ shapes Foxp3 regulatory T cell compartment through ART2-P2X7 pathway. *J Exp Med*. 2010;207:2561-2568.

200. Klein L, Khazaie K, von Boehmer H. In vivo dynamics of antigen-specific regulatory T cells not predicted from behavior in vitro. *Proc Natl Acad Sci U S A* . 2003;100:8886-8891.

201. Walker LS, Chodos A, Eggena M, et al. Antigen-dependent proliferation of

CD4+CD25+ regulatory T cells in vivo. *J Exp Med*. 2003;198:249-258.

202. Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. *Nat Immunol*. 2005;6:353-360.

203. McGeachy MJ, Stephens LA, Anderton SM. Natural recovery and protection from autoimmune encephalomyelitis: contribution of $CD4^+CD25^+$ regulatory T cells within the central nervous system. *J Immunol*. 2005;175:3025-3032.

204. Shevach EM, Stephens GL. The GITR-GITRL interaction: costimulation or contrasuppression of regulatory activity. *Nat Rev Immunol*. 2006;6:613-618.

205. Rubtsov YP, Rasmussen JP, Chi EY, et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity*. 2008;28:546-558.

206. Mamura M, Lee W, Sullivan TJ, et al. CD28 disruption exacerbates inflammation in TGF-$\beta 1^{-/-}$ mice: in vivo suppression by $CD4^+CD25^+$regulatory T cells independent of autocrine TGF-β1. *Blood*. 2002;103:4594-4601.

207. Fahlen L, Read S, Gorelik L, et al. T cells that cannot respond to TGF-β1 escape control by $CD4^+CD25^+$ regulatory T cells. *J Exp Med.* 2005;201:737-746.

208. Kullberg MC, Hay V, Cheever AW, et al. TGF-β1 production by CD4+CD25+ regulatory T cells is not essential for suppression of intestinal inflammation. *Eur J Immunol*. 2005;35:2886-2895.

209. Cao X, Cai SF, Fehniger TA, et al. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity*. 2007;27:635-646.

210. Boissonnas A, Scholer-Dahirel A, Simon-Blancal V, et al. $Foxp3^+$ T cells induce perforin-dependent dendritic cell death in tumor-draining lymph nodes. *Immunity*. 2010;32:266-278.

211. Bystry RS, Aluvihare V, Welch KA, et al. B cells and professional APC recruit regulatory T cells. *Nat Immunol*. 2001;2:1126-1132.

212. Lim HW, Hillsamer P, Banham AH, et al. Cutting edge: direct suppression of B cells by $CD4^+CD25^+$ regulatory T cells. *J Immunol*. 2005;175:4180-4183.

213. Lim HW, Hillsamer P, Kim CH. Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th and GC-Th cell driven B cell responses. *J Clin Invest*. 2002;114:1640-1649.

214. Chung Y, Tanaka S, Chu F, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med*. 2011;17:983-988.

215. Linterman MA, Pierson W, Lee SK, et al. Foxp3^{+} follicular regulatory T cells control the germinal center response. *Nat Med*. 2011;17:975-982.

216. Tang Q, Adams JY, Tooley AJ, et al. Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nat Immunol*. 2006;7:83-92.

217. Tadokoro CE, Shakhar G, Shen S, et al. Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo. *J Exp Med*. 2006;203:505-511.

218. Onishi Y, Fehervari Z, Yamaguchi T, et al. Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. *Proc Natl Acad Sci U S A* . 2008;105:10113-10118.

219. Davidson TS, Shevach EM. Polyclonal Treg cells modulate T effector cell trafficking. Eur *J Immunol*. 2011;41:2862-2870.

220. Billiard F, Litvinova E, Sadoun D, et al. Regulatory and effector T cell activation levels are prime determinants of in vivo immune regulation. *J Immunol*. 2006;177:2167-2174.

221. Hanig J, Lutz MB. Suppression of mature dendritic cell function by regulatory T cells in vivo is abrogated by CD40 licensing. *J Immunol*. 2008;180:1405-1413.

222. Shevach EM. Biological functions of regulatory T cells. *Adv Immunol*. 2011;112:137-176.

223. Morlacchi S, Dal Secco V, Soldani C, et al. Regulatory T cells target chemokine secretion by dendritic cells independently of their capacity to regulate T cell proliferation. *J Immunol*. 2011;186:6807-6814.

224. Chen Q, Kim YC, Laurence A, et al. IL-2 controls the stability of Foxp3 expression in TGF-β-induced Foxp3+ T cells in vivo. *J Immunol*. 2011;186:6329-337.

225. Read S, Greenwald R, Izcue A, et al. Blockade of CTLA-4 on CD4^{+}CD25^{+} regulatory T cells abrogates their function in vivo. *J Immunol*. 2006;177:4376-4383.

226. Schmidt EM, Wang, CJ, Ryan GA, et al. CTLA-4 controls regulatory T cell peripheral homeostasis and is required for suppression of pancreatic islet autoimmunity. *J Immunol*. 2009;182:274-282.

227. Stephens GL, McHugh RS, Whitters MJ, et al. Engagement of glucocorticoid-

induced TNFR family related receptor on effector T cells by its ligand mediates resistance to suppression by $CD4^+CD25^+$ T cells. *J Immunol*. 2004;173:5008-5020.

228. Kohm AP, Williams JS, Miller SD. Cutting edge: ligation of the glucocorticoid-induced TNF receptor enhances autoreactive $CD4^+$ T cell activation and experimental autoimmune encephalomyelitis. *J Immunol*. 2004;172:4686-4690.

229. Valzasina B, Guiducci C, Dislich H, et al. Triggering of OX40 (CD134) on $CD4^+CD25^+$ T cells blocks their inhibitory activity: a novel regulatory role for OX40 and its comparison with GITR. *Blood*. 2005;105: 2845-2851.

230. Piconese S, Valzasina B. Colombo MP Ox40 triggering blocks suppression by regulatory T cells and facilitates tumor rejection. *J Exp Med*. 2008;205:825-839.

231. Valencia X, Stephens G, Goldsbach-Mansky R, et al. TNF downmodulates the function of human $CD4^+CD25^{hi}$ T-regulatory cells. *Blood*. 2006;108:253-261.

232. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+ CD25+ T cell-mediated suppression by dendritic cells. *Science*. 2003; 299:1033-1036.

233. Schreiber TH, Wolf D, Tsai MS, et al. Therapeutic Treg expansion in mice by TNFRSF25 prevents allergic inflammation. *J Clin Invest*. 2010;120:3629-3640.

234. Wohlfert EA, Gorelik L, Mittler R, et al. Cutting edge: deficiency in the E3 ubiquitin ligase cbl-b results in a multifunctional defect in T cells TGF-β sensitivity in vitro and in vivo. *J Immunol*. 2006;176:1316-1320.

235. Koch MA, Tucker-Heard G, Perdue NR, et al. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol*. 2009;10:595-602.

236. Zheng Y, Chaudry A, Kas A, et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control Th2 responses. *Nature*. 2009;458:351-355.

237. Chaudry A, Rudra D, Treuting P, et al. CD4+ regulatory T cells control th17 responses in a Stat3-dependent manner. *Science*. 2009;986-991.

238. Oldenhove G, Bouladoux N, Wohlfert EA. Decrease of $Foxp3^+$ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity*. 2009;31:772-786.

239. Xu L, Kitani A, Fuss I, et al. Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells are self-induced to become Th17 cells in the absence of exogenous TGF-β. *J Immunol*. 2007:178:6725-6729.

240. Yang XO, Nurieva R, Martinez GJ, et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity*. 2008;29:44-56.

241. Osorio F, LeibundGut-Landmann S, Lochner M, et al. DC activated via dectin-1 convert Treg into IL-17 producers. *Eur J Immunol* . 2008;38:3724-3281.

242. Laurence A, Tato, CM, Davidson TS, et al. Interleukin-signaling via Stat5 constrains T-helper 17 generation. *Immunity*. 2007;26:371-381.

243. Bruno L, Mazzarella L, Hoogenkamp M, et al. Runx proteins regulate Foxp3 expression. *J Exp Med*. 2009;206:2329-2337.

244. Tsuji M, Komatsu N, Kawamoto S, et al. Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. *Science*. 2009;323:1488-1492.

245. Wei G, Wei L, Zhu J, et al. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating $CD4^+$ T cells. *Immunity*. 2009;30:155-167.

246. Wohlfert EA, Grainger JR, Bouladoux N, et al. GATA3 controls $Foxp3^+$ regulatory T cell fate during inflammation in mice. *J Clin Invest*. 2011;121:4503-4515.

247. Wang Y, Su MA, Wan YY. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. *Immunity*. 2011; 35:1-12.

248. Zhou X, Bailey-Bucktrout SI, Jeker LT, et al. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol*. 2009;10:1000-1007.

249. Rubtsov YP, Niec RE, Josefowicz S, et al. Stability of the regulatory T cell lineage in vivo. *Science*. 2010;329:1667-1671.

250. Komatsue N, Mariotti-Ferandiz MA, Wang Y, et al. Heterogeneity of natural $Foxp3^+$ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proc Natl Acad Sci U S A* . 2009;106:1903-1908.

251. Hori S. Regulatory T cell plasticity: beyond the controversies. *Trends Immunol*. 2011;32:295-300.

252. Weiner HL, Friedman A, Miller A, et al. Oral tolerance-immunological mechanisms and treatment of animal and human organ-specific auto immune-diseases by oral-administration of autoantigens. *Ann Rev Immunol*. 1994;12:809-837.

253. Chen Y, Kuchroo VK, Inobe JI, et al. Regulatory T cell clones induced by oral

tolerance: suppression of autoimmune encephalomyelitis. *Science*. 1994:265:1237-1240.

254. Weiner HL. The mucosal milieu creates tolerogenic dendritic cells and T R 1 and T_H3 regulatory cells. *Nat Immunol*. 2001;2:671-672.

255. Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat Immunol*. 2001;2:725-731.

256. Bacchetta R, Bigler M, Touraine JL, et al. High levels of interleukin 10 production in vivo are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. *J Exp Med*. 1994;179:493-502.

257. Roncarolo MG, Bacchetta R, Bordignon C, et al. Type 1 regulatory cells. *Immunol Rev*. 2001;182:68-79.

258. Groux H, O'Garra A, Bigler M, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature*. 1997;389:737-742.

259. Levings MK, Sangregorio R, Galbiati F, et al. INF-α and IL-10 induce the differentiation of human type 1 T regulatory cells. *J Immunol*. 2001;166:5530-5539.

260. Viera PL, Christensen JR, Minaee S, et al. IL-10 secreting T cells do not express Foxp3 but have comparable regulatory functions to naturally occurring $CD4^+CD25^+$ regulatory T cells. *J Immunol*. 2004;172: 5986-5993.

261. Cottrez F, Hurst SD, Coffman RL, et al. T regulatory cells 1 inhibit a TH2-specific response in vivo. *J Immunol*. 2000;165:4848-4853.

262. Barrat FJ, Cua DJ, Boonstra A, et al. In vitro generation of interleukin 10-producing regulatory CD4+ T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med*. 2002;195:603-616.

263. Jonuleit H, Schmitt E, Schuler G, et al. Induction of interleukin 10-producing, nonproliferating CD4+ T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med*. 2000;192:1213-1222.

264. Maynard CL, Harrington LE, Janowski KM, et al. Regulatory T cells expressing interleukin 10 develop from $Foxp3^+$ and $Foxp3^-$ precursor cells in the absence of interleukin 10. *Nat Immunol*. 2007;8:931-941.

265. Fitzgerald DC, Zhang GX, El-Behi, M, et al. Suppression of autoimmune inflammation of the central nervous system by interleukin 10 secreted by interleukin 27-stimulated T cells. *Nat Immunol*. 2007;8: 1372-1380.

266. Awasthi A, Carrier Y, Peron JPS, et al. A dominant function for interleukin 27 in generating interleukin10-producing anti-inflammatory cells. *Nat Immunol*. 2007;8:1380-1389.

267. Pot C, Jin H, Awasthi A, et al. Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. *J Immunol*. 2009;183:797-801.

268. Murugaiyan G, Mittal A, Lopez-Diego R, et al. IL-27 is a key regulator of IL-10 and IL-17 production by human $CD4^+$ T cells. *J Immunol*. 2009;183:2435-2443.

269. Liu Z, Tugulea S, Cortesini R, et al. Specific suppression of T helper alloreactivity by all-MHC class I-restricted CD8+CD28- T cells. *Int Immunol*. 1998;10:775-783.

270. Li J, Liu Z, Jiang S, et al. T suppressor lymphocytes inhibit NF-kBmediated transcription of CD86 gene in APC. *J Immunol*. 1999;163: 6386-6392.

271. Chang CC, Ciubotariu R, Manavalan JS, et al. Tolerization of dendritic cells by Ts cells: the crucial role of inhibitory receptors ILT3 and ILT4. *Nat Immunol*. 2002;3:237-243.

272. Vlad G, Cortesini R, Suciu-Foca N. License to heal: bidirectional interaction of antigen-specific regulatory T cells and tolerogenic APC. *J Immunol*. 2005;174:5907-5914.

273. Gilliet M, Liu YJ. Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. *J Exp Med*. 2002;195:695-704.

274. Dhodapkar MV, Steinman RM, Krasovsky J, et al. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med*. 2001;193:233-238.

275. Wei S, Kryczek I, Zou L, et al. Plasmacytoid dendritic cells induce CD8+ regulatory T cells in human ovarian carcinoma. *Cancer Res*. 2005;65:5020-5026.

276. Rifa'I M, Kawamoto Y, Nakashima I, et al. Essential roles of CD8+ CD122+ regulatory T cells in the maintenance of T cell homeostasis. *J Exp Med*. 2004;200:1123-1134.

277. Endharti AT, Rifa'I, Shi Z, et al. Cutting edge: CD8+CD122+ regulatory T cells produce IL-10 to suppress IFN-γ production and proliferation of CD8+ T cells. *J Immunol*. 2005;175:7093-7097.

278. Jiang H, Chess L. The specific regulation of immune responses by CD8+ T cells restricted by the MHC class IB molecule, Qa-1. *Ann Rev Immunol*. 2000;18:185-216.

279. Tang X, Maricic I, Purohit N, et al. Regulation of immunity by a novel population of Qa-1-restricted CD8αα^{+} TCRαβ^{+} T cells. *J Immunol*. 2006;177:7645-7655.

280. Li J, Goldstein I, Glickman-Nir, et al. Induction of TCR Vbetaspecific CD8^{+} CTLs by TCR Vbeta-derived peptides bound to HLA-E. *J Immunol*. 2001;167:3800-3808.

281. Ford MS, Zhang ZX, Chen W, et al. Double-negative T regulatory cells can develop outside the thymus and do not mature from CD8^{+} T cell precursors. *J Immunol.* 2006;177:2803-2809.

282. Fischer K, Voekl S, Heymann J, et al. Isolation and characterization of human antigen-specific TCR αβ^{+} CD4^{-}CD8^{-}double-negative regulatory T cells. *Blood*. 2005;105:2826-2835.

283. Zhang ZX, Yang L, Young KJ, et al. Identification of a previously unknown antigen-specific regulatory T cells and its mechanism of suppression. *Nat Med*. 2000;6:782-789.

284. McIver Z, Serio B, Dunbar A, et al. Double-negative regulatory T cells induce allotolerance when expanded after allogeneic haematopoietic stem cell transplantation. *Brit J Haem*. 2008;141:170-178.

285. Dieckmann D, Bruett CH, Ploettner H, et al. Human CD4+CD25+ regulatory, contact-dependent T cells induce interleukin 10-producing, contact-independent type1-like regulatory T cells. *J Exp Med*. 2002:196:247-253.

286. Jonuleit H, Schmitt E, Kakirman H, et al. Infectious tolerance: Human CD25+ regulatory T cells convey suppressor activity to conventional CD4+ T helper T cells. *J Exp Med*. 2002;196:255-260.

287. Qiao M, Thornton AM, Shevach EM. CD4+CD25+ T cells render naive CD4+CD25- T cells anergic and suppressive. *Immunology*. 2007;120: 447-455.

288. Andersson J, Tran DQ, Pesu, M, et al. CD4+FoxP3+ regulatory T cells confer infectious tolerance in a TGF-β-dependent manner. *J Exp Med*. 2008;205:1975-1981.

289. Kendal AR, Chen Y, Regateiro FS, et al. Sustained suppression by Foxp3^{+} regulatory T cells is vital for infectious transplantation tolerance. *J Exp Med*. 2011;208:2043-2053.

290. Powrie F, Leach MW, Mauze S, et al. Inhibition of TH1 responses prevents inflammatory bowel-disease in SCID mice reconstituted with CD45RB^{high} T cells. *Immunity*. 1994;1:553-562.

291. Asseman C, Read S, Fowrie F. Colitogenic Th1 cells are present in the antigen-experienced T cell pool in normal mice: control by $CD4^+$ regulatory T cells and IL-10. *J Immunol*. 2003;171:971-978.

292. Suri-Payer E, Cantor H. Differential cytokine requirements for regulation of autoimmune gastritis and colitis by CD4+CD25+ T cells. *J Autoimmunity*. 2001;16:115-123.

293. Malmstrom V, Shipton D, Singh B, et al. CD134L expression on dendritic cells in the mesenteric lymph nodes drives colitis in T cell restored SCID mice. *J Immunol*. 2001;166:6972-6981.

294. Mottet C, Uhlig HH, Powrie F. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. *J Immunol*. 2003;170:3939-3943.

295. Delovitch TL, Singh B. The non-obese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. *Immunity*. 1997:7:727-738.

296. Chatenoud L, Salomon B, Bluestone JA. Suppressor T cells-they're back and critical for regulation of autoimmunity. *Immunol Rev*. 2001;182:149-163.

297. Green EA, Choi Y, Flavell RA. Pancreatic lymph node-derived CD4+ CD25+ treg cells: highly potent regulators of diabetes that require TRANCE-RANK signals. *Immunity*. 2002;16:183-191.

298. Tang Q, Henriksen KJ, Bi M, et al. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med*. 2004; 199:1455-1465.

299. Masteller EL, Warner MR, Tang Q, et al. Expansion of functional endogenous antigen-specific $CD4^+CD25^+$ regulatory T cells from nonobese diabetic mice. *J Immunol*. 2005;175:3053-3059.

300. Sarween N, Chodos A, Raykundalia C, et al. CD4+CD25+ cells controlling a pathogenic CD4 response inhibit cytokine differentiation, CXCR-3 expression, and tissue invasion. *J Immunol*. 2004;173:2942-2951.

301. Chen Z, Herman AE, Matos M, et al. Where $CD4^+CD25^+$ T reg cells impinge on autoimmune diabetes. *J Exp Med*. 2005;202:1387-1397.

302. Tang Q, Adams JY, Penaranda C, et al. Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity*. 2010;28:687-697.

303. Grinberg-Bleyer Y, Baeyens A, You S, et al. IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells. *J Exp Med*.

2010;207:1871-1878.

304. Webster KE, Walters S, Kohler RE, et al. In vivo expansion of T reg cells with IL-2 mAb complexes: induction of resistance to EAE and longterm acceptance of islet allografts without immunosuppression. *J Exp Med*. 2009:206:751-760.

305. Kohm AP, Carpentier PA, Anger HA, et al. Cutting edge: $CD4^+CD25^+$ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J Immunol*. 2002;169:4712-4716.

306. McGeachy MJ, Stephens LA, Anderton SM. Natural recovery and protection from autoimmune encephalomyelitis: contribution of $CD4^+$ $CD25^+$ regulatory cells within the central nervous system. *J Immunol*. 2005;175:3025-3032.

307. Korn T, Reddy J, Gao, W, et al. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat Med*. 2007;13:423-437.

308. Asefa A, Gumy A, Launois P, et al. The early IL-4 response to *Leishmania major* and the resulting Th2 cell maturation steering progressive disease in BALB/c mice are subject to the control of regulatory $CD4^+CD25^+$ T cells. *J Immunol*. 2002;169:3232-3241.

309. Xu D, Lin H, Koami-Koma M, et al. $CD4^+CD25^+$ regulatory T cells suppress differentiation and functions of Th1 and Th2 cells, Leishmania infection, and colitis in mice. *J Immunol*. 2003;170:394-399.

310. Stassen M, Jonuleit H, Muller C, et al. Differential regulatory capacity of $CD25^+$ T regulatory cells and preactivated $CD25^+$ T regulatory cells on development, functional activation, and proliferation of Th2 cells. *J Immunol*. 2004;173:267-274.

311. Lewkowich IP, Herman NS, Schliefer KW, et al. $CD4^+CD25^+$T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J Exp Med*. 2005;202:1549-1561.

312. Jaffar S, Sivakuru T, Roberts K. $CD4^+CD25^+$ T cells regulate airway eosinophilic inflammation by modulating Th2 cell phenotype. *J Immunol*. 2004;172:3842-3849.

313. Wilson MS, Taylor MD, Balic A, et al. Suppression of allergic airway inflammation by helminth-induced regulatory T cells. *J Exp Med*. 2005; 202:1199-1212.

314. Stockinger B, Barthlott T, Kassiotis G. T cell regulation: a special job or everyone's responsibility. *Nat Immunol*. 2001;2:757-759.

315. Bourgeoios C, Stockinger B. $CD25^{+}CD4^{+}$ regulatory T cells and memory T cells prevent lymphopenia-induced proliferation of naive T cells in transient state of lymphopenia. *J Immunol*. 2006;177:4558-4566.

316. McHugh RS, Shevach EM. Cutting edge: depletion of CD4+CD25+ regulatory T cells is necessary, but not sufficient, for induction of organ-specific autoimmune diseases. *J Immunol*. 2002;168:5979-5983.

317. Annacker O, Pimenta-Araujo R, Burlen-Defranoux O, et al. $CD25^{+}$ $CD4^{+}$ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *J Immunol*. 2001;166:3008-3018.

318. Shin S, Ding Y, Tadokoro, CD, et al. Control of homeostatic proliferation by regulatory T cells. *J Clin Invest*. 2005;115:3517-3526.

319. Waldmann H, Cobbold S. Regulating the immune response to transplants. A role for $CD4^{+}$ regulatory cells? *Immunity*. 2001;14:399-406.

320. Hara M, Kingsley CI, Niimi M, et al. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J Immunol*. 2001;166:3789-3796.

321. Graca L, Thompson S, Lin CY, et al. Both CD4+CD25+ and CD4+ CD25- regulatory cells mediate dominant transplantation tolerance. *J Immunol*. 2002;168:5558-5567.

322. Quezada SA, Bennett K, Blazar BR, et al. Analysis of the underlying cellular mechanisms of anti-CD154-induced graft tolerance: the interplay of clonal anergy and immune regulation. *J Immunol*. 2005;175:771-779.

323. Lu LF, Lind EF, Gondek DC, et al. Mast cells are essential intermediaries in regulatory T cell tolerance. *Nature*. 2006; 442:997-1002.

324. Cobbold SP, Castejon R, Adams E, et al. Induction of *foxp3*+ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants. *J Immunol*. 2004;172;6003-6010.

325. Taylor PA, Lees CJ, Blazer BR. The infection of ex vivo activated and expanded CD4+CD25+ immune regulatory cells inhibits graft-vs-host disease (GVHD) lethality. *Blood*. 2002;99:4601-4609.

326. Cohen JL, Trenado A. Vasey D, et al. CD4+CD25+ immunoregulatory T cells: new therapeutics for graft-versus-host disease. *J Exp Med*. 2002;196:401-406.

327. Ermann J, Hoffman P, Edinger M, et al. Only the $CD62L^{+}$ subpopulation of CD4+CD25+ regulatory T cells protects from lethal GVHD. *Blood*. 2005;105:2220-2226.

328. Hoffman P, Ermann J, Edinger M, et al. Donor-type $CD4^+CD25^+$ regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med*. 2002;196:389-399.

329. Edinger M, Hoffman P, Ermann J, et al. $CD4^+CD25^+$ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med*. 2003;9:1144-1150.

330. Golovina, TN, Mikheeva T, Suboski MM, et al. CD28 costimulation is essential for human T regulatory expansion and function. *J Immunol*. 2008;181:2855-2868.

331. Nadig SN, Wieckiewicz J, Wu DC, et al. In vivo prevention of transplant arteriosclerosis by ex vivo-expanded human regulatory T cells. *Nat Med*. 2010;16:809-813.

332. Joffre O, Gorsse N, Romagnoli P, et al. Induction of antigen-specific tolerance to bone marrow allografts with $CD4^+CD25^+$ T lymphocytes. *Blood*. 2004;103:4216-4221.

333. Hanash AM, Levy RB. Donor $CD4^+CD25^+$ T cells promote engraftment and tolerance following MHC-mismatched hematopoietic cell transplantation. *Blood*. 2005;105:1828-1836.

334. Brunstein CG, Miller JS, Cao Q, et al. Infusion of ex vivo expanded T regulatory cells in aduslts transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood*. 2011;20:1061-1070.

335. Di Ianni M, Falzetti F, Carotti A, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood*. 2011;117:3921-3928.

336. North, RJ, Bursuker I. Generation and decay of the immune response to a progressive fibrosarcoma. I. Ly-1+2- suppressor T cells down-regulate the generation of Ly-1-2+ effector T cells. *J Exp Med*. 1984;159: 1295-1311.

337. Awwad M, North RJ. Immunologically mediated regression of a murine lymphoma after treatment with anti-L3T4 antibody. A consequence of removing L3T4+ suppressor T cells from a host generating predominantly Lyt-2+ T cell-mediated immunity. *J Exp Med*. 1988;168: 2193-2206.

338. Onizuka S, Tawara I, Shimizu J, et al. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor) monoclonal antibody. *Cancer Res*. 1999;59:3128-3133.

339. Sutmuller RPM, van Duivenvoorde LM, van Elsas A, et al. Synergism of cytotoxic T

lymphocyte-associated antigen 4 blockade and depletion of CD25+ regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J Exp Med*. 2001;194:823-832.

340. Antony PA, Piccirillo CA, Akpinarli A, et al. CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. *J Immunol*. 2005;174:2591-2601.

341. Turk MJ, Guevara-Patino JA, Rizzuto GA, et al. Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells. *J Exp Med*. 2004;200:771-782.

342. Woo, EY, Yeh H, Chu CS, et al. Cutting edge: regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. *J Immunol*. 2002;168:4272-4276.

343. Curiel TJ, Cooukos G, Zou LH, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*. 2004;10:942-949.

344. Kryczek I, Wei S, Zou LH, et al. Cutting edge: induction of B7-H4 on APCs through IL-10: novel suppressive mode for regulatory T cells. *J Immunol*. 2006;177:40-44.

345. Wang HY, Lee DA, Peng GY, et al. Tumor-specific human $CD4^+$ regulatory T cells and their ligands: implications for immunotherapy. *Immunity*. 2004;20:107-118.

346. Nishikawa H, Kato T, Tanida K, et al. $CD4^+CD25^+$ T cells responding to serologically defined autoantigens suppress antitumor immune responses. *Proc Natl Acad Sci U S A*. 2003;100:10902-10906.

347. Phan GQ, Yang JC, Sherry RM, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci U S A* . 2003;100:8372-8377.

348. Dannull J, Su Z, Rizzieri D, Yang BK, et al. Enhancement of vaccine -mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J Clin Invest*. 2005;115:3623-3633.

349. Attia P, Maker AV, Haworth LR, et al. Inability of a fusion protein of IL-2 and diptheria toxin (Denileukin Diftitox, DAB(389)IL-2, ONTAK) to eliminate regulatory T lymphocytes in patients with melanoma. *J Immunotherapy*. 2005;28:582-592.

350. de Vries IJ, Castelli C, Huygens C, et al. Frequency of circulating Tregs with demethylated FOXP3 intron 1 in melanoma patients receiving tumor vaccines and potentially Treg-depleting agents. *Clin Cancer Res.* 2011;17:841-848.

351. Erdman SE, Sohn JJ, Rao VP, et al. $CD4^{+}CD25^{+}$ regulatory lymphocytes induce regression of intestinal tumors in $APC^{Min/+}$ mice. *Cancer Res*. 2005;65:3998-4004.

352. Hori S, Carvalho TL, Demengeot J. CD25+CD4+ regulatory T cells suppress CD4+ T cell-mediated pulmonary hyperinflammation driven by Pneumocystis carinii in immunodeficient mice. *Eur J Immunol* . 2002;32:1282-1291.

353. Maloy KJ, Salaun L, Cahill R, et al. CD4+CD25+T-R cells suppress innate immune pathology through cytokine-dependent mechanisms. *J Exp Med*. 2003;197:111-119.

354. Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. *Nat Immunol*. 2005;6:353-360.

355. Belkaid Y, Tarbell K. Regulatory T cells in the control of host-microorganism interactions. *Ann Rev Immunol*. 2009;27:551-589.

356. Belkaid Y, Hoffmann KF, Mendez S, et al. The role of interleukin (IL)-10 in the persistence of Leishmania major in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure. *J Exp Med*. 2001;194:1497-1506.

357. Belkaid Y, Piccirillo CA, Mendez S, et al. CD4+CD25+ immunoregulatory T lymphocytes control *Leishmania major* persistence and the development of concomitant immunity. *Nature*. 2002;420:502-507.

358. Suffia IJ, Reckling SK, Piccirillo CA, et al. Infected site-restricted $Foxp3^{+}$ natural regulatory T cells are specific for microbial antigens. *J Exp Med*. 2006;203:777-788.

359. McGuirk P, McCann C, Mills KHG. Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by Bordetella pertussis. *J Exp Med*. 2002;195:221-231.

360. MacDonald AJ, Duffy M, Brady MT, et al. CD4 T helper type 1 and regulatory T cells induced against the same epitopes on the core protein in hepatitis C virus-infected persons. *J Infect Dis*. 2002;185:720-727.

361. Iwashiro M, Messer RJ, Peterson KE, et al. Immunosuppression by $CD4^{+}$ regulatory T cells induced by chronic retroviral infection. *Proc Natl Acad Sci U S A* . 2001;98:9226-9230.

362. Robertson SJ, Messer RJ, Carmody AB, et al. In vitro suppression of $CD8^{+}$ T cell function by Friend virus-induced regulatory T cells. *J Immunol*. 2006;176:3342-3349.

363. Zelinskyy G, Dietze K, Sparwasser T, et al. Regulatory T cells suppress antiviral immune responses and increase viral loads during acute infection with a lymphotropic retrovirus. *Plos Pathog*. 2009;5:e1000406.

364. Kinter AL, Hennessey M, Bell A, et al. $CD25^{+}CD4^{+}$ regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate $CD4^{+}$ and $CD8^{+}$ HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. *J Exp Med*. 2004;200:331-343.

365. Suvas S, Azkur AK, Kim BS, et al. $CD4^{+}CD25^{+}$ regulatory T cells control the severity of viral neuroinflammatory lesions. *J Exp Med*. 2004;172:4123-4132.

366. Shafiani S, Tucker-Heard G, Karoyone A, et al. Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. *J Exp Med*. 2010;207;1409-1420.

367. Lund JM, Hsing L, Pham TT, Rudensky AY. Coordination of early protective immunity to viral infection by regulatory T cells. *Science*. 2008;320:1220-1224.

368. Zhao J, Zhao J, Fett C et al. IFN-γ- and IL-10-expressing virus epitopespecific $Foxp3^{+}$ T reg cells in the central nervous system during encephalomyelitis. *J Exp Med*. 2011;208:1571-1577.

369. Danke NA, Koelle DM, Yee C, et al. Autoreactive T cells in healthy individuals. *J Immunol*. 2004;172:5967-5972.

370. Allen SE, Crome SQ, Crellin NK, et al. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int Immunol*. 2007;190:345-354.

371. Wang J, Ioan-Facsinay A, van der Voort AEIH, et al. Transient expression of FOXP3 in human activated nonregulatory $CD4^{+}$ T cells. *Eur J Immunol* . 2007;37:129-138.

372. Tran, DQ, Ramsey H, Shevach EM. Induction of FOXP3 expression in naive human $CD4+FOXP3^{-}$ T cells by T cell receptor stimulation is TGF-β-dependent but does not confer a regulatory phenotype. *Blood*. 2007;110:2983-2990.

373. Hippen KL, Merkel SC, Schirm DK, et al. Generation and large-scale expansion of human inducible regulatory T cells that suppress graftverus-host disease. *Am J Transplantation*. 2011;11:1148-1157.

374. Lu L, Ma J, Li Z, et al. All-trans retinoic acid promotes TGF-β-induced Tregs via histone modification but not DNA demethylation on Foxp3 gene locus. *PLoS One*. 201;6:e24590.

375. Buckner JH. Mechanisms of impaired regulation by $CD4^+CD25^+FOXP3^+$ regulatory T cells in human autoimmune diseases. *Nat Rev Immunol* . 2010;10:8499-8509.

376. Miyara M, Yoshioka Y, Kitoh A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxpP3 transcription factor. *Immunity*. 2009;30:899-911.

377. Viglietta V, Baecher-Allan C, Weiner HL, et al. Loss of functional suppression by $CD4^+CD25^+$ regulatory T cells in patients with multiple sclerosis. *J Exp Med*. 2004;199:971-979.

378. Baladina A, Lecart S, Dartevelle P, et al. Functional defect of regulatory $CD4^+CD25^+$ T cells in the thymus of patients with autoimmune myasthenia gravis. *Blood*. 2005;105:735-741.

379. Kriegel MA, Lohmann T, Gable C, et al. Defective suppressor function of human $CD4^+CD25^+$ regulatory T cells in autoimmune polyglandular syndrome type II. *J Exp Med*. 2004;199:1285-1291.

380. Valencia X, Yarboro C, Illei G, et al. Deficient $CD4^+CD25^{high}$ T regulatory cell function in patients with active systemic lupus erythematosus. *J Immunol* . 2007;178:2579-2588.

381. Sugiyama H, Gyulai R, Toichi E, et al. Dysfunctional blood and target tissue $CD4^+CD25^{high}$ regulatory T cells in psoriasis: mechanism underlying unrestrained pathogenic effector T cell proliferation. *J Immunol* . 2005;174:164-173.

382. Lindley S, Dayan CM, Bishop A, et al. Tree TI. Defective suppressor function in CD4+CD25+T cells from patients with type 1 diabetes. *Diabetes*. 2005;54:92-99.

383. Nilsson J, Boasso A, Velila PA, et al. HIV-1-driven regulatory T cell accumulation I lymphoid tissues is associated with disease progression in HIV/AIDS. *Blood*. 2006;108:3808-3817.

384. Van Amelsfort JM, Jacobs, Billsma JW, et al. CD4+CD25+ regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. *Arthritis Rheum*. 2004;50:2775-2785.

385. Notley CA, Ehrenstein MR. The yin and yang of regulatory T cells and inflammation in RA. *Nat Rev Rheum*. 2010;6:572-577.

386. Ehrenstein MR, Evans JG, Singh A, et al. Compromised function of regulatory T

cells in rheumatoid arthritis and reversal by anti-TNF alpha therapy. *J Exp Med*. 2004; 200:277-285.

387. Flores-Borja F, Jury EC, Mauri C, et al. Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis. *Proc Natl Acad Sci U S A* . 2008;105:19396-19401.

388. Tang QZ, Bluestone JA. Regulatory T-cell physiology and application to treat autoimmunity. *Immunol Rev*. 2006;212:217-237.

389. Bluestone JA, Thomson, AW, Shevach EM, Weiner HL. What does the future hold for cell-based tolerogenic therapy? *Nat Rev Immunol* . 2007;7:650-654.

390. Zanin-Zhorov A, Ding Y, Kumari S, et al. Protein kinase C-θ mediates negative feedback on regulatory T cell function. *Science*. 2010;328:372-376.

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Chapter 34

The Mucosal Immune System

Yasmine Belkaid

INTRODUCTION

Most antigens encountered by the immune system enter the body through the mucosal surfaces of the respiratory, gastrointestinal (GI), and urogenital tracts, and the vast majority of infectious microorganisms use the mucosae as portals of entry. These sites are, therefore, charged with the formidable task of protecting the host from environmental and pathogenic challenges while preserving vital physiologic tissue functions. The GI tract in particular represents the most reactive and complex immune environment of the host. Along with its constant exposure to food antigen and primary role in acquisition of metabolites, gut mucosal surfaces host complex microbial communities whose combined membership outnumbers host somatic cells. This enormous and highly variable antigenic load presents a significant challenge for the host immune system. When uncontrolled, reactivity against innocuous antigens such as those derived from food and intestinal flora poses a substantial risk that can lead to tissue damage and severe inflammatory disorders. Therefore, multiple highly specialized innate and adaptive immune cell types, as well as structural features, have been put in place to prevent overt reactivity and favor the induction of tolerogenic responses. However, tolerance is not the only fate of the immune response at mucosal sites as a certain degree of constitutive activation and inflammation is beneficial for the host, not only to reinforce the barrier, but also to allow for the development of protective responses when required. Indeed, the majority of infectious diseases worldwide either target or are acquired through mucosal surfaces of the GI, respiratory, and genital tracts. The complex and highly dynamic maintenance of immune tolerance to mucosal antigens concomitantly with the induction of protective responses to pathogens requires an arsenal of unique cells specifically conditioned by the mucosal environment. Some of the main actors in this control are unique antigen-presenting cells (APCs), various regulatory T (T_{reg}) cell populations, intraepithelial lymphocytes (IELs), as well as recently identified innate lymphoid cells (ILCs). Further, the mucosal environment is under dominant control by the microbiota and dietary metabolites that direct the development and function of both innate and adaptive mucosal responses.

MUCOSAL COMPARTMENTALIZATION

The Gut-Associated Lymphoid Tissue

The mucosal immune system of the GI tract contains unique cell subsets that are structured in such a way as to favor the coexistence of the host with its flora, support food absorption,

as well as induction of effector responses to pathogens.[1] The primary barrier preventing transit of microorganisms and large molecules from the environment into the body is a simple epithelium made up of a single layer of cells overlaid with mucus and connected by junctional complexes. The gutassociated lymphoid tissue (GALT) can be divided into effector sites consisting of lymphocytes scattered throughout the epithelium and lamina propria (LP) of the mucosa and organized inductive sites. These inductive sites include the largest lymph node of the body, the mesenteric lymph node (MLN), Peyer patches, as well as cryptopatches and isolated lymphoid follicles (ILFs) that are distributed along the wall of the small and large intestine.[1] ILFs are inducible structures recently described in both mice and humans that develop after birth in response to environmental signals, such as those derived form the microbiota.[2,3,4] Peyer patches are macroscopic lymphoid aggregates consisting of large B-cell follicles with interspersed T-cell areas found in the submucosa along the length of the small intestine. In these structures, the lymphoid areas are separated from the intestinal lumen by a single layer of epithelial cells known as the follicle-associated epithelium and a more diffuse area below the epithelium known as the subepithelial dome. Notably, the follicle-associated epithelium contains unique cells referred to as microfold cells that have the capacity to transport organisms and particles from the gut lumen across the epithelial barrier.[5] These cells also represent a major point of pathogen entry.[5]

Antimicrobial Peptides

A central strategy utilized by the mucosal immune system to maintain its homeostatic relationship with the microbiota and limit pathogen exposure is to minimize contact between luminal microorganisms and the epithelial cell surface. This is accomplished by promoting a physical barrier through the production of mucus, immunoglobulin (Ig)A, and antimicrobial proteins. All intestinal cell lineages, including enterocytes, goblet cells, and Paneth cells, can produce antimicrobial peptides. These molecules play a significant role in the control of pathogenic infections as well as in limiting exposure to the commensal microbiota. Epithelial cell-derived antimicrobial proteins are members of a diverse family including defensins, cathelicidins, and C-type lectins.[6] Some of these molecules, such as α-defensins, are constitutively expressed. In other cases, engagement of pattern recognition receptors (PRRs) by commensally derived products induce the expression of a variety of antimicrobial peptides, which are critical to prevent translocation of gut bacteria across mucosal barriers.[6] These proteins can exert antimicrobial functions by direct killing resulting from enzymatic attack of the bacterial cell wall or by

disrupting the bacterial inner membrane.[6] Additionally, some antimicrobial proteins can function by depriving bacteria of essential heavy metals, such as iron.[7] One of the best characterized mucosal antimicrobial peptides is RegIIIγ. This lectin is expressed soon after birth or following colonization of germfree mice.[8] Production of RegIIIγ is tightly controlled by the flora in an MyD88-dependent manner and has a direct microbicidal effect on gram-positive bacteria.[8,9,10] Similarly, the PRR nucleotide-binding oligomerization domain-containing protein (NOD) 2 controls expression of a subset of α-defensins and cryptdins by Paneth cells.[11] Critically, antimicrobial proteins are retained in the mucus layer and are virtually absent from the luminal content.[12] Such a feature allows antimicrobial activity to be

concentrated within a region bordering the epithelial cell layer. Recent findings demonstrate that the accumulation of RegIIIγ in the mucus contributes to the maintenance of the segregation between the microbiota and the host intestine.[13]

Mucus

The stratified mucus layer plays a crucial role in the maintenance of the physical segregation between the microbiota and host tissue, as well as preventing pathogen invasion and supporting their clearance. In particular, the mucus layer limits commensal and pathogenic bacterial contact with the epithelium preventing transport of bacteria to distal sites.[14] To date,[17] mucus (MUC) genes have been described,[7] of them coding for secreted mucins (MUC2, MUC5A, MUC5B, MUC6, MUCC19, MUC7, and MUC8) that are differentially expressed in various mucosal compartments.[14] The secreted, gel-forming, mucin glycoproteins that constitute the major macromolecular component of mucus are produced by goblet cells found throughout the GI tract and airways. In the airways, both mucus cells and serous cells produce defined mucins.[15] Paneth cells represent another major secretory cell type within the GI tract.[14] Mucus is found in the entire GI tract but varies in thickness ranging from 700 μm in the stomach and large intestine to 150 to 300 μm in the small intestine.[16] The inner layer of the colonic mucus is attached to the epithelium and has a stratified appearance, ranging in thickness from 50 to 100 μm. Based on its structural capacity, this layer acts as a filter and is impermeable to commensal bacteria.[17,18,19] The inner mucus layer is renewed by goblet cell secretion and at its luminal border is converted into an outer mucus layer. This outer layer expands in volume allowing commensal bacteria access to this zone.[18] Contrasting the large intestine, the small intestine is only covered by a single layer of mucus that is not attached to the epithelium and more permeable to bacteria.[16,20] Of note, commensal bacteria have a high proportion of their genome devoted to production of enzymes involved in glycan degradation and are able to utilize the released mucin monosaccharides as energy sources.[21,22]

MUCOSAL ANTIBODIES

About three-quarters of total antibody production is composed of IgA, in the order of 3 to 5 g produced per day in humans, with the vast majority secreted across barrier surfaces.[23,24,25] In contrast, IgG is dominant in urine, bile, genital, and bronchoalveolar secretions; IgD is detected in nasal lacrimal as well as bronchoalveolar secretions,[26,27,28]; and IgE in nasal bronchoalveolar and intestinal secretions in the context of allergy.[29] Human B cells produce two IgA subclasses, IgA1 and IgA2, whereas mice only produce one kind of IgA.[30,31] IgA1 can be detected at systemic and mucosal sites, whereas IgA2 is essentially only present at mucosal sites.[32] IgA can exist in a dimeric form called secretory IgA that is the main Ig found in mucus secretions, including tears, saliva, colostrum, and secretions from the genitourinary tract, GI tract, prostate, and respiratory epithelium.[23,24,25] IgA is a major factor involved in the host-microbe dialogue at mucosal sites. It is critically important for shaping the microbiota, mediating pathogen clearance, neutralizing toxins and inflammatory microbial molecules such as lipopolysaccharides, as well as preventing adhesion of commensal bacteria to the

epithelial surfaces by generating steric hindrance.[30,31] Moreover, it can also induce bacterial agglutination and interact with the mucus layer through secretory component.[33,34,35,36,37,38] One remarkable feature of IgA is its capacity to disseminate between mucosa-associated lymphoid structures. Such a feature allows specificities of IgA present in breast milk to be matched to the dominant microbes resident in the maternal microbiota.[39,40] IgA induction is exquisitely sensitive to the presence of commensal bacteria in the intestine, and germ-free mice have very low levels of IgA that can be restored by commensal colonization.[41,42,43,44]

Immunoglobulin A Generation and Secretion

More than 40 years ago, Peyer patches were identified as the major source of IgA-producing B cells.[16] IgA can also be generated in regional lymph nodes as well as in ILFs.[45] The diffuse tissue of the LP can also support IgA production in the absence of follicular structures.[46,47,48] In order to express IgA, B cells must undergo class switch recombination in which deoxyribonucleic acid (DNA) is spliced out of the heavy chain constant region loci to replace the μ/δ genes with the α gene segment downstream of the recombined VDJ segment. The GC-rich splice sites upstream of the gene segment for each Ig constant region are the targets for enzymes such as activation-induced (cytidine) deaminase (AID), which catalyze the reaction sequence. In humans, there are two Cα regions generating the two IgA isotypes, IgA1 and IgA2.[30,31,32] Following induction in the intestinal lymphoid structures, B cells recirculate through the mesentery, lymphatic systems, and the bloodstream to home back to the intestinal mucosa where they develop into IgA-secreting plasma cells. Secretory IgA is produced as a result of cooperation between plasma cells in the LP that secrete dimeric IgA and epithelial cells that transport IgA into the lumen. This mechanism of transport involves covalent binding of dimeric IgA to the polymeric Ig receptor, which then carries it within vesicles through intestinal epithelial cells to the luminal surface. At this point, IgA is released by proteolytic cleavage of the polymeric Ig receptor leaving a fragment of the transport protein (termed secretory component) that remains associated with the joining chain of the IgA dimer forming a secretory IgA complex with noninflammatory protective functions.[49,50,51] Indeed, secretory IgA can bind to bacteria without activating complement or stimulating the release of inflammatory mediators by innate cells.[52,53]

T-Dependent and -Independent Immunoglobulin A Generation

Mucosal IgA can recognize antigen with high- and lowaffinity binding modes.[54] Generally, high-affinity IgA neutralizes microbial toxins and invasive pathogens whereas low-affinity IgA confines commensals to the lumen.[32] Highaffinity IgA is believed to emerge from follicular B cells stimulated in a T cell-dependent manner, whereas low-affinity IgA can develop from extrafollicular B cells stimulated in a T cell-independent fashion.[55] However, such distinction of origin and function is not absolute.[56] Antigen at mucosal sites can stimulate IgA responses through T cell-dependent pathways that are initiated in mucosal lymphoid structures such as Peyer patches and MLN.[54]

Various mechanisms can promote the induction of IgA in a T cell-independent manner. In particular, transforming growth factor (TGF)-β that is highly enriched at mucosal surfaces controls B-cell responsiveness and promotes class switch recombination to IgA, in combination with interleukin (IL)-2 and IL-10 that act synergistically.[57] Mechanistically, TGF-β1 binds to the TGF-β receptor complex that has a sterile transcript for the Iα locus upstream of the Sα recombination segment. Such a phenomenon is associated with signaling via mothers against decapentaplegic-2, -3, and -4 that bind to the Iα promoter in combination with the runt DNA binding factor RUNX3.[58,59,60,61,62,63,64] A cytokine of the tumor necrosis factor (TNF) ligand family referred to as B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL) can also induce NF-κB activation and IgA induction.[65,66] Further, as will be described in more detail later in this chapter, synthesis of the vitamin A metabolite retinoic acid (RA) by GALT dendritic cells (DCs) is crucial for the generation of mucosal IgA.[67,68]

Immunoglobulin A in Host Microbe Interaction

IgA plays a major homeostatic role by controlling host interactions with the microbiota as well as a protective role against pathogens.[6,69] Although nonspecific IgA can limit epithelial invasion, pathogen-specific IgA plays an important role in the control of mucosal infections. For example, IgA production has been shown to control *Shigella flexneri* during infection by binding the bacteria at the mucosal layer,[37] whereas IgA against the O-antigen component of lipopolysaccharides can protect against *Salmonella* infection.[70] IgA can also shape the microbiota composition, as demonstrated in mice lacking IgA that have expansion of segmented filamentous bacteria (SFB) and other *Clostridium*-related species in the intestinal tract.[71] In the context of IgA-microbiota dialogue, IgA responses lack typical memory characteristics and respond rapidly to changes in gut flora composition. This point was demonstrated using reversible colonization of mice with a strain of auxotrophic *Escherichia coli* that required nutrients unavailable from mammalian host metabolites.[72] IgA responses persisted in the absence of bacteria; however, introduction of additional species of bacteria caused a rapid decline in IgA specific for the auxotrophic *E. coli*.[72] As the microbiota composition can fluctuate in the context of infection or due to dietary changes, such a strategy would promote a dynamic IgA repertoire, allowing IgA to swiftly adapt to the commensals present at a given time.[72]

TABLE 34.1 Subsets of Intraepithelial Lymphocytes in Mice and Humans

Subsets		Frequency	T-Cell Receptor Repertoire
Mouse	TCRαβ+CD4+	< 15%	MHC-class II restricted Oligoclonal
	TCRαβCD8αβ	20%-30%	MHC-class II restricted

	TCRαβCD8αβCD8αα		Oligoclonal
	TCRαβCD8αα	20%-50%	Nonclassical MHC class I restricted
	TCRαβCD8-CD4-		Oligoclonal
	TCRγδCD4-CD8-	40%-70%	Nonclassical MHC class I ?
	TCRγδCD8αα		
Human	TCRαβCD8αβ	70%-80%	MHC-class I restricted Oligoclonal
	TCRαβ+CD4+	10%-15%	MHC-class II restricted Oligoclonal
	TCRγδCD4-CD8-	5%-20%	Nonclassical MHC class I ?
	TCRγδCD8+		
	CD3-CD103+	< 10%	

CD, cluster of differentiation; MHC, major histocompatibility complex; TCR, T-cell receptor

INTRAEPITHELIAL LYMPHOCYTES

IELs reside within the epithelial layer and represent a highly heterogeneous group of cells distributed along the GI tract[73] (Table 34.1). IELs represent one of the most abundant T-cell populations, and it has been estimated that 1 IEL is present for every 4 to 10 epithelial cells in the small intestine and 30 to 50 in the large intestine.[74] These cells are important in the induction of both protective and regulatory responses at mucosal sites. They express features of adaptive and innate immune cells that allow them to survey tissue based not only on antigen recognition but also stress signals.[73] IELs can be divided based on their expression of αβ and γδ T-cell receptors (TCRs) and further subdivided on the basis of

their cluster of differentiation (CD)8 expression with a large fraction expressing CD8αα (see Table 34.1).[75]

Homeostasis of Intraepithelial Lymphocytes

In the small intestine, IELs are highly abundant and are essentially composed of TCRαβ+ or TCRγδ+ CD8+ T cells. In contrast, in the large intestine the frequency of IELs is reduced and mostly represented by TCRαβ CD4+ T cells.[74,76,77,78] Although the development and origin of the various IELs is the subject of long debate, all IEL subsets are progeny of bone marrow precursors that initially develop in the thymus.[79] Some IEL precursors go through alternative self-antigen-based thymic maturation processes that lead to the functional maturation of CD4 and CD8αβ double negative, TCRαβ-expressing or TCRγδ-expressing T cells that directly migrate to the intestinal epithelium.[80,81,82] The capacity of IELs to home to the intestinal epithelium is controlled by the expression of CCR9 on the surface of IEL precursors and its ligand CCL25.[83,84] IELs also express the integrin αEβ7 (CD103) that can interact with E-cadherin on epithelial cells.[85] Additionally, the mucosal environment leads to the induction of IELs from conventional TCRαβ+ cells that have matured as antigen-experienced cells in response to cognate antigen.[75] In particular TGF-β, highly enriched in the gut, leads to the development of TCRαβ+CD8αα+ IELs and the coexpression of CD8α in conventional CD4+ T cells.[86] IEL repetoire is polyclonal at birth and in response to exogenous stimuli, including luminal antigens and microbial composition, these cells expand to become oligoclonal in the adult.[87] The development and expansion of IELs is also highly controlled by the cytokine IL-15 and by dietary metabolites. In particular, vitamin D and the arylhydrocarbon receptor, whose ligands are metabolized from the vegetable-derived phytochemical indile-3-carbinol, are required for the proper homeostasis of these cells.[75,88,89] Although mice and human IELs share a number of features, some notable differences exist in terms of their composition and phenotype[90] (see Table 34.1).

Intraepithelial Lymphocyte Functions

IELs have complex functions that include the promotion of local immunity concomitant with the maintenance of barrier integrity. Moreover, a role for IELs in promoting host defense has been revealed in various models of mucosal infection. For instance, TCRαβ+ CD8αβ+ IELs can promote effector responses to a variety of pathogens such as *Eimeria vermiformis*, *Encephalitozoon cuniculi*, and *Toxoplasma gondii*.[91,92,93,94] In the context of bacterial or parasitic infections, IELs can mediate pathogen clearance via their capacity to produce various inflammatory mediators such as interferon (IFN)γ and TNF-α.[95] IELs can also promote immunity to nematodes by releasing cytokines such as IL-13 that can promote mucus formation.[96] Moreover, IELs express various natural killer (NK) receptors, endowing them with a strong cytolytic activity leading to the direct elimination of damaged or infected cells.[75] Additionally, their protective role can result from the capacity of these cells to maintain the integrity of tight junctions between epithelial cells, therefore preventing epithelial transmigration of pathogens.[97] In this regard, IELs have been associated with the maintenance of barrier function in the context of simian immunodeficiency virus (SIV) or human immunodeficiency virus (HIV).[98,99] IELs can also exert regulatory function at mucosal sites. In particular, TCRγδ IELs play a major anti-inflammatory role via their capacity to promote tissue repair upon injury and to release an array of factors with regulatory properties. In particular, γδ T cells produce large amounts of keratinocyte growth factor,

which plays an important role in supporting epithelial integrity.[100,101] Further, these cells can abundantly produce the immunoregulatory cytokines IL-10 and TGF-β that can both limit inflammatory responses at mucosal sites.[102,103,104] In mice, TCRγδ IELs express predominantly Vγ5 and Vγ1, whereas in humans TCRγδ IELs express essentially the Vγ1 gene segment.[38] To date, little is known about the specificity of these cells.

Although the role of IELs is to maintain mucosal homeostasis, various lines of evidence suggest that in defined settings, IELs can also contribute to tissue damage and inflammation. For instance, the number of TCRγδ+ cells correlates with disease severity in inflammatory bowel disease (IBD) patients and, experimentally, these cells have been shown to promote mucosal inflammation.[105,106,107,108] Further, CD8αβ+ IELs can also contribute to the initiation of experimental colitis. More particularly, IELs have been associated with the exacerbation of celiac disease, an inflammatory disorder in genetically susceptible individuals associated with the induction of inflammatory T-cell responses against dietary gluten.[90] Celiac disease is characterized by a dramatic increase of the IEL compartment.[90] TCR-activated CD8αβ+ TCRαβ+ IELs cause severe villous atrophy by targeting intestinal epithelial cells that express stress-induced major histocompatibility complex class I polypeptiderelated sequence antigen in an NKG2D-dependent manner.[109,110] Additionally, IL-15, a factor that is overexpressed in various mucosal disorders including celiac and Crohn diseases,[90] can trigger potent cytotoxic responses by CD8 αβ+ IELs via a NKG2D-DAP10-dependent signaling pathway.[109] Supporting a role for IELs in celiac disease pathogenesis, genome-wide association studies have identified a number of genes involved in the development, migration, and cytotoxic/NK function of IELs.[111]

Mucosal Mononuclear Phagocytes

Immune reactivity against nonpathogenic gut elements is not only wasteful but is also known to lead to severe tissue damage. However, the development of active immunity is required to protect the host against invasive pathogens. This tight equilibrium is under the control of a complex network of mucosal mononuclear phagocytes (MPs) with unique properties (Fig. 34.1). Mucosal MPs form an organized cellular network in the LP and are distributed throughout the GALT.[112] In the LP, major histocompatibility complex II^{hi} $CD11c^{hi}$ MPs can be subdivided into two main populations defined as CD103+CD11b+CX3CR1- and CD103-CD11b+CX3CR1+. Although some debate exists in the

literature, the current consensus is that CD103+ cells are mucosal DCs with capacity to rapidly migrate into lymphoid structures, whereas the CX3CR1+ populations are considered to be tissue resident macrophages.[113] The gut also contains a minor population of CD103+CD11b-CD8α+CX3CR1-DCs believed to be associated with ILFs.[114] Additionally, the gut is home to plasmacytoid DCs.[115] APC subset distribution varies anatomically with CD103+CD11b+ DCs mostly represented in the duodenum and CD103-CD11b+ DCs enriched in the large intestine.[116]

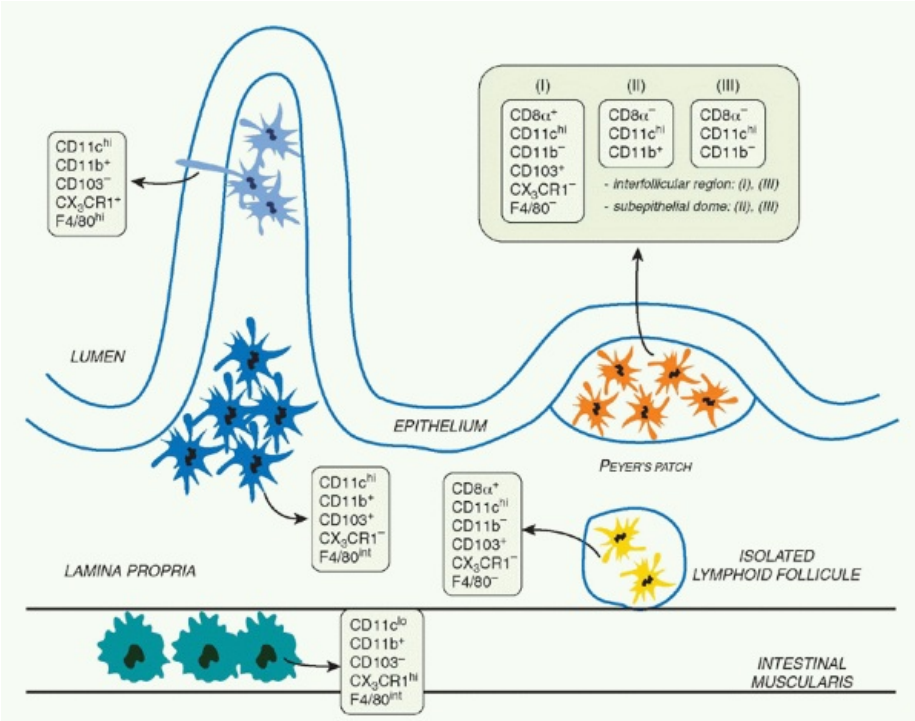

FIG. 34.1. Composition of the Gastrointestinal Mucosal Mononuclear Phagocyte Network.

Origin of Mucosal Mononuclear Phagocytes

In the intestine, DC-restricted progenitors (termed common DC precursors) and pre-DCs give rise to CD103+CD11b+ and CD103+CD11b- populations.[113,117] In contrast, CD103-CD11b+ cells are monocyte derived.[113,117] Various cytokines, including FMS-like tyrosine kinase 3 ligand, granulocyte macrophage-colony stimulating factor, and macrophagecolony stimulating factor, control MP development and homeostasis.[118] In the gut, these cytokines have a differential effect on the various MP subsets. Flt3L controls the differentiation of CD103+ DC (CD11b+ and CD11b-), granulocyte macrophage-colony stimulating factor the differentiation of CD103+CD11b+ DCs, and macrophage colony-stimulating factor receptor (M-CSFR) controls the homeostasis of CD103-CD11b+ cells.[113,117,119] The fractalkine receptor CX3CR1 also controls the development of CD103-CD11b+ CX3CR1+ cells, likely via its capacity to control the recruitment of progenitors to the tissue.[120] A number of transcription factors have been associated with DC lineage specification.[121] In the gut, CD103+CD11b- DCs are highly dependent upon the transcription factors Id2, Irf8, and Batf3, whereas CD103+CD11b+ DCs are Notch2 dependent.[113,122,123,124]

Antigen Uptake and Cell Trafficking

Luminal antigens, including macromolecules, bacteria, and viruses, gain access to the cells of Peyer patches and ILFs through microfold cells, present in the follicle-associated epithelium.[125,126,127] Microfold-cell transport is promiscuous and mediated by binding to surface-expressed carbohydrates in regions free of overlying mucus, but can be enhanced by

the presence of antigen-specific IgA by immune targeting with anti-M-cell antibodies[128,129] or by oral administration of toll-like receptor (TLR)2 or TLR4 ligands.[130] A second site for antigen entry into the intestine is the nonfollicular absorptive epithelium, where both soluble

antigens and bacteria can gain access to DCs in the LP. This can occur by trans- or paracellular transport or by receptormediated trafficking, such as occurs through the neonatal FcR expressed on absorptive epithelial cells in humans.[131] Luminal antigens can also be directly sampled by mucosal DCs. Indeed, subepithelial DCs can penetrate the epithelium monolayer by extending dendrites into the lumen, allowing them to sample particles and bacteria.[132,133,134] In the terminal ileum, this process is tightly dependent on the fractalkine receptor CX3CR1 and controlled by the microbiota in an MyD88-independent manner.[132,133,135] In addition to CX3CR1, CCL20 can also contribute to transepithelial dendrite formation.[133] Luminal antigen can gain access to mucosal sites by direct damage to the epithelium, as can occur during IBD or in response to infection with HIV[136] or *Shigella flexneri*.[137] Antigen sampling may also occur by uptake of exosomes from epithelial cells or across villous microfold cells.[138] Even in the absence of infection or inflammation, LP DCs constitutively traffic to MLN,[139] which appears to be a relatively active process. These migratory DCs can carry self- or cell-associated antigens from apoptotic epithelial cells[140] or soluble proteins given orally.[141] Soluble antigens given orally can be processed by LP DCs, which then migrate to the MLN in a CCR7-dependent manner.[142] Various lines of evidence support the idea that CD103+ DCs have enhanced migratory capacity to lymphoid structures compared to other mucosal MP subsets. Indeed, CD103+ DCs are highly represented in the gut afferent lymphatics,[119] constitutively migrating to the MLN at steady state, and are the first to reach the MLN under inflammatory conditions.[113,119] In response to inflammation, various signals including lipopolysaccharides and flagellin can influence the capacity of mucosal DCs to transport intestinal commensals and pathogenic bacteria to the MLN.[143,144] A remarkable feature of this process is that commensal loaded LP DC migration is restricted to the mucosal immune compartment by the MLN.[143] Such a process is believed to contribute to the compartmentalization of mucosal immune responses. Although as further discussed, mucosal MP can promote the induction of tolerance under steady-state conditions, the function of these cells is highly contextual, and under inflammatory settings mucosal MP can rapidly adopt a highly inflammatory phenotype and induce effector responses.[145]

VITAMIN A AND MUCOSAL IMMUNITY

In the early 20th century, various studies identified dietary constituents that were essential for mammalian health. A single factor present in lipids was essential for growth and survival, which they termed “fat soluble factor A.”[146] Subsequently designated vitamin A, studies over the years have demonstrated the pleiotropic influence of this nutrient, ranging from promoting eyesight and organogenesis to metabolism and immunologic fitness.[147,148,149,150] In particular, the vitamin A metabolite RA plays a central role in the control of various aspects of

innate and adaptive immune responses at mucosal sites.[151,152] The effects of RA in mucosal immunity range from promoting effector cell migration to mucosal tissue and IgA generation, to the control of oral tolerance.[153]

Acquisition, Storage, and Metabolism of Vitamin A

Vitamin A is a fat-soluble essential nutrient obtained from foods containing vitamin A precursors (ie, carotenoids) or vitamin A itself in the form of retinyl esters.[154,155] Following absorption and arrival into circulation, retinyl esters enter the liver, where most of the vitamin A in the body is stored (Fig. 34.2).[156] Liver retinyl esters are continually hydrolyzed into retinol and deployed into circulation.[157] Once inside a cell, widely expressed alcohol dehydrogenases oxidize retinol into retinal, which can then bind to more selectively expressed retinaldehyde dehydrogenases (RALDHs) for oxidation into RA. Prior to its action, RA binds to nuclear receptors, including retinoic acid receptors (RARs) and retinoid X receptors.[158,159] Notably, all-trans RA exclusively binds retinoid X receptors via heterodimers with the RAR family, which consists of three receptors: RAR alpha (RARα), beta (RARβ), and gamma (RARγ).[158] Although RA is constitutively present in serum at low levels,[160] RALDH expression is tightly controlled.[147] GALT DCs express messenger ribonucleic acid for Aldh1a2, the gene encoding RALDH2.[119,161,162] In particular, basal Aldh1a2 expression in GALT DCs is enriched in CD103+ DC subsets.[162,163,164] Vitamin A itself is absolutely required for DC production of Aldh1a2 during homeostasis.[162,165,166] Although dispensable at steady state, microbial stimuli may affect RALDH expression during an inflammatory or infectious response.[167,168,169] In addition to DCs, several nonhematopoietic lineages within the GI tract and GALT, such as epithelia and stromal cells, share the capacity to synthesize RA (see Fig. 34.2).[161,166,170,171,172]

Role of Vitamin A in Migration to Mucosal Sites

Appropriate immune responses depend on the ability of effector and regulatory lymphocytes to home to the site of infection or injury. In this regard, DCs have been shown to foster lymphocyte migration into tissues where antigen was initially encountered.[173] One of the dominant actions of vitamin A on the mucosal immune system is associated with the capacity of its metabolite RA to promote the migration of effector cells to mucosal tissues. In particular, CD103+ DCs from the GI tract and GALT can induce the mucosal homing markers—integrin heterodimer α4β7 and chemokine receptor CCR9—on stimulated effector T and B cells.[163,174,175] The insight that mucosal DCs triggered α4β7 and CCR9 through their capacity to synthesize RA was based on the seminal observation that adding RA to T cells during activation selectively induced these gut homing markers.[161] In a reciprocal fashion, blockade of RAR-mediated signaling and transcription in cultures containing GALT DCs reversed induction of α4β7 and CCR9.[161,176] Recently, RA was revealed to predominantly affect the α4 subunit of α4β7 via binding of RARα to a retinoic acid receptor response element within the regulatory region of the α4 gene.[177,178] An RA response element half-site was also recently discovered in the promoter region of CCR9, which RARα/retinoid X receptor heterodimers were able to bind to.[179] These data together with the prominent

expression of Rarα, the gene encoding RARα, in CD4+ T cells pinpoint RARα as a dominant mediator of CD4+ T-cell trafficking into mucosal sites.

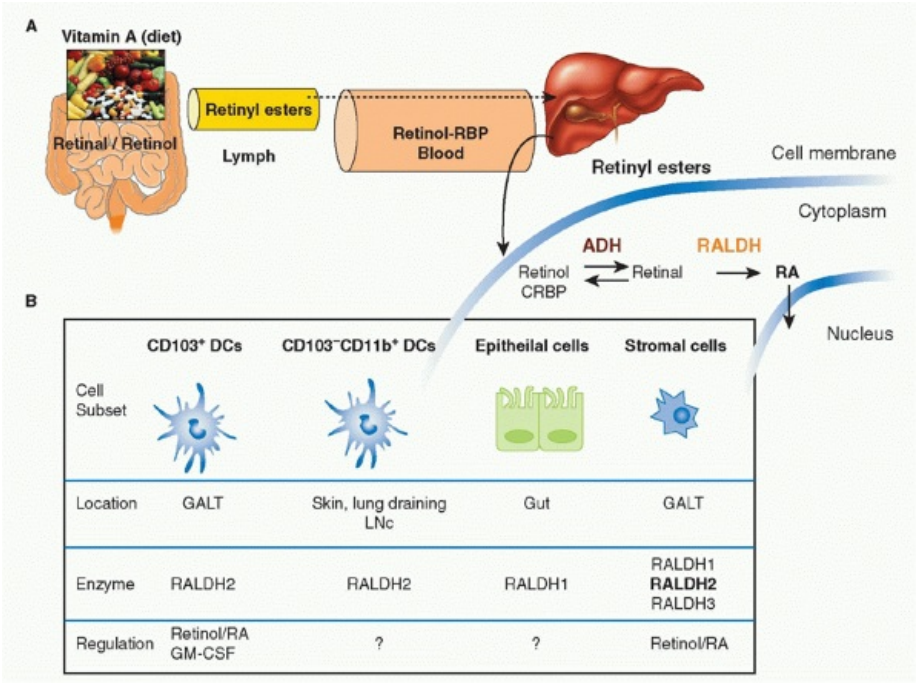

FIG. 34.2. Metabolism and Major Cellular Sources of Retinoic Acid (RA). Dietary vitamin A is absorbed in the intestine and transported through the lymphatics into the circulation where it enters the liver for storage. Retinol chaperoned by retinol binding protein is constitutively deployed from the liver into circulation. It is also secreted in bile that drains into the small intestine. Upon entry into cells, retinol is reversibly oxidized into retinal via the alcohol dehydrogenase enzyme family. Depending on the cell type (see Table 34.1), retinal can undergo irreversible metabolism into RA via retinal dehydrogenases (RALDH). Table 34.1 provides an overview of major cellular sources of RA at steady state including cellular location, the isoform(s) of RALDH that are expressed, and the factors known to induce RALDH expression in each cell type.

Retinoic Acid in Infection and Immunity

Recent human data highlight the correlation between vitamin A status and T-cell function.[180] Impaired and/or dysregulated T-cell responses have been observed in various models of infection and vaccination strategies in the context of vitamin A and retinoid receptor deficiency.[181,182,183,184,185] Experimentally, these results support a role for RA in the development of both Th1- and Th17-cell responses (Fig. 34.3).[181,182,183,184,185] RA signaling appears to control the fate of T-cell immunity largely through RARα and RARγ.[184] In particular RA controls the capacity of CD4+ T cells to respond to antigen by regulating TCR signaling.[184] Further, RARα regulates the maturation of DCs in the GALT[165] and their ability to drive inflammatory responses in certain pathologic settings.[186] These data illustrate

the ability of RA to regulate a network of innate and adaptive immune cell functions that, through nonredundant receptor signaling pathways, support functional immune responses. The discovery that RA served as a critical factor in the generation of IgA-secreting B cells offered further evidence of a multifactorial role for RA in mucosal immunity.[67] A number of studies have demonstrated the potent capacity of DCs from the intestinal LP, MLN, and Peyer patches to drive naïve B-cell differentiation into IgA+ B cells,[68,187,188] and the ability of stromal derived cells to support IgA+ class switching in activated B cells.[189,190] Synthesis of RA by GALT DCs was crucial for the generation of IgA by B cells, as antagonism of RA signaling significantly reduced IgA production.[68,188] Complementing this finding, addition of RA to DC cocultures in which DCs lacked the capacity to synthesize RA restored IgA+ production. Notably, microbial induced cytokines, such as IL-6, were also integral cofactors in this process.[68,188] This was also the case when stromal LP cells or stromal-derived follicular DCs from Peyer patches and MLN were assayed for their ability to foster the generation of IgA+ B cells.[189,190] In a manner analogous to peripheral DCs, peripheral follicular DCs were able to efficiently support IgA+ production only when treated with RA and in the context of Myd88-dependent microbial stimulus. 190 Intriguingly, this gain of function was dependent on the ability of RA signaling to induce secretion of TGF-β in peripheral follicular DCs.[191] RA signaling was shown to promote a similar effect (ie, induction of TGF-β production) in

bone marrow-derived DCs via inhibition of suppressor of cytokine signaling 3 activity.[192] These findings suggest interdependency between TGF-β- and RA-propagated signals in several cell lineages.

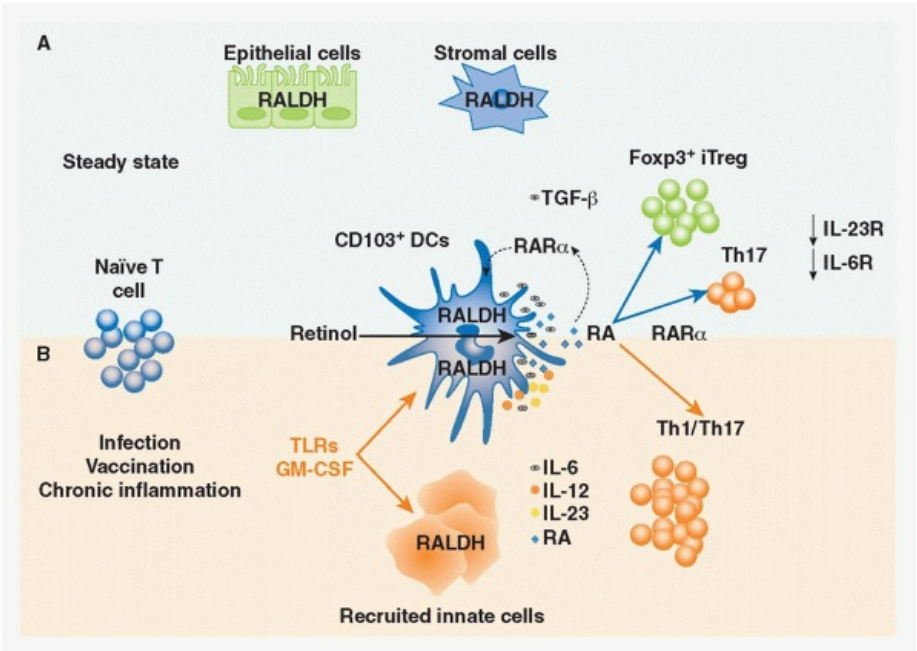

FIG. 34.3. Contextual Role of Retinoic Acid (RA) in Mucosal Immunity. Gut-associated lymphoid tissue is an RA-rich environment containing a large number of RA-synthesizing cells, including resident epithelia (intestinal epithelial cells) and stromal

cells, as well as migratory cluster of differentiation (CD)103+ dendritic cells (DCs). Under steady-state conditions (*top*), RA sustains oral tolerance and helps maintain barrier integrity. These processes are mediated in large part by the ability of RA to support the induction of Foxp3+ inducible regulatory T and Th17 cells. Lamina propria CD103+ DCs induce and recruit heterogeneous CD4+ T-cell populations at steady state as a result of their ability to respond to commensal derived signals and produce both RA and transforming growth factor-β. During inflammation or infection (*bottom*), the inflammatory milieu triggers altered cytokine production by CD103+ DCs, leading to retinoic acid receptor α-dependent effector CD4+ T-cell activation and differentiation. Innate cell populations, including antigen-presenting cells, are recruited during inflammation and also contribute to RA production. Factors in the inflammatory milieu, including toll-like receptor ligands and the cytokine granulocyte macrophage-colony stimulating factor, promote retinaldehyde dehydrogenase activity in CD103+ DCs and potentially in recruited innate cells.

Another significant source of IgA production is B1 B cells, which contribute to homeostatic mucosal integrity and early responses to pathogens. RA was recently shown to be required for the steady-state maintenance of this compartment through direct regulation of NFATc1.[86] Combined with the capacity of RA to generate IgA+ B cells and facilitate their mucosal localization, vitamin A deficiency leads to severe decreases both in intestinal and serum IgA levels.[86,188] Altogether, these findings underscore the importance of RA in IgA responses and humoral immunity.

Resulting from its pleiotropic effects on the mucosal immune system, RA can also, in a context-dependent manner, contribute to mucosal inflammation (see Fig. 34.3). Almost 30 years ago, mice fed a diet high in vitamin A were observed to exhibit more vigorous responses against grafts and tumors.[193,194] These findings suggested that elevated retinoid levels could potentially predispose an individual to exuberant immune responses. In this regard, exposure to RA during inflammation contributed to loss of oral tolerance and reactivity to dietary glutens in a mouse model of celiac disease.[186,195] More specifically, RA was demonstrated to synergize with IL-15 to promote mucosal DC production of IL-12p70, which exacerbated responses to the glutenderived antigen, gliadin.[186] These data resonate with several reports, describing a possible association between pharmacologic retinoid treatment and spontaneous development of IBD, and point to vitamin A metabolic pathways as potential instigators of chronic inflammation.[196,197]

IMMUNE REGULATION

The mucosal environment requires a complex network of immunoregulatory mediators to maintain its integrity at steady state and in the face of infection or inflammation. Because of the extraordinary antigenic pressure at these sites, many mucosal cell types can in a context-dependent manner exert

regulatory activities. Under homeostatic conditions, the coordinated action of these cells results in the induction of regulatory responses toward dietary or commensal derived antigens.

Oral Tolerance

The acquisition of oral tolerance—the active suppression of inflammatory responses against the myriad of antigens derived from food and the microbiota—is a fundamental aspect of the mucosal immune system. Multiple mechanisms are involved in the induction of oral tolerance.[198] Experimentally, oral administration of soluble proteins results in systemic tolerance that prevents the initiation of potentially damaging inflammatory responses upon rechallenge with the antigen in the periphery.[199,200,201] A complex regulatory network, including specialized populations of APCs, lymphocytes, and innate cytokines, controls GI tract homeostasis and converges to favor the induction of regulatory responses toward antigens present at mucosal sites. Early reports suggested that commensals played a role in this process as oral tolerance could not be induced in the absence of gut flora or gut flora-derived signals.[198,202,203,204,205] Over the last few years, various populations of T_{reg} cells have been ascribed a central role in the induction of oral tolerance. CD4+ T_{reg} cells, in particular, play an essential role in maintaining peripheral immune tolerance as well as preventing autoimmunity and chronic inflammation with several subsets described on the basis of their origin, generation, and mechanism of action. In a simplistic manner, these cells can be divided into endogenous T_{reg} cells, represented by thymically derived Foxp3+ T_{reg} cells and inducible T_{reg} cells, such as IL-10-producing T_{reg} cells[206] or inducible Foxp3+ T_{reg} (iT_{reg}) cells.[207,208] Although the precise mechanisms by which these cells coordinate their functions to maintain the delicate balance between immunity and tolerance remain incompletely understood, it can be conceived that collaboration and cross-talk among the aforementioned T_{reg} cell populations is required for the integrated control of mucosal immune responses (Fig. 34.4).

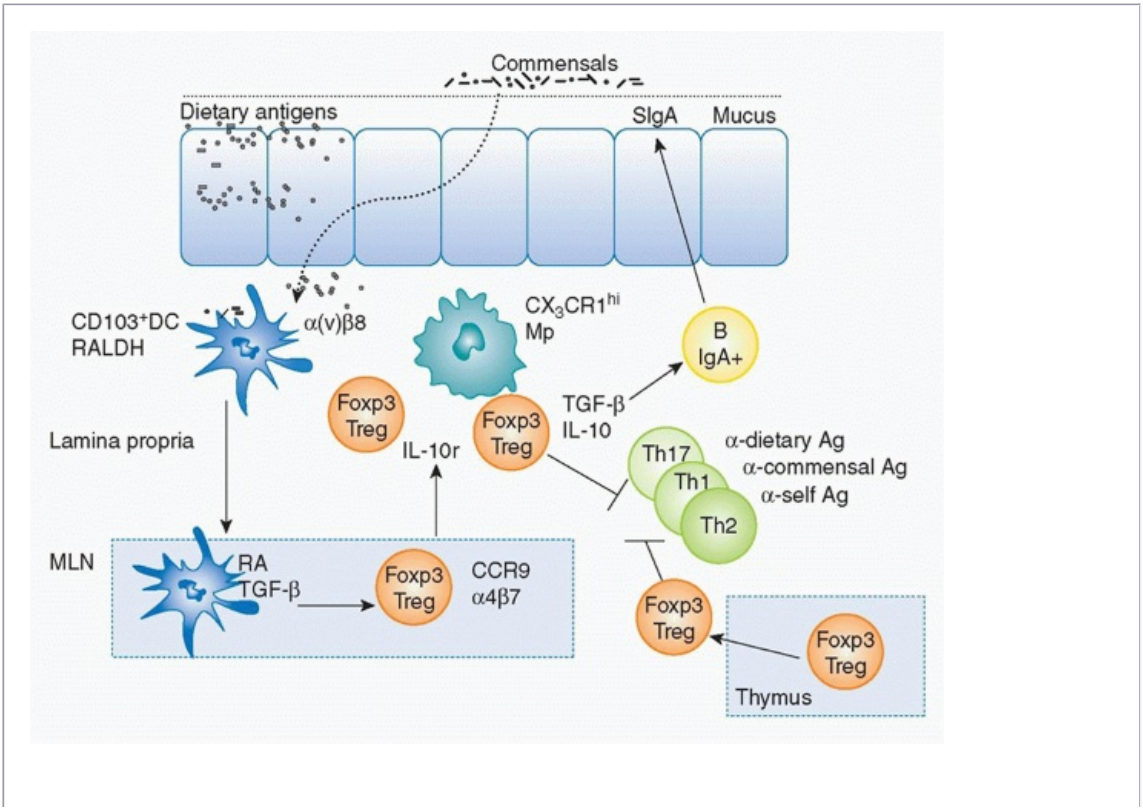

FIG. 34.4. Induction of Foxp3+ Regulatory T (T_{reg}) Cells at Mucosal Sites. Under steady-state conditions, cluster of differentiation (CD)103+ dendritic cells (DCs) uptake directly or indirectly commensals and dietary antigens, and constitutively migrate to the mesenteric lymph node (MLN). In the MLN, based on their capacity to metabolize vitamin A (retinaldehyde dehydrogenase) and produce retinoic acid (RA) and to activate transforming growth factor (TGF)-β (α(v)β8+); CD103+ DCs induce Foxp3+ T_{reg} cells from naïve T cells. Exposure to RA induces gut homing receptor CCR9 and α4β7 that allows the migration of these cells to the gut mucosa. In the gut, Foxp3+ T_{reg}s can be expanded by CX3CR1 macrophages in an interleukin-10-dependent manner. At mucosal sites, inducible T_{reg}s and thymically derived T_{reg}s can limit a range of effector responses directed against dietary or commensal-derived antigens. Further, T_{reg} cells in a TGF-β-dependent manner can also promote immunoglobulin A responses that in turn limit contact with the microbiota.

Thymically Derived Foxp3 Regulatory T Cells

Foxp3-expressing T_{reg} cells were initially described as a unique population of CD4+ T cells that prevent the expansion of self-reactive lymphocytes and subsequent autoimmune disease.[209] Foxp3+ T_{reg} cells maintain both peripheral and mucosal homeostasis throughout the lifespan of the host.[210] These cells are classically defined by their constitutive expression of the IL-2 receptor α-chain (CD25), cytotoxic T-lymphocyte antigen 4, the TNF receptor family member glucocorticoid-induced TNF-receptor-related protein), OX40,[211] CD39, CD73,[212] and high levels of folate receptor.[213] Sustained expression of Foxp3 in T_{reg} cells is critical for maintaining their regulatory function.[214] Several elements and in particular factors downstream of TCR signaling, such as calcium signaling, as well as the NF-κB and Ras-ERK pathways, have been shown to be important for both the induction and sustained expression of Foxp3 expression.[215] For instance, signaling through cooperation of NFAT/Foxp3 complexes and the NF-κb pathway through c-Rel and binding of Runx1-Cbf-β complex to the CNS2 loci in the Foxp3 gene are critical for T_{reg} cell function and sustained Foxp3 expression.[216,217,218,219,220] In addition to these factors, T_{reg} cells are exquisitely dependent on survival factors and in particular IL-2.[221,222,223] Deletion or loss of function mutation in the gene encoding FOXP3 results in fatal inflammatory disease in mice and humans, which is often associated with intestinal inflammation.[224] Indeed, Foxp3 T_{reg}s are highly enriched in the small and large intestine and play a central role in the control of mucosal homeostasis.[224] At these sites, Foxp3+ T_{reg} cells primarily act by controlling harmful

responses against dietary and microbial antigens via their capacity to directly or indirectly control a variety of immune cells including B cells, NK cells, NKT cells, CD4 and CD8+ T cells, as well as monocytes and DCs.[225,226] T_{reg} cells can exert their regulatory function using numerous and highly redundant mechanisms.[226] For example, Foxp3+ T_{reg} cells can

control DC function in a cytotoxic T-lymphocyte antigen 4-dependent manner.[227,228,229] These cells can also produce a variety of immunoregulatory mediators such as adenosine and IL-35.[212,230,231] In some instances, the capacity of Foxp3+ T_{reg}s to control effector responses has been ascribed to their capacity to compete for survival factors such as IL-2.[232] Notably, the cytokine TGF-β also contributes to the suppressive role of T_{reg} cells at mucosal sites. Mice deficient in molecules associated with TGF-β signaling such as Smad3 or Smad4, or in factors required for the cleavage of latent TGF-β, develop mucosal inflammation and colitis.[233,234,235,236] Mucosal T_{reg} cells express large amounts of TGF-β, and this production is required to mediate their regulatory function during experimental colitis.[237] The regulatory role of TGF-β derived from T_{reg}s can be direct, resulting from the capacity of this cytokine to target innate and adaptive cells,[238] as well as indirect. Indeed, TGF-β-dependent stimulation of intestinal IgA responses represents another mechanism by which T_{reg} cells can reinforce intestinal homeostasis.[239]

The cytokine IL-10, and in particular T cell-derived IL-10, plays a nonredundant role in limiting inflammatory responses in the intestine.[240] Mutations in the IL-10 receptor are associated with IBD in humans, and mice deficient in IL-10 or IL-10 receptor develop spontaneous intestinal inflammation.[240,241,242] In particular, the function and maintenance of Foxp3+ T_{reg}s is highly dependent on the presence of this cytokine.[243] The capacity of T_{reg}s to limit mucosal inflammation requires their sustained production of IL-10 at mucosal sites.[244,245,246,247] Further, IL-10 can act directly on Foxp3+ T_{reg} cells to maintain Foxp3 expression and the suppressive capacity of these cells.[243] T_{reg}-derived IL-10 can act on a large array of immune cells, including APCs, by limiting their activation status and capacity to present antigen.[248,249] Further, IL-10-derived T_{reg}s can directly target effector T cells including Th1 and Th17 cells.[250,251]

Regulatory T Cell Plasticity at Mucosal Sites

The capacity of T_{reg} cells to control defined polarized settings can be associated with the acquisition of specific transcription factors. For instance, interferon regulatory factor 4, T-bet, and signal transducer and activator of transcription 3 impact their capacity to control Th2, Th1, and Th17 responses, respectively.[252,253,254] These data support the idea that T_{reg} cells can partially mimic the phenotype of the effector T cells they have to regulate, a property that could endow them with finely tuned homing, survival, or functional properties. However, in some extreme settings, such plasticity can also be associated with the expression of effector cytokines.[255,256,257,258,259] Furthermore, both in humans and mice, T_{reg} cells that reside in the intestine can express RAR-related orphan receptor (ROR)γT, which can be associated with their production of IL-17.[260,261] Importantly, fate-mapping strategies revealed that a significant proportion of IL-17 effector T cells in the gut had expressed Foxp3.[262] To preserve tissue integrity at mucosal sites, mechanisms are in place to sustain T_{reg} cell survival, stability, and plasticity. For instance, in the gut, T_{reg} cells express high levels of the canonical Th2 transcription factor GATA-3 that promotes their

fitness during inflammation and limits their acquisition of effector cytokine production.[263,264]

Induction of Regulatory T Cells at Mucosal Sites

Although immunologic tolerance is likely to be achieved via multiple and redundant mechanisms,[198] over the past few years a role for induced Foxp3 T_{reg} cells has taken center stage in our understanding of this process. Aside from evidence that natural Foxp3+ T_{reg}s arise and mature in the thymus, Foxp3+ T_{reg}s can develop extrathymically under certain conditions. Both naïve murine[207,265] and human T cells express Foxp3 and acquire suppressive activity in vitro following TCR stimulation in the presence of TGF-β.[207,265,266] In vivo, delivery of subimmunogenic doses of antigen as well as endogenous expression of foreign antigens in a lymphopenic environment can also induce peripheral Foxp3+ T_{reg} development.[267,268,269] Notably, the GALT is a preferential site for the peripheral induction of Foxp3+ T_{reg} cells (see Fig. 34.4).[164,208,270,271,272] Mice in which Foxp3+ T_{reg} cells could not be induced failed to establish oral tolerance and develop aberrant Th2 responses at mucosal sites.[269,273,274] Development of iT_{reg} cells requires transcription factor binding to the intronic enhancer element (enhancer-1) of the foxp3 locus, also known as conserved noncoding sequence 1, and is dependent on several soluble mediators, including TGF-β, IL-2, and the vitamin A metabolite RA.[164,184,208,270,275,276,277] In particular, this process is tightly controlled at steady state by the capacity of a specialized population of gut tropic DCs expressing CD103 that activate latent TGF-β and produce RA.[164,208,278] Indeed, CD103+ DCs lacking the TGF-β-activating integrin alpha(v)beta8 fail to induce Foxp3+ T_{reg}s and develop colitis.[279,280,281] Further, because of their high level of RALDH2 expression, CD103+ DCs constitutively produce large amounts of RA.[119,161,162,163,164] RA derived from mucosal DCs serves as a potent cofactor in the generation of iT_{reg} cells in the presence of TGF-β.[164,208,270] In addition to supporting iT_{reg} differentiation, RA-initiated trafficking signals are required for a sustained accumulation of iT_{reg} cells in the gut.[282] Upon arrival at the mucosal site, Foxp3+ T_{reg} cell expansion and therefore proper acquisition of tolerance is propagated through IL-10-mediated interactions with LP resident CX3CR1+ macrophages.[243,278,282]

Foxp3+ T_{reg} conversion may be of particular importance during chronic mucosal infections. It is apparent from mouse models that augmentation of the Foxp3+ T_{reg} compartment is a feature common to a number of chronic infections including helminth infections.[283,284,285,286,287] Foxp3+ T_{reg} can both favor parasite persistence[287] and limit infection-induced immunopathology.[286] A key mechanism by which helminths are thought to regulate the host immune response is through the release of a complex mixture of excreted/secreted products.[288,289,290] Notably, helminth excreted/secreted products contain TGF-β homologues that have the potential to target DC and T cells to support Foxp3+ T_{reg} conversion.[291,292,293]

Immune regulation at mucosal sites is a complex task that requires the coordinated actions of numerous regulatory cells. Indeed, in addition to Foxp3+ T_{reg} cells, the mucosal environment promotes the differentiation of diverse population of T_{reg} cells. For instance, the gut contains a substantial population of CD4+ T cells producing IL-10, referred to as Tr1 cells,[206] that also contribute to the regulation of mucosal responses. Notably, several subsets of DCs with regulatory properties have been described with the capacity to induce IL-10 secretion from T cells or induce oral tolerance under steady-state conditions.[1,294,295,296] Although the precise mechanisms controlling the generation of these cells is not completely understood, various factors have been associated with their induction. For instance, IL-10, IFN-α, and IL-27 as well as inhibitory or costimulatory molecules such as inducible costimulator-L and CD46 can promote Tr1 differentiation.[297,298,299,300] Further, DCs modified by T_{reg} cells can produce IL-27 that in turn can induce the generation of Tr1 cells in a c-MAf-dependent manner.[301,302] Other cytokines produced by CD4+ T cells such as IL-35 have also been associated with mucosal T-cell regulation.[230] The cytokine IL-35 is a recently described heterodimeric cytokine consisting of Epstein-Barr virus-induced gene 3 and the p35 subunit of IL-12.[303] T_{reg}-mediated suppression induces, via the concerted action of IL-10 and IL-35, a population of T_{reg} cells characterized by their production of IL-35, referred to as iTR35. Although human natural T_{reg}s do not express IL-35,[304] human naïve CD4+T cells can be induced to become IL-35-producing T_{reg} cells in the presence of this cytokine or in the presence of virus-exposed DCs.[305]

CONTROL OF BARRIER FUNCTIONS AND MUCOSAL IMMUNITY BY TH17 CELLS

Cytokines of the IL-17 family play a central role in mucosal homeostasis and immunity. In humans and mice, the IL-17 cytokine family consists of six members: IL-17A (also referred to as IL-17), IL-17B, IL-17C, IL-17D, and IL-17E (also referred as IL-25). IL-17A and IL-17F share close sequence homology and can both signal through IL-17 receptor A and IL-17 receptor C expressed on both hematopoietic and nonhematopoietic cells.[306] The cytokine IL-17A is secreted by a subset of CD4+ T cells termed Th17 cells, that can also express the cytokines IL-22, IL-17F, and IL-21.[306] Differentiation of naïve CD4+ T cells into Th17 cells requires TCR recognition of its cognate antigen presented on major histocompatibility complex class II by professional APCs, such as DCs or monocytes. Moreover, Th17-cell differentiation in vivo is driven by the cytokines IL-6 and TGF-β, whereas IL-23 and IL-1β are required for the rapid and sustained activation of these cells.[306,307] Notably, the requirement for IL-6 is specific to mucosal sites and not required for Th17 cell priming in the spleen, whereas IL-1 plays a nonredundant role for priming of Th17 lineage cells in all tissues.[308]

Both Foxp3+ T_{reg}s and Th17 cells are enriched in the gut, and the balance between the two populations is tightly controlled by factors involving the cytokine milieu, the microbiota, and dietary elements.[309] The cytokine TGF-β plays a central role in this balance and in the maintenance of mucosal homeostasis.[309] In addition to the aforementioned role of TGF-β in

promoting T_{reg} cells and oral tolerance, TGF-β is also involved in the generation Th17 cells.[310] The induction of both cell types is highly sensitive to the concentration of TGF-β, with high doses favoring T_{reg} fate and low doses favoring Th17 differentiation.[311] TGF-β can promote this indirectly by suppressing naïve T-cell differentiation into Th1 or Th2 cells. The current understanding is that in the gut, IL-6 in combination with TGF-β leads to the generation of Th17 cells.[265] The transcription factors RORγt and RORα coordinate the differentiation of Th17 cells, whereas signal transducer and activator of transcription 3 (a transcription factor downstream of IL-6 receptor and IL-23 receptor) and the arylhydrocarbon receptor control RORγt expression levels and enhance cytokine secretion.[307,312]

Given the central role of IL-17A and in particular IL-22 in controlling innate barrier responses, these cytokines play a major role in mucosal immunity to microbial challenge and commensal containment[313,314] (see Fig. 34.4). IL-17, in concert with IL-22, which signals through the IL-22 receptor located on nonhematopoietic cells, can induce signal transducer and activator of transcription 3-dependent innate epithelial defense mechanisms.[315] These epithelial responses include a large array of inflammatory cytokines, chemokines, inducible nitric oxide synthase (iNOS), as well as antimicrobial peptides such as RegIIIγ and RegIIIβ, lipocalin-2, and defensins.[310,314,315,316,317] Further, stimulation via IL-17 or IL-22 reinforces tight junctions between enterocytes and enhances epithelial cell proliferation.[315] Indeed, lack of RORγT, IL-17, or IL-22 has been associated with poor containment of commensal bacteria and increased microbial translocation.[314,318] Additionally, via their capacity to act on epithelial cells and modulate inflammatory responses, IL-17 and IL-22 confer protection against a large range of GI pathogens including Helicobacter *pylori*, *Citrobacter rodentium*, and *Salmonella enterica*.[99,316,319,320,321,322,323] Further, *C. rodentium*-induced colitis triggers a robust colonic Th17 response by the second week after infection, which is required for full protection against this pathogen.[317,324] Defects in mucosal integrity have been linked to the pathogenesis of HIV and SIV. During chronic progressive HIV/SIV infection, there is a significant and progressive insult to the intestinal mucosa associated with massive depletion of CD4+ T cells, impaired epithelial structural integrity, and translocation of commensal products.[325,326] Notably, these changes are associated with preferential loss of IL-17- and IL-22-producing CD4+ and CD8+ T cells from mucosal sites, supporting a role for these cytokines in promoting mucosal barrier function during HIV/SIV infections.[327,328,329,330,331] The protective role of IL-17A during infection is also associated with the capacity of this cytokine to induce neutrophil granulopoiesis by stimulating epithelial cells to secrete granulocyte-colony stimulating factor and stimulate the recruitment of neutrophils by local stromal cells.[332] Furthermore, IL-17A and IL-17F can directly activate neutrophil cellular responses at sites of inflammation.[306]

Innate Lymphoid Cells

ILCs represent a novel family of hematopoietic effectors that serve protective or inflammatory roles in innate immune responses. The prototypic members of the ILC family

are NK cells and lymphoid tissue-inducer (LTi) cells that induce the development of lymph

nodes and Peyer patches during fetal life.[333] More recently, the ILC family expanded with the identification of ILC2s also referred as “natural helper” cells, or “nuocytes,” which have been shown to produce IL-5 and IL-13 that are associated with T helper type 2 responses. Additional ILC populations with characteristics of both NK cells and LTi cells have been referred to as NK22 cells, natural cytotoxicity receptor 22 cells, or NK receptorpositive LTi cells. These cells, which are essentially only present in tissue, are characterized by their rapid and abundant production of cytokines. At steady state, the intestinal LP harbors a large number of ILCs and in particular LTi cells, NK22 cells, and ILC2s.[334] Recent evidence demonstrates that these innate cells play a major role in the control of mucosal homeostasis as well as during immune responses.

Origin of Innate Lymphoid Cells

LTi cells arise from common lymphoid progenitors in an IL-7-dependent manner.[335] The inhibitor of DNA binding (Id2), which inhibits E-box proteins and the development of common lymphoid progenitors in B cells, also contributes to the development of LTi cells from common lymphoid progenitors. 336 NK22 cells arise from an α4β7 negative RORγT$_{low}$ precursor, and their final differentiation also depends upon Id2.[337,338] The development of RORγT ILCs depends on the expression of RORγT, whereas the development of ILC2s depends on the expression of RORα.[339,340,341,342] The transcription factors Tox and Runx1 are also required for the generation of LTi cells.[343,344] The arylhydrocarbon receptor is another factor involved in the generation of RORγT ILCs and their production of IL-22.[345,346,347] Notch signaling is additionally required for LTi, NK22 cells, and ILC2 generation.[337,342,345,348]

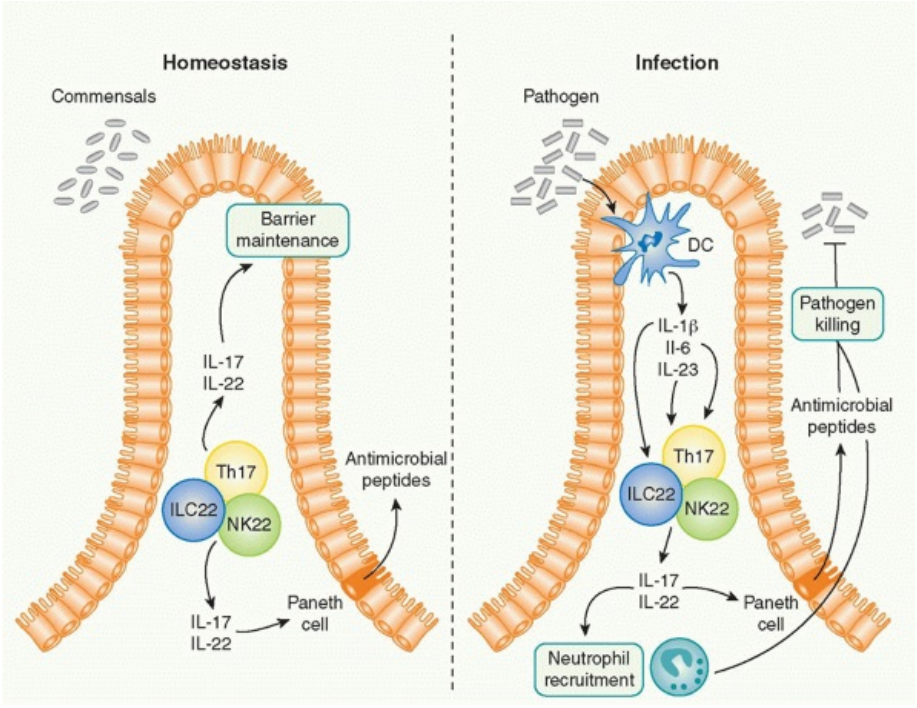

FIG. 34.5. Roles of Innate Lymphoid Cells (ILCs) and Interleukin (IL)-17 Family Members in Mucosal Immunity. Under steady-state conditions, IL-22 and IL-17 from

ILCs and Th17 cells promote commensal containment by promoting barrier integrity and the production of antibacterial peptides. In the context of infections with extracellular or intracellular pathogens, mucosal dendritic cells and recruited cells can release various mediators (transforming growth factor-β, IL-6, IL-23, and IL-1 β) that drive IL-17 and IL-22 production by innate lymphocytes. IL-22 primarily acts on epithelial cells (such as Paneth cells) to promote barrier functions such as enhancing production of antimicrobial peptides that control bacterial growth, whereas IL-17 acts to promote recruitment and activation of neutrophils that prevent bacterial spread.

Roles of Innate Lymphoid Cells in Mucosal Immunity and Homeostasis

RORγt expressing ILCs produce high levels of cytokines such as IL-17 and IL-22 that are known to trigger antimicrobial peptides by epithelial cells.[349,350] For instance, at steady state, RORγT ILCs are the major producers of IL-22 in the intestinal LP (Fig. 34.5).[350] As previously discussed, both IL-17 and IL-22 play a major role in mucosal homeostasis and immunity. 351 In the complete absence of RORγt+ cells, mice are less capable of controlling inflammation and display increased

bacterial translocation.[318] In particular, ILCs expressing RORγt are believed to play a major role in the containment of commensals.[314] ILCs (NK22 and LTi) can contribute to host protection against bacterial invasion via their capacity to produce large amounts of IL-22.[339,352] The expression of IL-22 by these cells is locally controlled by arylhydrocarbon receptor and IL-23.[345,347] As a result of their production of inflammatory cytokines, ILCs can also contribute to mucosal inflammation and pathology. For example, in mice lacking conventional lymphocytes, colitis induced by infection with *Helicobacter hepaticus* is triggered by a subset of Sca-1+ RORγt+ ILCs that express IFNγ and IL-17.[353] Notably, increases in ILCs expressing IL-17 and IL-22 has been reported in patients with IBD.[354] ILC2s, on the other hand, play a major role in responses to helminths.[336,355,356] Notably, helminths can trigger large amounts of IL-33 and IL-25 production by epithelial cells that in turn expand ILC2s and trigger their production of IL-4, IL-6, IL-5, and IL-13.[336,355,356] The production of these cytokines, and IL-13 in particular as a result of its capacity to trigger mucin production by goblet cells, contribute to worm expulsion.[336,355,356] A population similar to ILC2s has been described in the human gut and in the context of chronic respiratory inflammation.[357]

ROLE OF THE MICROBIOTA IN MUCOSAL IMMUNITY

All mucosal surfaces are covered by a diverse and abundant microbiota. The human intestine in particular harbors and is in constant contact with 1,000 trillion microbes, composed of an estimated 4,000 strains.[358,359] Recent studies have changed our perspective of commensal microbes from benign passengers to active participants in both the postnatal development of mucosal and systemic immunity, and in its long-term steady-state function (Fig. 34.6).

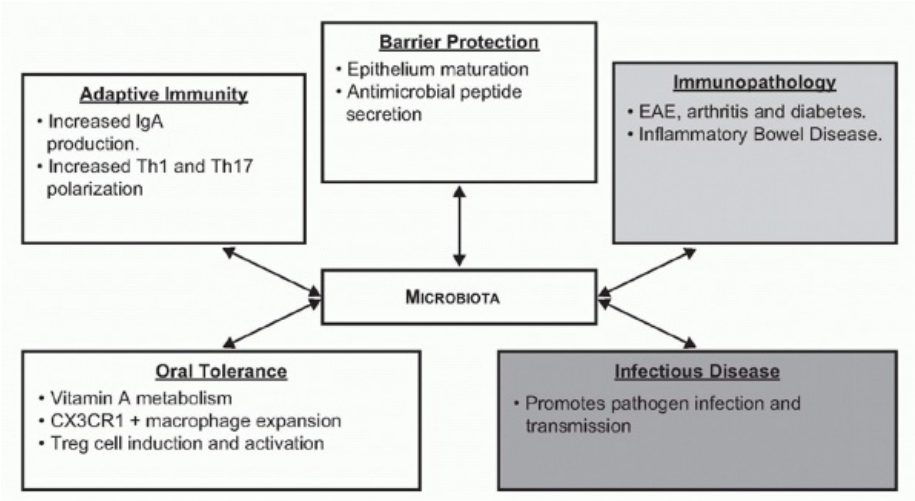

FIG. 34.6. The Microbiota Controls Various Aspects of Mucosal and Systemic Responses. The microbiota controls the intestinal barrier and antimicrobial peptide production. The microbota also controls effector responses by inducing immunoglobulin A and effector T-cell responses and induces oral tolerance through a multitude of mechanisms including toll-like receptor signaling, innate cell population recruitment, vitamin A metabolism, and the activation of regulatory T cells. On the negative side, the microbiota can contribute to inflammatory disorders such as inflammatory bowel disease, arthritis, or diabetes. Further, the microbiota is also required for transmission of a number of viruses and parasites. In a reciprocal manner, each of these response can influence the microbiota composition.

Role of the Microbiota in Mucosal Immune System Development

Various features of host protective structures and innate immunity develop extensively after birth, due in large part to the interaction with the recently acquired microbiota.[360] Studies performed in germ-free animals revealed that the microbiota plays a critical role in secondary lymphoid structure development.[361,362] This also includes fortification of the intestinal barrier through epithelial cell maturation and angiogenesis of a capillary network that facilitates transport of white blood cells.[363,364] The molecular mechanisms responsible for this development remain incompletely understood, but at least in part involves a variety of PRRs, which are capable of detecting microbe-associated molecular patterns including TLRs, NOD-like receptors, and RA inducible gene-like receptors.[365] Several lines of evidence indicate that microbial signals are also responsible for the induction and development of ILFs from cryptopatches in the intestinal tract. Notably, ILFs appear within the first weeks after birth of mice and can eventually number in the hundreds.[3] The current model hypothesizes that gram-negative bacterial-derived peptidoglycans are sensed by NOD1 expressed on intestinal epithelial cells.[366] The intestinal epithelial cells subsequently express CCL20 and β-defensin 3, which

activate LTi cells leading to the formation of ILFs.[367,368,369] An alternative but not exclusive model proposes that the activated LTi cells would engage lymphoid tissue organizer cells,

which are mesenchymal in origin, to express CCL20 for the recruitment for B cells. Other critical components of host defense are represented by the mucus layer and antimicrobial peptides both under the tight control of the flora, as discussed previously.[17,370,371] Engagement of PRRs by commensally derived products induces expression of a variety of antimicrobial peptides including RegIIIγ, which are critical to prevent translocation of bacteria through the rest of the host tissue.[6,8] Similarly, as previously discussed, the PRR NOD2 controls expression of a subset of α-defensins and cryptidins by Paneth cells.[11]

Control of Effector and Regulatory Responses by Commensals

Although mucosal surfaces have to constitutively integrate a multitude of microbial-derived signals, experimental evidence suggests that defined bacteria or microbial products can play a dominant role in the induction of a distinct class of immune responses (see Fig. 34.6). At steady state, the gut is home to a large number of lymphocytes, many of them with the potential to produce cytokines such as IL-17, IL-22, IFNγ, and/or IL-10.[185,372] The flora tightly controls this constitutive production of cytokines, as germ-free mice show extensive deficiencies in basal cytokine production.[187,372] Further, in the absence of flora, the CD4+ T-cell population is diminished, disproportionately affecting Th1 and Th17 cells, although T_{reg} frequencies are maintained or increased in the small intestine.[185,372] Colonization of germ-free mice with complex microbiota orchestrates a broad spectrum of T helper (Th1, Th17) and T_{reg} cell responses.[373] Recent advances revealed that the microbiota can directly promote the induction and/or activation of T_{reg} cells at mucosal sites. The first demonstration that a single symbiont molecule could promote regulatory responses was provided by the identification of polysaccharide A (PSA), which is produced by a prominent human symbiont *Bacteroides fragilis*.[362] Colonization of germ-free mice with *B. fragilis* or treatment with purified PSA directs immune system development.[362] Furthermore, via PSA expression, *B. fragilis* can protect mice from experimental colitis induced by *Helicobacter hepaticus*, a commensal bacterium with pathogenic potential.[374] This protective activity was associated with the capacity of PSA to induce or expand IL-10-producing CD4+ T cells.[374,375,376] Additionally, *B. fragilis* was able to promote T_{reg} cell function via a TLR2-dependent mechanism, a phenomenon associated with its capacity to limit IL-17 responses.[377] Induction of T_{reg} cells is not restricted to *B. fragilis* as the presence of *Clostridium* species also promotes T_{reg} cell accumulation via, at least in part, its capacity to create a TGF-β-rich environment. 378 This induction of T_{reg} function requires TCR-mediated signals, MyD88, and Ticam-1, as well as signaling via IL-10 receptor.[378] Based on the fundamental role of T_{reg} cells in maintaining mucosal homeostasis, it is likely that rather than being restricted to defined bacteria, most indigenous flora have evolved to favor this aspect of the regulatory network. Notably, colonic T_{reg} cells have a TCR usage distinct from those at other locations and a fraction of them can react preferentially to antigens derived from the commensal population.[379] These results suggest a major role for the microbiota in shaping the repertoire of tissue resident T_{reg} cells and in the maintenance of host-microbe mutualism at barrier sites.

Defined groups of bacteria have been associated with specific immunologic signatures. For example, mice lacking a key group of microbes (SFB) have severely decreased numbers of mucosal Th17 cells in the small intestine, although the T_{reg} compartment is expanded in this environment.[372,373,380] Remarkably, SFB in association with a specific-pathogen-free flora, can trigger intestinal inflammation in the lymphopenic host.[381] Indeed, SFB colonization of germ-free mice leads to increased T_{reg} cells as well as IFNγ- and IL-17-producing CD4 T cells in the small intestine and colon.[372,373,380] However, absolute numbers of these cells are not restored to the level of those mice with a complete microbiota, suggesting that as expected, SFB may be sufficient for such changes but needs to synergize with other microorganisms to coordinate the full maturation of the intestinal immune system. Scanning electron microscopy has revealed that SFB adhere tightly to Peyer patches and epithelial cells of the small intestine, a property that likely explains its dominant role on the mucosal immune system.

Specific commensal-derived ligands can also control effector T-cell balance at mucosal sites. Many of these ligands signal through the toll-like family of receptors.[382] TLRs are widely expressed by cells of hematopoietic origin, as well as nonhematopoietic cells, including the epithelial cells lining the intestinal tract.[383] Bacteria flagellin is a structural protein that forms the main portion of flagella and promotes bacterial chemotaxis, adhesion, and invasion of host tissue in the context of pathogenic bacteria. Many commensal bacteria also have flagella, and several lines of evidence indicate that interaction of commensal flagellin with TLR5 plays an important role in the GI tract. TLR5 is highly expressed by LP DCs from the small intestine and in particular on $CD11c^{hi}CD11b^{hi}$ DCs.[68,144] Flagellin-stimulated LP DCs express IL-6, IL-12, chemokines, prostaglandin, and antimicrobial peptides.[144] Further, LP DCs induce RORγT+ Th17 cells as well as Th1 cells from naïve CD4+ T cells in response to flagellin in vitro.[68] Of note, flagellin also represents an immunodominant antigen in patients with Crohn disease.[384] Several lines of evidence also support the idea that commensal derived flagellin plays a regulatory role in the GI tract as mice lacking TLR5 spontaneously develop colitis. 385 Furthermore, mice lacking TLR5 overexpress genes associated with innate and adaptive immunity, contributing not only to colitis development but also to their enhanced protection against infection to enteric pathogens such as *Salmonella*.[386]

TLR9 recognizes unmethylated cytosine phosphate guanosine dinucleotides, which are abundant in prokaryotic DNA found in intestinal flora. Using synthesized sequences containing cytosine phosphate guanosine, previous studies have shown that engagement of TLR9 expressed on DCs, T_{reg}s, and conventional T cells can limit T_{reg} cell suppressive

function.[387,388] In mice, gut flora DNA plays a major role in intestinal homeostasis through TLR9 engagement,[145] and constitutive interactions between gut flora DNA and TLR9 in the GI tract can act as an immunologic adjuvant and critically controls the balance between T_{reg} and effector T cells.[145] Notably, commensal bacteria also contain suppressive motifs that account for the differential effect of DNA from defined bacteria species.[389] It has also been noted that bacterially infected apoptotic cells trigger DC production of IL-6 and TGF-β, which are critical for Th17 induction in mice, through recognition of phosphatidylserine exposed on

the apoptotic cells.[390]

Adenosine 5′-triphosphate (ATP) can modulate immune cell function by means of activation of the ATP receptors, P2X and P2Y, and commensal bacteria have been shown to generate large amounts of ATP.[391] Consistent with this observation, germ-free animals have reduced ATP in their feces compared to conventional mice. ATP derived from commensal bacteria can activate LP DCs, leading to the differentiation of Th17 cells.[391] The critical role of commensalderived ATP is further underscored by the observation that administration of ATP exacerbates a T-cell-mediated colitis model with enhanced Th17 differentiation.[391] These data provide evidence that commensal-derived metabolites could directly affect T-cell polarization.

By virtue of their capacity to control immune responses, promising results have been obtained with probiotics— bacteria known to confer a health benefit to the host—in the treatment of human inflammatory diseases of the intestine and in the prevention and treatment of atopic eczema in neonates and infants. Some of the effect of probiotics is believed to be associated with the induction or expansion of T_{reg} cells. For instance, in mice, treatment with the probiotic *Lactobacillus* and/or *Bifidobacterium* suppressed trinitrobenzene sulfonic acid (TNBS)-induced colitis,[392,393] as well as allergic responses possibly resulting from the induction of TGF-β production[394] and stimulation of T_{reg} cells that are able to suppress allergic responses.[395] There is also good evidence of probiotic modulation of DCs toward a proregulatory phenotype.[396,397] Multiple reports indicate that bacteria with known anti-inflammatory properties such as *Bifidiobacteria*, *Lactobacilli*, and *Streptococcus salivarius* are capable of promoting both T_{reg}- and IL-10-producing FoxP3- Tr1 cells.[375,397,398,399,400,401]

Role of Commensals in Mucosal Inflammation

A role for the gut microbiota is now well established in the pathophysiology of mucosal inflammatory diseases and in particular IBD.[402] Despite differences in the target site and inflammatory processes associated with the two forms of IBD, compelling evidence generated from studies of human patients and experimental models indicate that both disorders depend upon the presence of the microbiota.[403] Early studies demonstrated that the critical effector cell in most models of experimental colitis that involve the adaptive immune system is the CD4 T cell.[403,404,405] One striking experimental example of the influence of the flora on mucosal disorders is that of mice that develop a spontaneous and transferrable form of ulcerative colitis.[406] Deficiency of T-bet in the innate immune system (T-bet-/- RAG2-/- ulcerative colitis [TRUC] mice) leads to exaggerated TNF production by DCs, which together with the absence of T_{reg} cells creates a chronic inflammatory state that modulates the composition of the microflora and eventually leads to the development of colorectal cancer.[406,407] Colitis in TRUC mice is caused by increased production of TNF-α by colonic DCs as restoration of T-bet expression specifically in the CD11c+ populations allowed for reduced TNF-α production and prevented neoplasia and excessive inflammation.[407] Interestingly, transfer of the microbiota from TRUC mice into wild-type recipients also

transfers the colitis, indicating that microbiota shifts are likely the cause of disease. Two species, *Proteus mirabilis* and *Klebsiella pneumoniae*, are found at increased frequency in TRUC mice and can induce colitis in specific-pathogen-free wild-type mice.[408]

Inflammasomes, complexes that function as sensors of endogenous or exogenous damage-associated molecular patterns, can also play a role in controlling the delicate homeostatic relationship with the flora.[409] Deficiency of NLRP6 inflammasome from colonic cells results in reduced IL-18 levels and shifts in microbiota composition characterized by expansion of the bacterial phyla *Bacteroidetes* (*Prevotellaceae*) and TM7. Further, these mice spontaneously develop colitis and are more susceptible to chemically induced colitis.[409] As observed in TRUC mice, colitogenic potential of this microbiota is transferable to neonatal or adult wild-type mice.

The gut also represents one of the primary sites of exposure to pathogenic microbes. In this environment, the proinflammatory properties of commensals can directly contribute to the pathogenesis of mucosal infection. One of the first examples of this scenario was demonstrated in an oral model of *Toxoplasma gondii* infection in which pathology is associated with exuberant sensing of commensals via TLRs.[410,411] This infection is also characterized by a reduction of the flora complexity and increase in gram-negative bacteria that in turn exacerbates the pathologic process.[410] Disruption of the microbiota composition has also been documented in the context of other enteric infections such as *Citrobacter rodentium* or *Salmonella typhimurium*.[412,413] In addition to microbial changes induced by infection, as previously discussed, accumulating evidence indicates that host genotypes reciprocally affect microbiota composition, which in turn alters host responses.

Bystander Effects of Gut Commensals on Peripheral Responses

One consequence of the immune system's reliance on microflora for optimal development and immunoregulation is that antibiotic therapies may result in unintended activation of immune effector mechanisms. In experimental models, antibiotic treatment renders mice more susceptible to induction of food allergy[414] as well as allergic airway inflammation.[415] For the human population, antibiotics are seen as major modifiers of beneficial human-microbe interactions[416] superimposed on alterations caused by other exogenous factors including urbanization, global travel, and dietary changes.[417] The acute effects of antibiotic treatment on the

native gut microbiota range from self-limiting diarrhea to life-threatening pseudomembranous colitis induced by bacteria filling the niches created by the reduction in bacterial diversity.[418] The long-term consequences of such perturbations for human-microbial symbiosis are more difficult to discern, but chronic conditions such as asthma and atopic disease have been associated with childhood antibiotic use and an altered intestinal microbiota.[419,420,421] More direct evidence of the systemic influence of the microbiota on peripheral immune responses were recently provided. Indeed, despite being confined to mucosal surfaces, gut microbiota can impact immune responses at distal sites. For instance, peptidoglycan from radio-labeled *E. coli* could be found in the serum and improved killing of *Streptococcus pneumoniae* and *Staphylococcus aureus* by bone-marrow derived neutrophils in a NOD1-

dependent manner.[422] Importantly, microbe-associated molecular patterns can be detected in the bone marrow where their signaling can alter hematopoiesis.[422] Further, bacterial products can induce monocyte emigration in response to very low levels of TLR ligands.[423] Whether the diffusion of commensal products into the bloodstream contributes to steady-state monocyte egress from the bone marrow remains to be determined. The microbiota also has the ability to regulate immune responses against various infections as mice given broad-spectrum antibiotics mounted a severely blunted T- and B-cell response against an intranasal infection with the A/PR8 strain of influenza, resulting in elevated viral titers.[424]

In addition to protective immunity, gut commensals can also alter autoimmune conditions. Mice lacking intestinal microbiota develop less severe disease in models of arthritis and experimental autoimmune encephalomyelitis.[376,425] Moreover, colonization with SFB promotes autoimmune arthritis through the induction of antigen-specific Th17 cells, which promote autoantibody production via B-cell expansion in germinal centers. A recent study further showed that recruitment and activation of autoantibody-producing B cells from the endogenous immune repertoire depends on availability of the target autoantigen and commensal microbiota.[426] The commensal microbiota can also help to reduce inflammation as colonization of mice with *B. fragilis* results in the expansion of IL-10-producing T_{reg} cells, which limit the proinflammatory mechanisms of experimental autoimmune encephalomyelitis in a TLR2-dependent manner.[376] Indeed, diabetes in the nonobese diabetic mouse model has been related to their housing conditions and presumably their commensal flora. When nonobese diabetic mice were genetically deficient for MyD88, induction of disease was delayed and associated with distinct floral changes to MyD88 intact controls.[427] Further, a recent study indicates that nonobese diabetic mice naturally colonized with SFB have a correlative protection from diabetes.[428] Finally, single chain fatty acids, such as acetate, are one of the most important metabolites provided by commensal organisms.[429] It has been shown that the recognition of these single chain fatty acids by innate immune cells is critical for the regulation of inflammation in response to not only intestinal injury but also in models of arthritis and allergy.[430]

MUCOSAL INFLAMMATION

Inflammatory Bowel Diseases

Chronic inflammatory syndromes arise as a consequence of genetic polymorphisms in concert with accumulating environmental exposure to toxins, pathogens, and diet.[431,432,433] IBDs refer to chronic inflammatory disorders affecting the GI tract.[434] There are two main clinical forms of IBD: Crohn disease, which can affect any part of the GI tract, and ulcerative colitis, the pathology of which is restricted to the colonic mucosa.[434] The etiology of these disorders is complex and believed to be the consequence of genetic factors, the host immune system, and environmental triggers such as the microbiota.[435] Individual genome-wide association study scans have identified more than 70 susceptibility loci in Crohn disease and 47 susceptibility loci for ulcerative colitis.[436,437,438,439,440] Some of these risk factors are associated with innate defenses. For instance, patients with Crohn

disease and those with NOD susceptibility have reduced α-defensin expression. Further, genetic variants in the transcription factor TCF4 that is involved in Paneth cell maturation and function have been associated with ileal Crohn diseases.[441] Paneth cell defects have also been associated with IBD. Patients who are homozygous for the T300A disease-risk allele of the autophagy gene ATG16L1 as well as in mice hypomorphic for ATG16L1 are more prone to inflammatory disorders.[442]

Effectors of Mucosal Inflammation: Tumor Necrosis Factor-α, Interleukin-23, TH1, Th17, and Nucleotide-Binding Oligomerization Domain-Containing Protein-Like Receptors

Multiple inflammatory mediators required to sustain mucosal homeostasis and immunity have also been associated with the etiology of IBD (Fig. 34.7). TNF-α, for instance, can contribute to mucosal tissue damage, and anti-TNF-α antibody treatment is highly effective in a large proportion of patients with IBD.[443] The cytokine IL-23 has been associated with a variety of autoimmune disorders and in particular intestinal inflammation.[444] Genome-wide association studies reported a strong association of polymorphisms in IL-23R and IL-12B gene loci with Crohn disease and ulcerative colitis.[436,437,438,439,440] The cytokine IL-23 can promote mucosal inflammation via its capacity to enhance proliferation of effector T cells, reduce Foxp3 T_{reg} cell induction, and induce pathogenic IL-17/IFNγ CD4+ T cells.[445] Notably, IL-17/IFNγ CD4+ T cells responding to IL-23 have been isolated from the guts of patients with Crohn disease,[446] and in phase II clinical trials anti-IL-12p40 monoclonal antibodies that target both IL-12 and IL-23 have shown clinical efficacy in a subset of patients.[443]

Studies in T-cell transfer colitis suggest that IL-17A and IL-17F can have redundant proinflammatory effects in the gut.[447] Th17 cells are highly enriched in the inflamed mucosa of mice and patients with IBD. Notably, chronic inflammation in the context of high levels of IL-17 has also been associated with the emergence of colitis-associated cancer.[448] A role for IL-22 in mucosal inflammation has been

recently proposed in the etiology of IBD. In patients with Crohn disease, high serum levels of this cytokine correlates with increased disease activity and susceptibility associated with IL-23R polymorphism.[449] Association of IBD with polymorphisms in NLRP3 and IL-18 receptor accessory protein strongly suggest role for IL-1β and IL-18 in mucosal inflammation.[449] Indeed, both IL-1β and IL-18 are increased in patients with IBD, and mice deficient in IL-18 are resistant to colitis.[450,451] In summary, although it is clear that various factors contribute to mucosal inflammation, much remains to be learned in order to understand the relative contributions and contextual role of these cytokines in mucosal inflammatory disorders (see Fig. 34.7).

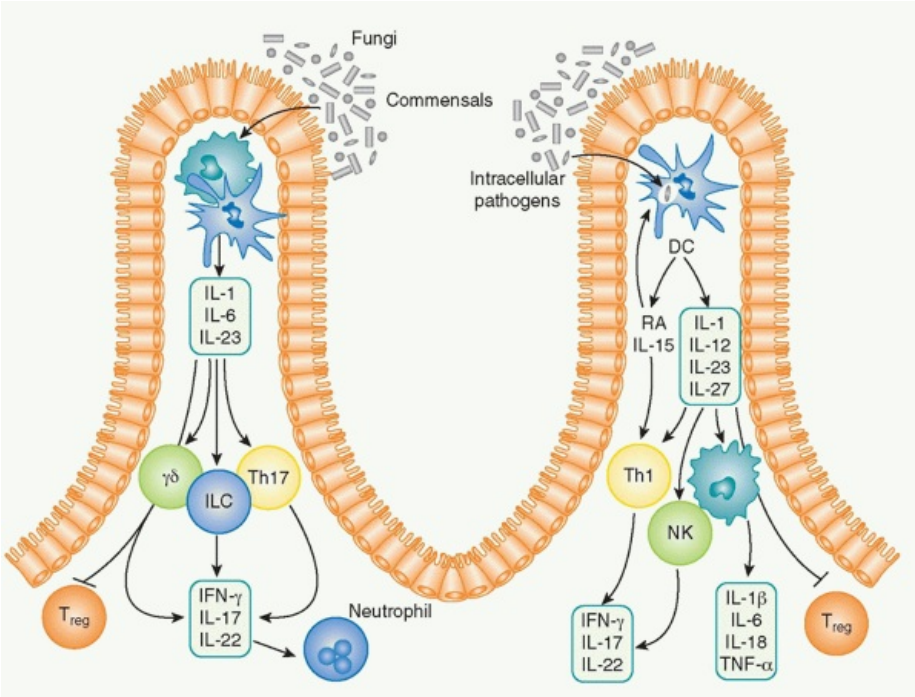

FIG. 34.7. Induction of Mucosal Effector Responses during Infection. Under inflammatory conditions, resident and recruited mononuclear phagocytes (MPs) produce inflammatory cytokines in a contextual-dependent manner. In responses to fungi and commensal bacteria, interleukin (IL)-1, IL-6, and IL-23 released by these cells can promote IL-17 and IL-22 responses by γδ T cells, innate lymphoid cells, and Th17 cells. These cytokines can act to promote intestinal barrier function as well as to promote the recruitment and activation of neutrophils. In the context of intracellular infections, mucosal and recruited MPs can produce various cytokines including IL-12 that can directly activate NK cells and promote the induction of Th1 responses. Effector T cells can in turn promote the effector function of resident macrophages. Commensal bacteria can promote both types of immune responses and in both settings, inflammatory cytokines limit regulatory T-cell induction. Overexpression of these effector responses combined with impairment of regulatory responses can lead to tissue damage and mucosal inflammation.

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REFERENCES

1. Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol*. 2003;3(4):331-341.

2. Moghaddami M, Cummins A, Mayrhofer G. Lymphocyte-filled villi: comparison with other lymphoid aggregations in the mucosa of the human small intestine.

Gastroenterology. 1998;115(6):1414-1425.

3. Hamada H, et al. Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *J Immunol* . 2002;168(1):57-64.

4. Eberl G, Lochner M. The development of intestinal lymphoid tissues at the interface of self and microbiota. *Mucosal Immunol* . 2009;2(6):478-485.

5. Miller H, et al. Intestinal M cells: the fallible sentinels? *World J Gastroenterol* . 2007;13(10):1477-1486.

6. Hooper LV, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol* . 2010;10(3): 159-169.

7. Flo TH, et al. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature*. 2004;432(7019):917-921.

8. Cash HL, et al. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science*. 2006;313(5790):1126-1130.

9. Mukherjee S, et al. Regulation of C-type lectin antimicrobial activity by a flexible N-terminal prosegment. *J Biol Chem*. 2009;284(8):4881-4888.

10. Ismail AS, et al. Gammadelta intraepithelial lymphocytes are essential mediators of host-microbial homeostasis at the intestinal mucosal surface. *Proc Natl Acad Sci U S A* . 2011;108(21):8743-8748.

11. Kobayashi KS, et al. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science*. 2005;307(5710):731-734.

12. Meyer-Hoffert U, et al. Secreted enteric antimicrobial activity localises to the mucus surface layer. *Gut*. 2008;57(6):764-771.

13. Vaishnava S, et al. The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. *Science*. 2011;334(6053):255-258.

14. McGuckin MA, et al. Mucin dynamics and enteric pathogens. *Nat Rev Microbiol* . 2011;9(4):265-278.

15. Thornton DJ, Rousseau K, McGuckin MA. Structure and function of the polymeric mucins in airways mucus. *Ann Rev Physiol* . 2008;70:459-486.

16. Atuma C, et al. The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. *Am J Physiol* . 2001;280(5):G922-G929.

17. Johansson ME, et al. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A* . 2008;105(39):15064-15069.

18. Johansson ME, Larsson JM, Hansson GC. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. *Proc Natl Acad Sci U S A* . 2011;108(suppl 1):4659-4665.

19. Ambort D, et al. Function of the CysD domain of the gel-forming MUC2 mucin. *Biochem J*. 2011;436(1):61-70.

20. Hansson GC, et al. Molecular cloning of a cDNA coding for a region of an apoprotein from the “insoluble” mucin complex of rat small intestine. *Biochem Biophys Res Comm*. 1994;198(1):181-190.

21. Qin J, et al. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature*. 2010;464(7285):59-65.

22. van Passel MW, et al. The genome of Akkermansia muciniphila, a dedicated intestinal mucin degrader, and its use in exploring intestinal metagenomes. *PLoS One*. 2011;6(3):e16876.

23. Delacroix DL, et al. Selective transport of polymeric immunoglobulin A in bile. Quantitative relationships of monomeric and polymeric immunoglobulin A, immunoglobulin M, and other proteins in serum, bile, and saliva. *J Clin Invest*. 1982;70(2):230-241.

24. Chodirker WB, Tomasi TB Jr. Gamma-globulins: quantitative relationships in human serum and nonvascular fluids. *Science*. 1963;142(3595): 1080-1081.

25. Tomasi TB Jr, et al. Characteristics of an immune system common to certain external secretions. *J Exp Med*. 1965;121:101-124.

26. Brandtzaeg P, et al. The B-cell system of human mucosae and exocrine glands. *Immunol Rev*. 1999;171:45-87.

27. Chen K, Cerutti A. New insights into the enigma of immunoglobulin D. *Immunol Rev*. 2010;237(1):160-179.

28. Preud'homme JL, et al. Structural and functional properties of membrane and secreted IgD. *Mol Immunol*. 2000;37(15):871-887.

29. Gould HJ, Sutton BJ. IgE in allergy and asthma today. *Nat Rev Immunol* . 2008;8(3):205-217.

30. Cerutti A, et al. Regulation of mucosal IgA responses: lessons from primary

immunodeficiencies. *Ann N Y Acad Sci* . 2011;1238:132-144.

31. Chen K, Cerutti A. AIDing the pursuit of IgA diversity. *Nat Immunol* . 2011;12(3):197-198.

32. Cerutti A. The regulation of IgA class switching. *Nat Rev Immunol* . 2008;8(6):421-434.

33. Hornquist E, Lycke N. Cholera toxin increases T lymphocyte responses to unrelated antigens. *Adv Exp Med Biol* . 1995;371B:1507-1512.

34. Fernandez MI, et al. Anti-inflammatory role for intracellular dimeric immunoglobulin a by neutralization of lipopolysaccharide in epithelial cells. *Immunity*. 2003;18(6):739-749.

35. Martinoli C, Chiavelli A, Rescigno M. Entry route of Salmonella typhimurium directs the type of induced immune response. *Immunity*. 2007;27(6):975-984.

36. Wade TK, Wade WF. Variable gene famiusage of protective and non-protective anti-Vibrio cholerae O1 LPS antibody heavy chains. *Microbiol Immunol* . 2008;52(12):611-620.

37. Boullier S, et al. Secretory IgA-mediated neutralization of Shigella flexneri prevents intestinal tissue destruction by down-regulating inflammatory circuits. *J Immunol* . 2009;183(9):5879-5885.

38. Wei M, et al. Mice carrying a knock-in mutation of Aicda resulting in a defect in somatic hypermutation have impaired gut homeostasis and compromised mucosal defense. *Nat Immunol* . 2011;12(3): 264-270.

39. Brandtzaeg P. Mucosal immunity: integration between mother and the breast-fed infant. *Vaccine*. 2003;21(24):3382-3388.

40. Kramer DR, Cebra JJ. Early appearance of “natural” mucosal IgA responses and germinal centers in suckling mice developing in the absence of maternal antibodies. *J Immunol* . 1995;154(5):2051-2062.

41. Benveniste J, Lespinats G, Salomon J. Serum and secretory IgA in axenic and holoxenic mice. *J Immunol* . 1971;107(6):1656-1662.

42. Benveniste J, et al. Immunoglobulins in intact, immunized, and contaminated axenic mice: study of serum IgA. *J Immunol* . 1971;107(6): 1647-1655.

43. Smith K, McCoy KD, Macpherson AJ. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Sem Immunol* . 2007;19(2):59-69.

44. Shroff KE, Meslin K, Cebra JJ. Commensal enteric bacteria engender a self-limiting humoral mucosal immune response while permanently colonizing the gut. *Infect Immunity*. 1995;63(10):3904-3913.

45. Hamada H, et al. Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *J Immunol*. 2002;168(1):57-64.

46. Tsuji M, et al. Requirement for lymphoid tissue-inducer cells in isolated follicle formation and T cell-independent immunoglobulin A generation in the gut. *Immunity*. 2008;29(2):261-271.

47. Crouch EE, et al. Regulation of AID expression in the immune response. *J Exp Med*. 2007;204(5):1145-1156.

48. Uematsu S, et al. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. *Nat Immunol*. 2008;9(7):769-776.

49. Mostov KE. Transepithelial transport of immunoglobulins. *Ann Rev Immunol*. 1994;12:63-84.

50. Phalipon A, Corthesy B. Novel functions of the polymeric Ig receptor: well beyond transport of immunoglobulins. *Trends Immunol*. 2003;24(2):55-58.

51. Favre L, Spertini F, Corthesy B. Secretory IgA possesses intrinsic modulatory properties stimulating mucosal and systemic immune responses. *J Immunol*. 2005;175(5):2793-2800.

52. Monteiro RC, Van De Winkel JG. IgA Fc receptors. *Ann Rev Immunol*. 2003;21:177-204.

53. Pasquier B, et al. Identification of FcalphaRI as an inhibitory receptor that controls inflammation: dual role of FcRgamma ITAM. *Immunity*. 2005;22(1):31-42.

54. Macpherson AJ, et al. The immune geography of IgA induction and function. *Mucosal Immunol*. 2008;1(1):11-22.

55. Fagarasan S, Honjo T. Intestinal IgA synthesis: regulation of front-line body defences. *Nat Rev Immunol*. 2003;3(1):63-72.

56. Suzuki K, et al. The sensing of environmental stimuli by follicular dendritic cells promotes immunoglobulin A generation in the gut. *Immunity*. 2010;33(1):71-83.

57. Coffman RL, Lebman DA, Shrader B. Transforming growth factor beta specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. *J Exp*

Med. 1989;170(3):1039-1044.

58. Shi MJ, Stavnezer J. CBF alpha3 (AML2) is induced by TGF-beta1 to bind and activate the mouse germline Ig alpha promoter. *J Immunol*. 1998;161(12):6751-6760.

59. Yang X, et al. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. *EMBO J*. 1999;18(5):1280-1291.

60. Pardali E, et al. Smad and AML proteins synergistically confer transforming growth factor beta 1 responsiveness to human germ-line IgA genes. *J Biol Chem*. 2000;275(5):3552-3560.

61. Zhang Y, Derynck R. Transcriptional regulation of the transforming growth factor-beta-inducible mouse germ line Ig alpha constant region gene by functional cooperation of Smad, CREB, and AML family members. *J Biol Chem*. 2000;275(22):16979-16985.

62. Park SR, Lee JH, Kim PH. Smad3 and Smad4 mediate transforming growth factor-beta1-induced IgA expression in murine B lymphocytes. *Eur J Immunol* . 2001;31(6):1706-1715.

63. Miyazono K, Maeda S, Imamura T. Coordinate regulation of cell growth and differentiation by TGF-beta superfamily and Runx proteins. *Oncogene*. 2004;23(24):4232-4237.

64. Cazac BB, Roes J. TGF-beta receptor controls B cell responsiveness and induction of IgA in vivo. *Immunity*. 2000;13(4):443-451.

65. Litinskiy MB, et al. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. *Nat Immunol*. 2002;3(9):822-829.

66. He B, et al. The transmembrane activator TACI triggers immunoglobulin class switching by activating B cells through the adaptor MyD88. *Nat Immunol*. 2010;11(9):836-845.

67. Mora JR, et al. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science*. 2006;314(5802):1157-1160.

68. Uematsu S, et al. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. *Nat Immunol*. 2008; 9(7):769-776.

69. Endt K, et al. The microbiota mediates pathogen clearance from the gut lumen after non-typhoidal Salmonella diarrhea. *PLoS Pathogens*. 2010;6(9):e1001097.

70. Iankov ID, et al. Protective efficacy of IgA monoclonal antibodies to O and H antigens in a mouse model of intranasal challenge with Salmonella enterica serotype Enteritidis.

Microbes Infect. 2004;6(10):901-910.

71. Suzuki K, et al. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. *Proc Natl Acad Sci U S A* . 2004;101(7):1981-1986.

72. Hapfelmeier S, et al. Reversible microbial colonization of germfree mice reveals the dynamics of IgA immune responses. *Science*. 2010;328(5986):1705-1709.

73. Cheroutre H, Lambolez F, Mucida D. The light and dark sides of intestinal intraepithelial lymphocytes. *Nat Rev Immunol* . 2011;11(7):445-456.

74. Beagley KW, et al. Differences in intraepithelial lymphocyte T cell subsets isolated from murine small versus large intestine. *J Immunol* . 1995;154(11):5611-5619.

75. Cheroutre H, Lambolez F, Mucida D. The light and dark sides of intestinal intraepithelial lymphocytes. *Nat Rev Immunol* . 2011;11(7): 445-456.

76. Lundqvist C, et al. Intra-epithelial lymphocytes. Evidence for regional specialization and extrathymic T cell maturation in the human gut epithelium. *Int Immunol* . 1995;7(9):1473-1487.

77. Ebert EC. Interleukin 15 is a potent stimulant of intraepithelial lymphocytes. *Gastroenterology*. 1998;115(6):1439-1445.

78. Guy-Grand D, et al. Complexity of the mouse gut T cell immune system: identification of two distinct natural killer T cell intraepithelial lineages. *Eur J Immunol* . 1996;26(9):2248-2256.

79. Cheroutre H, Lambolez F. The thymus chapter in the life of gut-specific intra epithelial lymphocytes. *Curr Opin Immunol* . 2008;20(2):185-191.

80. Leishman AJ, et al. Precursors of functional MHC class I- or class IIrestricted CD8alphaalpha(+) T cells are positively selected in the thymus by agonist self-peptides. *Immunity*. 2002;16(3):355-364.

81. Gangadharan D, et al. Identification of pre- and postselection TCRalphabeta+ intraepithelial lymphocyte precursors in the thymus. *Immunity*. 2006;25(4): 631-641.

82. Jensen KD, et al. Thymic selection determines gammadelta T cell effector fate: antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon gamma. *Immunity*. 2008;29(1):90-100.

83. Wurbel, M.A., et al., The chemokine TECK is expressed by thymic and intestinal epithelial cells and attracts double- and single-positive thymocytes expressing the TECK

receptor CCR9. *Eur J Immunol* . 2000. 30(1): p. 262-71.

84. Zabel BA, et al. Human G protein-coupled receptor GPR-9-6/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis. *J Exp Med*. 1999;190(9):1241-1256.

85. Cepek KL, et al. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. *Nature*. 1994;372(6502):190-193.

86. Konkel JE, et al. Control of the development of CD8alphaalpha+ intestinal intraepithelial lymphocytes by TGF-beta. *Nat Immunol* . 2011;12(4):312-319.

87. Probert CS, et al. Repertoire of the alpha beta T-cell receptor in the intestine. *Immunol Rev*. 2007;215:215-225.

88. Bruce D, Cantorna MT. Intrinsic requirement for the vitamin D receptor in the development of CD8alphaalpha-expressing T cells. *J Immunol* . 2011;186(5):2819-2825.

89. Li Y, et al. Exogenous stimuli maintain intraepithelial lymphocytes via aryl hydrocarbon receptor activation. *Cell*. 2011;147(3):629-640.

90. Abadie V, Discepolo V, Jabri B. Intraepithelial lymphocytes in celiac disease immunopathology. *Semin Immunopathol*. 2012;34(4):551-566.

91. Roberts SJ, et al. T-cell alpha beta + and gamma delta + deficient mice display abnormal but distinct phenotypes toward a natural, widespread infection of the intestinal epithelium. *Proc Natl Acad Sci U S A* . 1996;93(21):11774-11779.

92. Lepage AC, et al. Gut-derived intraepithelial lymphocytes induce long term immunity against Toxoplasma gondii. *J Immunol* . 1998;161(9):4902-4908.

93. Chardes T, et al. Toxoplasma gondii oral infection induces specific cytotoxic CD8 alpha/beta+ Thy-1+ gut intraepithelial lymphocytes, lytic for parasite-infected enterocytes. *J Immunol* . 1994;153(10): 4596-4603.

94. Moretto M, Weiss LM, Khan IA. Induction of a rapid and strong antigenspecific intraepithelial lymphocyte response during oral Encephalitozoon cuniculi infection. *J Immunol*. 2004;172(7):4402-4409.

95. Lefrancois L, Goodman T. In vivo modulation of cytolytic activity and Thy-1 expression in TCR-gamma delta+ intraepithelial lymphocytes. *Science*. 1989;243(4899):1716-1718.

96. Inagaki-Ohara K, et al. Gammadelta T cells play a protective role during infection with Nippostrongylus brasiliensis by promoting goblet cell function in the small intestine.

Immunology. 2011;134(4):448-458.

97. Dalton JE, et al. Intraepithelial gammadelta+ lymphocytes maintain the integrity of intestinal epithelial tight junctions in response to infection. *Gastroenterology*. 2006;131(3):818-829.

98. Epple HJ, et al. Acute HIV infection induces mucosal infiltration with CD4+ and CD8+ T cells, epithelial apoptosis, and a mucosal barrier defect. *Gastroenterology*. 2010;139(4):1289-1300.

99. Raffatellu M, et al. Simian immunodeficiency virus-induced mucosal interleukin-17 deficiency promotes Salmonella dissemination from the gut. *Nat Med*. 2008;14(4):421-428.

100. Chen Y, et al. Protection of the intestinal mucosa by intraepithelial gamma delta T cells. *Proc Natl Acad Sci U S A* . 2002;99(22):14338-14343.

101. Boismenu R, Havran WL. Modulation of epithelial cell growth by intraepithelial gamma delta T cells. *Science*. 1994;266(5188):1253-1255.

102. Kamanaka M, et al. Expression of interleukin-10 in intestinal lymphocytes detected by an interleukin-10 reporter knockin tiger mouse. *Immunity*. 2006;25(6):941-952.

103. Inagaki-Ohara K, et al. Intestinal intraepithelial lymphocytes sustain the epithelial barrier function against Eimeria vermiformis infection. *Infect Immun*. 2006;74(9):5292-5301.

104. Bhagat G, et al. Small intestinal CD8+ TCRgammadelta+ NKG2A + intraepithelial lymphocytes have attributes of regulatory cells in patients with celiac disease. *J Clin Invest*. 2008;118(1):281-293.

105. Giacomelli R, et al. Increase of circulating gamma/delta T lymphocytes in the peripheral blood of patients affected by active inflammatory bowel disease. *Clin Exp Immunol*. 1994;98(1):83-88.

106. Kanazawa H, et al. Multiple accumulation of Vdelta2+ gammadelta T-cell clonotypes in intestinal mucosa from patients with Crohn's disease. *Dig Dis Sci* . 2001;46(2):410-416.

107. Kawaguchi-Miyashita M, et al. An accessory role of TCRgammadelta (+) cells in the exacerbation of inflammatory bowel disease in TCRalpha mutant mice. *Eur J Immunol* . 2001;31(4):980-988.

108. Simpson SJ, et al. Expression of pro-inflammatory cytokines by TCR alpha beta+ and TCR gamma delta+ T cells in an experimental model of colitis. *Eur J Immunol* . 1997;27(1):17-25.

109. Meresse B, et al. Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity*. 2004;21(3):357-366.

110. Hue S, et al. A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. *Immunity*. 2004;21(3):367-377.

111. Abadie V, et al. Integration of genetic and immunological insights into a model of celiac disease pathogenesis. *Annu Rev Immunol* . 2011;29: 493-525.

112. Niess JH, Reinecker HC. Lamina propria dendritic cells in the physiology and pathology of the gastrointestinal tract. *Curr Opin Gastroenterol* . 2005;21(6):687-691.

113. Bogunovic M, et al. Origin of the lamina propria dendritic cell network. *Immunity*. 2009;31(3):513-525.

114. Jaensson E, et al. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. *J Exp Med*. 2008;205(9):2139-2149.

115. Wendland M, et al. CCR9 is a homing receptor for plasmacytoid dendritic cells to the small intestine. *Proc Natl Acad Sci U S A* . 2007;104(15):6347-6352.

116. Denning TL, et al. Functional specializations of intestinal dendritic cell and macrophage subsets that control Th17 and regulatory T cell responses are dependent on the T cell/APC ratio, source of mouse strain, and regional localization. *J Immunol* . 2011;187(2):733-747.

117. Varol C, et al. Intestinal lamina propria dendritic cell subsets have different origin and functions. *Immunity*. 2009;31(3):502-512.

118. Merad M, Manz MG. Dendritic cell homeostasis. *Blood*. 2009;113(15): 3418-3427.

119. Schulz O, et al. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *J Exp Med*. 2009;206(13):3101-3114.

120. Medina-Contreras O, et al. CX3CR1 regulates intestinal macrophage homeostasis, bacterial translocation, and colitogenic Th17 responses in mice. *J Clin Invest*. 2011;121(12):4787-4795.

121. Chow A, Brown BD, Merad M. Studying the mononuclear phagocyte system in the molecular age. *Nat Rev Immunol* . 2011;11(11):788-798.

122. Ginhoux F, et al. The origin and development of nonlymphoid tissue CD103+ DCs. *J Exp Med*. 2009;206(13):3115-3130.

123. Edelson BT, et al. Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells. *J Exp Med*. 2010;207(4):823-836.

124. Lewis KL, et al. Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. *Immunity*. 2011;35(5):780-791.

125. Kelsall B. Recent progress in understanding the phenotype and function of intestinal dendritic cells and macrophages. *Mucosal Immunol*. 2008;1(6):460-469.

126. Kraehenbuhl JP, Neutra MR. Epithelial M cells: differentiation and function. Annu Rev Cell Dev Biol. 2000;16:301-332.

127. Hathaway LJ, Kraehenbuhl JP. The role of M cells in mucosal immunity. *Cell Mol Life Sci*. 2000;57(2):323-332.

128. Mantis NJ, et al. Selective adherence of IgA to murine Peyer's patch M cells: evidence for a novel IgA receptor. *J Immunol*. 2002;169(4):1844-1851.

129. Nochi T, et al. A novel M cell-specific carbohydrate-targeted mucosal vaccine effectively induces antigen-specific immune responses. *J Exp Med.* 2007;204(12):2789-2796.

130. Chabot S, et al. TLRs regulate the gatekeeping functions of the intestinal follicle-associated epithelium. *J Immunol*. 2006;176(7):4275-4283.

131. Yoshida M, et al. Neonatal Fc receptor for IgG regulates mucosal immune responses to luminal bacteria. *J Clin Invest*. 2006;116(8):2142-2151.

132. Niess JH, et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science*. 2005;307(5707):254-258.

133. Chieppa M, et al. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J Exp Med*. 2006;203(13):2841-2852.

134. Rescigno M, et al. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol*. 2001;2(4):361-367.

135. Niess JH, Adler G. Enteric flora expands gut lamina propria CX3CR1+ dendritic cells supporting inflammatory immune responses under normal and inflammatory conditions. *J Immunol*. 2010;184(4):2026-2037.

136. Brenchley JM, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med*. 2006;12(12):1365-1371.

137. Sansonetti PJ. Rupture, invasion and inflammatory destruction of the intestinal barrier by Shigella: the yin and yang of innate immunity. *Can J Infect Dis Med Microbiol* . 2006;17(2):117-119.

138. Karlsson M, et al. “Tolerosomes” are produced by intestinal epithelial cells. *Eur J Immunol*. 2001;31(10):2892-2900.

139. Pugh CW, MacPherson GG, Steer HW. Characterization of nonlymphoid cells derived from rat peripheral lymph. *J Exp Med*. 1983;157(6):1758-1779.

140. Huang FP, et al. A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *J Exp Med*. 2000;191(3):435-444.

141. Liu LM, MacPherson GG. Lymph-borne (veiled) dendritic cells can acquire and present intestinally administered antigens. *Immunology*. 1991;73(3):281-286.

142. Worbs T, et al. Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. *J Exp Med*. 2006;203(3):519-527.

143. Macpherson AJ, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science*. 2004;303(5664): 1662-1665.

144. Uematsu S, et al. Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c+ lamina propria cells. *Nat Immunol* . 2006;7(8):868-874.

145. Hall JA, et al. Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses. *Immunity*. 2008;29(4):637-649.

146. Wolf G. A history of vitamin A and retinoids. *FASEB J.* 1996;10(9): 1102-1107.

147. Duester G. Retinoic acid synthesis and signaling during early organogenesis. *Cell*. 2008;134(6):921-931.

148. Ziouzenkova O, et al. Retinaldehyde represses adipogenesis and dietinduced obesity. *Nat Med*. 2007;13(6):695-702.

149. Acin-Perez R, et al. Control of oxidative phosphorylation by vitamin A illuminates a fundamental role in mitochondrial energy homoeostasis. *FASEB J.* 2010;24(2):627-636.

150. Underwood BA. Vitamin A deficiency disorders: international efforts to control a preventable “pox.” *J Nutr*. 2004;134(1):231S-236S.

151. Chun TY, Carman JA, Hayes CE. Retinoid repletion of vitamin A-deficient mice restores IgG responses. *J Nutr*. 1992;122(5):1062-1069.

152. Cantorna MT, Nashold FE, Hayes CE. Vitamin A deficiency results in a priming environment conducive for Th1 cell development. *Eur J Immunol* . 1995;25(6):1673-1679.

153. Hall JA, et al. The role of retinoic acid in tolerance and immunity. *Immunity*. 2011;35(1):13-22.

154. Yeum KJ, Russell RM. Carotenoid bioavailability and bioconversion. *Annu Rev Nutr*. 2002;22:483-504.

155. Harrison EH. Mechanisms of digestion and absorption of dietary vitamin A. *Annu Rev Nutr*. 2005;25:87-103.

156. Blomhoff R, Blomhoff HK. Overview of retinoid metabolism and function. *J Neurobiol*. 2006;66(7):606-630.

157. Wolf G. Identification of a membrane receptor for retinol-binding protein functioning in the cellular uptake of retinol. *Nutr Rev*. 2007;65(8, pt 1):385-388.

158. Chambon P. A decade of molecular biology of retinoic acid receptors. *FASEB J*. 1996;10(9):940-954.

159. Schug TT, et al. Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. *Cell*. 2007;129(4):723-733.

160. Kane MA, et al. Quantitative profiling of endogenous retinoic acid in vivo and in vitro by tandem mass spectrometry. *Anal Chem*. 2008;80(5):1702-1708.

161. Iwata M, et al. Retinoic acid imprints gut-homing specificity on T cells. *Immunity*. 2004;21(4):527-538.

162. Yokota A, et al. GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity. *Int Immunol*. 2009;21(4):361-377.

163. Johansson-Lindbom B, et al. Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. *J Exp Med.* 2005;202(8):1063-1073.

164. Coombes JL, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-{beta}- and retinoic acid-dependent mechanism. *J Exp Med*. 2007;204(8):1757-1764.

165. Jaensson-Gyllenback E, et al. Bile retinoids imprint intestinal CD103(+) dendritic cells with the ability to generate gut-tropic T cells. *Mucosal Immunol*. 2011;4(4):438-447.

166. Molenaar R, et al. Expression of retinaldehyde dehydrogenase enzymes in mucosal dendritic cells and gut-draining lymph node stromal cells is controlled by dietary vitamin A. *J Immunol*. 2011;186(4):1934-1942.

167. Manicassamy S, et al. Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity. *Nat Med*. 2009;15(4):401-409.

168. Guilliams M, et al. Skin-draining lymph nodes contain dermis-derived CD103(-) dendritic cells that constitutively produce retinoic acid and induce Foxp3(+) regulatory T cells. *Blood*. 2010;115(10):1958-1968.

169. Feng T, et al. Generation of mucosal dendritic cells from bone marrow reveals a critical role of retinoic acid. *J Immunol*. 2010;185(10): 5915-5925.

170. Hammerschmidt SI, et al. Stromal mesenteric lymph node cells are essential for the generation of gut-homing T cells in vivo. *J Exp Med*. 2008;205(11):2483-2490.

171. Edele F, et al. Cutting edge: instructive role of peripheral tissue cells in the imprinting of T cell homing receptor patterns. *J Immunol*. 2008;181(6):3745-3749.

172. Iliev ID, et al. Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. *Mucosal Immunol*. 2009;2(4):340-350.

173. Campbell DJ, Butcher EC. Rapid acquisition of tissue-specific homing phenotypes by CD4(+) T cells activated in cutaneous or mucosal lymphoid tissues. *J Exp Med*. 2002;195(1):135-141.

174. Mora JR, et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature*. 2003;424(6944):88-93.

175. Johansson-Lindbom B, et al. Selective generation of gut tropic T cells in gut-associated lymphoid tissue (GALT): requirement for GALT dendritic cells and adjuvant. *J Exp Med*. 2003;198(6):963-969.

176. Svensson M, et al. Retinoic acid receptor signaling levels and antigen dose regulate gut homing receptor expression on CD8+ T cells. *Mucosal Immunol*. 2008;1(1):38-48.

177. DeNucci CC, et al. Control of alpha4beta7 integrin expression and CD4 T cell homing by the beta1 integrin subunit. *J Immunol*. 2010;184(5): 2458-2467.

178. Kang SG, et al. Complementary roles of retinoic acid and TGF-beta1 in coordinated expression of mucosal integrins by T cells. *Mucosal Immunol*. 2011;4(1):66-82.

179. Ohoka Y, et al. Retinoic acid-induced CCR9 expression requires transient TCR stimulation and cooperativity between NFATc2 and the retinoic acid receptor/retinoid X receptor complex. *J Immunol*. 2011;186(2):733-744.

180. Ahmad SM, et al. Vitamin A status is associated with T-cell responses in Bangladeshi men. *Br J Nutr* . 2009;102(6):797-802.

181. Carman JA, et al. Immunity to Trichinella spiralis infection in vitamin Adeficient mice. *J Exp Med*. 1992;175(1):111-120.

182. Stephensen CB, Jiang X, Freytag T. Vitamin A deficiency increases the in vivo development of IL-10-positive Th2 cells and decreases development of Th1 cells in mice. *J Nutr*. 2004;134(10):2660-2666.

183. Dzhagalov I, Chambon P, He YW. Regulation of CD8+ T lymphocyte effector function and macrophage inflammatory cytokine production by retinoic acid receptor gamma. *J Immunol*. 2007;178(4):2113-2121.

184. Hall JA, et al. Essential role for retinoic acid in the promotion of CD4(+) T cell effector responses via retinoic acid receptor alpha. *Immunity*. 2011;34(3):435-447.

185. Hall JA, et al. Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses. *Immunity*. 2008;29(4):637-649.

186. Depaolo RW, et al. Co-adjuvant effects of retinoic acid and IL-15 induce inflammatory immunity to dietary antigens. *Nature*. 2011;471(7337): 220-224.

187. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol* . 2004;4(6):478-485.

188. Luhn K, et al. Increased frequencies of CD4+ CD25(high) regulatory T cells in acute dengue infection. *J Exp Med*. 2007;204(5):979-985.

189. Fagarasan S, et al. In situ class switching and differentiation to IgAproducing cells in the gut lamina propria. *Nature*. 2001;413(6856): 639-643.

190. Maruya M, et al. Vitamin A-dependent transcriptional activation of the nuclear factor of activated T cells c1 (NFATc1) is critical for the development and survival of B1 cells. *Proc Natl Acad Sci U S A* . 2011;108(2):722-727.

191. Inamoto Y, et al. Long-term outcome after bone marrow transplantation for aplastic

anemia using cyclophosphamide and total lymphoid irradiation as conditioning regimen. *Biol Blood Marrow Transplant* . 2008;14(1):43-49.

192. Feng T, et al. Generation of mucosal dendritic cells from bone marrow reveals a critical role of retinoic acid. J Immunol. 2010;185(10):5915-5925.

193. Malkovsky M, et al. Enhancement of specific antitumor immunity in mice fed a diet enriched in vitamin A acetate. *Proc Natl Acad Sci U S A* . 1983;80(20):6322-6326.

194. Malkovsky M, et al. T-cell-mediated enhancement of host-versus-graft reactivity in mice fed a diet enriched in vitamin A acetate. *Nature*. 1983;302(5906):338-340.

195. Jabri B, Sollid LM. Tissue-mediated control of immunopathology in coeliac disease. *Nat Rev Immunol* . 2009;9(12):858-870.

196. Reddy D, et al. Possible association between isotretinoin and inflammatory bowel disease. *Am J Gastroenterol* . 2006;101(7):1569-1573.

197. Crockett SD, et al. Isotretinoin use and the risk of inflammatory bowel disease: a case-control study. *Am J Gastroenterol* . 2010;105(9):1986-1993.

198. Weiner HL, et al. Oral tolerance. *Immunol Rev*. 2011;241(1):241-259.

199. Smith DW, Nagler-Anderson C. Preventing intolerance: the induction of nonresponsiveness to dietary and microbial antigens in the intestinal mucosa. *J Immunol* . 2005;174(7):3851-3857.

200. Kato H, et al. Oral tolerance revisited: prior oral tolerization abrogates cholera toxin-induced mucosal IgA responses. *J Immunol* . 2001;166(5):3114-3121.

201. Higgins PJ, Weiner HL. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein and its fragments. *J Immunol* . 1988;140(2):440-445.

202. Wannemuehler MJ, et al. Lipopolysaccharide (LPS) regulation of the immune response: LPS converts germfree mice to sensitivity to oral tolerance induction. *J Immunol* . 1982;129(3):959-965.

203. Kiyono H, et al. Lack of oral tolerance in C3H/HeJ mice. *J Exp Med*. 1982;155(2):605-610.

204. Michalek SM, et al. Lipopolysaccharide (LPS) regulation of the immune response: LPS influence on oral tolerance induction. *J Immunol* . 1982;128(5):1992-1998.

205. Bashir ME, et al. Toll-like receptor 4 signaling by intestinal microbes influences

susceptibility to food allergy. *J Immunol*. 2004;172(11): 6978-6987.

206. Roncarolo MG, et al. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev*. 2006;212:28-50.

207. Chen W, et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med*. 2003;198(12):1875-1886.

208. Sun CM, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med*. 2007;204(8):1775-1785.

209. Sakaguchi S, et al. Regulatory T cells and immune tolerance. *Cell*. 2008;133(5):775-787.

210. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol*. 2007;8(2):191-197.

211. Shevach EM, et al. The lifestyle of naturally occurring CD4CD25Foxp3 regulatory T cells. *Immunol Rev*. 2006;212:60-73.

212. Deaglio S, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med*. 2007;204(6):1257-1265.

213. Yamaguchi T, et al. Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor. *Immunity*. 2007;27(1): 145-159.

214. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol*. 2007;8(3):277-284.

215. Josefowicz SZ, Rudensky A. Control of regulatory T cell lineage commitment and maintenance. *Immunity*. 2009;30(5):616-625.

216. Wu Y, et al. FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell*. 2006;126(2):375-387.

217. Zheng Y, et al. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature*. 2010;463(7282):808-812.

218. Ruan Q, et al. Development of Foxp3(+) regulatory t cells is driven by the c-Rel enhanceosome. *Immunity*. 2009;31(6):932-940.

219. Long M, et al. Nuclear factor-kappaB modulates regulatory T cell development by

directly regulating expression of Foxp3 transcription factor. *Immunity*. 2009;31(6):921-931.

220. Isomura I, et al. c-Rel is required for the development of thymic Foxp3+ CD4 regulatory T cells. *J Exp Med*. 2009;206(13):3001-3014.

221. Burchill MA, et al. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. *J Immunol*. 2007;178(1):280-290.

222. Malek TR. The main function of IL-2 is to promote the development of T regulatory cells. *J Leukocyte Biol*. 2003;74(6):961-965.

223. Fontenot JD, et al. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol*. 2005;6(11):1142-1151.

224. Izcue A, Coombes JL, Powrie F. Regulatory lymphocytes and intestinal inflammation. *Annu Rev Immunol*. 2009;27:313-338.

225. Shevach EM. The resurrection of T cell-mediated suppression. *J Immunol*. 2011;186(7):3805-3807.

226. Shevach EM. Biological functions of regulatory T cells. *Adv Immunol*. 2011;112:137-176.

227. Wing K, et al. CTLA-4 control over Foxp3+ regulatory T cell function. *Science*. 2008;322(5899):271-275.

228. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med*. 2000;192(2):295-302.

229. Tang Q, et al. Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nat Immunol*. 2006;7(1):83-92.

230. Collison LW, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature*. 2007;450(7169):566-569.

231. Schmidt A, Oberle N, Krammer PH. Molecular mechanisms of tregmediated T cell suppression. *Front Immunol*. 2012;3:51.

232. Pandiyan P, et al. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol*. 2007;8(12):1353-1362.

233. Ashcroft GS, et al. Mice lacking Smad3 show accelerated wound healing and an

impaired local inflammatory response. *Nat Cell Biol*. 1999;1(5):260-266.

234. Fantini MC, et al. Smad7 controls resistance of colitogenic T cells to regulatory T cell-mediated suppression. *Gastroenterology*. 2009;136(4): 1308-1316, e1-e3.

235. Kim BG, et al. Smad4 signalling in T cells is required for suppression of gastrointestinal cancer. *Nature*. 2006;441(7096):1015-1019.

236. Pesu M, et al. T-cell-expressed proprotein convertase furin is essential for maintenance of peripheral immune tolerance. *Nature*. 2008;455(7210):246-250.

237. von Boehmer H. Mechanisms of suppression by suppressor T cells. *Nat Immunol*. 2005;6(4):338-344.

238. Fahlen L, et al. T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. *J Exp Med*. 2005;201(5): 737-746.

239. Cong Y, et al. A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. *Proc Natl Acad Sci U S A*. 2009;106(46):19256-19261.

240. Kuhn R, et al. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*. 1993;75(2):263-274.

241. Glocker EO, et al. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med*. 2009;361(21):2033-2045.

242. Spencer SD, et al. The orphan receptor CRF2-4 is an essential subunit of the interleukin 10 receptor. *J Exp Med*. 1998;187(4):571-578.

243. Murai M, et al. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat Immunol*. 2009;10(11):1178-1184.

244. Hara M, et al. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J Immunol*. 2001;166(6):3789-3796.

245. Uhlig HH, et al. Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis. *J Immunol*. 2006;177(9):5852-5860.

246. Rubtsov YP, et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity*. 2008;28(4):546-558.

247. O'Garra A, et al. IL-10-producing and naturally occurring CD4 + Tregs: limiting collateral damage. *J Clin Invest*. 2004;114(10):1372-1378.

248. Fiorentino DF, et al. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol*. 1991;146(10):3444-3451.

249. Fiorentino DF, et al. IL-10 inhibits cytokine production by activated macrophages. *J Immunol*. 1991;147(11):3815-3822.

250. Huber S, et al. Th17 cells express interleukin-10 receptor and are controlled by Foxp3(-) and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. *Immunity*. 2011;34(4):554-565.

251. Chaudhry A, et al. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity*. 2011;34(4):566-578.

252. Zheng Y, et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature*. 2009;458(7236):351-356.

253. Koch M, et al. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol*. 2009;10(5):1-7.

254. Chaudhry A, et al. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science*. 2009;326(5955):986-991.

255. Stock P, et al. Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. *Nat Immunol*. 2004;5(11):1149-1156.

256. Sawitzki B, et al. IFN-gamma production by alloantigen-reactive regulatory T cells is important for their regulatory function in vivo. *J Exp Med*. 2005;201(12):1925-1935.

257. Wei G, et al. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity*. 2009;30(1):155-167.

258. Oldenhove G, et al. Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity*. 2009;31(5):772-786.

259. Zhou X, et al. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol*. 2009;10(9):1000-1007.

260. Voo KS, et al. Identification of IL-17-producing FOXP3+ regulatory T cells in humans. *Proc Natl Acad Sci U S A*. 2009;106(12):4793-4798.

261. Beriou G, et al. IL-17-producing human peripheral regulatory T cells retain suppressive function. *Blood*. 2009;113(18):4240-4249.

262. Hirota K, et al. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nat Immunol*. 2011;12(3):255-263.

263. Wohlfert EA, et al. GATA3 controls Foxp3+ regulatory T cell fate during inflammation in mice. *J Clin Invest*. 2011;121(11):4503-4515.

264. Wang Y, Su MA, Wan YY. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. *Immunity*. 2011;35(3):337-348.

265. Bettelli E, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*. 2006;441(7090):235-238.

266. Fantini MC, et al. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and downregulation of Smad7. *J Immunol*. 2004;172(9):5149-5153.

267. Kretschmer K, et al. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol*. 2005;6(12):1219-1227.

268. Knoechel B, et al. Sequential development of interleukin 2-dependent effector and regulatory T cells in response to endogenous systemic antigen. *J Exp Med*. 2005;202(10):1375-1386.

269. Curotto de Lafaille MA, et al. Adaptive Foxp3+ regulatory T celldependent and -independent control of allergic inflammation. *Immunity*. 2008;29(1):114-126.

270. Mucida D, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science*. 2007;317(5835):256-260.

271. Mucida D, et al. Oral tolerance in the absence of naturally occurring Tregs. *J Clin Invest*. 2005;115(7):1923-1933.

272. Curotto de Lafaille MA, Lafaille JJ. Natural and adaptive foxp3 + regulatory T cells: more of the same or a division of labor? *Immunity*. 2009;30(5):626-635.

273. Josefowicz SZ, et al. Extrathymically generated regulatory T cells control mucosal TH2 inflammation. *Nature*. 2012;482(7385):395-399.

274. Cassani B, et al. Gut-tropic T cells that express integrin alpha4beta7 and CCR9 are required for induction of oral immune tolerance in mice. *Gastroenterology*. 2011;141(6):2109-2118.

275. Tone Y, et al. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nat Immunol*. 2008;9(2):194-202.

276. Zheng Y, et al. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature*. 2010;463(7282):808-812.

277. Benson MJ, et al. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of costimulation. *J Exp Med*. 2007;204(8):1765-1774.

278. Denning TL, et al. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nat Immunol*. 2007;8(10):1086-1094.

279. MA, et al. Loss of integrin alpha(v)beta8 on dendritic cells causes autoimmunity and colitis in mice. *Nature*. 2007;449(7160):361-365.

280. Lacy-Hulbert A, et al. Ulcerative colitis and autoimmunity induced by loss of myeloid alphav integrins. *Proc Natl Acad Sci U S A* . 2007;104(40):15823-15828.

281. Worthington JJ, et al. Intestinal dendritic cells specialize to activate transforming growth factor-beta and induce Foxp3+ regulatory T cells via integrin alphavbeta8. *Gastroenterology*. 2011;141(5):1802-1812.

282. Hadis U, et al. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity*. 2011;34(2):237-246.

283. Finney CA, et al. Expansion and activation of CD4(+)CD25(+) regulatory T cells in Heligmosomoides polygyrus infection. *Eur J Immunol* . 2007;37(7):1874-1886.

284. Wilson MS, et al. Suppression of allergic airway inflammation by helminth-induced regulatory T cells. *J Exp Med*. 2005;202(9):1199-1212.

285. McSorley HJ, et al. Expansion of Foxp3+ regulatory T cells in mice infected with the filarial parasite Brugia malayi. *J Immunol*. 2008;181(9): 6456-6466.

286. D'Elia R, et al. Regulatory T cells: a role in the control of helminthdriven intestinal pathology and worm survival. *J Immunol*. 2009;182(4): 2340-2348.

287. Taylor MD, et al. CTLA-4 and CD4+ CD25+ regulatory T cells inhibit protective immunity to filarial parasites in vivo. *J Immunol*. 2007;179(7): 4626-4634.

288. Hewitson JP, Grainger JR, Maizels RM. Helminth immunoregulation: the role of parasite secreted proteins in modulating host immunity. *Mol Biochem Parasitol*. 2009;167(1):1-11.

289. Balic A, et al. Selective maturation of dendritic cells by Nippostrongylus brasiliensis-secreted proteins drives Th2 immune responses. *Eur J Immunol* . 2004;34(11):3047-

3059.

290. Segura M, et al. Impairment of dendritic cell function by excretorysecretory products: a potential mechanism for nematode-induced immunosuppression. *Eur J Immunol* . 2007;37(7):1887-1904.

291. McSorley HJ, et al. daf-7-related TGF-beta homologues from Trichostrongyloid nematodes show contrasting life-cycle expression patterns. *Parasitology*. 2009:1-13.

292. Gomez-Escobar N, van den Biggelaar A, Maizels R. A member of the TGF-beta receptor gene family in the parasitic nematode Brugia pahangi. *Gene*. 1997;199(1-2):101-109.

293. Gomez-Escobar N, Gregory WF, Maizels RM. Identification of tgh-2, a filarial nematode homolog of Caenorhabditis elegans daf-7 and human transforming growth factor beta, expressed in microfilarial and adult stages of Brugia malayi. *Infect Immun*. 2000;68(11): 6402-6410.

294. Iwasaki A, Kelsall BL. Unique functions of CD11b+, CD8 alpha+, and double-negative Peyer's patch dendritic cells. *J Immunol* . 2001;166(8): 4884-4890.

295. Chirdo FG, et al. Immunomodulatory dendritic cells in intestinal lamina propria. *Eur J Immunol* . 2005;35(6):1831-1840.

296. Coombes JL, Powrie F. Dendritic cells in intestinal immune regulation. *Nat Rev Immunol* . 2008;8(6):435-446.

297. Stumhofer JS, et al. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat Immunol* . 2007;8(12):1363-1371.

298. Levings MK, et al. IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. *J Immunol* . 2001;166(9):5530-5539.

299. Akbari O, et al. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat Med* . 2002;8(9):1024-1032.

300. Kemper C, et al. Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. *Nature*. 2003;421(6921):388-392.

301. Awasthi A, et al. A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. *Nat Immunol* . 2007;8(12):1380-1389.

302. Pot C, et al. Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote

differentiation of IL-10-producing Tr1 cells. *J Immunol*. 2009;183(2):797-801.

303. Niedbala W, et al. IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *Eur J Immunol* . 2007;37(11):3021-3029.

304. Bardel E, et al. Human CD4+ CD25+ Foxp3+ regulatory T cells do not constitutively express IL-35. *J Immunol*. 2008;181(10):6898-6905.

305. Seyerl M, et al. Human rhinoviruses induce IL-35-producing Treg via induction of B7-H1 (CD274) and sialoadhesin (CD169) on DC. *Eur J Immunol* . 2010;40(2):321-329.

306. Korn T, et al. IL-17 and Th17 cells. *Annu Rev Immunol*. 2009;27:485-517.

307. Ivanov II, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell*. 2006;126(6):1121-1133.

308. Hu W, et al. Priming microenvironments dictate cytokine requirements for T helper 17 cell lineage commitment. *Immunity*. 2011;35(6): 1010-1022.

309. Konkel JE, Chen W. Balancing acts: the role of TGF-beta in the mucosal immune system. *Trends Mol Med*. 2011;17(11):668-676.

310. Mangan PR, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature*. 2006;441(7090):231-234.

311. Zhou L, et al. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature*. 2008;453(7192): 236-240.

312. Esser C, Rannug A, Stockinger B. The aryl hydrocarbon receptor in immunity. *Trends Immunol*. 2009;30(9):447-454.

313. Eberl G. Development and evolution of RORgammat+ cells in a microbe's world. *Immunol Rev*. 2012;245(1):177-188.

314. Sonnenberg GF, et al. Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. *Science*. 2012;336(6086):1321-1325.

315. Sonnenberg GF, Fouser LA, Artis S. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. *Nat Immunol*. 2011;12(5):383-390.

316. Ishigame H, et al. Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. *Immunity*.

2009;30(1):108-119.

317. Zheng Y, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med*. 2008;14(3):282-289.

318. Lochner M, et al. Microbiota-induced tertiary lymphoid tissues aggravate inflammatory disease in the absence of RORgamma t and LTi cells. *J Exp Med*. 2011;208(1):125-134.

319. Mayuzumi H, et al. Interleukin-17A is required to suppress invasion of Salmonella enterica serovar Typhimurium to enteric mucosa. *Immunology*. 2010;131(3):377-385.

320. Velin D, et al. Interleukin-17 is a critical mediator of vaccine-induced reduction of Helicobacter infection in the mouse model. *Gastroenterology*. 2009;136(7):2237-2246.

321. Godinez I, et al. T cells help to amplify inflammatory responses induced by Salmonella enterica serotype Typhimurium in the intestinal mucosa. *Infect Immun*. 2008;76(5):2008-2017.

322. Godinez I, et al. Interleukin-23 orchestrates mucosal responses to Salmonella enterica serotype Typhimurium in the intestine. *Infect Immun*. 2009;77(1):387-398.

323. Geddes K, et al. Identification of an innate T helper type 17 response to intestinal bacterial pathogens. Nat Med. 2011;17(7):837-844.

324. Symonds EL, et al. Involvement of T helper type 17 and regulatory T cell activity in Citrobacter rodentium invasion and inflammatory damage. *Clin Exp Immunol*. 2009;157(1):148-154.

325. Estes JD, et al. Damaged intestinal epithelial integrity linked to microbial translocation in pathogenic simian immunodeficiency virus infections. *PLoS Pathog*. 2010;6(8):e1001052.

326. Brenchley JM, Douek DC. The mucosal barrier and immune activation in HIV pathogenesis. *Curr Opin HIV AIDS* . 2008;3(3):356-361.

327. Brenchley JM, et al. Differential Th17 CD4 T-cell depletion in pathogenic and nonpathogenic lentiviral infections. *Blood*. 2008;112(7): 2826-2835.

328. Cecchinato V, et al. Altered balance between Th17 and Th1 cells at mucosal sites predicts AIDS progression in simian immunodeficiency virus-infected macaques. *Mucosal Immunol*. 2008;1(4):279-288.

329. Lederer S, et al. Transcriptional profiling in pathogenic and nonpathogenic SIV infections reveals significant distinctions in kinetics and tissue compartmentalization.

PLoS Pathog. 2009;5(2):e1000296.

330. Gordon SN, et al. Disruption of intestinal CD4+ T cell homeostasis is a key marker of systemic CD4+ T cell activation in HIV-infected individuals. *J Immunol*. 2010;185(9):5169-5179.

331. Klatt NR, et al. Loss of mucosal CD103+ DCs and IL-17+ and IL-22 + lymphocytes is associated with mucosal damage in SIV infection. *Mucosal Immunol*. 2012. Epub ahead of print.

332. Fossiez F, et al. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J Exp Med*. 1996;183(6): 2593-2603.

333. Mebius RE, et al. A developmental switch in lymphocyte homing receptor and endothelial vascular addressin expression regulates lymphocyte homing and permits CD4+ CD3- cells to colonize lymph nodes. *Proc Natl Acad Sci U S A*. 1996;93(20):11019-11024.

334. Cherrier M, et al. Development and function of intestinal innate lymphoid cells. *Curr Opin Immunol*. 2012;24(3):277-283.

335. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell*. 1997;91(5): 661-672.

336. Moro K, et al. Innate production of T(H)2 cytokines by adipose tissueassociated c-Kit(+)Sca-1(+) lymphoid cells. *Nature*. 2010;463(7280): 540-544.

337. Cherrier M, Sawa S, Eberl G. Notch, Id2, and RORgammat sequentially orchestrate the fetal development of lymphoid tissue inducer cells. *J Exp Med*. 2012;209(4):729-740.

338. Sawa S, et al. Lineage relationship analysis of RORgammat+ innate lymphoid cells. *Science*. 2010;330(6004):665-669.

339. Satoh-Takayama N, et al. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity*. 2008;29(6):958-970.

340. Sanos SL, et al. RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nat Immunol*. 2009;10(1):83-91.

341. Eberl G, et al. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. *Nat Immunol*. 2004;5(1):64-73.

342. Wong SH, et al. Transcription factor RORalpha is critical for nuocyte development.

Nat Immunol. 2012;13(3):229-236.

343. Aliahmad P, de la Torre B, Kaye J. Shared dependence on the DNAbinding factor TOX for the development of lymphoid tissue-inducer cell and NK cell lineages. *Nat Immunol*. 2010;11(10):945-952.

344. Tachibana M, et al. Runx1/Cbfbeta2 complexes are required for lymphoid tissue inducer cell differentiation at two developmental stages. *J Immunol*. 2011;186(3):1450-1457.

345. Lee JS, et al. AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. *Nat Immunol*. 2012;13(2):144-151.

346. Kiss EA, et al. Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. *Science*. 2011;334(6062): 1561-1565.

347. Qiu J, et al. The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. *Immunity*. 2012;36(1): 92-104.

348. Possot C, et al. Notch signaling is necessary for adult, but not fetal, development of RORgammat(+) innate lymphoid cells. *Nat Immunol*. 2011;12(10):949-958.

349. Wolk K, et al. IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis. *Eur J Immunol*. 2006;36(5):1309-1323.

350. Sawa S, et al. RORgammat+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. *Nat Immunol*. 2011;12(4):320-326.

351. Zenewicz LA, et al. Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. *Immunity*. 2008;29(6):947-957.

352. Sonnenberg GF, et al. CD4(+) lymphoid tissue-inducer cells promote innate immunity in the gut. *Immunity*. 2011;34(1):122-134.

353. Buonocore S, et al. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature*. 2010;464(7293):1371-1375.

354. Geremia A, et al. IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease. *J Exp Med*. 2011;208(6):1127-1133.

355. Neill DR, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature*. 2010;464(7293):1367-1370.

356. Price AE, et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci U S A* . 2010;107(25):11489-11494.

357. Mjosberg JM, et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol* . 2011;12(11):1055-1062.

358. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* . 2006;124(4): 837-848.

359. Eckburg PB, et al. Diversity of the human intestinal microbial flora. *Science*. 2005;308(5728):1635-1638.

360. Salzman NH, Underwood MA, Bevins CL. Paneth cells, defensins, and the commensal microbiota: a hypothesis on intimate interplay at the intestinal mucosa. *Semin Immunol* . 2007;19(2):70-83.

361. Talham GL, et al. Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system. *Infect Immun*. 1999;67(4):1992-2000.

362. Mazmanian SK, et al. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* . 2005;122(1): 107-118.

363. Hooper LV, et al. Molecular analysis of commensal host-microbial relationships in the intestine. *Science*. 2001;291(5505):881-884.

364. Stappenbeck TS, Hooper LV, Gordon JI. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proc Natl Acad Sci U S A* . 2002;99(24):15451-15455.

365. Lavelle EC, et al. The role of TLRs, NLRs, and RLRs in mucosal innate immunity and homeostasis. Mucosal Immunol. 2010;3(1):17-28.

366. Fritz JH, et al. Nod-like proteins in immunity, inflammation and disease. *Nat Immunol* . 2006;7(12):1250-1257.

367. Eberl G, Lochner M. The development of intestinal lymphoid tissues at the interface of self and microbiota. *Mucosal Immunol* . 2009;2(6):478-485.

368. Bouskra D, et al. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. *Nature*. 2008;456 (7221):507-510.

369. Yang D, et al. Beta-defensins: linking innate and adaptive immunity through dendritic

and T cell CCR6. *Science*. 1999;286(5439):525-528.

370. Mack DR, et al. Probiotics inhibit enteropathogenic E. coli adherence in vitro by inducing intestinal mucin gene expression. *Am J Physiol.* 1999;276(4, pt 1):G941-G950.

371. Van der Sluis M, et al. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. *Gastroenterology*. 2006;131(1):117-129.

372. Ivanov II, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe*. 2008;4(4):337-349.

373. Gaboriau-Routhiau V, et al. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. *Immunity*. 2009;31(4):677-689.

374. Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature*. 2008;453(7195):620-625.

375. O'Mahony C, et al. Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-kappaB activation. *PLoS Pathog*. 2008;4(8):e1000112.

376. Ochoa-Reparaz J, et al. Central nervous system demyelinating disease protection by the human commensal Bacteroides fragilis depends on polysaccharide A expression. *J Immunol*. 2010;185(7):4101-4108.

377. Round JL, et al. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science*. 2011;332(6032): 974-977.

378. Atarashi K, et al. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science*. 2011;331(6015):337-341.

379. Lathrop SK, et al. Peripheral education of the immune system by colonic commensal microbiota. *Nature*. 2011;478:250-254.

380. Ivanov II, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell*. 2009;139(3):485-498.

381. Stepankova R, et al. Segmented filamentous bacteria in a defined bacterial cocktail induce intestinal inflammation in SCID mice reconstituted with CD45RBhigh CD4+ T cells. *Inflamm Bowel Dis*. 2007;13(10):1202-1211.

382. Sansonetti PJ, Di Santo JP. Debugging how bacteria manipulate the immune response. *Immunity*. 2007;26(2):149-161.

383. Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol*. 2003;21:335-376.

384. Lodes MJ, et al. Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest*. 2004;113(9):1296-1306.

385. Vijay-Kumar M, et al. Deletion of TLR5 results in spontaneous colitis in mice. *J Clin Invest*. 2007;117(12):3909-3921.

386. Vijay-Kumar M, et al. Toll-like receptor 5-deficient mice have dysregulated intestinal gene expression and nonspecific resistance to Salmonellainduced typhoid-like disease. *Infect Immun*. 2008;76(3):1276-1281.

387. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells.[comment]. *Science*. 2003;299(5609):1033-1036.

388. Larosa DF, et al. CpG DNA inhibits CD4+CD25+ Treg suppression through direct MyD88-dependent costimulation of effector CD4+ T cells. *Immunol Lett*. 2007;108(2):183-188.

389. Bouladoux N, et al. Regulatory role of suppressive motifs from commensal DNA. *Mucosal Immunol*. 2012. Epub ahead of print.

390. Torchinsky MB, et al. Innate immune recognition of infected apoptotic cells directs T(H)17 cell differentiation. *Nature*. 2009;458(7234):78-82.

391. Atarashi K, et al. ATP drives lamina propria T(H)17 cell differentiation. *Nature*. 2008;455(7214):808-812.

392. Di Giacinto C, et al. Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-b-bearing regulatory cells. *J Immunol*. 2005;174:3237-3246.

393. Zoumpopoulou G, et al. Lactobacillus fermentum ACA-DC 179 displays probiotic potential in vitro and protects against trinitrobenzene sulfonic acid (TNBS)-induced colitis and Salmonella infection in murine models. *Int J Food Microbiol* . 2008;121(1):18-26.

394. Feleszko W, et al. Probiotic-induced suppression of allergic sensitization and airway inflammation is associated with an increase of T regulatory-dependent mechanisms in a murine model of asthma. *Clin Exp Allergy.* 2007;37(4):498-505.

395. Karimi K, et al. Lactobacillus reuteri-induced regulatory T cells protect against an allergic airway response in mice. *Am J Respir Crit Care Med* . 2009;179:186-193.

396. Smits HH, et al. Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. *J Allergy Clin Immunol* .

2005;115(6):1260-1267.

397. Foligne B, et al. A key role of dendritic cells in probiotic functionality. *PLoS ONE*. 2007;2(3):e313.

398. Di Giacinto C, et al. Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-betabearing regulatory cells. *J Immunol*. 2005;174(6):3237-3246.

399. Karimi K, et al. Lactobacillus reuteri-induced regulatory T cells protect against an allergic airway response in mice. *Am J Respir Crit Care Med* . 2009;179(3):186-193.

400. Ostman S, et al. Impaired regulatory T cell function in germ-free mice. *Eur J Immunol*. 2006;36(9):2336-2346.

401. Torii A, et al. Lactobacillus Acidophilus strain L-92 regulates the production of Th1 cytokine as well as Th2 cytokines. *Allergol Int.* 2007;56(3):293-301.

402. Strober W, Fuss I, Mannon P. The fundamental basis of inflammatory bowel disease. *J Clin Invest*. 2007;117(3):514-521.

403. Saleh M, Elson CO. Experimental inflammatory bowel disease: insights into the host-microbiota dialog. *Immunity*. 2011;34(3):293-302.

404. Powrie F, et al. Regulatory interactions between CD45RBhigh and CD45RBlow CD4+ T cells are important for the balance between protective and pathogenic cell-mediated immunity. *J Exp Med*. 1994;179(2): 589-600.

405. Izcue A, Coombes JL, Powrie F. Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. *Immunol Rev*. 2006;212:256-271.

406. Garrett WS, et al. Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. *Cell*. 2007;131(1):33-45.

407. Garrett WS, et al. Colitis-associated colorectal cancer driven by T-bet deficiency in dendritic cells. *Cancer Cell* . 2009;16(3):208-219.

408. Garrett WS, et al. Enterobacteriaceae act in concert with the gut microbiota to induce spontaneous and maternally transmitted colitis. *Cell Host Microbe*. 2010;8(3):292-300.

409. Elinav E, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell*. 2011;145(5):745-757.

410. Heimesaat MM, et al. Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with Toxoplasma gondii. *J Immunol*. 2006;177(12):8785-8795.

411. Benson A, et al. Gut commensal bacteria direct a protective immune response against Toxoplasma gondii. *Cell Host Microbe*. 2009;6(2): 187-196.

412. Lupp C, et al. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe*. 2007;2(3):204.

413. Stecher B, et al. Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota. *PLoS Biol*. 2007;5(10):2177-2189.

414. Bashir ME, et al. Toll-like receptor 4 signaling by intestinal microbes influences susceptibility to food allergy. *J Immunol*. 2004;172:6978-6987.

415. Noverr MC, et al. Development of allergic airway disease in mice following antibiotic therapy and fungal microbiota increase: role of host genetics, antigen, and interleukin-13. *Infect Immun*. 2005;73:30-38.

416. Sullivan A, Edlund C, Nord CE. Effect of antimicrobial agents on the ecological balance of human microflora. *Lancet Infect Dis.* 2001;1:101-114.

417. Dethlefsen L, McFall-Ngai M, Relman DA. An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature*. 2007;449(7164):811-818.

418. Beaugerie L, Petit JC. Microbial-gut interactions in health and disease. Antibiotic-associated diarrhoea. *Best Pract Res Clin Gastroenterol*. 2004;18(2):337-352.

419. Noverr MC, Huffnagle GB. The “microflora hypothesis” of allergic diseases. *Clin Exp Allergy*. 2005;35:1511-1520.

420. Prioult G, Nagler-Anderson C. Mucosal immunity and allergic responses: lack of regulation and/or lack of microbial stimulation? *Immunol Rev*. 2005;206:204-218.

421. Marra F, et al. Does antibiotic exposure during infancy lead to development of asthma?: a systematic review and metaanalysis. *Chest*. 2006;129(3):610-618.

422. Clarke TB, et al. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat Med*. 2010;16(2):228-231.

423. Shi C, et al. Bone marrow mesenchymal stem and progenitor cells induce monocyte emigration in response to circulating toll-like receptor ligands. *Immunity*. 2011;34(4):590-601.

424. Ichinohe T, et al. Microbiota regulates immune defense against respiratory tract influenza A virus infection. *Proc Natl Acad Sci U S A.* 2011;108(13):5354-5359.

425. Wu HJ, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity*. 2010;32(6): 815-827.

426. Berer K, et al. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature*. 2011;479(7374): 538-541.

427. Wen L, et al. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature*. 2008;455(7216):1109-1113.

428. Kriegel MA, et al. Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice. Proc *Natl Acad Sci U S A.* 2011;108(28):11548-11553.

429. Wolowczuk I, et al. Feeding our immune system: impact on metabolism. *Clin Dev Immunol*. 2008;2008:639803.

430. Maslowski KM, et al. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature.* 2009;461(7268): 1282-1286.

431. Yazdanbakhsh M, Kremsner PG, van Ree R. Allergy, parasites, and the hygiene hypothesis. *Science*. 2002;296(5567):490-494.

432. Wang J, Sampson HA. Food allergy. *J Clin Invest*. 2011;121(3): 827-835.

433. Garrett WS, Gordon JI, Glimcher LH. Homeostasis and inflammation in the intestine. *Cell*. 2010;140(6):859-870.

434. Kaser A, Zeissig S, Blumberg RS. Inflammatory bowel disease. *Annu Rev Immunol.* 2010;28:573-621.

435. Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature*. 2011;474(7351):298-306.

436. Barrett JC, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet*. 2008;40(8): 955-962.

437. Franke A, et al. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet*. 2010;42(12): 1118-1125.

438. McGovern DP, et al. Genome-wide association identifies multiple ulcerative colitis susceptibility loci. *Nat Genet*. 2010;42(4):332-337.

439. Anderson CA, et al. Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. *Nat Genet*. 2011;43(3):246-252.

440. Rivas MA, et al. Deep resequencing of GWAS loci identifies independent rare variants associated with inflammatory bowel disease. *Nat Genet*. 2011;43(11):1066-1073.

441. Koslowski MJ, et al. Innate antimicrobial host defense in small intestinal Crohn's disease. *Int J Med Microbiol* . 2010;300(1):34-40.

442. Cadwell K, et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature*. 2008; 456(7219):259-263.

443. Melmed GY, Targan SR. Future biologic targets for IBD: potentials and pitfalls. *Nat Rev Gastroenterol Hepatol*. 2010;7(2):110-117.

444. Tato CM, Cua DJ. Reconciling id, ego, and superego within interleukin-23. *Immunol Rev*. 2008;226:103-111.

445. Ahern PP, et al. Interleukin-23 drives intestinal inflammation through direct activity on T cells. *Immunity*. 2010;33(2):279-288.

446. Kleinschek MA, et al. Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation. *J Exp Med*. 2009;206(3):525-534.

447. Leppkes M, et al. RORgamma-expressing Th17 cells induce murine chronic intestinal inflammation via redundant effects of IL-17A and IL-17F. *Gastroenterology*. 2009;136(1):257-267.

448. Wu S, et al. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. *Nat Med*. 2009;15(9):1016-1022.

449. Wolk K, et al. Biology of interleukin-22. *Semin Immunopathol*. 2010; 32(1):17-31.

450. Siegmund B. Interleukin-18 in intestinal inflammation: friend and foe? *Immunity*. 2010;32(3):300-302.

451. Salcedo R, et al. MyD88-mediated signaling prevents development of adenocarcinomas of the colon: role of interleukin 18. *J Exp Med*. 2010;207(8):1625-1636.

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Chapter 35

Neurophysiologic Reflex Mechanisms in Immunology

Sangeeta S. Chavan

Kevin J. Tracey

INTRODUCTION

A fundamental principle in biology is that organ function is maintained within a narrow physiologic range, one optimal for health. Heart rate, body temperature, and the output of all organ systems are controlled by homeostatic mechanisms. The nervous system occupies a crucial role in establishing this healthy range of organ function. There is continuous communication between the nervous system and the other organ systems, linked by two major information highways (Fig. 35.1). One route is neural, which transmits action potentials via nerves that travel directly into the organs back and forth to the nervous system. The other is humoral, which utilizes the circulatory system to deliver hormones, inflammatory mediators, and other soluble factors that shuttle information from the immune system to the nervous system, and back again from the neuroendocrine organs to the immune system.

The immune system, the principle organ system of host defense, exerts significant influences on the function of the other organ systems, and the magnitude of these responses is crucial to physiologic homeostasis. For example, cytokines modulate the metabolism of hepatocytes, adipocytes, and skeletal muscle cells to mobilize fatty acids, glucose, and other energy stores required for new protein synthesis in lymphocytes and other immune cells to support antibody production, and other essential molecules. Inflammatory mediators also modify the activity of cells in the nervous system, disrupting homeostasis and producing fever, anorexia, and avoidance behavior. These protective responses, which together improve the odds of the organism surviving infection and injury, are frequently the major signs and symptoms of disease. Decades of prior work revealed how the immune system communicates with the nervous system by utilizing both communication routes, the humoral and neural, to change the behavior of the organisms. Quite recently, however, advances in neurophysiology and immunology elucidated mechanisms by which these incoming signals from the immune system stimulate regulatory signals that return from the nervous system to the immune system through neural networks that maintain immunologic homeostasis.

These neural reflexes are essential for maintaining a healthy immune response for optimal function during infection and injury, termed “immunologic homeostasis.” Impairment of these fundamental reflex mechanisms leads to unregulated immune responses, uncontrolled cytokine production, and deviations of organ system function outside of the homeostatic range. This chapter focuses on the basic principles underlying the neurophysiologic mechanisms that regulate immunologic homeostasis.

ORGANIZATION OF THE NERVOUS SYSTEM

To understand the role of neural reflexes and the neuroendocrine system in maintaining immunologic homeostasis, it may be illustrative to review the basic organization of the nervous system. It is comprised of the central nervous system (the brain and spinal cord) and the peripheral nervous system (Fig. 35.2). The peripheral nervous system is comprised of two parts: the voluntary or skeletal nervous system, which enables purposeful, conscious actions, like piano playing; and the involuntary or autonomic nervous system, which innervates organs to provide a major information conduit that regulates homeostasis. The autonomic nervous system normally functions without conscious involvement. There are two divisions of the autonomic nervous system: the sympathetic and parasympathetic. Most organs receive input from both of these autonomic divisions, which deliver information that adjusts organ output according to setpoints established in the brainstem and hypothalamus.

The neuron, the principle information-transmitting cell of the nervous system, communicates with other cells by action potentials that propagate unidirectionally along axons away from the neuronal cell body. Arrival of action potentials at the axon terminus causes the release of neurotransmitters into the synaptic cleft, the narrow space adjacent (< 20 nM) to the innervated neuron or somatic cell. This converts the electrical information carried in the axon into molecular (or pharmacologic) information, because secreted neurotransmitters bind to specific receptors expressed on the innervated cell. Ligand-receptor interaction leads to signal transduction that modulates the behavior of that cell in response to the arrival of a train of action potentials. Henry Dale, the Nobel Laureate discoverer of acetylcholine, recommended that autonomic nerves be described by the neurotransmitters they produce, not their function (ie, not as “sympathetic” or “parasympathetic”). This is an important point that will be emphasized again in this chapter. Autonomic neurons may be adrenergic (using catecholamines), cholinergic (using acetylcholine), or peptidergic (using neuropeptides) as their principle neurotransmitter.

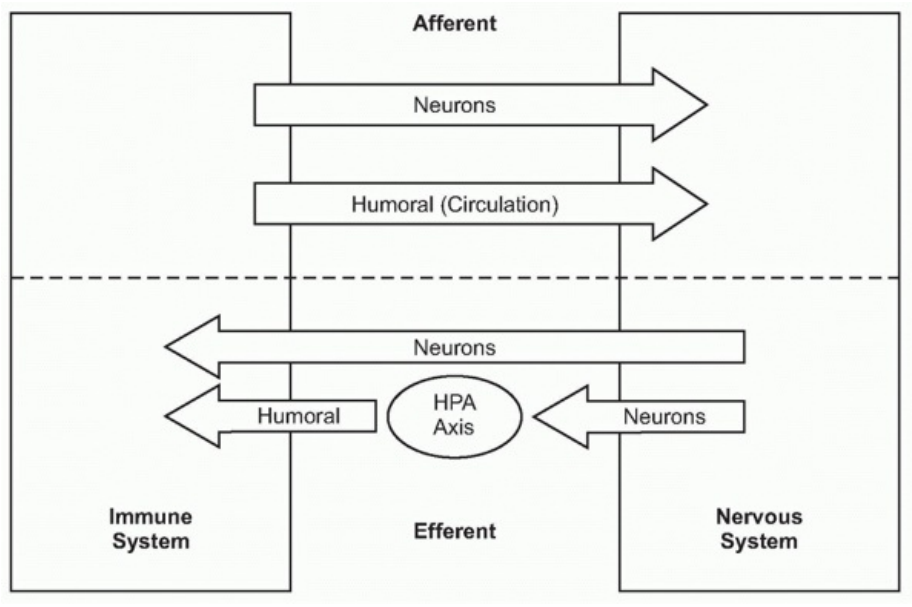

FIG. 35.1. Neural versus Humoral Communication Pathways between Nervous

System and Immune System. Neural pathways communicate to distant organ systems via action potentials transmitted through efferent nerves travelling to various organs. Alternatively, peripheral nerve endings get activated by systemic cytokines and transmit signals to the central nervous system. In humoral pathways, the central nervous system activates the hypothalamic-pituitary-adrenal axis to secrete glucocorticoid hormones in circulation that in turn modulates immune functions. The immune system utilizes the circulatory system to deliver inflammatory mediators to the central nervous system.

The autonomic nervous system provides the principle communication pathway by which the nervous system rapidly adjusts specific cellular functions to maintain homeostasis. The nervous system can also communicate to other organ systems via the neuroendocrine organs, a humoral pathway that relies on the bloodstream to deliver the final signal. As compared to the neural communication pathway, the additional steps of hormone release and transit through the circulatory system means that neuroendocrinederived homeostatic regulation signals are delivered much more slowly than neural action potentials. The primary signal in both systems, however, is neural, because neuroendocrine signaling is initiated by neurons in the brainstem and hypothalamus that relay action potentials to the autonomic nuclei, and the pituitary gland, to regulate the release of pituitary hormones into the bloodstream. This modulates the endocrine organ output of glucocorticoids (GCs), thyroid hormones, catecholamines, and other circulating hormones that influence cellular function and metabolism in other organ systems. Neural signaling mechanisms adjust cellular responses in an extremely rapid time frame, whereas the neuroendocrine system establishes longer-term, more chronic homeostatic setpoints.

THE NERVOUS SYSTEM MAINTAINS ORGAN SYSTEM HOMEOSTASIS

The basic physiologic unit that maintains organ homeostasis is the reflex. A neural reflex circuit is comprised of three components: a sensory arc, a relay or interneuron arc, and a motor arc (Fig. 35.3). The sensory (afferent) neural path relays information to the central nervous system. Sensory neurons respond to pressure, vibration, temperature, and other molecular signals (eg, taste) that trigger action potentials that travel to the spinal cord or brainstem. Molecular products of infection, injury, and inflammation including bacterial endotoxin, interleukin (IL)-1, and tumor necrosis factor (TNF) can also activate specific receptors on sensory neurons, which initiate afferent action potentials that transfer information to the central nervous system. Within the central nervous system, interneurons receive the incoming signals from sensory nerves and transfer the information to other brainstem nuclei,

which coordinate the efferent, or outgoing, signals back to the organs. In a neural reflex, which operates extremely quickly, the signals are projected back to the organs via neuronal action potentials. In a neuroendocrine reflex circuit, the efferent neurons project to the hypothalamic-pituitary-adrenal axis, which in turn regulates circulating hormone levels over more prolonged periods of time.

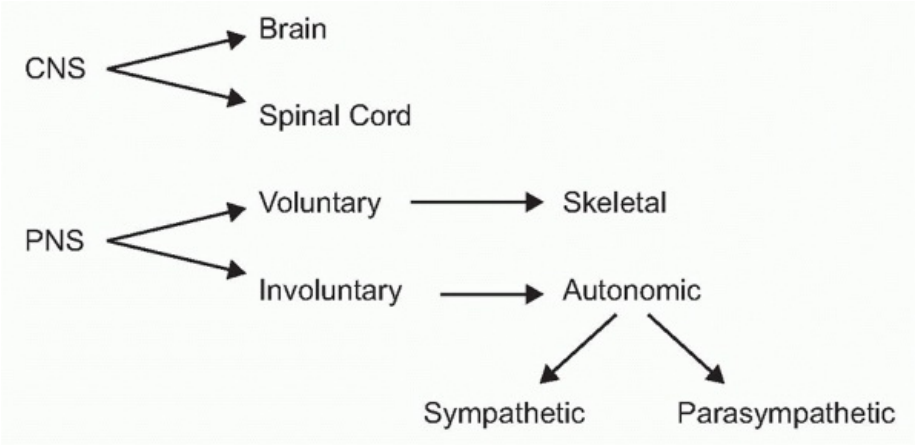

FIG. 35.2. Organization of the Nervous System. The nervous system is comprised of central nervous system (CNS) and peripheral nervous system (PNS). The CNS is comprised of two parts: the brain and the spinal cord. Most of the important processing of the information takes place in the CNS. The PNS is comprised of two divisions: the voluntary-somatic nervous system and the autonomic nervous system. The voluntary nervous system allows the brain to consciously (or voluntarily) monitor the environmental stimuli and respond to it. The autonomic nervous system maintains the physiologic balance of various visceral organs systems, such as respiratory, endocrine, cardiovascular, gastrointestinal, etc. The autonomic nervous system is further subdivided into sympathetic nervous system and the parasympathetic nervous system.

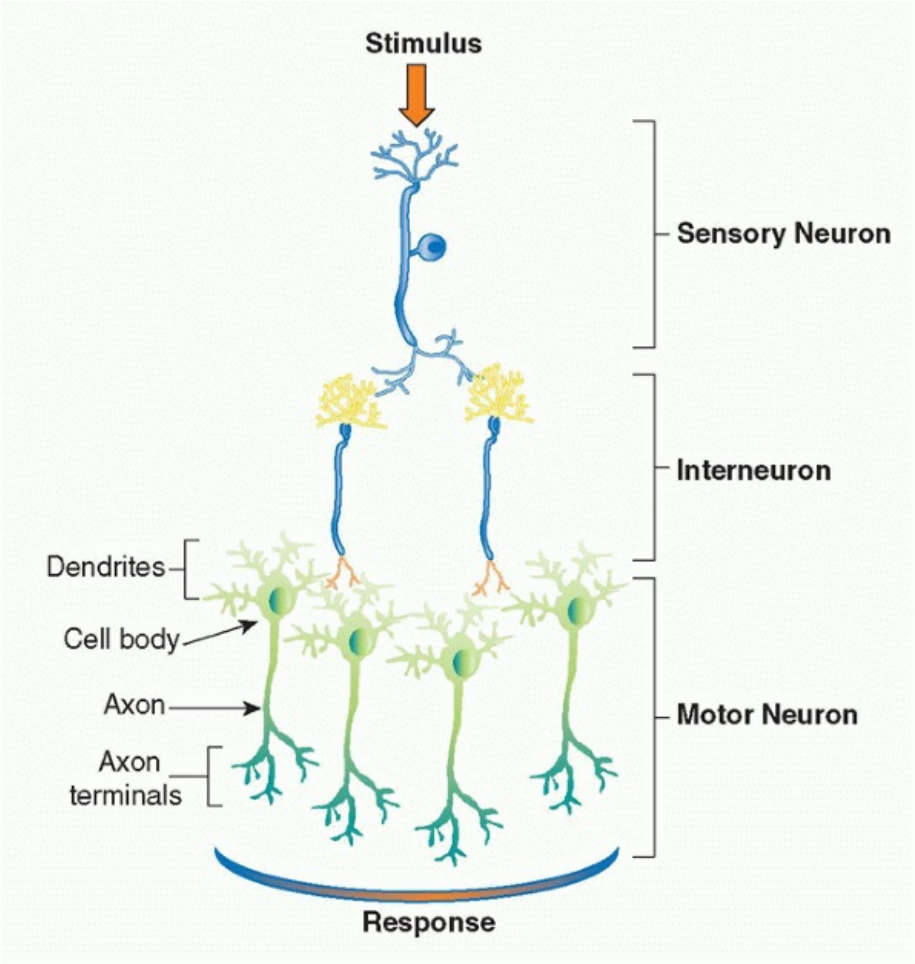

FIG. 35.3. The Neural Reflex Circuit. A neuron, the basic signaling unit of the nervous system, is comprised of a cell body containing the nucleus, dendrites that primarily receive chemical message from other neurons, an axon that can extend for significant length, and transmits electrochemical message to other cells. Three types of neurons comprise the neural reflex circuit: sensory neurons, interneurons, and motor neurons. The sensory neurons respond to stimulation from external or internal environment and transmit signals to the central nervous system. Motor neurons transmit information from the central nervous system to the periphery. Interneurons provide connections between sensory neurons and motor neurons, because they receive signals from the sensory nerves and project the outgoing signals via their axons to the dendrites of the motor neurons.

A useful example of reflex circuits that maintain homeostasis of organ function is the acute neural regulation of heart rate and the chronic neuroendocrine regulation of blood pressure. The heart rate control circuit begins with pressuresensitive neurons that transmit information about heart rate to interneurons in the brainstem. These relay information to efferent brainstem nuclei that activate either adrenergic or cholinergic nerves that project back to the heart. The descending neural signals either increase or decrease heart rate in response to deviations from the heart rate setpoint as established in the brainstem. Blood pressure also activates sensory neurons, which initiate signals that modulate the renin-angiotensin system, and the hypothalamic pituitary axis. These neuroendocrine responses establish hormone

levels that maintain chronic blood pressure levels. In both examples (heart rate and blood pressure), the reflex circuit is initiated by information traveling from the periphery to the nervous system; they differ in that neural signals elicit rapid changes in organ output, whereas the humoral pathway works to maintain long-term stability.

These reflex principles governing the cardiovascular and other relatively accessible organ system were established a long time ago. Only quite recently has it become possible to understand how these reflex principles operate in the more diffuse immune system. The remainder of this chapter reviews the neural and humoral mechanisms underlying the reflex control of immune homeostasis by the nervous system.

THE IMMUNE SYSTEM IS INNERVATED

As reviewed in Chapter 3 in this volume, the immune system is comprised of circulating cells and resident or tissue fixed cells residing primarily in the spleen, liver, lymph nodes, thymus, lungs, intestines, central nervous system, and bone marrow. These anatomic sites are richly innervated by the autonomic nervous system via neurons that are adrenergic, cholinergic, and peptidergic.[1,2,3] These neural circuits provide an anatomic and functional basis for direct neural communication between the nervous system and cells of the immune system. The majority of nerve fibers innervating the lymphoid tissues are adrenergic. They enter the lymphoid organs by traveling with the arterial vasculature; upon entering the tissue, they form networks that distribute to fields of lymphocytes and macrophages in the parenchyma. Innervation of lymphoid tissue is regional, primarily found in T-cell- and plasma-cell-rich zones, and lacking in nodular regions and zones of maturing B cells.[3]

Nerve terminals in the lymphoid tissues can undertake the appearance of a classic synapse on immune cells, which may be lymphocytes (Fig. 35.4),[4] eosinophils, mast cells, and

macrophages.[3,5] These innervated cells express receptors for cholinergic (acetylcholine), adrenergic (norepinephrine and epinephrine), and peptidergic (vasoactive intestinal peptide, pituitary adenylate cyclase activating peptide, calcitonin gene-related peptide), and other (substance P, histamine and serotonin) neurotransmitters.[6] Release of neurotransmitters in response to action potentials propagated along these neurons specifically alters the metabolism, biochemistry, and gene expression patterns in the immune cells. Thus, neurotransmitter release converts the electrical signals propagated along neurons to the immune system into molecular or pharmacologic signals that exert discrete action on the responding immunocompetent cells.

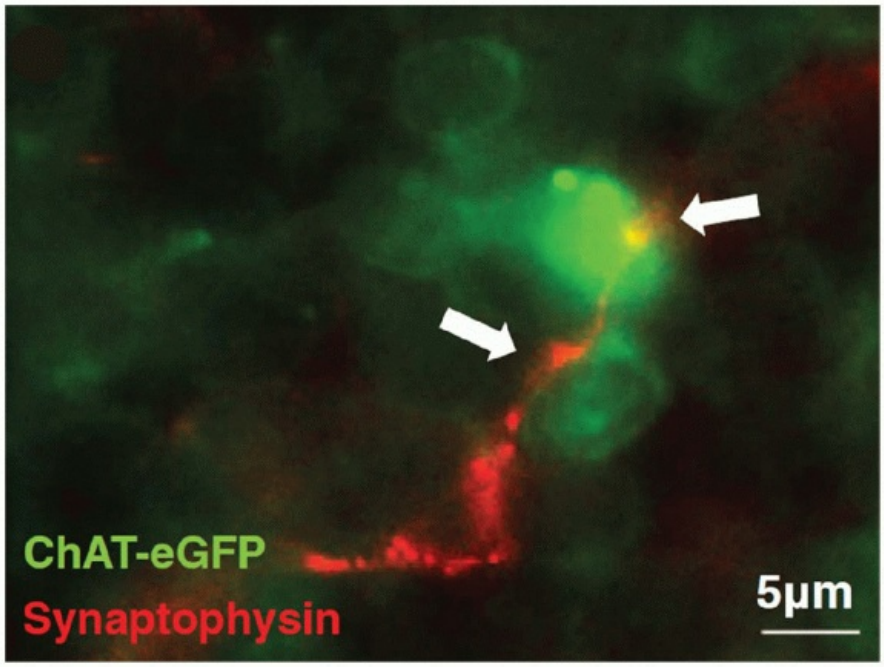

FIG. 35.4. Splenic Nerve Forms a Synapse with Lymphocyte. Immunohistochemical analysis of splenic nerve endings (synaptophysin, *red*) in direct contact with lymphocyte (choline acetyltransferase-enhanced green fluorescent protein+, *green*) in the white pulp of the spleen. Magnification × 630. Adapted from Rosas-Ballina et al.[4]

Innervation of the immune cells within lymphoid organs is not a static phenomenon, because the number, density, and distribution of nerve fibers can undergo dynamic changes during immune responses. The molecular products of an immune response significantly influence the remodeling of nerve fibers and the expression of neurotransmitter receptors in lymphoid organs. Nerve fiber density is increased in the spleen and thymus of SCID mice, which lack functional T and B cells, and in nude mice, which lack T cells.[7,8,9] Reconstitution of SCID or nude mice with T-lymphocytes restores the normal pattern of innervations, indicating that immune cells modulate neural network formation. Additionally, systemic or regional inflammatory challenges lead to increase in nerve fiber distribution in the lymphoid organs such as thymus, lymph nodes, and spleen.[10,11,12] A reduction in splenic nerve fibers is observed during adjuvant arthritis in rodents, a finding that is accompanied by significant changes in spleen cytokine release.[13,14] Co-culture studies have established that neurons can selectively establish and maintain contacts with immune cells, and that cytokines (IL3, IL6, granulocyte macrophage-colony-stimulating factor-1, and nerve growth factor) derived from immune cells promote neurite expansion.[15,16,17] Integrins and other cell adhesion molecules also participate in the dynamic nature of nerve and immune cell interaction. Thus, the changing milieu in the immune system organs provides important signals that prune or expand the neural network to these organs. As will be explained in more detail subsequently, these changing neural networks in turn modify the ongoing immune response.

Together with other data that are beyond the scope of this chapter, these findings indicate that the nervous system is hardwired to the major organs of the immune system and is

capable of conveying information that modulates immune cell function. These neural connections are plastic, operate rapidly, and are positioned to monitor and modify the immune response to products of infection, injury, or invasion.

THE NERVOUS SYSTEM MONITORS IMMUNE SYSTEM ACTIVITY

As the nervous system enjoys two routes to communicate with the immune system (neural and humoral), the immune system can also send information to the nervous system through both avenues (see Fig. 35.1). Indeed, peripheral inflammation profoundly affects the function of the brain and central nervous system (CNS) though both neural and humoral mechanisms. Molecular products of inflammation alter neurogenesis, neural plasticity, learning, and memory. They also produce fever, anorexia, and acutephase responses. Cytokines and other immune system-derived molecules traveling in the circulation can influence neurons in the central nervous system through five mechanisms that circumvent the blood-brain barrier (BBB), the tight endothelial junctions that normally partition large or hydrophilic molecules in blood from the extracellular fluid compartment in the CNS. First, cytokines can pass through circumventricular regions of the brain, anatomic areas where the BBB is absent or incompetent.[18,19,20] Cytokines carried in the circulation to these brain regions can directly interact with neurons residing in the hind brain and brainstem. Second, cytokines can be actively transported across the BBB via a saturable transporter system.[21,22,23,24] This depends upon endothelial cell expression of specific cytokine binding transporter receptors that mediate the active chaperoning of the cytokines across the BBB and into CNS extracellular fluid.[25,26,27] Third, endothelial cells and perivasculature macrophages can bind circulating cytokines or pathogenassociated stimulatory molecules on the intraluminal side, and respond by secreting cytokines and other inflammatory mediators into the brain parenchyma on the adluminal side.[28,29,30] Fourth, activated immune cells, including monocytes/macrophages, can traverse the microvasculature, crossing from the circulation and entering the CNS parenchyma, where they can produce cytokines.[31] Fifth, cytokines can act on peripheral sensory nerve endings at the site of infection or injury, and the ascending action potentials can activate neurons and glial cells in the CNS to secrete cytokines.[32,33,34,35] Together, the net influence of these pathways is to provide mechanisms by which cytokines and other soluble immune system mediators can directly influence the activity of neurons in the CNS, which modifies the activity of neural signaling networks.

For example, several cytokines have pyrogenic activity, meaning that they activate fever responses. Early work on these mechanisms focused on the roles of blood-borne IL-1 and TNF entering the hypothalamus and altering the function of hypothalamic thermoregulatory nuclei that regulate body temperature. More recent discoveries revealed that the vagus nerve also communicates direct neural signals to mediate IL-1-induced fever. The vagus nerve connects the major body organs to the brainstem, and as many as 80-90% of the neurons that travel in the vagus nerve are afferent (sensory). This provides an important, rapid, and efficient neural route for communicating information about the organ milieu to the brain.[36] These afferent vagus nerve fibers express receptors for IL-1 and other inflammatory mediators.[32,37,38,39,40,41,42] Activation of these receptors by binding to IL-1 is an important mechanism for initiating fever, because cutting the vagus nerve prevents the

development of fever in animals receiving of IL-1 by intra-abdominal injection.[38,43,44] Cytokine-activated afferent vagus nerve signals alter the function of thermoregulatory hypothalamic nuclei and the activity of other neurons in brainstem, hypothalamus, and limbic structures (Fig. 35.5). Other sensory nerve fibers including,

for example, the glossopharyngeal nerves, can be activated by cytokines to mediate fever.[35,45,46] Neurophysiologic studies have demonstrated that administration of IL-1 in visceral organs stimulates the generation of action potentials that travel in the vagus nerve sensory arc, ascend to the brainstem, and return to the spleen and other organs.[47,48,49] Signals ascending to the nucleus tractus solatarius can be relayed throughout the brain, to the parabrachial nucleus, the thalamus, the paraventricular nucleus, the central nucleus of the amygdala, the insula cortex, the infralimbic cortex, the anterior cingulate cortex, and the medial prefrontal cortex. Thus, cytokines do not have to enter the bloodstream to mediate the onset of fever and other behavioral responses, because they can also activate these responses by binding to sensory neurons in distant peripheral tissues.

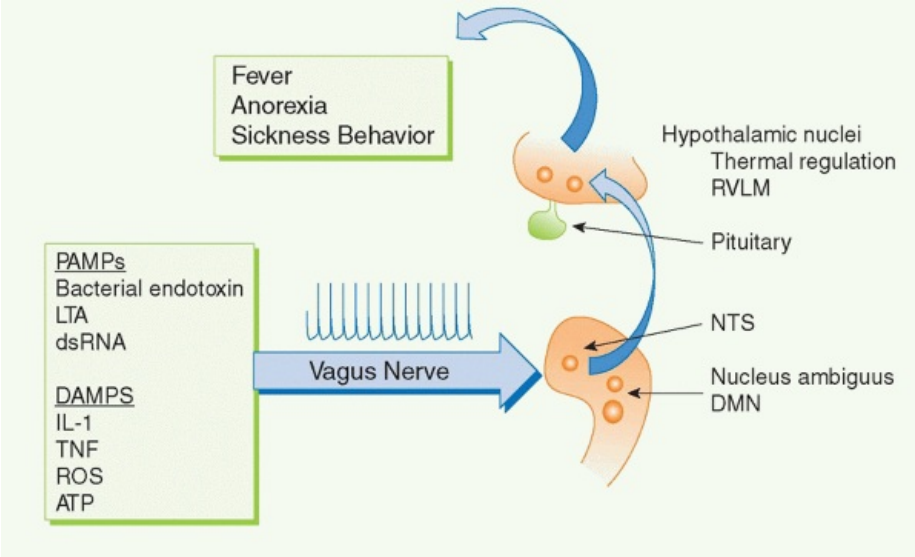

FIG. 35.5. Systemic Cytokines Modulate the Central Nervous System via Vagus Nerve. Products derived from infectious agents and sterile injury lead to cytokine release by the innate immune system. The systemic cytokines activate the afferent signals in the vagus nerve, which in turn transmits the information to the central nervous system, mediating the alteration of thermoregulatory hypothalamic nuclei resulting in fever, anorexia, and other symptoms related to sickness behavior. DMN, dorsal motor nucleus; NTS, nucleus tractus solatarius; RVLM, rostral ventrolateral medulla.

There are also immunocompetent cells residing within the CNS that can be activated to produce immunologically active molecules within the brain and spinal cord, where these mediators can exert their effects on neurons, locally. Numerous cell types in the CNS produce cytokines (and other inflammatory mediators) including astrocytes, microglia, lymphocytes, and dendritic cells. Immunocompetent cells are present in the choroid plexus, meninges, and brain parenchyma, and microglia form a regularly spaced network of resident cells throughout

the CNS. Microglia are morphologically, phenotypically, and functionally related to the monocyte/macrophage cell lineage.[50,51,52] They are quiescent in healthy conditions, but respond rapidly to pathological conditions associated with infection, injury, invasion, or neuronal degeneration.[53] Microglial activation leads to cell proliferation, enhanced expression of cytokines and other inflammatory mediators, and recruitment to inflammatory sites.[50,54] Activation of micoglia and neurons by molecular products of inflammation induces the release of cytokines (e.g., IL1, IL6, and TNF) and other inflammatory mediators (e.g., prostaglandins and nitric oxide) (Table 35.1). The local production of these mediators alters the activity of brainstem nuclei that mediate the onset of fever, anorexia, sickness behavior, acute-phase responses, and other neurologic responses.

Activated microglia upregulate the activity of phospholipase A2 and cyclooxygenases that catalyze the synthesis and release of prostaglandins and other eicosanoid mediators of inflammation. Other cells in the nervous system can be activated to release eicosanoid mediators during inflammation including astrocytes, neurons, and endothelial cells in the brain vasculature. Eicosanoid metabolites function in autocrine and paracrine signaling loops by binding to specific G-protein-coupled receptors expressed

on neurons. Receptor-ligand signal transduction culminates on pathways that increase intracellular cyclic adenosine monophosphate (cAMP) concentrations. Eicosanoid signaling in neurons has been implicated in the development of fever, acute-phase protein responses, and impaired memory and learning. Infection, trauma, neurodegenerative diseases, and acute and chronic inflammatory diseases are all associated with increased release of cytokines and eicosanoids in the brain. Importantly, low-level eicosanoid production is necessary for neuronal homeostasis and is essential in the mechanisms underlying memory formation and neural plasticity (Fig. 35.6). Eicosanoid levels that are either too high or too low impair memory development, thermoregulation, and feeding behavior. An inverted U-shaped doseresponse curve links IL-1 levels to memory function, which may be attributable to IL-1 signaling mechanisms that stimulate eicosanoid release in these mechanisms.[55,56] The neurophysiologic mechanisms underlying memory impairment in inflammatory conditions have been studied in brain slices, and addition of cyclooxygenase-2 inhibitors restores amyloid β-mediated impairment of long-term potentiation, the cellular neurophysiologic mechanism that links neuronal activity to memory formation.[57]

TABLE 35.1 Inflammatory Mediators Produced by Microglia and Other Immunocompetent Cells that Alter Neurologic Behavior

Inflammatory Mediator	Effects
IL1, IL2, IL6, IL8, TNF, MIP1, IFNα, IFNγ	Fever
IL1β, IL6, TNF, prostaglandins	Pain
IL1β, IL6, IFNγ, TNF	Anorexia

IL1β, IL6, TNF, IL2, IFNγ	Depression
IL1β, IL6, TNF, IL8, CRP	Anxiety
IL1, IL6, TNF, prostaglandins	Decreased neurogenesis
IL1, IL6, TNF, prostaglandins	Impaired memory and learning
IL1, IL6, TNF, prostaglandins	Impaired neural plasticity
IL1β, IL6, TNF, TGFβ, IL8, MCP1	Alzheimer disease pathogenesis
IL1β, TNF, HMGB1, IL6, IFNα	Epilepsy pathogenesis
IL1α, IL1β, IL6, TNF, IL18, ROS, TGFβ, IL10	Ischemia/stroke pathogenesis

CRP, C-reactive protein; HMGB, high mobility group box; IFN, interferon; IL, interleukin; MIP, macrophage inflammatory protein; ROS, reactive oxygen species; TNF, tumor necrosis factor.

Activated microglia are also stimulated to produce increased levels of reactive oxygen species (ROS), including hydrogen peroxide, superoxide anion, and hydroxyl radicals. Like other inflammatory mediators, ROS are homeostatically regulated because their activities can be both deleterious and beneficial.[58,59] At low concentrations, free radicals play an important role in several cellular signaling systems. ROS regulate redox sensitive transcription factors such as NF-κB, AP-1, and nuclear factor of activated T-cells, and the redox state of tyrosine phosphorylated proteins, thereby having an impact on many transcriptional networks and signaling cascades important for neurogenesis. Low concentrations of ROS play a significantly beneficial role in neurogenesis. However, elevated ROS levels are extremely neurotoxic because they oxidize essential macromolecules such as enzymes and cytoskeletal proteins, lipids, and nucleic acids leading to neuronal death by apoptosis. ROS-mediated neuronal toxicity and degeneration is implicated in the pathogenesis of Alzheimer disease, Parkinson disease, and other neurodegenerative disorders.[60,61] Physiological ROS levels facilitate synaptic plasticity and cognitive function, but elevated ROS levels impair these functions. Thus, even within the CNS itself, the regulation of immune responses establishes a critical level of immunologic homeostasis that is crucial for normal, healthy nervous system function.

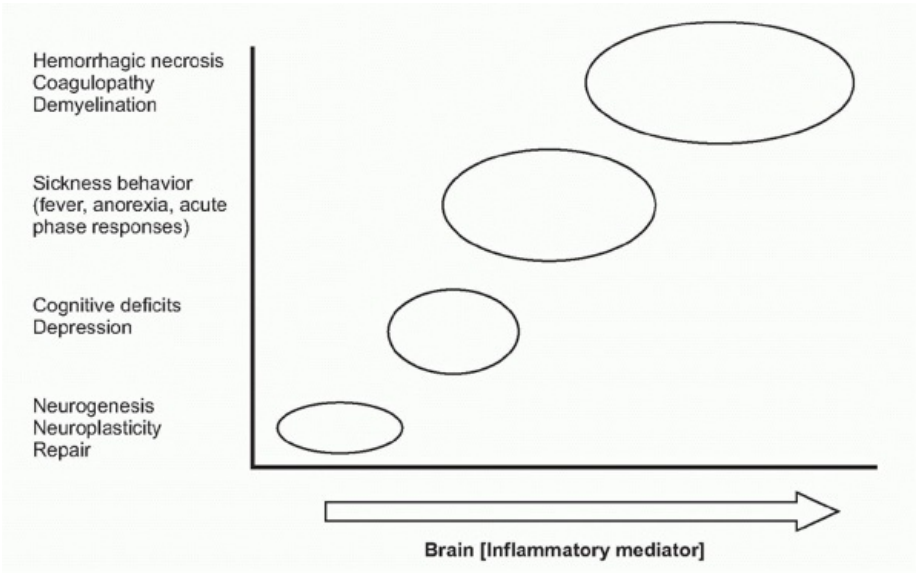

FIG. 35.6. Neural Responses to Inflammatory Mediators. Immune mediators can access the central nervous system and influence virtually every aspect of the brain function relevant to optimal brain functioning including neuronal development and function (neurogenesis and neural plasticity), behavior changes (cognitive deficits, sickness behavior), and hemorrhagic necrosis. The response of the nervous system is modulated by the nature of the immunologic mediators, as well as magnitude and duration of exposure.

NEURONS RESPOND SPECIFICALLY TO IMMUNE SYSTEM MOLECULES

Immune mediators that influence neuronal function in turn modify the behavior and physiology of the organism, because behavior and physiologic setpoints are primarily governed by the nervous system. The output of the nervous system is dependent upon the nature of the immunologic mediators, as well as magnitude and duration of exposure.

Immune system-derived signals can therefore produce a range of nervous system-mediated organism responses that cover a spectrum ranging from beneficial to deleterious (see Fig. 35.6). Some examples are presented in the following.

Neurogenesis produces thousands of new neurons every day in the mammalian brain, primarily within the subventricular zone and the subgranular zone. The activity and replication capacity of neural stem cells in these regions, which are multipotent and self-renewing, are regulated by immune system mediators including IL-1, IL-6, and TNF. Intracortical brain injury and systemic inflammation that activates microglial cells impairs neurogenesis. IL-1, IL-6, and TNF released by activated microglia play a pivotal role in this suppression of neurogenesis.[62,63] IL-1 induces impairment of hippocampal neurogenesis via two mechanisms. The first is by activation of hypothalamuspituitary-adrenal (HPA) axis and GC secretion that in turn lead to impaired hippocampal neurogenesis.[64,65,66] The second mechanism involves a direct inhibitory effect of IL-1 on neuronal progenitors via IL-1 receptor-mediated mechanism leading to NF-κB activation. This inhibitory effect of IL-1 can be attenuated by blockade of IL-1 signaling.[64,67,68] Elevated IL-1 levels have been implicated

in several syndromes associated with impaired neurogenesis, including depression, stroke, infection, and stress. TNF signaling through the TNF receptor type I inhibits neurogenesis and induces differentiation of neural stem cells toward astrocytic, rather than neuronal, phenotypes.[69,70,71] IL-6 inhibits neurogenesis by decreasing cell survival and reducing neuronal differentiation.[63,72]

Learning and memory are neuronal processes that produce persistent changes in brain neural networks. In the hippocampus, a brain region that occupies a critical role in these functions, neural networks are extremely plastic. The neurophysiologic basis of this plasticity requires long-term potentiation (LTP) and long-term depression, the activitydependent enhancement or attenuation synaptic efficacy. Under homestatic conditions, cytokines play a crucial role in the trophic support of neurons that enhances neuronal integrity. In inflammatory conditions, however, enhanced cytokine production is detrimental, because IL-1, IL-6, and TNF produced by activated microglia, or entering the hippocampus from the circulation, suppress LTP and impair synaptic plasticity.[73] This significantly impairs the organism's ability to learn and remember. Exposure of rat hippocampal slices to IL-1 or TNF leads to suppression of LTP and basal synaptic transmission.[73,74] Low IL-6 levels are involved in mediating transition between short- and longterm plasticity, but at increased levels, IL-6 interferes with LTP process.[75] LTP is accordingly suppressed in diverse inflammatory conditions including infection, trauma, neurological diseases, stress, and aging.[76,77,78,79,80]

Cytokines and prostaglandins are directly implicated in the impairment of learning and memory that accompanies infection, stress, and aging. Systemically and locally (brain) elevated levels of IL-1 specifically interferes with hippocampal dependent spatial learning.[81,82,83] Chronic transgenic expression of IL-1 in the hippocampus produces microglial activation and increased production of inflammatory mediators with impairment in spatial memory and long-term contextual fear memory, another hippocampal-dependent memory phenotype.[84,85] IL-1 has also been implicated in mediating the detrimental effects of stress and aging on memory in the hippocampus.[86,87,88] Age-related memory impairment is associated with significant increases of IL-6 in the circulation and brain, and administration of IL-6 inhibitors can prevent memory loss.[89,90] The acute administration of IL-6 failed to produce any significant memory impairment,[91] providing an example of the importance of exposure duration to determining the net response of the nervous system to immune system-derived mediators. The role of TNF in modifying memory is less clear, because knockout animals rendered deficient in TNF do not have memory impairment.[92] Elevated TNF levels do impair performance tasks that are hippocampus dependent, and these responses are dependent upon the age of the organism and the duration of TNF exposure, which may be attributable to TNF stimulating the release of IL-1 and IL-6.[93,94,95]

Higher levels of cytokines and other inflammatory mediators complicate infection and injury, resulting in subjective feelings of sickness in the form of malaise, lethargy, fatigue, coldness, numbness, reduced appetite, and muscle and joint pain. Together with fever, these responses are termed “sickness behavior.”[96,97,98] For many decades, sickness behavior was presumed to be directly mediated by the molecular products of pathogen invasion that

directly altered neuronal, and therefore, nervous system functions. Advances in understanding cytokine biology revised this view and demonstrated that sickness behavior is a highly organized immune and nervous system behavior that enhances the ability of the organism to recover.[96,97,98] The principle mediators of sickness behavior are produced by the immune system during infection or injury, including IL-1, IL-6, TNF, and dangerassociated molecular pattern molecules (DAMPs). These have a pivotal role in orchestrating the inflammatory response and in restoring homeostasis.

The effects of immune system-derived signaling molecules on nervous system function and behavior is not limited to acute and subacute infection or injury: they also contribute to the pathogenesis of autoimmune and autoinflammatory diseases. Depression and cognitive impairment are disabling complications of chronic autoimmune diseases, including systemic lupus erythematosis. Up to 30% of lupus patients have elevated levels of antideoxyribonucleic acid autoantibodies that cross-react with the N-methyl-D-aspartate receptor to mediate an agonistic signal in neurons. The BBB in these patients is porous to these antibodies, enabling high levels of these signaling antibodies to accumulate in the hippocampus, where the agonist N-methyl-D-aspartate activity is cytotoxic to neurons, impairs LTP, and adversely influences learning and memory.[99,100,101,102] This has significant implications for neural developmental disorders, because the pups of antibody-bearing pregnant dams have evidence of hippocampal cytotoxicity and developmental abnormalities associated with behavior and learning.

NEURAL SIGNALS MAINTAIN IMMUNE SYSTEM HOMEOSTASIS

At this point, we have reviewed how immune system-derived molecules deliver information to the nervous via humoral and neural routes, which reports the organism's immune status to the brain. Signaling is accomplished by activation of afferent neurons in the periphery, accomplished by the direct action of mediators that either cross the BBB, or are produced locally within the brain and spinal cord. Neurons respond to this information by generating action potentials and by altering their cellular physiology that modifies the behavior in neural networks and shapes physiological responses. The result is that the organism alters its behavior and physiological status. As discussed in the following, these incoming signals occupy a critical role in initiating reflex responses to the immune system. These outgoing signals from the nervous system to the immune system utilize both of the major communication routes, neural and humoral. These will be considered separately.

A NEURAL CIRCUIT THAT REGULATES IMMUNITY IS THE "INFLAMMATORY REFLEX"

In the 1990s, a surreptitious finding led to the discovery of the inflammatory reflex, a prototypical neural circuit that regulates the output of the innate immune system. The experiments that were being performed had originally set out to delineate the anti-inflammatory mechanisms of CNI-1493, a tetravalent guanylhydrazone that inhibits the release of TNF and other cytokines released during innate immune responses. The investigators observed that the intracerebral administration of extremely low quantities of CNI-1493 significantly inhibited serum TNF during endotoxemia. This was an extremely puzzling result because the quantities of CNI-1493 delivered were below the efficacy concentration threshold necessary to suppress cytokine release from macrophages in vitro.

Reasoning that the presence of CNI-1493 in the brain was activating neurons that in turn relayed these signals to the peripheral immune system, the investigators repeated the experiments, only this time the animals were first subjected to vagotomy, a procedure to surgically interrupt the vagus nerve. The vagus nerve (which is named for its wandering course) innervates all of the major organs of the reticuloendothelial system: liver, lung, spleen, kidneys, and gut. Selectively cutting the vagus nerve completely abolished the anti-inflammatory actions of CNI-1493 administered into the brain.[103,104] This first established that molecular signals originating in the brain could be transduced into neural signals that were transmitted by the vagus nerve to inhibit the innate immune system (Fig. 35.7).

The vagus nerve, the tenth cranial nerve, originates in the brainstem. It is named for its wandering path through the neck, thorax, and abdomen, where it terminates on ganglia in close proximity to target organs. Up to 90% of the neural fibers in the vagus nerve are sensory, which transmit sensory information to the brain. These afferent signals in response to IL-1 in peripheral tissues are both necessary and sufficient to initiate fever and other sickness behavioral responses to injury or infection.[32,38] In the inflammatory reflex, both the afferent and efferent nerve signals travel in the vagus nerve. It is a prototypical neural circuit that modulates inflammatory responses in regionally, tissue-specific manner. Inflammatory molecules of exogenous (ie, pathogen-associated molecular pattern molecules [PAMPs]) or endogenous (ie, DAMPs) origin activate afferent nerve fibers in the vagus nerve that travel to the brainstem. As the incoming signals arrive there, they are relayed to interneurons in the nucleus tractus solatarius, which relays signals to the motor nuclei of the vagus nerve, including the nucleus ambiguous and the dorsal motor nucleus of the vagus. This culminates in action potentials that return along the vagus nerve via efferent vagus nerve fibers to the reticuloendothelial organs. There is another relay station in the outgoing pathway, because the vagus neurons terminate in the celiac ganglion, a small collection of neurons that feed axons into the splenic nerve, the only efferent nerve to the spleen.[5] Electrical or pharmacologic stimulation of the efferent vagus nerve arc produces acetylcholine release in the spleen, the neurotransmitter that suppresses macrophage cytokine release.[105] This efferent circuit of the inflammatory reflex is termed the “cholinergic anti-inflammatory pathway.”

The inflammatory reflex controls immune cell function in the spleen by transmitting action potentials through two serially connected neurons. The first is preganglionic, originating in the dorsal motor nucleus and nucleus ambiguous in the brainstem, and the second is postganglionic, originating in the celiac ganglion, the location of neural cell bodies that project axons in the splenic nerve to innervate the spleen.[106,107] The secondary splenic nerve fibers are adrenergic, not cholinergic, and utilize norepinephrine as the primary neurotransmitter.[108] Ablation of splenic neurons impairs the regulation of spleen TNF by vagus nerve stimulation, indicating that splenic neurons are integral to the cholinergic anti-inflammatory pathway. This occurs because action potentials transmitted by the efferent vagus nerve fibers culminate in the release of acetylcholine from nonneuronal splenic cells. Recently, the cellular source of this splenic acetylcholine was identified as T-lymphocytes in the spleen.[105]

T- and B-lymphocytes possess the essential molecular components required to synthesize and release acetylcholine. These include choline acetyltransferase, the enzyme that

catalyzes the biosynthesis of acetylcholine, and the high-affinity choline transporter that exports acetycholine from the cells.[109,110,111] T-cell activation by phytohemagglutinin, phorbol ester, and dibutyryl cAMP can enhance the synthesis and release of acetylcholine.[112] Splenic nerve fibers terminate in close proximity with lymphocytes to form synapse-like structures, and these lymphocytes express beta-adrenergic receptors that can respond to the neurotransmitter released by splenic nerve action potentials.[1,3,113,114] The first proof that these acetylcholine producing T cells were essential to the inflammatory reflex came from studies in nude mice, which lack functional T cells but do have B cells. Reconstitution of T cells by adoptive

transfer restores the inflammatory reflex in nude mice.[105] The identity of these acetylcholine producing spleen T cells has recently been accomplished by studying transgenic mice that express enhanced green fluorescent protein under the control of transcriptional regulatory elements for choline acetyltransferase. Only 2% to 3% of total T cells express green fluorescent protein, indicating that only a relatively small subset of total spleen T cells synthesize acetylcholine. The majority of these acetylcholine producing T cells are cluster of differentiation (CD)4+, and predominantly express markers of the memory phenotype, $CD44^{high}$ $CD62L^{low}$. Although only 4% of CD4+ T cells express acetylcholine, a total of 10% to 11% of the $CD44^{high}$ $CD62L^{low}$ memory population express acetylcholine. These cells reside within close proximity to adrenergic nerve endings in splenic white pulp parenchyma. T-lymphocytes express β2 adrenergic receptors, and stimulation of these receptors enhances acetylcholine release from this T-cell subset.[105,115]

The molecular mechanism of acetylcholine inhibition of macrophage activation and cytokine release requires signaling through a specific cholinergic receptor, the " α7 nicotinic acetylcholine receptor" (α7 nAchR). It is expressed by macrophages and other cytokine-producing cells.[116,117] Animals deficient in vagus nerve signaling by cutting the vagus or splenic nerves, or lacking molecular signaling components of the cholinergic anti-inflammatory pathway by α7 nAchR gene knockout or administration of pharmacologic α7 nAchR antagonists, are rendered exquisitely sensitive to inflammatory challenges. Because the cholinergic anti-inflammatory pathway normally provides a braking mechanism to restrain excessive cytokine release, an incomplete inhibitory neural circuit (as occurs in α7 nAchR knockout mice or when the vagus nerve is surgically cut) produces exaggerated innate immune responses. Preclinical studies in animal models and humans have established that vagus nerve activity is impaired during inflammation, an observation that agrees with an important role played by this neural circuit in maintaining normal immunologic homeostasis.

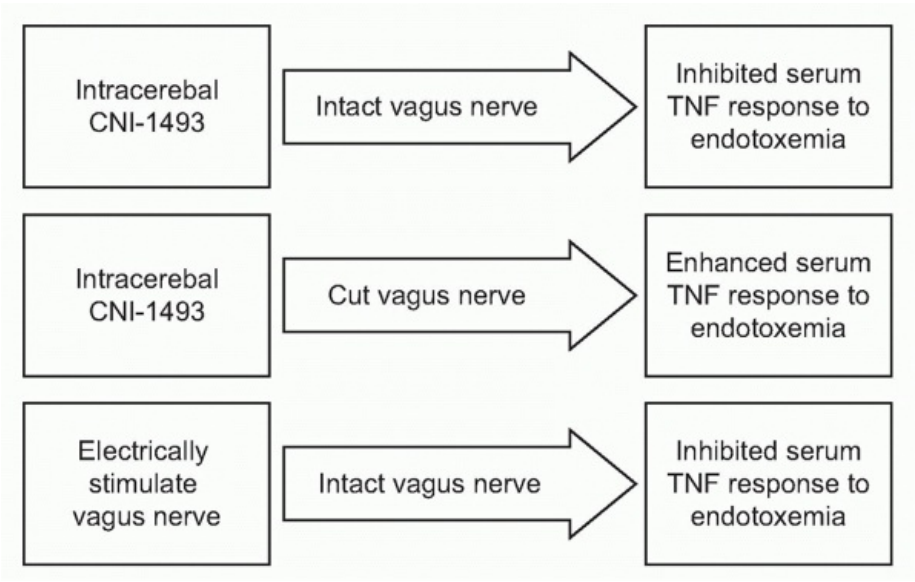

FIG. 35.7. Discovery of the "Inflammatory Reflex." Intracerebroventricular administration of CNI-1493, a tetravalent guanylhydrazone attenuates systemic inflammatory responses. This effect is abolished when animals are subjected to selective vagotomy, indicating that CNI-1493 relays signals to the periphery via the action potentials in the efferent vagus nerve. This was confirmed by activating the action potentials in the efferent vagus nerve by direct electrical stimulation. These observations provided the first experimental evidence for molecular signals generated in the brain and transmitted to the periphery by vagus nerve to inhibit the innate immune responses.

The identification of this neural circuit launched the development of selective α7 nAchR agonists as experimental therapeutics, and this has facilitated the first studies of signaling mechanisms. Selective α7 nAchR agonists significantly attenuate cytokine release by macrophages in response a range of PAMPs and DAMPs that interact with TLR2, TLR3, TLR4, TLR7, TLR9, and RAGE.[118] Signal transduction via α7 nAchR on macrophages leads to physical interaction between α7 nAchR and adenylate cyclase 6, which generates increased levels of intracellular cAMP. This leads to increase in of the transcription factor cAMP response element binding protein, and subsequent increase in expression of c-fos. Activation of c-fos attenuates NF-κB activity leading to inhibition of transcription of cytokines.

It is important to consider that the inflammatory reflex and the cholinergic anti-inflammatory pathway are neither "parasympathetic" nor "sympathetic" (Fig. 35.8). This point has created some confusion in the literature because there has been a tendency to ignore Henry Dale's original recommendation, as noted previously, that neural circuits should be described by their neurotransmitters, not their function. The inflammatory reflex has been incorrectly described as "parasympathetic," based on reasoning that the vagus nerve is classically considered to be the principal parasympathetic nerve. But as defined here and elsewhere, in the inflammatory reflex the neural signals are transmitted in the vagus nerve, then pass through the adrenergic splenic nerve before being converted back to a cholinergic signal by the T cells in the spleen.[5,105] The primary neuron (vagus) is cholinergic, the secondary neuron (splenic) is adrenergic, and terminal neurotransmitter (acetylcholine) is produced by T-lymphocytes. This circuit does not conform to either sympathetic or parasympathetic, a

point that cannot be overstressed. Perhaps the strongest argument for this point is that the cholinergic anti-inflammatory pathway can be activated

experimentally by electrically stimulating the adrenergic splenic nerve, which is often labeled (inappropriately) as a “sympathetic” nerve. Clearly, lucid understanding requires precise nomenclature, because the inflammatory reflex, while perhaps the most completely characterized neural regulatory circuit to the immune system, is not the only neural pathway that modulates immunological homeostasis.

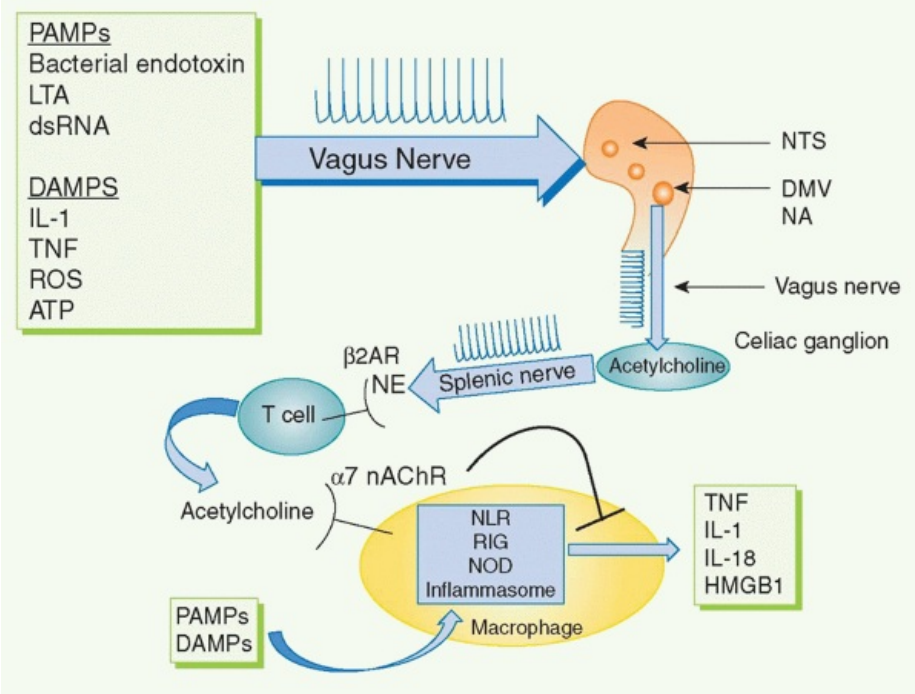

FIG. 35.8. The Inflammatory Reflex and the Cholinergic Anti-inflammatory Pathway are Neither “Parasympathetic” nor “Sympathetic.” In the inflammatory reflex, the cytokine mediators generated in response to infectious or sterile injury challenges are sensed by the afferent (sensory) vagus nerve, which transmits the information to the brain. The efferent arm of the inflammatory reflex is called the cholinergic anti-inflammatory pathway. The brain network generates the efferent (motor) vagus nerve signals that transmit via the cholinergic vagus nerve to secondary adrenergic splenic nerve, and terminate in acetylcholine production by T-lymphocytes. The terminal neurotransmitter, acetylcholine, inhibits cytokine production via pathways dependent on the α7 subunit of the nicotinic acetylcholine receptor on macrophages.

ADRENERGIC NEURONS MODULATE DIVERSE RESPONSES IN LYMPHOCYTES, MONOCYTES, AND OTHER IMMUNE CELLS

As described previously, many organs of the immune system receive innervation from adrenergic neurons that are postganglionic, residing in ganglia in the vicinity of the target organs. The preganglionic signals, which originate in neurons residing in the intermediolateral cell column of the lateral horn of the spinal cord adjacent to T1-L2, are

transmitted to the ganglia via myelinated axons that exit the spinal cord through ventral nerve roots. Within the brainstem, the major nuceli that control the activity of neurons in the sympathetic chain are located in the paraventricular nucleus of the hypothalamus, the rostral ventrolateral medulla, the ventromedial medulla, and the caudal raphe nucleus. Within the immune system organs, the major neurotransmitter of the adrenergic postganglionic neuron is norepinephrine, but other neurotransmitters (eg, vasoactive intestinal peptide) can also be released from these neurons. Within the spleen, these neurons can terminate as close as 6 nm from the resident lymphocyte (see Fig. 35.4).[1,113]

Norepinephrine biosynthesis from tyrosine is catalyzed by tyrosine hydroxylase, which is the rate-limiting enzyme in the process. Norepinephrine biosynthesis is completed by membrane-bound dopamine-hydroxylase residing in vesicles at the axon terminus. Once released by the arrival of action potentials in the postganglionic neuron, norepinephrine binds to three beta adrenergic receptors (β1-3), but the principle receptor implicated in modulating immune cell responses is β2, a member of the classic 7-transmembrane heterotrimeric guanine nucleotide-binding protein (G-protein) receptor family. β2 androgen receptor (AR) ligand-receptor interaction stimulates adenylyl cyclase activity and increases intracellular adenosine 3,5-cAMP and protein kinase A activity. This enhanced protein kinase A activity has been implicated as a critical step in signal transduction leading to downstream target responses and altered transcriptional activity. Norepinephrine signaling via βAR has been implicated as immunosuppressive on lymphocytes, monocytes, macrophages, natural killer cells, and neutrophils via β-adrenoceptors. In B cells, norepinephrine signaling via β2 has been implicated in enhancing proliferation and antibody responses.

On the other hand, norepinephrine can also interact with αARs, a receptor class that exerts proinflammatory and immunostimulatory effects. The expression of αAR and βAR within a given immune cell can be influenced by the activation status of the cell and by the presence of hormones and inflammatory factors in the extracellular milieu. The net effect of an immune cell response to norepinephrine is critically dependent upon the ratio of αAR and βAR expression. Therefore, because of absent information about AR expression and the specific contribution of αAR and βAR to signal transduction in a given cell, it is not generally possible to predict the net influence of norepinephrine on the immune response as inhibitory or stimulatory.

To this point, therefore, we have reviewed the principles governing the neural regulation of innate and adaptive immune responses. The first regulatory steps are initiated by incoming signals from PAMPs, DAMPs, hormones, and mediators derived from the immune system itself that signal to the nervous system via humoral and neural routes. This

incoming information is processed by brainstem nuclei, then relayed to neurons in the nuclei that regulate the output of the cholinergic and adrenergic input to the immune system. These neural circuits can be mapped and studied, one example being the prototypical inflammatory reflex that was defined by combining basic neurophysiology and immunobiology techniques to *in vivo* studies of immunologic homeostasis. It is likely that the future application of these techniques to other neural circuits will reveal the functional importance of other reflex mechanisms that operate through adrenergic and cholinergic neural signaling to modulate the cellular substrates of immunity.

A HUMORAL CIRCUIT THAT REGULATES INNATE IMMUNITY IS THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

Having reviewed the neural reflex regulation of the immune system, what follows is a review of a principle humoral pathway from the nervous system that maintains immunologic homeostasis. The neuroendocrine system provides a major regulatory influence on the immune system that operates chronically and transmits its terminal signals by modulating hormone levels in the circulation. This signaling network, comprised of the hypothalamus, the pituitary gland, and the adrenal glands, and termed the "HPA axis," is the major source of GCs. In the mid-20th century, Kendall, Reichstein, and Hench first demonstrated the anti-inflammatory and immunosuppressive effects of GCs, for which they later received the Nobel Prize, and since then effects of these hormones on immune function has been exhaustively studied. The HPA axis is activated by physical, psychological, and immunologic stress that cause release of corticotrophinreleasing hormone and arginine vasopressin by neurons arising in the paraventricular nucleus of the hypothalamus. Corticotrophin-releasing hormone and arginine vasopressin induce adrenocorticotropic hormone secretion from the anterior pituitary gland, which enters the circulation, travels to the adrenal gland, and stimulates GC release from the adrenal cortex. Thus, the HPA axis functions as another set of brakes on the immune system in order to prevent damage to the organism caused by an excessive immune system activation. It shares this role with the inflammatory reflex, except that it transmits anti-inflammatory signals via the humoral pathway, which can maintain long-term immunologic homeostasis, instead of through hardwired neural circuits that can operate much more rapidly to alter immune responses.

The predominant effector molecules of the HPA axis are GCs, and their major effect on the immune system is to produce immunosuppression. Chronic stress or infection is associated with chronically elevated GC levels that have been implicated in suppressed cytokine release, impaired antibody responses to vaccination, increased susceptibility to bacterial and viral infections, and a shift from Th1 to Th2 phenotype that can enhance delayed-type hypersensitivity responses. Deficiencies in HPA axis function are associated with increased susceptibility to autoimmune, autoinflammatory, infectious, and other injury syndromes associated with activation of innate immune responses. To extend the brake-failure analogy, impairment of the HPA axis by surgical lesions, genetic ablation, or pharmacologic intervention renders animals and humans exquisitely sensitive to exaggerated immune responses that damage host tissues and impair organ function. Deficient HPA axis restraint on immune responses has been implicated in the pathogenesis of systemic lupus erythematosis, rheumatoid arthritis, Sjogren syndrome, inflammatory bowel disease, psoriatic arthritis, severe sepsis, burn injury, septic shock, viral infection, and the response to a range of bacterial toxins. Thus, the neutrally regulated output of the HPA axis occupies an important role in maintaining immunologic homeostasis in the intact organism.

GC signal transduction is mediated by receptor ligand interaction with receptors that are widely expressed by immune system cells. There are two major isoforms of the GC receptor (GCR), termed α and β, formed by alternative splicing.[119] The αGCR form expressed in nearly cell types has a C-terminal ligand binding domain, and mainly mediates transcriptional modifying actions of GCs, whereas the βGCR isoform is a dominant negative inhibitor.[120,121] The diverse responses of different cell types to GC are partially dependent on the ratio of α and β GCRs expressed. GCs exert anti-inflammatory and

immunosuppressive effects by several mechanisms.[121,122] The GC-GCR complex is translocated to the nucleus where it binds to target deoxyribonucleic acid sequences that activate anti-inflammatory genes (transactivation) or attenuation of proinflammatory genes (transrepression). Nuclear GC-GCR complexes also mediate epigenetic modification by activating histone deacetylases, which remove acetyl groups from histone tails, leading to increased chromatin density resulting in inhibition of transcription factor binding and gene silencing. Moreover, GC-GCR complexes can activate human airway trypsin and induce activation of anti-inflammatory genes.[123] These nuclear actions and genomic effects of GC-GCR signaling occur within hours, but alternative GC-GCR signaling mechanisms have been implicated for more rapid responses to receptor-ligand interaction.[124,125]

GCs present in high concentrations can intercalate into plasma and mitochondrial membranes leading to altered physiochemical properties that produce changes in membrane associated protein function.[124,125,126] In immune cells, this produces reduced calcium and sodium cycling across the plasma membrane, and decreased mitochondrial adenosine triphosphate synthesis, which is essential for cytokine synthesis, chemotaxis, phagocytosis, and antigen processing and presentation.[127,128] These responses to GCR also alter cellular transmembrane potentials, which contribute to the immunosuppressive and anti-inflammatory phenotypes. Membrane-bound GCR (mGCR), a variant of cytosolic GCR formed by alternative splicing, a promoter of switching or posttranslational modification,[129] is found in peripheral blood mononuclear cells in patients with autoimmune diseases. The frequency of mGCR-positive monocytes is associated with increased disease activity, suggesting that mGCR may contribute to the pathogenesis of inflammatory and autoimmune diseases.[130,131] Targeting mGCR in T cells inhibits lymphocyte-specific protein kinase and Fyn, leading

to inhibition of T-cell receptor signaling and subsequent cytokine synthesis, cell migration, and proliferation.[132,133,134] GCs can mediate nongenomic effects by binding to cytosolic GCR (cGCR) without triggering either nuclear translocation of GC-cGCR complex or effect on transcription.[135] Proteins, such as heat shock proteins, chaperonins, and Src, released from a multiprotein GC-cGCR complex following binding with GCs are responsible for some of nongenomic effects of GCs. cGCR also attenuate arachidonic acid release, which is required for cell growth and several metabolic and inflammatory reactions.

Whereas chronically elevated GC, as occurs during stress or chronic illness, is primarily immunosuppressive, the acute release of GC occupies an important protective role in the early response to infection and injury. Increased GC levels mediate the redistribution, margination, and trafficking of lymphocytes, monocytes, and neutrophils. The resolution of the acute event is associated with a return to normal GC levels. Immunosuppressive complications occur when inflammation fails to resolve, the stimulus to GC release persists, and the side effects of excessive GC and dysregulated GCR responses become manifest.

TABLE 35.2. Therapeutic Strategies Targeting the Components of the Inflammatory Reflex

Disease Model	Activation Method	Result
Sepsis induced by lethal peritonitis	Transcutaneous stimulation of cervical vagus nerve	Inhibition of serum HMGB1 levels and improved survival
	Administration of the α7 nAChR agonist GTS21 and choline	Inhibition of serum HMGB1 levels and improved survival
Endotoxin-induced shock	Direct electrical stimulation of the cervical vagus nerve	Inhibition of serum TNF and attenuation of shock
Lethal endotoxemia	Administration of the α7nAChR agonist GTS21 and choline	Inhibition of serum TNF and improved survival
Pancreatitis	Administration of the α7 nAChR agonist GTS21	Decreased pancreatitis severity
	Vagotomy and administration of an α7 nAChR agonist	Increased pancreatitis severity
Peripheral inflammation in subcutaneous tissue	Direct electrical stimulation of the cervical vagus nerve	Inhibition of the neutrophil recruitment to the wound
Hemorrhagic shock	Direct electrical stimulation of the cervical vagus nerve	Decreased hypotension severity, inhibition of TNF secretion
Ischemia-repurfusion injury secondary to	Direct electrical stimulation of the	Inhibition of cytokine production in spleen, liver, and heart, and

suprarenal aortic clamping	cervical vagus nerve	attenuation of shock and tissue damage
Collagen-induced arthritis	Surgical stimulation of the cervical vagus nerve	Decreased arthritis severity
	Administration of the α7 nAChR agonist AR-R17779	Inhibition of TNF secretion and decreased arthritis severity
	Vagotomy	Increased arthritis severity
Ileus	Vagotomy	Increased ileus severity
	Administration of the α7 nAChR agonist AR-R17779	Attenuation of postoperative ileus and intestinal inflammation
Myocardial infarction	Direct electrical stimulation of the cervical vagus nerve	Inhibition of cardiac myoglobin release during reperfusion injury
Colitis	Administration of α7 nAChR agonists and vagotomy	Increased colitis severity
	Administration of α7 nAChR agonists	Decreased colitis severity
Ventilator-induced lung injury	Administration of α7 nAChR agonists	Inhibition of cytokine levels in lung and plasma, and amelioration of lung injury
Obesity	Administration of α7 nAChR agonists	Improves glucose homeostasis and insulin sensitivity Reduces weight gain

IMPAIRED NEUROPHYSIOLOGIC CONTROL OF IMMUNE HOMEOSTASIS CONTRIBUTES TO NONRESOLVING INFLAMMATION

Failure of the neural and humoral reflex mechanisms that operate to normally restrain the immune system can produce “nonresolving inflammation,” which contributes significantly to the pathogenesis of atherosclerosis, obesity, cancer, obstructive pulmonary disease, asthma, inflammatory bowel disease, neurodegenerative disease, multiple sclerosis, rheumatoid arthritis, and other important syndromes.[136] The treatment of inflammatory and autoimmune diseases, including rheumatoid arthritis and inflammatory bowel disease, has benefited significantly by clinical use of anti-inflammatory agents that prevent the

activity of ongoing inflammatory responses, but do not force it to resolve. Anti-TNF- and anti-IL-1-based therapeutics have been administered to millions of patients who have recognized improved qualities of life, diminished pain and tissue destruction, and disease remissions. These anti-inflammatory agents are distributed by the circulatory system and function systemically to modify the activity of specific cytokines or cellular targets. While these therapies have been successful in many, in some cases these agents are ineffective or even immunosuppressive, resulting in potentially serious secondary infections. There remains a strong mandate to develop new anti-inflammatory treatment approaches by continuing to focus on fundamental mechanisms that regulate inflammatory responses in order to restore processes that lead to the resolution of the immune response.

Understanding the neurophysiology of the inflammatory reflex may lead to an ability to exploit it for development of novel therapeutic strategies. Electrical and pharmacologic stimulation of the inflammatory reflex in experimental models have demonstrated the efficacy of this approach in preclinical models of inflammatory diseases. Electrical stimulation of the vagus nerve or pharmacological activation of α7 nAchR signaling reduces the magnitude of inflammatory response by 50% to 75%, but does not completely eliminate cytokine activity. These results suggest that while activation of inflammatory reflex attenuates the immune response significantly, it does not induce immunosuppression. This concept has been evaluated in a range of infectious and sterile injury experimental laboratory models including infection, hemorrhagic shock, and ischemia-reperfusion injury (Table 35.2).

There is also evidence that the inflammatory reflex is impaired in humans with nonresolving inflammation. Vagus nerve activity can be measured in humans by evaluating the instantaneous heart rate variability (the time duration between individual heart beats). Vagus nerve activity leads to slowing of heart rate under basal condition, which transiently increases heart rate variability. Clinical data reveals that vagus nerve activity is depressed or absent during inflammation, and inversely correlates with exaggerated immune responses and increased morbidity and mortality.[137,138,139,140] The association between impaired vagus nerve activity and increased inflammatory responses has been studied in numerous inflammatory diseases, including inflammatory bowel disease, systemic lupus erythematosus, rheumatoid arthritis, and sepsis.[141] In septic patients, mortality in subjects with decreased vagus nerve activity on admission was significantly higher than in septic patients with normal vagus nerve activity (63.6% vs. 0%, respectively).[142] In patients with rheumatoid arthritis, decreased vagus nerve activity has been associated with increased levels of circulating cytokines.[143] Decreased heart rate variability has been significantly associated with risk of

development of atherosclerosis, a disease where inflammatory responses play a pathogenic role in the onset and maintenance of disease.[144] Taken together, these clinical data indicate that the loss of the modulating effect of the inflammatory reflex may aggravate the immune responses, contributing to nonresolving inflammation and exacerbation of disease pathology.

It is plausible to activate the inflammatory reflex to therapeutic advantage by selectively stimulating the vagus nerve or pharmacologically activating the α7 nAchR pathway. Studies involving the use of direct vagus nerve stimulation, aimed at attenuating inflammatory responses but not leading to immunosuppression, are in progress.[145,146] Direct vagus nerve stimulation has been used widely to treat patients with medically refractory partial-onset seizures. So far, more than 50,000 individuals have received implantable vagus nerve stimulators for treatment of epilepsy. Vagus nerve stimulation, carried out by implanting a small pacemaker-like device in the chest, is safe, effective, and well tolerated. It is possible that a similar device, designed to periodically activate vagus nerve, can be used to treat chronic inflammatory diseases. In place of an implantable device, it may also be possible to develop pharmacologic approaches to activate α7 nAchR, a new anti-inflammatory drug target. Studies are in progress to evaluate the efficacy of vagus nerve stimulation and the use of α7 nAchR agonists in modulating the immunologic responses in human disease.

The first use of GCs to treat rheumatoid arthritis occurred prior to our current advanced understanding of the neuroendocrine regulation of the immune system. Today, it is possible to consider that in the future, patients may derive clinical benefit from medical neurostimulating devices that target the inflammatory reflex and other neural circuits that provide the basis for maintaining immunologic homeostasis. It is even possible that these devices will restore the resolution of inflammation for some patients and replace antiinflammatory drugs.

REFERENCES

1. Felten SY, Olschowka J. Noradrenergic sympathetic innervation of the spleen: II. Tyrosine hydroxylase (TH)-positive nerve terminals form synapticlike contacts on lymphocytes in the splenic white pulp. *J Neurosci Res.* 1987;18:37-48.

2. Bellinger DL, Felten SY, Collier TJ, Felten DL. Noradrenergic sympathetic innervation of the spleen: IV. Morphometric analysis in adult and aged F344 rats. *J Neurosci Res.* 1987;18:55-59.

3. Felten DL, Felten SY, Carlson SL, Olschowka JA, Livnat S. Noradrenergic and peptidergic innervation of lymphoid tissue. *J Immunol.* 1985;135:755s-765s.

4. Rosas-Ballina M, Olofsson PS, Ochani M, et al. Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. *Science.* 2011;334:98-101.

5. Rosas-Ballina M, Ochani M, Parrish WR, et al. Splenic nerve is required for cholinergic antiinflammatory pathway control of TNF in endotoxemia. *Proc Natl Acad Sci U S A.* 2008;105:11008-11013.

6. Steinman L. Connections between the immune system and the nervous system. *Proc Natl Acad Sci U S A.* 1993;90:7912-7914.

7. Kannan-Hayashi Y, Okamura K, Hattori S, et al. Neuritogenic effects of T cell-derived IL-3 on mouse splenic sympathetic neurons in vivo. *J Immunol.* 2008;180:4227-4234.

8. Besedovsky HO, del Rey A, Sorkin E, et al. T lymphocytes affect the development of sympathetic innervation of mouse spleen. *Brain Behav Immun.* 1987;1:185-193.

9. Mitchell B, Kendall M, Adam E, Schumacher U. Innervation of the thymus in normal and bone marrow reconstituted severe combined immunodeficient (SCID) mice. *J Neuroimmunol.* 1997;75:19-27.

10. Novotny GE, Heuer T, Schöttelndreier A, Fleisgarten C. Plasticity of innervation of the medulla of axillary lymph nodes in the rat after antigenic stimulation. *Anat Rec.* 1994;238:213-224.

11. Novotny GE, Schöttelndreier A, Heuer T. A light and electron microscopic quantitative analysis of the innervation of axillary lymph nodes in juvenile and old rats. *J Anat.* 1993;183(pt 1):57-66.

12. Yang H, Wang L, Huang CS, Ju G. Plasticity of GAP-43 innervation of the spleen during immune response in the mouse. Evidence for axonal sprouting and redistribution of the nerve fibers. *Neuroimmunomodulation.* 1998;5:53-60.

13. Straub RH, Rauch L, Fassold A, Lowin T, Pongratz G. Neuronally released sympathetic neurotransmitters stimulate splenic interferongamma secretion from T cells in early type II collagen-induced arthritis. *Arthritis Rheum.* 2008;58:3450-3460.

14. Lorton D, Lubahn C, Sweeney S, et al. Differences in the injury/sprouting response of splenic noradrenergic nerves in Lewis rats with adjuvant-induced arthritis compared with rats treated with 6-hydroxydopamine. *Brain Behav Immun.* 2009;23:276-285.

15. Kannan Y, Stead RH, Goldsmith CH, Bienenstock J. Lymphoid tissues induce NGF-dependent and NGF-independent neurite outgrowth from rat superior cervical ganglia explants in culture. *J Neurosci Res.* 1994;37:374-383.

16. Kannan Y, Bienenstock J, Ohta M, Stanisz AM, Stead RH. Nerve growth factor and cytokines mediate lymphoid tissue-induced neurite outgrowth from mouse superior cervical ganglia in vitro. *J Immunol.* 1996;157:313-320.

17. Kannan Y, Moriyama M, Sugano T, et al. Neurotrophic action of interleukin 3 and granulocyte-macrophage colony-stimulating factor on murine sympathetic neurons. *Neuroimmunomodulation.* 2000;8:132-141.

18. Komaki G, Arimura A, Koves K. Effect of intravenous injection of IL-1 beta on PGE2 levels in several brain areas as determined by microdialysis. *Am J Physiol.* 1992;262:E246-E251.

19. Ericsson A, Kovacs KJ, Sawchenko PE. A functional anatomical analysis of central pathways subserving the effects of interleukin-1 on stressrelated neuroendocrine neurons. *J Neurosci.* 1994;14:897-913.

20. Vitkovic L, Konsman JP, Bockaert J, Dantzer R, Homburger V, Jacque C. Cytokine signals propagate through the brain. *Mol Psychiatry.* 2000;5:604-615.

21. Banks WA. The blood-brain barrier in psychoneuroimmunology. *Neurol Clin.* 2006;24:413-419.

22. Plotkin SR, Banks WA, Kastin AJ. Comparison of saturable transport and extracellular pathways in the passage of interleukin-1 alpha across the blood-brain barrier. *J Neuroimmunol.* 1996;67:41-47.

23. Eskandari F, Webster JI, Sternberg EM. Neural immune pathways and their connection to inflammatory diseases. *Arthritis Res Ther.* 2003;5:251-265.

24. Banks WA, Erickson MA. The blood-brain barrier and immune function and dysfunction. *Neurobiol Dis.* 2010;37:26-32.

25. Banks WA, Ortiz L, Plotkin SR, Kastin AJ. Human interleukin (IL) 1 alpha, murine IL-1 alpha and murine IL-1 beta are transported from blood to brain in the mouse by a shared saturable mechanism. *J Pharmacol Exp Ther.* 1991;259:988-996.

26. Pan W, Kastin AJ. Cytokine transport across the injured blood-spinal cord barrier. *Curr Pharm Des.* 2008;14:1620-1624.

27. Pan W, Kastin AJ. Penetration of neurotrophins and cytokines across the blood-brain/blood-spinal cord barrier. *Adv Drug Deliv Rev.* 1999;36:291-298.

28. Cao C, Matsumura K, Watanabe Y. Induction of cyclooxygenase-2 in the brain by cytokines. *Ann N Y Acad Sci.* 1997;813:307-309.

29. Fabry Z, Fitzsimmons KM, Herlein JA, Moninger TO, Dobbs MB, Hart MN. Production of the cytokines interleukin 1 and 6 by murine brain microvessel endothelium and smooth muscle pericytes. *J Neuroimmunol.* 1993;47:23-34.

30. Schiltz JC, Sawchenko PE. Distinct brain vascular cell types manifest inducible cyclooxygenase expression as a function of the strength and nature of immune insults. *J Neurosci.* 2002;22:5606-5618.

31. D'Mello C, Le T, Swain MG. Cerebral microglia recruit monocytes into the brain in response to tumor necrosis factoralpha signaling during peripheral organ inflammation. *J Neurosci*. 2009;29:2089-2102.

32. Watkins LR, Goehler LE, Relton JK, et al. Blockade of interleukin-1 induced hyperthermia by subdiaphragmatic vagotomy: evidence for vagal mediation of immune-brain communication. *Neurosci Lett.* 1995;183:27-31.

33. Watkins LR, Maier SF. Implications of immune-to-brain communication for sickness and pain. *Proc Natl Acad Sci U S A.* 1999;96: 7710-7713.

34. Bluthe RM, Walter V, Parnet P, et al. Lipopolysaccharide induces sickness behaviour in rats by a vagal mediated mechanism. *C R Acad Sci III.* 1994;317:499-503.

35. Romeo HE, Tio DL, Rahman SU, Chiappelli F, Taylor AN. The glossopharyngeal nerve as a novel pathway in immune-to-brain communication: relevance to neuroimmune surveillance of the oral cavity. *J Neuroimmunol.* 2001;115:91-100.

36. Berthoud HR, Neuhuber WL. Functional and chemical anatomy of the afferent vagal system. *Auton Neurosci*. 2000;85:1-17.

37. Sternberg EM. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. *Nat Rev Immunol.* 2006;6:318-328.

38. Hansen MK, O'Connor KA, Goehler LE, Watkins LR, Maier SF. The contribution of the vagus nerve in interleukin-1beta-induced fever is dependent on dose. *Am J Physiol Regul Integr Comp Physiol.* 2001;280:R929-R934.

39. Goehler LE, Gaykema RP, Hansen MK, Anderson K, Maier SF, Watkins LR. Vagal immune-to-brain communication: a visceral chemosensory pathway. *Auton Neurosci.* 2000;85:49-59.

40. Goehler LE, Gaykema RP, Nguyen KT, et al. Interleukin-1beta in immune cells of the abdominal vagus nerve: a link between the immune and nervous systems? *J Neurosci.* 1999;19:2799-2806.

41. Goehler LE, Gaykema RP, Hammack SE, Maier SF, Watkins LR. Interleukin-1 induces c-Fos immunoreactivity in primary afferent neurons of the vagus nerve. *Brain Res.* 1998;804:306-310.

42. Goehler LE, Relton JK, Dripps D, et al. Vagal paraganglia bind biotinylated interleukin-1 receptor antagonist: a possible mechanism for immune-to-brain communication. *Brain Res Bull.* 1997;43:357-364.

43. Goehler LE, Gaykema RP, Nguyen KT, et al. Interleukin-1beta in immune cells of the

abdominal vagus nerve: a link between the immune and nervous systems? *J Neurosci.* 1999;19:2799-2806.

44. Watkins LR, Goehler LE, Relton JK, et al. Blockade of interleukin-1 induced hyperthermia by subdiaphragmatic vagotomy: evidence for vagal mediation of immune-brain communication. *Neurosci Lett.* 1995;183:27-31.

45. Romeo HE, Tio DL, Taylor AN. Effects of superior cervical ganglionectomy on body temperature and on the lipopolysaccharide-induced febrile response in rats. *J Neuroimmunol.* 2009;209:81-86.

46. Romeo HE, Tio DL, Taylor AN. Effects of glossopharyngeal nerve transection on central and peripheral cytokines and serum corticosterone induced by localized inflammation. *J Neuroimmunol.* 2003;136: 104-111.

47. Niijima A, Hori T, Katafuchi T, Ichijo T. The effect of interleukin-1 beta on the efferent activity of the vagus nerve to the thymus. *J Auton Nerv Syst.* 1995;54:137-144.

48. Niijima A. The afferent discharges from sensors for interleukin 1 beta in the hepatoportal system in the anesthetized rat. *J Auton Nerv Syst.* 1996;61:287-291.

49. Niijima A, Hori T, Aou S, Oomura Y. The effects of interleukin-1 beta on the activity of adrenal, splenic and renal sympathetic nerves in the rat. *J Auton Nerv Syst.* 1991;36:183-192.

50. Lull ME, Block ML. Microglial activation and chronic neurodegeneration. *Neurotherapeutics.* 2010;7:354-365.

51. Ling EA, Wong WC. The origin and nature of ramified and amoeboid microglia: a historical review and current concepts. *Glia.* 1993;7:9-18.

52. Dalmau I, Vela JM, Gonzalez B, Finsen B, Castellano B. Dynamics of microglia in the developing rat brain. *J Comp Neurol.* 2003;458:144-157.

53. Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci.* 1996;19:312-318.

54. Ransohoff RM, Perry VH. Microglial physiology: unique stimuli, specialized responses. *Annu Rev Immunol.* 2009;27:119-145.

55. Goshen I, Kreisel T, Ounallah-Saad H, et al. A dual role for interleukin-1 in hippocampal-dependent memory processes. *Psychoneuroendocrinol.* 2007;32:1106-1115.

56. Hein AM, O'Banion MK. Neuroinflammation and memory: the role of prostaglandins. *Mol Neurobiol.* 2009;40:15-32.

57. Kotilinek LA, Westerman MA, Wang Q, et al. Cyclooxygenase-2 inhibition improves amyloid-beta-mediated suppression of memory and synaptic plasticity. *Brain.* 2008;131:651-664.

58. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol.* 2007;39:44-84.

59. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact.* 2006;160:1-40.

60. Butterfield DA, Perluigi M, Sultana R. Oxidative stress in Alzheimer's disease brain: new insights from redox proteomics. *Eur J Pharmacol.* 2006;545:39-50.

61. Jomova K, Vondrakova D, Lawson M, Valko M. Metals, oxidative stress and neurodegenerative disorders. *Mol Cell Biochem.* 2010;345:91-104.

62. Ekdahl CT, Kokaia Z, Lindvall O. Brain inflammation and adult neurogenesis: the dual role of microglia. *Neuroscience.* 2009;158:1021-1029.

63. Monje ML, Toda H, Palmer TD. Inflammatory blockade restores adult hippocampal neurogenesis. *Science.* 2003;302:1760-1765.

64. Goshen I, Kreisel T, Menachem-Zidon O, et al. Brain interleukin-1 mediates chronic stress-induced depression in mice via adrenocortical activation and hippocampal neurogenesis suppression. *Mol Psychiatry.* 2008;13:717-728.

65. Leuner B, Gould E. Structural plasticity and hippocampal function. *Annu Rev Psychol.* 2010;61:111-113.

66. Song C, Wang H. Cytokines mediated inflammation and decreased neurogenesis in animal models of depression. *Prog Neuropsychopharmacol Biol Psychiatry.* 2011;35:760-768.

67. Koo JW, Duman RS. IL-1beta is an essential mediator of the antineurogenic and anhedonic effects of stress. *Proc Natl Acad Sci U S A.* 2008;105:751-756.

68. Menachem-Zidon O, Goshen I, Kreisel T, et al. Intrahippocampal transplantation of transgenic neural precursor cells overexpressing interleukin-1 receptor antagonist blocks chronic isolation-induced impairment in memory and neurogenesis. *Neuropsychopharmacol.* 2008;33:2251-2262.

69. Liu YP, Lin HI, Tzeng SF. Tumor necrosis factor-alpha and interleukin-18 modulate neuronal cell fate in embryonic neural progenitor culture. *Brain Res.* 2005;1054:152-158.

70. Iosif RE, Ekdahl CT, Ahlenius H, et al. Tumor necrosis factor receptor 1 is a negative regulator of progenitor proliferation in adult hippocampal neurogenesis. *J Neurosci.* 2006;26:9703-9712.

71. Keohane A, Ryan S, Maloney E, Sullivan AM, Nolan YM. Tumour necrosis factor-alpha impairs neuronal differentiation but not proliferation of hippocampal neural precursor cells: role of Hes1. *Mol Cell Neurosci.* 2010;43:127-135.

72. Nakanishi M, Niidome T, Matsuda S, Akaike A, Kihara T, Sugimoto H. Microglia-derived interleukin-6 and leukaemia inhibitory factor promote astrocytic differentiation of neural stem/progenitor cells. *Eur J Neurosci.* 2007;25:649-658.

73. Stellwagen D, Malenka RC. Synaptic scaling mediated by glial TNFalpha. *Nature.* 2006;440:1054-1059.

74. Bellinger FP, Madamba S, Siggins GR. Interleukin 1 beta inhibits synaptic strength and long-term potentiation in the rat CA1 hippocampus. *Brain Res.* 1993;628:227-234.

75. Li AJ, Katafuchi T, Oda S, Hori T, Oomura Y. Interleukin-6 inhibits long-term potentiation in rat hippocampal slices. *Brain Res.* 1997;748:30-38.

76. Dong J, Xiong H. Human immunodeficiency virus type 1 gp120 inhibits long-term potentiation via chemokine receptor CXCR4 in rat hippocampal slices. *J Neurosci Res.* 2006;83:489-496.

77. Hauss-Wegrzyniak B, Lynch MA, Vraniak PD, Wenk GL. Chronic brain inflammation results in cell loss in the entorhinal cortex and impaired LTP in perforant path-granule cell synapses. *Exp Neurol.* 2002;176:336-341.

78. Jacobsen JS, Wu CC, Redwine JM, et al. Early-onset behavioral and synaptic deficits in a mouse model of Alzheimer's disease. *Proc Natl Acad Sci U S A.* 2006;103:5161-5166.

79. Kim JJ, Foy MR, Thompson RF. Behavioral stress modifies hippocampal plasticity through N-methyl-D-aspartate receptor activation. *Proc Natl Acad Sci U S A.* 1996;93:4750-4753.

80. Lynch MA. Age-related impairment in long-term potentiation in hippocampus: a role for the cytokine, interleukin-1 beta? *Prog Neurobiol.* 1998;56:571-589.

81. Oitzl MS, van Oers H, Schobitz B, de Kloet ER. Interleukin-1 beta, but not interleukin-6, impairs spatial navigation learning. *Brain Res.* 1993;613:160-163.

82. Gibertini M. IL1 beta impairs relational but not procedural rodent learning in a water

maze task. *Adv Exp Med Biol*. 1996;402:207-217.

83. Song C, Horrobin D. Omega-3 fatty acid ethyl-eicosapentaenoate, but not soybean oil, attenuates memory impairment induced by central IL-1beta administration. *J Lipid Res.* 2004;45:1112-1121.

84. Hein AM, Stasko MR, Matousek SB, et al. Sustained hippocampal IL-1beta overexpression impairs contextual and spatial memory in transgenic mice. *Brain Behav Immun.* 2010;24:243-253.

85. Moore AH, Wu M, Shaftel SS, Graham KA, O'Banion MK. Sustained expression of interleukin-1beta in mouse hippocampus impairs spatial memory. *Neuroscience.* 2009;164:1484-1495.

86. Barrientos RM, Sprunger DB, Campeau S, et al. Brain-derived neurotrophic factor mRNA downregulation produced by social isolation is blocked by intrahippocampal interleukin-1 receptor antagonist. *Neuroscience.* 2003;121:847-853.

87. Pugh CR, Nguyen KT, Gonyea JL, et al. Role of interleukin-1 beta in impairment of contextual fear conditioning caused by social isolation. *Behav Brain Res.* 1999;106:109-118.

88. Maier SF, Watkins LR. Intracerebroventricular interleukin-1 receptor antagonist blocks the enhancement of fear conditioning and interference with escape produced by inescapable shock. *Brain Res.* 1995;695: 279-282.

89. Godbout JP, Johnson RW. Interleukin-6 in the aging brain. *J Neuroimmunol*. 2004;147:141-144.

90. Heyser CJ, Masliah E, Samimi A, Campbell IL, Gold LH. Progressive decline in avoidance learning paralleled by inflammatory neurodegeneration in transgenic mice expressing interleukin 6 in the brain. *Proc Natl Acad Sci U S A.* 1997;94:1500-1505.

91. Jones SA, Richards PJ, Scheller J, Rose-John S. IL-6 transsignaling: the in vivo consequences. *J Interferon Cytokine Res.* 2005;25:241-253.

92. Golan H, Levav T, Mendelsohn A, Huleihel M. Involvement of tumor necrosis factor alpha in hippocampal development and function. *Cereb Cortex.* 2004;14:97-105.

93. Fiore M, Probert L, Kollias G, Akassoglou K, Alleva E, Aloe L. Neurobehavioral alterations in developing transgenic mice expressing TNF-alpha in the brain. *Brain Behav Immun.* 1996;10:126-138.

94. Casolini P, Catalani A, Zuena AR, Angelucci L. Inhibition of COX-2 reduces the age-dependent increase of hippocampal inflammatory markers, corticosterone secretion, and

behavioral impairments in the rat. *J Neurosci Res.* 2002;68:337-343.

95. Matsumoto Y, Watanabe S, Suh YH, Yamamoto T. Effects of intrahippocampal CT105, a carboxyl terminal fragment of beta-amyloid precursor protein, alone/with inflammatory cytokines on working memory in rats. *J Neurochem.* 2002;82:234-239.

96. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat Rev Neurosci.* 2008;9:46-56.

97. Dantzer R. Cytokine, sickness behavior, and depression. *Immunol Allergy Clin North Am.* 2009;29:247-264.

98. Konsman JP, Parnet P, Dantzer R. Cytokine-induced sickness behaviour: mechanisms and implications. *Trends Neurosci.* 2002;25:154-159.

99. Kowal C, DeGiorgio LA, Nakaoka T, et al. Cognition and immunity; antibody impairs memory. *Immunity.* 2004;21:179-188.

100. Huerta PT, Kowal C, DeGiorgio LA, Volpe BT, Diamond B. Immunity and behavior: antibodies alter emotion. *Proc Natl Acad Sci U S A.* 2006;103:678-683.

101. Diamond B, Huerta PT, Mina-Osorio P, Kowal C, Volpe BT. Losing your nerves? Maybe it's the antibodies. *Nat Rev Immunol.* 2009;9:449-456.

102. Faust TW, Chang EH, Kowal C, et al. Neurotoxic lupus autoantibodies alter brain function through two distinct mechanisms. *Proc Natl Acad Sci U S A.* 2010;107:18569-18574.

103. Bernik TR, Friedman SG, Ochani M, et al. Pharmacological stimulation of the cholinergic antiinflammatory pathway. *J Exp Med.* 2002;195: 781-788.

104. Borovikova LV, Ivanova S, Nardi D, et al. Role of vagus nerve signaling in CNI-1493-mediated suppression of acute inflammation. *Auton Neurosci.* 2000;85:141-147.

105. Rosas-Ballina M, Olofsson PS, Ochani M, et al. Acetylcholine-synthesizing T cells relay neural signals in the inflammatory reflex. *Science.* 2011;334(6052):98-101.

106. Berthoud HR, Powley TL. Characterization of vagal innervation to the rat celiac, suprarenal and mesenteric ganglia. *J Auton Nerv Syst.* 1993;42:153-169.

107. Bellinger DL, Felten SY, Lorton D, Felten DL. Origin of noradrenergic innervation of the spleen in rats. *Brain Behav Immun.* 1989;3:291-311.

108. Bellinger DL, Lorton D, Hamill RW, Felten SY, Felten DL. Acetylcholinesterase

staining and choline acetyltransferase activity in the young adult rat spleen: lack of evidence for cholinergic innervation. *Brain Behav Immun.* 1993;7:191-204.

109. Fujii T, Takada-Takatori Y, Kawashima K. Basic and clinical aspects of non-neuronal acetylcholine: expression of an independent, non-neuronal cholinergic system in lymphocytes and its clinical significance in immunotherapy. *J Pharmacol Sci.* 2008;106:186-192.

110. Fujii T, Kawashima K. An independent non-neuronal cholinergic system in lymphocytes. *Jpn J Pharmacol.* 2001;85:11-15.

111. Fujii T, Okuda T, Haga T, Kawashima K. Detection of the high-affinity choline transporter in the MOLT-3 human leukemic T-cell line. *Life Sci.* 2003;72:2131-2134.

112. Fujii T, Yamada S, Watanabe Y, et al. Induction of choline acetyltransferase mRNA in human mononuclear leukocytes stimulated by phytohemagglutinin, a T-cell activator. *J Neuroimmunol.* 1998;82:101-107.

113. Felten DL, Ackerman KD, Wiegand SJ, Felten SY. Noradrenergic sympathetic innervation of the spleen: I. Nerve fibers associate with lymphocytes and macrophages in specific compartments of the splenic white pulp. *J Neurosci Res.* 1987;18:28-36, 118-121.

114. Williams JM, Felten DL. Sympathetic innervation of murine thymus and spleen: a comparative histofluorescence study. *Anat Rec.* 1981;199:531-542.

115. Vida G, Pena G, Kanashiro A, et al. Beta2-Adrenoreceptors of regulatory lymphocytes are essential for vagal neuromodulation of the innate immune system. *FASEB J.* 2011;25:4476-4485.

116. Wang H, Yu M, Ochani M, et al. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. *Nature.* 2003;421:384-388.

117. Wang H, Liao H, Ochani M, et al. Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis. *Nat Med.* 2004;10:1216-1221.

118. Rosas-Ballina M, Goldstein RS, Gallowitsch-Puerta M, et al. The selective alpha7 agonist GTS-21 attenuates cytokine production in human whole blood and human monocytes activated by ligands for TLR2, TLR3, TLR4, TLR9, and RAGE. *Mol Med.* 2009;15:195-202.

119. Lu NZ, Cidlowski JA. The origin and functions of multiple human glucocorticoid receptor isoforms. *Ann N Y Acad Sci.* 2004;1024:102-123.

120. Chikanza IC. Mechanisms of corticosteroid resistance in rheumatoid arthritis: a

putative role for the corticosteroid receptor beta isoform. *Ann N Y Acad Sci.* 2002;966:39-48.

121. Stahn C, Buttgereit F. Genomic and nongenomic effects of glucocorticoids. *Nat Clin Pract Rheumatol.* 2008;4:525-533.

122. Zen M, Canova M, Campana C, et al. The kaleidoscope of glucorticoid effects on immune system. *Autoimmun Rev.* 2011;10:305-310.

123. Goulding NJ. The molecular complexity of glucocorticoid actions in inflammation—a four-ring circus. *Curr Opin Pharmacol.* 2004;4: 629-636.

124. Buttgereit F, Scheffold A. Rapid glucocorticoid effects on immune cells. *Steroids.* 2002;67:529-534.

125. Daufeldt S, Lanz R, Allera A. Membrane-initiated steroid signaling (MISS): genomic steroid action starts at the plasma membrane. *J Steroid Biochem Mol Biol.* 2003;85:9-23.

126. Schmid D, Burmester GR, Tripmacher R, Kuhnke A, Buttgereit F. Bioenergetics of human peripheral blood mononuclear cell metabolism in quiescent, activated, and glucocorticoid-treated states. *Biosci Rep.* 2000;20:289-302.

127. Buttgereit F, Burmester GR, Brand MD. Therapeutically targeting lymphocyte energy metabolism by high-dose glucocorticoids. *Biochem Pharmacol.* 2000;59:597-603.

128. Sionov RV, Cohen O, Kfir S, Zilberman Y, Yefenof E. Role of mitochondrial glucocorticoid receptor in glucocorticoid-induced apoptosis. *J Exp Med.* 2006;203:189-201.

129. Bartholome B, Spies CM, Gaber T, et al. Membrane glucocorticoid receptors (mGCR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis. *FASEB J.* 2004;18:70-80.

130. Spies CM, Schaumann DH, Berki T, et al. Membrane glucocorticoid receptors are down regulated by glucocorticoids in patients with systemic lupus erythematosus and use a caveolin-1-independent expression pathway. *Ann Rheum Dis.* 2006;65:1139-1146.

131. Tryc AB, Spies CM, Schneider U, et al. Membrane glucocorticoid receptor expression on peripheral blood mononuclear cells in patients with ankylosing spondylitis. *J Rheumatol.* 2006;33:2249-2253.

132. Lowenberg M, Tuynman J, Bilderbeek J, et al. Rapid immunosuppressive effects of glucocorticoids mediated through Lck and Fyn. *Blood.* 2005;106:1703-1710.

133. Lowenberg M, Verhaar AP, Bilderbeek J, et al. Glucocorticoids cause rapid dissociation of a T-cell-receptor-associated protein complex containing LCK and FYN. *EMBO Rep.* 2006;7:1023-1029.

134. Boldizsar F, Talaber G, Szabo M, et al. Emerging pathways of nongenomic glucocorticoid (GC) signalling in T cells. *Immunobiology.* 2010;215:521-526.

135. Limbourg FP, Liao JK. Nontranscriptional actions of the glucocorticoid receptor. *J Mol Med (Berl).* 2003;81:168-174.

136. Nathan C, Ding A. Nonresolving inflammation. *Cell.* 2010;140:871-882.

137. Haensel A, Mills PJ, Nelesen RA, Ziegler MG, Dimsdale JE. The relationship between heart rate variability and inflammatory markers in cardiovascular diseases. *Psychoneuroendocrinol*. 2008;33:1305-1312.

138. Thayer JF, Sternberg E. Beyond heart rate variability: vagal regulation of allostatic systems. *Ann N Y Acad Sci*. 2006;1088:361-372.

139. Thayer JF, Fischer JE. Heart rate variability, overnight urinary norepinephrine and C-reactive protein: evidence for the cholinergic anti-inflammatory pathway in healthy human adults. *J Intern Med.* 2009;265:439-447.

140. Thayer JF, Lane RD. The role of vagal function in the risk for cardiovascular disease and mortality. *Biol Psychol.* 2007;74:224-242.

141. Tracey KJ. Physiology and immunology of the cholinergic antiinflammatory pathway. *J Clin Invest.* 2007;117:289-296.

142. Pontet J, Contreras P, Curbelo A, et al. Heart rate variability as early marker of multiple organ dysfunction syndrome in septic patients. *J Crit Care.* 2003;18:156-163.

143. Goldstein RS, Bruchfeld A, Yang L, et al. Cholinergic anti-inflammatory pathway activity and high mobility group box-1 (HMGB1) serum levels in patients with rheumatoid arthritis. *Mol Med.* 2007;13:210-215.

144. Hansson GK, Libby P. The immune response in atherosclerosis: a double-edged sword. *Nat Rev Immunol.* 2006;6:508-519.

145. Ben Menachem E. Vagus nerve stimulation, side effects, and long-term safety. *J Clin Neurophysiol.* 2001;18:415-418.

146. Schachter SC. Vagus nerve stimulation therapy summary: five years after FDA approval. *Neurology.* 2002;59:S15-S20.

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Chapter 36

Complement

B. Paul Morgan

DEFINITIONS AND HISTORY

Complement comprises a set of proteins present in plasma and other biological fluids, and on cell membranes, that together play key homeostatic roles in combating infection and disposal of waste. Complement is a central pillar of innate immunity, a ready-to-go, fast-response system that efficiently targets pathogens and toxic waste.

The discovery of complement dates back to the late 19th century when a number of pioneering biologists, including Josef Fodor, George Nuttal, and Hans Buchner, were exploring the bactericidal activities of plasma and serum.[1] They showed that fresh serum contained an activity that could efficiently kill some types of bacteria, and that this bactericidal activity decayed as the serum aged ex vivo and was rapidly lost when the serum was exposed to heat. To describe this heat-labile activity, Buchner coined the enigmatic term “alexin,” from the Greek and roughly translated as “without a name.” Jules Bordet extended these serum bactericidal studies using cholera bacilli and showed that sera from choleraimmune individuals efficiently killed the organism, whereas sera from nonimmune individuals did not; heating the immune serum caused loss of this bactericidal effect, suggesting it was related to Buchner's alexin.[2] Bordet then did a clever experiment: first incubating cholera bacilli with heat-treated immune serum (which alone did nothing), then adding fresh nonimmune serum; the bacilli were killed, demonstrating that killing of cholera bacilli required two serum components, a heat-stable component present only in immune serum which he termed “sensitiser,” and a heat-labile component present only in fresh serum, alexin. Around the same time, Paul Ehrlich was exploring how immune serum caused hemolysis of animal erythrocytes; he also found that these same two components were required. The heat-stable component present only in immune serum he termed “amboceptor” (and later immune body or antibody), while the heat labile alexin, he called “complement,” to indicate that it merely complemented the inherent hemolytic effect of amboceptor.[3]

Over the first few years of the 20th century, an intense debate continued regarding the nature of complement and its relationship to antibody, with Bordet and Ehrlich as the main protagonists. The “complement fixation test,” developed by Bordet and his coworkers around 1900 as a means of testing whether an individual possessed antibodies against a particular bacterium (ie, was immune), relied on the fact that complement was consumed when antibody bound its target and demonstrated conclusively that complement was a distinct activity in serum.[4] This test, the mainstay of immune diagnostics for a century, is still used for some

pathogens today. The famous Glasgow physician and pathologist Sir Robert Muir, writing in 1906, described complement as “that labile substance of normal serum that is taken up by the combination of an antigen and its antibody,”[5] a definition it would be hard to improve on today.

Over the next 20 or so years, a number of scientists used the serum fractionation techniques that were state of the art at the time to investigate the “substance” called complement. Euglobulin precipitation (by dialysing serum against water) revealed that neither the re-dissolved euglobulin precipitate nor the dialyzed serum supernatant alone possessed complement hemolytic activity; however, when re-combined, complement activity was restored, demonstrating the need for at least two components, termed C′1 and C′2. The precipitable euglobulin C′1 component was inactivated by heating to 56°C, while the soluble C′2 component was heat-stable. By the mid-1920s, other manipulations of serum, including adsorption on yeast particles, incubation with ammonia or treatment with cobra venom had shown that there were at least four separable components necessary for complement activity, termed C′1, C′2, C′3, and C′4.[6]

The physicochemical nature of complement also attracted much interest and debate until 1941, when a landmark paper from Louis Pillemer and his colleagues showed clearly that each of the components C′1 to C′4 was protein in nature.[7] Recognition of the protein nature of the complement components fueled a frenzy of protein chemistry activity that, by the mid-1960s, had further refined the components C′1 to C′4, in particular demonstrating that C′3 actually contained six separate proteins; the nine complement component proteins were therefore termed C′1, C′2, C′4, C′3a, C′3b, C′3c, C′3d, C′3e, and C′3f. The euglobulin, C′1, was also shown to be a complex of three different proteins. In 1968, a Committee on Complement Nomenclature met under the auspices of the World Health Organization to simplify and standardize, resulting in the modern terminology, in order of reaction, C1, C4, C2, C3, C5, C6, C7, C8, and C9.[8]

Up to the 1950s, complement research was focussed on the antibody-dependent activity recognised by Bordet and Ehrlich. Then, in 1954, Pillemer made a startling discovery: serum contained a protein that he called properdin (from the latin, *perdere*, which means to destroy) that could trigger complement attack on pathogens without the need for antibody.[9] This finding was hailed as a “magic bullet” against infection and was so newsworthy that it was featured in *Time* magazine under the banner “Medicine: Death to Germs.”[10]

Others strongly denied the existence of the extraordinary properdin system, accusing Pillemer of carelessness or worse; perhaps in part due to this criticism, Pillemer committed suicide in 1958, leaving others in the 1960s to confirm and extend his findings. In 1971, Hans Muller-Eberhard and Manfred Mayer independently provided definitive proof of the existence of the properdin system, which they called the alternate pathway (now alternative) to distinguish from the original antibody-dependent classical pathway of activation.[11,12] Identification of complement proteins unique to this pathway soon followed.

Up until the end of the 1950s, complement proteins were considered, with no good evidence, to be minor plasma components, present in trace amounts and hence difficult to work with. This misconception was laid bare in 1960 with the demonstration by Hans Muller-Eberhard

and colleagues that the β1c-globulin band visible on serum electrophoretograms, was in fact C3, a major component accounting for 1% to 2% of total plasma proteins.[13] Other complement proteins were soon shown to be relatively abundant in plasma, opening the door to their purification and functional characterization.

The next major leap forward was the recognition of the enzymatic nature of complement. Irwin Lepow and colleagues in the late 1950s had demonstrated that C′1 was associated with enzymatic (esterase) activity. By the mid-1960s, they had achieved a remarkable understanding of this first step of complement activation[14]; they showed that the enzyme comprised three distinct protein subunits (C′1q, C′1r, C′1s), that formation of the C′1 complex required calcium ions, that its substrates were C′4 and C′2 in that order, and that the enzyme was controlled by a plasma C′1 esterase inhibitor. It was soon recognized that a puzzling disease, hereditary angioedema, long associated with complement activation, was caused by a deficiency of this inhibitor,[15] launching the field of complement therapeutics.

Around the same time, Muller-Eberhard and colleagues showed that activation of C′3 was also enzymatic in nature. They showed that a cell-bound complex of C′1-activated C′4 and C′2 could cause activation of many molecules of C3 and their deposition on the cell surface in an active form.[16] It took another 15 years before the mechanism by which activated C′4 and C′3 attached to membranes was identified, when Brian Tack's group showed that both these molecules possess a labile, buried thioester group, exposed on activation, that covalently attached the proteins to surfaces.[17,18]

One problem with starting a history is knowing when to stop. The brief history above is incomplete and ends 40 years ago, and much that is noteworthy has occurred since then! However, that more recent history will form part of later descriptions, so I will draw a line here. Before moving on, I will remark that the use of “apostrophes” in complement nomenclature was quietly dropped in the late 1970s, so henceforth they will not be featured.

THE COMPONENTS AND PATHWAYS OF COMPLEMENT

In the 21st century, the term **complement** encompasses some 35 plasma and membrane proteins. The pathway components (Table 36.1) are outnumbered by regulatory proteins (Table 36.2) that limit activation in plasma and on self cells, and receptors (Table 36.3) that bind complement proteins to trigger a range of cellular responses. The complement proteins interact with one another to provide an effective and efficient antimicrobial defense system, along with a growing list of other roles, for example, in immune complex handling and priming for adaptive immune responses. Critical features include activation by diverse triggers, enzymatic amplification at multiple steps, and rigid control to prevent damage to self. Complement activation can be initiated in a variety of ways to target different pathogens and toxic agents. The literature describes three distinct activation pathways, although in reality, these pathways are closely interlinked. Antibodydependent activation, described by Ehrlich and others at the beginning of the 20th century, was for many years the only known pathway, now termed **the classical pathway (CP)**. The re-discovery by Pillemer in the 1950s of an antibodyindependent, pathogen-triggered pathway for complement activation laid the foundations for the **alternative pathway** (**AP**). A third activation pathway, antibody independent and triggered by pathogen-specific sugars, was described in the 1980s; at first given a variety of names to reflect the activating sugars, it is now universally known as the

lectin pathway (**LP**). These three activation systems share common components but also have pathway-specific ones.

The Classical Pathway

Antibody-dependent triggering of the CP begins with immunoglobulin (Ig)G or IgM antibody bound to its target antigen either on a pathogen or host cell membrane, or on an immune complex. IgM, a large, pentameric molecule, is the most efficient activator of complement; a single IgM molecule bound to antigen is, in theory, sufficient to generate a nidus for complement activation. Free IgM in plasma does not activate complement; however, binding to antigen through its five antigen binding domains generates major structural change affecting the whole molecule, causing it to transition from a planar to a staple conformation. These events expose complement binding sites in the constant (Fc) regions of each of the five subunits that initiate the process of complement activation. In contrast, multiple IgG molecules, bound close together on the target, termed an immune array, are needed to trigger complement activation. Again, conformational changes occur, exposing complement initiation sites in the Fc regions of the molecule. Not all IgG subclasses possess complement binding sites; IgG1 and IgG3 are strong complement activators, whereas IgG2 is a weak activator and IgG4 does not activate complement at all.

The first component of the CP is C1, a large multimeric protein comprising one copy of C1q and two copies each of C1r and C1s, held together noncovalently in a calcium-dependent complex. C1q, the recognition unit of the C1 complex, is itself multimeric, made up of six subunits, each comprising a collagenous stalk and a carboxy-terminal globular head; the six subunits are tightly associated along the collagenous stalks but separate in the head regions, giving the classical electron microscopy image of a “bunch

of tulips” (Fig. 36.1). To add further complexity to this, the most complex of complement proteins, each of the six C1q subunits is itself assembled from a trimer of homologous chains termed C1q-A, C1q-B, and C1q-C, intertwined in a triple helix in the stalk but distinct in the globular head. C1r and C1s, the proteolytic units of the C1 complex, are homologous serine proteases that associate with one-another in a calcium-dependent linear tetramer complex in the order C1s-C1r-C1r-C1s. When bound in the C1 complex, the tetramer folds upon itself in a figure-eight conformation and sits between the globular heads, held in place by ionic bonds between acidic residues in C1r/C1s and a single basic residue in each of the six C1q stalks (see Fig. 36.1).[19]

TABLE 36.1 Component Proteins of the Complement Pathway

Pathway/Component	Structure	Function	Plasma Level
Classical pathway			
C1q	460 kDa collectin, six subunits each of three	Binds immobilized	150 mg/L

	25 kDa chains	IgG/IgM to initiate the CP	
C1r	85 kDa single chain	In C1 complex, activates C1s	50 mg/L
C1s	85 kDa single chain	In C1 complex, cleaves C4/C2	50 mg/L
C4	S-S bonded heterotrimer, α, 97 kDa, β, 75 kDa, γ, 33 kDa	C4b fragment target-bound via thioester is the receptor for C2	500 mg/L
C2	100 kDa single chain	C2a fragment bound to C4b cleaves/activates C3	25 mg/L
Alternative pathway			
Factor B	110 kDa single chain, C2 homologue	Bb fragment bound to C3b cleaves and activates C3	200 mg/L
Factor D	25 kDa single chain protease	Cleaves factor B to activate	5 mg/L
Properdin	Oligomers of 53 kDa chain	Stabilizes the C3bBb complex	20 mg/L
Lectin pathway			
MBL	200-600 kDa collectin, two to six subunits each comprising three 32 kDa chains	Binds mannan sugars on pathogens to initiate the LP	0-5 mg/L (broad normal range)
Ficolin-1 (M-ficolin)	440 kDa lectin, 12 subunits each 36 kDa	Binds carbohydrate epitopes on pathogens to initiate the LP	0.05 mg/L

Ficolin-2 (L-ficolin)	420 kDa lectin, 12 subunits each 35 kDa	Binds carbohydrate epitopes on pathogens to initiate the LP	5 mg/L
Ficolin-3 (H-ficolin)	590 kDa lectin, 18 subunits each 3 kDa	Binds carbohydrate epitopes on pathogens to initiate the LP	5 mg/L
MASP-1	90 kDa single chain	Uncertain; in mouse, activates pro-fD to active fD	5 mg/L
MASP-2	74 kDa single chain	In complex with MBL or ficolin, cleaves C4/C2	0.4 mg/L
MASP-3	94 kDa single chain	Uncertain, perhaps as MASP-2	4 mg/L
MAp19(s-MAP)	19 kDa single chain	Suggested MASP-2 inhibitor	0.2 mg/L
MAp44(MAP-1)	44 kDa single chain	Suggested MASP-2 inhibitor	1.4 mg/L
Common			
C3	S-S bonded heterodimer, α, 110 kDa, β, 75 kDa	Central component in all pathways, C3b major opsonin	1200 mg/L
C5	S-S bonded heterodimer, α, 115 kDa, β, 75 kDa	Binds C6 to initiate TP, C5a major effector molecule	75 mg/L
Factor I	S-S linked heterodimer,	Serine proteasel	30 mg/L

	heavy (50 kDa) and light (38 kDa) chains	cleaves C3b/C4b in presence of cofactor	
Terminal pathway			
C6	110 kDa single chain	Binds C5b, C5b6 receives C7	50 mg/L
C7	100 kDa single chain	Binds C5b6, C5b-7 attaches to target cell	90 mg/L
C8	α (64 kDa) and γ(22 kDa) chains S-S linked, β chain (65 kDa) noncovalently associated	Binds C5b-7, C5b-8 receives C9	60 mg/L
C9	70 kDa single chain	Binds C5b-8 to form MAC	60 mg/L

CP, classical pathway; fD, factor D; Ig, immunoglobulin; LP, lectin pathway; MAC, membrane attack complex; MASP, mannan-binding lectin-associated serine protease; MBL, mannan-binding lectin; TP, terminal pathway.

For C1 activation to occur, at least two of the six head domains must be engaged simultaneously, explaining both the efficiency of activation by the multivalent IgM and the

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need for a critical surface density of IgG, the immune array, for activation; this latter limitation reduces the risk of inappropriate activation of complement on host tissues. Binding of the C1q globular heads to antibody Fc is itself a complex series of events; an exposed calcium ion in the head mediates the initial binding and induces further binding events that cause rotation of the head domain. These conformational changes stress the C1s-C1r-C1r-C1s tetramer tightly gripped between the C1q stalks, thereby triggering the autoactivation of C1r via cleavage at a single site to yield a two-chain disulphide-bonded active protease. Activated C1r then cleaves its homologous substrate, C1s, at a single site to generate the active C1s protease.[*,19]

TABLE 36.2 Regulatory Proteins of the Complement Pathway

		Plasma

Regulator	Structure	Function	Level/Cell Expression
Fluid phase			
C1 inhibitor	Single chain, 100 KDa, heavily glycosylated	Serine protease inhibitor binds and inactivates C1r, C1s, MASP-2, others	150 mg/L
Factor H	Single chain, 150 kDa, 20 SCRs	AP convertase decay accelerator and fI cofactor	300 mg/L
Factor H-like 1	Single chain, 42 kDa, seven SCRs	As for fH	10 mg/L
C4b binding protein	550 kDa oligomer comprising seven α chains (eight SCRs) and one β chain (three SCRs)*	CP convertase decay accelerator and fI cofactor	200 mg/L
Carboxypeptidase N	280 kDa dimer of heterodimers of 83 kDa and 55 kDa	Inactivates C3a/C5a by removing C-terminal Arg	30 mg/L
S protein/vitronectin	84 kDa single chain	Binds C5b67 in fluid phase to inhibit MAC formation	250 mg/L
Clusterin	70 kDa heterodimer of 35 kDa chains	Binds C5b67 in fluid phase to inhibit MAC formation	150 mg/L
Membrane-bound			
CR1/CD35	220 kDa single chain, 30 SCRs,† TM	CP/AP convertase decay accelerator and fI cofactor	RBC, WBC, renal, others

DAF/CD55	70 kDa single chain, four SCRs, GPI	CP/AP convertase decay accelerator	Broadly distributed
MCP/CD46	60 kDa, single chain, four SCRs, TM	CP/AP fI cofactor	Broad, absent from RBCs
CD59	20 kDa globular protein, four S-S bonds, heavy glycosylation, GPI	Binds C5b-8 on membrane to inhibit MAC formation	Broad, all blood cells, etc.

AP, alternative pathway; Arg, arginine; CP, classical pathway; DAF, decay accelerating factor; fH, factor H; fI, factor I; GPI, glycosyl phosphoinositol; LHR, long homologous repeat; MAC, membrane attack complex; MASP-2, mannan-binding lectin-associated serine protease-2; RBC, red blood cell; SCR, short consensus repeat; TM, transmembrane; WBC, white blood cell.

*Common isoform; other oligomers of C4bp, α7β0, and α6β1, are also found in plasma.

†Common isoform; forms comprising 37 SCRs (gain of LHR) and, rarely, 23 SCRs (loss of LHR) occur.

The fH-related proteins 1 to 5 are omitted for simplicity and because their functions are unconfirmed.

TABLE 36.3 Receptors for Products of Complement Activation

Receptor	Structure	Ligand; Function	Cell Expression
C3/C4/C5 fragment receptors			
CR1 (CD35)	TM, single chain, 30 SCRs	C3b/C4b; binds immune complexes; B-cell activation	RBC, WBC, FDC, renal, others

CR2(CD21)	TM, single chain, 15 or 16 SCRs	iC3b/C3dg/C3d; sensitizes B cells for response to antigen	B cells, some T cells, FDC
CR3 (CD11b/CD18)	Integrin heterodimer, α 160 kDa, β 95 kDa, TM proteins	iC3b; phagocytic receptor, leukocyte migration	Myeloid cells, NK cells
CR4 (CD11c/CD18)	Integrin heterodimer, α 150 kDa, β 95 kDa, TM proteins	iC3b; phagocytic receptor, leukocyte migration	Myeloid cells, T cells, NK cells
CRIg	45 kDa single chain Ig super-family member	C3b/iC3b; phagocytic receptor	Tissue macrophages
C3a receptor (C3aR)	54 kDa heptaspan G protein-coupled receptor	C3a; activates cell responses	WBC, brain and renal cells, etc.
C5a receptor (CD88)	45 kDa heptaspan G protein-coupled receptor	C5a > C5adesArg; activates cell responses	Broadly expressed
C5L2	37 kDa heptaspan; G protein coupling uncertain	C5a/C5adesArg/C3a/C3adesArg; activates cell responses?	Broadly expressed
Receptors for C1q			
CR1 (CD35)	See above	C1q; uncertain	See above

C1qRp(CD93)	125 kDa single chain TM	C1q/MBL/SP-A; phagocytic receptor	Myeloid cells, endothelium
gC1qR	Tetramer 33 kDa subunits	C1q; phagocytic receptor	WBC, platelets
α2β1 integrin	Integrin, heterodimer	C1q, collagens, laminins, decorin, etc.; cell activation	Broadly expressed

CR1, complement receptor 1; CR2, complement receptor 2; CR3, complement receptor 3; CR4, complement receptor 4; CRIg, complement receptor of the immunoglobulin superfamily; FDC, follicular dendritic cell; Ig, immunoglobulin; MBL, mannan-binding lectin; NK, natural killer; RBC, red blood cell; SCR, short consensus repeat; TM, transmembrane; WBC, white blood cell.

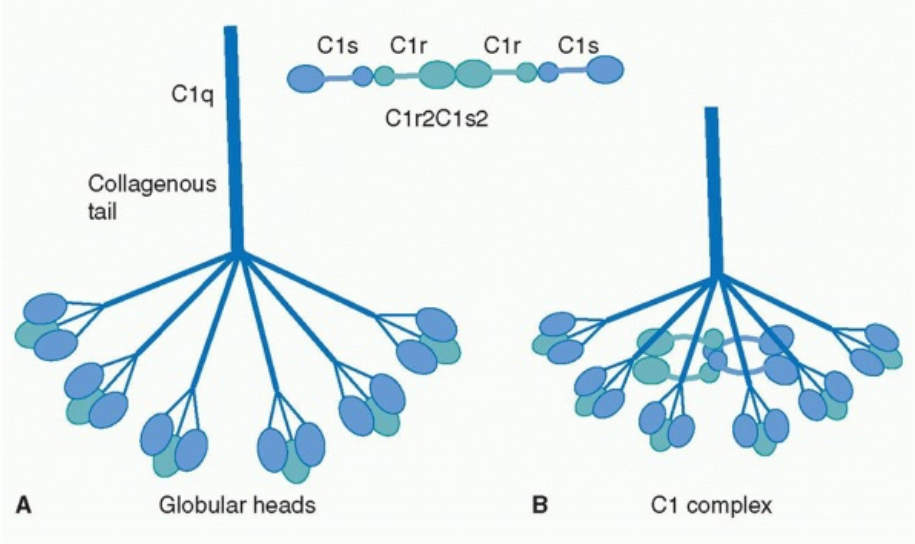

FIG. 36.1. Structure of C1. A: C1q comprises six units tightly associated through the collagenous tail, separating in the neck region to spread the globular heads out from the tail. Each unit is itself made up of three closely apposed subunits, C1q-A, -B, and -C. $C1r_2C1s_2$ is assembled in a linear complex in the order C1r-C1s-C1s-C1r. **B:** In the C1 complex, the $C1r_2C1s_2$ complex winds into a figure-of-eight conformation and binds among the globular heads in a calcium-dependent complex.

The C1s serine protease has two substrates, C4 and C2, the next two proteins in the classical pathway sequence. The illogical ordering here reflects the fact that complement components were named chronologically, according to the order of their discovery (C′1 > C′2 > C′3 > C′4), rather than according to their position in the reaction. **C4** is a relatively abundant protein, present in plasma at around 0.5 g/l. It is a large (210 kDa), disulphide-bonded

heterotrimer. Activated C1s captures C4 from the fluid phase, perhaps in part through interaction of its short consensus repeat (SCR) domains with C4, then cleaves the C4 α chain at a single site near the amino terminus, releasing a 77 amino-acid fragment, **C4a**, and exposing in the cleaved α′ chain of the large fragment **C4b** a labile thioester group (Fig. 36.2A). Although most of the nascent C4b formed will decay in the fluid phase through hydrolysis of the thioester, a small proportion will bind reactive hydroxyl or amino groups on the activating surface, creating a cluster of covalently bound C4b around the initiating IgG/C1 complex. Immobilized C4b binds the next component in the sequence, **C2**, in a magnesium-dependent complex. C2 is a single-chain plasma protein of mass 100 kDa and plasma concentration around 25 mg/L; it is the most heat-labile of the complement proteins, destroyed by brief incubation of plasma at 56°C. C4b-bound C2 is cleaved by activated C1s in an adjacent IgG/C1 complex, releasing a 30 kDa fragment **C2b**, while the 70 kDa **C2a** fragment, an active serine protease, remains associated with C4b on the surface.†,[20,21]

The magnesium-dependent **C4b2a** complex is the CP C3 convertase, the next activation enzyme in the sequence. C2a in the C4b2a complex is an active serine protease that cleaves **C3**, a two-chain 190 kDa protein, homologous to C4 and the most abundant of the complement proteins at around 1 g/l in plasma.[22] Cleavage releases a 77 amino-acid fragment, **C3a**, from the amino terminus of the α chain of C3, exposing in the large fragment, **C3b**, a labile thioester group essentially as described previously for C4b (Fig. 36.2B). Again, most of the C3b formed decays by thioester hydrolysis, but a small fraction covalently binds the activating surface, clustering around the site of activation. Some of the C3b formed will directly bind C4b2a through its thioester to form a trimolecular complex, **C4b2a3b**; this binding is not a random event but occurs at a single specific site in C4b, placing C3b in the correct orientation for succeeding steps of activation. The C4b2a3b complex contains a binding site for **C5** involving interactions with both C4b and C3b in the complex. C5, another homologue of C4 and C3, is a 200 kDa, two-chain molecule present in plasma at about 100 mg/L; importantly, C5 lacks the critical thioester group and so cannot bind covalently to targets. Once bound to C4b2a3b, the CP C5 convertase, C5 is cleaved by C2a in the complex, releasing a 74 amino-acid fragment, **C5a**, from the α chain of C5 and leaving the large fragment, **C5b**, loosely attached to the convertase. Cleavage of C5 is the final enzymatic step in the CP (Fig. 36.3).

Two features of CP activation are critical to its roles. First, amplification at each of the enzymatic steps is critical for efficient activation; thus, a single active IgG/C1 complex will deposit an abundance of C4b in the vicinity of the initiating

IgG/C1, each C4b2a complex formed will in turn cause deposition of many copies of C3b on the surrounding membrane, and each C5 convertase will also cleave multiple C5 molecules. Second, the nascent thioester groups in C4 and C3 are critical components of complement activation; without these entities, complement activation on surfaces would be an impossibility and the system would not function. The thioester group is formed from interaction between a glutamine and a cysteine residue that, in the intact molecule, are buried in the protein structure (Fig. 36.4).[17,18] When activated by the convertase, a major conformational change occurs that exposes the internal thioester bond in C3b and C4b, making it very unstable and highly susceptible to attack by nucleophiles such as hydroxyl groups (-OH) and amine groups

(-NH_2) in membrane proteins and carbohydrates, creating a covalent bond that locks C3b and C4b onto the surface.[23] The exposed thioester is highly labile and rapidly inactivated by hydrolysis, restricting binding of C3b and C4b to the immediate vicinity of the activating enzyme.

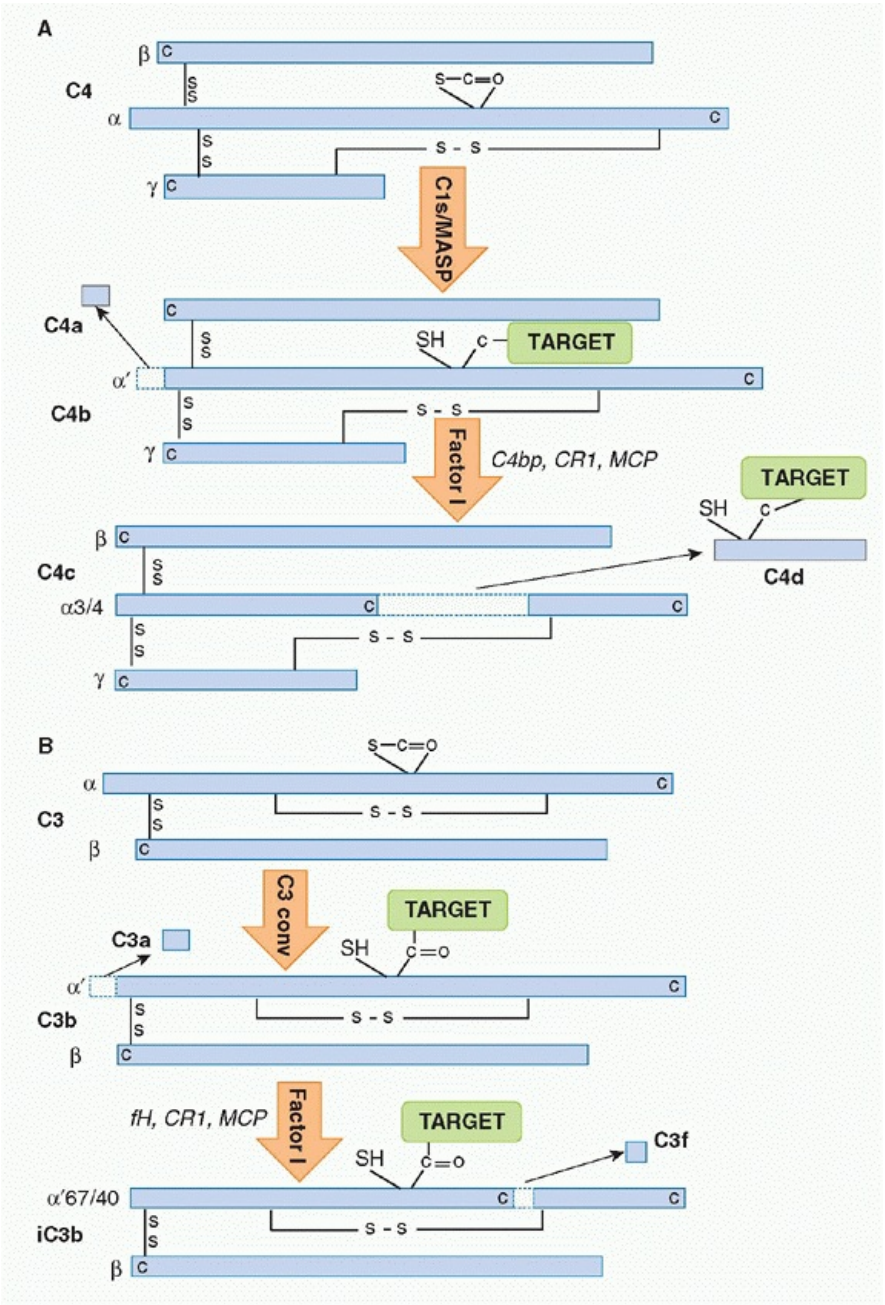

FIG. 36.2. Activation and Inactivation of C4 and C3. A: C4 is activated by cleavage of a 77 amino acid fragment, C4a, from the amino terminus of the α-chain, catalyzed by C1s in the classical pathway (CP) or MASP-2 in the lectin pathway (LP). Activation exposes the thioester in the α′-chain of C4b, enabling attachment to surfaces. The plasma enzyme factor I (fI) cleaves and inactivates C4b in the presence of appropriate cofactors, releasing the large, multichain C4c and leaving the C4d fragment attached to the surface. **B:** C3 is activated by cleavage of a 78 amino acid fragment, C3a, from the amino terminus of the α-chain, catalyzed by the C3 convertase of the CP/LP or

alternative pathway. Activation exposes the thioester in the α′-chain of C3b, enabling attachment to surfaces. The plasma enzyme fI cleaves and inactivates C3b in the presence of appropriate cofactors, releasing a small fragment, C3f, from the α′-chain, and leaving the iC3b fragment attached to the surface. Further cleavage events, not shown here, occur in the presence of CR1 as cofactor, releasing the large, multichain C3c and leaving the C3dg fragment attached to the surface. Finally, C3dg is further degraded by plasma proteases, releasing the small C3g peptide and leaving C3d attached to the target.

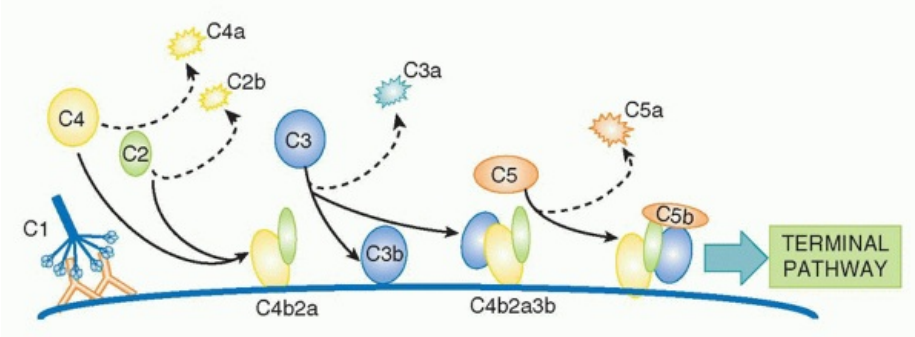

FIG. 36.3. Classical Pathway Activation. C1s in the activated C1 complex bound to immobilized antibody cleaves C4. C4b binds the surface through its thioester and acts as a receptor for C2, which is then cleaved by C1s to yield the C4b2a complex (classical pathway [CP] C3 convertase). C4b2a cleaves C3, depositing C3b on adjacent membrane. C3b binding in the C4b2a complex acts as a receptor for C5, presenting it for cleavage by C2a in the complex. Cleavage of C5 to form C5b is the last enzymatic step in the CP and initiates the terminal pathway.

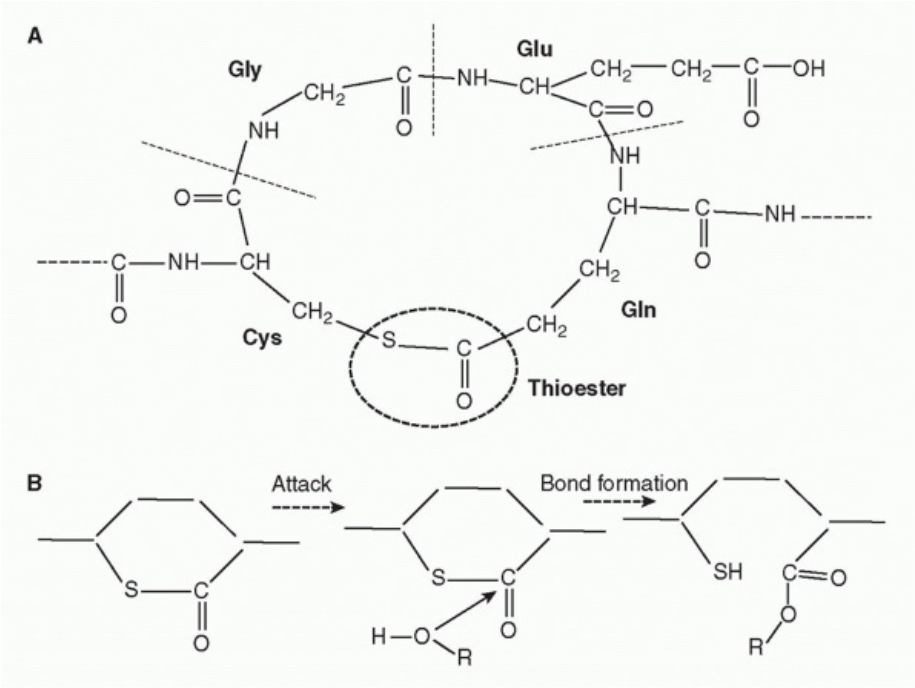

FIG. 36.4. Thioester Group Structure and Activation. A: The buried thioester in C3 and C4 is formed from the residues -Cys-Gly-Glu-Gln-. The bond is formed from a condensation reaction involving the Cys thiol group and the Glu carboxyl group in this four amino acid sequence. **B:** Hydroxyl groups on target proteins or sugars "attack" the exposed thioester in C3b or C4b in a reaction that is catalyzed by an adjacent histidine residue in the complement protein. The reaction creates a covalent bond, rigidly attaching C3b or C4b to the target.

The Alternative Pathway

The concept of an AP of complement activation grew out of the recognition that some pathogens activated complement without the need for antibody. The controversial history was briefly described previously, but today the existence of the AP is not in doubt, although whether it should be considered a separate pathway remains an area of debate. The AP functions in two interlinked ways, first as an efficient amplification loop to drive further complement activation whatever the initiating trigger, and second as an always-on pathogen sensor ready to attack foreign surfaces.[24,25] The trigger for AP activation is the presence of "activated" C3; this can be "classically activated" nascent C3b on membranes or in plasma, or C3 that has been hydrolyzed in plasma, **C3(H_2O)**. Spontaneous hydrolysis of C3 occurs under physiological conditions at approximately 5% of total C3 per day, driving a constant, low-grade "tickover" activation of the AP in plasma. **Factor B** (fB), a 110 kDa single-chain protein and structural homologue of C2, binds to form a magnesium-dependent complex, C3bB or C3(H_2O) B; in its bound state, fB is cleaved by a plasma serine protease, **factor D** (fD), releasing the 50kDa Ba fragment and leaving the serine protease fragment, Bb, in the complex to form the AP C3 convertase. The enzyme fD is a small (25 kDa) serine protease present in tiny amounts (1 mg/L) in plasma as an active enzyme with just one substrate, fB in complex with C3b/C3(H_2O). Bb in the C3bBb or C3(H_2O)Bb convertase cleaves C3 exactly

as occurs in the CP, releasing C3a and generating C3b that can bind to targets and/or bind more fB to continue the AP amplification cycle—a positive feedback amplification loop (Fig. 36.5). C3bBb on targets will catalyze the deposition of many C3b molecules as described for the CP convertase, and C3b binding to a specific site on C3b in the convertase will create a trimolecular C5 convertase in which C5 can be cleaved by the adjacent C2b, events that are analogous to those described in the CP. I have yet to mention the protein for which the AP was first named: **properdin**. This complicated molecule, an oligomer comprising two, three, or four copies of a 53Da single-chain protein, binds and stabilizes the AP convertases, reducing their inherent tendency to fall apart ("decay") and increasing markedly their capacity to perpetuate activation. Recently, a second role for properdin, binding to bacterial surfaces and forming a platform for AP convertase assembly, has been emphasised.[26]

The recognition that the AP is always on, in a constant tick-over state, explained how complement is so efficiently activated on pathogens. Tick-over ensures that all plasmaexposed surfaces are continuously showered with C3b; on self cells, inhibitory mechanisms described in the following sections ensure that no amplification occurs. However, on pathogens, lacking such protection, AP amplification kicks in, rapidly coating the surface with C3b. It is important

to note that the AP is inexorably linked to the CP in that C3b generated through the latter will feed into the former to amplify activation. It therefore does not matter whether the initial C3b is generated by the CP or AP (or, indeed, the LP that follows), the AP amplification loop will amplify the response, particularly when occurring on the intrinsically activating surfaces of pathogens.

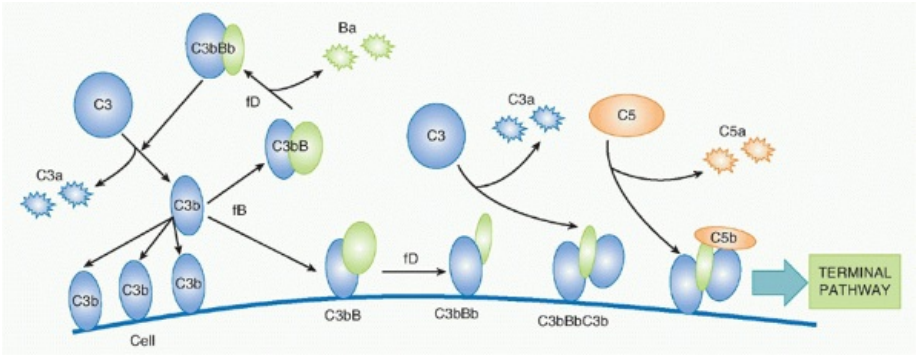

FIG. 36.5. Alternative Pathway Activation. The initiating activated C3 may be C3(H_2O) formed by spontaneous hydrolysis in the fluid phase, or C3b, formed from classical pathway/lectin pathway activation in the fluid or surface phase. Activated C3 binds factor B, rendering it susceptible to cleavage/activation by factor D. The resulting enzyme, C3(H_2O)Bb or C3bBb, cleaves more C3b, creating a positive feedback amplification loop in the fluid phase and depositing more C3b on the target. C3b binding into a target-bound C3bBb enzyme acts as a receptor for C5, presenting it for cleavage by Bb in the complex. Cleavage of C5 to form C5b is the last enzymatic step in the alternative pathway and initiates the terminal pathway.

The Lectin Pathway

The LP, first described only in 1987,[27,28] shares features with both the AP and CP. Like the AP, it provides antibody-independent "innate" immunity, activated by pathogens independent of antibody. Its similarities to the CP are legion; indeed, it only differs in the initiation step and is perhaps better considered as a different route to CP activation that bypasses the need for antibody.[29] The C1 complex is replaced by a structurally similar multimolecular complex, comprising a C1q-like recognition unit, either **mannan-binding lectin** (MBL) or **ficolin** (a family of three proteins in man), and **MBL-associated serine protease-2** (MASP-2) that provides the enzymatic activity. The recognition units bind carbohydrate epitopes, N-acetyl glucosamine for both ficolins and MBL, and mannose for MBL alone; these ligands are abundant in the cell walls of diverse pathogens, including bacteria, yeast, fungi, and viruses, making them targets for LP activation. Each MBL subunit comprises a homotrimer of 32 kDa chains, an amino-terminal collagen-like region responsible for trimerization in a triple helix, a short α-helical neck region, and a globular carbohydrate recognition domain. Subunits assemble into oligomers containing between two and six oligomers, the latter closely resembling the C1q hexamers. Indeed, C1q and MBL are members of the collectin family of proteins characterized by globular head regions with binding activities and long collagenous tail regions with diverse roles. Serum levels of MBL are highly variable in the population, from undetectable to 5 mg/L. **Ficolins** are novel lectins, structurally similar to C1q and MBL through the collagenous regions, but with head regions comprising fibrinogen-like domains.[30] Three ficolins are described in man, termed ficolin-1, ficolin-2, and ficolin-3.‡ Serum concentrations of all are low, ficolins-2 and -3 around 5 mg/L and ficolin-1 about 0.05 mg/L, although reported levels for each vary widely. **MASP-2** is a member of a family of homologous lectin-binding proteins, structural homologues of C1r/C1s, three of which express protease activity (MASP-1, MASP-2, MASP-3) while the other two do not (MAp19, also called sMAP; MAp44, also called MAP-1). After a decade of debate, claim and counterclaim, it is now generally agreed that MASP-2 is the critical enzyme of the lectin pathway: a MASP-2 dimer associates with the MBL or ficolin oligomer in a calcium-dependent manner to generate a complex that is necessary and sufficient to create the activation enzyme.[31] The biological roles of the other members of the MASP protein family remain obscure, although MASP-1 has recently been shown to be critical for AP activation in the mouse as it activates profD.[32] MASP-2 complexed with the MBL or ficolin oligomer captures C4 from the fluid phase, likely via its SCR domains, then cleaves C4 at a single site, identical to that targeted by C1s, to release C4a and generate C4b that binds through its thioester to the surface adjacent the initiating enzyme. The rest of the sequence mirrors that of the CP: C2 is captured onto C4b and presented for cleavage by MASP-2

in an adjacent complex, and the resultant enzyme, C4b2a, continues activation through C3 and C5.

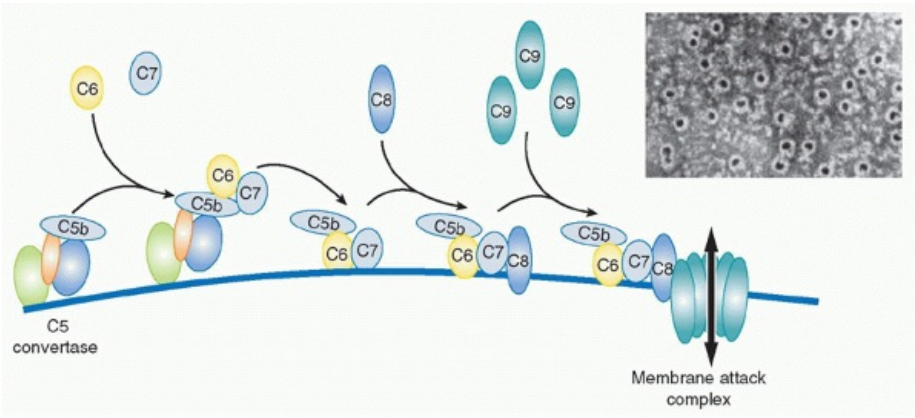

FIG. 36.6. Terminal Pathway. C5b, still attached to the convertase, binds C6, then C7. The trimolecular C5b67 complex is released to the fluid phase. A fraction of the complexes formed attach through hydrophobic interaction to the membrane. Membrane bound C5b67 recruits C8, then multiple copies of C9. C9 monomers unfold, insert into and through the membrane, and polymerize to form a transmembrane pore through which ions and water can freely flow. The inset shows an electron micrograph of a complement-lyzed cell; circular membrane attack complex lesions, a light protein rim surrounding a dark pore, are readily seen.

The Terminal Pathway

The terminal pathway (TP), sometimes referred to as the membrane attack pathway, is a final, common pathway for all activation routes. Cleavage of C5 is the last enzymatic step in the complement sequence and the final step of each activation pathway. The TP is a system almost unique in nature where five plasma proteins join to create an amphipathic membrane-inserted complex, the membrane attack complex (MAC), that creates a lytic pore in the membrane (Fig. 36.6).[33] The TP begins with the binding of the next component in the sequence, **C6**, to C5b still in the grip of the C5 convertase. C6 is a 100 kDa single-chain protein present in plasma at around 50 mg/L. Conformational changes during formation of the C5b6 complex weaken the grip of the convertase and create a binding site for the next component, C7, a 95 kDa single-chain molecule, plasma concentration about 90 mg/L. C6 and C7 are homologous molecules and are genetically linked, with genes adjacent on chromosome 5p. Incorporation of C7 causes further loosening of grip, releasing the trimolecular C5b67 complex into the fluid phase. The newly released C5b67 complexes shower down onto the lipid membrane surrounding the convertase and bind firmly to the surface via a hydrophobic site in the complex, thereby creating a nidus for continued assembly of the MAC. This is an inefficient process; the large majority of C5b67 complexes formed are inactivated in the fluid phase before they can bind membranes. Spontaneous inactivation occurs rapidly even when the C5b67 complex is assembled from pure proteins; in plasma, several proteins act as C5b-7 inhibitors to further accelerate inactivation. Those C5b67 complexes that do bind membranes then recruit the next protein in the sequence, **C8**, a heterotrimeric molecule (α and β chains each approximately 61 kDa, γ chain, 22 kDa; α and γ covalently linked, β noncovalently associated) present in plasma at about 80 mg/L. Binding of C8 introduces additional hydrophobicity, causing the resultant C5b-8 complex to embed

more firmly in the membrane. There is some evidence that the C5b-8 complex can cause membrane disruption and leakiness, but the major membrane disruption necessary to kill bacteria or other target cells requires the recruitment of multiple copies of the final component of the MAC, **C9**, a 70 kDa single-chain protein present in plasma at around 60 mg/L. The first globular C9 molecule binds C8 in the C5b-8 complex and undergoes major conformational rearrangement, unfolding to reveal a hydrophobic face that allows insertion into and through the membrane lipid bilayer. As additional C9 molecules are recruited, they in turn unfold and insert, aligning with the first C9, like barrel staves; with the recruitment of about 10 C9 molecules, the barrel is completed, creating a protein-lined channel through the membrane, the MAC (see Fig. 36.6). The C9 hydrophobic faces tightly lock the MAC in the membrane while the opposite, hydrophilic faces create a channel through which water and ions can flow, the MAC pore.** In electron micrographs of complement-lyzed targets, the MAC is readily visible as a

ring of about 10 nm internal diameter. The C5b-8 complex is displaced to the edge of the ring, resembling a pan handle.

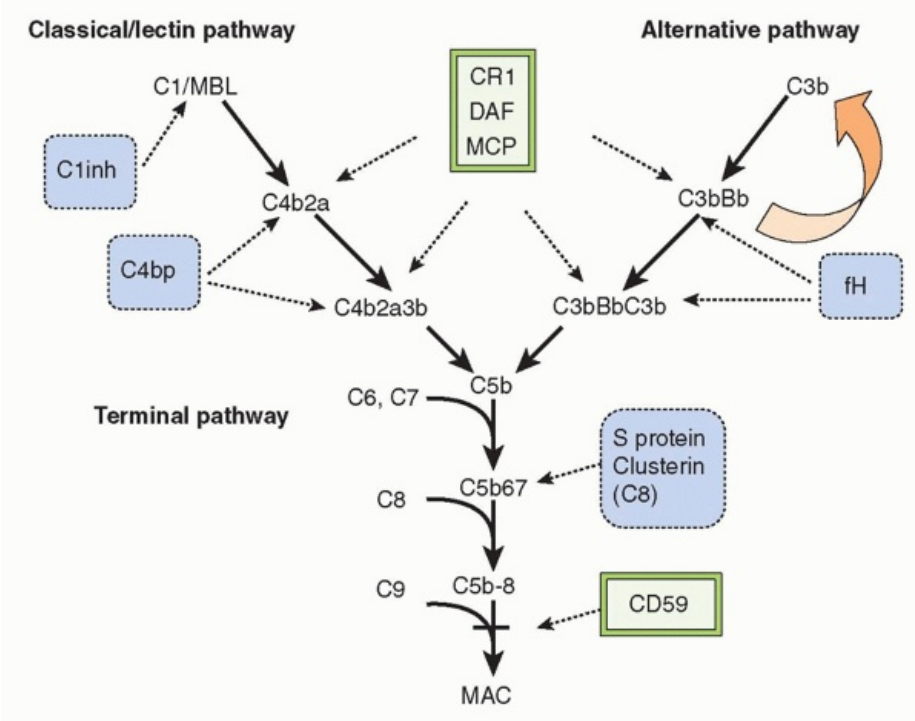

FIG. 36.7. Complement Regulation. Regulatory proteins are present both in plasma (*dotted boxes*) and on cell surfaces (*solid boxes*) to control complement activation at multiple stages in the activation and terminal pathways.

CONTROLLING COMPLEMENT ACTIVATION

Complement is designed to efficiently target and destroy pathogens and other foreign cells; however, the nature of the system dictates that it also targets host cells and thus has the potential to cause tissue damage and disease—a double-edged sword. Damage to self is minimized by the presence of numerous regulatory proteins and control mechanisms.

Complement regulatory proteins are present both on host cell membranes and in the fluid phase, collaborating to minimize activation and suppress amplification of complement. Each stage of each of the complement pathways is controlled through inhibition or accelerated decay of complement enzymes or by physical interference (see Table 36.2 and Fig. 36.7).

Control of Initiation of the Classical Pathway and Alternative Pathway

Activated C1 is regulated by a plasma serine protease inhibitor called **C1 inhibitor** (C1inh), a 100 kDa heavily glycosylated single-chain protein present in plasma at around 150 mg/L. C1inh binds C1r and C1s in activated C1 and forms a tight complex (C1inh-$C1r_2C1s_2$), simultaneously stripping them from the Ig-bound C1q. Like other serine protease inhibitors, C1inh undergoes an autocatalytic cleavage event during formation of the complex that stabilizes its binding to substrate; it thus behaves as a suicide inhibitor, inactivated during the process of inhibition. C1inh is the only plasma inhibitor for activated C1, but it has several other substrates, notably plasma kallikrein and the coagulation enzymes, factor XIa and factor XIIa.[34] C1inh also inhibits the LP, binding to and removing MASP-2 from the activated MBL/ficolin-MASP complex to switch off activation.

Control of the CP/LP C3 Convertase

The CP/LP C3 convertase, C4b2a, is regulated by inhibitors present in the fluid phase and on membranes. **C4b binding protein** (C4bp) is a complex, multi-chain plasma protein present in plasma at about 200 mg/L. The predominant structure for the C4bp oligomer comprises seven copies of an eight-SCR α-chain and one copy of a three-SCR β-chain (α7β1), locked together through disulphide bonds in the short, carboxy-terminal oligomerization domains, yielding a spider-like eight-armed structure (Fig. 36.8).††,[35] Oligomers missing the β-chain and containing seven (α7β0) or eight (α8β0) α-chains are found in various amounts in different individuals. Each of the α-chains in C4bp contains a binding site in its three amino-terminal SCRs that can bind C4b in the C4b2a complex. C4bp exerts two different inhibitory activities: first, it displaces C2a from C4b, termed “accelerated decay,” and second, it acts as a cofactor for enzymatic cleavage of C4b by the plasma enzyme **factor I** (fI), a two-chain (heavy, 50 kDa; light, 38 kDa), disulphide-bonded serine protease present at about 30 mg/L in plasma.‡‡ C4b is inactivated by cleavage at two sites in the α′-chain straddling the thioester, thereby releasing a large fragment, C4c, to the fluid phase and leaving the small C4d fragment attached to membrane (see Fig. 36.2A); neither C4c nor C4d are of particular biological

relevance. The β-chain of C4bp binds and inactivates Protein S, an important anticoagulation protein, one of many fascinating links between complement and coagulation.[36]

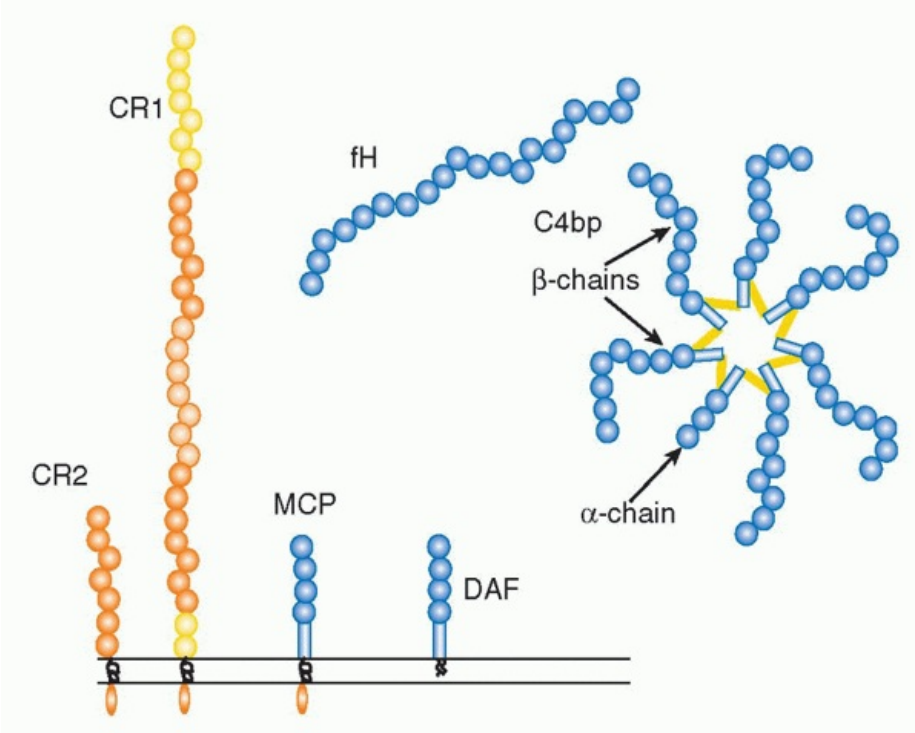

FIG. 36.8. Complement Regulators in the Regulators of Complement Activation Cluster. The complement regulators (and receptors) encoded in the regulators of complement activation cluster are all assembled from the same building block, the short consensus repeat (SCR) domain. The simplest, the fluid-phase regulator factor H, comprises just 20 SCRs arranged like beads on a string; C4bp is more complicated but is dominated by SCRs, the oligomer held together by short non-SCR joining sequences at their carboxy-termini. On the membrane, complement receptor 1 (CR1) is a very large molecule, comprising 30 SCRs in its most common isoform, with short transmembrane and cytoplasmic regions at its carboxy-terminus. The first 28 SCRs are arranged in four groups of seven called long homologous repeats, with repeating homology. Complement receptor 2 structurally resembles CR1 but comprises only seven SCRs in its most common isoform. Decay accelerating factor and membrane cofactor protein both comprise four amino terminal SCRs followed by a heavily carboxylated stalk and either a glycosyl phosphoinositol anchor (decay accelerating factor) or transmembrane and cytoplasmic regions (membrane cofactor protein).

Two homologous cell surface complement inhibitors act in tandem to regulate the C4b2a complex. **Decay accelerating factor** (DAF; CD55) is a broadly distributed glycosyl phosphoinositol (GPI)-linked membrane protein made up of four SCR domains and a heavily glycosylated stalk at the carboxy terminus that links to the GPI anchor.[37] As the name implies, DAF accelerates decay of the convertase; it binds membrane-associated C4b2a and displaces C2a, then releases because its affinity for C4b alone is low. Decay allows the second inhibitor, **membrane cofactor protein** (MCP; CD46), access to bind membrane-associated C4b. MCP, like DAF, is broadly distributed, though absent from erythrocytes, and comprises four SCR domains and a heavily glycosylated stalk, but differs in that it is a transmembrane protein with an intracellular domain that has important signalling roles,

described in a later section.[38] As its name implies, it is a cofactor for fI-mediated cleavage of C4b to C4c and C4d. This two-step regulation is important because decay alone leaves intact C4b on the surface, which can recruit more C2 and form a new convertase; in contrast, fI cleavage destroys C4b, thereby switching off activation. Another membrane protein, **complement receptor 1** (CR1; CD35), can also regulate the C4b2a enzyme through decay and cofactor activities. CR1 is an enormous molecule expressed on erythrocytes, the majority of leukocyte subsets, dendritic cells, glomerular podocytes, and a few other cell types. CR1 comprises, in its most common isoform, 30 SCRs, with a transmembrane region and short cytoplasmic domain at the carboxy-terminus.[39] The amino-terminal 28 SCRs are arranged in seven blocks, termed long homologous repeats (LHRs), in which the first SCR in each LHR is homologous to the first SCR in every other LHR, the second to every other second SCR, and so on. The functionally important parts of CR1 are the C3b-/C4b-binding sites contained in the first four SCRs of the first three LHRs; although differing in relative activities and ligand binding affinities, each of these sites can both decay the C4b2a convertase and bind C4b to catalyze its cleavage by fI. Because the functional sites are at the cell-distal end of a very large, elongated molecule, interaction with C4b or C4b2a on the same cell (intrinsic activity) is limited; however, interaction with C4b or C4b2a on adjacent surfaces (extrinsic activity), for example on circulating immune complexes, is favoured and is an important physiological role of erythrocyte CR1.[40]

Control of the Alternative Pathway C3 Convertase

The AP C3 convertase, C3bBb, is regulated in a very similar manner to the CP convertase. C4bp has only very low affinity for C3b or C3bBb, likely of no physiological relevance. Its role is taken by another plasma protein, **factor H** (fH), an elongated, single-chain protein made up entirely of 20 SCRs, present in plasma at about 300 mg/L.[41] The complement regulatory activity of fH resides in SCRs 1 to 4; these four domains bind the C3bBb convertase, displace Bb from C3b (accelerated decay), and act as cofactor for fI to cleave C3b at two internal sites in the α′-chain, generating the large membrane-bound fragment, iC3b, and releasing a small peptide, C3f. iC3b cannot drive further complement activation but is an important ligand for complement receptors, as described in a later section. On membranes, the C3bBb convertase is regulated by the same proteins that control C4b2a. DAF binds C3bBb, displaces Bb, and is then released (decay acceleration); MCP binds C3b and catalyzes its cleavage by fI to yield iC3b and C3f (see Fig. 36.2B). CR1, through its C3b-/C4b-binding sites, both decays C3bBb and binds C3b to catalyze its cleavage by fI. Importantly, CR1 catalyzes a second fI cleavage event, an additional cut that releases the large fragment, C3c to the fluid phase, leaving a small piece of the α′ chain, C3dg, attached to the membrane through the thioester. Plasma proteases then chew C3dg down further to C3d, an important ligand for complement receptors.

Control of the Anaphylatoxins

The fragments C3a and C5a released during activation of complement are extremely potent inflammatory mediators, attracting phagocytes to the site of activation (chemotaxis),

and activating them to release their cargoes of enzymes, reactive oxygen species, and other proinflammatory molecules. These powerful effects are limited in time and space by the rapid

inactivation of C3a and C5a in plasma and tissue fluids. The principal inactivator is **carboxypeptidase N** (CPN), a metallocarboxypeptidase made up of four noncovalently associated subunits, two identical 85 kDa regulatory subunits that stabilize the enzyme, and two identical 55 kDa catalytic subunits, present in plasma at 30 mg/L. CPN is a zinc-dependent enzyme that cleaves carboxy-terminal arginine or lysine residues from proteins or peptides; it removes the carboxy-terminal arginine (Arg; N) residue from both C3a and C5a.[42] The residual peptides, C5adesArg and C3adesArg have, respectively, much reduced or absent proinflammatory activities because of reduced or absent capacity to bind the receptors for the parent molecules. Plasma levels of these peptides can be measured and provide a useful index of ongoing complement activation in man and animal models. Although C3adesArg and C5adesArg are inactivated with respect to proinflammatory activity, they retain or acquire other activities that may be of equal biological importance. CPN also removes the carboxy-terminal Arg from bradykinin, altering receptor binding and biological properties of this important mediator.

Control in the Terminal Pathway

Control at the C3 convertase stage is the crucial pinch-point of complement activation; nevertheless, later stages are also subject to control. C5 convertases of both activation pathways are subject to the attentions of the same regulators described for the equivalent C3 convertases, limiting their lifespan through decay acceleration and cofactor collaboration with fI. As further insurance against damage to self, the TP is also regulated at multiple stages. Once released from the convertase, the C5b67 complex must make the perilous journey to the membrane—an entirely random migration that must be accomplished before the membrane-binding site decays or is blocked, all of a fraction of a second. The vast majority of the C5b67 complexes formed never bind membrane; the site is hydrolyzed or the plasma scavenger proteins **S-protein** (also called vitronectin) and/or **clusterin** bind and block the site.[43,44] Perhaps the most efficient fluidphase inhibitor of the TP is the next component in sequence, C8; if C8 binds C5b67 before it attaches to membrane, the binding site is lost. Because the process is so inefficient, large amounts of the waste product, the terminal complement complex (TCC) containing all the TP components, clusterin and S-protein, are found in plasma when complement activation occurs in vivo. TCC levels in plasma can be measured and provide an excellent index of ongoing complement activation. Despite the attention of these fluid-phase inhibitors, some C5b67 complexes will bind the membrane, usually on the same cell and close to the triggering convertase, but occasionally on adjacent, innocent bystander cells. C8 then binds, and with recruitment of multiple C9 molecules, the MAC forms. Membrane regulation of this process is provided by **CD59**, a small, compact GPI-anchored molecule widely expressed on most cell types.[45] CD59, by moving randomly in the membrane, will encounter the forming MAC at the C5b-8 stage and lock firmly to it, preventing the recruitment of multiple C9 molecules and the assembly of the MAC lytic pore (Fig. 36.9).

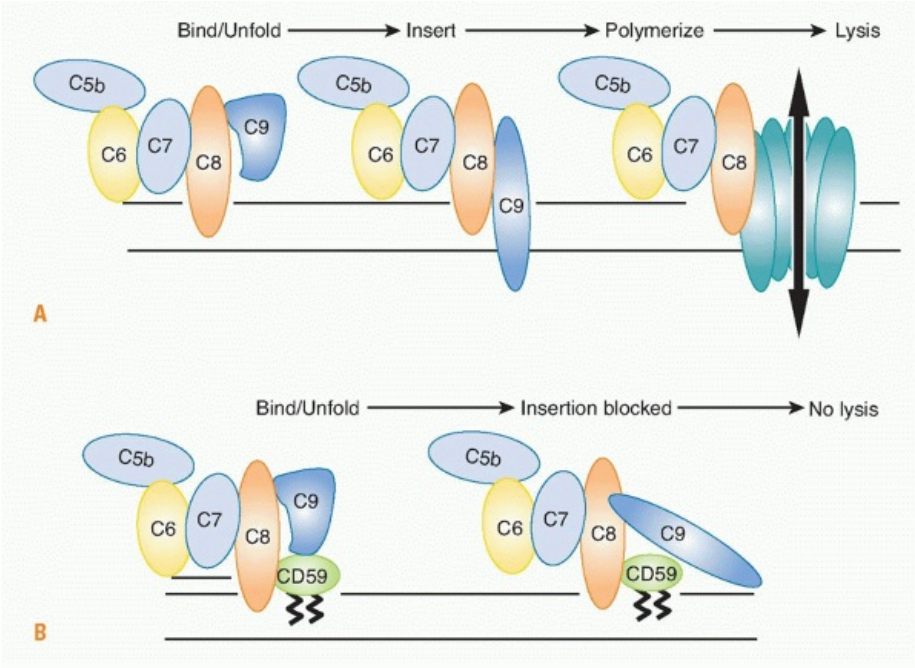

FIG. 36.9. Membrane Attack Complex Inhibition by CD59. A: In the absence of CD59, C5b67 bound to the membrane sequentially recruits C8 then multiple copies of C9 that unfold, insert, and polymerize to form the membrane attack complex pore. **B:** When CD59 is present on the membrane, it binds noncovalently into the C5b-8 complex. Although the first C9 can still bind, unfolding and insertion is sterically hindered, further C9 recruitment is halted and no pore forms.

Resistance and Recovery Mechanisms in Nucleated Cells

As a further failsafe to protect from MAC killing, metabolically active nucleated cells deploy recovery processes that eliminate the MAC lesion from the cell membrane. Recovery

was first described in neutrophils that, when attacked by complement, rapidly started to "bleb," releasing multiple vesicles to the fluid phase.[46] Electron microscopy and biochemical analyses showed that these vesicles were packed with MAC pores, while the rest of the cell membrane was free of lesions (Fig. 36.10). This packaging and removal was an active process and, when inhibited by metabolic poisons, neutrophil killing by MAC was increased. Similar mechanisms of MAC removal, either by shedding on vesicles or internalization, were soon discovered in many other nucleated cell lines and primary cells. Despite the fact that the recovery mechanism was discovered 25 years ago, the precise way in which the cell senses the MAC, packages MAC lesions into membrane patches, and sheds or internalizes the patches remain poorly defined. In neutrophils, calcium ion influx through the MAC pore is a key early event, but the downstream signaling pathways leading to recovery are unknown.

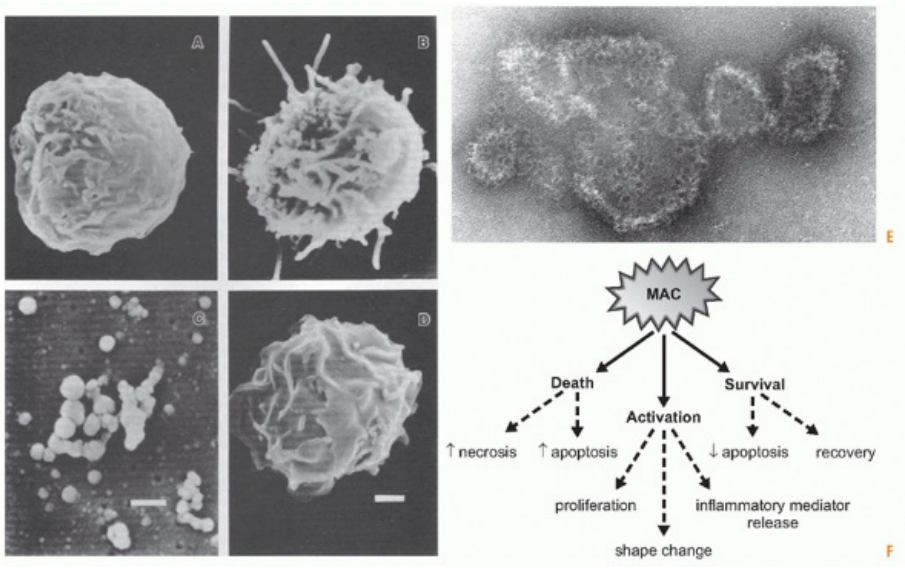

FIG. 36.10. Recovery Mechanisms in Nucleated Cells. A: Neutrophils prior to complement attack display a smooth, slightly ruffled appearance in scanning electron micrographs (SEMs). **B:** Within minutes of exposure to a nonlethal complement attack, the appearance is transformed: cells are covered in membrane projections. **C:** This frame shows SEM appearance of vesicles shed from attacked cells. **D:** After recovery, cells regain their tranquil appearance. **E:** This frame shows transmission electron micrographs of the same vesicles to show rich decoration with membrane attack complex (MAC) pores. **F:** Nonlethal MAC attack is not without consequences for the cell; changes in cell survival, proliferation, morphology, inflammatory mediator release, and many other events may be triggered in the cell.

COMPLEMENT RECEPTORS, OPSONINS, AND ANAPHYLATOXINS

Complement activation is a danger signal that alerts the host to infection or injury and initiates appropriate responses. Complement activation products flag danger by physically binding to pathogens, immune complexes, and other toxic bodies, and by their release into the surrounding milieu; subsequent events require the interaction of these fragments with specific receptors present on a variety of cell types (see Table 36.3).

Receptors for the Opsonic Fragments of C3 and C4

C3 is the most abundant complement component and the most important source of complement fragments. C3b and its degradation products iC3b and C3d coat the target, a process called opsonization, thereby tagging the target for recognition by cells bearing complement receptors.[47] **CR1,** described previously as a complement regulator, is an atypical receptor in that it modifies its own ligand. CR1 binds C3b (and C4b) coating immune complexes (ICs) via its binding sites in LHRs 1, 2, and 3, and catalyzes its cleavage by fI. Erythrocyte CR1 plays a critical role in IC handling; the C3b-coated IC binds CR1 on the erythrocyte and is held transiently until the ligated C3b is cleaved by fI to iC3b/C3dg; binding affinity is lost and the complex is released, only to bind again via another C3b.[40] This

dynamic binding permits the efficient sequestration of ICs on erythrocytes but without immobilizing them in a way that inhibits their efficient transfer to complement receptor-bearing tissue macrophages in the spleen and liver for final disposal. CR1 on other cell types also operates by binding and processing C3b-coated ICs. On dendritic cells, CR1 localizes opsonized ICs for presentation of antigen to T cells, while on B cells, C3b coating the IC binds CR1 and is cleaved to C3d, a ligand for another complement receptor, **complement receptor 2** (CR2; CD21), clustered with CR1 and the B-cell antigen receptor (BCR). Simultaneous engagement of CR2 and the BCR markedly lowers the threshold for antibody response.

CR2 is made up of either 15 or 16 SCR domains (alternative splicing removes SCR11 in the smaller isoform), a transmembrane region, and a cytoplasmic tail with important roles in signalling.[39] It is expressed on B cells, T-cell precursors and some mature T cells, dendritic cells, and some other cells involved in antigen presentation, basophils and epithelia. CR2 is a rather promiscuous receptor with numerous binding partners, including the IgE receptor (CD23), interferon-α, and the Epstein-Barr virus; its complement ligands are C3d or iC3b, binding at a single site in SCR1 and SCR2. The key role of CR2 is to enhance the immune response to antigens contained within the IC. On B cells, CR2 is clustered with the B cell-specific signaling molecule CD19 in a complex held together by the tetraspanin CD81; this tri-molecular complex interacts with the BCR to modulate the B cell response to antigen. CR2 on dendritic cells contributes to IC trapping in lymph node germinal centres.

Complement receptor 3 (CR3; CD11b/CD18) is a heterodimer comprising a 165 kDa α-chain (CD11b) and a 95 kDa β-chain (CD18); it is a member of the β_2-integrin family of leukocyte surface heterodimeric proteins sharing the common β-chain. CR3 is expressed on monocytes, neutrophils, mast cells, natural killer cells, dendritic cells, and some T cells.[48] It is notable for its promiscuity, binding adhesion molecules (intercellular adhesion molecule [ICAM]-1 and -2), coagulation proteins, microbial products, and carbohydrate antigens; its principal complement ligand is iC3b, although it binds weakly to C3d. Binding of an iC3b-coated particle to CR3 on phagocytic cells triggers the phagocytic process, leading to the elimination of the opsonized particle. Other phagocytic receptors, including the Ig Fc receptors and CD14, certainly contribute to the phagocytic process, and there is continuing debate around which of these triggers are required for and most important in the phagocytic process.

Complement receptor 4 (CR4; CD11c/CD18), another β_2-integrin expressed on myeloid cells, resembles CR3 with regard to distribution and complement ligand-binding properties. It is possible that CR4 plays important roles in some dendritic cell processes, although it remains something of an enigma.

Complement receptor of the immunoglobulin superfamily is a recently described receptor for C3b/iC3b expressed exclusively on tissue-resident macrophages, including Kupffer cells in the liver.[49] It plays important roles in the capture and clearance of opsonized ICs and pathogens from the circulation.

Receptors for the Anaphylactic Fragments of C3 and C5

Receptors for the small anaphylatoxin (AT) fragments released from C3 and C5 during activation are extremely important players in the recruitment of inflammatory cells and in a

growing list of other events. C3, C4, and C5 are structurally similar molecules and are all activated in similar ways: a single cut by the activating enzyme that releases a peptide, C3a, C4a, and C5a, respectively, from the amino terminus of the α-chain. Current opinion is that there are no receptors for C4a and that, as a consequence, it has no biological function. In contrast, specific receptors exist for the AT molecules C3a and C5a, which mediate important biological roles. Three AT receptors have been described to date, the **C3a receptor** (C3aR), the **C5a receptor** (C5aR; CD88), and the **C5a receptor-like 2** (C5L2).[50] They are homologous molecules, members of the G-protein-coupled receptor family of heptaspan membrane proteins. **C3aR** is expressed on all myeloid cells and some nonmyeloid, including activated T cells, astrocytes, endothelia, epithelia, and smooth muscle. C3aR binds C3a with nanomolar affinity but does not bind C3adesArg, C5a, or C5adesArg. Upon binding of C3a, intracellular signaling cascades are triggered through activation of heterotrimeric G-proteins that in turn cause increased intracellular free calcium and other downstream events. **C5aR** binds C5a with nanomolar affinity and C5adesArg with tenfold lower affinity; it does not bind C3a/C3adesArg. C5aR is expressed on all myeloid cells and numerous nonmyeloid including endothelia, neurones, and astrocytes; expression on lymphoid cells has been suggested but current evidence does not support this. Binding of C5a or C5adesArg triggers activation via heterotrimeric G-proteins, essentially as described for C3aR. **C5L2** was first described just 10 years ago and remains something of an enigma.[51] It is broadly expressed on myeloid and nonmyeloid cells and is often co-expressed with C5aR in many tissues. C5L2 binds both C5a and C5adesArg with nanomolar affinity, although the binding sites for these two ligands are not identical, suggesting that they may have different effects. Some researchers contend that C5L2 also binds C3a and C3adseArg, although this remains controversial. Critically, C5L2, unlike the other AT receptors, is not G-protein coupled and, as a consequence, is considered by many as a nonfunctional decoy receptor that dampens down local responses to C5a. In one specific site, adipose tissue, C5L2 appears to be functionally important, strongly implicated as the receptor triggering fat mobilization in response to locally produced C3adesArg (termed “acylation-stimulating protein” in this context).[52] Clarification will likely soon follow in this active research area.

Receptors for C1q

Identification of receptors for C1q has been a minefield of false leads because of the inherent stickiness of the protein. Several receptors have been reported over the years,

although many have not stood the test of time and the physiological relevance of the others remains uncertain. The most convincing candidate receptor is the **C1q receptor for phagocytic enhancement** (**C1qRp**; CD93), a heavily glycosylated 120 kDa transmembrane sialoglycoprotein expressed on myeloid cells and endothelia.[53] Its extracellular region comprises a collagen recognition domain followed by five epidermal growth factor (EGF) domains; the collagen recognition domain binds the collagenous regions of C1q, MBL, and another lectin molecule, surfactant protein A (SpA). It is suggested that this interaction is important in the clearance of apoptotic cells; these spontaneously bind C1q, MBL, and likely SpA, tagging them for binding C1qRp and phagocytic clearance. Antibodies against C1qRp inhibit apoptotic cell clearance in vitro, and mice deficient in C1qRp or C1q display delayed apoptotic cell clearance. Several members of the **integrin family** of cell adhesion molecules have been implicated as C1q binders; of the several collagen-binding integrins, α2β1 stands

out for its capacity to bind C1q and MBL.[54] A 60 kDa receptor for the collagenous tail of C1q, MBL, and other collectins, termed **cC1qR**, was shown to be identical to calreticulin, a cytoplasmic chaperone protein, raising questions about how this molecule could associate with membranes and act as a C1q receptor. It is now suggested that calreticulin binds the extracellular domains of CD91 to create a receptor. Other molecules suggested to act as receptors for C1q (± MBL) include CR1 (binding the collagenous region through its membrane-proximal SCRs) and a 33 kDa protein that binds the C1q globular heads (**gC1qR**).[55] The physiological roles of these interactions are uncertain.

COMPLEMENT ONTOGENY, GENETICS, AND PROTEIN FAMILIES

Complement is an evolutionarily ancient defense system; proteins with C3-like opsonic activities and fB-like enzymatic properties are present in the hemolymph of starfish, sea anemones, and even the horseshoe crab, but not in insects.[56,57] Early studies of primitive complement systems were limited because they relied upon the identification of opsonic or lytic activities in fluids derived from various species. A more recent approach to complement evolution has been to search for complement homologues in the genomes of primitive animals.[58] These studies confirm the very early arrival of C3- and fB-like molecules, show that C3 multiplication to yield C4 and C5 occurred prior to the emergence of cartilaginous fish, confirm that the fB/C2 duplication event preceded the split between Amphibia and Mammalia, and confirm the presence of C1q-like, MBL-like, and MASP-like proteins in lamprey. All four terminal components are present in all mammals, birds, and amphibians, but no genetic evidence for terminal components is found in sharks despite early claims of a C9-like molecule and lytic activity in shark serum.

Many of the complement components, regulators, and receptors share structural features with others, creating protein families within the complement system, usually a result of gene reduplication events during evolution.

The Thioester Protein Family

C3, C4, and C5, together with the abundant noncomplement plasma protease inhibitor, α2-macroglobulin (α-2M), comprise a family of homologous proteins each made up of multiple disulphide-linked chains derived from a single chain precursor and typified by the presence of an internal thioester, hence called the thioester protein family (TEP). These proteins have evolved from an ancestral protease inhibitor closely resembling α-2M[56] and have utilized the thioester, part of the α-2M protease inhibition machinery, to confer capacity to covalently bind surfaces. Loss of the thioester in C5, a requirement for the evolution of a lytic TP, is a relatively late evolutionary event. With some of the complement protein families, their common evolutionary origins are evidenced by conservation of genetic linkage— the duplicated genes remain adjacent in the genome; however, for the TEP family, gene linkage has been lost, with each gene on a different chromosome; *A2M* on chromosome 12, *C3* on chromosome 19, *C5* on chromosome 9, and *C4* on chromosome 6. The *C4* gene is particularly interesting. First, it is duplicated in man, the two genes, *C4A* and *C4B*, encoding highly homologous proteins, differing in just four amino acids clustered in the α-chain; second, it is situated in the major histocompatibility complex (MHC) on chromosome 6, linked to the genes encoding **C2** and **fB,** themselves homologous molecules, derived from a

common ancestor.[59] Co-location of these three genes into the MHC cluster is seen in all higher vertebrates, indicating that it was an early event and suggesting that the common ancestor of the TEP family proteins was linked to fB/C2 from the earliest origins of the complement system. Whether there is any functional consequence of the location of these complement genes in the MHC is unclear; however, the *C4* gene in zebrafish is not linked to MHC,[60] implying that its location within the MHC in higher vertebrates is an evolutionary accident of no functional importance.

The Terminal Pathway Protein Family

The TP components, **C6**, **C7**, **C8, and C9**, are structural homologues derived from a common C9-like ancestor protein and show increasing complexity as one travels back from C9 to C6. **C9** is the simplest, comprising, from the amino terminus, a thrombospondin (TSP) domain, a low-density lipoprotein-receptor A-type domain, a MAC-perforin domain, and an EGF domain. The **C8β** and **C8α** chains each contain the same domains as C9, but with the addition of an extra TSP domain at the carboxy-terminus; **C8γ** is a lipocalin family member with no homology to other complement proteins; **C7** contains all the domains present in the C8 chains, but has an additional four domains at its carboxy-end, two SCRs and two factor I-MAC domains, also found as a single domain in fI.[61] **C6** closely resembles C7 but has acquired an additional TSP domain at its amino terminus. The MAC-perforin domain in the T cell pore-forming protein perforin is homologous to that in C9 and the other TP proteins, and is also found in many bacterial cytolysins, suggesting that it is a common feature of pore-forming proteins in nature.[62] The genes encoding TP proteins are linked

in two groups. C6, C7, and C9 are linked on chromosome 5. The first two are immediately adjacent to one another, albeit in opposite orientations, while *C9* is more distantly linked. *C8A* and *C8B* are very closely linked and in opposite orientations on chromosome 1p.

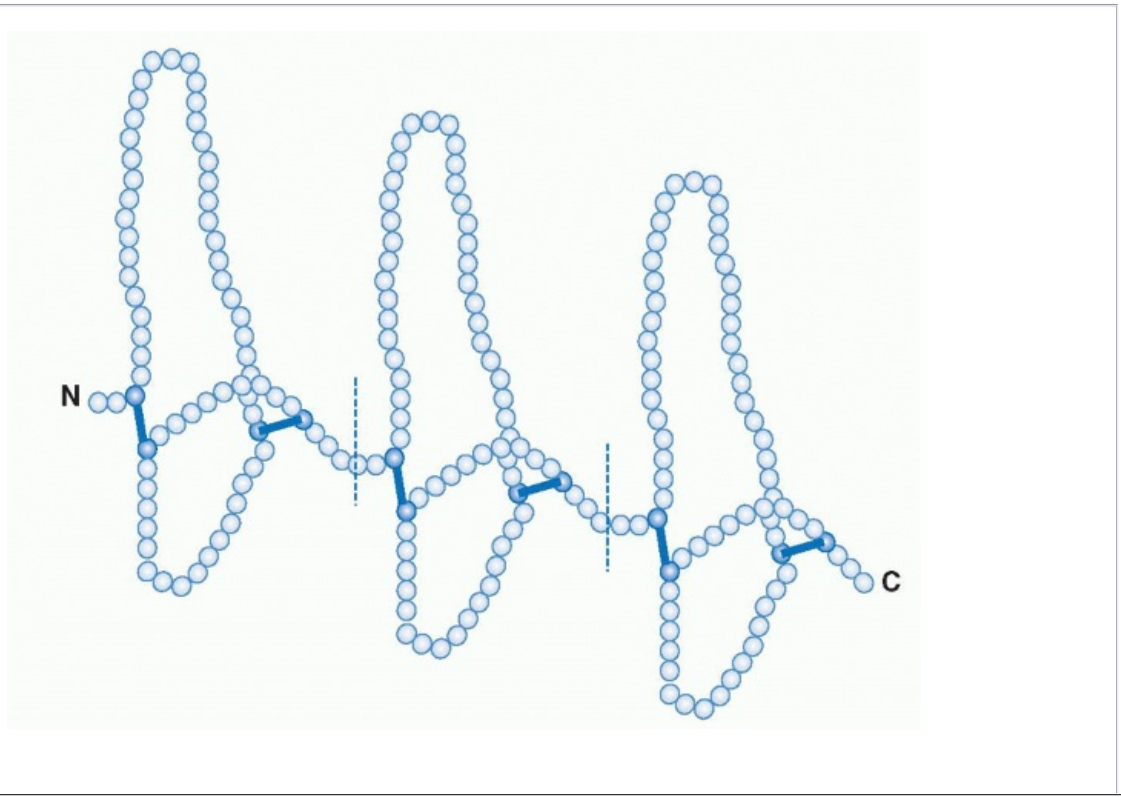

FIG. 36.11. Short Consensus Repeat Structure. The short consensus repeat comprises approximately 60 amino acids held in a rigid "bead" structure by intradomain disulphide bonds.

The Collectin Family

The **collectin** family includes C1q, MBL, and the ficolins. All have collagenous amino terminal stalks and globular recognition domains at their carboxy-termini.[63] Evolutionary tracking of this family is complicated by the frequency of collagenous proteins in nature, most of which are unrelated to complement. Clade analysis strongly suggests that lampreys (*agnathia*; jawless fish) have a *C1q* gene that is a likely ancestor of the mammalian *C1QA*, *C1QB*, and *C1qC* genes. This finding indicates that *C1q* predates the evolution of antibody and adaptive immunity, likely as a pathogen recognition molecule. MBL genes are found even in the earliest vertebrate genomes, but ficolin genes appear to be more recent, present in tetrapod genomes but not in lower vertebrates.[64] In man, the three *C1Q* genes are arranged in tandem in order A-C-B on chromosome 1p. The MBL gene resides on chromosome 10, clustered with the genes encoding the related collectins, surfactant proteins A and D (*SFTPA1*, *SFTPA2*, *SFTPD*). The genes encoding ficolins-1 and -2 are closely linked on chromosome 9q, while the ficolin-3 gene is on chromosome 1p.

The C1r/C1s/Mannan-Binding Lectin-Associated Serine Protease-2 Family

The initiating enzymes of the CP and LP comprise another family (MASPs-1, -2, and -3; C1r; and C1s) sharing the same domain structure comprising, from the amino terminus, a C1r/C1s/Uegf/Bmp1 (CUB) domain, an EGF domain, a second CUB domain, two SCRs, and a carboxy-terminal serine protease domain.[65] The *MASP1* gene, on chromosome 3, is alternatively spliced to yield MASP-1 itself, MASP-3 in which the serine protease domain is larger, and Map44, a product truncated after the first SCR. Alternative splicing of the *MASP2* gene, on chromosome 1, yields either MASP-2 itself or a truncated product, Map19, comprising the CUB and EGF domains with four unique amino acids at the carboxy-terminus. The common ancestor, present in ascidian worms, is a *MASP1* gene, although the precise pathway through which this gene reduplicated to yield the *MASP2*, *C1R*, and *C1S* genes is uncertain.[66] The *C1R* and *C1S* genes are adjacent in a tail-totail arrangement on chromosome 12p, suggesting that these two genes arose relatively recently from a common precursor.

The Regulators of Complement Activation Family

The last, and perhaps most interesting, of the complement protein families to be discussed in this section is the **regulators of complement activation** (RCA) family, characterized structurally by a preponderance of SCR domains and genetically by close linkage in the RCA cluster on chromosome 1q, suggesting that they have developed through serial duplication events.[67] The SCR domain, the signature of the family, is a globular domain of around 60 amino acids, including a number of highly conserved tryptophans, prolines, and cysteines that give the SCR its shape (Fig. 36.11). The conserved cysteines, four in each SCR, form two invariant disulphide bonds to lock the structure, and joining of domains end-toend creates the elongated, "beads on a string" appearance typical of this family. SCR domains

are also found in other complement proteins (C6, C7, fI, C1r, C1s, MASPs, etc.) and some noncomplement proteins (eg, selectins), but only in RCA proteins is the dominance of this domain seen. RCA proteins in lower vertebrates, though fewer in number (six in chicken, two in frog), are also made up of SCR domains, and their genes are clustered. The frog genes are orthologs of the human *DAF* and *C4BP* genes, indicating that these are the common ancestors of other RCA proteins. The RCA cluster comprises two blocks of genes encoding complement regulators and receptors, interspersed with a number of unrelated noncomplement genes (Fig. 36.12). The first block comprises, in sequence from telomeric end, the genes for MCP (*MCP*), a CR1-like pseudogene (*CR1-like*), CR1 (*CR1*), CR2 (*CR2*), DAF (*DAF*), a C4bp α-chain-like pseudogene (*C4BPA-like*), C4bp α-chain (*C4BPA*), and C4bp β-chain (*C4BPB*); the pseudogenes have no protein product. The second block, some distance from the first in a centromeric direction, comprises, in sequence, the genes for fH (*CFH*), fH-related 3 (*CFHL3*), fH-related 1 (*CFHR1*), fH-related 4 (*CFHR4*), fH-related 2 (*CFHR2*), and fH-related 5 (*CFHR5*). Alternative splicing of the *CFH* gene creates an additional protein product, fH-like 1 (fHL-1), comprising the first seven SCRs of fH and an additional six unrelated carboxy-terminal amino acids.[68]

Expression Patterns of Complement Proteins

The complement genes are not ubiquitously expressed; rather, they show cell type-specific expression patterns that are important in understanding how the system works.

Restricted expression of complement receptors, described previously, ensures that the appropriate cell types respond to complement activation products. Regulators are broadly expressed, on most host cells and particularly those exposed to plasma, to protect from damage; however, there are notable exceptions where one or more regulators are absent or nearly so; for example, erythrocytes lack MCP,[38] while some cells in the brain lack CD59.[69] Plasma contains an abundance of complement proteins, at concentrations varying from very high (1 g/L for C3) to very low (1 mg/L for fD). The major site of biosynthesis for most of these plasma components is the liver; abundant proteins like C3 and C4 are products of hepatocytes and, like the other hepatocytederived plasma complement proteins, behave as acute phase reactants, increasing in concentration in response to inflammatory cytokines. Hepatic synthesis of C3 and C4 can therefore increase two- or three-fold during inflammation, but plasma levels rarely increase as much because inflammation is also associated with increased complement activation and consumption of these proteins. A few complement proteins are mainly produced elsewhere; tissue macrophages are the major source of plasma C1q, adipocytes the primary source of fD, and leukocytes, likely neutrophils, the major source of plasma C7.[70] Other cell types and tissues can contribute to plasma complement protein production, and this local production is likely to be very important for complement activity within organs and tissues, particularly when inflammation is present. The kidney is a secondary source of many complement proteins; indeed, transplantation studies have shown that the kidney produces up to 5% of plasma C3, increasing to as high as 10% with renal inflammation.[71] There is very little complement synthesis in the noninflamed brain, fortunately so because brain cells are poorly protected from complement; however, inflammation switches on complement production in glia and neurons, contributing to brain cell loss in neurodegeneration.

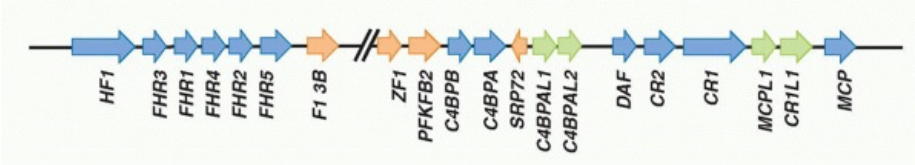

FIG. 36.12. The Regulators of Complement Activation Gene Cluster. The regulators of complement activation cluster comprise a long region (up to 7 Mb) on band q32 of human chromosome 1 that contains the genes for all the short consensus repeat-containing complement regulators and receptors. Two separate subclusters contain, respectively, the genes for factor H and the factor H-related proteins 1 to 5, and the genes encoding, in order, the β- and α-chains of C4bp, decay accelerating factor, complement receptor 2, complement receptor 1, and membrane cofactor protein. The "true" genes (*blue*) are interspersed with numerous pseudogenes (*green*) that have no protein product and several nonrelated noncomplement genes (*peach*).

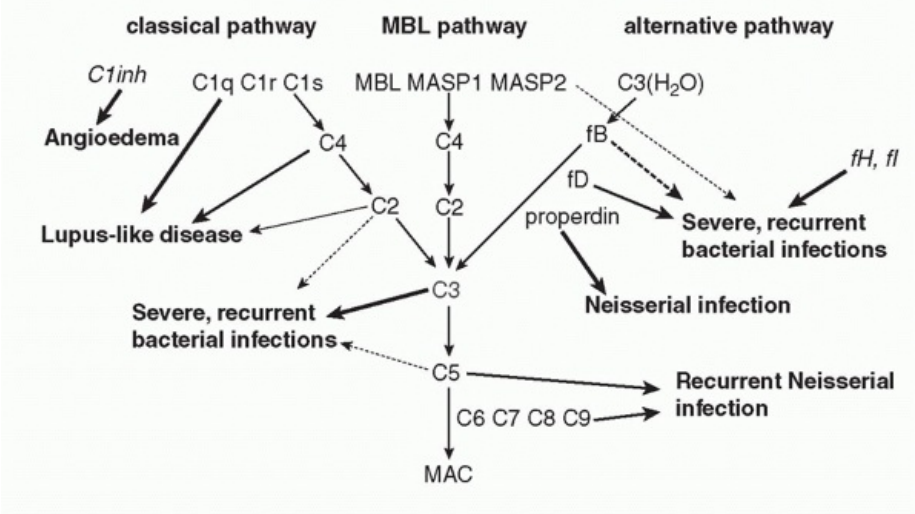

FIG. 36.13. Complement Deficiencies. Deficiencies of most of the components and plasma regulators of complement have been described in human subjects. The clinical presentations commonly associated with each of the deficiencies are summarized in this simplified figure.

COMPLEMENT MUTATIONS, POLYMORPHISMS, AND AUTOANTIBODIES

Mutations in complement proteins are rare events that can be catastrophic and cause partial or total deficiency of the protein, or more subtle, altering the expression level or functional

efficiency of the protein. Polymorphisms are common variations in protein composition that may or may not have functional consequences.

The most dramatic outcome of a mutation in a complement protein is **deficiency**, which may involve a loss of protein product (Type I deficiency) or the production of a functionally inactive protein (Type II deficiency). For almost every complement protein, regulator, and receptor, individuals totally and/or partially deficient in the component have been described, experiments of nature that have informed understanding of the biological roles of different complement pathways; indeed, deficiencies are best understood when grouped according to pathway or family affected (Fig. 36.13).

Classical Pathway Component Deficiencies

Deficiencies of **CP component** proteins (C1, C4, C2) remove the capacity for efficient activation of complement on ICs and are strongly associated with lupus-like IC disease and infections.[72] **C1q deficiency** is rare (only a dozen or so families have been described, and all affected homozygous individuals develop severe lupus-like disease with rashes, renal failure, and other sequelae before adulthood). Deficiencies of either **C1r** or **C1s** are similarly rare and often combined because of linkage and coordinate expression of the genes; symptoms and penetrance are as for C1q deficiency. **Total C4 deficiency** is also rare because there are two C4 genes (*C4A*, *C4B*) each with two alleles, requiring co-inheritance of four mutations for complete deficiency. These rare individuals will all develop lupus-like disease early. Mutations leading to loss of expression of one of more C4 alleles (null alleles) are relatively common in the population: 35% have one null allele, 8% two null alleles, and 1% three null alleles; these latter individuals will have low plasma C4 levels and an increased tendency to develop IC disease, greater in those with more null alleles and in those null at the *C4A* gene.[73] Genetic testing for C4 gene copy number is therefore a useful adjunct to measuring plasma levels in testing for C4 deficiency. **C2 deficiency** is the most common complement deficiency among Caucasians; C2 null alleles are present in some 1% of individuals, giving a predicted incidence of deficiency of 1:10,000 in the population. Although C2 deficiency does increase the risk of developing IC disease, the majority of C2-deficient individuals are healthy, detected at routine screening because their plasma lacks CP hemolytic activity.

The strong association of classical pathway deficiencies with lupus-like disease has led to the assertion that lupus, not only in complement deficiency but also in classical systemic lupus erythematosus (SLE), is caused by the defective clearance of immune complexes and apoptotic cells which then become sources of autoantigens.[74] This “waste disposal hypothesis” has altered perceptions of the disease and modified approaches to treatment.

Lectin Pathway Component Deficiencies

Deficiencies of **LP components**, partial and total, have been described. Plasma levels of MBL vary widely in the population due to a complex set of mutations and polymorphisms in the constituent chains.[75] Three mutations clustered in exon 1 of the *MBL* gene individually disrupt the capacity to form the higher oligomers necessary for MBL function and, in homozygosity or compound heterozygosity, result in complete deficiency of MBL. Complete MBL deficiency predisposes to bacterial infections, particularly in individuals who are

otherwise compromised (eg, neonates, the elderly, patients with cystic fibrosis, and patients with acquired immunodeficiency syndrome).[76] A number of common polymorphisms in MBL can also profoundly affect MBL plasma levels and oligomerization status, and those inheriting an extreme set of variants can have profoundly low MBL levels, with the same resultant problems as those totally deficient in MBL. Genetic mutations leading to deficiencies of **ficolin-2** and **ficolin-3** were recently reported and shown to cause defective LP activation in plasma.[30] Mutations in the *MASP2* gene leading to complete or subtotal deficiency of this key LP enzyme **MASP-2** were also described,[77] but their clinical relevance remains unclear.

C3 Deficiency

C3 is the most abundant and most critical of the complement proteins, essential for activation through all pathways. It is therefore not surprising that **total C3 deficiency**, a very rare finding restricted to a few dozen families, is devastating.[78] Individuals present in childhood with severe, recurrent bacterial infections that, if not promptly treated, will progress to uncontrolled sepsis and death. Supportive care and antibiotics can permit survival into adulthood, but IC disease and renal injury then become apparent.

Alternative Pathway Component Deficiencies

Deficiencies of the **AP components** are rare, with just a few individuals or families reported for each. Total deficiency of fB has not been reported, although a recent abstract described total or subtotal deficiency in a teenager with meningitis.[79] Each of the fD-deficient cases have presented with Neisserial infection, most often fulminant septicaemia, suggesting that AP amplification is particularly important in dealing with bacteria of this genus[80]; however, numbers of cases are small, and it is possible that AP deficiencies have broader consequences for susceptibility to bacterial infections. Deficiency of the AP stabilizer, properdin, is also associated with severe Neisserial infections, meningitis, and septicaemia.[81] Inherited in an X-linked manner, it is almost exclusively seen in young males, usually presenting in childhood. It is rare but probably underascertained. Properdin deficiency may be associated with a complete absence of the protein (Type I), presence of very low levels of normal protein (Type II), or presence of near-normal levels of a nonfunctional protein (Type III). Patients who survive the initial infectious episode tend to do reasonably well thereafter as anti-Neisserial antibodies develop, permitting recruitment of the CP in subsequent exposures.

Terminal Pathway Component Deficiencies

Deficiencies of **TP components** (C5, C6, C7, C8, and C9) are relatively common and all predispose to Neisserial infections. C5, though not a true TP component, is included here because consequences of deficiency are similar; **C5 deficiency** has been identified in a few dozen families from across the globe, perhaps more common in Africans and African Americans. Deficiency predisposes to Neisserial infections, including meningitis and septicaemia, and perhaps other infections[82]; unlike AP deficiencies, infections tend to be recurrent and often involve unusual serotypes of Neisseria. **C6 deficiency** is the second most common complement deficiency in Caucasians, predicted from null allele frequency to have an incidence of around 1:10,000 in the population; in African Americans, it may be even

more common, with a predicted incidence greater than 1:2,000.[83] C6 deficiency is

common among the Cape Coloured ethnic group in South Africa, believed to be the result of a founder mutation imported from Holland in the 17th century. Consequences are similar to those of C5 deficiency: recurrent Neisserial infections. Subtotal deficiency of C6 has also been described, with levels a few percent of normal and with or without linked deficiency of C7; the frequency and relevance of these is uncertain.[84] **C7 deficiency** is also common in some racial groups, with a predicted incidence of 1:10,000 in Israelis of Moroccan Jewish descent.[85] The frequency of total deficiency in Caucasians is not well defined, but personal experience suggests it is considerably less common that C6 deficiency in the United Kingdom. Clinical consequences are identical to C6 deficiency. Subtotal C7 deficiency is common in Caucasians, with a defined mutation at an allele frequency of about 1%.[84] A few cases of combined deficiency of C6 and C7 have been described as a consequence of the close linkage of the genes.[86] **C8-deficient** individuals can lack either C8αγ or C8β chain; the former is rare in Caucasians, the latter more common.[87] In all C8αγ-deficient individuals characterized to date, the mutation is in the *C8A* gene. Plasma contains no detectable C8αγ and very low amounts of C8β, indicating that this chain is labile in isolation in plasma. C8β-deficient individuals have no detectable C8β in plasma but variable amounts of C8αγ, often approaching normal. In either case, plasma haemolytic activity is completely lost. Presenting symptoms are identical to those of other TP deficiencies noted previously. **C9 deficiency** is rare in most ethnic groups with only a handful of cases reported in Caucasians, but it is by far the most common complement deficiency in Japanese and other Oriental races, the result of a single, ancient non-sense mutation (R95X) in the gene.[88] Frequency of the null mutation is about 2% in Koreans and 1% in Chinese; however, in Japanese, null allele frequency is 6.7%, giving a predicted incidence of C9 deficiency of about 1:260 of the population! This incredible frequency implies some selective advantage of the null allele in these populations; most compelling is the suggestion that C9 deficiency blunts the sometimes overvigorous complement response to infection, making C9-deficient individuals less susceptible to the development of sepsis.

Plasma Regulatory Protein Deficiencies

Deficiencies of many of the **complement regulatory proteins** have been described. The most common by far is **C1inh deficiency**, which has a prevalence in Caucasian populations of 1:50,000. Deficiency of C1inh is common primarily because it presents as an autosomal dominant, heterozygous deficiency is sufficient to cause disease.[89] Deficiency of C1inh causes the disease hereditary angioedema (HAE), characterized by recurrent, acute episodes of localized swelling of the skin and/or mucous membranes. As noted previously, C1inh is not only the sole plasma protease inhibitor of activated C1r/s and the MASP enzymes, but also inhibits enzyme cascades involved in coagulation, fibrinolysis, and the contact system. Inflammation or injury triggers activation of complement and these other proteolytic cascades in tissues, leading to the consumption of C1inh, a suicide inhibitor. In the presence of only a single normal *C1INH* gene, synthesis of C1inh cannot keep up with triggered consumption and plasma levels fall precipitously, leading to loss of control of the multiple cascades. Active products of the cascades, particularly the kinins, cause localized

tissue edema, the hallmark symptom of HAE. Many different mutations in *C1INH* have been described in patients with HAE.[90] In about 80% of cases, these are null mutations with no protein product (Type I); in about 20%, a protein is produced but is inactive (Type II). **Deficiency of fI**, the key enzyme responsible for the inactivation of the convertase enzymes of the AP and CP, is a rare but devastating problem.[78,91] Tickover complement activation and AP amplification proceed unchecked, leading to complete consumption of C3; this secondary C3 deficiency predisposes to bacterial infections and IC disease, precisely as in primary C3 deficiency. The plasma regulator of the AP convertase, fH, is essential for fluid-phase AP regulation. **Complete deficiency of fH** is rare and, like fI deficiency, results in uncontrolled AP amplification and consumption of C3.[78] Secondary C3 deficiency predisposes to infection and immune complex disease, but fH deficiency uniquely affects the kidney, causing a characteristic set of renal pathologies, including various subtypes of membranoproliferative glomerulonephritis and atypical hemolytic uremic syndrome (aHUS).[92] aHUS is much more strongly associated with mutations in the carboxy-terminal SCRs of fH that do not cause deficiency but do reduce or ablate the capacity of fH to bind surfaces; these binding mutants of fH are the most common genetic finding in aHUS, responsible for up to half of all cases and causing disease even in heterozygosity.[93] Deficiencies of other members of the fH gene family have recently been described and in some cases associated with disease. Combined deletion of the *FHR1* and *FHR3* genes is a common finding, present in about 5% of Caucasians and even more common in some racial groups. Absence of fHR1 and fHR3 is associated with aHUS, apparently because it predisposes to the development of anti-fH autoantibodies (described in the following)[94]; the same deficiency is protective for the common eye disease age-related macular degeneration (AMD). Deficiencies of other fHR proteins are much less common and of uncertain clinical relevance. **Deficiency of C4bp**, the fluid phase regulator of the CP, is extremely rare with only two case reports to date; in neither case were the presenting symptoms compatible with a complement defect, suggesting that they were ascertainment artifacts.

Membrane Regulatory Protein Deficiencies

Complete, global deficiencies of any of the cell surface regulators are very rare events. A single case of **complete deficiency of CD59** was reported, a young Japanese male who presented with a severe hemolytic syndrome.[95] A handful of families with **complete deficiency of DAF** have been described, apparently healthy apart from an apparent association with gut inflammation in some families.[96] Of note, both CD59 and DAF are GPI-anchored molecules and are missing from the GPI-negative hemopoietic clone in the hemolytic disorder **paroxysmal nocturnal hemoglobinuria** (PNH);

absence of these complement regulators is the cause of the erythrocyte lysis and activation of platelets and leukocytes that typify this disorder.[97] **Complete deficiency of MCP** has not been reported, perhaps reflecting the fact that this molecule plays key roles outside of complement regulation. Deficiencies of complement receptors are similarly rare or nonexistent. No complete inherited deficiencies of **CR1** have been described, although acquired deficiencies are common in those with IC diseases such as SLE. No deficiencies of **CR2** have been reported, although again, acquired low levels are found in SLE. A specific

deficiency of the **CR3** α-chain was described in one individual presenting with severe SLE; deficiency of the common β-chain causes loss of all four integrins that share this chain with profound consequences for leukocyte adhesion and immune function. No complete deficiencies of the AT receptors have been reported.

Complement Protein Point Mutations

Point mutations that affect function of a complement protein in more subtle ways are also sometimes of clinical importance. Perhaps the best example is that of fH mentioned previously, where the carboxy-terminal SCRs appear to be "hotspots" for point mutations that have no direct effect on the complementregulating capacity of fH, but profoundly reduce its capacity to bind surfaces.[98] Even in heterozygosity, these mutations predispose the individual to the development of aHUS, particularly when combined with other complement polymorphisms or mutations that alone have little effect. Another intriguing example is a recent report describing an unusual renal disease in Greek Cypriots; search for a genetic cause identified a mutation in **FHR5**.[99] The mutant protein was present in plasma but was dysfunctional, unable to bind efficiently surface-bound C3b; quite how this functional deficit in an apparently minor member of the fH family causes such major pathology remains to be determined.

Complement Protein Polymorphisms

Common polymorphisms in complement components and regulators have also been linked to disease, particularly chronic inflammatory diseases and infections. Among the inflammatory diseases linked to complement polymorphisms, two stand out; **AMD**, the most common cause of vision loss in the elderly in Western societies, and **dense deposit disease** (DDD), a rare renal disease. A common polymorphism in fH causing a single amino acid change in the seventh SCR (Y402H) was shown in many independent studies to be strongly linked to AMD, individuals homozygous for the less common H allele having a 6- to 10-fold higher risk of developing the disease than those homozygous for the more common Y allele.[100] Vision loss in AMD is caused by a buildup of an amorphous material called "drusen" beneath the retina that disrupts retinal architecture, causing loss of vision. Analysis of drusen shows that it contains an abundance of complement fragments, indicating that it is a site of complement activation, driving inflammation in the eye. Recent biochemical studies have suggested that the Y402 variant of fH binds better to drusen than the H402 variant—more fH binding will result in less complement activation, less inflammation, and less disease in Y402 homozygotes. A second fH polymorphism, V62I, is also linked to AMD, the I allele being protective. This polymorphism increases the C3b binding and cofactor activities of fH, making a more efficient inhibitor and thus restricting AP activation.[101] Other complement polymorphisms have also been linked to AMD; a common polymorphism in C3 (C3S/F; R102G) was associated with increased risk of AMD (two-fold higher for G allele homozygotes compared to R allele homozygotes), while a common polymorphism in factor B (R32Q) was associated with a near-two-fold decreased risk for Q allele homozygotes. The functional effects of the C3 and fB polymorphisms have been characterized[102,103]; in each case, the allele that is "risk" for AMD ($C3_{102G}$; factor B_{32R}) is more active in forming AP convertases and will hence drive more complement activation and inflammation in drusen. DDD is linked to both of the polymorphisms in fH described previously, Y402H (H allele risk, as for AMD

described previously), and V62I (I allele protective, as in AMD), and is also linked to the C3 R102G polymorphism.

The Complotype

The "set" of complement polymorphisms that an individual inherits can have dramatic effects on complement activity in his or her plasma. An individual inheriting in homozygosity the more active variants of C3 and fB and the less inhibitory variants of fH can have an AP activation loop in plasma that is 10-fold more active than that of an individual inheriting the less active components and more inhibitory fH variants.[103] This inherited **"complotype"** will markedly affect disease susceptibility—an individual with a more active AP will combat infection well but may be more susceptible to chronic inflammation, while those with a less active AP may be at risk of infection but protected from chronic inflammatory diseases (Fig. 36.14). To date, attention has focused on the AP; however, polymorphisms in any of the LP, CP, and TP components and regulators, that alter function and/or plasma levels, will impact upon the complotype of an individual.

Complement Autoantibodies

Autoantibodies against complement components, regulators, and complexes have been found in and linked with disease. **Nephritic factors** (NeFs) are autoantibodies that bind and stabilize the AP (and rarely CP) C3 convertase. They are, as the name implies, associated with renal disease, usually present in patients with DDD, and sometimes found in other types of membranoproliferative glomerulonephritis.[104] NeFs are also found in the rare disease partial lipodystrophy where peripheral adipose tissue is lost.[105] NeF stabilizes the convertase both by slowing natural decay and by preventing accelerated decay mediated by fH and other regulators. Complement is therefore consumed systemically and/or locally, and this in turn causes renal pathology. It is not at all clear why an immune response to the convertase enzyme occurs in NeF-positive individuals. One clue is provided by the demonstration that NeF are linked to the

convertase-stabilizing C3 polymorphism, $C3_{102G}$ (C3F)[103,106]; perhaps a more stable convertase is more likely to be immunogenic because it lasts longer? **Autoantibodies against fH** are increasingly recognized in association with renal disease, particularly aHUS.[94] Almost all anti-fH-positive aHUS patients lack fHR1 and fHR3; this appears to be necessary but not sufficient for anti-fH production because most individuals with this common gene deletion do not make anti-fH antibodies. The anti-fH antibodies predominantly target the carboxy-terminal SCRs of fH and inhibit fH binding to surfaces, mimicking the effects of the aHUS-associated mutations in fH. **Anti-C1q autoantibodies** are associated with a number of autoimmune and renal disease, notably lupus nephritis and poststreptococcal glomerulonephritis. Anti-C1q antibodies are a useful marker of disease activity, but their contribution to the disease process is uncertain. **Anti-C1inh autoantibodies** are associated with a rare, acquired form of angioedema mimicking HAE[107]; the autoantibodies target the reactive center of C1inh thereby blocking its function.

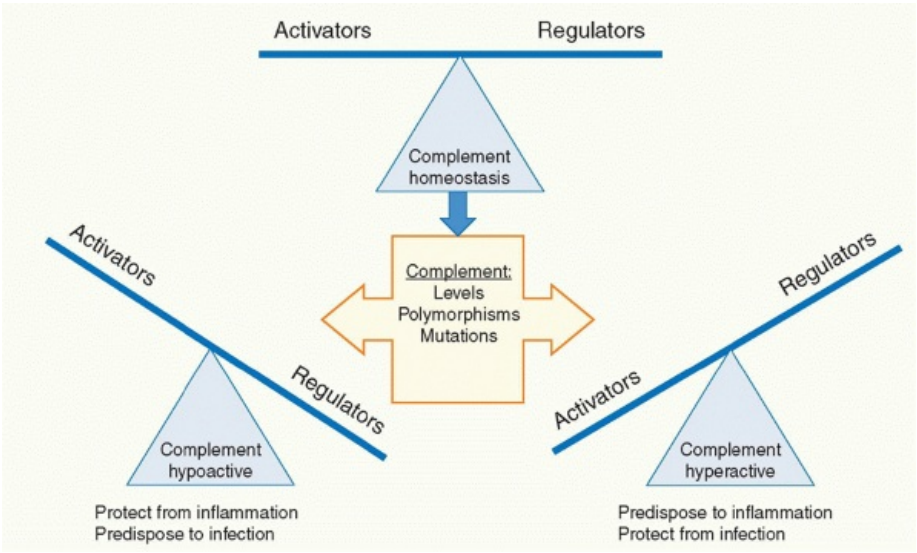

FIG. 36.14. The Complotype. Despite the tendency of complement to spontaneously activate, the balance of activators and regulators ensures that homeostasis prevails. Interindividual variability in terms of complement protein/regulator plasma levels, common polymorphisms, or rare mutations dictate that some individuals will have an intrinsically more active complement system (*right*), protective against infection but risk for chronic inflammation, while others have an intrinsically less active system (*left*), risky for infection but protective for chronic inflammatory disease.

COMPLEMENT IN DISEASE: ROLES AND TREATMENT OPPORTUNITIES

As noted previously, complement deficiencies, mutations in complement proteins, polymorphic variants in complement, and complement autoantibodies can all cause disease. In addition to these specific circumstances, complement contributes to a large number of diseases, triggered by diverse events, by driving inflammation and tissue damage. Some diseases are clearly complement mediated, while others present a mixed picture with multiple effectors contributing. The best example of the former is the hemolytic and thrombotic disorder **PNH**. A somatic mutation gives rise to a clonal population of hemopoietic cells that cannot make GPI anchors and thus lack all GPI-linked proteins from their surfaces.[97] Key among these are the complement regulators DAF and CD59. Absence of DAF and CD59 on erythrocytes, which intrinsically lack the other membrane regulator MCP, renders them highly susceptible to autologous complement activation and hemolysis, resulting in the cardinal signs anemia and hemoglobinuria. Platelets and leukocytes derived from the clone are also deficient in regulators and prone to complement activation and injury, resulting in increased platelet aggregation and leukocyte activation, which in turn predispose to thrombotic stroke.

Complement in Ischemia-Reperfusion Injuries

Ischemia-reperfusion (IR) injuries (eg, ischemic stroke, myocardial ischemia, or post-bypass) are highly dependent on complement activation. The ischemic tissue, when reperfused spontaneously or through clinical intervention, activates complement through the AP and likely also the LP, leading to injury and inflammation, increasing the extent of the injury.[108] Precisely why the ischemic tissue becomes an AP activator is still debated, although reactive

oxygen species generated during anoxia are implicated and the ischemic tissue shows decreased expression of complement regulatory proteins. The earliest studies of the efficacy of anticomplement drugs were performed in animal models of IR injury;

these, and many more since, have firmly demonstrated that complement inhibition is highly protective in this group of conditions.

Complement in Sepsis

Sepsis is a devastating disease for which there are few therapeutic options. The best available drug treatments, steroids, and/or activated protein C, only modestly impact outcome, and for most patients, all that can be offered is life support. As a consequence, mortality exceeds 25% of cases and has remained stubbornly unchanged for decades. The realization that sepsis was a consequence of overwhelming immune system activation provoked considerable interest and investment in anti-tumor necrosis factor and other agents targeting inflammatory cytokines; however, none have yet stood the test in clinical trials. There is an abundance of evidence implicating complement in sepsis. Serum complement levels fall, and complement activation products, including C3a, C5a, and TCC, are present at high levels and are predictive of outcome.[109] Animal models of sepsis have added to the evidence; in many studies, inhibition of complement activation improves the clinical outcome, animals genetically deficient in complement components have improved survival. The activation fragment C5a appears to be particularly important in driving the disease in man and models, at least in part because of its effects on neutrophils. Initially, a powerful attractant and activator, C5a in excess causes suppression of neutrophil phagocytosis and mediator release.[110] Antibodies that block C5a and several different agents that block the C5a receptor have been shown to be protective in sepsis models but have yet to be tested in man.

Complement in Transplant Rejection

The immune system treats transplanted organs in much the same way that it treats other foreign invaders: it attacks. Complement is no exception in this respect, although the full force of complement-mediated rejection is seen only when the organ is very foreign. When organs are transplanted across species barriers, **xenotransplantation**, the host response is instant: hyperacute rejection occurs, destroying the organ graft within minutes of initiating perfusion. The primary cause of this explosive rejection is activation of complement in the graft.[111] Pig-to-human transplantation is superficially attractive for anatomical, physiological, and ethical reasons; however, when pig organs are perfused with human blood, IgM natural antibodies in the plasma bind carbohydrate epitopes on the pig cells, activate complement, and destroy the graft. A decade ago, there was much interest in the production of pigs expressing transgenically the human complement regulators on their cells. This strategy, combined with a second genetic manipulation to modify the expressed carbohydrate epitopes, was successful in creating pig organs resistant to hyperacute rejection when perfused with human blood or transplanted into primates. This promising industry was mortally wounded by concerns about the transmission of pig viruses into the human population, but is slowly rising from the ashes on the back of even better pigs.[112]

Hyperacute rejection can also be seen in human-human (allo-) transplants where the donor and recipient are ABO blood group incompatible or where the recipient is presensitized to

donor antigens.[113] Here too, antibody binding activates complement in the graft, leading to its rapid destruction. As anticomplement drugs, described in the following, become more widely available, it is likely that they will be used to protect grafts transplanted into sensitized or incompatible individuals. Surprisingly, work in animal models suggests that even short-term complement inhibition can have long-term effects on graft survival, a phenomenon known as accommodation.

Allotransplants are also subject to acute vascular rejection, a slower process starting days or weeks after the transplant and taking days or weeks. Long considered a cell-mediated process, it is now clear that complement activation contributes; rejecting grafts stain for C4d, a marker of CP activation and inhibition of complement in animal models, is protective.

Complement in Autoimmunity

In many **autoimmune diseases**, although the initiating event is the generation of an immune response against self antigens, much of the observed pathology requires complement activation. In some diseases, complement activation is essential for pathology. **Myasthenia gravis** offers a particularly good example. The initial event is an autoimmune response against motor end plate sequestered antigens, notably the acetylcholine receptor. Binding of antibody in itself causes little damage, but the resultant activation of complement causes the destruction of the end plate and failure of neuromuscular conduction.[114] In the animal model, inhibition of complement completely prevents the development of weakness and paralysis.[115] In **multiple sclerosis**, an immune response against central nervous system-sequestered antigens leads to breaching of the blood-brain barrier, influx of inflammatory and immune cells, and the generation of antibodies that bind myelin and other structures. Complement activation in the brain is particularly harmful because myelin and many brain cell types are poorly protected by complement regulators and are thus easily damaged or destroyed.[69] Again, animal models have suggested that complement inhibition can suppress disease[116]; however, it is likely that multiple sclerosis is a heterogeneous disease, with complement as a major driver in only some patients.[117] A related autoimmune neurological disease, **neuromyelitis optica**, is associated with autoantibodies against aquaporin 4, a water channel expressed on astrocytes and some other cell types. Complement activation is a prerequisite for the myelin loss and cell damage that typifies this disease. In the autoimmune peripheral neuropathy, **Guillain-Barre syndrome**, antibodies target peripheral nerve myelin, fix complement, and drive axonal damage. **Recurrent fetal loss** is a distressing syndrome where women mount an immune response against fetal antigens leading to fetal rejection. Recent evidence has implicated antiphospholipid antibodies in a large proportion of affected women; evidence from human studies and mouse models implicates complement activation as the major effector of rejection in this syndrome.[118]

Complement in Renal Disease

Many **renal diseases** are associated with abundant complement activation in the kidney. Some of these are autoimmune in origin, with antibody or immune complex deposition in the glomerulus being the trigger for complement activation. The glomerular injuries associated with aHUS, fHR5 deficiency, and DDD were described previously. DDD, noted previously for

its association with anti-C3 convertase autoantibodies (NeF), is an archetypal disease of complement dysregulation, linked not only to NeF but also to mutations and polymorphisms in complement proteins that predispose to a loss of fluid-phase control of activation.[104] Precisely why these triggers target exclusively the kidney remains unknown. Other renal diseases in which complement activation plays a critical role include the various membranoproliferative glomerulonephritides, IgA nephropathy, antiglomerular basement membrane disease, lupus nephritis, and membranous nephritis.[119] In the last of these, MAC-induced activation of glomerular podocytes is the principal cause of the renal injury.

Complement in Degenerative Diseases

Complement also plays roles in many **degenerative disorders**, likely because of the accumulation of damage from low-grade complement activation over many years. The association of complement polymorphisms with AMD has been mentioned previously, a more active complement system increasing the risk of disease.[99,103] The brain and retina are particularly susceptible to this slow-burn injury, likely because these sites have poor waste disposal systems, allowing the accumulation of debris—drusen in AMD, plaques and tangles in Alzheimer's disease—that cause further complement activation thereby driving inflammation and injury. The recent demonstration that a common polymorphism in the AP regulator fH is the strongest known risk factor for AMD has focused attention; individuals homozygous for the risk allele (fH-H402) have up to 10-fold greater risk of developing AMD compared to those homozygous for the protective allele (fH-Y402).[99] Other AP protein polymorphisms have also been linked to AMD, leading many in the field to conclude that AMD is caused by AP dysregulation. Anticomplement therapies, described in the following section, are now being tested for treating AMD.

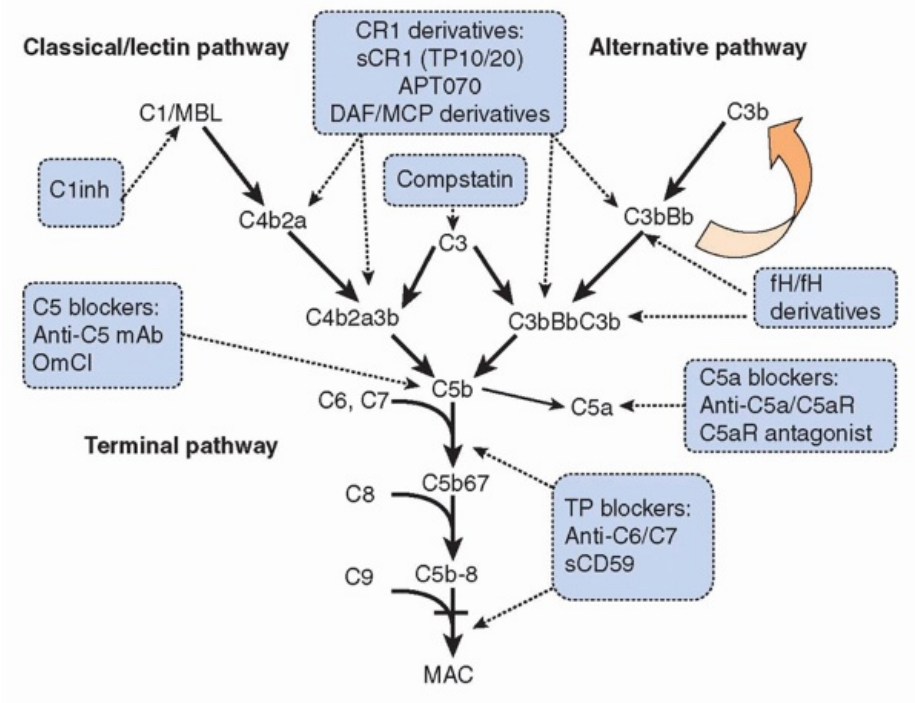

FIG. 36.15. Anticomplement Therapies. Drugs in the market place, in trials, or in development target each stage of complement activation. Agents are in *dotted boxes*;

dotted arrows illustrate primary targets in the complement pathways.

ANTICOMPLEMENT THERAPIES

General Principles

Drugs targeting the complement system have come of age in the last 10 years. Twenty years ago there were, with the exception of C1inh, described in the following, no clinically feasible ways of modulating complement activation in man. Cobra venom factor, a C3-like molecule that forms a stable AP convertase in serum and rapidly consumes all available complement activity, was used as a proof-of-concept in animal models of disease, but caused shock syndromes and occasional deaths in animals through precipitous release of activation products, making it unviable clinically. The scene has changed markedly in recent years with the arrival of an armory of agents that inhibit complement in different ways and in different parts of the pathway (Fig. 36.15; Table 36.4).

Modified Membrane Regulators as Anticomplement Drugs

The advent of clinically viable complement inhibitors came with the invention by Fearon and colleagues of the concept of modifying the naturally occurring membrane complement regulators to inhibit complement in plasma.[120] They expressed a soluble form of CR1 (sCR1) comprising the 30 SCRs of the extracellular portion and showed that it was a powerful fluid-phase inhibitor of C3 cleavage. When tested

in a myocardial ischemia model, sCR1 delivered at the time of reperfusion markedly reduced the size of the resultant infarct, effectively protecting the myocardium from further injury by complement. Soluble CR1 was tested in many other animal models of disease, spanning the spectrum from ischemia through autoimmune to degenerative, and proved effective in most conditions.[116,121] Despite its capacity to inhibit complement activation in vivo in man, clinical trials of sCR1 (developed latterly by Avant Therapeutics, Needham, MA, and known as TP10) have been disappointing. Development for the original application, adult respiratory distress syndrome, was abandoned after a single, small trial. Some success was obtained in use post-lung transplant where sCR1 treatment significantly shortened time on respirator postoperatively,[122] while in a large-scale trial in highrisk patients on cardiopulmonary bypass, sCR1 reduced the incidence of post-bypass myocardial infarction and death in men but not women—an unexpected finding confirmed in a second trial.[123] Although still in development for this and a few other applications, the history of sCR1 remains one of unfulfilled promise. Modified forms of sCR1, including addition of carbohydrate groups (sLex) to target sites of inflammation (TP20), and the engineering of small (three SCRs), membrane-localizing forms (APT070), may prove of more clinical benefit in the future. The success of sCR1, at least in animal models, led to the engineering of soluble forms of other membrane C3 convertase regulators, DAF, MCP, and hybrids of DAF and MCP (CAB-2), all of which have proved effective in at least some animal models, but none have progressed beyond the earliest stages of clinical testing. Similarly, attempts to develop soluble forms of CD59 as therapeutics have been unsuccessful, likely because of the small size of the molecule and its propensity to bind plasma proteins. Very recently, a fusion protein in which the C3d-binding

SCRs of CR2 are fused to the complement AP-inhibiting amino terminal SCRs of fH has been described.[124]

This agent, which binds C3d at sites of complement activation and there inhibits complement activation, has excellent AP-inhibitory activity in vitro and in vivo in monkeys, and is being fast-tracked for treatment of AP-mediated diseases.

TABLE 36.4 Complement Therapeutic Agents

Agent	Development Status	Mode of Action
Protease inhibitors		
Plasma-derived C1inh	Available for > 20 years for HAE, new formulations now FDA approved	Inhibitor of C1r/s, MASPs, kallikrein, and other plasma proteases
Recombinant C1inh	Made in Tg rabbits, in phase 3 trials for HAE (Rhucin)	As above
fD inhibitors; BCX1470, others	Models only, no significant human trials	Small molecules, specific inhibitors of fD protease activity
Soluble forms of natural complement regulators		
sCR1 (TP10; TP20)	Phase 2 trials CPB, early trials in stroke, MI, others	CP/AP C3 convertase regulator
APT070 (Microcept)	Preclinical in renal transplantation, development stages in others	SCR 1-3 of CR1 on membrane-targeting tail, convertase regulator
CAB-2 (MLN-2222)	Phase 1 trials in CPB	DAF:MCP hybrid, convertase regulator
Recombinant MBL	Preclinical as substitution	Replacement of deficient

	therapy	protein
Recombinant fH	Preclinical, substitution therapy in AMD	Increasing levels of “protective” fH; convertase regulator
TT30	Preclinical for AP-mediated diseases	CR2-fH hybrid, targeted convertase regulator
Antibodies		
Anti-C5 (Eculizumab)	Available for PNH, aHUS; other indications in development	Humanized mAb, C5 cleavage blocker; inhibits C5a production, MAC
Anti-C5 (Pexelizumab)	Short-acting variant of above; phase 2 for MI, CPB	As above
Anti-C5a (TNX-558)	Preclinical, inflammation	Humanized mAb, binds/blocks C5a
Anti-C5aR (Neutrazumab)	Preclinical, arthritis, stroke	Humanized mAb, blocks C5aR
Anti-fD (TNX-334)	Preclinical, AMD	Humanized mAb, blocks fD enzyme
Anti-fB (TA106)	Preclinical	Humanized mAb, blocks fB binding
Antiproperdin	Preclinical	Humanized mAb, blocks properdin
Complement protein blockers		
Compstatin (POT-4)	Phase 1, AMD	Cyclic peptide, binds C3, blocks cleavage
OmCI	Preclinical, AMD	Tick-derived C5-binder, inhibits C5a production, MAC
ARC1905	Preclinical, AMD	RNA aptamer; binds/blocks

		C5
PMX-53	Phase 2, arthritis, psoriasis	Peptide C5a antagonist
JPE-1375	Preclinical, inflammation	Peptide C5a antagonist

aHUS, atypical hemolytic uremic syndrome; AMD, age-related macular degeneration; AP, alternative pathway; CP, classical pathway; CPB, cardiopulmonary bypass; CR1, complement receptor 1; CR2, complement receptor 2; DAF, decay accelerating factor; fB, factor B; fD, factor D; FDA, U.S. Food and Drug Administration; fH, factor H; HAE, hereditary angioedema; MAC, membrane attack complex; MASP, mannan-binding lectin-associated serine protease; MBL, mannan-binding lectin; MCP, membrane cofactor protein; MI, myocardial infarction; PNH, paroxysmal nocturnal hemoglobinuria; RNA, ribonucleic acid; SCR, short consensus repeat.

Plasma Regulators as Anticomplement Drugs

The plasma regulators of complement activation are also potential therapeutics; indeed, the exception referred to previously is the enormous success achieved in treating HAE, caused by deficiency of C1inh, with plasma-derived C1inh. Frank and coworkers showed 30 years ago that C1inh purified from plasma could terminate acute attacks in patients with HAE.[125] Commercial preparations of pasteurized, purified C1inh were widely available within a few years and have been used since in Europe and some other locations to great effect, saving the lives of many sufferers. Unfortunately, use of C1inh in the United States fell foul of suspicions that it was a vector for transmission of hepatitis and other viruses, removing from use the best means of treating attacks. Events in the last few years have changed the scene. New formulations of plasma-derived C1inh, subjected to ultrafiltration to further reduce risk of virus contamination, and recombinant forms of C1inh are now approved by the U.S. Food and Drug Administration (FDA) and in use both for treatment of acute attacks and prophylaxis in HAE.[126] Both plasma-derived and recombinant fH are also in development as potential therapeutics, driven in large part by the strong genetic link, noted previously, between the fH-Y402H variants and AMD. Treatment of AMD patients with the “protective” Y402 variant, either local (intraocular) or systemic, is suggested but remains untested clinically.

Monoclonal Anticomplement Antibodies as Drugs

A number of blocking monoclonal antibodies, capable of switching off complement activation in vivo, have been tested with good effect in animal models of complementmediated disease. Monoclonal antibodies targeting either the AP (anti-fB, anti-fD) or TP (anti-C5, anti-C6) have proved particularly effective in models. Several of these reagents are being developed as human therapeutics; the first of these to reach the market is a humanized monoclonal anti-C5 antibody, eculizumab, marketed under the trade name Soliris™.[127] This monoclonal antibody binds human C5 with high affinity and prevents its cleavage by the convertase enzyme, thus blocking C5a generation and MAC assembly. Eculizumab was first approved for use in the rare hemolytic disorder PNH in which erythrocytes and other blood cells are rendered susceptible to complement damage because they lack the GPI-anchored regulators

DAF and CD59. Erythrocytes hemolyse spontaneously, releasing hemoglobin into the plasma and, via renal filtration, the urine, leading to the characteristic dark urine. Patients become anemic as erythrocyte loss exceeds rate of production and are often dependent on large, frequent blood transfusions. Thrombotic episodes and strokes can prove fatal. Administration of eculizumab dramatically reverses these symptoms; erythrocyte hemolysis ceases, patients become transfusion independent, and risk of stroke markedly decreases. Maintenance with twice-weekly dosing maintains this spectacular remission for many months or years.[128] In 2011, eculizumab was approved by the FDA for treatment of aHUS, a major advance for a disease that was previously untreatable except by dialysis.[129] Epidemic HUS, caused by infection with Shiga toxin-producing strains of *Escherichia coli*, closely resembles aHUS in terms of pathology but is an acute disease ending either in recovery or renal failure. Emergency use of eculizumab in a very large outbreak of HUS in Germany proved remarkably effective, rescuing many critically ill patients.[130]

Small Molecule Complement Inhibitors as Drugs

A limiting factor in the use of recombinant proteins and monoclonal antibodies as anticomplement agents is cost; eculizumab treatment of PNH currently costs in excess of $500,000 per patient per year, making it the most expensive FDA-approved drug! The quest for an inexpensive small molecule inhibitor of complement, preferably orally active, has been a long one, and there are still no ideal agents available. Most promising among the current candidates is a C3-/C3b-inhibiting peptide, compstatin, which tightly binds its ligand thereby blocking complement activation via all pathways.[131] Compstatin has been shown to be an effective complement inhibitor in vivo in primate models (it does not work in rodents). Compstatin is currently in phase I trials for treatment of AMD where local (intraocular), slowrelease formulations may prove particularly useful. A number of other synthetic and naturally occurring molecules have been shown to be effective inhibitors of complement activation in vitro and in models, but none have yet entered clinical trials. The G-protein-coupled heptaspan receptors for C3a and C5a are excellent drug targets, and a number of small molecule blockers of the C3a and/or C5a receptors have been reported.[132] In animal models, these act to reduce the inflammation associated with complement activation. A cyclic hexapeptide, F[OpdChaWR], blocks the C5a receptor and inhibits inflammation in a broad range of animal models and is in development for human use with sepsis likely to be the first target.[133]

NOVEL ROLES OF COMPLEMENT

In addition to its roles as innate immune effector, complement is increasingly being implicated as an important player in a number of other physiological and pathological processes. The list of novel roles is long and growing, and here I will briefly describe just a few as an illustration of this burgeoning field of interest.

Complement as a Regulator of B-Cell Responses

The first indication that complement influenced adaptive immunity came from the work of Pepys who, in the 1970s, showed that C3 depletion in mice treated with cobra venom factor or other complement activators caused a marked reduction in antibody response to a range of antigens.[134]

Although a number of mechanisms were suggested at the time, it was more than a decade before Fearon and coworkers showed that C3 fragment-coated (opsonized) antigen was a much more efficient activator of B cells than unopsonized antigen because it simultaneously engaged the BCR (surface IgM) and CR2, assembled in a signaling complex on the B-cell surface.[135] The signaling complex comprised surface IgM tightly linked to the phosphatase enzyme CD45, adjacent to CR2, and linked to the Ig superfamily member CD19 and the tetraspanin molecule tetraspanin CD81, which connects the complex to the intracellular signaling moiety PI-3 kinase (Fig. 36.16). Although antigen-binding BCR can alone cause B-cell activation, large amounts of antigen are needed; if the antigen is coated with iC3b/C3d, it binds both CR2 and the BCR, lowering the B-cell antigen response threshold by up to 1000-fold! CR1 on the B cell also plays an important role by first capturing C3b-coated antigen, then acting as cofactor for the fl-mediated cleavage of C3b into iC3b and C3d, the ligands for CR2. Recognition of the importance of CR2 in regulating B-cell responses led to a flurry of interest in using C3d, coupled to pathogen-derived antigens, as super-vaccines; promising results in animal models have yet to translate to humans.

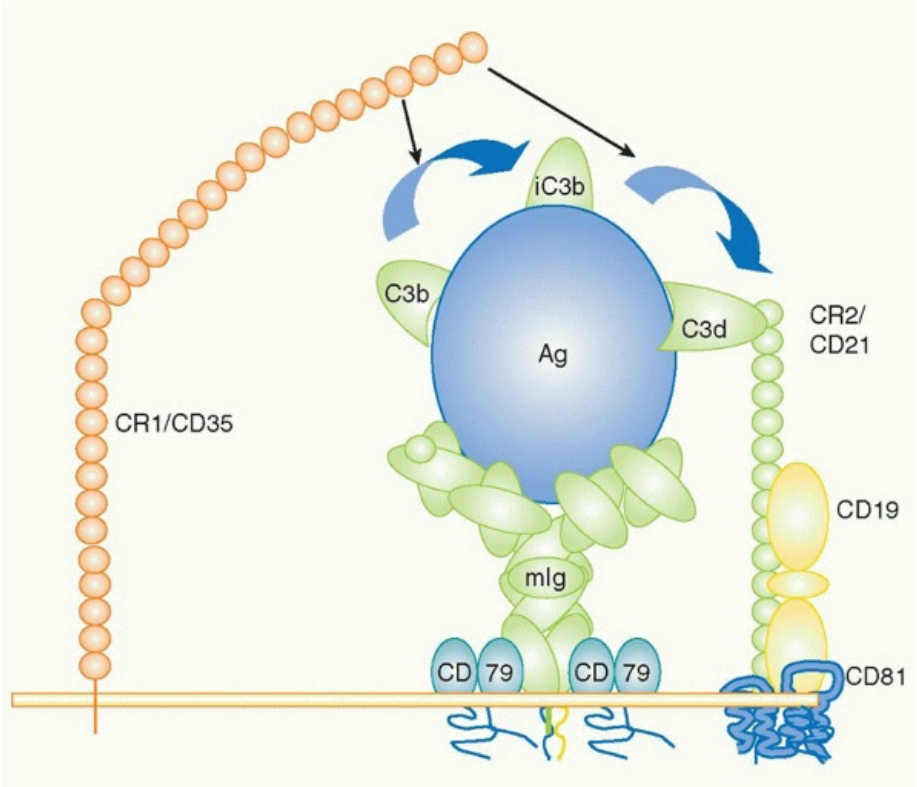

FIG. 36.16. The B-cell Signaling Complex. Antigen coated with C3 fragments (opsonized) binds the B-cell receptor (mIg) to trigger B-cell activation. C3d fragments on the antigen, produced by factor I degradation in the presence of complement receptor 1 as cofactor, bind complement receptor 2, present on the B cell in a signaling complex with CD19 and CD81. Co-ligation of the B-cell receptor and complement receptor 2 signaling complexes markedly lowers the threshold for B-cell response to antigen.

Complement and T-Cell Activation

Most published work suggests that T cells do not express classical complement receptors in significant amounts. Expression of CR2 is controversial, with some reporting expression, particularly on activated T cells, and suggesting that CR2 might play a role in driving T-cell activation. CD8 T cells certainly express receptors for C3a, and several studies have suggested that local activation of C3 and production of C3a, and C5a, might be an important part of T-cell help and a trigger to T-cell activation.[136] The complement regulator MCP (CD46) is expressed on T cells and also plays roles as a costimulator in T-cell activation; coligation of CD3 and CD46 in the presence of interleukin-10 drives T cells to differentiate into T regulatory cells that in turn dampen activation of bystander cells.[137] The natural ligand for MCP in this context is C3b, further implicating C3 products in T-cell control. Indeed, a growing body of evidence indicates that MCP and its ligand C3b are key controllers of T-cell fate and modulate T-cell cytokine production in health and disease.

Interactions with Toll-like Receptors

The toll-like receptor (TLR) family of pattern recognition molecules are an ancient immune defense system that act by sensing and signaling cell responses to infection. TLRs recognize a broad range of bacterial and viral products, present either outside or inside the cell, and set in train cytokine production and other appropriate cell responses to infection. Several of the pathogen TLR agonists, including lipopolysaccharide (from bacterial cell walls) and zymosan (from yeast cell walls) are efficient activators of complement; hence, these agonists trigger a double-hit through the TLR and through complement activation. Complement activation products, specifically C5a and, less strongly, C3a, enhance the cytokine response triggered by engagement of the TLRs, although the precise mechanism remains a subject of debate.[138] Some studies strongly implicate the C5L2 receptor in these events and, indeed, TLR activation of cells markedly increases their subsequent response to C5a in a C5L2-dependent manner.[139] Cross-talk between TLRs and complement via C5a has been implicated in T-cell

activation, and other complement molecules, including the gC1qR, CR3, and MCP, have been shown to independently interact with TLRs. The growing understanding of the interactions between these two innate immune effector systems will likely inform better approaches to therapeutic intervention in the future.

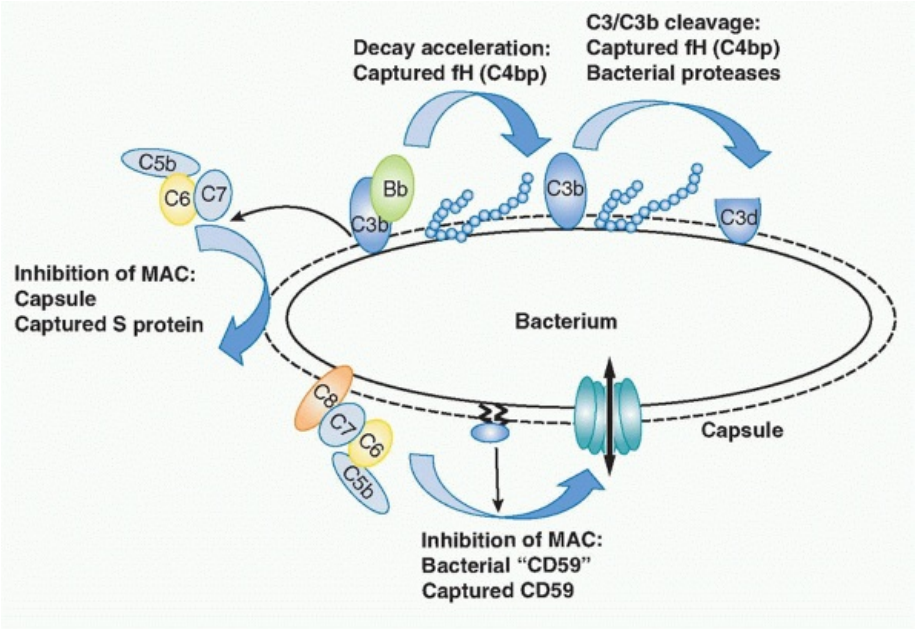

FIG. 36.17. Bacterial Complement Evasion Strategies. Many bacterial species have evolved elaborate defense strategies to protect against complement opsonization and killing; some of the more common are illustrated here. The capsule, where present, provides a physical barrier to bacterial membrane damage but may itself be a target for opsonization. C3 fragments and C3 convertases are targeted by secreted proteases and by C3 convertase regulators; they are either pirated from the host or expressed in the bacterial genome. Membrane attack complex formation is inhibited not only by capsule but also by terminal pathway regulators, either captured (such as S protein) or expressed by bacteria (CD59-like molecules).

Evasion and Hijacking of Complement by Pathogens

Complement exists to rapidly identify and destroy invading bacteria, a vital pillar of innate immune defense. Driven by this selective pressure, bacteria and some other pathogens have evolved numerous ways of evading complement activation and killing (Fig. 36.17).[140] First among these are the physical barriers; for example, gram-positive bacteria possess cell walls that protect from MAC killing. Many pathogens have evolved the capacity to mimic or steal the human complement regulators to subvert complement attack. Recruitment of host plasma complement regulators C4bp and/or fH is a particularly common occurrence and many pathogens, notably *Borellia*, *Neisseria*, and *Streptococcus*, have evolved specific membrane proteins, virulence factors that act to capture these regulators, thereby protecting the pathogen surface.[141] Some pathogens acquire membrane regulators such as CD59 and DAF from host cells, either by physical interaction with the cells, or for some viruses, during their release from infected cells. Several viruses have evolved soluble complement regulators that are structural and functional analogues of the human regulators; for example, vaccinia virus and smallpox virus both encode complement control proteins made up of SCRs that have both decay accelerating and cofactor activities,[142] while *Borellia burgdorferi* expresses a CD59- like MAC inhibitor.[143] Other pathogens have evolved enzymes that efficiently

degrade complement proteins or activation products as a means of avoiding complement damage; still others possess binding proteins that mop up complement proteins onto the pathogen surface. Among pathogens, *Staphylococcus aureus* stands out as an escape artist extraordinaire, expressing factors that inhibit complement activation, digest complement proteins, block complement receptors, and mimic complement regulators at multiple stages.[144]

Some viruses go one step further: not content with avoiding complement killing, they turn complement to their advantage. Several viruses use complement regulators and receptors as entry routes into the cell; the measles virus binds MCP, the Epstein-Barr virus binds CR2, and the Coxsackie virus binds DAF. MCP is used as a receptor by many different pathogens, leading to its colorful description as a "pathogen magnet."[145] Human immunodeficiency virus takes yet further liberties, inviting complement activation in order to acquire surface C3 fragments that are then used as a passport for cell entry by binding CR3 on host cells.[146]

Complement and Lipid Metabolism

In the late 1980s and early 1990s, two separate lines of research converged to implicate complement as a major regulator of lipid metabolism in adipose tissue. Speigelman's group showed that an adipose tissue-derived protein they termed adipsin, implicated in regulation of fat mass, was identical to the complement AP enzyme fD.[147] They also showed that adipocytes synthesized not only fD but also the AP components fB and C3, everything needed to generate AP activity locally. Meanwhile, Cianflone was investigating a remarkable plasma protein termed acylation-stimulating protein, which was a powerful stimulus for triacylglycerol synthesis in adipose tissue; molecular characterization showed that acylation-stimulating protein was identical to the complement activation product

C3adesArg, generated when the C3a anaphylatoxin is "inactivated" by carboxypeptidase-N.[148] It is now clear that local production of AP proteins and AP cycling in adipose tissue is a major regulator of lipid turnover through production of C3adesArg, which binds specific receptors (likely C5L2) on adipocytes to mediate its effects (Fig. 36.18).[149] The link between AP activation and adipose tissue activities might explain the association, between NeF-driven AP dysregulation and the loss of adipose mass in the disease partial lipodystrophy. The broader implications of the effects of complement products on lipid handling, adipose tissue mass, and atherosclerosis are now coming under the spotlight.[150]

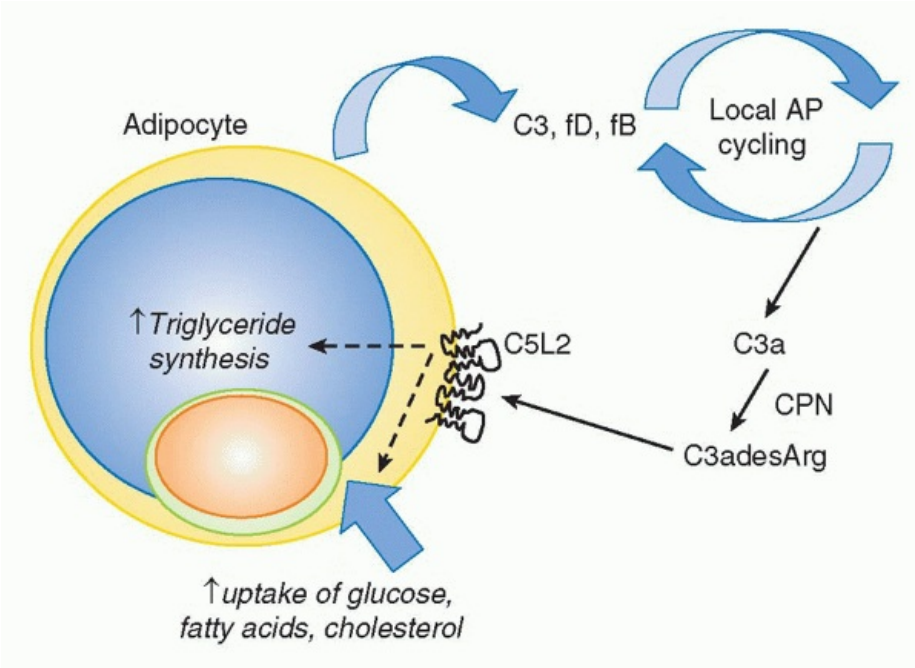

FIG. 36.18. Complement and Adipocyte Activation. Adipocytes synthesize and secrete the alternative pathway (AP) proteins factor D, C3, and factor B into the local environment. Local AP activation, initiated through tick-over or other triggers, generates C3a, which is rapidly processed to C3adesArg by carboxypeptidase N. C3adesArg binds C5L2 on adipocytes, initiating a signaling cascade that results in increased uptake of glucose, fatty acids, and cholesterol; increased triglyceride synthesis; and increased lipid accumulation in adipose tissue.

CONCLUSION

Complement, an evolutionarily ancient system discovered more than 120 years ago, continues to surprise with increasing realization of its importance in diverse diseases, growing capacity to regulate its activation in vivo, and expanding list of roles outside of innate immunity. The decades to come will likely bring further revelations. I hope that this brief chapter provides some of you with the background and enthusiasm to contribute to the quest.

REFERENCES

1. Mayer MM. Complement: historical perspectives and some current issues. *Complement.* 1984;1:2-26.

2. Bordet J. Sur le mode d'action des serums preventives. *Ann Inst Pasteur Paris.* 1896;10;193-219.

3. Ehrlich P, Morgenroth J. Zur theorie der lysinwirkung. *Berl Klin Wochenchr.* 1899;36:6-9.

4. Bordet J, Gengou O. Sur l'existence de substances sensibilisatirices dans le plupart des serums antimicrobiens. *Ann Inst Pasteur Paris.* 1901;15;289-302.

5. Muir R, Martin WR. On the deviation of complement by a serum and its anti-serum and its relations to the precipitin test. *J Hyg.* 1906;6:265-285.

6. Muller-Eberhard HJ. Chemistry and reaction mechanisms of complement. *Adv Immunol.* 1968;8:1-80.

7. Pillemer L, Ecker EE, Oncley JL, et al. The preparation and physicochemical characterization of the serum protein components of complement. *J Exp Med.* 1941;74:297-308.

8. Austen KF, Becker EL, Biro CE, et al. Nomenclature of complement. *Bull Worl Health Organ.* 1968;39:935-938.

9. Pillemer L, Blum L, Lepow IH, et al. The properdin system and immunity. I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena. *Science.* 1954;120:279-285.

10. Lepow IH. Louis Pillemer. Properdin and scientific controversy. *J Immunol.* 1980;125:471-475.

11. Gotze O, Muller-Eberhard HJ. The C3-activator system: an alternate pathway of complement activation. *J Exp Med.* 1971;134:90-108.

12. Marcus RL, Shin HS, Mayer MM. An alternate complement pathway: C3-cleaving activity, not due to C4,2a, on endotoxic lipopolysaccharide after treatment with guinea pig serum; relation to properdin. *Proc Natl Acad Sci U S A* . 1971;68:1351-1354.

13. Muller-Eberhard HJ, Nilsson U. Relation of a beta(1)-glycoprotein of human serum to the complement system. *J Exp Med.* 1960;111:217-234.

14. Naff GB, Pensky J, Lepow IH. The macromolecular nature of the first component of human complement. *J Exp Med.* 1964;119:593-613.

15. Donaldson VH, Evans RR. A biochemical abnormality in hereditary angioneurotic edema: absence of serum inhibitor of C1′-esterase. *Amer J Med* . 1963;35:37-44.

16. Muller-Eberhard HJ, Polley MJ, Calcott MA. Formation and functional significance of a molecular complex derived from the second and the fourth component of human complement. *J Exp Med.* 1967;125:359-380.

17. Tack BF, Harrison RA, Janatova J, et al. Evidence for presence of an internal thiolester bind in third component of complement. *Proc Natl Acad Sci U S A.*

1980;77:5764-5768.

18. Harrison RA, Thomas ML, Tack BF. Sequence determination of the thiolester site of the fourth component of human complement. *Proc Natl Acad Sci U S A.* 1981;78:7388-7392.

19. Gabouriad C, Thielens NM, Gregory LA, et al. Structure and activation of the C1 complex: unravelling the puzzle. *Trends Immunol.* 2004;25:368-373.

20. Nagasawa S, Stroud RM. Cleavage of C2 by C1s into the antigenically distinct fragments C2a and C2b: demonstration of binding of C2b to C4b. *Proc Natl Acad Sci U S A.* 1977;74:2998-3001.

21. Pangburn MK, Rawal N. Structure and function of complement C5 convertase enzymes. *Biochem Soc Trans.* 2001;30:1006-1010.

22. Kerr MA. The human complement system: assembly of the classical pathway C3 convertase. *Biochem J.* 1980;189:173-181.

23. Law SKA, Doss AW. The internal thioester and the covalent binding properties of the complement proteins C3 and C4. *Protein Sci.* 1997;6:263-274.

24. Lachmann PJ, Hughes-Jones NC. Inititation of complement activation. *Springer Semin Immunopathol.* 1984;7:143-162.

25. Harboe M, Mollnes TE. The alternative complement pathway revisited. *J Cell Mol Med.* 2008;12:1074-1084.

26. Hourcade DE. Properdin and complement activation: a fresh perspective. *Curr Drug Targets.* 2008;9:158-164.

27. Ikeda K, Sannoh T, Kawasaki N, et al. Serum lectin with known structure activates complement through the classical pathway. *J Biol Chem.* 1987;62:7451-7454.

28. Lu J, Thiel S, Wiedemann H, et al. Binding of the pentamer/hexamer forms of mannan-binding protein to zymosan activates the proenzyme $C1r_2C1s_2$ complex of the classical pathway of complement without involvement of C1q. *J Immunol.* 1990;144:2287-2294.

29. Wallis R, Mitchell DA, Schmid R, et al. Paths reunited: initiation of the classical and lectin pathways of complement activation. *Immunobiol.* 2010;215:1-11.

30. Endo Y, Matsushita M, Fujita T. The role of ficolins in the lectin pathway of innate immunity. *Int J Biochem Cell Biol.* 2011;43:705-712.

31. Gabouriad C, Teillet F, Gregory LA, et al. Assembly of C1 and the MBL- and ficolin-MASP complexes: structural insights. *Immunobiology.* 2007; 212:279-288.

32. Takahashi M, Ishida Y, Iwaki D, et al. Essential role of mannose-binding lectin-associated serine protease-1 in activation of the complement factor D. *J Exp Med.* 2010;207:29-37.

33. Muller-Eberhard HJ. The membrane attack complex of complement. *Ann Rev Immunol.* 1986;4:503-528.

34. Davis AE III, Lu F, Mejia P. C1 inhibitor, a multi-functional serine protease inhibitor. *Thromb Haemost.* 2010;104:886-893.

35. Blom AM. Structural and functional studies of the complement inhibitor C4b-binding protein. *Biochem Soc Trans.* 2002;30:978-982.

36. Dahlback B. C4-binding protein: a forgotten factor in thrombosis and hemostasis. *Semin Thromb Haemost.* 2011;37:355-361.

37. Nicholson-Weller A, Wang CE. Structure and function of decay accelerating factor. *J Lab Clin Med.* 1994;123:485-491.

38. Liszewski MK, Post TW, Atkinson JP. Membrane cofactor protein (MCP or CD46): newest member of the regulators of complement activation gene cluster. *Annu Rev Immunol.* 1991;9:431-55.

39. Ahearn JM, Fearon DT. Structure and function of the complement receptors CR1 (CD35) and CR2 (CD21). *Adv Immunol.* 1989;46:183-219.

40. Pascual M, Schifferli JA. The binding of immune complexes by the erythrocyte complement receptor 1 (CR1). *Immunopharmacol.* 1992;24:101-106.

41. Zipfel PF. Complement factor H: physiology and pathophysiology. *Semin Thromb Haemost.* 2001;27;191-199.

42. Skidgel RA, Erdos EG. Structure and function of human plasma carboxypeptidase N, the anaphylatoxin inactivator. *Int Immunopharmacol.* 2007;7:1888-1899.

43. Tschopp J, French LE. Clusterin: modulation of complement function. *Clin Exp Immunol.* 1994;97(suppl 2):11-14.

44. Sheehan M, Morris CA, Pussell BA, et al. Complement inhibition by human vitronectin involves non-heparin binding domains. *Clin Exp Immunol.* 1995;101:136-141.

45. Sugita Y, Masuho Y. CD59: its role in complement regulation and potential for therapeutic use. *Immunotechnol.* 1995;1:157-168.

46. Morgan BP. Complement membrane attack on nucleated cells: resistance, recovery and non-lethal effects. *Biochem J.* 1989;264:1-14.

47. van Lookeren Campagen M, Wiesmann C, Brown EJ. Macrophage complement receptors and pathogen clearance. *Cell Microbiol.* 2007;9:2095-2102.

48. Ross GD, Vetvicka V. CR3 (CD11b,CD18): a phagocyte and NK cell membrane receptor with multiple ligand specificities and functions. *Clin Exp Immunol.* 1993;92:181-184.

49. Helmy KY, Katschke KJ Jr, Gorgani NN, et al. CRIg: a macrophage complement receptor required for phagocytosis of circulating pathogens. *Cell.* 2006;124:915-927.

50. Klos A, Tenner AJ, Johswich KO, et al. The role of the anaphylatoxins in health and disease. *Mol Immunol.* 2009;46:2753-2766.

51. Monk PN, Scola AM, Madala P, et al. Function, structure and therapeutic potential of complement C5a receptors. *Br J Pharmacol.* 2007;152:429-448.

52. Johswich K. Klos A. C5L2—an anti-inflammatory molecule or a receptor for acylation stimulating protein (C3a-desArg)? *Adv Exp Med Biol.* 2007;598:159-180.

53. Greenlee MC, Sullivan SA, Bohlson SS. CD93 and related family members: their role in innate immunity. *Curr Drug Targets.* 2008;9:130-138.

54. Zutter MM, Edelson BT. The alpha2Bbeta1 integrin: a novel collectin/C1q receptor. *Immunobiology.* 2007;212:343-353.

55. Ghebrehiwet V, Peerschke EI. cC1q-R (calreticulin) and gC1q-R/p33: ubiquitously expressed multi-ligand binding cellular proteins involved in inflammation and infection. *Mol Immunol.* 2004;41:173-183.

56. Dodds AW, Law SK. The phylogeny and evolution of the thioester bond-containing proteins C3, C4 and alpha-2 macroglobulin. *Immunol Rev.* 1998;166:15-26.

57. Dodds AW, Matsushita M. The phylogeny of the complement system and the origins of the classical pathway. *Immunobiology.* 2007;212:233-243.

58. Nonaka M, Kimura A. Genomic view of the evolution of the complement system. *Immunogenetics.* 2006;58:701-713.

59. Martinez OP, Longman-Jacobsen N, Davies R, et al. Genetics of human complement

component C4 and evolution of the central MHC. *Front Biosci.* 2001;6:D904-D913.

60. Samonte IE, Sato A, Mayer WE, et al. Linkage relationships of genes encoding for alpha-2 macroglobulin, C3 and C4 in the zebrafish: implications for evolution of the complement and MHC systems. *Scand J Immunol.* 2002;56:344-352.

61. Hobart MJ, Fernie BA, DiScipio RG. Structure of the human C7 gene and comparison with the C6, C8A, C8B and C9 genes. *J Immunol.* 1995; 154:5188-5194.

62. Kondos SC, Hatfaludi T, Voskoboinik I, et al. The structure and function of mammalian membrane-attack complex/perforin-like proteins. *Tissue Antigens*. 2010;76:341-345.

63. Holmksov UL, Thiel S, Jensenius JC. Collectins and ficolins: humoral lectins of the innate immune defense. *Annu Rev Immunol.* 2003;21:547-578.

64. Fujita T. Evolution of the lectin-complement pathway and its role in innate immunity. *Nat Rev Immunol.* 2002;2:346-353.

65. Dobo J, Harmat V, Beinrhor L, et al. MASP-1, a promiscuous complement proteas: structure of its catalytic region reveals the basis of its broad specificity. *J Immunol.* 2009;183:1207-1214.

66. Endo Y, Takahashi M, Fujita T. Lectin complement system and pattern recognition. *Immunobiology*. 2006;211:283-293.

67. Houcade D, Holers VM, Atkinson JP. The regulators of complement activation (RCA) gene cluster. *Adv Immunol.* 1989;45:381-416.

68. Zipfel PF, Jokiranta TS, Hellwage J, et al. The factor H protein family. *Immunopharmacol.* 1999;42:53-60.

69. Singhrao SK, Neal JW, Rushmere NK, et al. Spontaneous classical pathway activation and deficiency of membrane regulators render human neurons susceptible to complement lysis. *Am J Pathol.* 2000;157:905-918.

70. Morgan BP, Gasque P. Extrahepatic complement biosynthesis: where, when and why? *Clin Exp Immunol*. 1997;107:1-7.

71. Tang S, Zhou W, Sheerin NS, et al. Contribution of renal secreted complement C3 to the circulating pool in humans. *J Immunol.* 1999;162: 4336-4341.

72. Truedsson L, Bengtsson AA, Sturfelt G. Complement deficiencies and systemis lupus erythematosus. *Autoimmunity*. 2007;40:560-566.

73. Yang Y, Chung EK, Wu Y, et al. The intricate role of complement component C4 in human systemic lupus erythematosus. *Curr Dir Autoimmun.* 2004;7:98-132.

74. Manderson AP, Botto M, Walport MJ. The role of complement in the development of systemic lupus erythematosus. *Annu Rev Immunol.* 2004;22:431-456.

75. Tsutsumi A, Takahashi R, Sumida T. Mannose binding lectin: genetics and autoimmune disease. *Autoimmun Rev.* 2005;4:364-372.

76. Bouwman LH, Roep BO, Roos A. Mannose-binding lectin: clinical implications for infection, transplantation and autoimmunity. *Hum Immunol.* 2006;67:247-256.

77. Thiel S, Steffensen R, Christensen IJ, et al. Deficiency of mannan-binding lectin associated serine protease-2 due to missense polymorphisms. *Genes Immun.* 2007;8:154-163.

78. Reis ES, Falcao DA, Isaac L. Clinical aspects and molecular basis of primary deficiencies of complement component C3 and its regulatory proteins factor I and factor H. *Scand J Immunol.* 2006;63:155-168.

79. Dehorne J, Haerynck F, Raes A, et al. Complement factor B deficiency associated with recurrent aseptic meningitis. *Paed Rheumatol.* 2008;6 (suppl 1):266.

80. Sprong T, Roos D, Weemaes C, et al. Deficient alternative pathway activation due to factor D deficiency by 2 novel mutations in the complement factor D gene in a family with meningococcal infections. *Blood*. 2006;107:4865-4870.

81. Fijen CA, van den Bogaard R, Schipper M, et al. Properdin deficiency: molecular basis and disease association. *Mol Immunol.* 1999;36:863-867.

82. Delgado-Cervino E, Fontan G, Lopez-Trascasa M. C5 complement deficiency in a Spanish family: molecular characterization of the double mutation responsible for the defect. *Mol Immunol.* 2005;42:105-111.

83. Zhu Z, Atkinson TP, Hovanky KT, et al. High prevalence of complement component C6 deficiency among African-Americans in the south-eastern USA. *Clin Exp Immunol.* 2000;119:305-310.

84. Wurzner R. Deficiencies of the complement MAC II gene cluster (C6, C7, C9): is subtotal C6 deficiency of particular evolutionary benefit? *Clin Exp Immunol.* 2003;133:156-159.

85. Halle D, Elstein D, Geudalia D, et al. High prevalence of complement C7 deficiency among healthy blood donors of Morrocan Jewish ancestry. *Am J Med Genet.* 2001;99:325-327.

86. Morgan BP, Vora JP, Bennett AJ, et al. A case of hereditary combined deficiency of complement components C6 and C7 in man. *Clin Exp Immunol.* 1989;75:396-401.

87. Rosa DD, Pasqualotto AC, de Quadros M, Prezzi SH. Deficiency of the eighth component of complement associated with recurrent meningococcal meningitis—case report and literature review. *Braz J Infect Dis.* 2004;8:328-330.

88. Khajoee V, Ihara K, Kira R, et al. Founder effect of the R95X mutation in Orientals. *Hum Genet*. 2003;112:244-248.

89. Davis AE III. The pathophysiology of hereditary angioedema. *Clin Immunol.* 2005;114:3-9.

90. Gosswein T, Kocot A, Emmert G, et al. Mutational spectrum of the C1INH (SERPING1) gene in patients with hereditary angioedema. *Cytogenet Genome Res.* 2008;121:181-188.

91. Nilsson SC, Sim RB, Lea SM, et al. Complement factor I in health and disease. *Mol Immunol.* 2011;48:1611-1620.

92. Pickering MC, Cook HT. Translational mini-review series on complement factor H: renal diseases associated with complement factor H; novel insights from humans and animals. *Clin Exp Immunol*. 2008;151:210-230.

93. Kavanagh D, Goodship TH. Atypical haemolytic uremic syndrome. *Curr Opin Haematol.* 2010;17:432-438.

94. Zipfel PF, Mache C, Muller D, et al. DEAP-HUS: deficiency of CFHR plasma proteins and autoantibody-positive form of haemolytic uremic syndrome. *Paediatr Nephrol.* 2010;25:2009-2019.

95. Yamashina M, Ueda E, Kinoshita T, et al. Inherited complete deficiency of 20-kilodalton homologous restriction factor (CD59) as a cause of paroxysmal nocturnal hemoglobinuria. *N Engl J Med.* 1990;323:1184-1189.

96. Telen MJ, Green AM. The Inab phenotype: characterization of the membrane protein and complement regulatory defect. *Blood.* 1989; 74:437-441.

97. Savage WJ, Brodsky RA. New insights into paroxysmal nocturnal hemoglobinuria. *Hematology.* 2007;12:371-376.

98. Perez-Caballero D, Gonzalev-Rubio C, Gallardo ME, et al. Clustering of missense mutations in the C-terminal region of factor H in atypical haemolytic uremic syndrome. *Am J Hum Genet.* 2001;68:478-484.

99. Gale DP, de Jorge EG, Cook HT, et al. Identification of a mutation in complement factor H-related protein 5 in patients of Cypriot origin with glomerulonephritis. *Lancet*. 2010;376:794-781.

100. Thaakkinstian A, Han P, McEvoy M, et al. Systematic review and meta-analysis of the association between complement factor H Y402H polymorphisms and age-related macular degeneration. *Hum Mol Genet.* 2006;15:2784-2790.

101. Tortajada A, Montes T, Martinez-Barricarte R, et al. The disease-protective complement factor H allotypic variant Ile62 shows increased binding affinity for C3b and enhanced cofactor activity. *Hum Mol Genet.* 2009;18:3452-3461.

102. Goicoechea de Jorge E, Harris CL, Esparza-Gordillo J, et al. Gain-offunction mutations in factor B are associated with atypical haemolytic uremic syndrome. *Proc Natl Acad Sci U S A.* 2007;104:240-245.

103. Heurich M, Martinez-Barricarte R, Francis NJ, et al. Common polymorphisms in C3, factor B and factor H collaborate to determine systemic complement activity and disease risk. *Proc Natl Acad Sci U S A.* 2011;108: 8761-8766.

104. Smith RJ, Harris CL, Pickering MC. Dense deposit disease. *Mol Immunol.* 2011;48:1604-1610.

105. Mathieson PW, Peters DK. Lipodystrophy in MPGN type II: the clue to links between the adipocyte and the complement system. *Nephrol Dial Transplant.* 1997;12:1804-1806.

106. Finn JE, Mathieson PW. Molecular analysis of C3 allotypes in patients with nephritic factor. *Clin Exp Immunol.* 1993;91:410-414.

107. Cicardi M, Zingale LC, Pappalardo E, et al. Autoantibodies and lymphoproliferative diseases in acquired C1-inhibitor deficiencies. *Medicine (Baltimore).* 2003;82:274-281.

108. Diepenhorst GM, van Gulik TM, Hack CE. Complement-mediated ischemia-reperfusion injury: lessons learned from animal and clinical studies. *Ann Surg.* 2009;249:889-899.

109. Markiewski MM, DeAngelis RA, Lambris JD. Complexity of complement activation in sepsis. *J Cell Mol Med.* 2008;12:2245-2254.

110. Ward PA. The harmful role of C5a on innate immunity in sepsis. *J Innate Immun.* 2010;2:439-445.

111. Cowan PJ, d'Apice AJ. Complement activation and coagulation in xenotransplantation. *Immunol Cell Biol.* 2009;87:203-208.

112. Ekser B, Ezzelarabad M, Hara H, et al. Clinical xenotransplantation: the next medical revolution? *Lancet.* 2012;379(9816):672-683.

113. Levine MH, Abt PL. Treatment options and strategies for antibodymediated rejection after renal transplantation. *Semin Immunol.* 2012; 24:136-142.

114. Gomez AM, van den Broeck J, Vrolox K, et al. Antibody effector mechanisms in myasthenia gravis-pathogenesis at the neuromuscular junction. *Autoimmunity*. 2010:43:353-370.

115. Hepburn NJ, Chamberlain-Banoub J, Williams AS, et al. Prevention of experimental autoimmune myasthenia gravis by rat Crry-Ig: a model agent for long-term complement inhibition in vivo. *Mol Immunol.* 2008; 45:395-405.

116. Piddlesden SJ, Storch MK, Hibbs M, et al. Soluble recombinant complement receptor 1 inhibits inflammation and demyelination in antibody-mediated demyelinating experimental allergic encephalomyelitis. *J Immunol.* 1994;152:5477-5484.

117. Lucchinetti C, Bruck W, Parisi J, et al. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol.* 2000;47:707-717.

118. Girardi G, Prohaszka Z, Bulla R, et al. Complement activation in human and animal pregnancies as a model for immunological recognition. *Mol Immunol.* 2011;48:1621-1630.

119. Brown KM, Sacks SH, Sheerin NS. Mechanisms of disease: the complement system in renal injury - new ways of looking at an old foe. *Nat Clin Pract Nephrol.* 2007;3:277-286.

120. Fearon DT. Anti-inflammatory and immunosuppressive effects of recombinant soluble complement receptors. *Clin Exp Immunol.* 1991; 86(suppl 1):43-46.

121. Goodfellow RM, Williams AS, Levin JL, et al. Soluble complement receptor 1 (sCR1) inhibits the development and progression of rat collagen-induced arthritis. *Clin Exp Immunol.* 2000;119:210-216.

122. Keshavjee S, Davis RD, Zamora MR, et al. A randomized placebo-controlled trial of complement inhibition in ischemia-reperfusion injury after lung transplantation in human beings. *J Thorac Cardiovasc Surg.* 2005;129:423-428.

123. Lazar HL, Keilani T, Fitzgerald CA, et al. Beneficial effects of complement inhibition with soluble complement receptor 1 (TP10) during cardiac surgery: is there a gender difference? *Circulation*. 2007;116(suppl 11):I83-I88.

124. Fridkis-Hareli, M, Storek M, Mazsaroff I, et al. Design and development of TT30, a novel C3d-targetted C3/C5 convertase inhibitor for treatment of human complement alternative pathway-mediated diseases. *Blood.* 2011;118:4705-4713.

125. Gadek JE, Hosea SW, Gelfand JA, et al. Replacement therapy in hereditary angioedema: successful treatment of acute episodes with partly purified C1 inhibitor. *N Engl J Med.* 1980;302:542-546.

126. Firszt R, Frank MM. An overview of novel therapies for acute hereditary angioedema. *Am J Clin Dermatol.* 2010;11:383-388.

127. Schrezenmeier H, Hochsmann B. Eculizumab opens a new era for treatment of paroxysmal nocturnal hemoglobinuria. *Expert Rev Hematol.* 2009;2:7-16.

128. Kelly RJ, Hill A, Arnold LM, et al. Long-term treatment with eculizumab in paroxysmal nocturnal hemoglobinuria: sustained efficacy and improved survival. *Blood.* 2011;117:6786-6792.

129. Waters AM, Licht C. aHUS caused by complement dysregulation: new therapies on the horizon. *Paediatr Nephrol.* 2011;26:41-57.

130. Lapeyraque AL, Malina M, Fremeaux-Bacchi V, et al. Eculizumab in severe shiga-toxin-associated HUS. *N Engl J Med.* 2011;364:2561-2563.

131. Ricklin D, Lambris JD. Compstatin: a complement inhibitor on its way to clinical application. *Adv Exp Med Biol.* 2008;632:273-292.

132. Kohl J. Drug evaluation: the C5a receptor antagonist PMX-53. *Curr Opin Mol Ther.* 2006;8:529-538.

133. Proctor LM, Woodruff TM, Sharma P. Transdermal pharmacology of small molecule cyclic C5a antagonists. *Adv Exp Med Biol.* 2006;586:329-345.

134. Pepys MB. Role of complement in induction of antibody production in vivo: effect of cobra factor and other C3-reactive agents on thymusdependent and thymus-independent antibody responses. *J Exp Med.* 1974;140:126-145.

135. Fearon DT. The CD19-CR2-TAPA-1 complex. CD45 and signalling by the antigen receptor of B lymphocytes. *Curr Opin Immunol.* 1993:5;341-348.

136. Vievra M, Leisman S, Raedler H, et al. Complement regulates T cell help to CD8 T cells required for murine allograft rejection. *Am J Pathol.* 2011;179:766-774.

137. Cope A, LeFriec G, Cardone J, et al. The TH1 life cycle: molecular control of IFN-γ to IL-10 switching. *Trends Immunol.* 2011;32:278-286.

138. Hajishengallis G, Lambris JD. Crosstalk pathways between Toll-like receptors and the complement system. *Trends Immunol.* 2011;31: 154-163.

139. Raby AC, Holst B, Davies J, et al. TLR activation enhances C5a-induced pro-inflammatory responses by negatively modulating the second C5a receptor, C5L2. *Eur J Immunol.* 2011;41:2741-2752.

140. Lambris JD, Ricklin D, Geisbrecht BV. Complement evasion by human pathogens. *Nat Rev Microbiol.* 2008;6:132-142.

141. Blom A, Hallstrom T, Riesbeck K. Complement evasion strategies of pathogens - acquisition of inhibitors and beyond. *Mol Immunol.* 2009;46:2808-2817.

142. Dunlop LR, Oehlberg KA, Reid JJ, et al. Variola virus immune evasion proteins. *Microbes Infect.* 2003;5:1049-1056.

143. Pausa M, Pellis V, Cinco M, et al. Serum resistant strains of Borellia burgdorferi evade complement-mediated killing by expressing a CD59- like complement inhibitory molecule. *J Immunol.* 2003;170:3214-3222.

144. Foster TJ. Immune evasion by staphylococci. *Nat Rev Microbiol.* 2005;3: 948-958.

145. Liszewski MK, Kemper C, Price JD, et al. Emerging roles and new functions of CD46. *Springer Semin Immunopathol.* 2005;27:345-358.

146. Huber G, Banki Z, Lengauer S, et al. Emerging roles for complement in HIV infection. *Curr Opin HIV AIDS.* 2011;6:419-426.

147. Rosen BS, Cook KS, Yaglom J, et al. Adipsin and complement factor D activity: an immune-related defect in obesity. *Science.* 1989;244: 1483-1487.

148. Baldo A, Sniderman AD, St. Luce S, et al. The adipsin-acylation stimulating protein system and regulation of intracellular triglyceride synthesis. *J Clin Invest.* 1993;92:1543-1547.

149. Cui W, Lapointe M. Gavreau D, et al. Recombinant C3adesArg/acylation stimulating protein (ASP) is highly bioactive: a critical evaluation of C5L2 binding and 3T3-L1 adipocyte activation. *Mol Immunol.* 2009; 46:3207-3217.

150. Lewis RD, Perry MJ, Guschina IA, et al. CD55 deficiency protects against atherosclerosis in ApoE-deficient mice via C3a modulation of lipid metabolism. *Am J Pathol.* 2011;179:1601-1607.

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Chapter 37

Cell-Mediated Cytotoxicity

Judy Lieberman

INTRODUCTION

The most effective way the immune system can control the threats of intracellular infection and cellular transformation is by destroying infected and cancerous cells.[1,2,3,4,5,6,7] When killer lymphocytes recognize harmful cells, they can target them for elimination by triggering programmed cell death. The main killer cells are natural killer (NK) cells of the innate immune sytem and cluster of differentiation (CD)8+ T lymphocytes of adaptive immunity, although some CD4+ T lymphocytes, particularly T_H1 and regulatory T (T_{reg}) cells, also express and deploy the specialized cell death machinery. All killer lymphocytes contain specialized secretory lysosomes, called cytotoxic granules, that are filled with deathinducing enzymes, called granzymes ("granule enzyme"). When the killer cell is activated, the cytotoxic granules move to the immune synapse formed with the target and fuse their membranes with the killer cell membrane, dumping their contents into the immune synapse in a process termed *granule exocytosis*. Perforin, a pore-forming protein in the granules, delivers the death-inducing granyzmes into the cytoplasm of the target cell to initiate its death. In this encounter, the killer cell remains unharmed.[8] It is a serial killer that can detach from one target to seek and destroy others.[9] Killer cells can also activate programmed cell death by using cell surface receptors to ligate cellular death receptors, such as Fas, on target cells. Granule-mediated cell death is key to control viral and intracellular bacterial infection and cancer because perforin-deficient mice and humans homozygous for perforin mutations or deficient in molecules needed for granule exocytosis are highly vulnerable to infection with intracellular pathogens and prone to develop spontaneous lymphomas.[10] The death receptor pathway regulates lymphocyte homeostasis. Patients genetically deficient in the death receptor Fas or its ligand FasL develop autoimmunity.[11] Target cells destroyed by cytotoxic granules or death receptor ligation die a highly regulated death (programmed cell death or apoptosis) rather than by necrosis. Programmed cell death minimizes inflammation and damage to nearby tissue as target cells undergoing programmed cell death are rapidly recognized and cleared by immune phagocytes, especially macrophages.[12] The topic of this chapter was reviewed in more depth in a recent issue of *Immunological Reviews*.[13]

In this chapter we first describe the killer cells: Which immune cells are able to kill and how they develop this capacity and are regulated. Because of its destructive potential, cytotoxicity needs to be carefully regulated. We next focus on the death machinery used for granule- and death receptor-mediated cytotoxicity and how it is mobilized and used to destroy the target

cell. We also discuss what is known about how killer cells are protected against their own weapons of destruction. Some granzymes are expressed without perforin in nonkiller cells. We also discuss the increasing evidence for noncytotoxic proinflammatory roles of killer molecules.

THE KILLER CELLS

The major killer cells are NK cells in innate immunity and CD8 T cells in adaptive immunity. Naïve T cells that have not previously seen antigen do not express either granule effector molecules or death receptors and are incapable of cell-mediated cytotoxicity.[14] Within about 5 days of activation, naïve CD8 T cells differentiate into effector cytotoxic T lymphocytes (CTLs) that express both types of cytotoxic molecules. At the same time, these cells downregulate adhesive and chemokine receptor molecules that retain them in lymph nodes and acquire receptors that allow them to traffic to tissue sites of infection and tumor invasion. Activation to cytotoxic effector cells is tightly regulated. It requires not only antigen-receptor activation but also costimulation, and is greatly enhanced when antigen-presenting cells are stimulated by danger and pathogen-associated pattern motif receptors or when naïve T cells are stimulated by exogenous inflammatory and antiviral cytokines, including type I interferons (IFNs), interleukin (IL)-1, and IFN γ. Upon activation, effector CD8 T cells also begin to express the Fcγ receptor CD16, also present on NK cells, which enables them to recognize and lyse target cells that have been coated with IgG antibodies in a process called antibody-dependent cell-mediated cytotoxicity.[15] In situations of persistent and extensive antigen, however, such as occur in tumors and chronic viral infection, many of the CD8 T cells that have the surface protein expression of CD8 effector cells no longer express perforin and are not cytotoxic.[16,17,18] Effector CD8 T cells that lack cytotoxicity have been termed "exhausted." Most effector cells in an immediate immune response die within a few weeks, but some survive and develop into memory cells. Memory cells downregulate expression of cytotoxic effector proteins, but the kinetics of downregulation varies with the molecule and with the particularities of the immunostimulatory environment.[14,19] In particular, activation of CD8 T cells without CD4 T-cell help leads to an unimpaired primary cytotoxic response but greatly impairs the development of antigen-specific memory cells.[20]

The immunosuppressive drug rapamycin directs antigenstimulated CD8 T cells to differentiate preferentially into memory cells rather than to effector CTLs.[21] Memory CD8 T cells rapidly reacquire cytotoxic capability within hours of restimulation. The molecular basis for this rapid response is not well understood, although recent studies suggest that in memory CD8 T cells, the chromatin of cytolytic effector gene promoters and of eomesodermin, the master transcription factor that regulates CD8 effector genes, bears epigenetic marks that poise them for transcription compared to naïve T cells.[22,23,24,25,26] These cells may also store messenger ribonucleic acids (mRNAs) for perforin and granzyme that can be rapidly translated upon activation. Some types of activated CD4 T cells, especially T_H1, NKT, and T_{reg} cells, also express granzymes and perforin and have cytotoxic activity. Murine T_{reg}s express granzyme B but probably not granzyme A.[27,28] Although immunosuppression by T_{reg}s is mediated by soluble factors, there is also a poorly understoood component that requires cell-to-cell contact. Direct lysis of cognate T cells and

potentially other immune cells by granule-mediated and death receptor pathways by T_{reg}s is likely an important mechanism for suppressing immune activation.[29,30,31]

Because it takes a week to 10 days for naïve CD8 T cells to proliferate and differentiate into a large population of antigen-specific CTLs, the immediate response to intracellular infection in individuals that have not been vaccinated or previously exposed is mediated by NK cells. Although freshly minted NK cells were previously thought to immediately express granzymes and perforin, it now seems clear that—at least in mice—resting NK cells have minimal cytotoxic activity.[32] They constitutively express mRNAs for granzymes A and B and perforin but only have granzyme A protein. Because they lack substantial perforin protein, cytotoxicity is limited. However, perforin and granzyme proteins and cytotoxicity are upregulated rapidly when NK cell-activating receptors are stimulated. Less differentiated NK cells that highly express the neural cell adhesion molecule or CD56 are poorly cytotoxic, whereas more differentiated $CD56^{dim}$ NK cells are potent killer cells.[33] In the circulation, $CD56^{dim}$ NK cells have about a log more perforin than $CD56^{bright}$ NK cells. NK-activating receptors recognize cell surface changes in tumors, stressed cells, and infected cells, such as downregulation of major histocompatibility complex/human leukocyte antigen molecules or cell surface expression of nonclassical major histocompatibility complex molecules, such as MICA and MICB, which are induced by stress. A longstanding dogma of innate immunity is that innate immune responses are not altered by antigen exposure. However, it is now clear that NK cytotoxicity to infection and other stimuli can be greatly increased by previous antigen exposure.[34] NK cell memory of prior exposure probably results from the expansion of NK cells bearing activating receptors specific for different important pathogens. These receptors, many of which are poorly conserved during mammalian evolution, may have coevolved with important pathogens. The link between individual NK receptors and pathogen recognition remains to be defined.

CYTOTOXIC GENE EXPRESSION

There are 5 human granzymes and 10 mouse granzymes expressed from three gene clusters that arose by gene duplication. In humans, the genes encoding granzymes A and K, tryptases that cleave after basic amino acids, are clustered on chromosome 5; the genes for granzyme B, which cleaves after aspartic acid residues such as the caspases; and granzyme H (or C in mice), which cleaves after hydrophobic residues, are clustered with myeloid cell proteases like mast cell chymase on chromosome 14; and the gene for granzyme M, which is highly expressed in NK cells and cleaves after Met or Leu, is found on chromosome 19 (Fig. 37.1). The mouse granzyme B cluster is uniquely expanded by multiple gene duplications to encode, in addition, granzymes D, E, F, G, L, and N. Nothing is known about these mouse-specific enzymes, but they may have evolved to defend against specific common mouse pathogens.[1] Granzyme A and B are the most abundant granzymes and the most studied. Killer cells, including NK cells, cytotoxic CD4 and CD8 T cells, and even some T_{reg} cells, express highly individualized and tightly regulated patterns of granzymes that depend on both cell type and mode of activation.[35,36]

Expression in Noncytolytic Cells

Perforin is only expressed by cytotoxic cells. Although granzymes were previously also

thought to have similarly restricted expression, noncytotoxic cells can express granzymes without perforin.[37] Granzyme transcripts can be amplified from prothymocytes in fetal liver and double negative thymocytes.[38] Although granzyme A transcripts are detected in thymocytes with the potential to develop into CD8+ cells, granzyme A activity is detected only in the most mature CD4-CD8+ thymocytes. These results suggest posttranscriptional regulation of granzyme translation (see the following for additional examples). Granzyme B, but not granzyme A, is expressed in T_{reg} cells and plays an important perforin-dependent role in T_{reg} function in mice. Benign and transformed B cells can be induced to express granzyme B by IL-21 alone or when combined with anti-B-cell receptor antibody.[39] Granzyme B is also expressed without perforin in many different types of myeloid cells. Within the immune system, granzyme B is expressed in human plasmacytoid dendritic cells (pDCs).[40] There are comparable levels of granzyme B transcripts in resting and activated pDCs but significantly higher amounts of granzyme B protein in activated cells, suggesting posttranscriptional regulation of expression. Granzyme B is also expressed in both normal and neoplastic human mast cells in vitro and in vivo.[41] It localizes to mast cell granules and is secreted when they are activated. In mice, skin-associated mast cells and bone marrow-derived in vitro differentiated mast cells express granzyme B but lung mast cells do not.[42] Neither granzyme A nor perforin are detected in mouse mast cells. The granzyme B gene is encoded within a few hundred kilobases of mast cell proteases. Thus, the granzyme B/mast cell chymase and tryptase genomic region is likely open and active in mast cells. In human basophils, IL-3 induces granzyme B, but not granzyme A or perforin,

expression.[43] Expression of granzyme B in mast cells and basophils suggests a role of granzyme B in mediating allergic disease. In fact, granzyme B has been found in bronchoalveolar lavage fluid after allergen exposure. Several studies have suggested that granzyme B and perforin are expressed in human neutrophils, but this is controversial.[44,45,46,47]

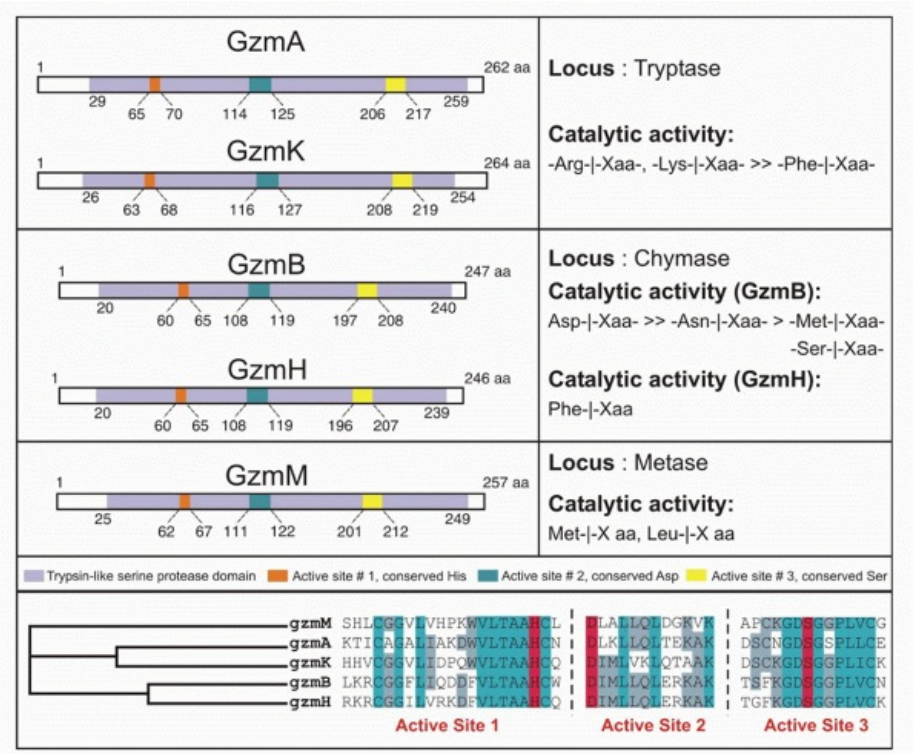

FIG. 37.1. The Human Granzymes are Encoded in Three Clusters. (Figure reprinted with permission from Chowdhury D, Lieberman J. Death by a thousand cuts: granzyme pathways of programmed cell death. *Annu Rev Immunol*. 2008;26: 389-420.)

Granzyme B is also expressed in the absence of perforin in the human reproductive system in developing spermatocytes and in placental trophoblasts[48] and by granulosa cells of the human ovary in response to follicle stimulating hormone.[49] In addition, granzyme B has been detected in a subset of primary human breast carcinomas and in chondrocytes of articular cartilage.[50] The granzyme M transcript is expressed at low levels in the photoreceptor cells of the retina in the mouse.[51] An alternatively spliced form (aGM) is exclusively expressed in these cells at much higher levels. Like granzyme M, granzyme K has an alternatively spliced form exclusively expressed in the brain.[52] The physiologic significance of the alternative transcripts of granzymes M and K is unclear.

Extracellular Signals Regulating Granzyme Expression

The kinetics and expression of the individual granzymes and perforin vary in different clonal populations in vitro and in vivo and depend on how they are activated.[53,54,55] Most circulating CD8+ T lymphocytes that express any granzyme express both granzyme A and granzyme B, but some cells are positive for only one granzyme. Single-cell expression profiles of granzymes, perforin, and IFNγ have been investigated in in vitro or in vivo activated CD8+ T cells using reverse transcription-polymerase chain reaction in mice[35] and intracellular staining and flow cytometry in humans.[56] Individual T cells show diverse expression of these genes. Although some pairs of genes (perforin and IFNγ) are coexpressed more frequently than others, no specific combination of genes is consistently

coexpressed. During in vitro activation of mouse naïve lymphocytes with antibodies to CD3, CD8, and CD11a and IL-2, the expression of granzyme A and granzyme C is delayed compared with cytolytic activity and expression of perforin and granzyme B.[35] When mouse CTLs are activated in vivo by influenza virus infection, most antigen-specific CD8 T cells found in the lung 1 week after infection express both granzymes A and B, and about a third of them also express perforin. Moreover, there is no in vivo difference in the kinetics of induction of granzyme A, granzyme B, or perforin. Granzyme C is not induced by influenza infection in vivo. The diversity of expression of individual granzyme and perforin genes suggests that each gene is regulated independently, although it is likely that these genes will share some common transcription factor recognition sites and epigenetic changes. Differences in T-cell receptor (TcR) avidity, costimulatory and inhibitory receptor engagement, danger and innate immune receptor activation, cytokine milieu, type and state of activation of the antigen-presenting cell, and presence of helper or regulatory CD4 T cells will likely influence the induction of the granzyme and perforin genes. Moreover, the cell's prior history of activation will affect cytolytic gene expression during subsequent encounters with antigen. Surprisingly, little is known about this subject.

The perforin and granzyme genes are induced during T-cell activation. However, the only signal shown consistently to upregulate granzyme A and B and perforin is IL-2.[57] IL-2 regulates perforin and granzyme expression directly and independently of its effect on CD8+ T-cell survival and proliferation.[58] Mice genetically deficient in IL-2 retain the ability to elicit a CTL response against many viruses, tumors, and allografts,[59,60] although there are deficiencies in cytotoxicity under certain conditions.[61] The other γ_C-dependent cytokines (IL-4, IL-7, IL-9, IL-15, and IL-21) likely substitute for IL-2 in its absence. IL-15 is particularly important because it also shares the γ-chain with the IL-2 receptor. IL-15 induces the expression of perforin, granzymes A and B, IFNγ, and Fas ligand in primary mouse lymphocytes.[62] IL-21 works synergistically with IL-15 to upregulate granzyme A and B expression in mouse CD8 T cells.[63] In vivo in mice, IL-21 exhibits potent antitumor function by enhancing NK and CD8 T-cell cytotoxicity.[64] Similarly in human peripheral blood CD8 T cells, IL-15 and IL-21 both activate granzyme B and perforin expression, but IL-21 does so without inducing CD8 T-cell proliferation.[65] Members of the IL-6/IL-12/IL-27 family also can upregulate granzyme and perforin expression.[66,67]

Transcriptional Regulation of Perforin and Granzymes

Two key transcription factors, T-bet (*TBX21*) and eomesodermin (*EOMES*), which belong to the T-box family, are the key master regulators of cytotoxic gene expression and survival of committed CD8 memory cells.[68,69,70,71] After naïve CD8 T-cell activation, T-bet is induced before eomesodermin.[72] Notch signaling and the Runx3 transcription factor upregulate eomesodermin, but also directly upregulate expression of perforin and granzyme B genes.[72,73] Mice deficient in both T-bet and eomesodermin genes are unable to control tumors and intracellular infection.[74,75,76] They develop a wasting syndrome caused by anomalous differentiation to IL-17-secreting cells, suggesting that these two genes not only

positively regulate cytotoxic gene expression and other genes required for CTL survival and function but also suppress differentiation to alternate lineages.

Chromosome transfer experiments have shown that expression of the perforin gene (*PRF1/prf1*) is regulated by cis-regulatory regions extending about 150 kb around the gene.[72,77,78] These include a core promoter located 120 bp upstream of the transcription start site and two enhancer regions and a locus control region (LCR) that are altered during T-cell differentiation and activation. The LCR is open for transcription specifically in cytotoxic cells. The region around the presumed LCR is more accessible to deoribonuclease (DNase I) digestion (and therefore its chromatin is open) in murine CD8 CTLs than in CD4 T_H1 cells, likely explaining their approximately 20-fold increase in *prf1* mRNA. Increased IL-2 does not enhance the accessibility of the LCR. The enhancers are both activated by IL-2R signaling mediated by signal transducer and activator of transcription (STAT)5 binding to two sites in each enhancer. Other STAT family members activated by alternate cytokines can also activate them. Activation of the more proximal enhancer also depends on IL-2-activated NF-κB binding. Both enhancers also contain binding sites for AP-1 and ETS transcription factors, whereas the distal enhancer has an E-box and NFAT binding site, and the proximal enhancer contains eomesodermin, Ikaros, and CREB binding sites. Recruitment of ribonucleic acid (RNA) pol II to the transcription start site and activation of transcription increase with IL-2 stimulation. The key factors involved in activating transcription at the prf1 promoter are Runx3 and eomesodermin. T-bet does not appear to play a direct role in activating prf1 transcription but likely acts indirectly by increasing IL2-Rβ expression and enhancing IL-2 signaling. The current model suggests that Runx3 is needed to open the extended prf1 locus during T-cell differentiation, whereas eomesodermin plays a more direct role in activating transcription near the promoter. Other transcription factors also likely participate in transactivating the perforin promoter, including an ETS transcription factor, probably myeloid elf-1-like factor (MEF).

Much less is known about the details of gene regulation of the granzymes. Enhancers or other long-range regulatory region of granzyme genes remain to be defined. Granzyme B is the only granzyme whose expression has been studied. A distal DNase hypersensitivity site 3.9 kb upstream of the granzyme B transcription start site is accessible only in activated, but not resting, CD8 T cells.[79] Inclusion of this region in a GFP reporter in transgenic mice enhances CTL-specific expression, suggesting that this region may have enhancer activity. Induction of the expression of granzyme transcripts requires at least two independent stimuli: activation of the TcR and costimulation by cytokines of the γ_c family. The signals from several distinct signal transduction pathways are integrated in the nucleus in the form of transcription factors that bind to granzyme gene regulatory elements and activate transcription. Early studies identified a 243-bp fragment upstream of the mouse granzyme B transcription start site that potentially regulates granzyme B transcription.[80] This region contains binding sites for two ubiquitous transcription factors: activating transcription factor/cyclic AMP-responsive element binding protein and activator protein-1, and two lymphoid specific factors, Ikaros and core-binding factor (PEBP2).[81] Several of these transcription factor binding sites are evolutionarily conserved between the human and mouse granzyme B promoters.[82,83] Analysis of reporter assays using promoters that had been systematically mutated at these sites in primary cells and cell lines revealed subtle differences in the importance of some

transcription factors in primary cells versus cell lines. For example, activator protein-1, cyclic AMP-responsive element binding protein, and core-binding factor were not as important for transcription in primary cells as they appeared to be in cell lines.[82,84] These studies suggested that combinations of transcription factors (particularly, activator protein-1 and corebinding factor) activate granzyme B expression in primary cells. The most compelling difference between the mouse and human granzyme B gene promoter is the importance of the Ikaros site only in human granzyme B expression.[82,84] Studies in *Stat1*-deficient mice indicate that STAT1 mediates

granzyme B induction by IFNα or IL-27.[67,85] IL-27-induced augmentation of granzyme B expression also depends on T-bet.[67] Eomesodermin also drives granzyme B expression.[71] Direct binding of T-bet and eomesodermin to the granzyme B promoter has not been examined.

Posttranscriptional Regulation

Several examples of cells expressing perforin and/or granzyme transcripts, but not protein, were described previously, including resting NK cells, thymocytes, and unactivated pDCs and mast cells. Murine memory CTLs also express abundant granzyme B mRNA but no protein.[86] All these results point toward a general mechanism of "prearming" cytotoxic lymphocytes with effector mRNAs, allowing these cells to rapidly respond to external stimuli. This type of gene regulation is well known to regulate cytokine expression, presumably for the same purpose. Two recent studies provide evidence for negative regulation of granzyme B and perforin expression by microRNAs miR-27* and miR-223 in NK cells.[87,88] It will be interesting to see if expression or processing of these micro RNAs declines rapidly after NK cell activation.

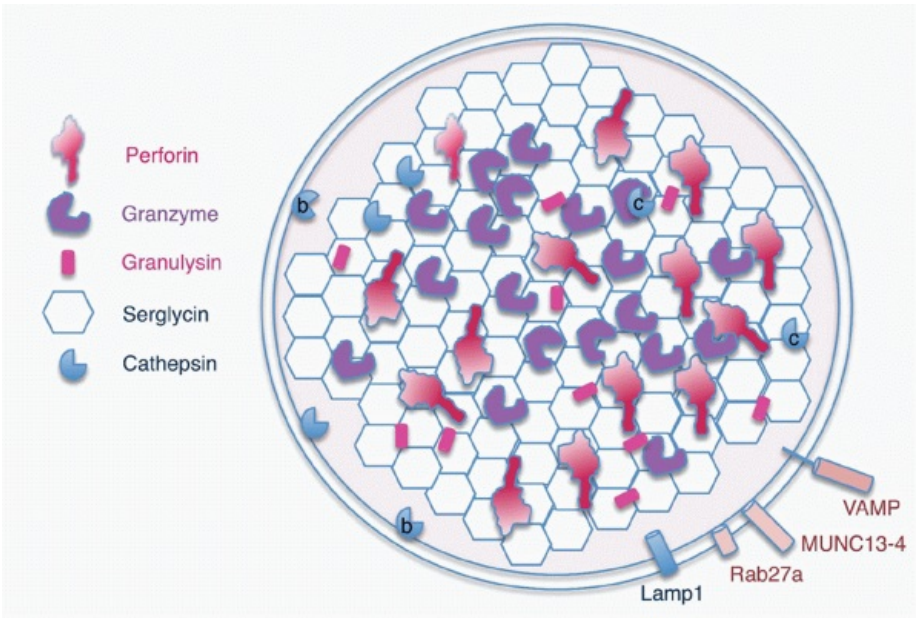

FIG. 37.2. Key Components of Cytotoxic Granules. The cytolytic effector molecules, perforin, granzymes, and granulysin, are bound to the serglycin proteoglycan. Cytotoxic granules also contain molecules found in all lysosomes, such as Lamp1 (CD107a) and

cathepsins, as well as membrane-associated proteins specific to secretory lysosomes, such as vesicle-associated soluble N-ethylmaleimide-sensitive factor accessory complex component (VAMP)7 or VAMP8, Munc13-4, and Rab27a, which are essential for granule exocytosis. Cathepsins B and C play a special role in cytotoxic granules: Cathepsin C processes the progranzymes to the active enzyme, and membrane-associated cathepsin B helps protect the killer cell from membrane damage in the immune synapse by perforin. Other cathepsins may substitute for these cathepsins when they are absent or mutated.

GRANULE-MEDIATED CELL DEATH

Killer Cell Granules

Killer cells contain cytotoxic granules that are acidic, electron-dense, specialized secretory lysosomes[89] (Fig. 37.2). These granules are mobilized like secretory vesicles in other secretory cells, such as neurotransmitter-containing vesicles near the synapses of neurons and melanin-containing vesicles of melanocytes. Cytotoxic granules contain the granzymes, trypsin-like serine proteases, whose major job is to initiate programmed cell death in cells marked for immune elimination. Cytotoxic granule proteins also regulate the survival of activated lymphocytes and may also cause inflammation by acting on extracellular substrates. The granzymes are trypsin-like serine proteases that use a classic histidine, serine, aspartic acid catalytic triad to cleave their substrates. Human granzymes A, B, C, and M; rat granzyme B; and human progranzyme K have all now been crystallized with high resolution.[90,91,92,93,94,95] The active granzymes are produced by cleavage of a dipeptide from the N-terminus of the proenzyme. Activation is accompanied by a radical conformational change. Progranzyme K has a more rigid structure lacking an open active site than the active granzymes.

Detailed information about the conformation surrounding the active sites of granzyme A and B has provided the structural basis for understanding how subtle differences in the active site conformation lead to substantial differences in substrate specificity. As a consequence, mouse granzyme B is preferentially able to cleave mouse procaspase-3, whereas human granzyme B is better able to cleave the human orthologue. Granzyme A differs from the other granzymes in forming a covalent homodimer; the other granzymes are monomeric. Dimerization creates an extended site for substrate binding that is believed to confer a high degree of specificity to granzyme A for its substrates.[90,96] In particular, because of the extended exosite for substrate binding, granzyme A substrates do not share a common short peptide sequence around the cleavage site.

The cytotoxic granules also contain perforin, a poreforming molecule that delivers the granzymes into the target cell. Another pore-forming molecule, granulysin, which is homologous to the saposins, is cationic and selectively active at disrupting negatively charged bacterial and possibly fungal and parasite cell membranes. Granulysin is expressed in humans and nonhuman primates, and orthologues are found in some other species (pigs, cows, and horses), but not in mice. The positively charged cytotoxic effector molecules are bound in the granule to an acidic proteoglycan, called serglycin, after its many Ser-Gly repeats.[97,98] In addition to these specialized molecules, the cytotoxic granules also contain

lysosomal enzymes, the cathepsins, and internal lysosomal membrane proteins, such as CD107 (Lamp1). The outside of the granule membrane binds soluble N-ethylmaleimidesensitive factor accessory protein receptor (SNARE) proteins, synaptotagmins and Rab GTPases, which regulate vesicular trafficking and cytotoxic granule release. Some of these molecules, including Rab27a and Munc13-4, which are important for granule exocytosis, are only incorporated into cytotoxic granules as they mature by fusion of cytotoxic granules with specialized exocytic vesicles, formed in secretory cells by fusion of late endosomes and recycling endosomes. Some of the granule-associated molecules associate with lysosomes in all cells, whereas some have a specialized function in killer cells.

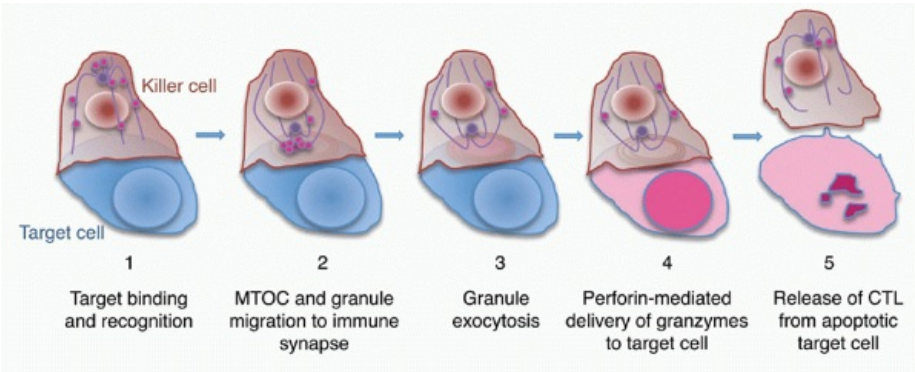

FIG. 37.3. Steps in Granule-Mediated Cytotoxicity. After the killer cell recognizes a target cell ***(1)***, an immune synapse is formed at the interface and the microtubule organizing center (MTOC) moves to the synapse, reorganizing the microtubule network ***(2)***. Cytotoxic granules move along microtubules to dock at the killer cell membrane in the central supramolecular activation complex (c-SMAC) of the immune synapse. Granule membranes fuse with the killer cell plasma membrane, releasing their contents *(magenta)* into the immune synapse ***(3)***. Perforin delivers the granzymes into the cytosol of target cells **(*4*) where they initiate apoptotic death *(5)*. The granzymes concentrate in the nucleus of target cells. The killer cell then detaches from the dying cell and is free to seek out additional targets.**

Steps in Granule Exocytosis

When CTLs and NK cells form an immune synapse with a target cell, engagement of activating receptors, including the TcR, NK cell-activating receptors, and Fc receptors, stimulates the killer cell to destroy the target cell[7] (Figs. 37.3 and 37.4). Activation for cytolysis is enhanced by binding of CD8 or CD4, costimulatory receptors, and adhesion molecules such as LFA-1, which cluster in well-defined concentric rings within the immune synapse. Killer cell activation causes a calcium flux that induces lytic granules to cluster around the microtubule organizing center and then align along the immunologic synapse.[99,100,101,102,103] Granules move to the immune synapse via both the microtubule network and actin cytoskeleton. The latter interaction is via myosin IIA in NK cells.[104] The actin meshwork thins around the site of the synapse to make room for granules to move through it.[105,106,107] Cytotoxic granules then dock to the killer cell plasma membrane in the

central region of the immune synapse (c-SMAC). In T cells, granule docking and fusion may localize to a distinct (secretory) region of the central cluster (c-SMAC) of the immune synapse that is separate from the signaling domain containing the T-cell receptor and associated kinases.[108] Recent studies did not observe a separation of signaling and secretory domains in the c-SMAC of NK cells. Cytotoxic granule docking is orchestrated by binding

of Rab27a on the cytosolic side of the mature granule membrane with synaptotagmin-like proteins, SLP1 or SLP2, which are anchored in the cell membrane. Docked granules are then primed for fusion by the interaction of Munc13-4 on their surface with syntaxin 11 on the killer cell membrane. This triggers the formation of a SNARE complex, the molecular machine for granule membrane fusion, between a cytotoxic granule vesicle-associated SNARE complex component (VAMP) protein with syntaxin 11 and SNAP23 on the cell membrane. Of the seven human VAMP proteins, studies in cytotoxic T cells have suggested that VAMP8 is required, whereas in NK cells both VAMP4 and VAMP7 are needed for different steps leading to granule exocytosis.[109,110] Granule membrane fusion also requires participation of Munc18-2 to trigger the conformational activation of the SNARE complex. Although the general mechanism of granule exocytosis described previously is used by all killer cells, some of the details of granule trafficking and fusion at the synapse may differ between killer T cells and NK cells (although apparent differences may disappear when the same high-resolution techniques are applied to both types of killer cells). Cytotoxicity and granule fusion may occur even in the absence of a stable synapse.[111]

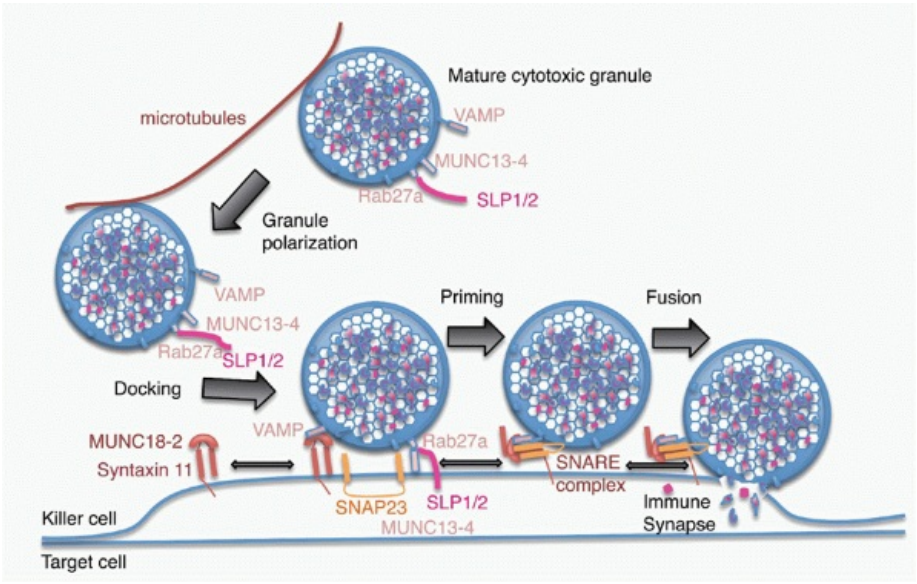

FIG. 37.4. Model of Granule Exocytosis. In response to antigen recognition, the mature cytotoxic granule moves along microtubules, probably with assistance from the actin-myosin cytoskeleton (*not shown*) to dock at the cell membrane at the immune synapse. A cytotoxic granule vesicle-associated soluble N-ethylmaleimide-sensitive factor accessory (SNARE) complex component (VAMP) protein binds to Munc18-2, which is associated with plasma membrane syntaxin 11. Cytotoxic granule proteins Rab27a and Munc13-4, in association with a synaptotagmin SLP1 or SLP2, help anchor the granule to the membrane. A SNARE complex forms between plasma membrane SNAP23 and

syntaxin 11 and granule membrane VAMP to initiate fusion of the granule membrane to the plasma membrane. Following membrane fusion, the cytotoxic granule contents are released into the immune synapse. After fusion, granule membrane-associated cathepsin B (*not shown*) is displayed on the killer cell membrane and protects it from perforin membrane damage. Figure adapted from de Saint Basile et al.[7]

Genetic Diseases Caused by Defects in Perforin or Granule Exocytosis

Inherited deficiencies in perforin or the genes encoding syntaxin 11, Munc13-4, and Munc18-2 that orchestrate cytotoxic granule trafficking and release are linked to defective cytotoxicity and profound immunodeficiency.[112,113,114,115,116,117,118] Patients with mutations in these genes develop the familial hemophagocytic lymphohistocytosis (FHL) syndrome. These patients are handicapped in controling viral infections and develop a severe immune activation syndrome that is often fatal in childhood unless treated with bone marrow transplantation. Some patients with milder perforin mutations that do not completely eliminate cytotoxic function are not diagnosed until adulthood. These adult patients with FHL not only have impaired antiviral immunity but are also more prone to develop lymphoma (such as perforin-deficient mice). The most prominent and sometimes fatal clinical manifestation of FHL is an inflammatory syndrome caused by uncontrolled activation and expansion of CD8 T cells, often in response to poorly controlled herpes virus infections, which leads to systemic activation of macrophages, which infiltrate tissues and overproduce proinflammatory cytokines. Macrophage activation is driven by excessive production of IFNγ by activated CD8 T cells.[10,119] Sequencing

of perforin mutations in patients with FHL has identified nonsense, frameshift, and missense mutations that disrupt perforin synthesis, folding, or activity.[5,120] The importance of some of these have been validated by testing cytolytic function of rat basophilic leukemia cells engineered to express mutant perforin and granzyme B. Defects in genes encoding the AP-3 adaptor—needed to shuttle cargo from the Golgi to secretory lysosomes—the lysosomal trafficking regulator LYST, or Rab27 lead to human syndromes (Hermansky-Pudlak syndrome type 2, Chediak-Higashi syndrome, and Griscelli syndrome type 2, respectively) and corresponding mouse models (*pearl*, *beige*, and *ashen* mice, respectively) in which cytotoxicity as well as other processes involving secretory lysosomes are defective. In fact, mice and humans with defects in these genes have defects in pigmentation due to defective melanosome transport.

Lessons from Knockout Mice

Mice genetically deficient for granzymes A, B (and the granzyme B cluster), and M and perforin provide important tools for probing the importance of these effector molecules in immune defense.[1] Perforin-deficient mice[112] closely recapitulate the symptoms of humans with genetic perforin deficiency. They are severely immunodeficient and compromised in their ability to defend against viruses and tumors and develop the inflammatory syndrome of FHL when infected with mouse cytomegalovirus.[121] Mice deficient in any 1 of the 10 granzymes, or even of the granzyme B cluster, only have subtle differences compared to wild-type

animals. These experiments highlight the functional redundancy of the granzymes. While only one molecule (perforin) effectively delivers the granzymes into target cells, each of the granzymes can trigger cell death. However, target cells may be selectively resistant to one or another of the granzymes (ie, by bcl-2 overexpression or by expression of viral serpins). Requirements for a single granzyme have been shown in some cases by specific immune challenges. For example, granzyme A-deficient mice are more susceptible to the poxvirus ectromelia[122] and granzyme B-deficient mice have less GvHD.[123] In constructing genetically deficient mice, genetic alterations of one gene can affect the expression of nearby granzyme genes. In the original granzyme B knockout mice, the PGK-neo cassette remaining in the granzyme B locus impedes the expression of other granzyme cluster genes (granzymes C, D, and F). The granzyme B gene has also been deleted keeping the expression of granzymes C, D, and F intact.[124] Cytotoxic T cells from the granzyme B-specific deletion mouse are significantly more effective at inducing apoptosis than those from the granzyme B-cluster knockout animal, underlining the importance of the other granzyme B cluster granzymes, especially when granzyme B is absent.

Because granzyme A and B are the most abundant granzymes in T cells, granzyme A/B doubly deficient mice are more immunodeficient than the single knockouts.[125,126,127] CTLs from granzyme A/B-deficient mice, although somewhat impaired in cytotoxicity relative to wild-type cells, nonetheless largely retain the ability to kill target cells.[128,129,130] However, the timing of key molecular events during apoptosis, such as externalization of phosphatidylserine (annexin V staining), is delayed during cell death induced by granzyme A/B-deficient CTLs versus wild-type CTLs.[131] Cytotoxic T cells lacking granzyme A and B induce a modified form of cell death that seems morphologically distinct from either perforin-mediated necrosis or wild-type CTL-mediated apoptosis.[131] Granzyme A/B-deficient animals do not develop spontaneous tumors and clear many viruses normally. The likely explanation of these results is that the other “orphan” granzymes (particularly H/C, K, and M),[132,133,134] substitute for granzyme A and B.

Although granzyme M is highly expressed in innate immune killer cells, including NK cells, NKT cells, and γδ T cells, granzyme M-deficient mice have normal NK and T cell numbers and NK activity against tumors.[135] Defense against the mouse poxvirus ectromelia and implanted NK-sensitive tumors is unimpaired in granzyme M-deficient mice compared to wild-type mice. Deficient mice are somewhat impaired in responding to mouse cytomegalovirus infection as they have higher viral levels, but they are eventually able to clear the infection. Thus, granzyme M does not appear to be essential for NK cell-mediated cytotoxicity.

Perforin Delivery of Cytotoxic Molecules into Target Cells

When the granule membrane fuses with the killer cell membrane, the granule contents are released into the synapse. Granzymes and perforin probably dissociate from serglycin in the immune synapse before they enter target cells.[136] Granzymes bind to the target cell membrane by electrostatic interactions (granzymes are very positively charged and the cell surface is negatively charged)[137,138,139] and also by specific receptors, such as the cation-independent mannose-6-phosphate receptor.[140] However, specific receptors are not required for binding, internalization, or cytotoxicity.[137,139,141,142] The lack of a receptor

enables all types of cells to be eliminated and limits escape from immune surveillance. The granzymes are delivered into the target cell (but not the killer cell) by perforin where they initiate at least three distinct pathways of programmed cell death. Although perforin is essential for granule-mediated cytotoxicity to deliver the granzymes into cells, the granzymes are redundant, as each granzyme can independently activate cell death. Although genetic deficiency of one or a few granzymes does not lead to severe immunodeficiency, mice lacking one or another granzyme display subtle differences in their ability to control specific viral infections. Why are there so many granzymes? The immune system needs to contend with a wide variety of tumors and infections, some of which have elaborated strategies to evade apoptosis and immune destruction. Some of the granzymes may have evolved to disarm specific intracellular pathogens. The interplay between granzyme B and H and adenovirus illustrates how multiple granzymes may have evolved to eliminate important pathogens.[143,144,145] Although both enzymes can cleave and inactivate at least two adenoviral proteins, the virus has also developed a way of inactivating granzyme B. Granzyme H potentiates the effect of granzyme B by destroying an adenoviral granzyme B inhibitor.

Perforin delivers granzymes and other effector molecules into the target cell cytosol.[146,147] At high concentrations,

perforin multimerizes in a cholesterol- and calcium-dependent manner in the plasma membrane of cells to form 5- to 30-nm pores.[148,149,150,151,152] Recent cross-linking and biophysical studies suggest that perforin may form at least two types of pores in membranes: small pores composed of about seven monomers that are not stable and much larger stable pores.[153,154] Cryoelectron microscopy reconstructions suggest that the large pores are composed of approximately 19 to 24 subunits and have a lumen large enough for granzyme monomers or granzyme A dimers to readily pass through. The precursor of human perforin is a 555 amino acid protein synthesized with a 21 amino acid leader sequence. The N-terminal region of the mature 67-kDa protein (residues 44-410 of the human protein) is homologous to domains in complement proteins C6, C7, C8a, C8b, and C9 that form the complement membrane attack complex (MAC). The crystal structure of monomeric mouse perforin was recently solved.[152] The complement homology domain, termed the *MAC/perforin domain* (MACPF), is similar in structure to that of bacterial pore-forming cholesteroldependent cytolysins, although they insert into membranes in opposite orientations. The MACPF domain of perforin is followed by an epidermal growth factor (EGF)-like domain; a C2 domain, a domain present in synaptotagmins and other calcium-dependent proteins, which becomes able to bind to lipid membranes after a conformational change in response to calcium; and a short 12 amino acid C-terminal peptide. The docking of the calcium-bound C2 domain is the first step in pore formation. Docking likely triggers both multimerization and a major conformational change in which two clusters of α-helices in the MACPF domain jackknife into the membrane. It is unclear whether multimerization to form a pore occurs before or after this conformational change. Perforin is glycosylated at two sites: one in the MACPF domain and one in the C-terminal peptide. Glycosylation of at least one site is needed for targeting perforin to cytotoxic granules, probably via binding of the glycan to the mannose-6-phosphate receptor.[155] En route to or in the granule, the glycosylated C-terminal peptide is removed from human (but not mouse) perforin by an undefined cysteine protease to produce the

mature active protein.[156]

The original model for how perforin delivers granzymes into cells was that granzymes entered cells through perforin pores in the target cell plasma membrane. This model predicts that granzymes directly pass and disperse into the target cell cytosol. However, granzymes do not directly enter the cytosol but instead are first endocytosed into clathrincoated vesicles and transported to endosomes.[154,157,158] Thus, the original model is not correct. Current data suggest that perforin indeed forms target cell plasma membrane pores, but these pores are small and transient (Fig. 37.5). However, calcium flows into the target cell through these pores and remains elevated for a few minutes. Because intracellular calcium is low in cells with an intact cell membrane, the cell senses a calcium influx as a sign of disruption of the plasma membrane. The elevated calcium triggers a cellular membrane damage response (also known as cellular wound healing) in which intracellular vesicles move to the plasma membrane and fuse their membranes to patch holes, and any damaged membrane is rapidly removed and internalized into endosomes.[158,159,160,161] At the same time, membrane-bound granzymes, granulysin, and perforin are endocytosed. Elevated cytosolic calcium activates endosomal fusion, and granzyme- and perforin-containing endosomes fuse to form giant endosomes approximately 10 times larger than normal endosomes that have been termed gigantosomes. In the endosomal membrane, perforin forms larger and more stable pores through which granzymes begin to leak out into the cytosol. About 15 minutes after cell death has been triggered, the gigantosomes rupture, releasing any remaining cargo to the cytosol where they begin to activate programmed cell death. When the membrane repair response is inhibited, because the cell remains leaky, target cells die by necrosis instead of by slower, regulated, and energy- dependent programmed cell death.

Although perforin is the major molecule responsible for granzyme delivery, under some circumstances other molecules might serve that function. For example, bacterial and viral endosomolysins can substitute for perforin in vitro (and are widely used as laboratory reagents for intracellular delivery[162]) and potentially might play a similar role in vivo. The heat shock protein (Hsp)70, which chaperones some peptides across cell membranes, can also carry granzyme B (and presumably other granyzmes) into cells.[163] Hsp70 is on the surface of some stressed cells or tumor cells and might help to remove these cells from the body.

Programmed Cell Death Pathways Activated by Granzymes

Once in the cytosol, the granzymes independently activate several parallel pathways of programmed cell death[6] (Table 37.1). Granzyme B cleaves and activates the caspases and also directly cleaves many important caspase substrates. Granzyme B can activate cell death that mimics caspase activation, even when the caspases are inhibited or in cells in which the caspase mitochondrial pathway is deficient. Granzyme A activates a distinct programmed cell death pathway that does not involve the caspases or disrupt the mitochondrial outer membrane. The substrates of the two major granzymes are largely nonoverlapping. The exceptions, lamin B and PARP-1, may indicate common features needed for cells undergoing all forms of programmed cell death, such as disruption of the nuclear membrane, inhibition of deoxyribonucleic acid (DNA) repair, or maintaining cellular adenosine triphosphate levels. What is known about cell death executed by the other (so-called orphan) granzymes is briefly

described in the following. The orphan granzymes may be more highly expressed under conditions of prolonged immune activation.[164] The orphan granzymes are functionally important as mice genetically deficient in the whole granzyme B cluster are less efficient at clearing allogeneic tumors than mice deficient in just granzyme B.[124] Although some key granzyme proteolytic substrates are in the cytosol (ie, Bid, caspase-3, and ICAD for granzyme B), other important targets are in other membrane-bound cellular compartments, especially the nucleus and mitochondrion. In the cytosol, granzymes B, H, and possibly K also directly cleave the proapoptotic BH3-only Bcl-2

family member Bid to initiate the classical mitochondrial apoptotic pathway that leads to mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome c and other proapoptotic proteins from the intermembrane space.[165,166,167,168,169,170,171] Granzymes A and B (and possibly other granzymes) enter mitochondria through an unknown mechanism to cleave important substrates including electron transport proteins.[172,173,174] Granzyme C (in mice) and granzyme H (in humans) activate caspase-independent cell death with a pronounced mitochondrial phenotype. All of these events cause mitochondrial depolarization and production of superoxide anions and other reactive oxygen species, which is a key first step in killer lymphocyte-mediated death, as superoxide scavengers block granzyme-mediated cell death.[173] Granzyme A and B rapidly translocate to and concentrate in the nucleus,[175,176] where proteolytic cleavage of key substrates is important to induce programmed cell death by both granzyme A (SET, Ape1, lamins, histones, Ku70, PARP-1) and B (lamin B, PARP-1, NuMa, DNA-PK_{cs}). Nuclear translocation of the granzymes may be mediated by importin-α.[177]

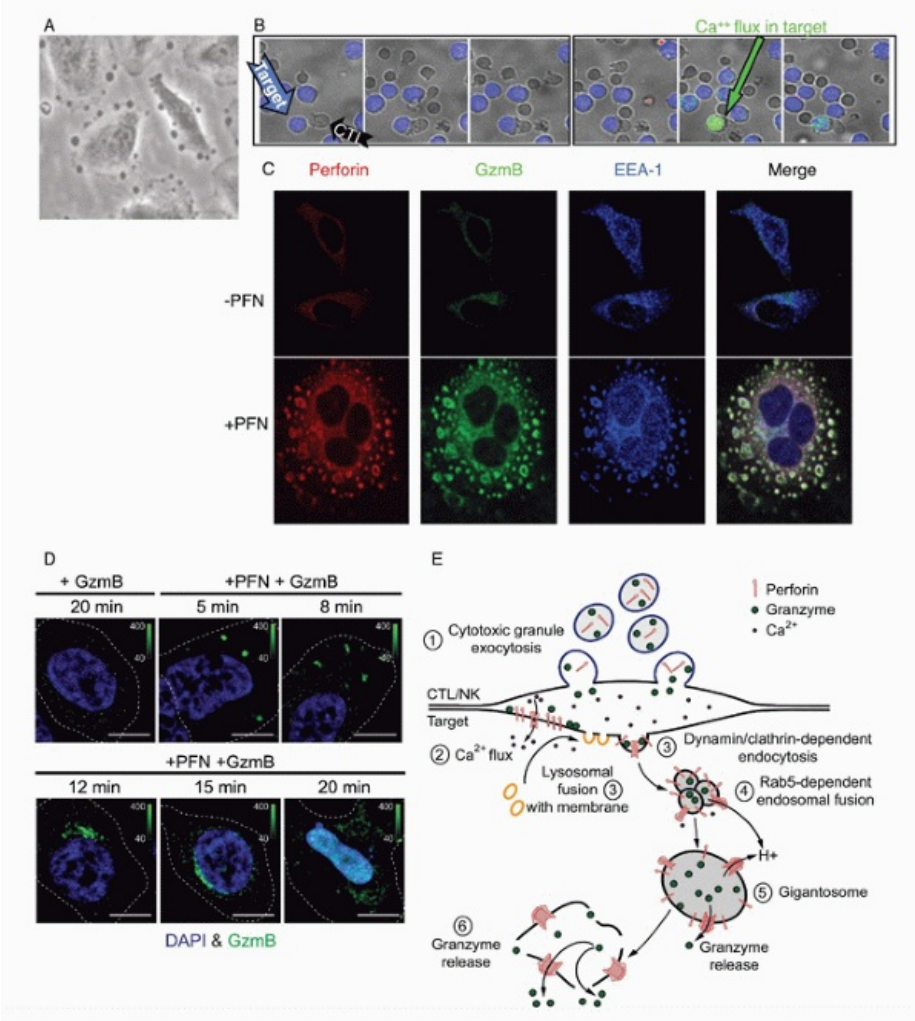

FIG 37.5. Current Model of Perforin Delivery of Granzymes into the Target Cell. **A:** Perforin treatment of HeLa cells causes dramatic membrane perturbation and blebbing. **B:** Killer cell degranulation causes a transient calcium influx in target cells that persists for a few minutes. In this experiment from Keefe et al.,[157] PHA-activated human cytotoxic T lymphocytes were incubated with Fura-2-loaded, anticluster of differentiation three-coated U937 cells, and images were obtained every 30 seconds. The Fura-2 indicator dye is *blue* when calcium is low and *green* when it is elevated. **C:** Perforin and granzyme B are endocytosed into giant EEA-1-staining endosomes (image courtesy of Jerome Thiery). **D:** When HeLa cells are treated with perforin and granzyme B, within 5 minutes, granzyme B (*green*) concentrates in gigantosomes and is released beginning after about 12 minutes. The released granzyme concentrates in the target cell nucleus. **E:** Model for perforin delivery. After cytotoxic granule exocytosis into the immunologic synapse *(1)*, perforin multimerizes in the target cell membrane to form small transient pores through which calcium enters *(2)*, triggering a plasma membrane repair response *(3)* in which lysosomes fuse with the damaged plasma membrane and perforin and granzymes are rapidly internalized by endocytosis. Perforin and granzyme-containing endosomes then fuse in response to the transient calcium flux *(4)* to form gigantosomes. Within gigantosomes, perforin continues to multimerize to form larger pores, preventing acidification and causing some granzyme release *(5)* before inducing endosomal rupture and complete granzyme release into the target cell cytoplasm *(6).* (**D,E:** Reprinted from

Thiery J, Keefe D, Boulant S, et al. Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. *Nat Immunol*. 2011;12:770-777, with permission.)

TABLE 37.1 Features of the Distinct Cell Death Pathways Induced by the Granzymes

Granzyme	A	B	C/H	K	M
Expression					
Cytolytic CD8 T cells	++	++	+	+	+/−
Cytolytic CD4 T cells	+	+			
CD4 T_{reg}s	−	+			
NK cells	+	+/−			++
Myeloid cells	−	+			
Common features					
Rapid loss of membrane integrity	+	+	+	+	?
Annexin V staining	+	+	+	+	?
Chromatin condensation	+	+	+	+	?
DNA damage	+	+	+	+	?
Mitochondrial depolarization	+	+	+	+	?
Caspase activation	−	+	−	−	?
Type of DNA damage					
Oligonucleosomal DNA fragmentation	−	+	−	−	?
Single-stranded DNA nicks	+	−	+	+	−

TdT labeling	+	+	+	+	?
Klenow labeling	+	+	+	+	−?
Type of mitochondrial damage					
Inhibition by Bcl-2 overexpression	−	+	?	?	?
Cytochrome c release	−	+	+?	?	?
Mitochondrial swelling	+	+	++	+	+
Autophagy	−	−	−	−	+?

CD, cluster of differentiation; DNA, deoxyribonucleic acid; NK, natural killer; TdT, terminal deoxynucleotidyl transferase; T_{reg}, regulatory T.

Table modified from Chowdhury and Lieberman.6

Granzyme A

Granzyme A induces caspase-independent cell death, which is morphologically indistinguishable from apoptosis[178,179,180] (Fig. 37.6). Granzyme A is the most ancient of the granzymes; tryptases homologous to granzyme A are found in cytotoxic cells in bony fish.[181] Granzyme A was the first granzyme described and is the most widely expressed. Cells treated with granzyme A and perforin die rapidly: Within minutes, they undergo membrane blebbing and have evidence of mitochondrial dysfunction (increased reactive oxygen species, loss of mitochondrial transmembrane potential [$\Delta\Psi_m$], disruption of mitochondrial morphology).[172,173] Within half an hour, externalization of phosphatidyl serine (measured by annexin V staining) occurs; and DNA damage, chromatin condensation, and nuclear fragmentation become apparent within 1 to 2 hours. DNA is damaged by single-stranded cuts into megabase fragments that are much larger than the oligonucleosomal fragments generated during caspase or granzyme B-activated cell death.[182] Because the DNA fragments are too large to be released from the nucleus, assays that measure DNA release into culture supernatants are typically negative. Mitochondria are damaged without MOMP or release of proapoptotic mediators, such as cytochrome c, from the mitochondrial intermembrane space.[173] In mitochondria, granzyme A cleaves Ndufs3 in electron transport chain complex I to interfere with mitochondrial redox function, adenosine triphosphate generation and maintenance of $\Delta\Psi_m$ and to generate superoxide anion.[129,172,173] The superoxide generated

by damaged mitochondria drives an endoplasmic reticulum (ER)-associated oxidative stress response complex, called the SET complex, into the nucleus where it plays a critical role in

granzyme A-induced nuclear damage.[173,182] The SET complex contains three nucleases (the base excision repair endonuclease Ape1, an endonuclease NM23-H1, and a 5′-3′-exonuclease Trex1), the chromatin modifying proteins SET and pp32, which are also inhibitors of the tumor suppressor protein phosphatase 2A, and a DNA binding protein that recognizes distorted DNA, HMGB2.[183,184,185,186,187] One of the normal functions of the complex is to repair abasic sites in DNA generated by oxidative damage. Recent studies also implicate the cytosolic SET complex as binding to the human immunodeficiency virus preintegration complex and facilitating human immunodeficiency virus infection.[188] The SET complex exonuclease Trex1 digests cytosolic DNA produced by endogenous retroviruses and infectious viruses to inhibit the innate immune response to cytosolic DNA.[189,190,191,192] Mutations in *Trex1* that inactivate its nuclease activity or cause its mislocalization are linked to human inflammatory and autoimmune diseases, including Aicardi-Goutiere syndrome and systemic lupus erythematosis.[193,194,195,196,197] Granzyme A, which traffics to the nucleus by an unknown mechanism, converts this DNA repair complex into an engine for DNA destruction by cleaving SET, an inhibitor of the endonuclease NM23-H1.[185] This allows NM23-H1 to nick DNA; the exonuclease Trex1 then extends the break.[184] At the same time, granzyme A cleaves and inactivates HMGB2 and Ape1 to interfere with base excision repair.[186,187] In addition to disabling base excision repair, granzyme A also interferes with DNA repair more generally by interfering with the recognition of damaged DNA by cleaving and inactivating Ku70[198] and PARP-1.[199] Within the

nucleus, granzyme A also opens up chromatin by cleaving the linker histone H1 and removing the tails from the core histones, making DNA more accessible to any nuclease, and disrupts the nuclear envelope by cleaving lamins.[200,201]

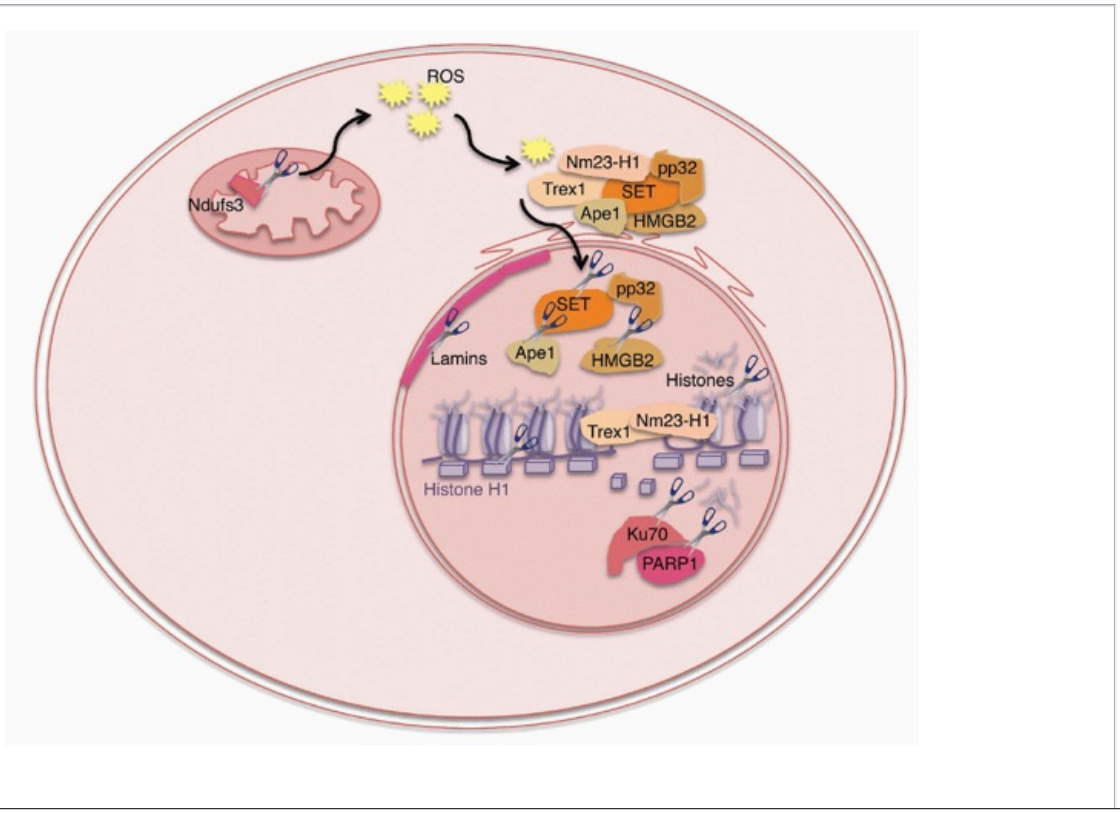

FIG 37.6. The Granzyme A Pathway of Cell Death. Reactive oxygen species generated by granzyme A (represented by *scissors*) cleavage of Ndufs3 in electron transport complex I in mitochondria drives the endoplasmic reticulum-associated SET complex into the nucleus. Granzyme A enters the nucleus by an unknown pathway. In the nucleus, Granzyme A cleaves three components of the SET complex (SET, HMGB2, and Ape1) to activate two nucleases in the complex to make single-stranded deoxyribonucleic acid (DNA) lesions; NM23-H1 makes a nick, which is extended by the exonuclease Trex1. Granzyme A also degrades the lamins and the linker histone H1 and removes the tails from the core histones, opening up chromatin and making it more accessible to these nucleases. DNA repair proteins Ku70 and PARP-1 are also targets.

Granzyme B

Granzyme B is unique among serine proteases because it cleaves after aspartic acid residues like the caspases[202,203] (Fig. 37.7). It induces target cell apoptosis by activating the caspases, particularly the key executioner caspase, caspase-3.[204,205] Human granzyme B, but not the mouse enzyme, also activates cell death by directly cleaving the key caspase substrates, Bid and ICAD, to activate the same mitochondrial and DNA damage pathways, respectively, as the caspases.[92,166,167,168,206,207,208] As a consequence, caspase inhibitors have little effect on human granzyme B-mediated cell death and DNA fragmentation while the same inhibitors significantly block the action of the mouse enzyme. Thus, human CTLs and NK cells may be more effective than mouse killer cells at eradicating virus-infected cells or tumors that have developed methods for evading the caspases. Both human and mouse enzymes cleave many of the same substrates as the caspases (including PARP-1, lamin B, NuMa, DNA-PK$_{CS}$, tubulin) and have substrate specificity close to that of caspases-6, -8, and -9.[209] However, human granzyme B cleaves optimally after the tetrapeptide IEPD, whereas mouse granzyme B has somewhat different peptide specificity, preferring to cleave after IEFD.[92,206] Moreover, other regions including the P' region (C-terminal to the cleavage site) and more distal regions contribute to substrate specificity. Because of subtle

differences in sequence, the human and mouse granzyme B can differ in important ways with respect to their substrates and the efficiency with which they are cleaved.

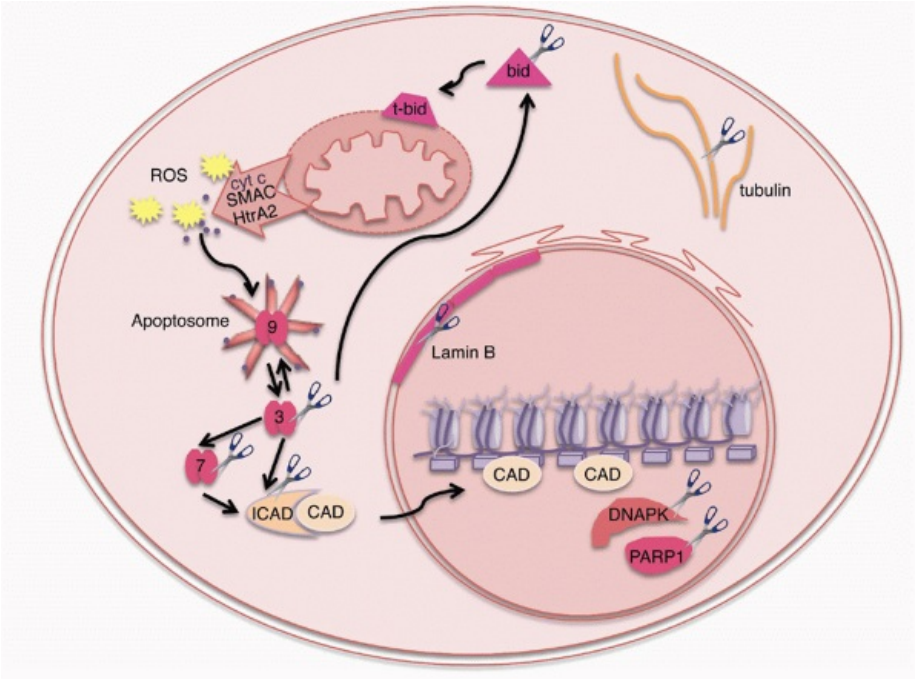

FIG 37.7. The Granzyme B Pathway of Cell Death. Human granzyme B (represented by *scissors*) cleaves Bid to produce truncated Bid (tBid), which initiates the classical mitochondrial apoptotic pathway characterized by reactive oxygen species generation, loss of the mitochondrial transmembrane potential, and disruption of the mitochondrial outer membrane to release proapoptotic mediators in the intermembrane space, including cytochrome c (*blue balls*, cyt c), SMAC/Diablo, and HtrA2/Omi. Cytochrome c binds to apoptotic protease activating factor 1 (Apaf-1) to form the apoptosome that activates caspase-9. Caspase-9 activates caspase-3 and the apoptotic cascade that includes cleavage of ICAD, the inhibitor of the caspase-activated DNase (CAD), allowing CAD to enter the nucleus and make oligosomal DNA double strand breaks. Human granzyme B on its own can directly activate caspase-3 and some key downstream caspase targets, including ICAD, lamin B, tubulin, and the DNA repair proteins DNA-PK_{CS} and PARP-1. Mouse granzyme B lacks direct proteolytic activity on some important substrates (see text).

The granzyme B (and caspase) mitochondrial pathway leads to reactive oxygen species (ROS) generation, dissipation of $\Delta\Psi_m$, and MOMP with release of cytochrome c and other proapoptotic molecules from the mitochondrial intermembrane space. Human granzyme B activates this pathway directly by cleaving Bid, whereas mouse granzyme B activates it indirectly. However, granzyme B targets mitochondria in other ways, including by cleaving antiapoptotic Mcl-1 and Hax-1, a protein that helps maintain the mitochondrial transmembrane potential.[174,210] Loss of $\Delta\Psi_m$, but not cytochrome c release, occurs in the presence of pancaspase- inhibitors (even using mouse granzyme B) and in mice genetically deficient for Bid, Bax, and Bak (the latter two Bcl-2 family members are required for Bid-induced mitochondrial damage).[132,169,211,212] Granzyme B can also activate ROS by activating extramitochondrial nicotinamide adenine dinucleotide phosphate-oxidase.[213] DNA damage by granzyme B is mediated primarily by the activation of the caspase-activated

DNase (CAD) following proteolytic cleavage of its inhibitor ICAD either directly by human granzyme B or indirectly by executioner caspases, such as caspase-3.

In humans, there is a common polymorphism of granzyme B in which three amino acids (Q^{48}, P^{88}, Y^{245}) are mutated to $R^{48}A^{88}$ H^{24}.[214] This polymorphism does not seem to affect cytotoxicity and does not have any known clinical significance.[215]

Granzymes C and H

Mouse granzyme C and human granzyme H, homologous granzymes encoded downstream from granzyme B, are predicted to cleave after aromatic residues.[133,216] Granzyme H arose during primate evolution, independently of granzyme C, in an intergenic recombination event between granzyme B and a mast cell chymase.[217] Both induce caspase-independent death with hallmarks of programmed cell death: ROS generation, dissipation of $\Delta\Psi_m$, chromatin condensation, and nuclear fragmentation.[133,216] DNA destruction by granzyme C (and probably granzyme H as well) is via single-stranded nicks and does not involve CAD. Rapid mitochondrial swelling and disruption of mitochondrial ultrastructure are particularly striking in cells treated with granzyme C. The mitochondrial pathways activated by granzyme C and H may be different; granzyme C triggers cytochrome c release, a sign of MOMP, whereas granzyme H does not.[133,216]

Granzyme H cleaves two adenoviral proteins: a DNA binding protein (also a granzyme B substrate) and the adenovirus 100K assembly protein, a previously described inhibitor of granzyme B.[143,145] Cleavage of DNA binding protein interferes with viral DNA replication, whereas cleavage of 100K restores granzyme B function in adenovirus-infected cells. Granzyme H also cleaves the cellular La protein, an RNA binding protein that participates in the posttranscriptional processing of mRNAs transcribed by RNA polymerase III and some transfer RNA (tRNA) and viral RNAs.[218] Cleavage mislocalizes La from the nucleus and decreases translation of hepatitis C virus proteins. Therefore, granzyme H may play a special role in immune defense against certain viruses. Because granzyme H is expressed in NK cells, it may help eliminate these viruses early in infection before adaptive immunity has had a chance to develop.

Granzyme K

Granzyme K is another tryptase in mice, rats, and humans that is encoded downstream near granzyme A on human 5q11 to 5q12 (or the syntenic region of mouse chromosome 13). It is much less expressed than granzyme A, and unlike granzyme A, is a monomer, not a dimer. Mice genetically deficient in granzyme A express granzyme K, which may explain the lack of a significant phenotype of granzyme A-/- mice, except when challenged with some viruses.[219,220] Purified rat and recombinant human granzyme K have been available for some time,[221,222] but little was known about its cell death activation until recently. Like granzyme A, purified rat granzyme K efficiently induces caspase-independent cell death, characterized by mitochondrial dysfunction without MOMP (ROS and loss of $\Delta\Psi_m$, but without cytochrome c release).[132] However, unlike granzyme A, rat granzyme K-induced cell death was originally reported to be inhibited in cells overexpressing Bcl-2.[132] This finding

was surprising, as Bcl-2 inhibits MOMP, which leads to cytochrome c release, which was not detected in granzyme K-treated cells. In fact, a more recent study found that cell death by recombinant human granzyme K did not activate caspase-3 and was unaffected by caspase inhibitors or Bcl-x_L overexpression.[171] Granzyme K mimics granzyme A DNA damage [171]: It causes caspaseindependent nuclear fragmentation and nuclear condensation and single-stranded DNA breaks by targeting the SET complex. Like granzyme A, granzyme K causes SET complex nuclear translocation and hydrolyzes and inactivates SET, Ape1, and HMGB2 in the SET complex.[171] Presumably, cleavage of SET, the inhibitor of NM23-H1, triggers DNA damage by the granzyme A-activated DNases, NM23-H1, and Trex1 in the SET complex.[184,185] The same group recently reported that granzyme K causes mitochondrial damage that includes not only ROS generation and dissipation of $\Delta\Psi_m$ but also Bid cleavage (to a fragment that appears to be the same size as is generated by granzyme B) and MOMP with release of cytochrome c and endonuclease G (endoG).[171] This needs to be verified because rat granzyme K does not cause cytochrome c release,[132] and this same group showed that caspases are not activated by granzyme K, and overexpression of Bcl-x_L does not interfere with human granzyme K-induced cell death,[171] as would be expected if MOMP is triggered. Although granzyme K appears to duplicate the nuclear damage pathway of granzyme A, further studies are needed to determine whether the mitochondrial granzyme K pathway resembles that activated by granzyme A (no MOMP) or granzyme B (Bid cleavage, MOMP), or is a hybrid of both. A proteomics analysis that compared granzyme A and K suggested that although the two enzymes share many substrates, some may be unique to granzyme K.[223] In fact, recent studies suggest that granzyme K cleaves and inactivates p53, which should interfere with cellular repair pathways, and interferes with the ER unfolded protein response by cleaving multiple components of the ER degradation complex.[224,225]

Granzyme M

Granzyme M is the most distinctive of the granzymes. It likely arose from a gene duplication of a neutrophil protease, as it is encoded near a cluster of other neutrophil proteases in human chromosome 19p13.3 (or a synteic region of mouse chromosome 10) and is slightly more homologous to one of them (complement factor D) than to the other granzymes.[226] Unlike the other granzymes, granzyme M cuts after Met or Leu.[227,228] None of the serine protease inhibitors that block the other granzymes, including the pan-granzyme inhibitor 3,4-dichloroisocoumarin, effectively inhibit granzyme M.[229] Moreover, granzyme M appears to function primarily in innate immunity, as it is expressed mostly in NK cells and γδ T cells and only in the subset of CD56+ T cells.[230,231] Until recently, it was not clear whether granzyme M induces cell death.[134] Granzyme M-/- mice have unimpaired NK- and T-cell development and NK cell-mediated cytotoxicity but are less able to defend against mouse cytomegalovirus infection.[135]

The literature does not agree about the type of cell death activated by granzyme M. Kelly et al.,[134] using recombinant human granzyme M expressed from baculovirus in insect cells, found that granzyme M induced rapid, caspase-independent cell death that looked like

autophagic death and did not find evidence for DNA fragmentation, mitochondrial depolarization, phosphatidyl serine externalization, or caspase activation. On the other hand, using human granzyme M expressed in yeast, the Fan laboratory argued that granzyme M activated caspase-dependent cell death, in part by cleaving and inactivating both the apoptosis inhibitor survivin and ICAD, with phosphatidyl serine externalization, caspase activation, CAD activation with oligonucleosomal DNA laddering, PARP cleavage, and mitochondrial disruption with MOMP (mitochondrial swelling, dissipation of $\Delta\Psi_m$, ROS generation, cytochrome c release).[232,233,234] This group also suggested that another granzyme M substrate may be TRAP75, a Hsp that inhibits granzyme M-induced ROS generation.[233] However, one aspect of this study that may not be completely consistent with what is known about granzyme M is that the Fan paper[232,233] claims that granzyme M cleaves ICAD after a Ser residue, whereas peptides containing Ser at the P1 site are not substrates of granzyme M expressed in yeast. Therefore, further work will be needed to determine whether granzyme M activates granzyme B- like caspase-dependent cell death or a novel pathway distinct from that activated by the other granzymes. The mouse and human isoforms may also have diffeent substrates.[235] Examining cell death induced by native purified granzyme M may be necessary to determine what type of cell death is induced by these enzymes. One intriguing other activity of granzyme M might be to cleave and inactivate the granzyme B serpin inhibitor SerpinB9 (PI-9), which it has been shown to do in vitro.[228] If this proves to be a physiologically relevant substrate in cells, then one function of granzyme M might be to potentiate the activity of granzyme B. Mice genetically deficient in granzyme M are more susceptible to cytomegalovirus infection, and granzyme M cleaves a cytomegalovirus structural protein and inhibits its replication.[236] Thus, an important function of granzyme M may be to help protect us from this important human pathogen.

Granulysin

Human cytotoxic granules of cytotoxic T cells and NK cells also contain another effector molecule: the membrane perturbing saposin-like molecule granulysin.[237] The granulysin gene (*GNLY*) was first identified as a late activation gene expressed 3 to 5 days after T-cell activation, which coincides with the expression of the other cytotoxic effector molecule genes in naïve T cells.[238] Granulysin is synthesized as a 15-kDa protein that is cleaved at both ends to produce a 9-kDa peptide. Both forms can form membrane pores in membranes. The larger form is secreted by NK cells and cytotoxic T cells, whereas the 9-kDa form is stored and released from cytotoxic granules during NK-cell or cytotoxic T-cell attack. Granulysin preferentially disrupts bacterial membranes and has been postulated to play a role in immune elimination of bacteria, fungi, and parasites.[239,240,241,242] It may also have some antitumor activity, but this requires very high granulysin concentrations in vitro that may not be physiologically relevant. There is also some evidence that secreted granulysin can act as a chemoattractant for dendritic cells and other immune cells and can induce them to express proinflammatory cytokines.[243,244] Purified granulysin is only active as a cytotoxic agent against bacteria and other pathogens when experiments are performed at high micromolar concentrations of granulysin under hypotonic or acidic conditions that are not found extracellularly. Thus, granulysin's membrane perturbing activity likely only operates within cells, perhaps to target intracellular bacteria and other pathogens located in acidic

intracellular vesicles, such as phagolysosomes. Perforin is needed to deliver granulysins into target cells.[239,245,246] Understanding the importance of granulysin in antibacterial defense and immunopathology (it is overexpressed at sites of immune activation and in blistering skin diseases) will be facilitated by the recent generation of transgenic mice that express granulysin.[247]

How is the Killer Cell Protected from its Cytotoxic Molecules?

The killer cell is not injured by its own granules. It delivers the "kiss of death" and escapes the encounter with the cell targeted for elimination unharmed and then can find and destroy other targets. How the killer cell determines that it has killed its target and is ready to detach is unknown. Several mechanisms ensure that the killer molecules are inactive during protein synthesis, processing, and storage within the granule. Within the killer cell, the cytotoxic molecules are synthesized as proenzymes that are only processed to their active form within the granule. The granzymes and perforin are expressed with a signal sequence that directs them to the ER. The high concentration of calreticulin in the ER likely serves as a sink for free calcium, which prevents perforin activation.[248,249,250,251] Cleavage of the signal peptide of the granzymes produces an inactive proenzyme that contains an N-terminal dipeptide that needs to be removed to produce an active protease. During synthesis, perforin is also rapidly transported from the ER to the Golgi. This is facilitated by a conserved C-terminal tryptophan residue by an unknown mechanism.[155] Mutation of the terminal tryptophan leads to enhanced death of the killer cell. In the Golgi, mannose-6-phosphate-containing glycans are added

to progranzymes and perforin, which serve as sorting signals for transport to lysosomes. Within the cytotoxic granule, the N-terminal dipeptide on all progranzymes is removed by cathepsin C (dipeptidyl peptidase I) to generate the active enzyme.[252] However, mice and humans genetically deficient in cathepsin C have only partially reduced granzyme activity and cytolytic function and modestly reduced immune defense against viral infection.[253,254] This suggests that alternate ways can activate progranzymes. In fact, IL-2 treatment stimulates cathepsin C-independent dipeptide cleavage in NK cells from patients with Papillon-Lefevre syndrome, who have loss of function of cathepsin C.[255] Cathepsin H and probably other cathepsins can process progranzyme B.[256] Granzymes, which are highly basic, are bound, as are perforin and presumably granulysin, to the acidic serglycin proteoglycan within the granule, which helps keep them inactive. Serglycin is responsible for the electron dense core and may also enhance effector protein storage in the granules.[257] Granzyme proteolytic activity and perforin pore formation is also negligible at the acidic pH (pH 5.1 to 5.4) of the granule.

Although granzyme and perforin trafficking within cytotoxic cells minimizes leakage of active death effector molecules out of granules, any stray molecules in the cytoplasm could cause cell death.[258] During granule exocytosis, some granzymes might inadvertently reenter effector cells. Because CTLs typically kill several targets in succession without harming themselves, an important question is how CTLs protect themselves from their own cytotoxic molecules. An important protective mechanism against killer cell suicide is serpin expression

in the killer cell cytoplasm.[259] Serpins are members of a superfamily of protease inhibitors with more than 1,500 family members.[260,261] Serpins inactivate their target proteases either by covalently and irreversibly binding to the active site of the enzyme or by forming noncovalent complexes that are so strong they resist the denaturing conditions of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).[261,262] Serpins that inactivate granzyme B (SerpinB9, also known as PI-9, in human cells[263] or its ortholog Spi6 in mouse cytotoxic cells[264]) are expressed in killer cells. Mice genetically deficient in *Spi6* have reduced numbers of memory CD8 T cells, suggesting that CTL survival is compromised by their own granzyme B.[265] In fact, cytotoxic T cells from these mice show granule membrane instability and have increased cytosolic granzyme B and apoptosis.[266] However, no killer cell serpins are known that inactivate the other granzymes. A recent report suggests that SerpinB4 is a potent granzyme M inhibitor that may render some tumors resistant to granzyme M.[267] However, NK cells or activated killer lymphocytes are not known to express this serpin.

When perforin and granzymes are released into the immune synapse, why is killing unidirectional? How is the killer cell membrane protected from perforin damage? During granule exocytosis, the cytotoxic granule membrane fuses with the killer cell plasma membrane, exposing internal granule membrane-associated proteins. These include cathepsin B, which inactivates by proteolysis perforin redirected toward the killer cell.[8] However, killer cells genetically deficient in cathepsin B survive unscathed when they kill targets.[268] This suggests that other membrane-bound granule cathepsins (or perhaps other CTL surface proteases or other perforin inhibitors) might also inactivate perforin redirected at the killer cell.

Cellular Resistance to Granule-Mediated Death

The human granzyme B SerpinB9 is not only expressed by lymphocytes[259,269] but also by dendritic cells,[270] cells at immune privileged sites (testis and placenta),[48,271,272] endothelial and mesothelial cells,[273] and mast cells.[274] Similar results hold for Spi6 in mice.[264,265,275] Modulators of inflammation such as LPS, IFNγ and IL-1β[276,277] induce *SerpinB9* expression. SerpinB9 expression is enhanced by estrogen and hypoxia because of estrogen responsive elements and hypoxia-inducible factor 2 binding sites, respectively, in its promoter.[278,279] In particular, SerpinB9 is induced by hypoxia in neuroblastomas.[280] This expression pattern suggests that SerpinB9 not only protects killer cells and myeloid cells that express granzyme B from autodestruction but also may protect antigen-presenting cells, bystander cells at sites of inflammation, and cells in immune privileged sanctuaries from granzyme B-mediated killing. It may also be a mechanism for tumor evasion of immune surveillance.

No intracellular inhibitors of granzyme A have yet been identified. However, some trypsin inhibitors also inhibit granzyme A. Granzyme A is bound and irreversibly inhibited in the circulation by two trypsin inhibitors, α-2 macroglobulin and antithrombin III.[281] Extracellular granzyme A complexed to proteoglycans is resistant to these two protease inhibitors.[282] A

recent study identified another granzyme A inhibitor, pancreatic secretory trypsin inhibitor, from pancreatic secretions.[283] Pancreatic secretory trypsin inhibitor is found in the blood, particularly in patients with severe inflammation and tissue destruction.[284,285] Unlike the other two granzyme A inhibitors, pancreatic secretory trypsin inhibitor inhibits granzyme A complexed to proteoglycans.[283] It is unclear whether any of these granzyme A inhibitors are expressed in cytotoxic lymphocytes.

Viral Granzyme Inhibitors

A number of viruses produce inhibitors of apoptosis or Bcl-2-like proteins that inhibit caspase-mediated apoptosis, which consequently also inhibit granzyme B-mediated cell death. The pox virus-encoded cytokine response modifier A gene (CrmA) inhibits granzyme B.[286] CrmA directly binds and inhibits granzyme B both in vitro and in vivo. Overexpression of CrmA in target cells inhibits CTL-mediated cell death. CrmA also strongly binds and inhibits caspases-1 and -8 and weakly inhibits other caspases such as caspase-3.[287] Parainfluenza virus type 3 specifically inhibits granzyme B by degrading granzyme B mRNA in infected T cells.[288] Importantly, granzyme A transcripts are not affected by this virus. The mechanism of virus-mediated granzyme B mRNA decay is not known.

Human granzyme B is inhibited by the adenoviral assembly protein (Ad5-100K) by a unique "unserpin"-like mechanism.[143] Ad5-100K rapidly complexes with granzyme B and gets cleaved very slowly at specific sites. Granzyme B that enters the infected target cell during killer cell attack is

saturated by the abundant Ad5-100K protein. Importantly, the slow kinetics of the cleavage reaction ensures that there is always a molar excess of Ad5-100K protein relative to granzyme B. Ad5-100K, which is also needed for virus assembly,[143] does not inhibit caspases or other apoptotic pathways.[144] In fact, the inhibitory activity of Ad5-100K is specific for human granzyme B and not its mouse or rat ortholog.

EXTRACELLULAR ROLES OF GRANZYMES

Although most research has focused on the cell death-inducing properties of granzymes, there is increasing evidence of extracellular functions of granzymes in promoting inflammation and coagulation and degrading extracellular matrix. Low amounts of granzymes A, B, and K are detected in the serum of healthy donors.[289] During inflammation and infection, elevated levels of granzymes are found in serum and other bodily fluids, including the serum of patients undergoing acute cytomegalovirus infection and chronic human immunodeficiency virus infection, the joints of patients with rheumatoid arthritis, and the bronchoalveolar lavage fluid of allergen-challenged patients with asthma and patients with chronic obstructive pulmonary disease.[43,282,290,291,292,293,294] Elevated granzyme levels have also been found in the serum of patients with endotoxemia and bacteremia, reflecting the fact that granzymes (but not perforin) are expressed and secreted by activated myeloid cells and a few other cell types, not just by lymphocytes.[41,44,45,295,296,297,298] In fact, in patients with sepsis, not only is serum granzyme K elevated, but its natural inhibitor (inter-α protein) is depleted, so the free active form of the enzyme is circulating and might cause damage.[299]

Granzyme B has also been detected in macrophages of atheromatous lesions and rheumatoid joints.[296] Proteolysis by extracellular granzymes will be inhibited by serum and extracellular protease inhibitors, such as the trypsin inhibitors, antithrombin III and α-2 macroglobulin.[282] Some conditions that induce extracellular granzymes may also increase the release of intracellular serpins.[300]

Extracellular granzymes might arise from direct secretion —bypassing granule exocytosis—or by leakage from the immune synapse or by release from necrotic cells. Most directly secreted granzymes are secreted as proenzymes, which are inactive.[301] However, some of the proenzymes might be activated extracellularly by serum proteases. It is not known whether the immune synapse forms a perfectly tight gasket that completely prevents granzymes from leaking into the extracellular space during degranulation. Asymmetric synapses termed *kinapses*, which are less stable and less tight, and are formed by cytolytic CD4 T cells and probably under circumstances where the integrated activating signal from the target cell is weaker, may be leakier than the canonical stable synapse.[302] Although extracellular granzymes are not likely to get into the cytoplasm of cells to induce cell death without a high local concentration of perforin, they can proteolyze cell surface receptors or extracellular proteins. Recent studies suggest extracellular granzymes A and K activate macrophages to produce and secrete inflammatory cytokines, although the mechanism for this is not known.[303,304,305] These experiments performed with recombinant and purified granzymes need to be confirmed using cytotoxic cells because macrophages are exquisitely sensitive to immune activation by endotoxin, and it is not possible to verify the absence of endotoxin in preparations of these trypsin-like enzymes because the endotoxin assay measures trypsin activity.[180] Some of the reported extracellular functions/substrates of the granzymes are summarized subsequently, but it is likely that these proteases, despite their high degree of substrate specificity, could have multiple, as yet unappreciated, destructive effects, particularly if present at high concentrations at inflamed sites in the absence of natural inhibitors. The physiologic significance of these extracellular activities is still unclear. One provocative study found a dramatic increase in granzyme B-sufficient versus granzyme B-deficient mice in the rate of rupture of aortic aneurysms in atherogenic mice (deficient in apolipoprotein E [Apo E]) that were also perforin deficient.[306] This result suggests that extracellular granzyme B contributes to the pathogenesis of atherosclerosis.

The known extracellular activities of granzymes suggest a proinflammatory effect. Granzyme A can activate the proinflammatory cytokine IL-1β directly,[307] and granzyme B can convert pro-IL-18 to its active form.[308] Granzyme K can activate proinflammatory cytokine production from lung fibroblasts, probably by cleaving their surface protease-activated receptor-1.[309] Granzymes also degrade extracellular matrix. Granzyme A may be able to degrade heparin sulfate proteoglycans, collagen type IV, and fibronectin.[310,311,312] Granzyme B can remodel the extracellular matrix by cleaving vitronectin, fibronectin, and laminin.[313] Proteolysis of the extracellular matrix might facilitate lymphocyte migration to sites of infection or inflammation or cause tissue destruction at sites of inflammation.[313,314] Granzyme A may also inhibit clotting by cleaving the thrombin receptor and von Willibrand factor[315,316,317] or by activating prourokinase to activate plasminogen.[318] In the central nervous system, granzyme B cleaves

a glutamate receptor (GluR3), potentially contributing to immunoneurotoxicity, excitation, and autoimmunity in the brain.[319,320] Granzyme B on its own causes death of neurons in a pertussis toxin-sensitive manner, suggesting possible cleavage or involvement of G protein-coupled receptors.[321] Other potential granzyme B receptor targets are Notch1 and fibroblast growth factor receptor 1 (FGFR1), which might inhibit growth signals to developing or malignant cells.[322]

DEATH RECEPTOR PATHWAYS

NK cells and cytotoxic T cells can also trigger apoptosis by ligating and activating cell surface tumor necrosis factor (TNF) receptor family members that contain a cytoplasmic approximately 80 amino acid long death domain on target cells[323,324] (Fig. 37.8). Death-by-death receptor ligation can be distinguished from granule-mediated cell death because it is calcium independent and is not inhibited by calcium chelation. The death receptors on target cells form trimers when they are activated. In humans, six members of the larger TNF receptor family contain death domains: Fas (CD95, activated by Fas ligand [FasL, CD95L]), TNFR1 (activated by TNF), DR3 (activated by TNF ligand-related molecule 1 [TL1 or TNFS15]), DR4 and DR5 (activated by TNF-related apoptosisinducing ligand [TRAIL]), and DR6 (unknown ligand). There

are mouse orthologs for all of these, except DR5. The death domains recuit one of two adapter molecules, Fas-associated death domain (FADD) or TNF receptor-associated death domain (TRADD), which serve as a platform for recruiting signaling complexes. Depending on the cellular context, signaling by the death receptors can either trigger caspasemediated apoptosis or proliferative and proinflammatory responses. In general, the receptors that predominantly recruit FADD (Fas, DR4, and DR5) are more likely to trigger apoptosis, whereas signaling from the TRADD-associated receptors (TNR1, DR3, and possibly DR6) is more likely to activate cell survival and proliferation pathways via activation of the NF-κB transcription factor and the JNK and p38 MAP kinase pathways. When cell death is triggered in cells in which the caspase pathway is inhibited, targeted cells undergo an alternate programmed cell death pathway termed necroptosis, mediated by a kinase (receptor-interacting protein 1 [RIP1]) that is recruited by TRADD.[325,326]

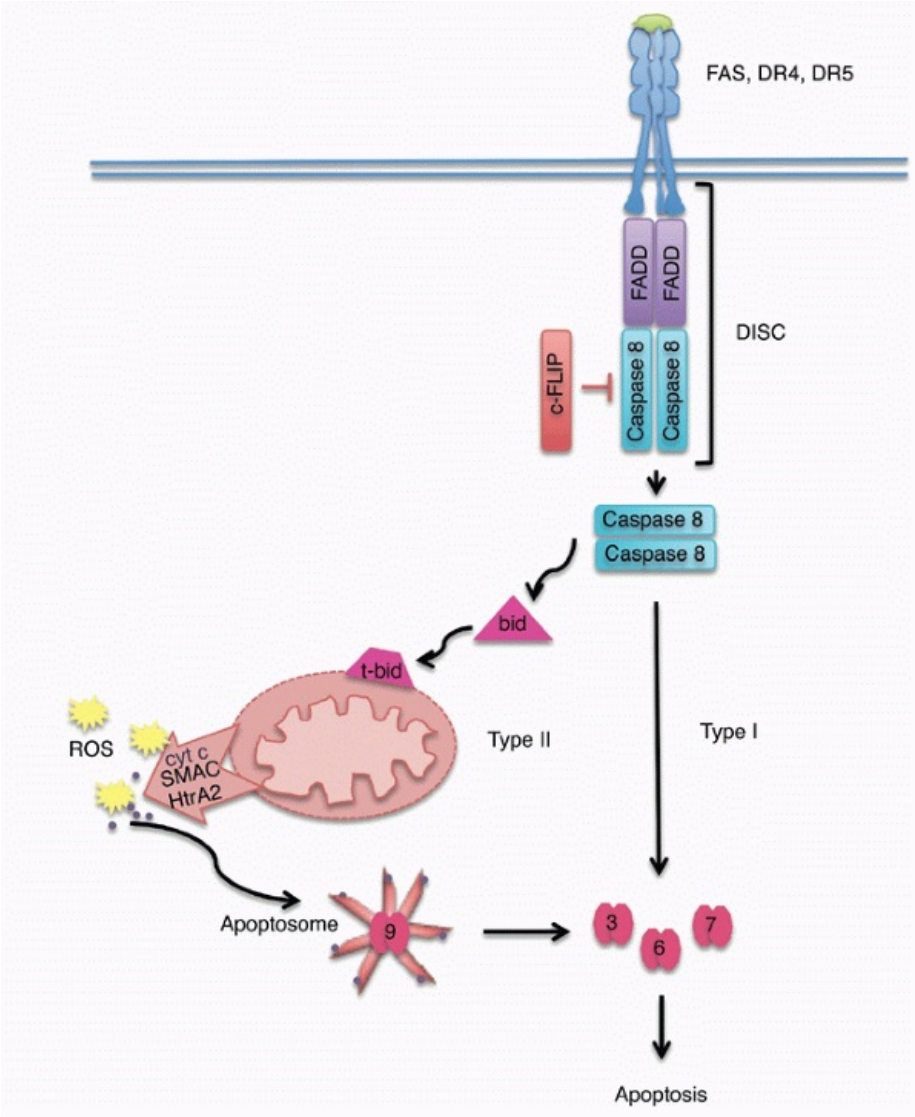

FIG. 37.8. Death Receptor Pathways of Apoptosis. Ligation of a death receptor trimer on target cells recruits the deathinduced signaling complex, which activates caspase-8, releasing it to the cytoplasm where it can cleave Bid to activate mitochondrial apoptotic pathways and cleave and activate the effector caspases-3, -6, and -7. In type I cells, apoptosis does not require mitochondrial amplification, whereas type II cells die only if mitochondrial mediators of apoptosis are released. The caspases activated downstream of caspase-8 are represented by numbered dimers. Cytochrome c required for caspase-9 activation in the apoptosome is represented by a *blue ball*. Caspase-8 activated by death receptor signaling and granzyme B-mediated death are very similar, although the granzyme B-mediated death is much more rapid. Fas-associated death domain can also recruit an alternate signaling complex that leads to cell activation rather than apoptosis (*not shown*).

After death receptor activation and recruitment of FADD, FADD recruits the apical caspases-8 and/or -10 (the latter has no mouse ortholog) to form the death-induced signaling complex (DISC) at the cell membrane. Within the DISC, these caspases are autoproteolyzed and activated. The activated caspases are then released to the cytoplasm where they can cleave

the Bcl-2 family member Bid to activate mitochondrial damage and also cleave and activate the executioner caspases (3, 6, and 7). The mitochondrial pathway amplifies caspase activation by activating caspase-9. Some cells (called type I [eg, thymocytes]) undergo apoptosis without requiring activation of the mitochondrial pathway, whereas others (type II cells [eg, B lymphocytes]) are resistant to cell death if the mitochondrial pathway is blocked.

Humans and mice that are genetically deficient in either FAS or its ligand are able to defend against intracellular pathogens but develop an autoimmune syndrome called autoimmune lymphoproliferative syndrome.[11,327] Fas-mediated death is required to eliminate chronically activated T cells and contributes to elimination of self-reactive immune cells. Mice with genetic deficiencies in these genes develop similar symptoms. Although caspase-8 is considered the main initiator caspase that associates with and is activated by death receptor signaling, humans bearing caspase-8 mutations have defects in T-cell activation and immunodeficiency rather than autoimmunity, which highlights the importance of the nonapoptotic signaling that results from death receptor engagement.[328] Of note, humans with caspase-10 mutations develop autoimmune lymphoproliferative syndrome, suggesting that under certain circumstances, caspase-10 substitutes for caspase-8 in initiating death receptor-mediated apoptosis.[329] Nonapoptotic death receptor signaling, mediated by activation of NF-κB, JNK, and MAP kinase pathways, not only promotes cell proliferation but also has a proinflammatory effect, which involves activating chemokine and cytokine production by macrophages and dendritic cells. The relative strength of proapoptotic and nonapoptotic signaling is determined in part by cellular expression of c-FLIP, an inhibitor of caspase- 8, which is recruited to the DISC and promotes recruitment of RIP1 and TNF receptor-associated factor 2 (TRAF2) to activate nonapoptotic pathways. The c-FLIP mRNA can be spliced into alternate isoforms: c-$FLIP_L$ (long) and c-$FLIP_S$ (short). DISC recruitment of c-$FLIP_S$, which is homologous to caspase-8 but is enzymatically inactive, inhibits apoptosis, whereas the concentration of c-$FLIP_L$ determines whether it is proapoptotic (at low concentrations) or antiapoptotic (at high concentrations). Some tumor cells as well as some activated T cells and NK cells overexpress c-$FLIP_L$, which renders them insensitive to death receptor-mediating apoptosis and promotes their survival.

Mouse studies suggest that DR5 (the ortholog of human DR4 and DR5) and its TRAIL may play an important role in innate immune tumor surveillance.[330] TRAIL may also participate in eliminating activated CD8 CTLs that were primed in the absence of effective CD4 help.[20] Immature NK cells express TRAIL, and DR5-deficient mice are prone to develop tumors and metastases in several endogenous mouse tumor models. As a consequence, soluble TRAIL and agonistic antibodies to DR4 or DR5 are currently being developed for potential tumor immunotherapy.[331,332]

CONCLUSION

Killer lymphocytes in the innate and adaptive immune responses protect us from infection and cellular transformation by releasing cytotoxic granules and help control immune cell proliferation and autoimmunity by both cytotoxic granule release and death receptor-activated cell death. Killer cells trigger multiple programs of cell death, which ensures that the immune system can control pathogens that have devised strategies to resist individual cell death pathways. Lymphocyte-targeted cells are recognized by scavenger cells such as

macrophages that rapidly engulf them and remove them to limit inflammation that occurs when cells die by necrosis. Research in the next few years should provide a better understanding of how cytotoxic gene expression is regulated, how killer cells are protected from their own molecules of destruction, the alternate cell death pathways activated by the multiple granzymes, and how the granzymes overcome the strategies by which viruses and tumors try to evade elimination. Further research will clarify the mechanisms and physiologic importance of inflammatory noncytotoxic effects of killer cell enzymes.

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REFERENCES

1. Russell JH, Ley TJ. Lymphocyte-mediated cytotoxicity. *Annu Rev Immunol.* 2002;20:323-370.

2. Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol.* 2002;2:735-747.

3. Lieberman J. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat Rev Immunol.* 2003;3:361-370.

4. Catalfamo M, Henkart PA. Perforin and the granule exocytosis cytotoxicity pathway. *Curr Opin Immunol.* 2003;15:522-527.

5. Voskoboinik I, Smyth MJ, Trapani JA. Perforin-mediated target-cell death and immune homeostasis. *Nat Rev Immunol.* 2006;6:940-952.

6. Chowdhury D, Lieberman J. Death by a thousand cuts: granzyme pathways of programmed cell death. *Annu Rev Immunol.* 2008;26:389-420.

7. de Saint Basile G, Menasche G, Fischer A. Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. *Nat Rev Immunol.* 2010;10:568-579.

8. Balaji KN, Schaschke N, Machleidt W, et al. Surface cathepsin B protects cytotoxic lymphocytes from self-destruction after degranulation. *J Exp Med.* 2002;196:493-503.

9. Bossi G, Trambas C, Booth S, et al. The secretory synapse: the secrets of a serial killer. *Immunol Rev.* 2002;189:152-160.

10. Pachlopnik Schmid J, Cote M, Menager MM, et al. Inherited defects in lymphocyte cytotoxic activity. *Immunol Rev.* 2010;235:10-23.

11. Bidere N, Su HC, Lenardo MJ. Genetic disorders of programmed cell death in the immune system. *Ann Rev Immunol.* 2006;24:321-352.

12. Savill J, Fadok V. Corpse clearance defines the meaning of cell death. *Nature.* 2000;407:784-748.

13. Lieberman J. Anatomy of a murder: how cytotoxic T cells and NK cells are activated, develop, and eliminate their targets. *Immunol Rev.* 2010;235:5-9.

14. Rutishauser RL, Kaech SM. Generating diversity: transcriptional regulation of effector and memory CD8 T-cell differentiation. *Immunol Rev.* 2010;235:219-233.

15. Houot R, Kohrt HE, Marabelle A, et al. Targeting immune effector cells to promote antibody-induced cytotoxicity in cancer immunotherapy. *Trends Immunol.* 2011;32:510-516.

16. Zajac AJ, Blattman JN, Murali-Krishna K, et al. Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med.* 1998;188:2205-2213.

17. Trimble LA, Lieberman J. Circulating CD8 T lymphocytes in human immunodeficiency virus-infected individuals have impaired function and downmodulate CD3 zeta, the signaling chain of the T-cell receptor complex. *Blood.* 1998;91:585-594.

18. Wherry EJ. T cell exhaustion. *Nature Immunol.* 2011;12:492-499.

19. Cui W, Kaech SM. Generation of effector CD8+ T cells and their conversion to memory T cells. *Immunol Rev.* 2010;236:151-166.

20. Janssen EM, Droin NM, Lemmens EE, et al. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature.* 2005;434:88-93.

21. Araki K, Turner AP, Shaffer VO, et al. mTOR regulates memory CD8 T-cell differentiation. *Nature.* 2009;460:108-112.

22. Zediak VP, Johnnidis JB, Wherry EJ, et al. Cutting edge: persistently open chromatin at effector gene loci in resting memory CD8+ T cells independent of transcriptional status. *J Immunol.* 2011;186:2705-2709.

23. Araki Y, Wang Z, Zang C, et al. Genome-wide analysis of histone methylation reveals chromatin state-based regulation of gene transcription and function of memory CD8+ T cells. *Immunity.* 2009;30:912-925.

24. Northrop JK, Wells AD, Shen H. Cutting edge: chromatin remodeling as a molecular basis for the enhanced functionality of memory CD8 T cells. *J Immunol.* 2008;181:865-

868.

25. Araki Y, Fann M, Wersto R, et al. Histone acetylation facilitates rapid and robust memory CD8 T cell response through differential expression of effector molecules (eomesodermin and its targets: perforin and granzyme B). *J Immunol.* 2008;180:8102-8108.

26. Fann M, Godlove JM, Catalfamo M, et al. Histone acetylation is associated with differential gene expression in the rapid and robust memory CD8(+) T-cell response. *Blood.* 2006;108:3363-3370.

27. Gondek DC, Lu LF, Quezada SA, et al. Cutting edge: contactmediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol*. 2005;174:1783-1786.

28. Grossman WJ, Verbsky JW, Barchet W, et al. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity.* 2004;21:589-601.

29. Czystowska M, Strauss L, Bergmann C, et al. Reciprocal granzyme/perforin-mediated death of human regulatory and responder T cells is regulated by interleukin-2 (IL-2). *J Mol Med.* 2010;88:577-588.

30. Gondek DC, Devries V, Nowak EC, et al. Transplantation survival is maintained by granzyme B+ regulatory cells and adaptive regulatory T cells. *J Immunol.* 2008;181:4752-4760.

31. Cao X, Cai SF, Fehniger TA, et al. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity.* 2007;27:635-636.

32. Fehniger TA, Cai SF, Cao X, et al. Acquisition of murine NK cell cytotoxicity requires the translation of a pre-existing pool of granzyme B and perforin mRNAs. *Immunity.* 2007;26(6):798-811.

33. Vivier E, Sorrell JM, Ackerly M, et al. Developmental regulation of a mucinlike glycoprotein selectively expressed on natural killer cells. *J Exp Med.* 1993;178:2023-2033.

34. Paust S, von Andrian UH. Natural killer cell memory. *Nat Immunol.* 2011;12:500-508.

35. Kelso A, Costelloe EO, Johnson BJ, et al. The genes for perforin, granzymes A-C and IFN-gamma are differentially expressed in single CD8(+) T cells during primary activation. *Int Immunol.* 2002;14:605-613.

36. Johnson BJ, Costelloe EO, Fitzpatrick DR, et al. Single-cell perforin and granzyme expression reveals the anatomical localization of effector CD8+ T cells in influenza virus-

infected mice. *Proc Natl Acad Sci U S A.* 2003;100:2657-2662.

37. Maurice M, Verhoeyen E, Salmon P, et al. Efficient gene transfer into human primary blood lymphocytes by surface-engineered lentiviral vectors that display a T cell-activating polypeptide. *Blood.* 2002;99:2342-2350.

38. Ebnet K, Levelt CN, Tran TT, et al. Transcription of granzyme A and B genes is differentially regulated during lymphoid ontogeny. *J Exp Med.* 1995;181:755-763.

39. Jahrsdorfer B, Blackwell SE, Wooldridge JE, et al. B-chronic lymphocytic leukemia cells and other B cells can produce granzyme B and gain cytotoxic potential after interleukin-21-based activation. *Blood.* 2006;108:2712-2719.

40. Rissoan MC, Duhen T, Bridon JM, et al. Subtractive hybridization reveals the expression of immunoglobulin-like transcript 7, Eph-B1, granzyme B, and 3 novel transcripts in human plasmacytoid dendritic cells. *Blood.* 2002;100:3295-3303.

41. Strik MC, de Koning PJ, Kleijmeer MJ, et al. Human mast cells produce and release the cytotoxic lymphocyte associated protease granzyme B upon activation. *Mol Immunol.* 2007;44:3462-3472.

42. Pardo J, Wallich R, Ebnet K, et al. Granzyme B is expressed in mouse mast cells in vivo and in vitro and causes delayed cell death independent of perforin. *Cell Death Differ.* 2007;14:1768-1779.

43. Tschopp CM, Spiegl N, Didichenko S, et al. Granzyme B, a novel mediator of allergic inflammation: its induction and release in blood basophils and human asthma. *Blood.* 2006;108:2290-2299.

44. Hochegger K, Eller P, Huber JM, et al. Expression of granzyme A in human polymorphonuclear neutrophils. *Immunology.* 2007;121:166-173.

45. Wagner C, Iking-Konert C, Denefleh B, et al. Granzyme B and perforin: constitutive expression in human polymorphonuclear neutrophils. *Blood.* 2004;103:1099-1104.

46. Martin P, Wallich R, Pardo J, et al. Quiescent and activated mouse granulocytes do not express granzyme A and B or perforin: similarities or differences with human polymorphonuclear leukocytes? *Blood*. 2005;106:2871-2878.

47. Metkar SS, Froelich CJ. Human neutrophils lack granzyme A, granzyme B, and perforin. *Blood.* 2004;104:905-906; author reply 907-908.

48. Hirst CE, Buzza MS, Sutton VR, et al. Perforin-independent expression of granzyme B and proteinase inhibitor 9 in human testis and placenta suggests a role for granzyme B-mediated proteolysis in reproduction. *Mol Hum Reprod.* 2001;7:1133-1142.

49. Sasson R, Dantes A, Tajima K, et al. Novel genes modulated by FSH in normal and immortalized FSH-responsive cells: new insights into the mechanism of FSH action. *FASEB J.* 2003;17:1256-1266.

50. Horiuchi K, Saito S, Sasaki R, et al. Expression of granzyme B in human articular chondrocytes. *J Rheumatol.* 2003;30:1799-1810.

51. Taniguchi M, Tani N, Suemoto T, et al. High expression of alternative transcript of granzyme M in the mouse retina. *Neurosci Res.* 1999;34:115-123.

52. Suemoto T, Taniguchi M, Shiosaka S, et al. cDNA cloning and expression of a novel serine protease in the mouse brain. *Brain Res Mol Brain Res.* 1999;70:273-281.

53. Prendergast JA, Helgason CD, Bleackley RC. Quantitative polymerase chain reaction analysis of cytotoxic cell proteinase gene transcripts in T cells. Pattern of expression is dependent on the nature of the stimulus. *J Biol Chem.* 1992;267:5090-5095.

54. Garcia-Sanz JA, MacDonald HR, Jenne DE, et al. Cell specificity of granzyme gene expression. *J Immunol.* 1990;145:3111-3118.

55. Ebnet K, Chluba-de Tapia J, Hurtenbach U, et al. In vivo primed mouse T cells selectively express T cell-specific serine proteinase-1 and the proteinase-like molecules granzyme B and C. *Int Immunol.* 1991;3:9-19.

56. Grossman WJ, Verbsky JW, Tollefsen BL, et al. Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood.* 2004;104:2840-2848.

57. Liu CC, Rafii S, Granelli-Piperno A, et al. Perforin and serine esterase gene expression in stimulated human T cells. Kinetics, mitogen requirements, and effects of cyclosporin A. *J Exp Med.* 1989;170:2105-2118.

58. Janas ML, Groves P, Kienzle N, et al. IL-2 regulates perforin and granzyme gene expression in CD8+ T cells independently of its effects on survival and proliferation. *J Immunol.* 2005;175:8003-8010.

59. Kundig TM, Schorle H, Bachmann MF, et al. Immune responses in interleukin-2-deficient mice. *Science.* 1993;262:1059-1061.

60. Steiger J, Nickerson PW, Steurer W, et al. IL-2 knockout recipient mice reject islet cell allografts. *J Immunol.* 1995;155:489-498.

61. Kramer S, Mamalaki C, Horak I, et al. Thymic selection and peptide-induced activation of T cell receptor-transgenic CD8 T cells in interleukin-2-deficient mice. *Eur J*

Immunol. 1994;24:2317-2322.

62. Ye W, Young JD, Liu CC. Interleukin-15 induces the expression of mRNAs of cytolytic mediators and augments cytotoxic activities in primary murine lymphocytes. *Cell Immunol.* 1996;174:54-62.

63. Zeng R, Spolski R, Finkelstein SE, et al. Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function. *J Exp Med*. 2005;201:139-148.

64. Leonard WJ, Spolski R. Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. *Nat Rev Immunol.* 2005;5: 688-698.

65. White L, Krishnan S, Strbo N, et al. Differential effects of IL-21 and IL-15 on perforin expression, lysosomal degranulation, and proliferation in CD8 T cells of patients with human immunodeficiency virus-1 (HIV). *Blood.* 2007;109:3873-3880.

66. Yamamoto K, Shibata F, Miyasaka N, et al. The human perforin gene is a direct target of STAT4 activated by IL-12 in NK cells. *Biochem Biophys Res Commun.* 2002;297:1245-1252.

67. Morishima N, Owaki T, Asakawa M, et al. Augmentation of effector CD8+ T cell generation with enhanced granzyme B expression by IL-27. *J Immunol.* 2005;175:1686-1693.

68. Szabo SJ, Kim ST, Costa GL, et al. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell.* 2000;100:655-669.

69. Glimcher LH, Townsend MJ, Sullivan BM, et al. Recent developments in the transcriptional regulation of cytolytic effector cells. *Nat Rev Immunol.* 2004;4:900-911.

70. Werneck MB, Lugo-Villarino G, Hwang ES, et al. T-bet plays a key role in NK-mediated control of melanoma metastatic disease. *J Immunol*. 2008;180:8004-8010.

71. Pearce EL, Mullen AC, Martins GA, et al. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. *Science*. 2003;302:1041-1043.

72. Cruz-Guilloty F, Pipkin ME, Djuretic IM, et al. Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs. *J Exp Med.* 2009;206:51-59.

73. Cho OH, Shin HM, Miele L, et al. Notch regulates cytolytic effector function in CD8+ T cells. *J Immunol.* 2009;182:3380-3389.

74. Intlekofer AM, Banerjee A, Takemoto N, et al. Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin. *Science.* 2008;321:408-411.

75. Zhu Y, Ju S, Chen E, et al. T-bet and eomesodermin are required for T cell-mediated antitumor immune responses. *J Immunol.* 2010;185: 3174-3183.

76. Banerjee A, Gordon SM, Intlekofer AM, et al. Cutting edge: the transcription factor eomesodermin enables CD8+ T cells to compete for the memory cell niche. *J Immunol.* 2010;185:4988-4992.

77. Pipkin ME, Ljutic B, Cruz-Guilloty F, et al. Chromosome transfer activates and delineates a locus control region for perforin. *Immunity.* 2007;26:29-41.

78. Pipkin ME, Rao A, Lichtenheld MG. The transcriptional control of the perforin locus. *Immunol Rev.* 2010;235:55-72.

79. Duggan BL, Cabilio NR, Dickie P, et al. A novel lineage-specific hypersensitive site is essential for position independent granzyme B expression in transgenic mice. *Biochem Biophys Res Commun.* 2008;368:357-363.

80. Fregeau CJ, Bleackley RC. Transcription of two cytotoxic cell protease genes is under the control of different regulatory elements. *Nucleic Acids Res.* 1991;19:5583-5590.

81. Babichuk CK, Duggan BL, Bleackley RC. In vivo regulation of murine granzyme B gene transcription in activated primary T cells. *J Biol Chem.* 1996;271:16485-16493.

82. Wargnier A, Legros-Maida S, Bosselut R, et al. Identification of human granzyme B promoter regulatory elements interacting with activated T-cell-specific proteins: implication of Ikaros and CBF binding sites in promoter activation. *Proc Natl Acad U S A.* 1995;92:6930-6934.

83. Haddad P, Wargnier A, Bourge JF, et al. A promoter element of the human serine esterase granzyme B gene controls specific transcription in activated T cells. *Eur J Immunol.* 1993;23:625-629.

84. Babichuk CK, Bleackley RC. Mutational analysis of the murine granzyme B gene promoter in primary T cells and a T cell clone. *J Biol Chem.* 1997;272:18564-18571.

85. Zimmerer JM, Lesinski GB, Radmacher MD, et al. STAT1-dependent and STAT1-independent gene expression in murine immune cells following stimulation with interferon-alpha. *Cancer Immunol Immunother.* 2007;56:1845-1852.

86. Kaech SM, Hemby S, Kersh E, et al. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell.* 2002;111:837-851.

87. Fehniger TA, Wylie T, Germino E, et al. Next-generation sequencing identifies the natural killer cell microRNA transcriptome. *Genome Res.* 2010;20:1590-1604.

88. Kim TD, Lee SU, Yun S, et al. Human microRNA-27a* targets Prf1 and GzmBexpression to regulate NK cell cytotoxicity. *Blood.* 2011;118: 5476-5486.

89. Peters PJ, Borst J, Oorschot V, et al. Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *J Exp Med.* 1991;173:1099-1109.

90. Bell JK, Goetz DH, Mahrus S, et al. The oligomeric structure of human granzyme A is a determinant of its extended substrate specificity. *Nat Struct Biol.* 2003;10:527-534.

91. Hink-Schauer C, Estebanez-Perpina E, Wilharm E, et al. The 2.2-A crystal structure of human pro-granzyme K reveals a rigid zymogen with unusual features. *J Biol Chem.* 2002;277:50923-50933.

92. Casciola-Rosen L, Garcia-Calvo M, Bull HG, et al. Mouse and human granzyme B have distinct tetrapeptide specificities and abilities to recruit the bid pathway. *J Biol Chem.* 2007;282:4545-4552.

93. Estebanez-Perpina E, Fuentes-Prior P, Belorgey D, et al. Crystal structure of the caspase activator human granzyme B, a proteinase highly specific for an Asp-P1 residue. *Biol Chem.* 2000;381:1203-1214.

94. Wu L, Wang L, Hua G, et al. Structural basis for proteolytic specificity of the human apoptosis-inducing granzyme M. *J Immunol.* 2009;183: 421-429.

95. Kaiserman D, Buckle AM, Van Damme P, et al. Structure of granzyme C reveals an unusual mechanism of protease autoinhibition. *Proc Natl Acad U S A.* 2009;106:5587-5592.

96. Hink-Schauer C, Estebanez-Perpina E, Kurschus FC, et al. Crystal structure of the apoptosis-inducing human granzyme A dimer. *Nat Struct Biol.* 2003;10:535-540.

97. Masson D, Peters PJ, Geuze HJ, et al. Interaction of chondroitin sulfate with perforin and granzymes of cytolytic T-cells is dependent on pH. *Biochemistry.* 1990;29:11229-11235.

98. Metkar SS, Wang B, Aguilar-Santelises M, et al. Cytotoxic cell granulemediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation. *Immunity.* 2002;16:417-428.

99. Bossi G, Griffiths GM. CTL secretory lysosomes: biogenesis and secretion of a harmful organelle. *Semin Immunol.* 2005;17:87-94.

100. Orange JS. The lytic NK cell immunological synapse and sequential steps in its formation. *Adv Exp Med Biol.* 2007;601:225-233.

101. Liu D, Bryceson YT, Meckel T, et al. Integrin-dependent organization and bidirectional vesicular traffic at cytotoxic immune synapses. *Immunity.* 2009;31:99-109.

102. Dustin ML, Long EO. Cytotoxic immunological synapses. *Immunol Rev.* 2010;235:24-34.

103. Dustin ML. Modular design of immunological synapses and kinapses. *Cold Spr Harb Persp Biol.* 2009;1:a002873.

104. Andzelm MM, Chen X, Krzewski K, et al. Myosin IIA is required for cytolytic granule exocytosis in human NK cells. *J Exp Med.* 2007;204:2285-2291.

105. Rak GD, Mace EM, Banerjee PP, et al. Natural killer cell lytic granule secretion occurs through a pervasive actin network at the immune synapse. *PLoS Biol.* 2011;9:e1001151.

106. Kanwar N, Wilkins JA. IQGAP1 involvement in MTOC and granule polarization in NK-cell cytotoxicity. *Eur J Immunol.* 2011;41: 2763-2773.

107. Brown AC, Oddos S, Dobbie IM, et al. Remodelling of cortical actin where lytic granules dock at natural killer cell immune synapses revealed by super-resolution microscopy. *PLoS Biol.* 2011;9:e1001152.

108. Stinchcombe JC, Bossi G, Booth S, et al. The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity*. 2001;15:751-761.

109. Krzewski K, Gil-Krzewska A, Watts J, et al. VAMP4- and VAMP7- expressing vesicles are both required for cytotoxic granule exocytosis in NK cells. *Eur J Immunol.* 2011;41(11):3323-3329.

110. Loo LS, Hwang LA, Ong YM, et al. A role for endobrevin/VAMP8 in CTL lytic granule exocytosis. *Eur J Immunol.* 2009;39:3520-3528.

111. Purbhoo MA, Irvine DJ, Huppa JB, et al. T cell killing does not require the formation of a stable mature immunological synapse. *Nat Immunol.* 2004;5:524-530.

112. Kagi D, Ledermann B, Burki K, et al. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice [see comments]. *Nature.* 1994;369:31-37.

113. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science*. 1999;286:1957-1959.

114. Goransdotter Ericson K, Fadeel B, Nilsson-Ardnor S, et al. Spectrum of perforin

gene mutations in familial hemophagocytic lymphohistiocytosis. *Am J Hum Genet.* 2001;68:590-597.

115. Feldmann J, Le Deist F, Ouachee-Chardin M, et al. Functional consequences of perforin gene mutations in 22 patients with familial haemophagocytic lymphohistiocytosis. *Br J Haematol.* 2002;117:965-972.

116. Menasche G, Feldmann J, Fischer A, et al. Primary hemophagocytic syndromes point to a direct link between lymphocyte cytotoxicity and homeostasis. *Immunol Rev.* 2005;203:165-179.

117. Feldmann J, Menasche G, Callebaut I, et al. Severe and progressive encephalitis as a presenting manifestation of a novel missense perforin mutation and impaired cytolytic activity. *Blood.* 2005;105:2658-2663.

118. Dufourcq-Lagelouse R, Pastural E, Barrat FJ, et al. Genetic basis of hemophagocytic lymphohistiocytosis syndrome (Review). *Int J Mol Med.* 1999;4:127-133.

119. Pachlopnik Schmid J, Ho CH, Chretien F, et al. Neutralization of IFNgamma defeats haemophagocytosis in LCMV-infected perforinand Rab27a-deficient mice. *EMBO Mol Med.* 2009;1:112-124.

120. Voskoboinik I, Thia MC, De Bono A, et al. The functional basis for hemophagocytic lymphohistiocytosis in a patient with co-inherited missense mutations in the perforin (PFN1) gene. *J Exp Med.* 2004;200: 811-816.

121. van Dommelen SL, Sumaria N, Schreiber RD, et al. Perforin and granzymes have distinct roles in defensive immunity and immunopathology. *Immunity.* 2006;25:835-848.

122. Mullbacher A, Ebnet K, Blanden RV, et al. Granzyme A is critical for recovery of mice from infection with the natural cytopathic viral pathogen, ectromelia. *Proc Natl Acad Sci U S A.* 1996;93:5783-5787.

123. Graubert TA, Russell JH, Ley TJ. The role of granzyme B in murine models of acute graft-versus-host disease and graft rejection. *Blood.* 1996;87:1232-1237.

124. Revell PA, Grossman WJ, Thomas DA, et al. Granzyme B and the downstream granzymes C and/or F are important for cytotoxic lymphocyte functions. *J Immunol.* 2005;174:2124-2131.

125. Simon MM, Hausmann M, Tran T, et al. In vitro- and ex vivo-derived cytolytic leukocytes from granzyme A × B double knockout mice are defective in granule-mediated apoptosis but not lysis of target cells. *J Exp Med.* 1997;186:1781-1786.

126. Davis JE, Smyth MJ, Trapani JA. Granzyme A and B-deficient killer lymphocytes are defective in eliciting DNA fragmentation but retain potent in vivo anti-tumor capacity. *Eur J Immunol.* 2001;31:39-47.

127. Pardo J, Balkow S, Anel A, et al. The differential contribution of granzyme A and granzyme B in cytotoxic T lymphocyte-mediated apoptosis is determined by the quality of target cells. *Eur J Immunol.* 2002;32:1980-1985.

128. Krenacs L, Smyth MJ, Bagdi E, et al. The serine protease granzyme M is preferentially expressed in NK-cell, gamma delta T-cell, and intestinal T-cell lymphomas: evidence of origin from lymphocytes involved in innate immunity. *Blood.* 2003;101:3590-3593.

129. Pardo J, Bosque A, Brehm R, et al. Apoptotic pathways are selectively activated by granzyme A and/or granzyme B in CTL-mediated target cell lysis. *J Cell Biol.* 2004;167:457-468.

130. Waterhouse NJ, Sedelies KA, Sutton VR, et al. Functional dissociation of DeltaPsim and cytochrome c release defines the contribution of mitochondria upstream of caspase activation during granzyme B-induced apoptosis. *Cell Death Differ.* 2006;13:607-618.

131. Waterhouse NJ, Sutton VR, Sedelies KA, et al. Cytotoxic T lymphocyte-induced killing in the absence of granzymes A and B is unique and distinct from both apoptosis and perforin-dependent lysis. *J Cell Biol.* 2006;173:133-144.

132. MacDonald G, Shi L, Vande Velde C, et al. Mitochondria-dependent and -independent regulation of Granzyme B-induced apoptosis. *J Exp Med.* 1999;189:131-144.

133. Johnson H, Scorrano L, Korsmeyer SJ, et al. Cell death induced by granzyme C. *Blood.* 2003;101:3093-3101.

134. Kelly JM, Waterhouse NJ, Cretney E, et al. Granzyme M mediates a novel form of perforin-dependent cell death. *J Biol Chem.* 2004;279:22236-22242.

135. Pao LI, Sumaria N, Kelly JM, et al. Functional analysis of granzyme M and its role in immunity to infection. *J Immunol.* 2005;175:3235-3243.

136. Raja SM, Metkar SS, Honing S, et al. A novel mechanism for protein delivery: granzyme B undergoes electrostatic exchange from serglycin to target cells. *J Biol Chem.* 2005;280:20752-20761.

137. Kurschus FC, Bruno R, Fellows E, et al. Membrane receptors are not required to deliver granzyme B during killer cell attack. *Blood.* 2005;105:2049-2058.

138. Bird CH, Sun J, Ung K, et al. Cationic sites on granzyme B contribute to cytotoxicity by promoting its uptake into target cells. *Mol Cell Biol.* 2005;25:7854-7867.

139. Shi L, Keefe D, Durand E, et al. Granzyme B binds to target cells mostly by charge and must be added at the same time as perforin to trigger apoptosis. *J Immunol.* 2005;174:5456-5461.

140. Motyka B, Korbutt G, Pinkoski MJ, et al. Mannose 6-phosphate/insulin-like growth factor II receptor is a death receptor for granzyme B during cytotoxic T cell-induced apoptosis. *Cell.* 2000;103:491-500.

141. Dressel R, Raja SM, Honing S, et al. Granzyme-mediated cytotoxicity does not involve the mannose 6-phosphate receptors on target cells. *J Biol Chem.* 2004;279:20200-20210.

142. Trapani JA, Sutton VR, Thia KY, et al. A clathrin/dynamin- and mannose-6-phosphate receptor-independent pathway for granzyme B-induced cell death. *J Cell Biol.* 2003;160:223-233.

143. Andrade F, Bull HG, Thornberry NA, et al. Adenovirus L4-100K assembly protein is a granzyme B substrate that potently inhibits granzyme B-mediated cell death. *Immunity.* 2001;14:751-761.

144. Andrade F, Casciola-Rosen LA, Rosen A. A novel domain in adenovirus L4-100K is required for stable binding and efficient inhibition of human granzyme B: possible interaction with a species-specific exosite. *Mol Cell Biol.* 2003;23:6315-6326.

145. Andrade F, Fellows E, Jenne DE, et al. Granzyme H destroys the function of critical adenoviral proteins required for viral DNA replication and granzyme B inhibition. *EMBO J.* 2007;26:2148-2457.

146. Pipkin ME, Lieberman J. Delivering the kiss of death: progress on understanding how perforin works. *Curr Opin Immunol.* 2007;19:301-308.

147. Voskoboinik I, Dunstone MA, Baran K, et al. Perforin: structure, function, and role in human immunopathology. *Immunol Rev.* 2010;235: 35-54.

148. Sauer H, Pratsch L, Tschopp J, et al. Functional size of complement and perforin pores compared by confocal laser scanning microscopy and fluorescence microphotolysis. *Biochim Biophys Acta.* 1991;1063: 137-146.

149. Millard PJ, Henkart MP, Reynolds CW, et al. Purification and properties of cytoplasmic granules from cytotoxic rat LGL tumors. *J Immunol*. 1984;132:3197-3204.

150. Podack ER, Young JD, Cohn ZA. Isolation and biochemical and functional

characterization of perforin 1 from cytolytic T cell granules. *Proc Natl Acad Sci U S A.* 1985;82:8629-8633.

151. Tschopp J, Masson D, Stanley KK. Structural/functional similarity between proteins involved in complement- and cytotoxic T-lymphocyte-mediated cytolysis. *Nature.* 1986;322:831-834.

152. Law RH, Lukoyanova N, Voskoboinik I, et al. The structural basis for membrane binding and pore formation by lymphocyte perforin. *Nature.* 2010;468:447-451.

153. Praper T, Sonnen A, Viero G, et al. Human perforin employs different avenues to damage membranes. *J Biol Chem.* 2011;286:2946-2955.

154. Thiery J, Keefe D, Boulant S, et al. Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. *Nat Immunol.* 2011;12:770-777.

155. Brennan AJ, Chia J, Browne KA, et al. Protection from endogenous perforin: glycans and the C terminus regulate exocytic trafficking in cytotoxic lymphocytes. *Immunity.* 2011;34:879-892.

156. Uellner R, Zvelebil MJ, Hopkins J, et al. Perforin is activated by a proteolytic cleavage during biosynthesis which reveals a phospholipidbinding C2 domain. *EMBO J.* 1997;16:7287-7296.

157. Keefe D, Shi L, Feske S, et al. Perforin triggers a plasma membranerepair response that facilitates CTL induction of apoptosis. *Immunity*. 2005;23:249-262.

158. Thiery J, Keefe D, Saffarian S, et al. Perforin activates clathrin- and dynamin-dependent endocytosis, which is required for plasma membrane repair and delivery of granzyme B for granzyme-mediated apoptosis. *Blood.* 2010;115:1582-1593.

159. Terasaki M, Miyake K, McNeil PL. Large plasma membrane disruptions are rapidly resealed by Ca2+-dependent vesicle-vesicle fusion events. *J Cell Biol.* 1997;139:63-74.

160. Idone V, Tam C, Goss JW, et al. Repair of injured plasma membrane by rapid Ca2+-dependent endocytosis. *J Cell Biol.* 2008;180:905-914.

161. McNeil PL, Kirchhausen T. An emergency response team for membrane repair. *Nature Rev Mol Cell Biol.* 2005;6:499-505.

162. Browne KA, Blink E, Sutton VR, et al. Cytosolic delivery of granzyme B by bacterial toxins: evidence that endosomal disruption, in addition to transmembrane pore formation, is an important function of perforin. *Mol Cell Biol.* 1999;19:8604-8615.

163. Gross C, Koelch W, DeMaio A, et al. Cell surface-bound heat shock protein 70 (Hsp70) mediates perforin-independent apoptosis by specific binding and uptake of granzyme B. *J Biol Chem.* 2003;278:41173-41181.

164. Getachew Y, Stout-Delgado H, Miller BC, et al. Granzyme C supports efficient CTL-mediated killing late in primary alloimmune responses. *J Immunol.* 2008;181:7810-7817.

165. Barry M, Heibein JA, Pinkoski MJ, et al. Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. *Mol Cell Biol* . 2000;20:3781-3794.

166. Heibein JA, Goping IS, Barry M, et al. Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members bid and Bax. *J Exp Med.* 2000;192:1391-1402.

167. Sutton VR, Davis JE, Cancilla M, et al. Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation. *J Exp Med.* 2000;192:1403-1414.

168. Alimonti JB, Shi L, Baijal PK, et al. Granzyme B induces BID-mediated cytochrome c release and mitochondrial permeability transition. *J Biol Chem.* 2001;276:6974-4982.

169. Thomas DA, Scorrano L, Putcha GV, et al. Granzyme B can cause mitochondrial depolarization and cell death in the absence of BID, BAX, and BAK. *Proc Natl Acad Sci U S A.* 2001;98:14985-14990.

170. Hou Q, Zhao T, Zhang H, et al. Granzyme H induces apoptosis of target tumor cells characterized by DNA fragmentation and Biddependent mitochondrial damage. *Mol Immunol.* 2008;45:1044-1055.

171. Zhao T, Zhang H, Guo Y, et al. Granzyme K directly processes bid to release cytochrome c and endonuclease G leading to mitochondriadependent cell death. *J Biol Chem.* 2007;282:12104-12111.

172. Martinvalet D, Dykxhoorn DM, Ferrini R, et al. Granzyme A cleaves a mitochondrial complex I protein to initiate caspase-independent cell death. *Cell.* 2008;133:681-692.

173. Martinvalet D, Zhu P, Lieberman J. Granzyme A induces caspase-independent mitochondrial damage, a required first step for apoptosis. *Immunity.* 2005;22:355-370.

174. Han J, Goldstein LA, Hou W, et al. Deregulation of mitochondrial membrane potential by mitochondrial insertion of granzyme B and direct Hax-1 cleavage. *J Biol Chem.* 2010;285:22461-22472.

175. Jans DA, Briggs LJ, Jans P, et al. Nuclear targeting of the serine protease granzyme A (fragmentin-1). *J Cell Sci.* 1998;111(pt 17):2645-2654.

176. Jans DA, Jans P, Briggs LJ, et al. Nuclear transport of granzyme B (fragmentin-2). Dependence of perforin in vivo and cytosolic factors in vitro. *J Biol Chem.* 1996;271:30781-30789.

177. Blink EJ, Jiansheng Z, Hu W, et al. Interaction of the nuclear localizing cytolytic granule serine protease granzyme B with importin alpha or beta: modulation by the serpin inhibitor PI-9. *J Cell Biochem.* 2005;95:598-610.

178. Beresford PJ, Xia Z, Greenberg AH, et al. Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. *Immunity.* 1999;10:585-594.

179. Shresta S, Graubert TA, Thomas DA, et al. Granzyme A initiates an alternative pathway for granule-mediated apoptosis. *Immunity.* 1999;10: 595-605.

180. Lieberman J. Granzyme A activates another way to die. *Immunol Rev.* 2010;235:93-104.

181. Praveen K, Evans DL, Jaso-Friedmann L. Evidence for the existence of granzyme-like serine proteases in teleost cytotoxic cells. *J Mol Evol.* 2004;58:449-459.

182. Beresford PJ, Zhang D, Oh DY, et al. Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks. *J Biol Chem.* 2001;276:43285-43293.

183. Beresford PJ, Kam CM, Powers JC, et al. Recombinant human granzyme A binds to two putative HLA-associated proteins and cleaves one of them. *Proc Natl Acad Sci U S A.* 1997;94:9285-9290.

184. Chowdhury D, Beresford PJ, Zhu P, et al. The exonuclease TREX1 is in the SET complex and acts in concert with NM23-H1 to degrade DNA during granzyme A-mediated cell death. *Mol Cell.* 2006;23: 133-142.

185. Fan Z, Beresford PJ, Oh DY, et al. Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. *Cell.* 2003;112: 659-672.

186. Fan Z, Beresford PJ, Zhang D, et al. HMG2 interacts with the nucleosome assembly protein SET and is a target of the cytotoxic T-lymphocyte protease granzyme A. *Mol Cell Biol.* 2002;22:2810-2820.

187. Fan Z, Beresford PJ, Zhang D, et al. Cleaving the oxidative repair protein Ape1 enhances cell death mediated by granzyme A. *Nat Immunol*. 2003;4:145-153.

188. Yan N, Cherepanov P, Daigle JE, et al. The SET Complex Acts as a barrier to autointegration of HIV-1. *PLoS Pathog.* 2009;5:e1000327.

189. Morita M, Stamp G, Robins P, et al. Gene-targeted mice lacking the Trex1 (DNase III) 3′—>5′ DNA exonuclease develop inflammatory myocarditis. *Mol Cell Biol.* 2004;24:6719-6727.

190. Stetson DB, Ko JS, Heidmann T, et al. Trex1 prevents cell-intrinsic initiation of autoimmunity. *Cell.* 2008;134:587-598.

191. Yan N, Regalado-Magdos AD, Stiggelbout B, et al. The cytosolic exonuclease TREX1 inhibits the innate immune response to human immunodeficiency virus type 1. *Nat Immunol.* 2010;11:1005-1013.

192. Yan N, Lieberman J. Gaining a foothold: how HIV avoids innate immune recognition. *Curr Opin Immunol.* 2011;23:21-28.

193. Crow YJ, Hayward BE, Parmar R, et al. Mutations in the gene encoding the 3′-5′ DNA exonuclease TREX1 cause Aicardi-Goutieres syndrome at the AGS1 locus. *Nat Genet.* 2006;38:917-920.

194. Lee-Kirsch MA, Chowdhury D, Harvey S, et al. A mutation in TREX1 that impairs susceptibility to granzyme A-mediated cell death underlies familial chilblain lupus. *J Mol Med.* 2007;85:531-537.

195. Lee-Kirsch MA, Gong M, Chowdhury D, et al. Mutations in the gene encoding the 3′-5′ DNA exonuclease TREX1 are associated with systemic lupus erythematosus. *Nat Genet.* 2007;39:1065-1067.

196. Rice G, Newman WG, Dean J, et al. Heterozygous mutations in TREX1 cause familial chilblain lupus and dominant Aicardi-Goutieres syndrome. *Am J Hum Genet.* 2007;80:811-815.

197. Richards A, van den Maagdenberg AM, Jen JC, et al. C-terminal truncations in human 3′-5′ DNA exonuclease TREX1 cause autosomal dominant retinal vasculopathy with cerebral leukodystrophy. *Nat Genet.* 2007;39:1068-1070.

198. Zhu P, Zhang D, Chowdhury D, et al. Granzyme A, which causes single-stranded DNA damage, targets the double-strand break repair protein Ku70. *EMBO Rep.* 2006;7:431-437.

199. Zhu P, Martinvalet D, Zhang D, et al. The cytotoxic T lymphocyte protease granzyme A cleaves and inactivates poly(adenosine 5 ′-diphosphateribose) polymerase-1. *Blood.* 2009;114:1205-1216.

200. Zhang D, Beresford PJ, Greenberg AH, et al. Granzymes A and B directly cleave lamins and disrupt the nuclear lamina during granulemediated cytolysis. *Proc Natl Acad Sci U S A.* 2001;98:5746-5751.

201. Zhang D, Pasternack MS, Beresford PJ, et al. Induction of rapid histone degradation by the cytotoxic T lymphocyte protease granzyme A. *J Biol Chem.* 2001;276:3683-3690.

202. Lord SJ, Rajotte RV, Korbutt GS, et al. Granzyme B: a natural born killer. *Immunol Rev.* 2003;193:31-38.

203. Trapani JA, Sutton VR. Granzyme B: pro-apoptotic, antiviral and antitumor functions. *Curr Opin Immunol.* 2003;15:533-543.

204. Darmon AJ, Nicholson DW, Bleackley RC. Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature.* 1995;377:446-448.

205. Adrain C, Murphy BM, Martin SJ. Molecular ordering of the caspase activation cascade initiated by the cytotoxic T lymphocyte/natural killer (CTL/NK) protease granzyme B. *J Biol Chem.* 2005;280:4663-4673.

206. Cullen SP, Adrain C, Luthi AU, et al. Human and murine granzyme B exhibit divergent substrate preferences. *J Cell Biol.* 2007;176:435-444.

207. Thomas DA, Du C, Xu M, et al. DFF45/ICAD can be directly processed by granzyme B during the induction of apoptosis. *Immunity.* 2000;12:621-632.

208. Sharif-Askari E, Alam A, Rheaume E, et al. Direct cleavage of the human DNA fragmentation factor-45 by granzyme B induces caspase-activated DNase release and DNA fragmentation. *EMBO J.* 2001;20:3101-3113.

209. Thornberry NA, Rano TA, Peterson EP, et al. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J Biol Chem.* 1997;272:17907-17911.

210. Han J, Goldstein LA, Gastman BR, et al. Degradation of Mcl-1 by granzyme B: implications for Bim-mediated mitochondrial apoptotic events. *J Biol Chem.* 2004;279:22020-22029.

211. Heibein JA, Barry M, Motyka B, et al. Granzyme B-induced loss of mitochondrial inner membrane potential (Delta Psi m) and cytochrome c release are caspase independent. *J Immunol.* 1999;163:4683-4693.

212. Wang GQ, Wieckowski E, Goldstein LA, et al. Resistance to granzyme B-mediated cytochrome c release in Bak-deficient cells. *J Exp Med.* 2001;194:1325-1337.

213. Aguilo JI, Anel A, Catalan E, et al. Granzyme B of cytotoxic T cells induces extramitochondrial reactive oxygen species production via caspasedependent NADPH oxidase activation. *Immunol Cell Biol.* 2010;88:545-554.

214. McIlroy D, Cartron PF, Tuffery P, et al. A triple-mutated allele of granzyme B incapable of inducing apoptosis. *Proc Natl Acad Sci U S A.* 2003;100:2562-2567.

215. Sun J, Bird CH, Thia KY, et al. Granzyme B encoded by the commonly occurring human RAH allele retains pro-apoptotic activity. *J Biol Chem.* 2004;279:16907-16911.

216. Fellows E, Gil-Parrado S, Jenne DE, et al. Natural killer cell-derived human granzyme H induces an alternative, caspase-independent celldeath program. *Blood.* 2007;110:544-552.

217. Haddad P, Jenne D, Tschopp J, et al. Structure and evolutionary origin of the human granzyme H gene. *Int Immunol.* 1991;3:57-66.

218. Romero V, Fellows E, Jenne DE, et al. Cleavage of La protein by granzyme H induces cytoplasmic translocation and interferes with La-mediated HCV-IRES translational activity. *Cell Death Diff*. 2009;16:340-348.

219. Shresta S, Goda P, Wesselschmidt R, et al. Residual cytotoxicity and granzyme K expression in granzyme A-deficient cytotoxic lymphocytes. *J Biol Chem.* 1997;272:20236-20244.

220. Ebnet K, Hausmann M, Lehmann-Grube F, et al. Granzyme A-deficient mice retain potent cell-mediated cytotoxicity. *EMBO J.* 1995;14:4230-4239.

221. Wilharm E, Parry MA, Friebel R, et al. Generation of catalytically active granzyme K from Escherichia coli inclusion bodies and identification of efficient granzyme K inhibitors in human plasma. *J Biol Chem*. 1999;274:27331-27337.

222. Shi L, Kam CM, Powers JC, et al. Purification of three cytotoxic lymphocyte granule serine proteases that induce apoptosis through distinct substrate and target cell interactions. *J Exp Med.* 1992;176:1521-1529.

223. Bovenschen N, Quadir R, van den Berg AL, et al. Granzyme K displays highly restricted substrate specificity that only partially overlaps with granzyme a. *J Biol Chem.* 2009;284:3504-3512.

224. Hua G, Wang S, Zhong C, et al. Ignition of p53 bomb sensitizes tumor cells to granzyme K-mediated cytolysis. *J Immunol.* 2009;182:2152-2159.

225. Guo Y, Chen J, Shi L, et al. Valosin-containing protein cleavage by granzyme K accelerates an endoplasmic reticulum stress leading to caspase-independent cytotoxicity

of target tumor cells. *J Immunol*. 2010;185:5348-5359.

226. Smyth MJ, Sayers TJ, Wiltrout T, et al. Met-ase: cloning and distinct chromosomal location of a serine protease preferentially expressed in human natural killer cells. *J Immunol.* 1993;151:6195-6205.

227. Smyth MJ, O'Connor MD, Trapani JA, et al. A novel substrate-binding pocket interaction restricts the specificity of the human NK cell-specific serine protease, Met-ase-1. *J Immunol.* 1996;156:4174-4181.

228. Mahrus S, Kisiel W, Craik CS. Granzyme M is a regulatory protease that inactivates proteinase inhibitor 9, an endogenous inhibitor of granzyme B. *J Biol Chem.* 2004;279:54275-54282.

229. Rukamp BJ, Kam CM, Natarajan S, et al. Subsite specificities of granzyme M: a study of inhibitors and newly synthesized thiobenzyl ester substrates. *Arch Biochem Biophys.* 2004;422:9-22.

230. Sayers TJ, Brooks AD, Ward JM, et al. The restricted expression of granzyme M in human lymphocytes. *J Immunol.* 2001;166:765-771.

231. de Koning PJ, Tesselaar K, Bovenschen N, et al. The cytotoxic protease granzyme M is expressed by lymphocytes of both the innate and adaptive immune system. *Mol Immunol.* 2010;47:903-911.

232. Lu H, Hou Q, Zhao T, et al. Granzyme M directly cleaves inhibitor of caspase-activated DNase (CAD) to unleash CAD leading to DNA fragmentation. *J Immunol.* 2006;177:1171-1178.

233. Hua G, Zhang Q, Fan Z. Heat shock protein 75 (TRAP1) antagonizes reactive oxygen species generation and protects cells from granzyme M-mediated apoptosis. *J Biol Chem.* 2007;282(28):20553-20560.

234. Hu D, Liu S, Shi L, et al. Cleavage of survivin by Granzyme M triggers degradation of the survivin-X-linked inhibitor of apoptosis protein (XIAP) complex to free caspase activity leading to cytolysis of target tumor cells. *J Biol Chem.* 2010;285:18326-18335.

235. de Poot SA, Westgeest M, Hostetter DR, et al. Human and mouse granzyme M display divergent and species-specific substrate specificities. *Biochem J.* 2011;437:431-442.

236. van Domselaar R, Philippen LE, Quadir R, et al. Noncytotoxic inhibition of cytomegalovirus replication through NK cell protease granzyme M-mediated cleavage of viral phosphoprotein 71. *J Immunol.* 2010;185:7605-7613.

237. Krensky AM, Clayberger C. Biology and clinical relevance of granulysin. *Tissue Antigens.* 2009;73:193-198.

238. Jongstra J, Schall TJ, Dyer BJ, et al. The isolation and sequence of a novel gene from a human functional T cell line. *J Exp Med.* 1987;165:601-614.

239. Stenger S, Hanson DA, Teitelbaum R, et al. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science.* 1998;282:121-125.

240. Zheng CF, Ma LL, Jones GJ, et al. Cytotoxic CD4+ T cells use granulysin to kill Cryptococcus neoformans, and activation of this pathway is defective in HIV patients. *Blood.* 2007;109:2049-2057.

241. Farouk SE, Mincheva-Nilsson L, Krensky AM, et al. Gamma delta T cells inhibit in vitro growth of the asexual blood stages of Plasmodium falciparum by a granule exocytosis-dependent cytotoxic pathway that requires granulysin. *Eur J Immunol.* 2004;34:2248-2256.

242. Ma LL, Spurrell JC, Wang JF, et al. CD8 T cell-mediated killing of Cryptococcus neoformans requires granulysin and is dependent on CD4 T cells and IL-15. *J Immunol.* 2002;169:5787-5795.

243. Deng A, Chen S, Li Q, et al. Granulysin, a cytolytic molecule, is also a chemoattractant and proinflammatory activator. *J Immunol.* 2005;174: 5243-5248.

244. Tewary P, Yang D, de la Rosa G, et al. Granulysin activates antigenpresenting cells through TLR4 and acts as an immune alarmin. *Blood.* 2010;116:3465-3474.

245. Walch M, Latinovic-Golic S, Velic A, et al. Perforin enhances the granulysin-induced lysis of Listeria innocua in human dendritic cells. *BMC Immunol.* 2007;8:14.

246. Stegelmann F, Bastian M, Swoboda K, et al. Coordinate expression of CC chemokine ligand 5, granulysin, and perforin in CD8+ T cells provides a host defense mechanism against Mycobacterium tuberculosis. *J Immunol.* 2005;175:7474-7483.

247. Huang LP, Lyu SC, Clayberger C, et al. Granulysin-mediated tumor rejection in transgenic mice. *J Immunol.* 2007;178:77-84.

248. Dupuis M, Schaerer E, Krause KH, et al. The calcium-binding protein calreticulin is a major constituent of lytic granules in cytolytic T lymphocytes. *J Exp Med.* 1993;177:1-7.

249. Fraser SA, Karimi R, Michalak M, et al. Perforin lytic activity is controlled by calreticulin. *J Immunol.* 2000;164:4150-4155.

250. Fraser SA, Michalak M, Welch WH, et al. Calreticulin, a component of the

endoplasmic reticulum and of cytotoxic lymphocyte granules, regulates perforin-mediated lysis in the hemolytic model system. *Biochem Cell Biol.* 1998;76:881-887.

251. Andrin C, Pinkoski MJ, Burns K, et al. Interaction between a Ca2 +-binding protein calreticulin and perforin, a component of the cytotoxic T-cell granules. *Biochemistry.* 1998;37:10386-10394.

252. Kummer JA, Kamp AM, Citarella F, et al. Expression of human recombinant granzyme A zymogen and its activation by the cysteine proteinase cathepsin C. *J Biol Chem.* 1996;271:9281-9286.

253. Pham CT, Ivanovich JL, Raptis SZ, et al. Papillon-Lefevre syndrome: correlating the molecular, cellular, and clinical consequences of cathepsin C/dipeptidyl peptidase I deficiency in humans. *J Immunol.* 2004;173:7277-7281.

254. Sutton VR, Waterhouse NJ, Browne KA, et al. Residual active granzyme B in cathepsin C-null lymphocytes is sufficient for perforin-dependent target cell apoptosis. *J Cell Biol.* 2007;176:425-433.

255. Meade JL, de Wynter EA, Brett P, et al. A family with Papillon-Lefevre syndrome reveals a requirement for cathepsin C in granzyme B activation and NK cell cytolytic activity. *Blood.* 2006;107:3665-3668.

256. D'Angelo ME, Bird PI, Peters C, et al. Cathepsin H is an additional convertase of pro-granzyme B. *J Biol Chem.* 2010;285:20514-20519.

257. Grujic M, Braga T, Lukinius A, et al. Serglycin-deficient cytotoxic T lymphocytes display defective secretory granule maturation and granzyme B storage. *J Biol Chem.* 2005;280:33411-33418.

258. Ida H, Nakashima T, Kedersha NL, et al. Granzyme B leakage-induced cell death: a new type of activation-induced natural killer cell death. *Eur J Immunol.* 2003;33:3284-3292.

259. Sun J, Bird CH, Sutton V, et al. A cytosolic granzyme B inhibitor related to the viral apoptotic regulator cytokine response modifier A is present in cytotoxic lymphocytes. *J Biol Chem.* 1996;271:27802-27809.

260. Law RH, Zhang Q, McGowan S, et al. An overview of the serpin superfamily. Geno*me Biol.* 2006;7:216.

261. Silverman GA, Bird PI, Carrell RW, et al. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. *J Biol Chem.* 2001;276:33293-33296.

262. Potempa J, Korzus E, Travis J. The serpin superfamily of proteinase inhibitors: structure, function and regulation. *J Biol Chem.* 1994;269:15957-15960.

263. Bird CH, Sutton VR, Sun J, et al. Selective regulation of apoptosis: the cytotoxic lymphocyte serpin proteinase inhibitor 9 protects against granzyme B-mediated apoptosis without perturbing the Fas cell death pathway. *Mol Cell Biol.* 1998;18:6387-6398.

264. Sun J, Ooms L, Bird CH, et al. A new family of 10 murine ovalbumin serpins includes two homologs of proteinase inhibitor 8 and two homologs of the granzyme B inhibitor (proteinase inhibitor 9). *J Biol Chem.* 1997;272:15434-15441.

265. Phillips T, Opferman JT, Shah R, et al. A role for the granzyme B inhibitor serine protease inhibitor 6 in CD8+ memory cell homeostasis. *J Immunol.* 2004;173:3801-3809.

266. Zhang M, Park SM, Wang Y, et al. Serine protease inhibitor 6 protects cytotoxic T cells from self-inflicted injury by ensuring the integrity of cytotoxic granules. *Immunity.* 2006;24:451-461.

267. de Koning PJ, Kummer JA, de Poot SA, et al. Intracellular serine protease inhibitor SERPINB4 inhibits granzyme M-induced cell death. *PloS One.* 2011;6:e22645.

268. Baran K, Ciccone A, Peters C, et al. Cytotoxic T lymphocytes from cathepsin B-deficient mice survive normally in vitro and in vivo after encountering and killing target cells. *J Biol Chem.* 2006;281:30485-30491.

269. Bird CH, Blink EJ, Hirst CE, et al. Nucleocytoplasmic distribution of the ovalbumin serpin PI-9 requires a nonconventional nuclear import pathway and the export factor Crm1. *Mol Cell Biol.* 2001;21:5396-5407.

270. Hirst CE, Buzza MS, Bird CH, et al. The intracellular granzyme B inhibitor, proteinase inhibitor 9, is up-regulated during accessory cell maturation and effector cell degranulation, and its overexpression enhances CTL potency. *J Immunol.* 2003;170:805-815.

271. Buzza MS, Hosking P, Bird PI. The granzyme B inhibitor, PI-9, is differentially expressed during placental development and up-regulated in hydatidiform moles. *Placenta.* 2006;27:62-69.

272. Bladergroen BA, Strik MC, Bovenschen N, et al. The granzyme B inhibitor, protease inhibitor 9, is mainly expressed by dendritic cells and at immune-privileged sites. *J Immunol.* 2001;166:3218-3225.

273. Buzza MS, Hirst CE, Bird CH, et al. The granzyme B inhibitor, PI-9, is present in endothelial and mesothelial cells, suggesting that it protects bystander cells during immune responses. *Cell Immunol.* 2001;210:21-29.

274. Bladergroen BA, Strik MC, Wolbink AM, et al. The granzyme B inhibitor proteinase inhibitor 9 (PI9) is expressed by human mast cells. *Eur J Immunol.* 2005;35:1175-1183.

275. Medema JP, Schuurhuis DH, Rea D, et al. Expression of the serpin serine protease inhibitor 6 protects dendritic cells from cytotoxic T lymphocyte-induced apoptosis: differential modulation by T helper type 1 and type 2 cells. *J Exp Med.* 2001;194:657-667.

276. Kannan-Thulasiraman P, Shapiro DJ. Modulators of inflammation use nuclear factor-kappa B and activator protein-1 sites to induce the caspase-1 and granzyme B inhibitor, proteinase inhibitor 9. *J Biol Chem.* 2002;277:41230-41239.

277. Barrie MB, Stout HW, Abougergi MS, et al. Antiviral cytokines induce hepatic expression of the granzyme B inhibitors, proteinase inhibitor 9 and serine proteinase inhibitor 6. *J Immunol.* 2004;172:6453-6459.

278. Jiang X, Orr BA, Kranz DM, et al. Estrogen induction of the granzyme B inhibitor, proteinase inhibitor 9, protects cells against apoptosis mediated by cytotoxic T lymphocytes and natural killer cells. *Endocrinology.* 2006;147:1419-1426.

279. Kanamori H, Krieg S, Mao C, et al. Proteinase inhibitor 9, an inhibitor of granzyme B-mediated apoptosis, is a primary estrogen-inducible gene in human liver cells. *J Biol Chem.* 2000;275:5867-5873.

280. Holmquist-Mengelbier L, Fredlund E, Lofstedt T, et al. Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. *Cancer Cell.* 2006;10:413-423.

281. Kam CM, Hudig D, Powers JC. Granzymes (lymphocyte serine proteases): characterization with natural and synthetic substrates and inhibitors. *Biochim Biophys Acta.* 2000;1477:307-323.

282. Spaeny-Dekking EH, Kamp AM, Froelich CJ, et al. Extracellular granzyme A, complexed to proteoglycans, is protected against inactivation by protease inhibitors. *Blood.* 2000;95:1465-1472.

283. Tsuzuki S, Kokado Y, Satomi S, et al. Purification and identification of a binding protein for pancreatic secretory trypsin inhibitor: a novel role of the inhibitor as an anti-granzyme A. *Biochem J.* 2003;372:227-233.

284. Kitahara T, Takatsuka Y, Fujimoto KI, et al. Radioimmunoassay for human pancreatic secretory trypsin inhibitor: measurement of serum pancreatic secretory trypsin inhibitor in normal subjects and subjects with pancreatic diseases. *Clin Chim Acta.* 1980;103:135-143.

285. Matsuda K, Ogawa M, Shibata T, et al. Postoperative elevation of serum pancreatic secretory trypsin inhibitor. *Am J Gastroenterol.* 1985;80: 694-698.

286. Quan LT, Caputo A, Bleackley RC, et al. Granzyme B is inhibited by the cowpox virus serpin cytokine response modifier A. *J Biol Chem.* 1995;270:10377-10379.

287. Cassens U, Lewinski G, Samraj AK, et al. Viral modulation of cell death by inhibition of caspases. *Arch Immunol Ther Exp (Warsz).* 2003;51:19-27.

288. Sieg S, Xia L, Huang Y, et al. Specific inhibition of granzyme B by parainfluenza virus type 3. *J Virol.* 1995;69:3538-3541.

289. Bade B, Boettcher HE, Lohrmann J, et al. Differential expression of the granzymes A, K and M and perforin in human peripheral blood lymphocytes. *Int Immunol.* 2005;17:1419-1428.

290. Bade B, Lohrmann J, ten Brinke A, et al. Detection of soluble human granzyme K in vitro and in vivo. *Eur J Immunol.* 2005;35:2940-2948.

291. Spaeny-Dekking EH, Hanna WL, Wolbink AM, et al. Extracellular granzymes A and B in humans: detection of native species during CTL responses in vitro and in vivo. *J Immunol.* 1998;160:3610-3616.

292. Tak PP, Spaeny-Dekking L, Kraan MC, et al. The levels of soluble granzyme A and B are elevated in plasma and synovial fluid of patients with rheumatoid arthritis (RA). *Clin Exp Immunol.* 1999;116:366-370.

293. Bratke K, Bottcher B, Leeder K, et al. Increase in granzyme B + lymphocytes and soluble granzyme B in bronchoalveolar lavage of allergen challenged patients with atopic asthma. *Clin Exp Immunol*. 2004;136:542-548.

294. Hodge S, Hodge G, Nairn J, et al. Increased airway granzyme b and perforin in current and ex-smoking COPD subjects. *COPD.* 2006;3:179-187.

295. Lauw FN, Simpson AJ, Hack CE, et al. Soluble granzymes are released during human endotoxemia and in patients with severe infection due to gram-negative bacteria. *J Infect Dis.* 2000;182:206-213.

296. Kim WJ, Kim H, Suk K, et al. Macrophages express granzyme B in the lesion areas of atherosclerosis and rheumatoid arthritis. *Immunol Lett.* 2007;111:57-65.

297. Hernandez-Pigeon H, Jean C, Charruyer A, et al. UVA induces granzyme B in human keratinocytes through MIF: implication in extracellular matrix remodeling. *J Biol Chem.* 2007;282:8157-8164.

298. Hernandez-Pigeon H, Jean C, Charruyer A, et al. Human keratinocytes acquire cellular cytotoxicity under UV-B irradiation. Implication of granzyme B and perforin. *J Biol Chem.* 2006;281:13525-13532.

299. Rucevic M, Fast LD, Jay GD, et al. Altered levels and molecular forms of granzyme k in plasma from septic patients. *Shock.* 2007;27:488-493.

300. Rowshani AT, Strik MC, Molenaar R, et al. The granzyme B inhibitor SERPINB9 (protease inhibitor 9) circulates in blood and increases on primary cytomegalovirus infection after renal transplantation. *J Infect Dis.* 2005;192:1908-1911.

301. Prakash MD, Bird CH, Bird PI. Active and zymogen forms of granzyme B are constitutively released from cytotoxic lymphocytes in the absence of target cell engagement. *Immunol Cell Biol.* 2009;87:249-254.

302. Dustin ML. T-cell activation through immunological synapses and kinapses. *Immunol Rev.* 2008;221:77-89.

303. Sower LE, Klimpel GR, Hanna W, et al. Extracellular activities of human granzymes. I. Granzyme A induces IL6 and IL8 production in fibroblast and epithelial cell lines. *Cell Immunol.* 1996;171:159-163.

304. Metkar SS, Menaa C, Pardo J, et al. Human and mouse granzyme A induce a proinflammatory cytokine response. *Immunity.* 2008;29:720-733.

305. Joeckel LT, Wallich R, Martin P, et al. Mouse granzyme K has proinflammatory potential. *Cell Death Diff.* 2011;18:1112-1119.

306. Chamberlain CM, Ang LS, Boivin WA, et al. Perforin-independent extracellular granzyme B activity contributes to abdominal aortic aneurysm. *Am J Path.* 2010;176:1038-1049.

307. Irmler M, Hertig S, MacDonald HR, et al. Granzyme A is an interleukin 1 beta-converting enzyme. *J Exp Med.* 1995;181:1917-1922.

308. Omoto Y, Yamanaka K, Tokime K, et al. Granzyme B is a novel interleukin-18 converting enzyme. *J Dermatol Sci.* 2010;59:129-135.

309. Cooper DM, Pechkovsky DV, Hackett TL, et al. Granzyme K activates protease-activated receptor-1. *PloS One.* 2011;6:e21484.

310. Simon MM, Kramer MD, Prester M, et al. Mouse T-cell associated serine proteinase 1 degrades collagen type IV: a structural basis for the migration of lymphocytes through vascular basement membranes. *Immunology.* 1991;73:117-119.

311. Simon MM, Simon HG, Fruth U, et al. Cloned cytolytic T-effector cells and their malignant variants produce an extracellular matrix degrading trypsin-like serine proteinase. *Immunology.* 1987;60:219-230.

312. Vettel U, Brunner G, Bar-Shavit R, et al. Charge-dependent binding of granzyme A (MTSP-1) to basement membranes. *Eur J Immunol.* 1993;23:279-282.

313. Buzza MS, Zamurs L, Sun J, et al. Extracellular matrix remodeling by human granzyme B via cleavage of vitronectin, fibronectin, and laminin. *J Biol Chem.* 2005;280:23549-23558.

314. Choy JC, Hung VH, Hunter AL, et al. Granzyme B induces smooth muscle cell apoptosis in the absence of perforin: involvement of extracellular matrix degradation. *Arterioscler Thromb Vasc Biol.* 2004;24: 2245-2250.

315. Suidan HS, Bouvier J, Schaerer E, et al. Granzyme A released upon stimulation of cytotoxic T lymphocytes activates the thrombin receptor on neuronal cells and astrocytes. *Proc Natl Acad Sci U S A.* 1994;91: 8112-8116.

316. Suidan HS, Clemetson KJ, Brown-Luedi M, et al. The serine protease granzyme A does not induce platelet aggregation but inhibits responses triggered by thrombin. *Biochem J.* 1996;315:939-945.

317. Buzza MS, Dyson JM, Choi H, et al. Antihemostatic activity of human granzyme B mediated by cleavage of von Willebrand factor. *J Biol Chem.* 2008;283:22498-22504.

318. Brunner G, Simon MM, Kramer MD. Activation of pro-urokinase by the human T cell-associated serine proteinase HuTSP-1. *FEBS Lett*. 1990;260:141-144.

319. Gahring L, Carlson NG, Meyer EL, et al. Granzyme B proteolysis of a neuronal glutamate receptor generates an autoantigen and is modulated by glycosylation. *J Immunol.* 2001;166:1433-1438.

320. Ganor Y, Teichberg VI, Levite M. TCR activation eliminates glutamate receptor GluR3 from the cell surface of normal human T cells, via an autocrine/paracrine granzyme B-mediated proteolytic cleavage. *J Immunol.* 2007;178:683-692.

321. Wang T, Allie R, Conant K, et al. Granzyme B mediates neurotoxicity through a G-protein-coupled receptor. *FASEB J.* 2006;20:1209-1211.

322. Loeb CR, Harris JL, Craik CS. Granzyme B proteolyzes receptors important to proliferation and survival, tipping the balance toward apoptosis. *J Biol Chem.* 2006;281:28326-28335.

323. Wertz IE, Dixit VM. Regulation of death receptor signaling by the ubiquitin system. *Cell Death Diff.* 2010;17:14-24.

324. Wilson NS, Dixit V, Ashkenazi A. Death receptor signal transducers: nodes of coordination in immune signaling networks. *Nat Immunol*. 2009;10:348-355.

325. Christofferson DE, Yuan J. Necroptosis as an alternative form of programmed cell death. *Curr Opin Cell Biol.* 2010;22:263-268.

326. Degterev A, Huang Z, Boyce M, et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat Cell Biol.* 2005;1:112-119.

327. Fisher GH, Rosenberg FJ, Straus SE, et al. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell.* 1995;81:935-946.

328. Chun HJ, Zheng L, Ahmad M, et al. Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency. *Nature.* 2002;419:395-399.

329. Wang J, Chun HJ, Wong W, et al. Caspase-10 is an initiator caspase in death receptor signaling. *Proc Natl Acad Sci U S A.* 2001;98: 13884-13888.

330. Takeda K, Hayakawa Y, Smyth MJ, et al. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nat Med.* 2001;7:94-100.

331. Smyth MJ, Takeda K, Hayakawa Y, et al. Nature's TRAIL—on a path to cancer immunotherapy. *Immunity.* 2003;18:1-6.

332. Johnstone RW, Frew AJ, Smyth MJ. The TRAIL apoptotic pathway in cancer onset, progression and therapy. *Nat Rev Cancer.* 2008;8:782-798.

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Chapter 38

The Immune Response to Parasites

Thomas A. Wynn
David L. Sacks
Alan Sher
Eleanor M. Riley

PARASITES AND THE IMMUNE SYSTEM

Distinct Features and Global Health Importance of Parasitic Pathogens

The term "parasite" is formally used as a designation for eukaryotic protozoan and metazoan pathogens residing within or upon their hosts. The origin of this usage is not clear but almost certainly relates to the common historical period and tropical disease context in which many of these agents were identified. Indeed, parasites are the most phylogenetically diverse category of pathogens and at the lower end of their evolutionary tree are often difficult to distinguish from fungi and other protista in both their morphology and genomic organization.

Although the taxonomic basis of their classification into a single group is under question, parasites as infectious agents do share many biologic characteristics. They frequently (although not always) display complex life cycles consisting of morphologically and antigenically distinct stages and produce long-lived or chronic infections to ensure transmission between their hosts. The induction of severe morbidity or mortality is an atypical outcome. However, in the tropical and subtropical regions where transmission is high, the low frequency of disease translates into a major global health and economic problem because of the sheer numbers of people exposed and because of the confounding issues of malnutrition and coinfection. As illustrated by outbreaks in the past decades of disease caused by the protozoa *Giardia*, *Cryptosporidia*, *Cyclospora*, and *Toxoplasma*, parasites also represent a continuing threat to populations in wealthier countries. Indeed, all of the major food- and water-borne protozoa have been classified as Category B bioterrorism pathogens because of their potential to cause acute epidemic illness. The human immunodeficiency virus (HIV)/acquired immune deficiency syndrome epidemic has also increased the impact of parasitic disease in both developed and developing regions because immunocompromised hosts become highly susceptible to some normally tolerated parasites such as *Cryptosporidia*, *Toxoplasma gondii*, and *Leishmania*. The danger also exists for many of these organisms to spread into new geographical regions, as environmental degradation and climate change become ever-increasing threats. Finally, parasitic disease remains an important problem in livestock, causing annual economic losses in the billions of dollars and, in the case of trypanosomiasis, limiting the agricultural development of huge areas of potential grazing lands on the African continent.

The immune system plays a central role in determining the outcome of parasitic infection establishing a critical balance meant to ensure both host and pathogen survival. As with other infectious agents, disease emerges when the scales tip toward either a deficient or excessive immune response. Manipulation of that response by means of vaccination or immunotherapy remains a key approach for global intervention in parasitic disease. A list of the most important parasitic infections of humans, along with estimates of their prevalence, annual mortality, and current control methods, is presented in Table 38.1. The data testify to the continued enormity of the problem reflected in the numbers of people annually infected and dying of diseases such as malaria, schistosomiasis, and trypanosomiasis as well as the high level of morbidity in those surviving. A striking situation reflected in the data is the complete absence of effective vaccines for protecting human populations. In the case of malaria, the need for a global immunization strategy has become particularly acute as drug resistance spreads worldwide. Clearly, the development of vaccines to prevent parasitic diseases remains one of the major unachieved goals of modern immunology and one of its greatest and most difficult challenges. The scientific challenge lies with the extraordinary complexity of parasites as immunologic targets and their remarkable adaptability to immunologic pressure. The field of immunoparasitology is focused on developing a basic understanding of this important host-pathogen interface for

the ultimate purpose of intervention. At the same time, the work in this area—particularly in recent years—has provided immunology with a series of major insights concerning effector and regulatory responses as they occur in vivo. Indeed, because of their years of close encounter with and adaptation to the vertebrate immune system, parasites can be thought of as the "ultimate immunologists," and there is much to be learned from them about the fundamental nature of immune responses.

TABLE 38.1 Global Impact of Parasitic Disease and Current Control Measures[a]

	Estimated Prevalence (Millions)	Annual Deaths (Thousands)	Control Methods Currently Used
Malaria	216	1200	Vector control, chemotherapy
Schistosomiasis	207	20-280	Chemotherapy, hygiene
Soil-transmitted helminths[b]	1200	12-135	Chemotherapy, hygiene
Leishmaniasis	12	50-80	Vector control, chemotherapy
African trypanosomiasis	0.3-0.5	10-48	Vector control
Chagas disease	10	10	Vector control
Lymphatic filariasis	120	0	Vector control, chemotherapy
Onchocerciasis	18	0	Vector control, chemotherapy

a Data compiled from World Health Organization Fact Sheets (www.who.int/mediacentre/factsheets/en/) and other miscellaneous sources.

b Hookworm, ascariasis, and trichuriasis. The data are combined values for all three infections.

Some Hallmarks of the Immune Response to Parasites

The interaction of parasites with the immune system has several distinguishing features that

are of special interest to fundamental immunologists. Most parasitic pathogens are able to survive the initial host response and produce longlasting or chronic infections designed to promote transmission. In the case of many protozoa (eg, *Toxoplasma*, *Leishmania*), *chronicity* is characterized by a state of *latency* in which replication of the parasite is minimal and infection cryptic. The development of chronicity depends not only on the ability of the parasite to escape protective immune responses (*immune evasion*) but also on the generation of finely tuned mechanisms of *immunoregulation* that serve both to prevent parasite elimination and suppress host immunopathology. As discussed in detail later in this chapter, the study of these immunomodulatory pathways in both human and experimental parasitic infections has yielded important insights concerning the mechanisms by which regulatory cells and cytokines control immune effector functions in vivo.

An additional prominent feature of the immune response to parasites is *Th1/Th2 polarization.* For reasons that are not entirely clear, parasitic infections often induce cluster of differentiation (CD)4+ T-cell responses that are highly polarized in terms of their Th1/Th2 lymphokine profiles. This phenomenon is particularly striking in the case of helminths, which in contrast to nearly all other pathogens, routinely trigger strong Th2 responses leading to high immunoglobulin (Ig)E levels, eosinophilia, and mastocytosis. At the opposite pole, many intracellular protozoa induce CD4+ T-cell responses with Th1-dominated lymphokine secretion patterns. This striking difference presents a beautiful example of immunologic class selection. Interestingly, in murine *Leishmania major* infection, CD4+ cells polarize to either Th1 or Th2 depending on the strain of parasite or strain of mouse infected, and the association of these responses with healing or exacerbation provided the first demonstration of a functional role for this dichotomy.[1,2] Parasite models have also been used to reveal new effector functions, such as the ability of eosinophils to kill pathogens and, as discussed subsequently, are now being used extensively to study microbial innate recognition and immune response initiation. This ability to uncover and investigate basic immune and immunopathogenic mechanisms while studying the host response to a group of phylogenetically unique pathogens of global importance is perhaps the most engaging and rewarding aspect of research in immunoparasitology.

INNATE RECOGNITION AND HOST DEFENSE

Innate recognition plays an important role in determining the outcome of the host-parasite encounter by both providing an initial barrier to infection and by influencing the magnitude and class of the subsequent adaptive immune response. At the same time, from the parasite's point of view, innate immune defenses must be subverted for infection to be established; it is clear that many parasitic pathogens have evolved specific mechanisms for evading them, and these evasion mechanisms can also provide an explanation for virulence differences amongst parasite strains. Moreover, in some cases, parasites appear to actually hijack the process of innate recognition to deviate adaptive immunity to facilitate their own persistence.

Humoral Mechanisms

Innate resistance against parasitic infection is mediated in part by preexisting, soluble factors that recognize and destroy invading developmental stages or target them for killing by effector cells. The alternative pathway of complement activation provides a first line of defense against extracellular parasites and because of this, the infective stages of parasitic protozoa and helminths have developed a various strategies to subvert complement-mediated attack. In some instances, blood and tissue parasites have evolved redundant mechanisms to ensure their survival

during serum exposure. For example, infective metacyclic and bloodstream trypomastigotes of *Trypanosoma cruzi* express multiple stage-specific surface glycoproteins, such as gp160 and the 87-93kDa trypomastigote decay accelerating factor, which are actively released by the parasite and are functional homologues of human decay accelerating factor that interferes with assembly of C3 convertases by binding to C3b.[3] Another trypomastigote glycoprotein, gp58/gp68, inhibits alternative pathway C3 convertase assembly by binding to factor B. *T. cruzi* trypomastigotes have also been found to continuously shed acceptor molecules with covalently bound C3 fragments, thought to be due to an endogenous phospholipase that cleaves glycosylphosphatidylinositol (GPI)-anchored membrane proteins. In addition, trypomastigotes export calreticulin to the parasite surface where, by binding C1, the protein can both inhibit activation of the classical pathway and promote parasite invasion.[4] *T. cruzi* amastigotes, on the other hand, have been shown to resist complement lysis by preventing membrane insertion of the membrane attack complex (MAC), C5b-9. An analogous mechanism of resistance has been observed for the infective metacyclic promastigote stage of *Leishmania*, which expresses an elongated form of the major surface and released glycolipid on *Leishmania* promastigotes, lipophosphoglycan (LPG), such that it behaves as an effective barrier to membrane insertion and pore formation by MAC.[5] Metacyclics also increase expression of the surface metalloproteinase gp63,[6] which can cleave C3b to the inactive iC3b form, thus preventing deposition of MAC.[7] Both C3b and iC3b effectively opsonize the complement resistant forms for uptake by macrophages, its host cell of choice. Tissue-invasive strains of *Entamoeba histolytica* also activate the alternative complement pathway but are resistant to lysis due to the action of a Gal/GalNAc lectin, which mediates adherence of trophozoites to host cells and binds to C8 and C9 terminal components.[8] Interestingly, the lectin shares sequence similarities with CD59, a membrane inhibitor of MAC in human blood cells.

The damage caused to worms as a consequence of alternative pathway activation is due primarily to the bound C3 activation products that act as ligands for cellular adherence and killing by eosinophils, neutrophils, and macrophages. In addition to synthesizing their own complement regulatory proteins to subdue the activation cascade, helminths also acquire endogenous regulatory molecules from the host. For example, schistosomes can inhibit complement activation through surface-expressed parasite proteins that bind C2, C3, C8, and C9 but also do so by acquiring decay accelerating factor from the host and incorporating it into their teguments.[9] Similarly, the infective L3 stage larvae of *Onchocerca volvulus*, the causative agent of river blindness, were shown to bind the main human fluid phase regulator factor H, thereby promoting C3b inactivation.[10] Other parasitic nematodes such as *Toxocara canis*, *Brugia malayi*, and *Trichinella spiralis* appear to block complement attack by secreting proteases that attack the complement pathway[11] or regulatory proteins that inhibit its function.[12]

A well-characterized set of soluble mediators providing a barrier to parasitic infection are the primate-specific trypanosome lysis factors (TLF1 and TLF2) present in serum that contribute to the innate resistance of humans to *Trypanosoma brucei* infection.[13] The key active components of these serum complexes are haptoglobin-related protein and apoliprotein L (ApoL)-1 that together are cytotoxic to *T. b. brucei* and act synergistically to provide enhanced trypanosome killing when assembled into the same high-density lipoprotein (HDL) particle. Haptoglobin-related protein and ApoL-1 have different proposed activities; ApoL-1 is able to form ion pores in lysosomal membranes, whereas haptoglobin-related protein is able to accelerate lysosomal membrane peroxidation.

Whereas the TLFs are capable of killing *T. brucei*, the species that infect humans, *T. b. gambiense* and *T. b. rhodesiense*, are both refractory to TLF-mediated cytolysis. This property has been correlated with the expression of a serum resistance-associated gene that is homologous to the variant surface glycoprotein. Importantly, transfection of serum resistance -associated gene from *T. b. rhodesiense* into *T. brucei* confers resistance to lysis by human serum, arguing that its expression may have been a critical step in the adaptation of the former parasite for infection of primates.[14] Interestingly, mice cotransfected with TLF components display enhanced resistance to *T. brucei* infection, suggesting that such a genetic modification strategy might be useful in protecting livestock against this parasite, which still is a major impediment to cattle farming in many parts of Africa.[15]

Cellular Mechanisms

Phagocytosis by macrophages represents an innate first line of defense against protozoan pathogens. Macrophages possess primary defense mechanisms, including activation of

oxidative metabolism, which are induced by the attachment and engulfment of microbial agents, and the early survival of intracellular parasites will depend on their ability to avoid or withstand oxidative stress conditions. The major source of reactive oxygen species is the multimeric enzyme complex, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Early studies suggested that *Leishmania* parasites avoid triggering the oxidative burst by actively inhibiting macrophage (PKC) activation,[16] which is required for phosphorylation of several sites on the cytosolic oxidase subunit, p47phox. The inhibition of the respiratory burst has also been linked to leishmanial LPG, which is rapidly transferred to the inner leaflet of the phagosomal membrane and prevents translocation of the NADPH oxidase cytosolic components.[17,18] As some LPG-deficient *Leishmania* strains still manage to survive in macrophages, it is clear that redundant mechanisms exist for the parasite to avoid macrophage triggering. These include opsonic ingestion through receptors that are uncoupled from the activation of NADPH oxidase. A number of "silent" entry receptors have been described that are variably used by different species and developmental stages of *Leishmania*, including complement and mannose receptors,[19] and receptors for apoptotic cells.[20,21]

In contrast to *Leishmania*, *Toxoplasma* enters all nucleated cells, including macrophages, by an active invasion mechanism that excludes most host cell proteins, including membrane components of the NADPH oxidase, from the

parasitophorous vacuole.[22] Malaria parasites are also sensitive to oxidative stress, and knockout mice lacking NADPH oxidase suffer more rapid increases in malaria parasite densities than wild-type mice.[23] In this case, reactive oxygen species are generated primarily as a result of the degradation of host hemoglobin within parasitized red blood cells (RBCs).[24] The detoxification of reactive oxygen species is achieved with a range of low-molecular weight antioxidants, including the tripeptide glutathione, and a number of host- and parasite-encoded enzymes.[25]

The maturation of phagosomes into digestive organelles represents the heart of the defensive machinery of macrophages, and intracellular parasites have evolved diverse strategies to avoid, escape from, or withstand the acidified, hydrolytic environment of phagolysosomes. For *Toxoplasma*, the integral membrane proteins that are excluded from the nascent vacuole include those involved in acidification and fusion with the endosomal network.[26] If instead the parasite is forced to enter the cell by a phagocytic pathway, as a consequence of, for example, antibody opsonization, it is targeted through the normal phagolysosomal system and is killed.[27] For *T. cruzi*, which trigger a wound repair pathway involving lysosome exocytosis to enter into cells,[28] the early vacuole is acidified and potentially fusogenic. Intracellular survival of *T. cruzi* is dependent on its ability to escape from the vacuole, a process facilitated by its expression of a putative pore-forming protein that is immunologically cross-reactive with human C9, and that can disrupt the phagosome membrane allowing egress of the parasite into the cytoplasm.[29] *Leishmania* promastigotes, again via transfer of their surface LPG which increases the periphagosomal accumulation of F-actin and disrupts phagosome microdomains, transiently inhibit normal phagosome maturation.[30] The delay in phagosome maturation may be necessary to allow sufficient time for metacyclic promastigotes transmitted by the sand fly to differentiate into more acidophilic, hydrolase-resistant amastigotes. The various strategies employed by parasitic protozoa to evade the innate defenses of host macrophages are depicted in Figure 38.1.

Neutrophils have been an understudied component of the innate cellular response to protozoan pathogens, despite the fact that they are rapidly and massively recruited to the site of parasite delivery by the bites of arthropod vectors. They have been clearly revealed by intravital two-photon microscopy to be the first cells to take up *Leishmania* in the skin during the first hours of infection following inoculation by needle or vector sand flies.[31,32] Their localized recruitment is triggered by the vascular damage caused by the needle injection or the sand fly bite in addition to signals derived from sand fly saliva and the parasite.[33,34] The survival of *Leishmania* following their phagocytosis by neutrophils, similarly to macrophages, is dependent on their ability to inhibit fusion of tertiary granules with the parasite-containing phagosome, which was again shown to be linked to the expression of the promastigote surface LPG.[35,36] Interestingly, macrophages have been observed to phagocytose infected neutrophils in vitro,[35,37] and the exploitation of the apoptotic cell clearance function of macrophages is the basis for the "Trojan horse" infection model whereby infected, apoptotic neutrophils are proposed to silently deliver *Leishmania* to host macrophages to initiate productive infections in these cells. At later stages of infection, neutrophils may help to defend against *Leishmania*, in part, by releasing fibrous deoxyribonucleic acid (DNA)-based extracellular traps that ensnare and kill the parasite.[38] DNA-based extracellular traps are also elicited by *T. gondii* tachyzoites, with evidence that they exert direct microbicidal effects and interfere with host cell invasion and parasite spread.[39] Although neutrophils are not required to control malaria infections (and may, indeed, contribute to tissue damage), their oxidative burst is severely compromised following exposure to the heme detoxifying enzyme heme oxygenase, leaving malaria patients at increased risk for systemic, gram-negative, bacterial infections.[40]

Unlike protozoa, helminths are too big to be engulfed by phagocytes and can only be killed by these cells when the latter have been activated by products of the adaptive immune response. Instead, eosinophils, which frequently accumulate in tissues soon after worm invasion, may mediate innate cellular defense against helminth larvae by means of discharge of the major basic protein and cationic proteins present in the granules of these cells.[41,42]

In contrast to intracellular killing by phagocytes and extracellular killing by eosinophils, some innate cellular defenses do not eliminate parasites directly but instead trigger other effector cells to do so. Perhaps the best studied example of this form of innate immunity is the natural killer (NK) cell pathway of cytokine production. NK cells become activated as a consequence of various parasitic infections; *Leishmania* promastigotes; *Plasmodium falciparum* -infected RBCs; components of *T. gondii*, *T. cruzi*, *E. histolytica*, and *Cryptosporidium parvum*; and excretory-secretory proteins of the hookworm *Necator americanus*[43] all activate human peripheral blood NK cells to produce interferon (IFN)γ. Despite occasional reports of direct binding of parasite ligands to NK-cell receptors, the emerging consensus is that NK-cell activation is secondary to pattern recognition receptor (PRR)-mediated activation of myeloid dendritic cells (DCs) and monocyte/macrophages and requires both contact-dependent and cytokine-mediated (interleukin [IL]-12, IL-18) signals.[44] Moreover, in the absence of Th2 responses, NK cells may become an important source of the protective type-2 cytokine, IL-13, during murine gastrointestinal nematode infections.[45] These findings suggest that NK cells may provide a T-lymphocyte independent pathway for cytokinemediated defense and as such serve to prevent parasites from overwhelming the host prior to the development of adaptive responses. Nevertheless, there is increasing evidence that NK responses are markedly enhanced by T-cell-derived IL-2, revealing a novel pathway by which adaptive immune responses may augment innate responses.[46,47] Trafficking of NK cells to parasite-infected tissues is critically dependent upon chemokines binding to CCR5.[48] Both IL-10 and transforming growth factor (TGF)-β have been shown to serve as negative regulators of NK cell IFNγ production by means of their suppression of monokine and B7 expression by antigen-presenting cells (APCs) or, in the case of TGF-β by directly affecting NK-cell function.[49] Such suppression

may be important in protecting the host against the tissue damaging effects of excessive NK-cell-derived IFNγ and tumor necrosis factor (TNF)-α. NK-cell responses are further regulated by calibration of signals from activating and inhibitory receptors for major histocompatibility complex (MHC) molecules; moderation of NK responses to malariainfected RBCs by inhibitory receptors such as NKG2A/CD94 and polymorphic killer-cell Ig-like receptors has been proposed.[50] Although NK-cell-derived IFNγ can limit the initial phase of protozoal replication[51] and may a play a role in the polarization and expansion of Th1 cells, in some

situations, adaptive T-cell immunity is sufficient to control infection even in the absence of this early NK response.[52,53] The role of NK-cell cytotoxicity in resistance to protozoan infection is less well understood; for murine malaria, it is not required for NK-mediated resistance to blood stages,[52] but cytotoxic NK killing of malaria-infected liver cells has been reported.[54]

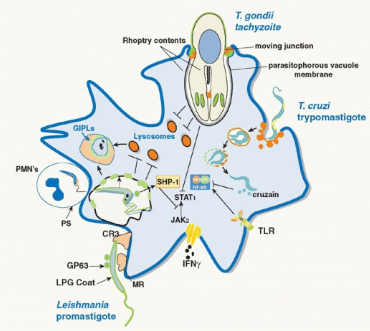

FIG. 38.1. Evasion of Innate Immune Mechanisms in Infected Macrophages by Parasitic Protozoa. Macrophages possess potent antimicrobial functions that are initiated by uptake of pathogens. Receptor-mediated phagocytosis of *Leishmania* promastigotes is accomplished by CR3 or mannose receptors that are unlinked from the signaling pathways involved in induction of reactive oxygen intermediates or proinflammatory cytokines. Silent uptake using receptors for apoptotic, phosphatidyl serine positive neutrophils delivering viable promastigote has also beeen described. The transfer of surface lipophosphoglycan to the phagosome membrane results in delayed fusion with lysosomes. Following transformation to amastigotes, which are more hydrolase resistant due to an abundance of surface glycoinositolphopsholipids, phagosome maturation proceeds. Host cell phosphatases such as SHP-1 can be directly activated by *Leishmania* surface and released molecules, including GP63, to inactivate janus kinase 2 (JAK2) and inhibit interferon (IFN)γ-inducible macrophage functions. *Toxoplasma* actively invades by rapid discharge of adhesive proteins from secretory organelles called rhoptries, then by inserting and squeezing past a moving junction in the plasma membrane that acts as a molecular sieve, excluding from its vacuole host proteins required for acidification and fusion with the endosomal network. Various *Toxoplasma*-induced defects in IFNγ signaling have been described, including proteolysis of signal transducer and activator of transcription 1. *T. cruzi* trypomastigotes enter the macrophage by inducing the recruitment of lysosomes to the plasma membrane; they only transiently reside in the vacuole before escape into the cytoplasm via secretion of a pore-forming molecule. *T. cruzi* inhibits toll-like receptor-mediated macrophage activation by directly targetting nuclear factor-kappaB (NF-κB) p65 for cleavage by a released protease, cruzain.

Two other cell populations that may function to provide a rapid cytokine response to invading parasites are γδT cells and NK T cells. These "unconventional T lymphocytes" express T-cell receptor chains of limited diversity, which may be designed for innate recognition of microbial structures or self-components revealed by infection of host cells. Although the function of NK T cells in innate resistance to parasites is currently under debate, there is considerable evidence supporting a protective role for γδT cells. Although representing a small percentage of lymphocytes in the periphery, γδT cells are abundant in epithelial and mucosal tissues, the sites of initial host invasion by many parasites. Moreover, their numbers increase in peripheral blood in response to a number of protozoan infections[55] where they can contribute effector cytokines or, in the case of extracellular *P. falciparum* merozoites, mediate direct granulysin-dependent killing.[56] Nevertheless, rather than being essential for host resistance, it is likely that γδT lymphocytes (in common with NK cells) provide an adjunct to conventional αβ CD4+ and CD8+ T cells in restricting parasite growth during the vulnerable period when the adaptive responses mediated by these lymphocyte subsets is emerging.[57,58]

A recently discovered group of effectors in the early cellular response to parasites are the innate lymphoid cells (ILCs). This cell population belongs to a heterogeneous family of innate non-T, non-B cells that are not antigen restricted. However, as they express CD45 and are dependent on traditional T-cell growth factor signaling pathways, they have been called ILCs. In common with NK cells, which themselves have now been reclassified as ILC, ILC2 produce cytokines important in T-cell subset differentiation and amplification. In the case of ILC2, these cytokines are IL-4 and IL-13, which promote Th2 development and function. As outlined later in this chapter, ILC2 (which may comprise as many as four distinct cell populations) play an important role as a major source of IL-13 in the intestinal response to nematodes and in worm expulsion.[59] Also as discussed subsequently, basophils can provide an innate source of IL-4, thereby driving Th2 differentiation, and have been proposed to do so while simultaneously serving as APCs for T-cell activation.

TABLE 38.2 Parasite Molecular Patterns Recognized by Toll-Like Receptors

MAMPs	Parasite	Structure	TLR Stimulated	Reference
GPI anchors	*Leishmania* ssp.	LPG	TLR2	60
	Trypanosoma cruzi	GPI anchors containing unsaturated alkylacylglycerol		
TLR2				
		GIPLs containing ceramide	TLR4	
	Trypanosoma brucei	GPI anchors of VSG	Undefined	
	Plasmodium falciparum	GPI anchors of MSPs	TLR2	118
			TLR4	
	Toxoplasma gondii	GIPLs and GPI anchors	TLR2	

			TLR4	
Genomic DNA	*T. brucei*	Contain unmethylated CpG motifs	TLR9	472
	L. major	Contain unmethylated CpG motifs	TLR9	64
	L. braziliensis	Leishmania RNA virus-1	TLR3	65
	T. cruzi	Contain unmethylated CpG motifs	TLR9	61
	P. falciparum	AT-rich stem-loop DNA motif	STING, TBK1, and IRF3-IRF7	80
Hemazoin	*P. falciparum*	Polymerized heme from degradation of hemoglobin	TLR9	
Protein	*T. gondii* (and related apicomplexa)	Profilin molecules	TLR11	68
Phospholipid	*Schistosoma mansoni*	Lysophosphatidylserine in tegument	TLR2	73
Phosphorylcholine	Filarial nematodes	Phosphorylcholine-containing glycoconjugates on ES-62 glycoprotein	TLR4	72
RNA	*S. mansoni*	Double-stranded RNA in parasite ova	TLR3	74

DNA, deoxyribonucleic acid; ES-62, filarial excretory-secretory antigen; GlPL, glycoinositolphospholipid; GPI, glycosylphosphatidylinositol; LPG, lipophosphoglycan; MAMP, microbe-associated molecular pattern; MSP, merozoite surface protein; RNA, ribonucleic acid; TLR, toll-like receptor; VSG, variant surface glycoproteins. Adapted from Gazzinelli RT, Denkers EY. Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. *Nat Rev Immunol* . 2006;6:895-906.

Role of Pattern Recognition Receptors in Innate Recognition of Parasites

The innate immune system, in addition to providing a natural barrier that limits infection, also plays a critical role in the initial recognition of parasites and the triggering of adaptive immunity. Invading parasites, such as other pathogens, are sensed by host PRRs that recognize microbe-associated molecular patterns shared by different groups of organisms (Table 38.2). These PRRs are highly expressed

on both epithelial cells and APCs and, when ligated, trigger cytokine and costimulatory signals that initiate both innate and adaptive cellular responses.

Toll-like receptors (TLR) are, so far, the major group of PRRs known to be triggered by parasites. The study of TLR involvement in parasitic infection began with the identification of parasite ligands that stimulate cytokine production from macrophages and DCs. In the case of protozoa, important classes of such ligands are the GPI lipid anchors present on many parasite surface proteins and phosphoglycans, and the membrane-associated glycoinositolphospholipids. Thus, GPIs from *T. brucei*, *Leishmania*, *T. gondii*, and *P. falciparum* can stimulate macrophages to upregulate inducible nitrogen oxide synthase expression and produce proinflammatory cytokines. Similarly, the GPI anchor fraction of mucin-like molecules from *T. cruzi* trypomastigotes triggers macrophage production of IL-12 and TNF. Glycoinositolphospholipids from *T. cruzi* and *T. brucei* possess similar agonist activities.[60] Studies employing reporter cell lines transfected with specific TLRs, or TLR-specific knockouts, have demonstrated that the responses induced by these parasite glycolipids are due to stimulation of TLR2 and to a lesser extent TLR4 (preferentially triggered by glycoinositolphospholipids in the case of *T. cruzi*).

Parasite nucleic acids also represent important ligands for TLR recognition. Thus, genomic DNAs from several protozoan species have been shown to stimulate host proinflammatory cytokine production presumably through the recognition of unmethylated CpG motifs by TLR9. A major role for nucleic acids in stimulation of host resistance to *T. cruzi* is evidenced by the diminished host resistance of both TLR9 and TLR2/TLR9 double knockout animals[61] and by more recent studies on mice deficient in UNC93B1, a protein that mediates translocation of TLR3, 7, and 9 to endolysosomes. These animals showed greater susceptibility to infection than TLR9-deficient mice, a finding that points to an additional role for TLR7 recognition of parasite RNA in the innate response to *T. cruzi*.[62] TLR9 signaling also contributes to host resistance to *Leishmania* apparently through DNAd-riven activation of both DCs and NK cells, although there is debate as to whether this stimulation also plays a role in Th1 response development.[63,64] *Leishmania* strains isolated from patients with mucocutaneous leishmaniasis were found to harbor high amounts of a ribonucleic acid virus that induced a TLR3-mediated hyperinflammatory response in mice that may explain the destructive metastatic lesions associated with mucosal disease.[65] In the case of *P. falciparum*, hemazoin, a product of malaria-induced hemoglobin degradation is a TLR9 agonist,[66] although at present there is controversy as to whether this results from contamination with immunostimulatory DNA fragments of parasite origin.[67]

A chemically different class of TLR ligands are the profilin proteins expressed by apicomplexan protozoa. These molecules are unique to eukaryotes and are typically associated with intracellular actin. Profilins from *T. gondii*, *Eimeria*, and *C. parvum* potently trigger IL-12 production from murine DCs as well as systemically following in vivo inoculation.[68] Experiments in the murine *T. gondii* model established that this response is due to the stimulation of TLR11, a TLR that although present in mice and other small animals is not functionally expressed in primates. Recent studies have demonstrated that UNC93B1 is required for triggering of TLR11 activation and IL-12 production by *T. gondii* profilin in mice, thus revealing an endosomal localization for this TLR.[69] Nevertheless, whereas UNC93B1-deficient mice are highly susceptible to *T. gondii* infection, animals deficient in TLR11[68] (as well as TLR3, 7, and 9) show no or only partial impairment of resistance, indicating that UNC93B1 must serve additional functions in innate immunity to the parasite beyond its role in TLR11 localization and activation.[70]

Helminth parasites also express TLR ligands, although as discussed subsequently, their role in the immune response has been harder to define than those characterized in protozoa. Moreover, in many studies, the possibility of contamination by bacterial or viral TLR ligands from symbionts has not been systematically ruled out. Indeed, *Wolbachia* symbionts confer strong TLR4 and TLR2 agonist activity on filarial parasites due to their endotoxin-like components.[71] Well-studied examples of helminth TLR microbe-associated molecular patterns include the phosphorylcholine containing moieties of the filarial glycoprotein ES-62[72] and the lysophosphatidylserine components of schistosome membranes[73] that trigger TLR4 and TLR2, respectively. In addition, schistosome eggs possess double stranded

ribonucleic acid molecules that stimulate TLR3 in DCs.[74]

The role played by TLRs in the host response to parasites is complex and not fully delineated in any of the host-parasite models studied. The main evidence for TLR involvement comes from experiments in mice deficient in myeloid differentiation primary response gene 88 (MyD88), a major adaptor protein required for signaling by most TLRs as well as by the IL-1 and IL-18 receptors. MyD88-deficient animals exhibit a loss in resistance to *T. gondii*, *T. cruzi*, *L. major*, *T. brucei*, and—in the case of *Plasmodium berghei*—decreased immunopathology[60] that likely reflects the role of TLR signaling in accessory cells (eg, DCs, epithelial cells) in the initiation and maintenance of Th1 responses. However, the altered susceptibility of these mice could also involve non-TLR-related effects of MyD88 deficiency and/or T-cell intrinsic functions of the gene, as demonstrated in the *T. gondii* mouse model.[75] Significantly, no major alterations in helminth-induced immune responses have as yet been described in MyD88- deficient hosts on susceptible genetic backgrounds, arguing against a significant role for TLR signaling in Th2-dependent host resistance and pathology in worm infections.

In contrast to the dramatically increased susceptibility often observed in MyD88-/- mice following protozoan infection, mice deficient in single TLRs exposed to the same parasites rarely show pronounced changes in resistance, even when such animals display major immune response impairments. For example, while TLR11-/- mice infected with *T. gondii* develop severely blunted IL-12 responses both in vitro and in vivo, they nevertheless survive the acute-phase infection and show only a minor increase in parasite load in comparison to fully susceptible IL-12-deficient animals.[68] Such findings may reflect redundant functions for different TLR or a requirement for multiple MyD88-dependent TLR (or IL-1/IL-18) signals in host resistance. An additional

complexity is that mice deficient in single TLRs may show unaltered (or even enhanced) resistance because the TLR in question controls an immunopathologic response or downregulates host effector functions.[76] Moreover, as exemplified by recent studies in the peroral *T. gondii* infection model, TLR signals triggered by host commensal flora (presumably as a result of intestinal barrier breakdown) can also influence the outcome of parasitic infection.[77] A major challenge of current research in this area is to decipher such positive and negative TLR signals triggered by parasite, commensal, or host ligands and establish how their integration governs host resistance.

There is also a dearth of knowledge concerning the function of PRR signaling pathways outside the TLR family. A topic of particular interest in this regard is the role of inflammasome activation in parasite-induced immunopathology. Two recent studies have demonstrated that malaria hemazoin is a potent activator of the NLRP3 inflammasome in vitro but have yielded conflicting results on the involvement of this pathway in cerebral malaria in vivo.[78,79] Most recently, sensing of AT-rich malarial DNA by an an unknown receptor that signals via the stimulator of IFN gene (STING), TANK-binding kinase 1 (TBK1), and interferon regulatory factor (IRF)3 to IRF7 signaling pathway has emerged as an entirely novel pathway of pattern recognition.[80]

DCs play a major role in linking parasite pattern recognition signals to the induction of both NK-mediated innate responses and T-cell-dependent adaptive immunity. As discussed previously, in the case of many protozoa, DC-derived IL-12 provides a major stimulus for the initiation of host defense pathways. The critical role of DCs is underscored by the impaired IL-12 production as well as impaired control of protozoan (*T. gondii*) infection[81] in mice in which CD11c+ DC populations have been genetically depleted. Such DC-depleted mice are also unable to generate CD8+ cytotoxic T-lymphocyte responses against *Plasmodium yoelii* sporozites.[82] The requirement for protozoan invasion in DC activation is complex and depends on the parasite species and DC subset in question. Indeed, as discussed subsequently, infection of DCs can result in suppressed responsiveness to activation stimuli. Nevertheless, infection of DCs with, for example, live *Leishmania*[83] or *T. gondii* appears to be important for efficient priming of CD8+ T cells despite the sequestration of many of the protozoa in question within parasitophorous vacuoles physically removed from the class I MHC antigen presentation machinery of the host cell.[84] In addition, it appears that under certain situations (eg, immunization with irradiated malarial sporozoites[85]) infected, apoptotic host cells are taken up by DCs as a mechanism of CD8+ T-cell priming. Thus, although live infection is clearly important for efficient T-cell response induction, direct infection of DCs (as opposed to other host cells) may not be required in vivo. The failure to observe parasite-containing DCs engaged in long-lived contact with T cells in in vivo imaging studies lends credence to this hypothesis but is subject to alternative interpretations.[86,87]

In addition to their role in initiating immune responses, DCs appear to be efficient vehicles for dissemination of parasites into different tissues as illustrated by studies in the *T. gondii* mouse model.[88] Infection of DCs can also serve as a survival strategy for protozoa and as a means for maintaining cryptic infection, as suggested by recent studies in which DCs from mice with rodent malaria were found to be capable of initiating infection when inoculated into naïve hosts.[89]

Downregulation of Innate Signaling Pathways by Parasites: Role in Virulence Determination

In addition to upregulating APC function, parasite products can also dampen their activity either as a mechanism of immune evasion or for the purpose of protecting the host against the pathology associated with an uncontrolled immune response. *Leishmania*, *T. cruzi*, and *Toxoplasma* have in common their ability to inhibit proinflammatory and IFNγ inducible responses in infected macrophages, such that the parasites might not only prevent or delay the induction of Th1 responses but also render infected macrophages unresponsive to activation signals during the effector phase of the immune response (see Fig. 38.1). In many cases, the parasites exploit the presence of host cell phosphatases whose normal role is to temper the magnitude and duration of the proinflammatory response. For *Leishmania*, the inhibition is due in part to activation by the *Leishmania* metalloproteinase gp63 of host cell protein tyrosine phosphatases, including SHP-1 and other nonreceptor protein tyrosine phosphatases, which inactivate Janus kinase (JAK) family members involved in the IFNγ inducible phosphorylation cascade.[90] Other host cell phosphatases were found to be activated by *Leishmania* cysteine proteinases to regulate the Mitogen-activated protein kinase (MAPK) family members p38 and extracellular signal-regulated kinase (Erk)1/2, resulting in the upregulation of IL-10 and downregulation of nitrogen oxide (NO) and TNF-α production.[91] *T. cruzi* trypomastigotes, via expression of GPI-anchored mucin-like molecules, also activate macrophage phosphatases that target downstream elements of the TLR pathway, including MAPK and nuclear factor-kappaB (NF-κB), to establish a state of tolerance in the infected cells.[92] Furthermore, NF-κB p65 was found to be targeted directly for cleavage by the *T. cruzi* protease cruzain, and cruzain-deficient parasites rapidly activated macrophages via NF-κB p65 for IL-12 expression.[93] A homologue of cruzain, cysteine peptidase B, is expressed in *Leishmania mexicana* and was likewise found to degrade NF-κB p65 to inhibit IL-12 production by infected macrophages.[94] The need to counterbalance the excessive inflammation that can be triggered by *T. gondii* infection extends to the modulation of IFNγ-induced responses, and in particular the signal transducer and activator of transcription (STAT)1 signaling cascade that is critical for resistance to *T. gondii*. \arious defects in IFNγ-initiated STAT1 signaling have been described in *T. gondii*-infected cells, including proteolytic cleavage of STAT1, dephosphorylation, and prevention of nuclear translocation.[95] Thus, although both *T. cruzi* and *T. gondii* initiate strong proinflammatory responses in host macrophages, these signaling pathways appear to be subsequently impaired to avoid reaching pathologic levels that may be detrimental to the host and/or lethal to the parasite during the adaptive phase of the immune response.

The differential ability of parasites to dampen cellular activation signals appears to be a major factor in virulence

determination. This has been elegantly studied in recent work on *T. gondii* examining the basis of the marked virulence differences in mice between Type I, Type II, and Type III strains of the parasite. By examining the progeny of genetic crosses between Type I and III or Type II and III parasites, two highly polymorphic genes ROP18 and ROP16 were identified as major virulence determinants.[96,97] Both genes encode serine-threonine kinases associated with a parasite organelle known as the rhoptry. During the process of invasion, rhoptry proteins are discharged into the host cell. Studies with ROP18 indicate that in Type I strains the kinase inactivates effectors known as immunity-related GTPase (IRG) proteins (discussed subsquently) that mediate parasite killing by disrupting the parasitophorous vacuole.[98] By contrast, the Type 1 variant of ROP16 transits to the host cell nucleus where it phosphorylates and activates STAT3 and STAT6, which in turn downregulate the proinflammatory IL-12, IL-6, and inducible NO synthase response to the parasite via their roles as positive regulators of silencers of cytokine signaling and/or IL-10.[96] Recently, a third virulence factor has been identified, in part, through a new genetic cross between Types II and I. ROP5 is a pseudokinase and serves as a scaffold to enable ROP18 activity.[99,100] The mechanism(s) by which these three rhoptry proteins interact in suppressing host cellular function is a major area of interest in the field. A current model of the function of the major *T. gondii* virulence determinants is presented in Figure 38.2.

In addition to their suppressive effects on macrophages and other host cells, parasites or their products can negatively regulate DC function, both as a means to delay the onset of the adaptive response or to restrain an ongoing response to prevent immunopathology. For example, IL-12 production by splenic DCs is rapidly suppressed following initial in vivo stimulation with soluble *T. gondii* antigen and cannot be restimulated for approximately 1 week thereafter. The antiinflammatory outcome appears to result from the induction, by parasite products, of lipoxin A4, an arachadonic acid metabolite that upregulates silencers of cytokine signaling-2 to inhibit soluble *T. gondii* antigen-induced DC migration and IL-12 production.[101] Similarly, in murine malaria, the initial burst of proinflammatory cytokines is down regulated as DCs become refractory to further TLR signaling.[102] The ligands implicated in these interactions include a conserved domain of *P. falciparum* erythrocyte membrane antigen and hemozoin that has been shown to directly inhibit the maturation of human DCs.[103] This TLR tolerance is similar to that induced by endotoxin and may explain the prevalence of "regulatory" $CD11c^{low}CD45RB^{high}$ DCs later in infection that have been shown to induce IL-10-secreting T cells as a negative feedback mechanism to control immunopathology.[104] The relevance of these findings to human disease is supported by the observation that the interethnic differences in malaria infection outcome in Malian children are associated with reduced expression of activation markers and reduced TLR-induced responses in DCs from *P. falciparum*-infected Dogon children who experience more severe disease.[105] Also, the percentage of human leukocyte antigen-DR + DCs has been reported to be significantly lower in Kenyan children with severe or mild malaria compared to healthy controls, and they also had increased frequencies of DCs expressing BDCA-3, a marker that is upregulated on IL-10 treated monocyte-derived dendritic cells (MDDC).[106] In Chagas disease, compromised DC function has been linked to immune suppression in chronically infected mice. *T. cruzi* blood stages inhibited the lipopolysaccharide (LPS)-induced activation of mouse bone marrow-derived DCs, with both IL-10 and TGF-β important in the induction of the regulatory DC phenotype.[107,108] Together, these studies involving *T. gondii*, malaria, and *T. cruzi* suggest that parasites drive DC activation and proinflammatory reactions during the acute stages of infection, followed by the emergence of regulatory DCs that modulate the adaptive response, limiting both immunopathology and pathogen clearance.

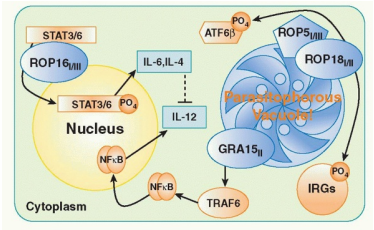

FIG. 38.2. Proposed Cellular Roles for *Toxoplasma gondii* Secretory Proteins in Determining Acute Parasite Virulence. The diagram depicts a cell infected with *T. gondii* showing the major tachyzoite secretory proteins implicated as virulence factors and the host functional pathways they regulate. The rhoptry protein ROP16 is secreted into the host cell and accumulates in the nucleus. There, it is thought to phosphorylate signal transducer and activator of transcription (STAT)3 and STAT6 transcription factors that upregulate interleukin (IL)-4- and IL-6-dependent responses, leading in turn to impaired IL-12 production. The granule protein GRA15 is also released following invasion and associates with the parasitophorous vacuole membrane (PVM). In the case of Type II strains, this results in the induction of IL-12 through a TNF receptor associated factor 6 (TRAF6), NF-kB-dependent pathway. ROP18 is secreted into the host cell where it remains tethered to the PVM and can directly phosphorylate immunity-related guanine triphosphatase (GTPase), blocking their recruitment to vacuoles resulting in impaired parasite clearance. ROP18 has also been shown to phosphorylate activating transcription factor 6 beta (ATF6b), an endoplasmic reticulum (ER) stress response transcription factor involved in control of *T. gondii*-induced dendritic cell function. Recent genetic studies indicate that ROP5, a polymorphic, tandemly duplicated pseudokinase, also plays a major role in acute virulence, likely through interaction with ROP18. See Fentress and Sibley[470] for a recent review. (Adapted from Sibley LD, Boothroyd JC. Genetic mapping of acute virulence in *Toxoplasma gondii*. In: Sibley LD, Howlett BJ, Heitman J, eds. *Evolution of Virulence in Eukaryotic Microbes.* Hoboken, NJ: Wiley Blackwell; 2012, with permission.)

By comparison, studies with *Leishmania* suggest that for most strains their initial encounters with DCs fail to activate these cells and, as a consequence, proinflammatory responses and cell-mediated immune mechanisms are effectively inhibited or delayed even during the acute stage of infection. In the case of *Leishmania major*, the infective

promastigotes deposited in the skin by vector sand flies were found to be poorly taken up by DCs in vitro. However, later in infection, efficient uptake of amastigotes by DC in vitro and in vivo is dependent on parasite-reactive IgG binding to FcγRI and FcγRIII; this primes DCs for efficient production of IL-12.[109] Furthermore, the initial encounter of other *Leishmania* species associated with nonhealing infections in mice not only failed to activate DC but also inhibited their subsequent responses to activation signals.[110] For example, DCs with clear regulatory properties and bearing the phenotype of $CD11c^{lo}$ CD45RB+ CD11b+ IL-10-producing cells, emerge as the predominant DC subset in the spleen of *Leishmania donovani*-infected mice and induce antigenspecific tolerance in vivo.[111]

Mechanisms Underlying Th1/Th2 Response Selection

Because parasites often stimulate CD4+ T-cell responses that are highly polarized in either the Th1 or Th2 direction, parasitic infection models have become important tools for studying the cellular basis of Th1/Th2 response selection. DCs are thought to be an important source of the signals that determine CD4+ T-cell effector choice, and their role is best understood for Th1 responses. *T. gondii*, *T. cruzi*, and *Plasmodium* have been shown upon their initial encounter with DCs to upregulate expression of IL-12 and costimulatory molecules. The

nature of these encounters has been extensively studied for *T. gondii*, whose possession of important Th1-inducing TLR ligand(s) has been inferred by the high susceptibility to infection of mice lacking MyD88[112] and shown to be selectively acting on DCs.[93] Importantly, the high susceptibility of MyD88-/- mice is comparable to that observed in IL-12-/- mice and is not due to the absence of IL-1/IL-18 signaling.[113] As noted previously, the stimulation of TLR11 by parasite profilin appears to be the major MyD88 pathway that triggers this IL-12 response in the murine model.[56] *T. cruzi* induces a delayed, although ultimately strongly and persistently polarized, Th1 response in infected mice that is also MyD88 dependent and, as already mentioned, appears to be induced largely by nucleic acid ligands.[91] The Th1 responses that contribute to immunopathology during blood-stage malaria infections are driven, at least in part, by TLR ligands as MyD88-/- mice have decreased production of IL-12 and attenuated pathology,[114] whereas—in acutely infected mice and humans—hyperresponsiveness has been linked to IFNγ-induced enhancement of TLR expression on DCs.[115] As previously discussed, hemozoin or a hemozoin-DNA complex hemozoin acting through TLR9, and GPI binding to TLR2, appear to be the main malaria component(s) that activate mouse and human blood DCs to secrete IL-12.[116,117,118]

Unlike the protozoan pathogens just described, *Leishmania* appears to trigger TLR signaling in DC poorly, and in most cases, their ability to activate these APCs—for upregulated expression of costimulatory molecules and especially for IL-12 production—requires additional signals that are host derived. For example, the interaction of CD40L on T cells with CD40 on infected DCs enhances IL-12p70 secretion in vitro[119] and is essential for *L. major* -specific Th1 activation and immunity in vivo.[120] In addition to IL-12, there are signals delivered by other cytokines, including IL- 18, IL-27, IFN α, and IL-1, which have been shown to bias *Leishmania*-specific CD4+ T-cell priming toward a Th1 cell fate. Mouse strains with intrinsic deficiencies in host factors necessary to augment Th1-polarized responses against *Leishmania* are highly susceptible to infection, and as noted previously, the first direct demonstrations of the relevance of the Th1/Th2 balance to the regulation of disease outcome in vivo were based on studies of *L. major* infection outcome in different inbred mouse strains. The Th2 polarization that determines the extreme susceptibility of BALB/c mice to *L. major* is due, at least in part, to an intrinsically poor Th1-differentiating capacity, as even in the absence of IL-4 or IL-4 receptor signaling, IFNγ responses remain relatively weak.[121,122] An additional strain difference that may influence Th subset development relates to the finding of very rapid dissemination of parasites from the site of inoculation to the draining lymph nodes and spleen in BALB/c mice, whereas early parasite containment is observed in resistant mice.[123] These differences in parasite dissemination raise the possibility that distinct populations of DCs, with the capacity to induce preferential priming for either Th1 or Th2 cells, are present in different tissue environments and become differentially activated in resistant versus susceptible mice. In support of this hypothesis, targeting to selective DC subsets can be achieved by altering the site of antigen delivery; *L. major* parasites delivered intravenously, intranasally, or even to different skin environments can elicit Th2 responses and nonhealing infections in normally resistant mice.[124,125]

Although *L. major* infection in BALB/c mice remains an extraordinary tool for investigating the factors controlling Th2 response selection in vivo, the fact that the model reflects an aberrant response arising, at least in part, from inherent Th1 developmental defects suggests that these defects might not reflect the mechanisms underlying the Th2 polarization that is a hallmark of helminth infections in virtually all mammalian hosts. Furthermore, although Th2 immune deviation is clearly an inappropriate host response to an intracellular pathogen such as *Leishmania*, Th2 responses are an evolutionarily driven, integral aspect of acquired resistance to most parasitic worms or to the containment of the immunopathologic reactions that worms or their products can provoke.

DCs conditioned by exposure to helminth products polarize naïve T cells toward a Th2 phenotype.[126,127] Importantly, Th2 polarization driven by most helminth antigens is MyD88 independent,[128] whereas Th1 differentiation is in most instances MyD88 dependent. Furthermore, activation of DCs by helminth antigens appears to be minimal as judged by the absence of upregulated MHC and costimulatory markers, cytokine production, and transcriptional or proteomic signatures.[129] In the case of DCs exposed to schistosome egg antigens (SEAs) or *Fasciola hepatica* (liver fluke) tegumental antigen, their maturation and IL-12 p70 production in response to TLR ligands become severely impaired, associated with defective MAPK and NF-κB activation.[130,131,132] In human filariasis, live microfilariae of *B. malayi* modulate DC function by altering TLR3 and TLR4 expression and function.[132] The major component of *Schistosoma mansoni* eggs responsible for conditioning DCs for Th2 polarization

is a secreted T2 ribonuclease omega-1 that is hypothesized to act by limiting conjugate formation between DCs and CD4+ T cells.[133,134] N-glycans containing fucose and binding to DC-specific intercellular adhesion molecule 3-grabbing nonintegrin on DCs have also been implicated in the induction of Th2 responses by schistosomes.[135]

Many of these observations would seem consistent with a model of Th2 induction by DCs that, in the absence of a threshold of instructional, positive signals for Th1 priming occurs via a default pathway. However, defaulting to Th2 is not observed when IL-12-deficient mice are infected with either *T. gondii* or *Mycobacterium avium*.[136] Furthermore, in the case of DCs exposed to SEA, there remains a requirement for NF-κB signaling and costimulation (CD40 and OX40L) for the induction of Th2 responses.[137] Therefore, it is more likely that helminth-conditioned DCs, rather than initiating a default pathway in naïve T cells, provide a set of active, instructive signals that result in Th2 priming. The exact nature of these instructive signals is, however, currently unclear.

Surprisingly, IL-4 does not appear to provide an essential instructive signal for Th2 differentiation, as helminth-conditioned DCs will polarize a Th2 response in vitro in the absence of IL-4,[126] and mice deficient in IL-4, IL-4R, or STAT 6 develop diminished but still physiologically significant Th2 responses when infected with *Nippostrongylus brasiliensis* or *S. mansoni*.[138,139] Using T-cell-specific gene ablation of Notch1 and 2 receptors, it was revealed that Notch is required for Th2 responses to *Trichuris muris*[140] and also drives IL-4R/STAT 6 independent Th2 differentiation in vitro in response to SEAs.[141] Although DC expression of the Notch ligand, Jagged-2, has been shown to be required for Th2 differentiation in response to SEA in vitro, it is apparently not required for Th2 polarization induced by SEA-pulsed DCs in vivo.[142]

Although IL-4R/STAT 6 signaling is not essential for priming of IL-4+CD4+ T lymphocytes in many helminth infection models, it is clear that IL-4 plays a critical role in the maturation and stabilization of Th2 cells once their phenotype has been decided. In this context, Th2 cells need not be the only source of IL-4 for maturation of the Th2 response; basophils committed to express IL-4 are recruited to the liver and lungs of mice infected with *N. brasiliensis*,[143] and a direct role for basophils in helminth-induced type 2 immunity was confirmed by the capacity of adoptively transferred IL-4 producing, adult-derived basophils to restore the ability of juvenile mice to expel *N. brasiliensis*.[144] It has been argued that basophils rather than DCs are the critical cells responsible for helminth-induced Th2 polarization (see following discussion), but this hypothesis has been challenged and may be limited in validity to those experimental models where IL-4 appears necessary for Th2 priming.

EFFECTOR MECHANISMS OF HOST RESISTANCE

Once parasites have successfully evaded innate host defenses and their antigens have been processed and presented by APCs, adaptive cellular and humoral immune responses are invariably induced, usually against a wide array of antigenic constituents of each pathogen. The problem is that because of the nature of the host-parasite adaptation, these responses are rarely orchestrated in a manner that will completely eliminate the parasite or restrict its growth. The design of successful immune intervention strategies depends on the identification of relevant target antigens but even more importantly on an understanding of

the type of immune responses and protective mechanisms that must be induced. These effector mechanisms can be broadly classified based on the type of parasite (intracellular or extracellular) against which they are directed.

Intracellular Parasites

Because of their primary habitat within host cells, intracellular parasites are thought to be particularly susceptible to cell-mediated immune effector mechanisms, often involving a mixture or succession of CD4+ and CD8+ T-cell responses. The extent of CD8+T-cell involvement appears to be related to the degree of class II versus class IMHC expression on the host cells infected. CD8+ T cells are especially critical effector cells for the control of *T. cruzi* or *T. gondii* infections, as well as the liver stages of malaria, as these parasites infect nucleated cell types that express only MHC class I molecules. Although slow to develop during the early stages of *T. cruzi* infection, CD8+ T cells with specificity for immunodominant antigens encoded by the trans-sialidase gene family reach enormous numbers in mice (30% of the total CD8+ T-cell population) and following a contraction phase, persist throughout the chronic stage of infection.[145] Unlike some chronic viral infections, *T. cruzi* -specific CD8+ T cells in chronically infected mice do not appear to become exhausted and bear the phenotype of effector memory cells that require persistent antigen to be maintained.[146] In mice chronically infected with *T. gondii*, an effectormemory population of CD8+ T cells is especially critical for long-term resistance to toxoplasmic encephalitis.[147] Even in *Leishmania* infection, where parasites reside almost exclusively in macrophages, CD8+ T cells can be highly protective against both primary infection and reexposure.[148,149] In addition to their contribution of IFNγ to the effector response, CD8+ T cells might also control intracellular parasitic infection through the lysis of host cells. In every protozoan infection analyzed, however, including *T. gondii*,[150] malaria,[151] and *T. cruzi*,[152] mice deficient in the lytic molecules perforin or granzyme B showed no or minimal loss of host resistance. In fact, perforin-mediated lysis of vascular endothelial cells was found to contribute not to protection but to the severity of experimental cerebral malaria in mice.[153] These observations suggest that, as already noted for NK cells (see previous discussion), the protective functions of CD4+ and CD8+ T cells against intracellular parasites are mediated primarily through cytokine production rather than target cell lysis.

IFNγ is the key cytokine involved in control of intracellular protozoan parasites, as demonstrated by the extreme susceptibility of IFNγ-deficient mouse strains to infections with *Leishmania*,[154] *T. cruzi*,[155] *T. gondii*,[156] *Plasmodium*,[157] and even *C. parvum*,[158] which dwells in epithelial cells inside the

gut. Its mechanism of action is perhaps clearest in the case of *Leishmania*, which replicate primarily in macrophages— a cell type readily activated by this cytokine. The major function of IFNγ in restricting parasite growth appears to be the induction of inducible nitrogen oxide synthase (also known as NOS2) and the subsequent generation of toxic reactive nitrogen intermediates (RNI) within infected macrophages. Thus, disruption of the NOS2 gene in a normally resistant strain leads to a susceptible phenotype, and macrophages from the same knockout strain show defective IFNγ-induced control of parasite growth.[159] In addition to IFNγ, optimal production of RNIs is dependent on costimulation with TNF-α or triggering by alternative signals such as IFN-αβ or by CD40L produced or expressed by activated CD4+ T cells.[160] The production of RNI by IFNγ-activated macrophages is inhibited by IL-4, IL-10, IL-13, and TGF-β,[161] and this is likely to be a major mechanism by which the Th2 response prevents healing in *Leishmaniasis*.

Cytokine-mediated control of intracellular *T. gondii* infection involves a more complicated mechanism than induction of RNIs. The immunity induced cannot be attributed solely to activated macrophages as originally thought, as the parasite infects multiple host cell types and host resistance requires IFNγ signaling in cells of both hemapoietic and nonhemapoietic origin.[162] Accordingly, the role of RNIs in resistance has been shown to be limited, functioning predominantly in the chronic stage of infection, although the outcome may vary following oral infection with *T. gondii*. An important clue concerning the mechanism controlling acute infection came from studies in mice deficient for members of the p47 GTPase family, now referred to as IRG genes. The IRG proteins involved are induced by IFNγ in a variety of hemapoietic as well as nonhemapoietic cell types. Mice deficient in either Irgm3 (IGTP) or a second family member, Irgm1 (LRG-47), were found to be highly susceptible to infection with *T. gondii* while developing a normal IFNγ response.[163] Upon IFNγ stimulation of *T. gondii*-infected cells, Irgm3 has been shown to traffic from the endoplasmic reticulum to the parasitophorous vacuole where it participates in a process involving disruption of the vacuole, stripping of the tachyzoite membrane, and autophagic elimination of the parasites in the host cell cytosol.[164,165] Although Igrm1 is similarly required for effective IFNγ-dependent control of *T. gondii*, it is not recruited to the parasitophorous vacuole and is thought instead to serve as a negative regulator protecting immune cells from a variety of cytotoxic functions.[166] In this regard, Irgm1 also plays a major role in IFNγ-dependent host resistance to *T. cruzi* where, in addition to regulating intracellular killing of the parasite, it is required for a normal hematopoietic response to the infection.[143] As noted previously, IRG proteins (eg, Irgm3) are targeted by *T. gondii* virulence factors, and thus, the IRG system may be coevolving in response to pathogen pressure.[98,166] Another IFNγ-dependent mechanism of intracellular parasite killing that limits *T. gondii* replication in human but not mouse nonhemapoietic cells is the induction of indolamine 2,3-dioxygenase, an enzyme that catabolizes tryptophan, an essential amino acid for growth of this protozoan.[167] These examples underscore the complexity of the effector pathways triggered by IFNγ, which act against different parasites in different host cells.

Although resistance to the erythrocytic stages of malaria is largely mediated by antibodies, they likely act in concert with T cells. Thus, even when the primary role of antibody is clear, as with the passive transfer of immune serum, the extent of protection is reduced by prior splenectomy or T-cell depletion. The relevant pathways seem to function through cytokine (eg, IFNγ, TNF-α) activation of macrophages that phagocytose and destroy infected RBCs in the spleen. This process is augmented when infected RBCs are opsonised by Fc receptor (FcR)-binding antibodies, providing an excellent example of cooperation between cellular and humoral arms of the immune response. In support of this concept, resistance to human malaria has been correlated with T-cell production of IFNγ[168] and generation of NO[169] in vitro and with FcR-binding antibody subclasses.[170] Moreover, blood-stage immunity can be established in the absence of antibody, for example, after infection in B-cell-deficient mice, and can be transferred with defined CD4+ T cell lines and clones, indicating that cellmediated effector mechanisms can operate independently of antibodies.[171] Experimental infection studies in humans further support this idea. Ultra-low dose challenge with *P. falciparum*-infected RBCs[172] or sporozoites[173] confer immunity to reinfection; Th1 cell responses are induced, but little or no malaria-specific antibody was detected. However, experimental vaccines designed to induce potent T-cell responses have been associated with high levels of immunopathology. For example, 60% of mice receiving a vaccine designed to induce CD4+ Th1 responses to an immunodominant blood stage antigen of *Plasmodium yoelii* died despite being able to effectively eliminate infected erythrocytes.[174]

Intracellular protozoa live briefly in the extracellular milieu during initial host infection and when they invade new cells during their in vivo multiplication. During this period, they are vulnerable to attack by antibody. In addition, while not directly killing free parasites, antibodies can block their invasion of new cells, thereby suppressing infection. These forms of humoral immunity are of special interest in vaccine development. After repeated infection, humans living in areas endemic for *P. falciparum* gradually develop immunity to asexual blood stages, preventing high-density parasitemia, and thereby preventing clinical disease. The contribution of antibody to this resistance was demonstrated in experiments in which serum from highly immune adults was transferred to acutely infected children, resulting in a temporary but highly significant reduction in parasitemia.[175] The generally accepted explanation for slow acquisition of immunity to malaria is that it is strain specific, and that an individual becomes immune only after being exposed to the strains circulating in his or her

community. Furthermore, humoral immunity to malaria is likely to depend upon an array of antibodies of differing antigen specificities and functions, including agglutination of sporozoites, merozoites, or parasitized RBCs; inhibition of parasitized RBC cytoadherence to small blood vessels; and/or blocking of hepatocyte or red cell invasion by sporozoites or free merozoites.[176] For the latter mechanism, the fine specificity of the

antibody is crucial as some antibody specificities are able to block merozoite invasion into erythrocytes, whereas others, with distinct but overlapping specificities, either have no effect or, in the worst case, impede the activity of invasion inhibitory specificities.[177]

A well-studied example of antibody-mediated protection is the response to the circumsporozoite (CS) protein present on preerythrocytic stages of malaria.[178] Monoclonal antibodies directed against the CS protein prevent invasion and development of sporozoites in cultured hepatocytes and in vivo, in passive transfer studies, confer protective immunity against *P. berghei*, *P. yoelii*, *Plasmodium vivax*, or *Plasmodium knowlesi* sporozoite challenge.[179] With the advent of intravital imaging techniques, it has become apparent that sporozoites are initially inoculated into dermal connective tissues where they may take up to 30 minutes to locate and invade a blood vessel. In immunized animals, or those that have been passively transfused with anti-CS antibodies, sporozoites become immobilized within minutes and fail to invade blood vessels (see Vanderberg and Frevert[180] for excellent video footage of these effects). Although incomplete sporozoite neutralization or inhibition of hepatocyte invasion allows the development of forms that can infect red cells, reducing the number of developing hepatic schizonts can significantly reduce the size of the blood inoculum, delay the onset of patent parasitemia, and may allow the host more time to develop effective anti-blood-stage immunity. Sporozoite antigens can confer significant protection in their own right (as described subsequently, see vaccines) and may also prove to be very valuable components of a multivalent vaccine.

Extracellular Parasites

Extracellular parasites are a highly diverse group of pathogens that include nematoda (round worms) and platyhelminthes (trematode and cestode flat worms) as well as some extracelluar protozoa such as *Giardia* spp. and African trypanosomes. Unlike bacteria, viruses and protozoa that replicate within their hosts and most helminth parasites require an intermediate host or a period outside the mammalian host to replicate and complete their life cycle. Extracellular parasites exhibit variability in size, tissue tropism, and mechanism of immune evasion; helminths, in particular, often live in their definitive host for several years, hiding out in the gut, blood, lymphatics, and a variety of other host tissues. Together, these life history traits likely explain why a distinct set of complementary immune effector mechanisms are required to combat these large, multicellular pathogens. A variety of specialized innate and adaptive immune cells and mediators are triggered during infection including ILCs, T cells, eosinophils, mast cells, basophils, macrophages, and antibodies, and together these effectors mount a multipronged attack. Thus, immunity is achieved through a variety of mechanisms that include antibody-dependent cellular cytotoxicity, mucus secretion, alterations in gut physiology, and exposure to toxic mediators produced by epithelial cells, eosinophils, and alternatively activated macrophages (AAMs). Intestinal microflora have also been shown to influence parasite fecundity[181] and host immunity.[182]

Most extracellular helminth parasites induce highly polarized CD4+ Th2 cell responses (IL-3, IL-4, IL-5, IL-9, IL-10, and IL-13) that promote immunity by triggering mast cell, eosinophil, giant cell, IgG1/IgE/IgA, and mucosal cell responses. Mice deficient in the IL-4 receptor alpha-chain (IL-4Rα), STAT6, or the transcription factor GATA-binding protein 3 (GATA3) display increased susceptibility to a wide variety of helminths, identifying a critical role for the IL-4 and IL-13 signaling cascade in antihelminth immunity.[183,184] In addition to antigen-specific adaptive immune responses, it has become clear that type 2 cytokines secreted by ILCs are also involved in the development of resistance to some helminth parasites.[59,185,186,187,188]

Although the mechanisms that trigger the differentiation and development of Th1 and Th17 type responses are well defined,[189,190] the mediators that drive Th2 development appear to be more complex, although DCs,[191] select parasite antigens,[133,134,192] basophil-derived IL-4,[193,194] and epithelium-derived alarmins such as thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 released during parasite invasion[188,195,196,197,198] are all involved to varying degrees depending on the particular helminth. Whereas basophils were recently reported to be the critical drivers of Th2 responses during helminth infection—by serving both as APCs and by producing IL-4[199,200]—subsequent studies have questioned the ability of basophils to serve as APCs[201] and instead identified DCs as the primary APCs and basophils as the dominant source of IL-4.[144,191,194,202,203,204,205,206] Thus, conventional DCs and basophils are both involved in the development of helminth-induced Th2 immunity.

Many helminths cause significant tissue damage as they migrate through tissues, triggering the release of alarmins by mucosal epithelial cells. One of the first epithelial-derived alarmins shown to play a role in helminth-induced Th2 immunity was epithelial cell-derived TSLP,[197,207,208] with a recent study demonstrating that TSLP can elicit a unique population of basophils that promotes Th2 cytokine-mediated immunity.[209] Nevertheless, other studies have suggested that the contribution of TSLP to Th2 response development may be limited to *T. muris* infection.[196,198] Indeed, several Th2-promoting helminth antigens have been shown to bypass the need for TSLP in the induction of Th2 immunity because they can directly inhibit the production of IL-12 p40 in DCs.[196,210,211] IL-1R4 (T1/ST2) and its ligand IL-33, an alarmin released from the nucleus of necrotic epithelial and endothelial cells and fibroblasts, have also been shown to contribute to Th2 response development,[212] again by suppressing the Th1 arm of immunity.[213] A similar role has also been identified for the IL-17 family cytokine IL-25 (IL-17E).[214,215,216] Studies conducted with IL-25-/- mice showed that by controlling the downstream actions of IL-13,[217] epithelial cell-derived IL-25 is required for the development of immunity to both *T. muris* and *N. brasiliensis*.[215,216] Thus, multiple alarmins released by epithelial cells are involved in the generation of protective IL-4/IL-13 responses during helminth infection, with the relative contribution of each mediator being largely dictated by the pathogen and its site of infection.

When these alarmins are released during parasite invasion, they induce the recruitment of ILCs called nuocytes or

innate helper cells, which are poor producers of IL-4 but secrete large quantities of IL-13, thus quickly activating downstream protective type 2 effector responses.[59,186,187,188] Their rapid production of type 2 cytokines also provides a feedforward mechanism to activate the adaptive immune response, further amplifying type 2 immunity. The combined innate and adaptive type 2 response activates a broad range of downstream antiparasite effector mechanisms. Epithelial cells and goblet cells in the gut express the type II IL-4 receptor,[218] which binds IL-4 and IL-13, triggering goblet cell differentiation and mucus secretion.[219,220,221] Indeed, the mucins Muc2 and Muc5AC were recently shown to be critically required for the development of resistance to several intestinal nematodes.[222,223] Intestinal epithelial cells also secrete resistin-like molecule-β (Relmβ),[224] which regulates both the spontaneous and IL-4-induced expulsion of the luminal dwelling parasites *N. brasiliensis* and *Heligmosomoides bakeri* (formerly called *Heligmosomoides polygyrus*).[225] IL-4 and IL-13 also have effects on intestinal physiology, causing decreased peristalsis, increased mucosal permeability, reduced sodium-linked glucose absorption, and decreased chloride secretion in response to 5-hydroxytryptamine and acetylcholine,[226,227] which facilitates the expulsion of nematodes from the gut. IL-4Rα signaling also stimulates intestinal smooth muscle contractility and epithelial cell proliferation and turnover,[228,229] which operate together with epithelial secretions to promote parasite entrapment in mucus and

ultimately expulsion from the gut via a combined “weep and sweep” mechanism.[230,231] Intestinal permeability and smooth muscle contractility are also regulated by the actions of mast cell-derived proteases and protease-activated receptors that are expressed throughout the small intestine. An overview of antihelminth effector mechanisms is shown in Table 38.3.

TABLE 38.3 Major Antihelminth Effector Mechanisms

Parasite[a]	CD4+Th2 cells	Eosinophils	Mast Cells	Basophils	ILC2[b]	M2 Mø[c]	M1 Mø/NO[d]	Neutrophils	IgE	Mucins goblet cells	Relm β	TSLP	IL-25	T1/ST2[e]-IL-33	IL-4/IL-13	IL-5	IL-9	IL-10	IFN-gamma
Trichuris muris	Yes	NO	NO	Yes	Yes	?	NO	NO	NO	Yes	NO	Yes	Yes	Yes	Yes	NO	Yes	Yes	NO
Nippostrongylus brasiliensis	Yes	NO	NO	Yes	Yes	NO	NO	NO	NO	Yes	Yes	NO	Yes	NO	Yes	NO	NO	Yes	NO
Heligmosomoides bakeri (Heligmosomoides polygyrus)	Yes	NO	Yes (fecundity)[f]	?	?	Yes	NO	NO	NO	?	Yes	NO	?	?	Yes	NO	?	NO	NO
Litomosoides sigmodontis	Yes	NO	NO	NO	?	?	NO	Yes	NO	?	?	?	?	?	Yes	Yes	?	NO	Yes
Trichinella spiralis	Yes	NO	Yes	?	?	NO	NO	NO	Yes	Yes	?	?	?	?	Yes	Yes (minor)[h]	Yes	Yes	NO
Schistosoma mansoni	NO(Yes[g] human)	NO(Yes human)	NO	NO	?	NO	NO	NO	NO (Yes human)	?	?	NO	NO	NO	NO(Yes human)	Yes (minor)	NO	NO	NO

CD, cluster of differentiation; IFN, interferon; Ig, immunoglobulin; IL, interleukin; NO, not needed; TSLP, thymic stromal lymphopoietin; YES, required; ?, indicates not investigated.

a Effector mechanisms involved in parasite clearance/expulsion determined from animal studies.

b ILC2: innate lymphoid cells producing Th2 cytokines (IL-13).

c M2 Mø: M2 alternatively activated macrophages.

d M1 Mø: M1 classically activated nitric oxide-producing macrophages.

e T1/ST2: IL-33 receptor.

f Decreases parasite fecundity only.

g No role in immunity in rodents but strong correlative evidence of a role in acquired immunity in humans.

h Only a minor role observed in some animal studies.

Several worms have been studied in detail in this regard: *T. muris*, a natural parasite of the mouse and closely related to human whipworm, *H. bakeri*, *Litomosoides sigmodontis*, *T. spiralis*, *N. brasiliensis* (the rat hookworm), and *Strongyloides stercoralis*. Although Th2 cytokines are clearly involved in resistance to many of the intestinal nematodes, the importance of Th2 immunity is less certain with many of the filarial (*B. malayi*, *Wuchereria bancrofti*, *L. sigmodontis*)[231,232,233] and schistosome species,[234] as discussed in detail in the following text.

T. muris and *H. bakeri* are both transmitted by the oralfecal route independently of an intermediate host; in some strains of mice, they are capable of establishing chronic infections. In the case of *T. muris*, development of immunity is dependent on the genetic background and sex of the definitive host, with resistant animals rejecting the parasite shortly after exposure and susceptible animals developing chronic infections.[235,236] With this parasite, resistant mice develop type 2 responses, whereas susceptible mice mount type 1 responses, with a variety of immunoregulatory cytokines and mediators influencing this decision. For example, in susceptible strains of mice, IFNγ, IL-12, MyD88, and TLR4 deficiencies[235,237] can promote the expansion of type 2 cytokine responses and facilitate the clearance of the parasites.

For *N. brasiliensis*, *H. bakeri*, and *T. muris*, primary immunity in most strains of mice depends on the development of a parasite-specific CD4+T-cell response,[238,239] with recent studies demonstrating that a broad T-cell receptor repertoire is needed for the development of efficient immunity to *N. brasiliensis*.[240] However, exogenous IL-4 or IL-13 can cure

primary *N. brasiliensis*, *T. muris*, and *H. bakeri* infections in T-cell-deficient mice.[241] Recent studies demonstrated that IL-25 and IL-33 induce IL-13-expressing nuocytes that are capable of mediating immunity independently of CD4+T cells, suggesting that the production of IL-13 by these ILC may be sufficient, particularly during primary infections.[59] The rapid immunity that develops during secondary infections, however, is likely facilitated by antigen-specific CD4+T cells. Interestingly, the individual roles of IL-4 and IL-13 in these infections appear to be dependent on the genetic background of the host as C57BL/6 IL-4 knockout mice are susceptible to *T. muris* infection, whereas BALB/c IL-4 knockout mice are resistant.[242,243] These latter studies and other comparative studies conducted in IL-4Rα-, STAT6-, and GATA3-deficient hosts revealed an IL-4-independent role for IL-13 in resistance,[183,184] which was confirmed by treating BALB/c IL-4-deficient mice with a soluble IL-13 inhibitor. IL-13- and IL-13Rα1-deficient mice also develop chronic infections despite generating relatively normal IL-4 responses,[244] further emphasizing the critical role of IL-13 in antinematode immunity.[242]

In contrast to *T. muris* where the requirement for IL-4 depends on the genetic background of the host, development of immunity to *N. brasiliensis* appears to be almost entirely IL-4 independent, as demonstrated in experiments with IL-4-/- and anti-IL-4 monoclonal antibody (mAb)-treated mice.[183] The IL-4-independent mechanism was, however, dependent upon IL-4 receptor and STAT6 signaling, again identifying a critical role for IL-13, which was confirmed in IL-13 blocking studies. IL-13- and IL-4/IL-13-deficient mice are also more susceptible to *N. brasiliensis* than IL-4-/- mice, further emphasizing the dominant role of IL-13.[245] Type 2 immunity was suggested to be controlled predominantly by an innate, IL-13-expressing, noneosinophil type cell of hematopoietic origin,[185] which has subsequently been hypothesized to be one or more of the recently described lineage-negative (CD4-CD127+IL-1RL1+ IL-17RB+RoRgt-) innate lymphoid cell populations: natural helper cells or fat-associated lymphoid clusters, nuocytes, innate natural helper type 2 cells, multipotent progenitor type 2 cells, and/or basophils.[59,186,187,188,203,204] Studies are ongoing to decipher the relative contributions of each of these populations in antinematode immunity.

Unlike infections with *N. brasiliensis* and *T. muris*, most mouse strains are susceptible to primary *H. bakeri* infections, but following drug clearance, the animals develop strong type 2 responses and become highly resistant to secondary infection. The maintenance of immunity during recall infections is crucially dependent on the presence of memory CD4+ Th2 cells at the site of infection,[246] with the lung being an important location for the initial priming of

memory CD4+ T cells.[247] The Th2 response triggers the development of arginase-1-expressing AAMs, which help clear primary *N. brasiliensis* and secondary *H. bakeri* infections.[218,248] The relative importance of AAMs in immunity to other nematode infections, however, remains less clear.[249]

Other cytokines, chemokines, and signaling pathways, including TNF, IL-18, IL-1, IL-10, IL-21, IL-27R (WSX-1), CCL2, Notch, and the NF-κB family have also been identified as important regulators of protective IL-13 responses during intestinal nematode infection. For example, mice treated with neutralizing antibodies to TNF develop relatively normal Th2 responses following infection with *T. muris*, yet they fail to expel their parasites, illustrating a critical downstream role for TNF in the mechanism of IL-13-mediated immunity.[250] IL-1α and IL-1β are also protective, although unlike TNF deficiency, which does not affect the Th2 response, IL-1 deficiency has a profound inhibitory effect on IL-13 production, thus identifying a unique role for IL-1 in the development of Th2-dependent antinematode immunity.[251,252] IL-21 also appears to play a critical role by augmenting Th2 cytokine production and the differentiation of AAMs.[253,254,255] Resistance to *T. muris* is also highly dependent on IL-10, which actively downregulates expression of the antagonistic type 1 cytokines IL-12 and IFN-γ .[220,256] IL-10-deficient mice also display marked morbidity and mortality following *T. muris* infection, identifying an additional role for IL-10 in the suppression of immune-mediated pathology during nematode infection. Interestingly, broad-spectrum antibiotics were found to partially protect *T. muris*-infected IL-10-deficient mice from morbidity and mortality, suggesting that an outgrowth of opportunistic bacteria in the gut exacerbates disease.[220]

Immunity to *T. spiralis* also requires STAT6-[257] and IL-4R signaling but not IL-4,[231] again identifying a dominant role for IL-13-mediated signaling in the development of immunity.[258] The fact that exogenous IL-13 can promote resistance in immunodeficient hosts suggests that a non-bone marrowderived cell in the gastrointestinal tract expressing the IL-4R is required for immunity, which was confirmed in elegant bone marrow chimera experiments.[259] The contribution of IL-13-producing innate effector cells in immunity to *T. spiralis*, however, has not yet been examined. Mast cells, which have been linked with IL-4/IL-13-induced changes in intestinal physiology, also play an important role in immunity to *T. spiralis*, as mice treated with mAbs against stem cell factor (a non-T-cell-derived cytokine) or c-kit (both of which play a central role in mast cell development) are unable to expel the parasites.[260] In these experiments, there was no inhibitory effect on the CD4+ Th2 cell response, and once the mAb treatment was stopped, the parasites were quickly expelled. These studies suggested that the CD4+ Th2 cell response cooperates with stem cell factor to promote mastocytosis, which in turn facilitates parasite expulsion by secreting proteases that degrade tight junctions, leading to increased fluid production in the gut.[261] Several cytokines made by CD4+ T cells, including IL-3, IL-4, IL-9, and IL-10, have been implicated in the development of the protective mast cell response during *T. spiralis* infection.[256,262,263,264,265,266] IL-9 is particularly important as high concentrations of IL-9 can accelerate the clearance of *T. spiralis*[267] and *T. muris*,[268] whereas anti-IL-9 mAb treatment significantly inhibits immunity to *T. muris*.[269] IL-9-producing DCs also generate protective CD4+ Th2 responses,[270] raising the possibility that IL-4 derived from mast cells operates in a feedback loop to enhance Th2 cell differentiation.[268] Mast cell-derived tryptases (eg, mMCP-6) are also be involved in the development of anti-*T. spiralis* immunity.[271]

Eosinophils are frequently associated with helminth infections, and AAMs have been shown to play an important role in eosinophil recruitment to peripheral sites.[272] Surprisingly, however, eosinophils do not appear to play

a significant role in immunity to *T. muris*, *H. bakeri*, *T. spiralis*, or *N. brasiliensis*.[273] Nevertheless, there is evidence from experiments with anti-IL-5 mAb treated, IL-5 transgenic, and IL-5Rα-chain knockout mice that these cells participate in immunity against tissue-invasive larval forms of *Strongyloides* spp. and *Angiostrongylus cantonensis*.[274,275,276,277] One study suggested that eosinophils promote immunity by serving as APCs.[277] There is also a large body of literature, primarily from studies in rats and humans, which suggests that eosinophils play a critical role in protection against (non-gut dwelling) schistosomes,[278,279] although anti-IL-5-treated and eosinophil-deficient mice displayed similar parasite burdens as their wild-type counterparts, possibly suggesting host-specific roles for eosinophils in the development of immunity.[278,280] In contrast to *S. mansoni* infections, immunity against primary *T. spiralis* infection in mice is impaired in IL-5-deficient mice and associated with decreased muscle hypercontractility.[281] However, recent studies conducted with eosinophil-deficient mice suggested that eosinophils promote the survival of muscle stage larvae in chronic *T. spiralis* infection by suppressing IFNγ and NO production.[282] Overall, despite the prevailing dogma, the majority of studies have failed to support a critical function for eosinophils as direct effector cells in immunity to intestinal helminths and instead suggest more of an immunoregulatory role for these cells.[283,284,285] In addition, because tissue eosinophils have been shown to produce IL-4 and IL-13,[280,286] one of their primary functions may be to participate in type 2-cytokine-mediated wound repair,[287,288] which appears to be a critical determinant in the successful establishment of chronic infections by many tissue-destructive helminths.[289]

A similar Th2-inducing mechanism has also been proposed for tissue basophils, which were identified as an important source of IL-4 in schistosome, filarial, and hookworm infections in both mice and humans.[143,290,291] In individuals infected with filarial parasites, the presence of antigen-specific IgE appears to be critical for the secretion of IL-4 by basophils.[291] Transgenic mice with constitutive and selective deletion of basophils are highly susceptible to secondary but not primary *N. brasiliensis* infection,[203] suggesting that the activation of basophils by antigen-specific IgE is critically required for the development of secondary immunity. Thus, CD4+ Th2 cells, ILCs (eg, nuocytes), eosinophils, mast cells, IgE, and basophils all contribute to the generation of protective type 2 responses to varying degrees.

Not withstanding the clear results in mice, the relative importance of the type 2 effector response in the development of immunity in humans remains uncertain. The most straight forward hypothesis, and one predicted for many years, that Th2-induced increases in IgE antibody production is protective against intestinal helminths, has either been refuted following intensive investigation using mouse models, or at least received little direct confirmation.[273,292] Indeed, B cell- and Ig-deficient mice display only modest increases in susceptibility to most helminth infections,[293] although parasite-specific IgG and IgA antibodies have been found to inhibit the fecundity of adult *H. bakeri*.[294] There is, however, a great deal of epidemiologic evidence that type 2 antibody responses, particularly in the form of antigen-specific IgE, mediate the resistance that develops with age in endemic areas. The possibility exists that there are host species differences in this regard because in rats the rapid expulsion of a secondary *T. spiralis* infection is easily transferred to naïve animals with IgE.[295] IgE-/- mice also expel adult *T. spiralis* worms at a much slower pace and develop nearly twice as many muscle larvae as their wild-type counterparts.[296] Although the exact mechanism by which IgE mediates protection is unclear, it is possible that parasitespecific IgE cooperates with eosinophils or macrophages in an antibody-dependent cellular cytotoxicity-type antiworm effector mechanism.[297,298,299] It may also play an important role in the production of IL-4 and IL-13 by mast cells and basophils. IgE-primed basophils secreting IL-4 may also enhance immunity by promoting the differentiation of CD4+ Th2 cells.[143,290,291] Consistent with the potential protective role of IgE, carriage of the human leukocyte antigen-DRB1*13 class II allele is associated with increased posttreatment IgE levels against *S. mansoni* antigens and decreased reinfection levels.[300] Further support for this mechanism comes from field studies in Brazil, where it was discovered that resistance to schistosome infection is controlled by a major gene that localizes to a region of chromosome 5, which

encodes the type 2 cytokines.[301] IgE and eosinophils are also both required for vaccine-mediated immunity against larval *O. volvulus* in mice immunized with irradiateiated larvae. Thus, in the case of secondary helminth infection, IgE, eosinophils, and basophils all collaborate to enhance immunity. Further work is required to elucidate the precise roles of these effector mechanisms in primary versus secondary infections and in different host species, with particular attention being paid to testing of hypotheses derived from model organisms in naturally infected hosts and vice versa.

In contrast to the intestinal parasites, where immunity is clearly dependent on type 2 cytokines, immunity to filarial parasites appears to be dependent on both type 1- and type 2-associated effector mechanisms.[302] In the *Brugia* mouse model of filariasis, B cells are also required for the development of immunity,[303] with IgM antibodies participating in the clearance of both primary and secondary infections and IgE assisting in clearance of primary but not secondary infections.[304] Parasite-specific IgA also plays a protective role in human bancroftan filariasis.[305] Genetic polymorphisms in genes encoding endothelin-1 and TNF-RII have also been correlated with the development of chronic human disease,[306] whereas studies in mouse models have identified important roles for IL-10 and possibly TGF-β1 in the establishment of chronic infections with *B. malayi*.[307] Eosinophils and antibody are required for resistance to primary *Brugia pahangi* infection,[308] suggesting an antibody-dependent cellular cytotoxicity-type effector mechanism is required for parasite clearance. However, studies with *Onchocerca ochengi*, a filarial parasite of cattle related to *O. volvulus*, have suggested that eosinophil degranulation on the parasite cuticle can in some cases actually promote worm viability.[309] Worm viability is also increased by endosymbiotic *Wolbachia* bacteria, which play a critical role in the growth, development, and survival of *B. malayi*.[310] *Wolbachia* also convert potentially deleterious eosinophil responses into neutrophildominated responses, which are incapable of contributing to

antifilarial immunity.[309] In the *L. sigmodontis* model of filariasis, IL-4 blocks nematode development in resistant hosts but not in susceptible strains of mice, further suggesting that Th2- dependent and Th2-independent mechanisms are involved in resistance.[311] Some studies have also identified important synergistic activity between IFNγ and IL-5 in antifilarial immunity, although the role of the Th1-associated cytokine IFNγ remains unclear.[312] Finally, IL-10-expressing CD4 +Foxp3+regulatory T cells have been shown to suppress immunity to *L. sigmodontis*, identifying a critical role for regulatory T cells in the establishment of chronic filarial infections.[313]

As with many of the extracellular parasites, resistance to protozoan trypanosomes requires elements of both cell-mediated and humoral immunity. The African trypanosomes are tsetse-transmitted parasites that inhabit the extracellular compartment of their host's blood and avoid detection by the humoral immune system by switching among antigenically distinct variant surface glycoproteins (VSGs) (see following discussion). Trypanosome-infected hosts typically do not produce antibodies that destroy the parasite, other than those that are VSG specific. Parasitemias manifest as recurring waves, with each wave of parasites expressing a different VSG, and are cleared following development of VSG-specific antibodies. Before an effective antibody response can be generated, however, the host develops quite high parasitemia, severe trypanosomiasis-associated pathology, generalized immunosuppression, and, in some circumstances, debilitating secondary infections.[314] It is clear that overproduction of IL-12, IFNγ, and NO is the primary trigger for these deleterious side effects[315] while also contributing to host resistance,[316] as parasite control within the extravascular tissue compartment requires a parasite antigen-specific Th1 response, associated with IFNγ-dependent activation of macrophages.[317,318] Type I IFNs have also been shown to play a role in early resistance to African trypansomes, although they may contribute to downregulation of IFNγ production and subsequent loss of host resistance later in infection.[319] In humans, resistance to most African trypanosomes, including *T. b. brucei*, is mediated by the TLFs, as previously discussed.[320] Interestingly, the higher rates of kidney disease in infected Africans compared to Americans of European descent appears to be linked to the expression of disease-associated variants of the apolipoprotein L (Apo L) components in TLF that are able to lyse *T. b. rhodesiense* in vitro.[321]

Another important extracellular protozoan, *Giardia*, is a flagellated intestinal parasite that causes both acute and chronic diarrheal disease. Despite its intestinal habitat, *Giardia* appears to be controlled by mechanisms distinct from those mediating resistance to most gastrointestinal nematodes, although recent studies suggest that mast cellderived IL-6 maybe important for the rapid elimination of *Giardia* in mice.[322,323] While a T-cell-dependent mechanism involving TNF-α is essential for resistance to acute infections,[324] numerous studies have suggested that antibodies, particularly the IgA isotype, are required to control chronic *Giardia lamblia* infections.[325] Moreover, it has been proposed that neuronal NOS (NOS1) might facilitate clearance of *Giardia* from the gut by increasing gastrointestinal motility and parasite-induced diarrhea.[326]

These examples clearly demonstrate that although intracellular and extracellular parasites often stimulate distinct immune responses, their immune control may involve overlapping immunologic effector arms.

MECHANISMS OF IMMUNE EVASION

Pathogens that rely on an insect vector to complete their life cycle, or are only sporadically transmitted from one host to another, are under strong evolutionary pressure to prolong their survival within their host. As the adaptive immune response is the principal barrier to the persistence of pathogens in mammalian hosts, parasites have evolved diverse strategies to evade immune control, either by evading immune recognition or by suppressing immune effector mechanisms. The former strategy refers to the ability of some parasites to sequester within sites that are inaccessible to immune attack, to mask themselves with host antigens, to shed their own target antigens, or most notably, to undergo antigenic variation. The latter strategy refers to the active suppression of established, ongoing immune responses that may contribute to the state of equilibrium that is established between host and parasite in sites of chronic infection.

Evasion of Immune Recognition

The asexual, blood stage of malaria would seem the most obvious example of a well-hidden parasite. Its ability to invade mature erythrocytes, which lack both class I and II histocompatibility molecules, in theory at least should protect it from recognition by antibodies or effector T cells. However, because parasitized erythrocytes are efficiently cleared by the spleen, additional immune evasion strategies (ie, antigenic variation) are required (described subsequently). Other intracellular protozoa appear to hide within immunologically privileged sites. The persistence of *T. cruzi* within heart or skeletal muscle, which is believed to underlie the pathogenesis of Chagas disease, occurs despite the fact that parasites are cleared from most other tissue.[327] Infected muscle cells may be only poorly recognized as targets for cytotoxic T lymphocytes, poorly accessible to their homing, or they may have intrinsic defects in immune-mediated killing mechanisms. A similar form of sequestration has been proposed to explain the long-term persistence of *Leishmania* within fibroblasts and dendritic cells following their efficient killing by activated macrophages during the acute stage of infection.[328] While persistent low-level infection of host cells has been proposed as an explanation for latency in *T. gondii* infection, the major parasite reservoir during chronic infection is undoubtedly provided by the tissue cyst, essentially a modified host cell carrying a specialized dormant parasite stage, the bradyzoite. Helminths (with the exception of *Trichinella*, which develop long-lived muscle stage larvae) do not invade host cells and, therefore, cannot use this strategy for evading immune recognition. Furthermore, because most multicellular helminth parasites do not replicate within their mammalian hosts, they are not equipped to evade immune recognition by undergoing antigenic variation. Instead, they employ alternative mechanisms

such as disguising their surfaces with host molecules and rapidly shedding membrane (tegument)-bound immune complexes.[329] In addition, helminths have evolved a series of elaborate processes for inactivating antibody, complement, and cellular effector elements that threaten the parasite surface.[330] Interestingly, recent data suggest that helminths may take advantage of host T lymphocyte and cytokine signals as developmental triggers; if these signals are in low abundance or lacking in vivo, parasite growth may be aborted or severely attenuated.[331,332,333] In the case of schistosomes, development of female parasites is not directly influenced by the adaptive immune system, whereas male development is.[334] In this sequential model, adaptive immune signals trigger development of mature males, which subsequently stimulate development of mature females.

Although the need for antigenic variation might be obvious for extracellular protozoa such as trypanosomes and *Giardia*, it is less obvious why malaria parasites—hiding inside RBCs—should have evolved a similar strategy of immune evasion.[335] The most plausible explanation is as follows. Developing schizonts cause erythrocyte distortion, loss of flexibility, and abnormal surface exposure of various membrane components, all of which make infected erythrocytes vulnerable to clearance from the circulation during passage through the spleen in a manner very similar to that by which normally aged red cells are removed and destroyed. In order to avoid passage through the spleen, the parasite exports to the red cell surface a number of molecular anchors—of which the best characterized is *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1)—that bind to endothelial receptors, allowing the parasite to sequester in peripheral tissues. Parasite sequestration contributes to malaria pathology, clogging blood vessels and triggering focal inflammation, giving rise to cerebral, respiratory, and renal symptoms; sequestration in the placenta gives rise to pregnancy-associated malaria. Because these molecular anchors are parasite-derived, they are recognized by the immune system; antibodies bind to them, prevent endothelial sequestration (and ameliorate disease), and allow parasitized RBCs to be cleared in the spleen.[336] Thus, in order for the parasite to establish a chronic infection, a system of clonal antigenic variation is required. The importance of these molecular anchors as a parasite survival strategy is demonstrated by the fact that there are upward of 50 copies of the gene for PfEMP-1 (*var* genes) per parasite, more than 200 copies of other clonally variant surface protein genes,[337] and innumerable allelic variants of each gene in the global parasite population.

Evasion by Immune Suppression

Generalized immunodepression, which is a feature of many chronic parasitic infections, including malaria, African trypanosomiasis, and visceral leishmaniasis, appears in most instances to be secondary to other immune evasion strategies and results from the need to control inflammation (see the next section) or from a variety of immune dysfunctions that high-systemic parasite burdens can produce. These dysfunctions include disruption of normal lymphoid architecture, such as occurs in the mouse spleen during acute malaria infection[338] and during chronic infection with *L. donovani*.[339] In the case of *T. cruzi*, which can express and secrete multiple members of the highly polymorphic surface sialidase superfamily at one time, epitope-specific T-cell responses are suppressed either by altered peptide ligand inhibition or because immune recognition is impeded by a flood of competing targets.[340] Similarly, lymphocyte polyclonal activation, which can result in depression of antigen-specific responses, is a feature of many parasitic protozoan infections, including blood and tissue trypanosomes, *L. donovani*, *T. gondii*, and rodent malarias, but again, a causal link between polyclonal activation and immune evasion has not been established. The original hypothesis—that these organisms possess mitogenic or superantigenic moieties[341]— has been substantiated only in the case of *T. cruzi* from which the B-cell mitogen has been cloned and characterized as a eukaryotic proline racemase.[342]

Regulatory T Cells and Parasite Persistence

The modulation of ongoing immune responses in sites of chronic infection, such as the gut or the skin, may in many instances reflect not only parasite survival strategies but also normal mechanisms of immune homeostasis that operate to control immunopathology. Accumulating evidence has implicated a crucial role for regulatory T cells in the dynamic equilibrium that is established between parasites and their hosts. Regulatory T cell is the name given generally to the subsets of CD4+ T cells, and more recently CD8+ T cells, which negatively regulate multiple immune functions. Among the different subsets of CD4+ regulatory T cells that have been described, the best characterized are the so-called naturally occurring CD4+CD25+Foxp3+ T cells (nT_{reg}) that are present in naive animals. T cells with suppressive function can also be generated from conventional naïve cells, and include induced regulatory T cells (iT_{reg}), which are converted from activated CD4+ CD25-Foxp3- T cells to CD25+Foxp3+ suppressor cells following encounter with antigen and TGF-β in the periphery, and adaptive regulatory T or Tr1 cells, also generated from antigen activated cells in the periphery but which remain Foxp3- and secrete high levels of IL-10. There is data linking one or more of these populations to the suppression of immune responses to all of the major classes of pathogenic protozoa and helminths.

The evidence that nT_{reg}s contribute to pathogen persistence was initially demonstrated in the *L. major* mouse model, in which CD4+CD25+ T cells accumulated in the dermal lesion and promoted subclinical persistence of amastigotes following clinical cure in resistant mice.[343] Similarly, in a model of mouse malaria characterized by extremely rapid parasite growth (*P. yoelii* YM or 17XL), very early induction of TGF-β and IL-10 appears to inhibit the development of responses that are required for parasite clearance. Neutralization of IL-10 and TGF-β[344]—or, in some studies, depletion of nT_{reg}[345]—at the onset of infection allows parasite replication to be contained and mice are able to resolve their infections and survive. A similar scenario has been observed in experimental human infections within the context of malaria vaccine trials.[346] More recently, however, it has

begun to be appreciated that classical Foxp3+ regulatory T cells and adaptive IL-10-producing T cells have rather different roles in malaria-infected hosts. Thus, the major role of Foxp3+ regulatory T cells seems to be in maintaining immune homeostasis during asymptomatic infection, whereas IL-10-secreting cells are required to restore immune homeostasis after acute clinical infection.[347,348]

The role of IL-10-producing T cells in controlling immunopathology and parasite persistence is especially striking in the case of *T. gondii* and *Leishmania*. IL-10 production by CD4+CD25-Foxp3- T cells that also produced IFNγ was required for the control of Th1-driven immune pathology associated with *T. gondii* infection in mice[349] (discussed in more detail in the following section). A similar phenotype of IL-10-producing Th1 cells was associated with the inability to heal infection with a substrain of *L. major* that induces a polarized Th1 response in C57Bl/6 mice.[350] The clinical data has consistently supported an association of human visceral leishmaniasis with elevated levels of IL-10, which in the visceral leishmaniasis spleen was found to be expressed primarily by CD25-Foxp3-T cells.[351] It is important to emphasize that even in patients with active visceral leishmaniasis in whom systemic infections go uncontrolled, their elevated levels of IL-10 are accompanied by augmented production of IFNγ, which in the visceral leishmaniasis spleen is also expressed primarily by CD25-Foxp3-T cells. Together, these data suggest that in sites of strong inflammation, IL-10-producing Th1 cells may be activated as a powerful mechanism of feedback control to prevent collateral tissue damage. The regulation of IL-10 expression in these inflammatory settings has recently been described. IL-27, an IL-12 family member, was shown to upregulate IL-10 expression by Th1 cells in *T. gondii*-infected mice in a STAT1- and STAT3-dependent manner.[352] Accordingly, *IL-27ra*-/- mice developed lethal inflammation that was associated with defective IL-10 production by Th1 cells isolated from the brains of infected mice. *IL-27ra*-/- mice efficiently control *P. berghei* NK65 infections but have reduced capacity to produce IL-10 and develop severe, frequently fatal, Th-1-mediated liver pathology.[353] *IL-27ra*-/- mice infected with *L. major* also developed more severe pathology, which in the case of nonhealing *L. major* infections is associated with fewer numbers of T cells coexpressing IL-10 and IFNγ, and an increased frequency of Th17 cells.[354] The *L.*

major mouse model has also revealed a potential role for suppression mediated by IL-10 and TGF-β produced by macrophages following uptake of Ig-opsonized parasites.[355,356] Thus, redundant mechanisms of homeostatic control, including innate cells, natural and adaptive regulatory T cells, appear to be activated to control persistent immune pathology associated with antimicrobial immune responses in tissues that are especially susceptible to injury (eg, the liver, brain, skin, and mucosa).

IMMUNOPATHOLOGIC MECHANISMS AND THEIR REGULATION

If the appropriate protective response fails to develop or if the host is not able to achieve sterile immunity, then inflammation and other pathologic changes may be unavoidable consequences of persistent infection. This is the situation for many parasitic diseases. This does not mean that all infections with the same parasite species lead to the same immune pathology. One of the most striking features of human parasitic disease is the great variability in clinical outcome, ranging from asymptomatic infection to fatal disease. Esophageal disease due to *T. cruzi*, liver fibrosis, portal hypertension, and hepatosplenomegaly due to *S. mansoni*, and cerebral malaria due to *P. falciparum* are a few examples of the many immunopathologic complications that may occur in some individuals but not others. Part of this variability is determined by host genetics, whereas other potential determinants include parasite virulence factors, infection intensity, and the prior level of immunity. The picture is further complicated in individuals that are coinfected with other pathogens: For example, the severity of *P. falciparum* malaria is increased by concomitant bacteremia but reduced by concomitant *P. vivax* infection.[357] Antiretroviral drugs also impair CD36-mediated cytoadherence and nonopsonic phagocytosis of parasitized erythrocytes by human macrophages, which may lead to severe malaria disease outcomes in antiretroviral treated coinfected individuals.[358] The pathology associated with chronic *S. mansoni* infections reduces the CD4+ T cell-count, which can exacerbate the effects of HIV-1 infection.[359] *S. mansoni* infection also increases susceptibility to HIV infection, transmission, and replication in nonhuman primates,[360] and similar observations have been made in malaria-infected patients.[361] Concomitant exposure to malaria can affect the regression of hepatosplenomegaly in drug-cured *S. mansoni* patients.[362] Coinfections of *T. gondii* and *S. mansoni* in mice are also more lethal,[363] whereas preexisting infections with *N. brasiliensis* have been shown to inhibit innate pulmonary antituberculosis defense by activating the IL-4 receptor pathway in murine macrophages.[364] Finally, although endosymbiotic *Wolbachia* bacteria are believed to increase the fitness of filarial parasties, *Wolbachia* LPS is believed to be the principle driver of river blindness in chronic filariasis.[365]

It is difficult to do justice to the remarkably broad range of immunologic mechanisms that contribute to the pathology of parasitic disease. Twenty-five years ago, much of the research in this field concerned the role of immune complexes, complement, and anaphylaxis. These areas remain important, but the focus has shifted to the molecular basis of cellular processes such as inflammation, granuloma formation, wound repair, and immune regulation. An important issue that is the focus of intensive research is how the host maintains the fine balance between a protective immune response and one that causes pathologic complications. It is becoming increasingly clear that achieving balance in the immune response is the most critical determinant in the establishment of a long-term host-parasite relationship and, therefore, should be of considerable interest to vaccinologists. Interestingly, in many chronic parasitic infections, this balance appears to be regulated by the coordinated actions of several distinct immunoregulatory mediators, including regulatory T cells, IL-10-expressing regulatory B cells, and AAM, to name just a few.

Achieving Balance between the Anti-infective Immune Response and Host Pathology

Whereas Th1 responses are required to control intracellular-infections, there is a need to balance these potentially tissue-destructive responses. The harmful effector molecules induced by Th1 responses include NO, reactive oxygen intermediates, and TNF, which operate in a synergistic fashion to promote antimicrobial immunity but are often associated with undesired inflammatory and toxic side effects. Proinflammatory Th17 responses have also been shown to contribute to the acute pathology seen in some schistosomeinfected mice.[366,367] IL-10, TGF-β, arginase-1, and IL-4, produced by distinct subsets of regulatory cells including myeloid-derived suppressor cells, AAMs, regulatory T cells, IL-4/IL-13-producing Th2 cells, IL-10-producing regulatory T cells (Tr1), FoxP3-expressing conventional Th1, and IL-10+IFNγ+Foxp3- CD4+ T cells, may all help prevent Th1 and Th17 immune responses from overshooting and becoming pathologic during infection with intracellular pathogens.[349,368,369,370,371] There are also several examples in which persistent helminth-induced Th2 responses appear to be detrimental to the host. Strong antibody responses can lead to the formation of antigen-antibody complexes or complement activation resulting in bystander lysis.[372] Eosinophils, typically associated with the Th2 response, are involved in hypersensitivity reactions to the filarial worm *O. volvulus* and promote Th2 pathology in schistosomiasis.[280] Th2 cytokines are also important drivers of hepatic fibrosis and morbidity in chronic schistosome infections.[373] Similar to chronic Th1 and Th17 responses, a variety of regulatory mediators including IFNγ, IL-12, IL-10, the IL-13 decoy receptor, regulatory T cells, regulatory B cells, and silencers of cytokine signaling proteins have all been shown to contribute to the suppression of pathogenic Th2 responses.[374,375,376] In summary, the successful resolution of infection typically requires precise titration of the specific T helper cell response induced by the pathogen. This is not just in terms of amount but also where, when, and for how long the polarized Th1, Th2, or Th17 response persists.

Pathogenesis of Chronic Th1 Responses

As discussed previously, control of intracellular pathogens such as *Leishmania* spp., *T. gondii*, and *T. cruzi* requires the coordinated activation of both antigen-specific cells (T lymphocytes) and less specific responses (NK cells, neutrophils, and macrophages) with IFNγ and TNF playing critical roles by upregulating macrophage activation and nitric oxide production. Interestingly, IL-10-deficient mice inoculated with a normally avirulent *T. gondii* strain or with a virulent strain of *T. cruzi* succumb to infection within the first 2 weeks of infection.[377,378] In both of these infections, animals lacking IL-10 show increased suppression of parasite growth and, in the case of *T. cruzi*, inflammation and necrosis within the endocardium and interstitium of the myocardium is reduced. The increase in mortality is caused by systemically high levels of IL-12, IFNγ, and TNF produced in large part by activated CD4+ T lymphocytes and macrophages. The livers of both *T. gondii*- and *T. cruzi* -infected mice show numerous and prominent necrotic foci together with dramatically increased mononuclear cell infiltration. Similarly, macrophages from the mutant mice activated in vitro or in vivo with *T. gondii* secrete higher levels of TNF, IL-12, and inducible NO than macrophages from IL-10-sufficient animals. The combined clinical manifestations suggest that the IL-10-/- mice die in response to an overwhelming systemic immune response, resembling that observed during septic shock. In support of this conclusion, administration of anti-CD4, anti-IL-12, or anti-TNF mAbs reduces mortality in IL-10-/- mice.[377,378] Thus, in these models, IL-10 plays a major role in protecting the host against an excessive and lethal type 1 cytokine response.

Much has been written about the protective versus pathologic consequences of proinflammatory cytokine production in malaria (Fig. 38.3[379]). During the preerythrocytic stage in the liver, when parasite burden is relatively low and the infection is clinically asymptomatic, there is evidence that IFNγ and NO each play an important role in preventing the infection from progressing further.[380] Once the parasites invade erythrocytes and grow to large numbers, the riskbenefit equation is less clear. At this stage, the inflammatory cytokine response is systemic, and some pathologic side effects are inevitable. The most common clinical consequence in humans is fever, whereas life-threatening complications such as profound anemia and cerebral malaria occur in a proportion of infections. Depending on the specific host-parasite combination, mice may develop anemia, fatal neurologic

symptoms, or multiorgan failure; IL-1, IL-6, TNF, and, more recently, lymphotoxin-α have been the cytokines most consistently associated with severe pathology in these models.[381,382]

Conversely, experimental studies suggest that IL-10 and TGF-β cooperate to downregulate potentially pathogenic proinflammatory cytokine responses in malaria.[348] IL-10-deficient mice have increased mortality compared with normal littermates; this results not from fulminant parasitemia but from a sustained and enhanced proinflammatory cytokine response[383] and severe liver necrosis. Immunopathology in IL-10-/- mice can be prevented simply by adoptive transfer of wild-type (ie, IL-10 competent) CD4+ T cells, indicating that they are a sufficient source of IL-10 to prevent severe disease in the latter stages of acute infection.[383] Similarly, treatment of infected mice with a neutralizing antibody to TGF-β exacerbates the virulence of *P. berghei* and *Plasmodium chabaudi* infection; it was concluded that the protective effects of this cytokine are also due to downregulation of inflammatory responses.[384] However, as described previously, very early induction of TGF-β and IL-10 can inhibit the effector response that is required for parasite clearance[344,345]; thus, the outcome of malaria infections is determined, in part at least, by the sequence and timing of proinflammatory and anti-inflammatory (immune regulatory) responses. This is likely to depend upon sequential activation of innate immune cells, such as NK cells and γδ T cells, then Th-1 CD4+ T cells, and finally IL-10-secreting T cells. Preliminary evidence suggesting that CD4+ T cells that secrete both IFNγ and IL-10 may be a signature of

protection against severe malaria in African children clearly needs to be explored further.[385]

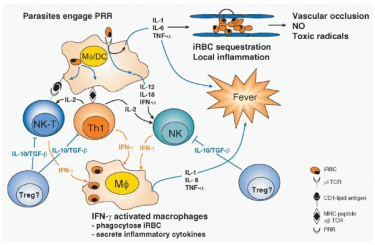

FIG. 38.3. Immunopathogenesis of Malaria. Parasitized red blood cells (pRBC) and parasite products bind to pattern recognition receptors (PRRs; eg, toll-like receptor [TLR]2 and TLR9) on monocytes and dendritic cells, and induce a cascade of proinflammatory cytokines (most importantly interleukin [IL]-12, interferon-γ, and tumor necrosis factor [TNF]-α). These inflammatory signals initiate numerous pathologic processes. They cause upregulation of endothelial adhesion molecule expression on vascular endothelium, promoting vascular sequestration of pRBCs, which clogs the vessels, reduces blood flow, and simultaneously raises intracranial pressure. The pRBC products also bind to PRRs in tissue, initiating local inflammatory loops, which amplify pRBC sequestration. Vascular occlusion and subsequent tissue damage contribute to cerebral malaria, respiratory distress, and multiorgan failure. Other cytokines such as IL-1, TNF-α, and IL-6 induce the fever response, which includes elevated temperature, nausea, headache, prostration, and muscle pain. The actions of inflammatory cytokines are antagonized by anti-inflammatory cytokines, principally IL-10 and transforming growth factor-β, which inhibit both production of, and cellular responses to, inflammatory signals.

Pathogenesis of Chronic Th2 Responses

During nematode infection, the protective Th2 response can cause significant pathologic changes in the intestine including inflammation, epithelial cell damage, excess mucus production, and diarrhea. However, these effects are often transient and resolve quickly once the parasite is expelled from the gut. If the parasites are not expelled, the immune response typically shifts to a more Th1/Th17-polarized reaction,[386] which can lead to an even more severe inflammation with characteristics similar to Crohn disease.[387] Thus, there are only a few chronic helminth infections where persistent Th2 reactions are established. Perhaps the most widely studied experimental model in this regard is the mouse model of schistosomiasis. Upon infection, adult parasites of *S. mansoni* migrate to the mesenteric veins where they live up to 10 years or more, laying hundreds of eggs per day. Some of the eggs become entrapped in the microvasculature of the liver and once there, they induce a granulomatous response.[388] Subsequently, hepatic fibrosis, portal hypertension, hepatosplenomegaly, and bleeding from esophageal and gastric varices may develop, which in some cases may ultimately lead to the death of the individual. Consequently, much of the symptomatology of schistosomiasis is attributed to the egg-induced inflammatory response and associated fibrotic pathology.[389,390]

Granulomas are pathogenic, not because they cause hepatic failure in the short-term, but rather because they induce hepatosplenomegaly and contribute to liver scarring.[390,391] CD4+ Th2 cells are essential for granuloma formation, whereas many other lymphocytes appear less critical including B cells, CD8 cells, NK T cells, and γδ T cells. B cell-deficient (μMT) mice, however, fail to downmodulate granuloma formation in the latter stages of the disease, suggesting that B cells and/or antibody might play an important immunoregulatory role in chronic infections.[392] In support of regulatory role for B cells and antibody, perinatal exposure to specific anti-SEA idiotypes has been shown to regulate survival, pathology, and immune response patterns in mice

subsequently infected with *S. mansoni*.[393] Cross-reactive regulatory idiotypes can also been used to predict clinical outcomes in chronically infected mice.[394]

Th2 cells also play an important role in the pathogenesis of schistosomiasis.[283] Indeed, a critical role for Th2 cells was confirmed in experiments in which mice vaccinated with egg antigen plus IL-12 to induce an egg-specific Th1 response upon subsequent infection developed smaller granulomas and less severe fibrosis than did nonvaccinated infected controls.[395] The decreased pathology was associated with a diminished Th2 and increased Th1 response. IL-4-/-, IL-4Rα-/-, and IL-13Rα1-/- mice as well as anti-IL-4 mAb-treated wild type (WT) mice were also shown to develop smaller granulomas and less fibrosis than similarly infected WT mice, further emphasizing the critical role of Th2 immunity in the pathogenesis of chronic schistosomiasis.[138,244,396,397]

Although the Th2-driven granulomatous response is widely believed to be detrimental in chronic infections, it is clear that IL-4-driven granuloma formation also serves an important host-protective role, particularly during acute infections with *S. mansoni*. During infection, the schistosome eggs that are produced in large quantities by adult parasites provide a continuous antigenic stimulus for the immune response. If these antigens are not sequestered or neutralized effectively, toxic components produced by the eggs are known to damage hepatocytes and reduce barrier immunity in the intestine.[396,398] Indeed, T cell-deprived, nude, severe combined immunodeficiency defect, and egg-tolerized mice infected with *S. mansoni* all die earlier than comparably infected immunologically intact mice because they are incapable of mounting a protective CD4+ Th2 cell-driven granulomatous response.[399] Widespread microvesicular hepatic and intestinal damage induced by toxic egg products contributes to the poorer survival of the infected immunosuppressed mice. IL-4R signaling is also required for the efficient passage of eggs through the intestine into the lumen.[398] Consequently, increased numbers of eggs are trapped in the intestinal wall when IL-4 is deficient, causing localized inflammation and increased systemic exposure to bacterial

toxins such as LPS. This response combined with the decreased Th2- and enhanced Th1-type response results in increased proinflammatory cytokine production that contributes to weight loss in and death of IL-4-deficient animals.[388] Therefore, although Th2-driven granuloma formation and fibrosis are detrimental in the long-term, they are also critically important in the short-term because they allow the establishment of a successful host-parasite relationship.[400]

Many of the pathologic complications associated with chronic helminth infections (portal hypertension, bleeding collateral vessels, anemia, mucosal barrier dysfunction, lymphatic blockage, etc.) do, however, result from the persistent expression of type 2 cytokines.[283] Whereas IL-4 was identified as the principle inducer of the protective Th2 response during acute schistosome infection,[396] IL-13 has been identified as the key driver of hepatic fibrosis and the fibrosis-associated complications observed in chronically infected mice.[374,398,401] In mice, the progression of hepatic fibrosis correlates with the intensity of the type 2 cytokine response,[402] and immunologic interventions that impair IL-13 activity have been shown to reduce collagen deposition and improve survival during chronic infection[374,398,401] as long as IL-4 production is preserved. Mediators produced during polarized type 1 cytokine responses have been shown to inhibit IL-13-dependent fibrosis, including TNF-α, IL-12, and NO.[403,404] Because macrophages and DCs are important sources of these mediators and have been shown to be important drivers of Th2 immunity in vivo, a major effort has been undertaken to elucidate the roles of distinct myeloid cell populations in the pathogenesis of schistosomiasis and other helminth infections.[191,400]

Numerous studies have shown that IL-4/IL-13-induced macrophages are found in many helminth infections. Gordon[405] first noted that macrophages treated with IL-4 and IL-13 develop an alternative activation state that is distinct from "classically activated macrophages" exposed to IFNγ. In vitro, the AAM or M2 phenotype[405] is characterized by elevated MHC class II, mannose receptor (CD206), YM1, Fizz1/Relm-α, and arginase (Arg) activity.[406,407,408,409] Studies of murine schistosomiasis have found that 20% to 30% of the cells in granulomas are AAMs,[410] and studies conducted with IL-4Rα conditional knockout mice have suggested that AAMs actively suppress the development of IL-12/IL-23p40-driven intestinal pathology during acute infection with *S. mansoni*.[371,411] Related studies conducted in conditional Arg1-deficient mice have also identified an important role for Arg1-expressing macrophages in the suppression of hepatic fibrosis during chronic *S. mansoni* infection.[407] These findings were supported in studies with mice deficient in cationic amino transporter-2, which imports L-arginine for use in NO production in macrophages. Cationic amino transporter-2-/- mice develop exacerbated liver fibrosis following schistosome infection associated with decreased NO production and increased Arg1 activity in AAMs and fibroblasts.[412] Together, these studies support the hypothesis that the phenotype of the macrophage plays a critical role in the pathogenesis of schistosomiasis. Similar studies are now underway to dissect the role of AAMs in other helminth infections.

PARASITE VACCINE STRATEGIES

Aside from the recent encouraging results involving the RTS,S malaria vaccine (discussed subsequently), there is still no safe, uniformly effective vaccine against any human parasitic infection. The lack of progress in this field is due to many factors, including the low priority that has historically been given to development of vaccines against diseases confined mainly to the developing world. By contrast, the perceived economic benefit to the agricultural industry of vaccines against livestock parasites has led to the licensing of several antihelminthic vaccines for veterinary use.[413] The greater impediments, however, may be related to the nature of parasitic infections themselves. In contrast to those bacterial and viral infections for which highly effective vaccines exist, and for which there is complete immunity induced by primary infection, most antiparasite vaccines will need to outperform the immune response to natural infection. Further, virtually all bacterial and viral vaccines that are currently in use mediate their protection by inducing a strong, long-lived,

humoral response that inhibits attachment or invasion, promotes clearance, or neutralizes released toxins. By contrast, there are no vaccines that are uniformly effective against diseases caused by intracellular pathogens that require cellular immunity to mediate protection. Thus, the manner in which potentially protective antigens can be administered to generate and maintain appropriate T-cell responses has yet to be proven in a clinical setting. Consequently, for the development of vaccines against intracellular protozoa (eg, malaria, *Leishmania*, *T. cruzi*, *Toxoplasma*), it will not be sufficient to simply identify target antigens; novel and rational approaches to vaccine design and delivery will need to be explored. In fact, from the examples discussed in the following, it is clear that ample numbers of potentially protective antigens have been identified and cloned from most of the major human parasitic disease agents, and the completion of their genomic sequences has already led to the identification of additional vaccine candidates based on their predicted developmental stage specificity, surface expression, secretion, or virulence associations.[414,415] The vaccination strategies that are currently being explored to meet the challenge of both antigen selection and delivery will be considered in the general context of B- and T-cell antiparasite vaccines. Note that the examples provided are by no means exhaustive but reflect general principles of vaccination against extracellular and intracellular targets.

B-Cell Vaccines

Vaccination Against Intestinal Protozoa

Parasitic protozoa that have an exclusive extracellular lifestyle in their mammalian hosts and are sensitive to antibody-mediated control include the intestinal pathogens *E. histolytica* and *G. lamblia*. Most deaths from *E. histolytica* arise from amebic liver abscess, the major extraintestinal manifestation of disease. Clinical studies suggest that the presence of mucosal antibodies to the surface Gal/GalNAc lectin of *E. histolytica* capable of blocking amebic adherence to intestinal epithelial cells, correlate with reduced risk of recolonization.[416] Furthermore, active immunization with the Gal/GalNAc lectin can induce IgA antilectin antibodies and provide protection against intestinal amebiasis in a mouse model of disease.[417] An amebic serine-rich protein (SREHP) and an alkyl hydroperoxide reductase (Eh29) are also highly immunogenic surface antigens of *E. histolytica* that can protect against amebic liver abscess in animal models via induction of intestinal IgA antibodies and together with the Gal/GalNAc lectin, represent the most promising candidates for oral vaccines against amoebiasis.[418] Specific serum and mucosal antibodies targeting surface antigens are also known to be important in elimination of *Giardia* from the host intestine. *Giardia* vaccines containing whole trophozoite preparations protected animals even when challenged with heterologous strains,[419] suggesting that an immune response to variant surface proteins, which are known to be targets of cytotoxic antibodies, are not essential for control of acute infection. However, by interfering with the mechanism controlling variant surface protein switching in *Giardia*, it was more recently shown that primary infection with trophozoites expressing many variant surface proteins was necessary to protect gerbils from subsequent infection.[420] More importantly, such genetically manipulated parasites might be used as cross-protective vaccine. Genes coding for *Giardia* cyst wall proteins, which could be used for developing a transmission-blocking vaccine, have been cloned, and a recombinant protein induced IgA antibodies such that immunized mice shed fewer cysts following challenge with live cysts.[421]

Vaccines Targeting Extracellular Stages of Malaria

Because both preerythrocytic- and erythrocytic-stage malaria parasites are at least transiently exposed to humoral antibody, vaccine strategies based on eliciting high-titered antibodies that can inhibit their invasion of RBCs or hepatocytes have long been favored (Fig. 38.4). As discussed previously, antibodies to sporozoite surface proteins can immobilize invading parasites, preventing them from reaching or invading hepatocytes; such antibodies can be protective, and the dominant antibody epitope is represented by the CS central repeat

sequences (NANP *n* in *P. falciparum*). These observations have made the CS protein the most extensively studied of all the malaria vaccine candidates.[422] The latest version of the vaccine—RTS,S—comprises a recombinant CS polypeptide fused to the surface protein of hepatitis B virus and administered together with an adjuvant containing LPS (monophosphoryl lipid A) and a water-soluble glycoside obtained from tree bark (*Quillaja saponaria*).[423] The vaccine has been designed to induce both antibodies and T cell-mediated effector mechanisms, and it is not clear which of these mechanisms is most important in conferring immunity. This vaccine is discussed in more detail in a following section.

Antibodies that inhibit the invasion of erythrocytes by the extracellular merozoite stage of malaria in vitro are found in many, but not all, individuals living in malaria endemic regions. Although the significance of these inhibitory antibodies to naturally acquired resistance remains unclear, their target antigens nonetheless remain prime candidates for asexual malaria vaccines[422] (see Fig. 38.4). Numerous antigens that form the surface coat of the merozoite or that are essential components of the parasite's cell invasion mechanism (apical complex and rhoptry proteins) have been evaluated as potential vaccine candidates. Although antibodies to several of these antigens have been found to be able to inhibit parasite development in vitro, or are associated with clinical immunity in vivo, the results of these studies tend to be rather inconsistent,[424] and the results of vaccine trials have been disappointing.[422] One of the problems seems to be that when one pathway of merozoite invasion into an erythrocyte is blocked by antibody, the parasite is able to switch to an alternative pathway using entirely different proteins. Recently, however, a novel receptor-ligand interaction has been identified that seems to be absolutely essential for merozoite invasion. *P. falciparum* rhoptry protein-5 (PfRh5) binds to the blood group antigen basigin on the red cell surface; blocking of this interaction with antibasigin antibody completely prevents parasite invasion and replication.[425] Just as importantly, antibodies to PfRh5 also block invasion and PfRh5 seems to

be a remarkably conserved protein among different parasite isolates, raising hopes that PfRh5 might be a credible new candidate for a blood stage malaria vaccine. Despite their extreme polymorphism, the variant surface antigens present on infected erythrocytes that mediate adhesion to endothelial cells are potential vaccine candidates.[426] Epidemiologic data suggest that the risk of severe manifestations of the disease is reduced after only a very few clinical episodes,[427] and that parasites causing severe disease tend to express a subset of variant surface antigens.[428] Thus, a finite number of variant antigens might be sufficient to elicit broad immunity against severe disease. Similarly, relatively few PfEMP-1 variants appear to mediate parasite sequestration in the placenta, and a pregnancy malaria vaccine might work by targeting the domains that bind to chondroitin sulphate, the major placental ligand for parasitized red blood cell (pRBC) sequestration.

Antimalarial transmission-blocking immunity works primarily by antibody-mediated, complement-dependent lysis of extracellular sexual stages of the parasite within the midgut of a blood-feeding mosquito[429] (see Fig. 38.4). Transmission-blocking immunity has been induced in vivo by immunization with gametes of avian, rodent, and monkey malarias. Several potential transmission-blocking vaccine candidates have been identified, and the genes encoding these surface proteins have been isolated and sequenced, but their production as recombinant proteins is hampered by failure to recreate the highly complex tertiary structures that are the targets of inhibitory antibodies. Furthermore, for those antigens expressed only by invertebrate stages of the parasite, lack of natural boosting may prevent maintenance of sufficiently high antibody titers.

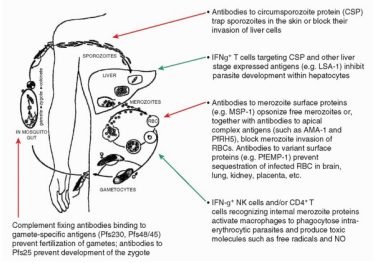

FIG. 38.4. Stage-Specific Vaccine Targets in Malaria Parasites. The *left* figure depicts the life cycle of malaria and the immune effector mechanisms that target the different developmental forms of the parasite (reproduced from Malaria Vaccine Initiative Web Site [www.malariavaccine.org/mal-what_is_malaria.htm]). (Adapted from Richie TL, Saul A. Progress and challenges for malaria vaccines. Nature. 2002;415:694-701, with permission.).

Clearly, an optimal vaccine against malaria would need to target multiple antigens and induce immunity against all stages. However, by targeting certain antigens confined to asexual blood stages, the induction of an adult-like immune status among high-risk infants in sub-Saharan Africa could greatly diminish severe disease and death caused by *P. falciparum*.

T-Cell Vaccines

Vaccination Against Leishmaniasis

Vaccines against intracellular parasites will need to induce long-lived cellular immune responses. As already discussed, for diseases such as Leishmaniasis, Chagas disease, and toxoplasmosis, Th1 and/or CD8+ T-cell responses are the effector mechanisms required for protective immunity. An inherent problem with most nonliving vaccines is their relative inefficiency in generating and/or sustaining these sorts of cellular responses. A major advance in T-cell vaccine development was the demonstration that proteins derived from *L. major* could elicit a powerful Th1 response and protective immunity if given with recombinant IL-12 as adjuvant.[430] IL-12 or IL-12-inducing adjuvants such as BCG, CpG-oligodinucleotides, or CD40L have since been used extensively in animal models to potentiate the efficacy of whole cell killed or a diversity of recombinant protein *Leishmania* vaccines.[431] A polyprotein containing several

Leishmania antigens (TSA, LmSTI1, and LelF), formulated with the TLR4 agonist MPL®, a detoxified derivative of 4'-monophosphoryl lipid A of LPS, is the only defined, subunit vaccine against leishmaniasis currently being tested in human trials.

The gold standard of the protection that can be achieved by vaccination in mice, and the only vaccination strategy against leishmaniasis that has worked so far in humans is "leishmanization," which is based on the lifelong convalescent immunity that is acquired following induction of a lesion at a selected site with a cutaneous strain of *L. major*. The nature of the acquired resistance that develops following healing of a primary lesion seems especially important to consider as only healed mice were protected against *L. major* transmitted by sand fly bites, whereas mice vaccinated with killed parasites plus CpG-oligodinucleotides were protected against needle but not sand fly challenge.[432] Importantly, the strong and durable protection against secondary challenge conferred by live vaccination in mice and humans is associated with the persistence of parasites long after clinical cure. The presence at the time of the challenge exposure of a population of effector memory cells that are rapidly tissue homing and readily secrete IFNγ upon encounter with antigen in the

challenge site appears to be critical for the full expression of acquired immunity.[432,433] The maintenance of effector memory T cells in live vaccinated hosts, and the loss of these cells following antigen clearance in antigen vaccinated hosts, likely explains the failures of a large number of human trials involving whole cell killed vaccines to significantly reduce the incidence of cutaneous leishmaniasis in individuals living in areas of relatively low transmission, where natural boosting is unlikely to have occurred.[434] The requirement for persistent antigens reinforces the rationale for live attenuated *Leishmania* vaccines, a number of which have been generated by targeted deletion of genes involved in parasite survival or virulence. Although the efficacy of live attenuated vaccines against needle challenge in mice has been shown,[431] none have been evaluated using infected sand flies and more generally pose greater difficulties in standardization and delivery in field settings.

Similar to *Leishmania*, a diversity of protein-, DNA-, and viral vector-based vaccines have been developed that successfully induce protective immunity against an experimental *T. cruzi* infection in mice, typically measured as a CD8+ T-cell-dependent reduction in acute-phase parasitemia or associated mortality.[435] In contrast to an anti-*Leishmania* vaccine, however, a prophylactic vaccine for human Chagas disease would almost certainly have to provide sterile immunity in order to be effective, as the cardiac pathologies are associated with persistent infection. Whereas a number of experimental vaccines were shown to reduce the tissue inflammation and parasitism associated with late chronic phase infections, it does not seem that infection itself, or even infection and drug cure, confers sterilizing immunity against reinfection,[146] and there is so far no evidence that a vaccine can achieve a better result. Furthermore, the immune response seems to be focused on epitopes encoded by genes of the large and strain variant trans-sialidase gene family,[436] which would require that a massive number of target epitopes be included in an effective vaccine. Thus, the rationale for the development of a safe and effective vaccine against human Chagas disease remains suspect, particularly as vector control methods have proven to be so highly effective.

Vaccines to prevent toxoplasma infection are needed primarily to protect livestock and prevent transmission to humans from felines. As with *T. cruzi*, CD8+T cells working in conjunction with IFNγ-producing CD4+T cells appear to provide optimal immunity to challenge infection and so far this type of protection is best induced by live attenuated strains of the parasite.[437] Recently, modern tetramer sceening and bioinformatic approaches have been used to identify dominant CD8+ epitopes for both mice[438] and humans[439] and in the latter case have been tested for their vaccine potential in transgenic mice expressing supertype human leukocyte antigen-A molecules. These findings offer new promise to peptide-based approaches for vaccination against *T. gondii*.

Vaccination Against Malaria Liver Stages

Because malaria parasites infect and replicate in hepatocytes, which express MHC class I, infected hepatocytes are potential targets of CD8+ T-cell responses (see Fig. 38.4). Irradiated or genetically attenuated *Plasmodium* sporozoites, which can infect hepatocytes but do not progress to a blood-stage infection, have been shown to protect rodents, monkeys, and humans against malaria and are believed to work by inducing IFNγ-producing CD8+ T cells specific for preerythrocytic antigen.[440] Overirradiation of sporozoites, or attenuation of sporozoites very early in their development inside the hepatocyte, negates their ability to immunize, suggesting that the targets of protective mechanisms are novel antigens expressed only if sporozoites are to undergo partial differentiation within hepatocytes. Efforts to mimic the protection generated by irradiated sporozoites using nonliving protein- and DNA-based vaccines have yielded encouraging results. A recombinant polypeptide representing the central repeat and C-terminal portions of the *P. falciparum* CS protein and fused to hepatitis B surface antigen (HBsAg) elicits IL-2-secreting CD4+ T-cell responses as well as antisporozoite antibodies in human volunteers, particularly when given with the GSK proprietary adjuvant AS01 (containing liposomes, MPL and QS21). The vaccine, RTS,S, induced antibody and T-cell responses in adults already primed to CS by preexposure to malaria. Most significantly, in this first phase II trial, vaccine efficacy during the first 9 weeks of follow-up was 71% but decreased to 0% over the next 6 weeks.[441] In the intervening decade, more than 50 clinical trials of RTS,S have been undertaken—many of them in Africa—refining the dose, the adjuvant, the immunization schedule, and age of vaccination,[442] and a phase III trial (the very first for a malaria vaccine) was begun in 2009. The preliminary results of this trial confirm that the vaccine has approximately 50% efficacy against clinical malaria attacks and against malaria-related hospital admissions,[443] but the duration of protection is still an area of concern. Moreover, as there are still no clear immunologic

correlates of vaccine-induced immunity, refinement of the vaccine to enhance its efficacy may mean that clinical trials need to continue for many years to come.

Despite initial enthusiasm that DNA vaccination strategies might improve the durability and potency of the cellular response, clinical trials have been disappointing, and the use of sequential immunizations using various heterologous prime/boost protocols are being tested to enhance the effectiveness of preerythrocytic vaccines.[178] Priming with plasmid DNA encoding CS and various liver stage-specific proteins and boosting with recombinant adenovirus or pox virus, such as modified vaccinia virus Ankara or fowlpox, has induced complete protection and very high levels of IFNγ-secreting CD8+ T cells in mice. Although protection in human trials has been less convincing, delays in time to emergence of blood-stage parasites have been consistently obtained, suggesting that the vaccination substantially reduces, but does not eliminate, liver stage parasites.

Vaccination to Prevent Pathology

In many parasitic infections, disease is a consequence of the host immune response. Because these pathogens are so well adapted to their hosts, it may be easier and more efficient to design immune interventions that prevent parasite-induced immunopathology rather than eliminate the infection itself. Although this approach will not lead to eradication of the parasite, it would likely reduce or alleviate the health consequences of infection. The feasibility of antipathology vaccines was demonstrated in a murine models of schistosomiasis.[395] Because disease in schistosomiasis is largely due to the granulomatous pathology that develops around parasite eggs trapped in target host tissues, a valid approach toward immunoprophylaxis for schistosomiasis is to vaccinate to minimize granulomatous pathology[395] or reduce parasite fecundity.[444,445] In mice, granuloma size and collagen deposition are correlated with the intensity of the type 2 response, and immunologic interventions, such as the administration of IL-4 and IL-13 antagonists, reduce both the size of granulomas and magnitude of fibrosis (discussed previously).[401,446] In extensions of these studies, mice immunized with parasite egg antigens plus IL-12[447] or CpGoligonucleotides[448] to induce an egg antigen-specific type 1 response, upon subsequent infection, exhibited far less severe egg-associated liver disease than did infected nonimmunized controls. Importantly, several immunodominant egg antigens have been described;[449,450] thus, it may be possible to design recombinant antipathology vaccines that duplicate the promising results produced with crude parasite extracts.

Parasite-derived GPI has been implicated in much of the pathology of malaria,[451] binding to TLRs on DCs and macrophages, and inducing release of TNF-α and other proinflammatory cytokines. As a proof of principle that GPI might serve as a target of an antidisease vaccine, mice immunized with synthetic GPI were protected against the acute immune pathology associated with *P. berghei* infection; however, as the mice were not immune to the parasite itself, they were unable to control parasite replication and eventually died of overwhelming parasitemia.[452] These observations suggest that although malaria vaccines that prevent immune pathology might reduce some aspects of morbidity, they are unlikely to be deployed in the absence of vaccine components that also limit parasite burden.

Vaccines Against Helminths

Infection with helminthic parasites remains a significant health problem in many tropical

countries. Whereas control measures are available in some areas, in most cases, patients living in endemic regions are quickly reinfected. Therefore, vaccines that reduce parasite and/or egg burdens would be a valuable tool to complement existing disease prevention programs and could represent a less costly and more practical approach than repeated chemotherapy. Although many subunit vaccines have been described and tested in various animal models, suboptimal levels of protection have hindered the development of all but a few of these candidate vaccines.[453] Significant advances in vaccination technology over the past decade have made it possible to identify novel targets using immunomics[454] and engineer vaccines that elicit strong cellular and humoral immunity.[455] Novel DNA vectors, improved delivery systems, new adjuvants, and immunomodulatory cytokines allow significant augmentation of the immune response to vaccines and preferential induction of specific effector mechanisms, including antibody isotypes, T helper cell subsets and cytotoxic T cells. However, in order to effectively harness and implement these advances, it will be necessary to fully understand the mechanisms of resistance to helminth parasites.

Vaccine models using radiation-attenuated larval parasites have provided the best examples of successful immunization against helminths. With the irradiated schistosome vaccine, although complete sterilizing immunity appears to be an unachievable goal, immunity approaching 60% to 80% is possible with the addition of adjuvants such as IL-12.[456] This model has served as the gold standard for schistosome vaccine development.[457] The cumulative evidence from vaccine studies conducted in numerous gene knockout mice suggests that irradiated parasites induce protection via both Th1- and Th2-dependent pathways,[234] and that both humoral and cellular mechanisms will be required for the generation of optimal immunity against most helminth parasites.[458,459]

While there has been extensive research on defined vaccines against helminth infection,[460,461] none of the candidate antigens have been shown to induce protective immunity in humans, although a few are actively being tested in clinical trials.[462] At least one of the antigens—glutathione-S-transferase (P28/GST)—has moved through a phase I trial, and phase II trials are now underway in Africa. Other antigens of interest include paramyosin (Sm97), Irv5 (myosin-like 62-kDa protein), triose phosphate isomerase, Sm23, the integral membrane protein tetraspanin-2, and Sm14, a fatty acidbinding protein.[463,464] With the recent advances in schistosome genomics, proteomics, and immunomics,, a new panel of vaccine antigens is being identified, and these antigens will warrant further investigation in animal models.[465,466]

A number of filarial vaccine candidates have also been described and are being tested, including chitinase, tropomyosin, paramyosin, and several larval antigens called the "abundant larval transcript family."[460] Finally, several experimental antihookworm vaccines are also being investigated.[467] While these accomplishments represent a significant advance for the field of helminthology, it is important to note that all of the candidate antihelminth vaccine antigens will at best provide only partial protection. It is hoped, however, that ongoing improvements in vaccination technology, combined with greater knowledge of the mechanisms controlling resistance, will allow development of more efficacious and better defined vaccines for these complex organisms.

CONCLUSION

In the 4 years since the publication of the last edition of *Fundamental Immunology*, studies on the host-parasite interaction have contributed important advances to our understanding of the immune response and its regulation. Interestingly, many of these discoveries emerged from the study of worm infection models and led to new insights into the mechanism of Th2 polarization, the orchestration of mucosal immune responses, and the function of ILCs, basophils, and AAMs in promoting adaptive immunity. Indeed, although once regarded as a less sophisticated topic than protozoan immunology, the study of immunity to helminths has undergone a major resurgence in part because of the growing appreciation of the phylogenetic uniqueness of worm pathogens and their host interaction. Research on the immune response to helminths has also been stimulated by the growing interest in neglected tropical diseases, where worm infections account for the most affected individuals. Finally, there has been a growing awareness of the important evolutionary role played by helminth infection in conditioning the mammalian immune system, in the maintenance of immunologic homeostasis at mucosal tissue barriers, and of the likely impact of the loss of this symbiotic relationship on immune function due to modern improvements in human hygiene.[468]

A second trend in immunoparasitology in recent years has been the widespread use of genetic and genomic screening technologies to identify genes with functional roles in the host-parasite relationship. Interestingly, many of these projects[98,438,439] have brought together molecular and immunoparasitologists with the common goal of simultaneously identifying both parasite epitopes/virulence determinants and their host receptors and signaling pathway targets. The findings gained from such studies should provide important groundwork for future systems biology approaches that seek to develop a broader vision of the interaction of parasites with the immune system.

Another important development since the publication of the last version of this chapter is the increased emphasis on the study of the human immune response to parasitic infection. The most notable advances have occurred in the field of malaria, which benefits from a wealth of clinical material and the ability to use peripheral blood as a physiologically relevant source of immune cells. The study of human malaria is yielding novel insights into the nature of B-cell effector and memory responses to the parasite as well as the role of innate cellular function in regulating disease outcome. Similarly, as noted previously in this chapter, human visceral leismaniasis has proved a fertile ground for the study of the role of T cells and cytokines in immunosuppression and, in the case of the disease itself, implicated IL-10 production as target for immunotherapeutic intervention.

Perhaps the most heralded recent advance in the field of immunoparasitology has been the success of the field trials of the RTS,S vaccine for falciparum malaria.[442] While still clearly limited as a tool for controlling malaria because of its partial efficacy, important lessons have been learned from the experience of bringing the RTS,S vaccine to its current stage of development. Ironically, although RTS,S was originally designed to simultaneously trigger humoral and cell responses to the circumsporozoite protein (CSP) Ag, it is not all clear that the protection induced by the vaccine depends on this multipronged response. Instead, its improved efficacy appears to have stemmed from changes to the GlaxoSmithKline (GSK) adjuvant formulations and dosing regimen employed.[441] Indeed, the vaccine construct itself has not changed significantly from the time of the earlier unsuccessful trials. Thus, the RTS,S trials have both affirmed the importance of antigen delivery and presentation in the induction of parasite immunity while emphasizing our continued ignorance of the rules linking these parameters to the induction of host resistance. Nevertheless, having a defined antigen vaccine in which at least a proportion of the immunized individuals become protected offers a rare opportunity to identify correlates of immunity to malaria triggered by a single immunogen through the use of a "systems vaccinology" approach.[469] Such studies, in addition to providing valuable information needed for the design of a more effective RTS,S vaccine, could supply an important precedent and platform for analyzing human immunity to parasitic pathogens at a wider level. It is hoped that exciting new approaches and opportunities of this kind will bring us closer to our ultimate goal of protecting human populations against the scourge of parasitic disease.

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REFERENCES

1. Heinzel FP, Sadick MD, Holaday BJ, et al. Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *J Exp Med.* 1989;169:59-72.

2. Scott P, Natovitz P, Coffman RL, et al. Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. *J Exp Med.* 1988;168:1675-1684.

3. Norris KA, Bradt B, Cooper NR, et al. Characterization of a Trypanosoma cruzi C3 binding protein with functional and genetic similarities to the human complement regulatory protein, decay-accelerating factor. *J Immunol.* 1991;147:2240-2247.

4. Ramirez G, Valck C, Molina MC, et al. Trypanosoma cruzi calreticulin: a novel virulence factor that binds complement C1 on the parasite surface and promotes infectivity. *Immunobiology.* 2011;216:265-273.

5. Puentes SM, Da Silva RP, Sacks DL, et al. Serum resistance of metacyclic stage Leishmania major promastigotes is due to release of C5b-9. *J Immunol.* 1990;145:4311-4316.

6. Kweider M, Lemesre JL, Santoro F, et al. Development of metacyclic Leishmania promastigotes is associated with the increasing expression of GP65, the major surface antigen. *Parasite Immunol.* 1989;11:197-209.

7. Brittingham A, Morrison CJ, McMaster WR, et al. Role of the Leishmania surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. *J Immunol.* 1995;155:3102-3111.

8. Braga LL, Ninomiya H, McCoy JJ, et al. Inhibition of the complement membrane attack complex by the galactose-specific adhesion of Entamoeba histolytica. *J Clin Invest.* 1992;90:1131-1137.

9. Skelly PJ. Intravascular schistosomes and complement. *Trends Parasitol.* 2004;20:370-374.

10. Meri T, Jokiranta TS, Hellwage J, et al. Onchocerca volvulus microfilariae avoid complement attack by direct binding of factor H. *J Infect Dis.* 2002;185:1786-1793.

11. Rees-Roberts D, Mullen LM, Gounaris K, et al. Inactivation of the complement anaphylatoxin C5a by secreted products of parasitic nematodes. *Int J Parasitol.* 2010;40:527-532.

12. Badley JE, Grieve RB, Rockey JH, et al. Immune-mediated adherence of eosinophils to Toxocara canis infective larvae: the role of excretorysecretory antigens. *Parasite Immunol.* 1987;9:133-143.

13. Thomson R, Samanovic M, Raper J. Activity of trypanosome lytic factor: a novel component of innate immunity. *Future Microbiol.* 2009;4: 789-796.

14. Xong HV, Vanhamme L, Chamekh M, et al. A VSG expression site-associated gene confers resistance to human serum in Trypanosoma rhodesiense. *Cell.* 1998;95:839-846.

15. Molina-Portela MP, Samanovic M, Raper J. Distinct roles of apolipoprotein components within the trypanosome lytic factor complex revealed in a novel transgenic mouse model. *J Exp Med.* 2008;205: 1721-1728.

16. Olivier M, Brownsey RW, Reiner NE. Defective stimulus-response coupling in human monocytes infected with Leishmania donovani is associated with altered activation and translocation of protein kinase C. *Proc Natl Acad Sci U S A.* 1992;89:7481-7485.

17. Lodge R, Diallo TO, Descoteaux A. Leishmania donovani lipophosphoglycan blocks NADPH oxidase assembly at the phagosome membrane. *Cell Microbiol.* 2006;8:1922-1931.

18. Tolson DL, Turco SJ, Pearson TW. Expression of a repeating phosphorylated disaccharide lipophosphoglycan epitope on the surface of macrophages infected with Leishmania donovani. *Infect Immun.* 1990;58:3500-3507.

19. Wilson ME, Pearson RD. Roles of CR3 and mannose receptors in the attachment and ingestion of Leishmania donovani by human mononuclear phagocytes. *Infect Immun.* 1998;56:363-369.

20. van Zandbergen G, Bollinger A, Wenzel A, et al. Leishmania disease development depends on the presence of apoptotic promastigotes in the virulent inoculum. *Proc Natl Acad Sci U S A.* 2006;103:13837-13842.

21. Wanderley JL, Moreira ME, Benjamin A, et al. Mimicry of apoptotic cells by exposing phosphatidylserine participates in the establishment of amastigotes of Leishmania (L) amazonensis in mammalian hosts. *J Immunol.* 2006;176:1834-1839.

22. Mordue DG, Hakansson S, Niesman I, et al. Toxoplasma gondii resides in a vacuole that avoids fusion with host cell endocytic and exocytic vesicular trafficking pathways. *Exp Parasitol.* 1999;92:87-99.

23. Sanni LA, Fu S, Dean RT, et al. Are reactive oxygen species involved in the pathogenesis of murine cerebral malaria? *J Infect Dis.* 1999;179:217-222.

24. Atamna H, Ginsburg H. Origin of reactive oxygen species in erythrocytes infected with Plasmodium falciparum. *Mol Biochem Parasitol.* 1993;61:231-241.

25. Becker K, Tilley L, Vennerstrom JL, et al. Oxidative stress in malaria parasite-infected erythrocytes: host-parasite interactions. *Int J Parasitol.* 2004;34:163-189.

26. Sibley LD, Weidner E, Krahenbuhl JL. Phagosome acidification blocked by intracellular Toxoplasma gondii. *Nature.* 1985;315:416-419.

27. Joiner KA, Fuhrman SA, Miettinen HM, et al. Toxoplasma gondii: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. *Science.* 1990;249:641-646.

28. Fernandes MC, Cortez M, Flannery AR, et al. Trypanosoma cruzi subverts the sphingomyelinase-mediated plasma membrane repair pathway for cell invasion. *J Exp Med.* 2011;208:909-921.

29. Andrews NW. The acid-active hemolysin of Trypanosoma cruzi. *Exp Parasitol.* 1990;71:241-244.

30. Lodge R, Descoteaux A. Modulation of phagolysosome biogenesis by the lipophosphoglycan of Leishmania. *Clin Immunol.* 2005;114:256-265.

31. Peters NC, Egen JG, Secundino N, et al. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science.* 2008;321:970-974.

32. Thalhofer CJ, Chen Y, Sudan B, et al. Leukocytes infiltrate the skin and draining lymph nodes in response to the protozoan Leishmania infantum chagasi. *Infect Immun.* 2011;79:108-117.

33. Muller K, van Zandbergen G, Hansen B, et al. Chemokines, natural killer cells and granulocytes in the early course of Leishmania major infection in mice. *Med Microbiol Immunol.* 2001;190:73-76.

34. Teixeira CR, Teixeira MJ, Gomes RB, et al. Saliva from Lutzomyia longipalpis induces CC chemokine ligand 2/monocyte chemoattractant protein-1 expression and macrophage recruitment. *J Immunol.* 2005;175:8346-8353.

35. Gueirard P, Laplante A, Rondeau C, et al. Trafficking of Leishmania donovani promastigotes in non-lytic compartments in neutrophils enables the subsequent transfer of parasites to macrophages. *Cell Microbiol.* 2008;10:100-111.

36. Mollinedo F, Janssen H, de la Iglesia-Vicente J, et al. Selective fusion of azurophilic granules with Leishmania-containing phagosomes in human neutrophils. *J Biol Chem.* 2010;285:34528-34536.

37. van Zandbergen G, Klinger M, Mueller A, et al. Cutting edge: neutrophil granulocyte serves as a vector for leishmania entry into macrophages. *J Immunol.* 2004;173:6521-6525.

38. Guimaraes-Costa AB, Nascimento MT, Froment GS, et al. Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps. *Proc Natl Acad Sci U S A.* 2009;106:6748-6753.

39. Abi Abdallah DS, Lin C, Ball CJ, et al. Toxoplasma gondii triggers release of human and mouse neutrophil extracellular traps. *Infect Immunity.* 2012;80:768-777.

40. Cunnington AJ, de Souza JB, Walther M, et al. Malaria impairs resistance to Salmonella through heme- and heme oxygenase-dependent dysfunctional granulocyte mobilization. *Nat Med.* 2011;18:120-127.

41. Galioto AM, Hess JA, Nolan TJ, et al. Role of eosinophils and neutrophils in innate and adaptive protective immunity to larval strongyloides stercoralis in mice. *Infect Immun.* 2006;74:5730-5738.

42. Klion AD, Nutman TB. The role of eosinophils in host defense against helminth parasites. *J Allergy Clin Immunol.* 2004;113:30-37.

43. Horowitz A, Stegmann KA, Riley E. Activation of natural killer cells during microbial infections. *Front NK Cell Biol.* 2012;2:1-10.

44. Newman KC, Riley EM. Whatever turns you on: accessory-celldependent activation of NK cells by pathogens. *Nat Rev Immunol.* 2007;7:279-291.

45. Hepworth MR, Grencis RK. Disruption of Th2 immunity results in a gender-specific expansion of IL-13 producing accessory NK cells during helminth infection. *J Immunol.* 2009;183:3906-3914.

46. Bihl F, Pecheur J, Breart B, et al. Primed antigen-specific CD4+ T cells are required for NK cell activation in vivo upon Leishmania major infection. *J Immunol.* 2010;185:2174-2181.

47. Horowitz A, Newman KC, Evans JH, et al. Cross-talk between T cells and NK cells generates rapid effector responses to Plasmodium falciparum-infected erythrocytes. *J Immunol.* 2010;184:6043-6052.

48. Khan IA, Thomas SY, Moretto MM, et al. CCR5 is essential for NK cell trafficking and host survival following Toxoplasma gondii infection. *PLoS Pathog.* 2006;2:e49.

49. Hunter CA, Bermudez L, Beernink H, et al. Transforming growth factor-beta inhibits interleukin-12-induced production of interferon-gamma by natural killer cells: a role for transforming growth factorbeta in the regulation of T cell-independent resistance to Toxoplasma gondii. *Eur J Immunol.* 1995;25:994-1000.

50. Artavanis-Tsakonas K, Eleme K, McQueen K, et al. Activation of a subset of human natural killer cells upon contact with Plasmodium falciparum-infected erythrocytes. *J Immunol.* 2003;171:5396-5405.

51. Stevenson MM, Riley EM. Innate immunity to malaria. *Nat Rev Immunol.* 2004;4:169-180.

52. Mohan K, Moulin P, Stevenson MM. Natural killer cell cytokine production, not cytotoxicity, contributes to resistance against blood-stage Plasmodium chabaudi AS infection. *J Immunol.* 1997;159:4990-4998.

53. Satoskar AR, Stamm LM, Zhang X, et al. Mice lacking NK cells develop an efficient Th1 response and control cutaneous Leishmania major infection. *J Immunol.* 1999;162:6747-6754.

54. Roland J, Soulard V, Sellier C, et al. NK cell responses to Plasmodium infection and control of intrahepatic parasite development. *J Immunol.* 2006;177:1229-1239.

55. Hviid L, Kurtzhals J, Adabayeri V, et al. Perturbation and proin-flammatory type activation of V delta 1(+) gamma delta T cells in African children with Plasmodium falciparum malaria. *Infect Immun.* 2001;69:3190-3196.

56. Costa G, Loizon S, Guenot M, et al. Control of Plasmodium falciparum erythrocytic cycle: gamma-delta T cells target the red blood cell-invasive merozoites. *Blood.* 2011;118(26):6952-6962.

57. Tsuji M, Mombaerts P, Lefrancois L, et al. Gamma delta T cells contribute to immunity against the liver stages of malaria in alpha beta T-cell-deficient mice. *Proc Natl Acad Sci U S A.* 1994;91:345-349.

58. Weidanz W, Kemp J, Batchelder J, et al. Plasticity of immune responses suppressing parasitemia during acute Plasmodium chabaudi malaria. *J Immunol.* 1999;162:7383-7388.

59. Neill DR, Wong SH, Bellosi A, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature.* 2010;464: 1367-1370.

60. Gazzinelli RT, Denkers EY. Protozoan encounters with toll-like receptor signalling pathways: implications for host parasitism. *Nat Rev Immunol.* 2006;6:895-906.

61. Bafica A, Santiago HC, Goldszmid R, et al. Cutting edge: TLR9 and TLR2 signaling together account for MyD88-dependent control of parasitemia in Trypanosoma cruzi infection. *J Immunol*. 2006;177:3515-3519.

62. Caetano BC, Carmo BB, Melo MB, et al. Requirement of UNC93B1 reveals a critical role for TLR7 in host resistance to primary infection with Trypanosoma cruzi. *J Immunol*. 2011;187:1903-1911.

63. Liese J, Schleicher U, Bogdan C. TLR9 signaling is essential for the innate NK cell response in murine cutaneous leishmaniasis. *Eur J Immunol*. 2007;37:3424-3434.

64. Abou Fakher FH, Rachinel N, Klimczak M, et al. TLR9-dependent activation of dendritic cells by DNA from Leishmania major favors Th1 cell development and the resolution of lesions. *J Immunol*. 2009;182: 1386-1396.

65. Ives A, Ronet C, Prevel F, et al. Leishmania RNA virus controls the severity of mucocutaneous leishmaniasis. *Science*. 2011;331:775-778.

66. Coban C, Ishii KJ, Kawai T, et al. Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J Exp Med*. 2005;201:19-25.

67. Parroche P, Lauw FN, Goutagny N, et al. Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to toll-like receptor 9. *Proc Natl Acad Sci U S A*. 2007;104:1919-1924.

68. Yarovinsky F, Zhang D, Andersen JF, et al. TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science*. 2005;308:1626-1629.

69. Pifer R, Benson A, Sturge CR, et al. UNC93B1 is essential for TLR11 activation and IL-12-dependent host resistance to Toxoplasma gondii. *J Biol Chem*. 2011;286:3307-3314.

70. Melo MB, Kasperkovitz P, Cerny A, et al. UNC93B1 mediates host resistance to infection with Toxoplasma gondii. *PLoS Pathog*. 2010;6:e1001071.

71. Brattig NW, Bazzocchi C, Kirschning CJ, et al. The major surface protein of Wolbachia endosymbionts in filarial nematodes elicits immune responses through TLR2 and TLR4. *J Immunol*. 2004;173:437-445.

72. Goodridge HS, Marshall FA, Else KJ, et al. Immunomodulation via novel use of TLR4 by the filarial nematode phosphorylcholine-containing secreted product, ES-62. *J Immunol*. 2005;174:284-293.

73. van der Kleij D, Latz E, Brouwers JF, et al. A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. *J Biol Chem*. 2002;277:48122-48129.

74. Aksoy E, Zouain CS, Vanhoutte F, et al. Double-stranded RNAs from the helminth parasite Schistosoma activate TLR3 in dendritic cells. *J Biol Chem*. 2005;280:277-283.

75. LaRosa DF, Stumhofer JS, Gelman AE, et al. T cell expression of MyD88 is required for resistance to Toxoplasma gondii. *Proc Natl Acad Sci U S A*. 2008;105:3855-3860.

76. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell*. 2006;124:783-801.

77. Benson A, Pifer R, Behrendt CL, et al. Gut commensal bacteria direct a protective immune response against Toxoplasma gondii. *Cell Host Microbe*. 2009;6:187-196.

78. Dostert C, Guarda G, Romero JF, et al. Malarial hemozoin is a Nalp3 inflammasome activating danger signal. *PLoS One*. 2009;4:e6510.

79. Reimer T, Shaw MH, Franchi L, et al. Experimental cerebral malaria progresses independently of the Nlrp3 inflammasome. *Eur J Immunol*. 2010;40:764-769.

80. Sharma S, DeOliveira RB, Kalantari P, et al. Innate immune recognition of an AT-rich stem-loop DNA motif in the Plasmodium falciparum genome. *Immunity*. 2011;35:194-207.

81. Liu CH, Fan YT, Dias A, et al. Cutting edge: dendritic cells are essential for in vivo IL-12 production and development of resistance against Toxoplasma gondii infection in mice. *J Immunol*. 2006;177:31-35.

82. Jung S, Unutmaz D, Wong P, et al. In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cellassociated antigens. *Immunity*. 2002;17:211-220.

83. Bertholet S, Debrabant A, Afrin F, et al. Antigen requirements for efficient priming of CD8+ T cells by Leishmania major-infected dendritic cells. *Infect Immun*. 2005;73:6620-6628.

84. Goldszmid RS, Sher A. Processing and presentation of antigens derived from intracellular protozoan parasites. *Curr Opin Immunol*. 2010;22:118-123.

85. Leiriao P, Mota MM, Rodriguez A. Apoptotic Plasmodium-infected hepatocytes provide antigens to liver dendritic cells. *J Infect Dis*. 2005;191:1576-1581.

86. Chtanova T, Schaeffer M, Han SJ, et al. Dynamics of neutrophil migration in lymph nodes during infection. *Immunity*. 2008;29:487-496.

87. John B, Harris TH, Tait ED, et al. Dynamic imaging of CD8(+) T cells and dendritic cells during infection with Toxoplasma gondii. *PLoS Pathog*. 2009;5:e1000505.

88. Lambert H, Hitziger N, Dellacasa I, et al. Induction of dendritic cell migration upon Toxoplasma gondii infection potentiates parasite dissemination. *Cell Microbiol*. 2006;8:1611-1623.

89. Wykes MN, Kay JG, Manderson A, et al. Rodent blood-stage Plasmodium survive in dendritic cells that infect naive mice. *Proc Natl Acad Sci U S A*. 2011;108:11205-11210.

90. Gomez MA, Contreras I, Halle M, et al. Leishmania GP63 alters host signaling through cleavage-activated protein tyrosine phosphatases. *Sci Signal*. 2009;2:ra58.

91. Kar S, Ukil A, Sharma G, et al. MAPK-directed phosphatases preferentially regulate pro- and anti-inflammatory cytokines in experimental visceral leishmaniasis: involvement of distinct protein kinase C isoforms. *J Leukoc Biol*. 2010;88:9-20.

92. Ropert C, Closel M, Chaves AC, et al. Inhibition of a p38/stress-activated protein kinase-2-dependent phosphatase restores function of IL-1 receptor-associate kinase-1 and reverses Toll-like receptor 2- and 4-dependent tolerance of macrophages. *J Immunol*. 2003;171:1456-1465.

93. Doyle PS, Zhou YM, Hsieh I, et al. The Trypanosoma cruzi protease cruzain mediates immune evasion. *PLoS Pathog.* 2011;7:e1002139.

94. Cameron P, McGachy A, Anderson M, et al. Inhibition of lipopolysaccharide-induced macrophage IL-12 production by Leishmania mexicana amastigotes: the role of cysteine peptidases and the NF-kappaB signaling pathway. *J Immunol*. 2004;173:3297-3304.

95. Leng J, Butcher BA, Denkers EY. Dysregulation of macrophage signal transduction by Toxoplasma gondii: past progress and recent advances. *Parasite Immunol*. 2009;31:717-728

96. Saeij JP, Coller S, Boyle JP, et al. Toxoplasma co-opts host gene expression by injection of a polymorphic kinase homologue. *Nature.* 2007;445:324-327.

97. Taylor S, Barragan A, Su C, et al. A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen Toxoplasma gondii. *Science.* 2006;314:1776-1780.

98. Fentress SJ, Behnke MS, Dunay IR, et al. Phosphorylation of immunity-related GTPases by a Toxoplasma gondii-secreted kinase promotes macrophage survival and virulence. *Cell Host Microbe.* 2010;8:484-495.

99. Behnke MS, Khan A, Wootton JC, et al. Virulence differences in Toxoplasma mediated by amplification of a family of polymorphic pseudokinases. *Proc Natl Acad Sci U S A.* 2011;108:9631-9636.

100. Reese ML, Zeiner GM, Saeij JP, et al. Polymorphic family of injected pseudokinases is paramount in Toxoplasma virulence. *Proc Natl Acad Sci U S A.* 2011;108:9625-9630.

101. Machado FS, Johndrow JE, Esper L, et al. Anti-inflammatory actions of lipoxin A4 and aspirin-triggered lipoxin are SOCS-2 dependent. *Nat Med.* 2006;12:330-334.

102. Perry JA, Olver CS, Burnett RC, et al. Cutting edge: the acquisition of TLR tolerance during malaria infection impacts T cell activation. *J Immunol.* 2005;174:5921-5925.

103. Skorokhod OA, Alessio M, Mordmuller B, et al. Hemozoin (malarial pigment) inhibits differentiation and maturation of human monocytederived dendritic cells: a peroxisome proliferator-activated receptorgamma-mediated effect. *J Immunol.* 2004;173:4066-4074.

104. Wong KA, Rodriguez A. Plasmodium infection and endotoxic shock induce the expansion of regulatory dendritic cells. *J Immunol.* 2008;180:716-726.

105. Arama C, Giusti P, Bostrom S, et al. Interethnic differences in antigenpresenting cell activation and TLR responses in Malian children during Plasmodium falciparum malaria. *PLoS One.* 2011;6:e18319.

106. Urban BC, Cordery D, Shafi MJ, et al. The frequency of BDCA3-positive dendritic cells is increased in the peripheral circulation of Kenyan children with severe malaria. *Infect Immun.* 2006;74:6700-6706.

107. Poncini CV, Gimenez G, Pontillo CA, et al. Central role of extracellular signal-regulated kinase and toll-like receptor 4 in IL-10 production in regulatory dendritic cells induced by Trypanosoma cruzi. *Mol Immunol*. 2010;47:1981-1988.

108. Van Overtvelt L, Vanderheyde N, Verhasselt V, et al. Trypanosoma cruzi infects human dendritic cells and prevents their maturation: inhibition of cytokines, HLA-DR, and costimulatory molecules. *Infect Immun.* 1999;67:4033-4040.

109. Woelbing F, Kostka SL, Moelle K, et al. Uptake of Leishmania major by dendritic cells is mediated by Fcgamma receptors and facilitates acquisition of protective immunity. *J Exp Med.* 2006;203:177-188.

110. Xin L, Li K, Soong L. Down-regulation of dendritic cell signaling pathways by Leishmania amazonensis amastigotes. *Mol Immunol*. 2008;45:3371-3382.

111. Svensson M, Kaye PM. Stromal-cell regulation of dendritic-cell differentiation and function. *Trends Immunol*. 2006;27:580-587.

112. Scanga CA, Aliberti J, Jankovic D, et al. Cutting edge: MyD88 is required for resistance to Toxoplasma gondii infection and regulates parasite-induced IL-12 production by dendritic cells. *J Immunol*. 2002;168:5997-6001.

113. Egan CE, Sukhumavasi W, Butcher BA, et al. Functional aspects of Toll-like receptor/MyD88 signalling during protozoan infection: focus on Toxoplasma gondii. *Clin Exp Immunol*. 2009;156:17-24.

114. Adachi K, Tsutsui H, Kashiwamura S, et al. Plasmodium berghei infection in mice induces liver injury by an IL-12- and toll-like receptor/myeloid differentiation factor 88-dependent mechanism. *J Immunol.* 2001;167:5928-5934.

115. Franklin BS, Parroche P, Ataide MA, et al. Malaria primes the innate immune response due to interferon-gamma induced enhancement of toll-like receptor expression and function. *Proc Natl Acad Sci U S A.* 2009;106:5789-5794.

116. Parroche P, Lauw FN, Goutagny N, et al. Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to toll-like receptor 9. *Proc Natl Acad Sci U S A.* 2007;104(6):1919-1924.

117. Wu X, Gowda NM, Kumar S, et al. Protein-DNA complex is the exclusive malaria parasite component that activates dendritic cells and triggers innate immune responses. *J Immunol.* 2010;184:4338-4348.

118. Gowda DC. TLR-mediated cell signaling by malaria GPIs. *Trends Parasitol*. 2007;23:596-604.

119. McDowell MA, Marovich M, Lira R, et al. Leishmania priming of human dendritic cells for CD40 ligand-induced interleukin-12p70 secretion is strain and species dependent. *Infect Immun.* 2002;70: 3994-4001.

120. Campbell KA, Ovendale PJ, Kennedy MK, et al. CD40 ligand is required for protective cell-mediated immunity to Leishmania major. *Immunity.* 1996;4:283-289.

121. Kopf M, Brombacher F, Kohler G, et al. IL-4-deficient Balb/c mice resist infection with Leishmania major. *J Exp Med.* 1996;184: 1127-1136.

122. Noben-Trauth N, Lira R, Nagase H, et al. The relative contribution of IL-4 receptor signaling and IL-10 to susceptibility to Leishmania major. *J Immunol*. 2003;170:5152-5158.

123. Laskay T, Diefenbach A, Rollinghoff M, et al. Early parasite containment is decisive for resistance to Leishmania major infection. *Eur J Immunol*. 1995;25:2220-2227.

124. Constant SL, Lee KS, Bottomly K. Site of antigen delivery can influence T cell priming: pulmonary environment promotes preferential Th2- type differentiation. *Eur J Immunol*. 2000;30:840-847.

125. Nabors GS, Nolan T, Croop W, et al. The influence of the site of parasite inoculation on the development of Th1 and Th2 type immune responses in (BALB/c x C57BL/6) F1 mice infected with Leishmania major. *Parasite Immunol*. 1995;17:569-579.

126. MacDonald AS, Straw AD, Bauman B, et al. CD8- dendritic cell activation status plays an integral role in influencing Th2 response development. *J Immunol*. 2001;167:1982-1988.

127. Whelan M, Harnett MM, Houston KM, et al. A filarial nematode-secreted product signals dendritic cells to acquire a phenotype that drives development of Th2 cells. *J Immunol*. 2000;164:6453-6460.

128. Kane CM, Jung E, Pearce EJ. Schistosoma mansoni egg antigenmediated modulation of toll-like receptor (TLR)-induced activation occurs independently of TLR2, TLR4, and MyD88. *Infect Immun*. 2008;76:5754-5759.

129. Ferret-Bernard S, Curwen RS, Mountford AP. Proteomic profiling reveals that Th2-inducing dendritic cells stimulated with helminth antigens have a "limited maturation" phenotype. *Proteomics*. 2008;8: 980-993.

130. Hamilton CM, Dowling DJ, Loscher CE, et al. The Fasciola hepatica tegumental antigen suppresses dendritic cell maturation and function. *Infect Immun*. 1999;77:2488-2498.

131. Kane CM, Cervi L, Sun J, et al. Helminth antigens modulate TLR-initiated dendritic cell activation. *J Immunol*. 2004;173:7454-7461.

132. Semnani RT, Venugopal PG, Leifer CA, et al. Inhibition of TLR3 and TLR4 function and expression in human dendritic cells by helminth parasites. *Blood*. 2008;112:1290-1298.

133. Everts B, Perona-Wright G, Smits HH, et al. Omega-1, a glycoprotein secreted by Schistosoma mansoni eggs, drives Th2 responses. *J Exp Med*. 2009;206:1673-1680.

134. Steinfelder S, Andersen JF, Cannons JL, et al. The major component in schistosome eggs responsible for conditioning dendritic cells for Th2 polarization is a T2 ribonuclease (omega-1). *J Exp Med*. 2009;206: 1681-1690.

135. Appelmelk BJ, van Die I, van Vliet SJ, et al. Cutting edge: carbohydrate profiling identifies new pathogens that interact with dendritic cellspecific ICAM-3-grabbing nonintegrin on dendritic cells. *J Immunol*. 2003;170:1635-1639.

136. Jankovic D, Kullberg MC, Hieny S, et al. In the absence of IL-12, CD4(+) T cell responses to intracellular pathogens fail to default to a Th2 pattern and are host protective in an IL-10(-/-) setting. *Immunity*. 2002;16:429-439.

137. Perona-Wright G, Jenkins SJ, MacDonald AS. Dendritic cell activation and function in response to Schistosoma mansoni. *Int J Parasitol*. 2006;36:711-721.

138. Jankovic D, Kullberg MC, Noben-Trauth N, et al. Single cell analysis reveals that IL-4 receptor/Stat6 signaling is not required for the in vivo or in vitro development of CD4+ lymphocytes with a Th2 cytokine profile. *J Immunol*. 2000;164:3047-3055.

139. Noben-Trauth N, Shultz LD, Brombacher F, et al. An interleukin 4 (IL-4)-independent pathway for CD4+ T cell IL-4 production is revealed in IL-4 receptor-deficient mice. *Proc Natl Acad Sci U S A*. 1997;94: 10838-10843.

140. Tu L, Fang TC, Artis D, et al. Notch signaling is an important regulator of type 2 immunity. *J Exp Med*. 2005;202:1037-1042.

141. Amsen D, Antov A, Jankovic D, et al. Direct regulation of Gata3 expression determines the T helper differentiation potential of Notch. *Immunity*. 2007;27:89-99.

142. Worsley AG, LeibundGut-Landmann S, Slack E, et al. Dendritic cell expression of the Notch ligand jagged2 is not essential for Th2 response induction in vivo. *Eur J Immunol*. 2008;38:1043-1049.

143. Min B, Prout M, Hu-Li J, et al. Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite. *J Exp Med*. 2004;200:507-517.

144. Nel HJ, Hams E, Saunders SP, et al. Impaired basophil induction leads to an age-dependent innate defect in type 2 immunity during helminth infection in mice. *J Immunol*. 2011;186:4631-4639.

145. Padilla AM, Bustamante JM, Tarleton RL. CD8+ T cells in Trypanosoma cruzi infection. *Curr Opin Immunol*. 2009;21:385-390.

146. Bustamante JM, Bixby LM, Tarleton RL. Drug-induced cure drives conversion to a stable and protective CD8+ T central memory response in chronic Chagas disease. *Nat Med*. 2008;14:542-550.

147. Gazzinelli R, Xu Y, Hieny S, et al. Simultaneous depletion of CD4 + and CD8+ T lymphocytes is required to reactivate chronic infection with Toxoplasma gondii. *J Immunol*. 1992;149:175-180.

148. Belkaid Y, Von Stebut E, Mendez S, et al. CD8+ T cells are required for primary immunity in C57BL/6 mice following low-dose, intradermal challenge with Leishmania major. *J Immunol*. 2002;168:3992-4000.

149. Muller I, Kropf P, Louis JA, et al. Expansion of gamma interferon-producing CD8+ T cells following secondary infection of mice immune to Leishmania major. *Infect Immun*. 1994;62:2575-2581.

150. Denkers EY, Yap G, Scharton-Kersten T, et al. Perforin-mediated cytolysis plays a limited role in host resistance to Toxoplasma gondii. *J Immunol*. 1997;159:1903-1908.

151. Doolan DL, Hoffman SL. The complexity of protective immunity against liver-stage malaria. *J Immunol*. 2000;165:1453-1462.

152. Kumar S, Tarleton RL. The relative contribution of antibody production and CD8+ T cell function to immune control of Trypanosoma cruzi. *Parasite Immunol*. 1998;20:207-216.

153. Nitcheu J, Bonduelle O, Combadiere C, et al. Perforin-dependent brain-infiltrating cytotoxic CD8+ T lymphocytes mediate experimental cerebral malaria pathogenesis. *J Immunol*. 2003;170:2221-2228.

154. Belosevic M, Finbloom DS, Van Der Meide PH, et al. Administration of monoclonal anti-IFN-gamma antibodies in vivo abrogates natural resistance of C3H/HeN mice to infection with Leishmania major. *J Immunol*. 1989;143:266-274.

155. Torrico F, Heremans H, Rivera MT, et al. Endogenous IFN-gamma is required for resistance to acute Trypanosoma cruzi infection in mice. *J Immunol*. 1991;146:3626-3632.

156. Scharton-Kersten TM, Wynn TA, Denkers EY, et al. In the absence of endogenous IFN-gamma, mice develop unimpaired IL-12 responses to Toxoplasma gondii while failing to control acute infection. *J Immunol*. 1996;157:4045-4054.

157. van der Heyde HC, Pepper B, Batchelder J, et al. The time course of selected malarial infections in cytokine-deficient mice. *Exp Parasitol*. 1997;85:206-213.

158. McDonald V. Host cell-mediated responses to infection with Cryptosporidium. *Parasite Immunol*. 2000;22:597-604.

159. Wei XQ, Charles IG, Smith A, et al. Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature*. 1995;375:408-411.

160. Nashleanas M, Kanaly S, Scott P. Control of Leishmania major infection in mice lacking TNF receptors. *J Immunol*. 1998;160:5506-5513.

161. Bogdan C, Rollinghoff M, Diefenbach A. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. *Curr Opin Immunol*. 2000;12:64-76.

162. Yap GS, Sher A. Effector cells of both nonhemopoietic and hemopoietic origin are required for interferon (IFN)-gamma- and tumor necrosis factor (TNF)-alpha-dependent host resistance to the intracellular pathogen, Toxoplasma gondii. *J Exp Med*. 1999;189:1083-1092.

163. Taylor GA, Feng CG, Sher A. p47 GTPases: regulators of immunity to intracellular pathogens. *Nat Rev Immunol*. 2004;4:100-109.

164. Ling YM, Shaw MH, Ayala C, et al. Vacuolar and plasma membrane stripping and autophagic elimination of Toxoplasma gondii in primed effector macrophages. *J Exp Med*. 2006;203:2063-2071.

165. Martens S, Parvanova I, Zerrahn J, et al. Disruption of Toxoplasma gondii parasitophorous vacuoles by the mouse p47-resistance GTPases. *PLoS Pathog*. 2005;1:e24.

166. Hunn JP, Feng CG, Sher A, et al. The immunity-related GTPases in mammals: a fast-evolving cell-autonomous resistance system against intracellular pathogens. *Mamm Genome*. 2011;22:43-54.

167. Pfefferkorn ER. Interferon gamma blocks the growth of Toxoplasma gondii in human fibroblasts by inducing the host cells to degrade tryptophan. *Proc Natl Acad Sci U S A*. 1984;81:908-912.

168. Riley EM, Allen SJ, Wheeler JG, et al. Naturally acquired cellular and humoral immune responses to the major merozoite surface antigen (PfMSP1) of Plasmodium falciparum are associated with reduced malaria morbidity. *Parasite Immunol*. 1992;14:321-337.

169. Anstey NM, Weinberg JB, Hassanali MY, et al. Nitric oxide in Tanzanian children with malaria: inverse relationship between malaria severity and nitric oxide production/nitric oxide synthase type 2 expression. *J Exp Med*. 1996;184:557-567.

170. Bouharoun-Tayoun H, Attanath P, Sabchareon A, et al. Antibodies that protect humans against Plasmodium falciparum blood stages do not on their own inhibit parasite growth and invasion in vitro but act in co-operation with monocytes. *J Exp Med*. 1990;172:1633-1641.

171. Good MF. Towards a blood-stage vaccine for malaria: are we following all the leads? *Nat Rev Immunol*. 2001;1:117-125.

172. Pombo DJ, Lawrence G, Hirunpetcharat C, et al. Immunity to malaria after administration of ultra-low doses of red cells infected with Plasmodium falciparum. *Lancet*. 2002;360:610-617.

173. Roestenberg M, McCall M, Hopman J, et al. Protection against a malaria challenge by sporozoite inoculation. *N Engl J Med*. 2009;361:468-477.

174. Makobongo MO, Riding G, Xu H, et al. The purine salvage enzyme hypoxanthine guanine xanthine phosphoribosyl transferase is a major target antigen for cell-mediated immunity to malaria. *Proc Natl Acad Sci U S A*. 2003;100:2628-2633.

175. Cohen S, McGregor IA, Carrington S. Gamma globulin and acquired immunity to malaria. *Nature*. 1961;192:733-737.

176. Beeson JG, Osier FH, Engwerda CR. Recent insights into humoral and cellular immune responses against malaria. *Trends Parasitol*. 2008;24: 578-584.

177. Uthaipibull C, Aufiero B, Syed S, et al. Inhibitory and blocking monoclonal antibody epitopes on merozoite surface protein 1 of the malaria parasite Plasmodium falciparum. *J Molecular Biol*. 2001;307:1381-1394.

178. Hill AV. Pre-erythrocytic malaria vaccines: towards greater efficacy. *Nat Rev Immunol*. 2006;6:21-32.

179. Hoffman SL, Franke ED. Inducing protective immune responses against the sporozoite and liver stages of Plasmodium. *Immunol Lett*. 1994;41:89-94.

180. Vanderberg JP, Frevert U. Intravital microscopy demonstrating antibody-mediated immobilisation of Plasmodium berghei sporozoites injected into skin by mosquitoes. *Int J Parasitol*. 2004;34:991-996.

181. Hayes KS, Bancroft AJ, Goldrick M, et al. Exploitation of the intestinal microflora by the parasitic nematode Trichuris muris. *Science*. 2010;328:1391-1394.

182. Pesce JT, Liu Z, Hamed H, et al. Neutrophils clear bacteria associated with parasitic nematodes augmenting the development of an effective Th2-type response. *J Immunol*. 2008;180:464-474.

183. Urban JF Jr, Noben-Trauth N, Donaldson DD, et al. IL-13, IL-4Ralpha, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite Nippostrongylus brasiliensis. *Immunity*. 1998;8:255-264.

184. Zhu J, Min B, Hu-Li J, et al. Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. *Nat Immunol*. 2004;5:1157-1165.

185. Voehringer D, Reese TA, Huang X, et al. Type 2 immunity is controlled by IL-4/IL-13 expression in hematopoietic non-eosinophil cells of the innate immune system. *J Exp Med*. 2006;203:1435-1446.

186. Moro K, Yamada T, Tanabe M, et al. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature*. 2010;463:540-544.

187. Price AE, Liang HE, Sullivan BM, et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci U S A*. 2010;107:11489-11494.

188. Saenz SA, Siracusa MC, Perrigoue JG, et al. IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature*. 2010;464:1362-1366.

189. Korn T, Bettelli E, Oukka M, et al. IL-17 and Th17 Cells. *Annu Rev Immunol*. 2009;27:485-517.

190. Zhou L, Chong MM, Littman DR. Plasticity of CD4+ T cell lineage differentiation. *Immunity*. 2009;30:646-655.

191. Phythian-Adams AT, Cook PC, Lundie RJ, et al. CD11c depletion severely disrupts Th2 induction and development in vivo. *J Exp Med*. 2010;207:2089-2096.

192. Donnelly S, Stack CM, O'Neill SM, et al. Helminth 2-Cys peroxiredoxin drives Th2 responses through a mechanism involving alternatively activated macrophages. *FASEB J*. 2008;22:4022-4032.

193. Sokol CL, Barton GM, Farr AG, et al. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat Immunol*. 2008;9: 310-318.

194. van Panhuys N, Prout M, Forbes E, et al. Basophils are the major producers of IL-4 during primary helminth infection. *J Immunol*. 2011;186:2719-2728.

195. Smithgall MD, Comeau MR, Yoon BR, et al. IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int Immunol*. 2008;20: 1019-1030.

196. Massacand JC, Stettler RC, Meier R, et al. Helminth products bypass the need for TSLP in Th2 immune responses by directly modulating dendritic cell function. *Proc Natl Acad Sci U S A*. 2009;106:13968-13973.

197. Taylor BC, Zaph C, Troy AE, et al. TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. *J Exp Med*. 2009;206:655-667.

198. Ramalingam TR, Pesce JT, Mentink-Kane MM, et al. Regulation of helminth-induced Th2 responses by thymic stromal lymphopoietin. *J Immunol*. 2009;182:6452-6459.

199. Perrigoue JG, Saenz SA, Siracusa MC, et al. MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. *Nat Immunol*. 2009;10:697-705.

200. Yoshimoto T, Yasuda K, Tanaka H, et al. Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. *Nat Immunol*. 2009;10:706-712.

201. Sullivan BM, Liang HE, Bando JK, et al. Genetic analysis of basophil function in vivo. *Nat Immunol*. 2011;12:527-535.

202. Hammad H, Plantinga M, Deswarte K, et al. Inflammatory dendritic cells—not basophils—are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J Exp Med*. 2010;207:2097-2111.

203. Ohnmacht C, Schwartz C, Panzer M, et al. Basophils orchestrate chronic allergic dermatitis and protective immunity against helminths. *Immunity*. 2010;33:364-374.

204. Ohnmacht C, Voehringer D. Basophils protect against reinfection with hookworms independently of mast cells and memory Th2 cells. *J Immunol*. 2010;184:344-350.

205. van Liempt E, van Vliet SJ, Engering A, et al. Mansoni soluble egg antigens are internalized by human dendritic cells through multiple C-type lectins and suppress TLR-induced dendritic cell activation. *Mol Immunol*. 2007;44:2605-2615.

206. Ohnmacht C, Voehringer D. Basophil effector function and homeostasis during helminth infection. *Blood*. 2009;113:2816-2825.

207. Ziegler SF, Artis D. Sensing the outside world: TSLP regulates barrier immunity. *Nat Immunol*. 2010;11:289-293.

208. Zaph C, Troy AE, Taylor BC, et al. Epithelial-cell-intrinsic IKK-beta expression regulates intestinal immune homeostasis. *Nature*. 2007;446:552-556.

209. Siracusa MC, Saenz SA, Hill DA, et al. TSLP promotes interleukin-3-independent basophil haematopoiesis and type 2 inflammation. *Nature*. 2011;477(7363):229-233.

210. Segura M, Su Z, Piccirillo C, et al. Impairment of dendritic cell function by excretory-secretory products: a potential mechanism for nematodeinduced immunosuppression. *Eur J Immunol*. 2007;37:1887-1904.

211. Balic A, Harcus Y, Holland MJ, et al. Selective maturation of dendritic cells by Nippostrongylus brasiliensis-secreted proteins drives Th2 immune responses. *Eur J Immunol*. 2004;34:3047-3059.

212. Townsend MJ, Fallon PG, Matthews DJ, et al. T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *J Exp Med*. 2000;191:1069-1076.

213. Humphreys NE, Xu D, Hepworth MR, et al. IL-33, a potent inducer of adaptive immunity to intestinal nematodes. *J Immunol*. 2008; 180:2443-2449.

214. Wang YH, Angkasekwinai P, Lu N, et al. IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC- activated Th2 memory cells. *J Exp Med*. 2007;204:1837-1847.

215. Owyang AM, Zaph C, Wilson EH, et al. Interleukin 25 regulates type 2 cytokine-dependent immunity and limits chronic inflammation in the gastrointestinal tract. *J Exp Med*. 2006;203:843-849.

216. Fallon PG, Ballantyne SJ, Mangan NE, et al. Identification of an interleukin (IL)-25-

dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J Exp Med*. 2006;203: 1105-1116.

217. Zhao A, Urban JF Jr, Sun R, et al. Critical role of IL-25 in nematode infection-induced alterations in intestinal function. *J Immunol*. 2010;185:6921-6929.

218. Anthony RM, Urban JF Jr, Alem F, et al. Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. *Nat Med*. 2006;12:955-960.

219. Khan WI, Blennerhasset P, Ma C, et al. Stat6 dependent goblet cell hyperplasia during intestinal nematode infection. *Parasite Immunol*. 2001;23:39-42.

220. Schopf LR, Hoffmann KF, Cheever AW, et al. IL-10 is critical for host resistance and survival during gastrointestinal helminth infection. *J. Immunol*. 2002;168(5):2383-2392.

221. Zhao A, McDermott J, Urban JF Jr, et al. Dependence of IL-4, IL-13, and nematode-induced alterations in murine small intestinal smooth muscle contractility on Stat6 and enteric nerves. *J Immunol*. 2003;171:948-954.

222. Hasnain SZ, Evans CM, Roy M, et al. Muc5ac: a critical component mediating the rejection of enteric nematodes. *J Exp Med*. 2011;208:893-900.

223. Hasnain SZ, Wang H, Ghia JE, et al. Mucin gene deficiency in mice impairs host resistance to an enteric parasitic infection. *Gastroenterology*. 2010;138:1763-1771.

224. Artis D, Wang ML, Keilbaugh SA, et al. RELMbeta/FIZZ2 is a goblet cell-specific immune-effector molecule in the gastrointestinal tract. *Proc Natl Acad Sci U S A*. 2004;101:13596-13600.

225. Herbert DR, Yang JQ, Hogan SP, et al. Intestinal epithelial cell secretion of RELM-beta protects against gastrointestinal worm infection. *J Exp Med*. 2009;206:2947-2957.

226. Madden KB, Yeung KA, Zhao A, et al. Enteric nematodes induce stereotypic STAT6-dependent alterations in intestinal epithelial cell function. *J Immunol*. 2004;172:5616-5621.

227. Shea-Donohue T, Sullivan C, Finkelman FD, et al. The role of IL-4 in Heligmosomoides polygyrus-induced alterations in murine intestinal epithelial cell function. *J Immunol*. 2001;167:2234-2239.

228. Cliffe LJ, Humphreys NE, Lane TE, et al. Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. *Science*. 2005;308:1463-1465.

229. Zhao A, Urban JF Jr, Morimoto M, et al. Contribution of 5-HT2A receptor in nematode infection-induced murine intestinal smooth muscle hypercontractility. *Gastroenterology*. 2006;131:568-578.

230. Finkelman FD, Shea-Donohue T, Morris SC, et al. Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. *Immunol Rev*. 2004;201:139-155.

231. Finkelman FD, Wynn TA, Donaldson DD, et al. The role of IL-13 in helminth-induced inflammation and protective immunity against nematode infections. *Curr Opin Immunol*. 1999;11:420-426.

232. Maizels RM, Balic A, Gomez-Escobar N, et al. Helminth parasites— masters of regulation. *Immunol Rev*. 2004;201:89-116.

233. Nutman TB, Kumaraswami V. Regulation of the immune response in lymphatic filariasis: perspectives on acute and chronic infection with Wuchereria bancrofti in South India. *Parasite Immunol*. 2001;23:389-399.

234. Wynn TA, Hoffmann KF. Defining a schistosomiasis vaccination strategy—is it really Th1 versus Th2? *Parasitol Today*. 2000;16:497-501.

235. Grencis RK. Cytokine regulation of resistance and susceptibility to intestinal nematode infection—from host to parasite. *Vet Parasitol*. 2001;100:45-50.

236. Hepworth MR, Hardman MJ, Grencis RK. The role of sex hormones in the development of Th2 immunity in a gender-biased model of Trichuris muris infection. *Eur J Immunol*. 2010;40:406-416.

237. Helmby H, Grencis RK. Essential role for TLR4 and MyD88 in the development of chronic intestinal nematode infection. *Eur J Immunol*. 2003;33:2974-2979.

238. Bancroft AJ, Else KJ, Sypek JP, et al. Interleukin-12 promotes a chronic intestinal nematode infection. *Eur J Immunol*. 1997;27:866-870.

239. Katona IM, Urban JF Jr, Finkelman FD. The role of L3T4+ and Lyt-2+ T cells in the IgE response and immunity to Nippostrongylus brasiliensis. *J Immunol*. 1988;140:3206-3211.

240. Seidl A, Panzer M, Voehringer D. Protective immunity against the gastrointestinal nematode Nippostrongylus brasiliensis requires a broad T-cell receptor repertoire. *Immunology*. 2011;134:214-223.

241. Mohrs M, Shinkai K, Mohrs K, et al. Analysis of type 2 immunity in vivo with a bicistronic IL-4 reporter. *Immunity*. 2001;15:303-311.

242. Bancroft AJ, McKenzie AN, Grencis RK. A critical role for IL-13 in resistance to intestinal nematode infection. *J Immunol*. 1998;160: 3453-3461.

243. Bancroft AJ, Artis D, Donaldson DD, et al. Gastrointestinal nematode expulsion in IL-4 knockout mice is IL-13 dependent. *Eur J Immunol*. 2000;30:2083-2091.

244. Ramalingam TR, Pesce JT, Sheikh F, et al. Unique functions of the type II interleukin 4 receptor identified in mice lacking the interleukin 13 receptor alpha1 chain. *Nat Immunol*. 2008;9:25-33.

245. McKenzie GJ, Fallon PG, Emson CL, et al. Simultaneous disruption of interleukin (IL)-4 and IL-13 defines individual roles in T helper cell type 2-mediated responses. *J Exp Med*. 1999;189:1565-1572.

246. Mohrs K, Harris DP, Lund FE, et al. Systemic dissemination and persistence of Th2 and type 2 cells in response to infection with a strictly enteric nematode parasite. *J Immunol*. 2005;175:5306-5313.

247. Harvie M, Camberis M, Tang SC, et al. The lung is an important site for priming CD4 T-cell-mediated protective immunity against gastrointestinal helminth parasites. *Infect Immun*. 2010;78:3753-3762.

248. Zhao A, Urban JF Jr, Anthony RM, et al. Th2 cytokine-induced alterations in intestinal smooth muscle function depend on alternatively activated macrophages. *Gastroenterology.* 2008;135:217-225, e1.

249. Bowcutt R, Bell LV, Little M, et al. Arginase-1-expressing macrophages are dispensable for resistance to infection with the gastrointestinal helminth Trichuris muris. *Parasite Immunol*. 2011;33:411-420.

250. Artis D, Humphreys NE, Bancroft AJ, et al. Tumor necrosis factor alpha is a critical component of interleukin 13-mediated protective T helper cell type 2 responses during helminth infection. *J Exp Med.* 1999;190:953-962.

251. Helmby H, Grencis RK. Interleukin 1 plays a major role in the development of Th2-mediated immunity. *Eur J Immunol*. 2004;34:3674-3681.

252. Humphreys NE, Grencis RK. IL-1-dependent, IL-1R1-independent resistance to gastrointestinal nematodes. *Eur J Immunol*. 2009;39: 1036-1045.

253. King IL, Mohrs K, Mohrs M. A nonredundant role for IL-21 receptor signaling in plasma cell differentiation and protective type 2 immunity against gastrointestinal helminth infection. *J Immunol*. 2010;185: 6138-6145.

254. Frohlich A, Marsland BJ, Sonderegger I, et al. IL-21 receptor signaling is integral to the development of Th2 effector responses in vivo. *Blood.* 2007;109:2023-2031.

255. Pesce J, Kaviratne M, Ramalingam TR, et al. The IL-21 receptor augments Th2 effector function and alternative macrophage activation. *J Clin Invest.* 2006;116:2044-2055.

256. Helmby H, Grencis RK. Contrasting roles for IL-10 in protective immunity to different life cycle stages of intestinal nematode parasites. *Eur J Immunol*. 2003;33:2382-2390.

257. Khan WI, Vallance BA, Blennerhassett PA, et al. Critical role for signal transducer and activator of transcription factor 6 in mediating intestinal muscle hypercontractility and worm expulsion in Trichinella spiralis-infected mice. *Infect Immun.* 2001;69:838-844.

258. Scales HE, lerna MX, Lawrence CE. The role of IL-4, IL-13 and IL-4Ralpha in the development of protective and pathological responses to Trichinella spiralis. *Parasite Immunol*. 2007;29:81-91.

259. Urban JF Jr, Noben-Trauth N, Schopf L, et al. Cutting edge: IL-4 receptor expression by non-bone marrow-derived cells is required to expel gastrointestinal nematode parasites. *J Immunol*. 2001;167: 6078-6081.

260. Donaldson LE, Schmitt E, Huntley JF, et al. A critical role for stem cell factor and c-kit in host protective immunity to an intestinal helminth. *Int Immunol*. 1996;8:559-567.

261. McDermott JR, Bartram RE, Knight PA, et al. Mast cells disrupt epithelial barrier function during enteric nematode infection. *Proc Natl Acad Sci U S A.* 2003;100:7761-7766.

262. Finkelman FD, Urban JF Jr. The other side of the coin: the protective role of the TH2 cytokines. *J Allergy Clin Immunol*. 2001;107:772-780.

263. Helmby H, Grencis RK. IL-18 regulates intestinal mastocytosis and Th2 cytokine production independently of IFN-gamma during Trichinella spiralis infection. *J Immunol*. 2002;169:2553-2560.

264. Sasaki Y, Yoshimoto T, Maruyama H, et al. IL-18 with IL-2 protects against Strongyloides venezuelensis infection by activating mucosal mast cell-dependent type 2 innate immunity. *J Exp Med.* 2005;202: 607-616.

265. Bliss SK, Bliss SP, Beiting DP, et al. IL-10 regulates movement of intestinally derived CD4+ T cells to the liver. *J Immunol*. 2007;178: 7974-7983.

266. Beiting DP, Gagliardo LF, Hesse M, et al. Coordinated control of immunity to muscle stage Trichinella spiralis by IL-10, regulatory T cells, and TGF-beta. *J Immunol*. 2007;178:1039-1047.

267. Faulkner H, Humphreys N, Renauld JC, et al. Interleukin-9 is involved in host protective immunity to intestinal nematode infection. *Eur J Immunol*. 1997;27:2536-2540.

268. Faulkner H, Renauld JC, Van Snick J, et al. Interleukin-9 enhances resistance to the intestinal nematode Trichuris muris. *Infect Immun.* 1998;66:3832-3840.

269. Richard M, Grencis RK, Humphreys NE, et al. Anti-IL-9 vaccination prevents worm expulsion and blood eosinophilia in Trichuris murisinfected mice. *Proc Natl Acad Sci U S A.* 2000;97:767-772.

270. Leech MD, Grencis RK. Induction of enhanced immunity to intestinal nematodes using IL-9-producing dendritic cells. *J Immunol*. 2006;176:2505-2511.

271. Shin K, Watts GF, Oettgen HC, et al. Mouse mast cell tryptase mMCP-6 is a critical link between adaptive and innate immunity in the chronic phase of Trichinella spiralis infection. *J Immunol.* 2008;180:4885-4891.

272. Voehringer D, van Rooijen N, Locksley RM. Eosinophils develop in distinct stages and are recruited to peripheral sites by alternatively activated macrophages. *J Leukoc Biol*. 2007;81:1434-1444.

273. Finkelman FD, Shea-Donohue T, Goldhill J, et al. Cytokine regulation of host defense against parasitic gastrointestinal nematodes: lessons from studies with rodent models. *Annu Rev Immunol*. 1997;15: 505-533.

274. Korenaga M, Hitoshi Y, Takatsu K, et al. Regulatory effect of anti-interleukin-5 monoclonal antibody on intestinal worm burden in a primary infection with Strongyloides venezuelensis in mice. *Int J Parasitol*. 1994;24:951-957.

275. Rotman HL, Yutanawiboonchai W, Brigandi RA, et al. Strongyloides stercoralis: eosinophil-dependent immune-mediated killing of third stage larvae in BALB/cByJ mice. *Exp Parasitol*. 1996;82:267-278.

276. Yoshida T, Ikuta K, Sugaya H, et al. Defective B-1 cell development and impaired immunity against Angiostrongylus cantonensis in IL-5R alpha-deficient mice. *Immunity.* 1996;4:483-494.

277. Padigel UM, Hess JA, Lee JJ, et al. Eosinophils act as antigen-presenting cells to induce immunity to Strongyloides stercoralis in mice. *J Infect Dis.* 2007;196:1844-1851.

278. Ganley-Leal LM, Mwinzi PN, Cetre-Sossah CB, et al. Correlation between

eosinophils and protection against reinfection with Schistosoma mansoni and the effect of human immunodeficiency virus type 1 coinfection in humans. *Infect Immun.* 2006;74:2169-2176.

279. Reimert CM, Fitzsimmons CM, Joseph S, et al. Eosinophil activity in Schistosoma mansoni infections in vivo and in vitro in relation to plasma cytokine profile pre- and posttreatment with praziquantel. *Clin Vaccine Immunol*. 2006;13:584-593.

280. Reiman RM, Thompson RW, Feng CG, et al. Interleukin-5 (IL-5) augments the progression of liver fibrosis by regulating IL-13 activity. *Infect Immun.* 2006;74:1471-1479.

281. Vallance BA, Blennerhassett PA, Deng Y, et al. IL-5 contributes to worm expulsion and muscle hypercontractility in a primary T. spiralis infection. *Am J Physiol*. 1999;277:G400-G408.

282. Fabre V, Beiting DP, Bliss SK, et al. Eosinophil deficiency compromises parasite survival in chronic nematode infection. *J Immunol*. 2009;182:1577-1583.

283. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat Rev Immunol*. 2004;4:583-594.

284. Jacobsen EA, Taranova AG, Lee NA, et al. Eosinophils: singularly destructive effector cells or purveyors of immunoregulation? *J Allergy Clin Immunol*. 2007;119:1313-1320.

285. Spencer LA, Weller PF. Eosinophils and Th2 immunity: contemporary insights. *Immunol Cell Biol*. 2010;88:250-256.

286. Rumbley CA, Sugaya H, Zekavat SA, et al. Activated eosinophils are the major source of Th2-associated cytokines in the schistosome granuloma. *J Immunol*. 1999;162:1003-1009.

287. Padigel UM, Lee JJ, Nolan TJ, et al. Eosinophils can function as antigen-presenting cells to induce primary and secondary immune responses to Strongyloides stercoralis. *Infect Immun.* 2006;74: 3232-3238.

288. Sabin EA, Kopf MA, Pearce EJ. Schistosoma mansoni egg-induced early IL-4 production is dependent upon IL-5 and eosinophils. *J Exp Med.* 1996;184:1871-1878.

289. Allen JE, Wynn TA. Evolution of Th2 immunity: a rapid repair response to tissue destructive pathogens. *PLoS Pathog.* 2011;7:e1002003.

290. Kullberg MC, Berzofsky JA, Jankovic DL, et al. T cell-derived IL-3 induces the production of IL-4 by non-B, non-T cells to amplify the Th2-cytokine response to a non-parasite antigen in Schistosoma mansoni-infected mice. *J Immunol*. 1996;156:1482-1489.

291. Mitre E, Taylor RT, Kubofcik J, et al. Parasite antigen-driven basophils are a major source of IL-4 in human filarial infections. *J Immunol*. 2004;172:2439-2445.

292. Faulkner H, Turner J, Kamgno J, et al. - and infection intensity-dependent cytokine and antibody production in human trichuriasis: the importance of IgE. *J Infect Dis.* 2002;185:665-672.

293. Liu Q, Kreider T, Bowdridge S, et al. B cells have distinct roles in host protection against different nematode parasites. *J Immunol*. 2010;184:5213-5223.

294. McCoy KD, Stoel M, Stettler R, et al. Polyclonal and specific antibodies mediate protective immunity against enteric helminth infection. *Cell Host Microbe.* 2008;4:362-373.

295. Bell RG. The generation and expression of immunity to Trichinella spiralis in laboratory rodents. *Adv Parasitol*. 1998;41:149-217.

296. Gurish MF, Bryce PJ, Tao H, et al. IgE enhances parasite clearance and regulates mast cell responses in mice infected with Trichinella spiralis. *J Immunol.* 2004;172:1139-1145.

297. Dombrowicz D, Capron M. Eosinophils, allergy and parasites. *Curr Opin Immunol*. 2001;13:716-720.

298. Dunne DW, Butterworth AE, Fulford AJ, et al. Immunity after treatment of human schistosomiasis: association between IgE antibodies to adult worm antigens and resistance to reinfection. *Eur J Immunol*. 1992;22:1483-1494.

299. Capron M, Capron A. Immunoglobulin E and effector cells in schistosomiasis. *Science.* 1994;264:1876-1877.

300. Booth M, Shaw MA, Carpenter D, et al. Carriage of DRB1*13 is associated with increased posttreatment IgE levels against Schistosoma mansoni antigens and lower long-term reinfection levels. *J Immunol*. 2006;176:7112-7118.

301. Marquet S, Abel L, Hillaire D, et al. Genetic localization of a locus controlling the intensity of infection by Schistosoma mansoni on chromosome 5q31-q33. *Nat Genet.* 1996;14:181-184.

302. Babu S, Ganley LM, Klei TR, et al. Role of gamma interferon and interleukin-4 in host defense against the human filarial parasite Brugia malayi. *Infect Immun.* 2000;68:3034-3035.

303. Paciorkowski N, Shultz LD, Rajan TV. Primed peritoneal B lymphocytes are sufficient to transfer protection against Brugia pahangi infection in mice. *Infect Immun.* 2003;71:1370-1378.

304. Spencer LA, Porte P, Zetoff C, et al. Mice genetically deficient in immunoglobulin E are more permissive hosts than wild-type mice to a primary, but not secondary, infection with the filarial nematode Brugia malayi. *Infect Immun.* 2003;71:2462-2467.

305. Sahu BR, Mohanty MC, Sahoo PK, et al. Protective immunity in human filariasis: a role for parasite-specific IgA responses. *J Infect Dis.* 2008;198:434-443.

306. Panda AK, Sahoo PK, Kerketta AS, et al. Human lymphatic filariasis: genetic polymorphism of endothelin-1 and tumor necrosis factor receptor II correlates with development of chronic disease. *J Infect Dis.* 2011;204:315-322.

307. Simons JE, Gray CA, Lawrence RA. Absence of regulatory IL-10 enhances innate protection against filarial parasites by a neutrophil-independent mechanism. *Parasite Immunol.* 2010;32:473-478.

308. Ramalingam T, Porte P, Lee J, et al. Eosinophils, but not eosinophil peroxidase or major basic protein, are important for host protection in experimental Brugia pahangi

infection. *Infect Immun.* 2005;73:8442-8443.

309. Hansen RD, Trees AJ, Bah GS, et al. A worm's best friend: recruitment of neutrophils by Wolbachia confounds eosinophil degranulation against the filarial nematode Onchocerca ochengi. *Proc Biol Sci.* 2011;278:2293-2302.

310. Taylor MJ, Hoerauf A, Bockarie M. Lymphatic filariasis and onchocerciasis. *Lancet.* 2010;376:1175-1185.

311. Le Goff L, Lamb TJ, Graham AL, et al. IL-4 is required to prevent filarial nematode development in resistant but not susceptible strains of mice. *Int J Parasitol.* 2002;32:1277-1284.

312. Saeftel M, Arndt M, Specht S, et al. Synergism of gamma interferon and interleukin-5 in the control of murine filariasis. *Infect Immun.* 2003;71:6978-6985.

313. Taylor MD, van der Werf N, Harris A, et al. Early recruitment of natural CD4+ Foxp3+ Treg cells by infective larvae determines the outcome of filarial infection. *Eur J Immunol.* 2009;39:192-206.

314. Black SJ, Seed JR, Murphy NB. Innate and acquired resistance to African trypanosomiasis. *J Parasitol.* 2001;87:1-9.

315. Sternberg JM. Immunobiology of African trypanosomiasis. *Chem Immunol.* 1998;70:186-199.

316. Barkhuizen M, Magez S, Atkinson RA, et al. Interleukin-12p70- dependent interferon-gamma production is crucial for resistance in African trypanosomiasis. *J Infect Dis.* 2007;196:1253-1260.

317. Hertz CJ, Filutowicz H, Mansfield JM. Resistance to the African trypanosomes is IFN-gamma dependent. *J Immunol.* 1998;161: 6775-6783.

318. Magez S, Radwanska M, Drennan M, et al. Interferon-gamma and nitric oxide in combination with antibodies are key protective host immune factors during trypanosoma congolense Tc13 Infections. *J Infect Dis.* 2006;193:1575-1583.

319. Lopez R, Demick KP, Mansfield JM, et al. Type I IFNs play a role in early resistance, but subsequent susceptibility, to the African trypanosomes. *J Immunol.* 2008;181:4908-4917.

320. Kieft R, Capewell P, Turner CM, et al. Mechanism of Trypanosoma brucei gambiense (group 1) resistance to human trypanosome lytic factor. *Proc Natl Acad Sci U S A.* 2010;107: 16137-16141.

321. Genovese G, Friedman DJ, Ross MD, et al. Association of trypanolytic ApoL1 variants with kidney disease in African Americans. *Science.* 2010;329:841-845.

322. Li E, Zhou P, Petrin Z, et al. Mast cell-dependent control of Giardia lamblia infections in mice. *Infect Immun.* 2004;72:6642-6649.

323. Zhou P, Li E, Zhu N, et al. Role of interleukin-6 in the control of acute and chronic Giardia lamblia infections in mice. *Infect Immun.* 2003;71:1566-1568.

324. Zhou P, Li E, Shea-Donohue T, et al. Tumour necrosis factor alpha contributes to protection against Giardia lamblia infection in mice. *Parasite Immunol.* 2007;29:367-374.

325. Faubert G. Immune response to Giardia duodenalis. *Clin Microbiol Rev.* 2000;13:35-54, table of contents.

326. Li E, Zhou P, Singer SM. Neuronal nitric oxide synthase is necessary for elimination of Giardia lamblia infections in mice. *J Immunol.* 2006;176:516-521.

327. Tarleton RL, Zhang L. Chagas disease etiology: autoimmunity or parasite persistence? *Parasitol Today.* 1999;15:94-99.

328. Bogdan C, Gessner A, Solbach W, et al. Invasion, control and persistence of Leishmania parasites. *Curr Opin Immunol.* 1996;8: 517-525.

329. Pearce EJ, Sher A. Mechanisms of immune evasion in schistosomiasis. *Contrib Microbiol Immunol.* 1987;8:219-232.

330. Maizels RM, Bundy DA, Selkirk ME, et al. Immunological modulation and evasion by helminth parasites in human populations. *Nature.* 1993;365:797-805.

331. Davies SJ, Grogan JL, Blank RB, et al. Modulation of blood fluke development in the liver by hepatic CD4+ lymphocytes. *Science.* 2001;294:1358-1361.

332. Osman A, Niles EG, Verjovski-Almeida S, et al. Schistosoma mansoni TGF-beta receptor II: role in host ligand-induced regulation of a schistosome target gene. *PLoS Pathog.* 2006;2:e54.

333. Wolowczuk I, Nutten S, Roye O, et al. Infection of mice lacking interleukin-7 (IL-7) reveals an unexpected role for IL-7 in the development of the parasite Schistosoma mansoni. *Infect Immun.* 1999;67:4183-4190.

334. Hernandez DC, Lim KC, McKerrow JH, et al. Schistosoma mansoni: sex-specific modulation of parasite growth by host immune signals. *Exp Parasitol.* 2004;106:59-61.

335. Mackinnon MJ, Marsh K. The selection landscape of malaria parasites. *Science.* 2010;328:866-871.

336. Buffet PA, Safeukui I, Deplaine G, et al. The pathogenesis of Plasmodium falciparum malaria in humans: insights from splenic physiology. *Blood.* 2011;117:381-392.

337. Scherf A, Lopez-Rubio JJ, Riviere L. Antigenic variation in Plasmodium falciparum. *Annu Rev Microbiol.* 2008;62:445-470.

338. Cadman ET, Abdallah AY, Voisine C, et al. Alterations of splenic architecture in malaria are induced independently of toll-like receptors 2, 4, and 9 or MyD88 and may affect antibody affinity. *Infect Immun.* 2008;76:3924-3931.

339. Kaye PM, Svensson M, Ato M, et al. The immunopathology of experimental visceral leishmaniasis. *Immunol Rev.* 2004;201:239-253.

340. Millar AE, Wleklinski-Lee M, Kahn SJ. The surface protein superfamily of Trypanosoma cruzi stimulates a polarized Th1 response that becomes anergic. *J Immunol.* 1999;162:6092-6099.

341. Greenwood BM. Possible role of a B-cell mitogen in hypergammaglobulinaemia in malaria and trypanosomiasis. *Lancet.* 1974;1: 435-436.

342. Reina-San-Martin B, Degrave W, Rougeot C, et al. A B-cell mitogen from a pathogenic trypanosome is a eukaryotic proline racemase. *Nat Med.* 2000;6:890-897.

343. Belkaid Y, Piccirillo CA, Mendez S, et al. CD4+CD25 + regulatory T cells control Leishmania major persistence and immunity. *Nature.* 2002;420:502-507.

344. Omer FM, de Souza JB, Riley EM. Differential induction of TGF-beta regulates proinflammatory cytokine production and determines the outcome of lethal and nonlethal Plasmodium yoelii infections. *J Immunol.* 2003;171:5430-5436.

345. Hisaeda H, Maekawa Y, Iwakawa D, et al. Escape of malaria parasites from host immunity requires CD4(+)CD25(+) regulatory T cells. *Nat Med.* 2004;10:29-30.

346. Walther M, Tongren JE, Andrews L, et al. Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. *Immunity.* 2005;23: 287-296.

347. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. *J Immunol.* 2008;180:5771-5777.

348. Finney OC, Riley EM, Walther M. Regulatory T cells in malaria— friend or foe? *Trends Immunol.* 2010;31:63-70.

349. Jankovic D, Kullberg MC, Feng C, et al. Conventional T-bet Foxp3 Th1 cells are the major source of regulatory IL-10 during intracellular protozoan infection. *J Exp Med.* 2007;204(2):273-283.

350. Anderson CF, Oukka M, Kuchroo VJ, et al. CD4+CD25-Foxp3- Th1 cells are the source of IL-10 mediated immune suppression in chronic cutaneous leishmaniasis. *J Exp Med.* 2007;204(2):285-297.

351. Nylen S, Sacks D. Interleukin-10 and the pathogenesis of human visceral leishmaniasis. *Trends Immunol.* 2007;28:378-384.

352. Stumhofer JS, Hunter CA. Advances in understanding the anti-inflammatory properties of IL-27. *Immunol Lett.* 2008;117:123-130.

353. Findlay EG, Greig R, Stumhofer JS, et al. Essential role for IL-27 receptor signaling in prevention of Th1-mediated immunopathology during malaria infection. *J Immunol.* 2010;185:2482-2492.

354. Anderson CF, Stumhofer JS, Hunter CA, et al. IL-27 regulates IL-10 and IL-17 from CD4+ cells in nonhealing Leishmania major infection. *J Immunol.* 2009;183:4619-4627.

355. Miles SA, Conrad SM, Alves RG, et al. A role for IgG immune complexes during infection with the intracellular pathogen Leishmania. *J Exp Med.* 2005;201:747-754.

356. Padigel UM, Farrell JP. Control of infection with Leishmania major in susceptible BALB/c mice lacking the common gamma-chain for FcR is associated with reduced production of IL-10 and TGF-beta by parasitized cells. *J Immunol.* 2005;174:6340-6345.

357. Wynn TA, Kwiatkowski D. Pathology and pathogenesis of parasitic disease. In: Kaufmann S, ed. *Immunology of Infectious Diseases.* Washington, DC: ASM Press; 2001:293-306.

358. Nathoo S, Serghides L, Kain KC. Effect of HIV-1 antiretroviral drugs on cytoadherence and phagocytic clearance of Plasmodium falciparum-parasitised erythrocytes. *Lancet.* 2003;362:1039-1041.

359. Mwinzi PN, Karanja DM, Kareko I, et al. Short report: evaluation of hepatic fibrosis in persons co-infected with Schistosoma mansoni and human immunodeficiency virus 1. *Am J Trop Med Hyg.* 2004;71:783-786.

360. Chenine AL, Buckley KA, Li PL, et al. Schistosoma mansoni infection promotes SHIV clade C replication in rhesus macaques. *AIDS.* 2005;19:1793-1797.

361. Kublin JG, Steketee RW. HIV infection and malaria—understanding the interactions. *J Infect Dis.* 2006;193:1-3.

362. Booth M, Vennervald BJ, Butterworth AE, et al. Exposure to malaria affects the regression of hepatosplenomegaly after treatment for Schistosoma mansoni infection in Kenyan children. *BMC Med.*2004;2:36.

363. Marshall AJ, Brunet LR, van Gessel Y, et al. Toxoplasma gondii and Schistosoma mansoni synergize to promote hepatocyte dysfunction associated with high levels of plasma TNF-alpha and early death in C57BL/6 mice. *J Immunol.* 1999;163:2089-2097.

364. Potian JA, Rafi W, Bhatt K, et al. Preexisting helminth infection induces inhibition of innate pulmonary anti-tuberculosis defense by engaging the IL-4 receptor pathway. *J Exp Med.* 2011;208:1863-1874.

365. Andre AvS, Blackwell NM, Hall LR, et al. The role of endosymbiotic wolbachia bacteria in the pathogenesis of river blindness. *Science.* 2002;295:1892-1895.

366. Rutitzky LI, Lopes da Rosa JR, Stadecker MJ. Severe CD4 T cellmediated immunopathology in murine schistosomiasis is dependent on IL-12p40 and correlates with high levels of IL-17. *J Immunol.* 2005;175:3920-3926.

367. Shainheit MG, Smith PM, Bazzone LE, et al. Dendritic cell IL-23 and IL-1 production in response to schistosome eggs induces Th17 cells in a mouse strain prone to severe immunopathology. *J Immunol.* 2008;181:8559-8567.

368. El Kasmi KC, Qualls JE, Pesce JT, et al. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. *Nat Immunol.* 2008;9:1399-1406.

369. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol.* 2009;9:162-174.

370. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol.* 2002;2:389-400.

371. Herbert DR, Orekov T, Roloson A, et al. Arginase I suppresses IL-12/IL-23p40-driven intestinal inflammation during acute schistosomiasis. *J Immunol.* 2010;184:6438-6446.

372. Infante-Duarte C, Kamradt T. Th1/Th2 balance in infection. *Springer Semin Immunopathol.* 1999;21:317-338.

373. Hoffmann KF, Cheever AW, Wynn TA. IL-10 and the dangers of immune polarization:

excessive type 1 and type 2 cytokine responses induce distinct forms of lethal immunopathology in murine schistosomiasis. *J Immunol*. 2000;164:6406-6416.

374. Mentink-Kane MM, Cheever AW, Wilson MS, et al. Accelerated and progressive and lethal liver fibrosis in mice that lack interleukin (IL)-10, IL-12p40, and IL-13Ralpha2. *Gastroenterology.* 2011;141(6):2200-2209.

375. Wilson MS, Taylor MD, O'Gorman MT, et al. Helminth-induced CD19+CD23hi B cells modulate experimental allergic and autoimmune inflammation. *Eur J Immunol*. 2010;40:1682-1696.

376. Mangan NE, Fallon RE, Smith P, et al. Helminth infection protects mice from anaphylaxis via IL-10-producing B cells. *J Immunol*. 2004;173:6346-6356.

377. Gazzinelli RT, Wysocka M, Hieny S, et al. In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. *J Immunol*. 1996;157:798-805.

378. Holscher C, Mohrs M, Dai WJ, et al. Tumor necrosis factor alpha-mediated toxic shock in Trypanosoma cruzi-infected interleukin 10-deficient mice. *Infect Immun*. 2000;68:4075-4083.

379. Schofield L, Grau GE. Immunological processes in malaria pathogenesis. *Nat Rev Immunol*. 2005;5:722-735.

380. Li C, Seixas E, Langhorne J. Rodent malarias: the mouse as a model for understanding immune responses and pathology induced by the erythrocytic stages of the parasite. *Med Microbiol Immunol (Berl)*. 2001;189:115-126.

381. Engwerda C, Mynott T, Sawhney S, et al. Locally up-regulated lymphotoxin alpha, not systemic tumor necrosis factor alpha, is the principle mediator of murine cerebral malaria. *J Exp Med*. 2002;195: 1371-1377.

382. de Souza JB, Hafalla JC, Riley EM, Couper KN. Cerebral malaria: why experimental murine models are required to understand the pathogenesis of disease. *Parasitology*. 2010;137(5):755-772.

383. Couper KN, Blount DG, Wilson MS, et al. IL-10 from CD4CD25Foxp3CD127 adaptive regulatory T cells modulates parasite clearance and pathology during malaria infection. *PLoS Pathog*. 2008;4:e1000004.

384. Omer FM, Kurtzhals JA, Riley EM. Maintaining the immunological balance in parasitic infections: a role for TGF-beta? *Parasitol Today.* 2000;16:18-23.

385. Walther M, Jeffries D, Finney OC, et al. Distinct roles for FOXP3 and FOXP3 CD4 T cells in regulating cellular immunity to uncomplicated and severe Plasmodium falciparum malaria. *PLoS Pathog*. 2009;5:e1000364.

386. Hasnain SZ, Thornton DJ, Grencis RK. Changes in the mucosal barrier during acute and chronic Trichuris muris infection. *Parasite Immunol*. 2011;33:45-55.

387. Cliffe LJ, Grencis RK. The Trichuris muris system: a paradigm of resistance and susceptibility to intestinal nematode infection. *Adv Parasitol*. 2004;57:255-307.

388. Pearce EJ, MacDonald AS. The immunobiology of schistosomiasis. *Nat Rev Immunol*. 2002;2:499-511.

389. Wynn TA, Thompson RW, Cheever AW, et al. Immunopathogenesis of schistosomiasis. *Immunol Rev*. 2004;201:156-167.

390. Wilson S, Vennervald BJ, Dunne DW. Chronic hepatosplenomegaly in african school children: a common but neglected morbidity associated with schistosomiasis and malaria. *PLoS Negl Trop Dis*. 2011;5:e1149.

391. Cheever AW, Hoffmann KF, Wynn TA. Immunopathology of schistosomiasis mansoni in mice and men. *Immunol Today.* 2000;21:465-466.

392. Jankovic D, Cheever AW, Kullberg MC, et al. CD4+ T cell-mediated granulomatous pathology in schistosomiasis is downregulated by a B cell-dependent mechanism requiring Fc receptor signaling. *J Exp Med*. 1998;187:619-629.

393. Montesano MA, Colley DG, Eloi-Santos S, et al. Neonatal idiotypic exposure alters subsequent cytokine, pathology, and survival patterns in experimental Schistosoma mansoni infections. *J Exp Med*. 1999;189:637-645.

394. Montesano MA, Colley DG, Willard MT, et al. Idiotypes expressed early in experimental Schistosoma mansoni infections predict clinical outcomes of chronic disease. *J Exp Med*. 2002;195:1223-1228.

395. Wynn TA, Cheever AW, Jankovic D, et al. An IL-12-based vaccination method for preventing fibrosis induced by schistosome infection. *Nature*. 1995;376:594-596.

396. Brunet LR, Finkelman FD, Cheever AW, et al. IL-4 protects against TNF-alpha-mediated cachexia and death during acute schistosomiasis. *J Immunol*. 1997;159:777-785.

397. Cheever AW, Williams ME, Wynn TA, et al. Anti-IL-4 treatment of Schistosoma mansoni-infected mice inhibits development of T cells and non-B, non-T cells expressing Th2 cytokines while decreasing egginduced hepatic fibrosis. *J Immunol*. 1994;153:753-759.

398. Fallon PG, Richardson EJ, McKenzie GJ, et al. Schistosome infection of transgenic mice defines distinct and contrasting pathogenic roles for IL-4 and IL-13: IL-13 is a profibrotic agent. *J Immunol*. 2000;164:2585-2591.

399. Fallon PG, Richardson EJ, Smith P, et al. Elevated type 1, diminished type 2 cytokines and impaired antibody response are associated with hepatotoxicity and mortalities during Schistosoma mansoni infection of CD4-depleted mice. *Eur J Immunol*. 2000;30:470-480.

400. Barron L, Wynn TA. Macrophage activation governs schistosomiasisinduced inflammation and fibrosis. *Eur J Immunol*. 2011;41:2509-2514.

401. Chiaramonte MG, Donaldson DD, Cheever AW, et al. An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. *J Clin Invest.* 1999;104: 777-785.

402. Chiaramonte MG, Cheever AW, Malley JD, et al. Studies of murine schistosomiasis reveal interleukin-13 blockade as a treatment for established and progressive liver fibrosis. *Hepatology.* 2001;34:273-282.

403. Hesse M, Cheever AW, Jankovic D, et al. NOS-2 mediates the protective anti-inflammatory and antifibrotic effects of the Th1-inducing adjuvant, IL-12, in a Th2 model of granulomatous disease. *Am J Pathol*. 2000;157:945-955.

404. Hoffmann KF, Caspar P, Cheever AW, et al. IFN-gamma, IL-12, and TNF-alpha are required to maintain reduced liver pathology in mice vaccinated with Schistosoma mansoni eggs and IL-12. *J Immunol*. 1998;161:4201-4210.

405. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol*. 2003;3:23-35.

406. Nair MG, Gallagher IJ, Taylor MD, et al. Chitinase and Fizz family members are a generalized feature of nematode infection with selective upregulation of Ym1 and Fizz1 by antigen-presenting cells. *Infect Immun*. 2005;73:385-394.

407. Pesce JT, Ramalingam TR, Mentink-Kane MM, et al. Arginase-1-expressing macrophages suppress Th2 cytokine-driven inflammation and fibrosis. *PLoS Pathog*. 2009;5:e1000371.

408. Pesce JT, Ramalingam TR, Wilson MS, et al. Retnla (relmalpha/fi zz1) suppresses helminth-induced Th2-type immunity. *PLoS Pathog*. 2009;5:e1000393.

409. Modolell M, Corraliza IM, Link F, et al. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrowderived macrophages by TH1 and TH2 cytokines. *Eur J Immunol*. 1995;25:1101-1104.

410. Hesse M, Modolell M, La Flamme AC, et al. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J Immunol*. 2001;167:6533-6544.

411. Herbert DR, Holscher C, Mohrs M, et al. Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. *Immunity*. 2004;20:623-635.

412. Thompson RW, Pesce JT, Ramalingam T, et al. Cationic amino acid transporter-2 regulates immunity by modulating arginase activity. *PLoS Pathog*. 2008;4:e1000023.

413. Vercruysse J, Knox DP, Schetters TP, et al. Veterinary parasitic vaccines: pitfalls and future directions. *Trends Parasitol*. 2004;20:488-492.

414. Doolan DL. Plasmodium immunomics. *Int J Parasitol*. 2011;41:3-20.

415. Dumonteil E. Vaccine development against Trypanosoma cruzi and Leishmania species in the post-genomic era. *Infect Genet Evol*. 2009;9:1075-1082.

416. Petri WA Jr, Chaudhry O, Haque R, et al. Adherence-blocking vaccine for amebiasis. *Arch Med Res*. 2006;37:288-291.

417. Houpt E, Barroso L, Lockhart L, et al. Prevention of intestinal amebiasis by vaccination with the Entamoeba histolytica Gal/GalNac lectin. *Vaccine*. 2004;22:611-617.

418. Carrero JC, Cervantes-Rebolledo C, Aguilar-Diaz H, et al. The role of the secretory immune response in the infection by Entamoeba histolytica. *Parasite Immunol*. 2007;29:331-338.

419. Olson ME, Ceri H, Morck DW. Giardia vaccination. *Parasitol Today*. 2000;16:213-217.

420. Rivero FD, Saura A, Prucca CG, et al. Disruption of antigenic variation is crucial for effective parasite vaccine. *Nat Med*. 2010;16:551-557, 1p following 7.

421. Larocque R, Nakagaki K, Lee P, et al. Oral immunization of BALB/c mice with Giardia duodenalis recombinant cyst wall protein inhibits shedding of cysts. *Infect Immun*. 2003;71:5662-5669.

422. Crompton PD, Pierce SK, Miller LH. Advances and challenges in malaria vaccine development. *J Clin Invest*. 2010;120:4168-4178.

423. Heppner DG Jr, Kester KE, Ockenhouse CF, et al. Towards an RTS,S-based, multi-stage, multi-antigen vaccine against falciparum malaria: progress at the Walter Reed Army Institute of Research. *Vaccine*. 2005;23:2243-2250.

424. Fowkes FJ, Richards JS, Simpson JA, et al. The relationship between anti-merozoite antibodies and incidence of Plasmodium falciparum malaria: a systematic review and meta-analysis. *PLoS Med*. 2010;7: e1000218.

425. Crosnier C, Bustamante LY, Bartholdson SJ, et al. Basigin is a receptor essential for erythrocyte invasion by Plasmodium falciparum. *Nature*. 2011;480:534-537.

426. Hviid L. The role of Plasmodium falciparum variant surface antigens in protective immunity and vaccine development. *Hum Vaccin*. 2010;6:84-89.

427. Gupta S, Snow RW, Donnelly CA, et al. Immunity to non-cerebral severe malaria is acquired after one or two infections. *Nat Med*. 1999;5:340-343.

428. Bull PC, Kortok M, Kai O, et al. Plasmodium falciparum-infected erythrocytes: agglutination by diverse Kenyan plasma is associated with severe disease and young host age. *J Infect Dis*. 2000;182:252-259.

429. Saul A. Mosquito stage, transmission blocking vaccines for malaria. *Curr Opin Infect Dis*. 2007;20:476-481.

430. Afonso LCC, Scharton TM, Vieira LQ, et al. The adjuvant effect of interleukin 12 in a vaccine against Leishmania major. *Science*. 1993;263:235-237.

431. Okwor I, Uzonna J. Vaccines and vaccination strategies against human cutaneous leishmaniasis. *Hum Vaccin*. 2009;5:291-301.

432. Peters NC, Kimblin N, Secundino N, et al. Vector transmission of leishmania abrogates vaccine-induced protective immunity. *PLoS Pathog*. 2009;5:e1000484.

433. Zaph C, Uzonna J, Beverley SM, et al. Central memory T cells mediate long-term immunity to Leishmania major in the absence of persistent parasites. *Nat Med*. 2004;10:1104-1110.

434. Noazin S, Khamesipour A, Moulton LH, et al. Efficacy of killed wholeparasite vaccines in the prevention of leishmaniasis: a meta-analysis. *Vaccine*. 2009;27:4747-4753.

435. Rodrigues MM, de Alencar BC, Claser C, et al. Swimming against the current: genetic vaccination against Trypanosoma cruzi infection in mice. *Mem Inst Oswaldo*

Cruz. 2009;104(suppl 1):281-287.

436. Martin DL, Weatherly DB, Laucella SA, et al. CD8+ T-Cell responses to Trypanosoma cruzi are highly focused on strain-variant trans-sialidase epitopes. *PLoS Pathog.* 2006;2:e77.

437. Jongert E, Roberts CW, Gargano N, et al. Vaccines against Toxoplasma gondii: challenges and opportunities. *Mem Inst Oswaldo Cruz.* 2009; 104:252-266.

438. Frickel EM, Sahoo N, Hopp J, et al. Parasite stage-specific recognition of endogenous Toxoplasma gondii-derived CD8+ T cell epitopes. *J Infect Dis.* 2008;198:1625-1633.

439. Cong H, Mui EJ, Witola WH, et al. Towards an immunosense vaccine to prevent toxoplasmosis: protective Toxoplasma gondii epitopes restricted by HLA-A*0201. *Vaccine.* 2011;29:754-762.

440. Borrmann S, Matuschewski K. Targeting Plasmodium liver stages: better late than never. *Trends Mol Med.* 2011;17:527-536.

441. Bojang KA, Milligan PJ, Pinder M, et al.Efficacy of RTS,S/AS02 malaria vaccine against Plasmodium falciparum infection in semi-immune adult men in The Gambia: a randomised trial. *Lancet.* 2001;358: 1927-1934.

442. Ballou WR. The development of the RTS,S malaria vaccine candidate: challenges and lessons. *Parasite Immunol*. 2009;31:492-500.

443. Agnandji ST, Lell B, Soulanoudjingar SS, et al. First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. *N Engl J Med.* 2011;365:1863-1875.

444. Hurd H. Host fecundity reduction: a strategy for damage limitation? *Trend Parasitol*. 2001;17:363-368.

445. McManus DP, Bartley PB. A vaccine against Asian schistosomiasis. *Parasitol Int.* 2004;53:163-173.

446. Cheever AW, Yap GS. Immunologic basis of disease and disease regulation in schistosomiasis. *Chem Immunol*. 1997;66:159-176.

447. Wynn TA, Eltoum I, Oswald IP, et al. Endogenous interleukin 12 (IL-12) regulates granuloma formation induced by eggs of Schistosoma mansoni and exogenous IL-12 both inhibits and prophylactically immunizes against egg pathology. *J Exp Med.* 1994;179:1551-1561.

448. Chiaramonte MG, Hesse M, Cheever AW, et al. CpG oligonucleotides can prophylactically immunize against Th2-mediated schistosome egginduced pathology by an IL-12-independent mechanism. *J Immunol*. 2000;164:973-985.

449. Chen Y, Boros DL. Polarization of the immune response to the single immunodominant epitope of p38, a major Schistosoma mansoni egg antigen, generates Th1- or Th2-type cytokines and granulomas. *Infect Immun.* 1999;67:4570-4577.

450. Asahi H, Osman A, Cook RM, et al. Schistosoma mansoni phosphoenolpyruvate carboxykinase, a novel egg antigen: immunological properties of the recombinant protein and identification of a T-cell epitope. *Infect Immun.* 2000;68:3385-3393.

451. Schofield L, Mueller I. Clinical immunity to malaria. *Curr Mol Med.* 2006;6:205-221.

452. Schofield L, Hewitt M, Evans K, et al. Synthetic GPI as a candidate antitoxic vaccine in a model of malaria. *Nature.* 2002;418:785-789.

453. Hagan P, Doenhoff MJ, Wilson RA, et al. Schistosomiasis vaccines: a response to a devils' advocate's view. *Parasitol Today.* 2000;16:322-323.

454. Driguez P, Doolan DL, Loukas A, et al. Schistosomiasis vaccine discovery using immunomics. *Parasites Vectors.* 2010;3:4.

455. Carvalho JA, Rodgers J, Atouguia J, et al. DNA vaccines: a rational design against parasitic diseases. *Expert Rev Vaccines.* 2010;9:175-191.

456. Wynn TA, Jankovic D, Hieny S, et al. IL-12 enhances vaccine-induced immunity to Schistosoma mansoni in mice and decreases T helper 2 cytokine expression, IgE production, and tissue eosinophilia. *J Immunol*. 1995;154:4701-4709.

457. Wilson RA, Coulson PS. Strategies for a schistosome vaccine: can we manipulate the immune response effectively? *Microbes Infect.* 1999;1:535-543.

458. Jankovic D, Wynn TA, Kullberg MC, et al. Optimal vaccination against Schistosoma mansoni requires the induction of both B cell- and IFN-gamma-dependent effector mechanisms. *J Immunol*. 1999;162: 345-351.

459. Anderson S, Coulson PS, Ljubojevic S, et al. The radiation-attenuated schistosome vaccine induces high levels of protective immunity in the absence of B cells. *Immunology.* 1999;96:22-28.

460. Maizels RM, Holland MJ, Falcone FH, et al. Vaccination against helminth parasites —the ultimate challenge for vaccinologists? *Immunol Rev.* 1999;171:125-147.

461. Allen JE, Maizels RM. Diversity and dialogue in immunity to helminths. *Nature reviews. Immunology.* 2011;11:375-388.

462. Capron A, Riveau G, Capron M, et al. Schistosomes: the road from host-parasite interactions to vaccines in clinical trials. *Trends Parasitol*. 2005;21:143-149.

463. Bergquist NR, Colley DG. Schistosomiasis vaccines: research to development. *Parasitol Today.* 1998;14:99-104.

464. Tran MH, Pearson MS, Bethony JM, et al. Tetraspanins on the surface of Schistosoma mansoni are protective antigens against schistosomiasis. *Nat Med.* 2006;12:835-840.

465. Dalton JP, Brindley PJ, Knox DP, et al. Helminth vaccines: from mining genomic information for vaccine targets to systems used for protein expression. *Int J Parasitol*. 2003;33:621-640.

466. McManus DP, Loukas A. Current status of vaccines for schistosomiasis. *Clin Microbiol Rev.* 2008;21:225-242.

467. Hotez PJ, Bethony JM, Diemert DJ, et al. Developing vaccines to combat hookworm infection and intestinal schistosomiasis. *Nat Rev Microbiol*. 2010;8:814-826.

468. Yazdanbakhsh M, Kremsner PG, van Ree R. Allergy, parasites, and the hygiene hypothesis. *Science.* 2002;296:490-494.

469. Pulendran B, Li S, Nakaya HI. Systems vaccinology. *Immunity.* 2010;33:516-529.

470. Fentress SJ, Sibley LD. The secreted kinase ROP18 defends Toxoplasma's border. *Bioessays.* 2011;33:693-700.

471. Richie TL, Saul A. Progress and challenges for malaria vaccines. *Nature.* 2002;415:694-701.

472. Drennan MB, Stijlemans B, Van den Abbeele J, et al. The induction of a type 1 immune response following a Trypanosoma brucei infection is MyD88 dependent. *J Immunol.* 2005;175:2501-2509.

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Chapter 39

Immunity to Viruses

Hildegund C.J. Ertl

INTRODUCTION

Veni, vidi, vici—I came, I saw, I conquered. If viruses could talk or see, this would be their hendiatris. Although viruses are minute particles composed only of a genome surrounded by a few proteins, they have a fiendish way of wreaking havoc not only on humans, as everyone who ever had the flu knows, but also on animals, plants, and even bacteria. Each year, more people die of viral infections than of natural disasters such as hurricanes, earthquakes, and tsunamis combined, or even manmade tragedies such as war. For example, it is estimated that variola major, the causative agent for smallpox, killed nearly half of the population of Native Americans after the virus was introduced into the western hemisphere by European colonizers. The Spanish Flu caused by an H1N1 influenza A virus caused the death of 50 to 100 million humans between 1918 and 1919, which is well in excess of the 16 million casualties of World War I. Human immunodeficiency virus (HIV)-1 has killed more than 25 million humans since 1981 and continues to spread, threatening the already frail economic structures of the most afflicted countries in sub-Saharan Africa, where more than 30% of the adult population carries the virus.

Not only do viruses cause acute or chronic infections with potentially fatal outcome, but they also contribute to other diseases. Viruses are associated with 20% of cancers, they have been implicated in the pathogenesis of human arteriosclerosis and autoimmune diseases, and they are linked to an overall reduction in life expectancy.

How can something so small be so deadly? Viruses, which range in size from 10 to 300 nm in diameter with genomes of minimally 2 kilobases to over 1.2 megabases, are unable to propagate themselves, but require a host cell to replicate. Once a cell becomes infected, viruses hijack its transcription and translation machinery to promote their own replication. The physiologic functions of the infected cell are disrupted as it is being turned into a virus production facility. However, the cell fights back as soon as it senses the virus. This fight initially takes place intrinsically within the infected cells but then rapidly spreads extrinsically once the immune system has been alerted. In turn, many viruses mount defenses against the attack from their host by encoding proteins that actively subvert innate and adaptive immune responses. In acute virus infections, the fight between virus and host literally lasts until the death of one of the adversaries. In chronic infections, a truce is eventually reached where virus and host coexist, generally at the expense of the well-being of the latter.

Fewer than 200 viruses are known to cause disease in humans. Over the last 50 years, on average two new species of human viruses have been discovered annually; one can expect that this number will continue to rise.[1] Where does this ever-increasing number of viruses come from? We are not certain about the origin of viruses, although we know from ancient texts as well as more modern data-driven genomic analyses that viruses have been around for a very long time.[2] Whether they originated from cells, concomitantly with cells, or even primordially from some genetic soup remains debated.[3] No matter how they evolved, their lack of common genes argues for a polyphyletic rather than monophyletic evolution. The constant discovery of new viruses may simply reflect improvements of detection technologies

that traditionally were based on cell culture and that are now being replaced with high-throughput genomics. Notwithstanding, many of the newly discovered viruses seem to evolve from animal reservoirs through mutations that allow for an extension of their host range. Of importance is that viral genomes have far higher mutation rates than, for example, mammalian genomes as they fail to correct errors during replication; such errors most commonly lead to loss of viral fitness but occasionally benefit the virus in its quest for replication. One example of a stable host range altering mutation that caught global attention was that of a coronavirus, which caused the outbreak of severe acute respiratory syndrome (SARS) in 2003 and 2004. This virus, termed SARS-CoV, which in its wild-type form infects civets, a cat-like carnivore, mutated and became infectious to humans and then within this host rapidly underwent further positive selection.[4] Other viruses such as pathogenic H5N1 avian influenza viruses have been isolated since the late 1990s repeatedly from humans, who commonly died as sequela of the infection. Pathogenic H5N1 viruses, which were, and by some still are, feared to evolve into pandemic viruses, have thus far failed to mutate to achieve sustained human-to-human transmission, while concomitantly, another influenza A virus arose from a triple reassortment between viruses that naturally infect humans, avians, and swine, and caused the 2007 influenza pandemic. Considering that our knowledge of animal viruses remains limited, the sudden emergence of new and potentially deadly viruses from other species continues to threaten global health.

While most other deadly disease can be treated with drugs such as antibiotics to resolve bacterial infections or can be prevented by lifestyle choices, our arsenal to combat viral infections remains limited. Vaccines, which can be effective

in preventing viral infections, and even achieved the eradication of small poxvirus, are only available for 15 viruses. Drugs to specifically treat infections are only effective for some viruses such as HIV-1, herpes, hepatitis B and C, and influenza viruses. Our main defense thus remains the immune system. Over eons, it has evolved to sense viruses as pathogens, to produce factors that stop viral replication, and to develop lymphocytes that destroy those cells that serve as viral production factories. Like an elephant, the immune system never forgets. Nevertheless, this defense like that in every war, even if victorious, comes at a price: a runny nose at best and death due to insufficient or overwhelming responses at worst.

BASICS IN VIROLOGY

Virus Classification

According to the International Committee of Taxometry of Viruses, all viruses are classified into order, family, subfamily, genus, and species. Names of serotypes, genotypes, strains, variants, or isolates of virus species or artificial viruses are not ruled by the International Committee of Taxometry of Viruses. A species is defined as a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecologic niche. A genus defines a group of species that share common characteristics, while subfamilies and families define a group of genera with common characteristics. An order, in turn, defines families with shared characteristics. Currently, 6 orders, 87 families, 19 subfamilies, 348 genera, and 2288 species of virus have been defined.[5] The Baltimore classification differs and divides viruses according to their genome or their mode of replication. Viruses contain either a deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) genome, which can be single stranded (ss) or double stranded (ds). SsRNA viruses carry either a negative- or positive-stranded RNA. During their lifecycle, some DNA and RNA viruses undergo an intermediate step in which the RNA genome is converted by reverse transcription (RT) into DNA or vice versa their DNA genome into an RNA genome. Accordingly, viruses are classified into dsDNA viruses (eg, adeno or poxviruses), ssDNA viruses (eg, parvoviruses), dsRNA viruses (eg, reovirus), positive-sense ssRNA viruses (eg, picornaviruses), negativesense ssRNA viruses (eg, rhabdoviruses), positive-sense ssRNA-RT viruses (eg, retroviruses), or dsDNA-RT viruses (eg, hepadnaviruses). Other classifications are based on host range (Holmes

classification) or structural characteristics (Lworff-Horne-Tournier [LHT] system).

The viral genome can be linear (eg, poxvirus), circular (eg, papillomaviruses), or segmented (eg, influenza virus). Viruses are further classified into enveloped (eg, rhabdoviruses) or naked (eg, picornaviruses) viruses. Viruses can also be divided according to their morphology, which can be polymorphic or structured, the latter having either icosahedral or helical symmetry.

To give an example of the taxonomic division of viruses: the 2007 pandemic H1N1 virus belongs to the species of influenza A virus, the genus of influenza virus, the family of Orthomyxoviridae with a negative-stranded ssRNA genome covered by a helical envelope. A list of common human viruses and additional viruses repeatedly referred to in this text and their classification is shown in Table 39.1. Other characteristics of virus families, such as their genomes and surface structures, are shown in Table 39.2.

Virus Transmission

Viruses have a single-track mind; their only goal is to replicate. The goal of their unwilling hosts is to get rid of them by mounting an immune response. Viruses have evolved a multitude of mechanisms to evade this immune response, and their hosts have adapted countermeasures accordingly, as only those that survived the onslaught of infections reached reproductive age. Highly contagious viruses can afford to not be overly concerned if their hosts, which are essential for their replication, survive, die, or mount a rapid and successful immune response because their ease of transmission ensures their continued existence. A typical example for such a virus is influenza virus, which is transmitted by aerosols before its host becomes sufficiently ill to seek solace in bed, which would limit contact with others and thus reduce the chance for the virus to spread. An example for a virus that is not overly contagious is rabies virus; it is transmitted by the bite of an infected animal, and it ensures its transmission by literally driving its host into an insane rage so that it will randomly attack and bite everyone in sight. Other viruses evolved to ensure the continued survival of their hosts, which enables their own continued replications without necessitating rapid transmission to a new individual. Such viruses are usually more complex, as much of their genome is devoted to combat immune responses.

Many viruses can only replicate in one species and therefore human infections require human-to-human contact. For viruses that are heat labile, such contact needs to be close, while more stable viruses can remain on surfaces or in water until the opportunity for infection arises. Viruses that only replicate in humans can potentially be eradicated once a vaccine becomes available as exemplified by smallpox virus. Other viruses are less discriminatory, and they replicate in multiple species, not necessarily only mammals but also birds or invertebrates. For example, influenza viruses can infect aquatic birds, chicken, swine, horses, humans, and even cats. Although they are most commonly transmitted to humans from other humans, spread from infected animals can occur, such as infections of humans with pathogenic H5N1 from chickens or ducks. Some viruses, such as rabies virus, infect all warm-blooded mammals, while other viruses alternate between mosquitoes and vertebrate animals. An example for the latter is Japanese encephalitis virus, which, in addition to mosquitoes of the *Culex tritaeniorhynchus* species, can infect humans, birds, most domestic animals, snakes, and frogs. The host range of a specific virus affects the mode of transmission, which is further influenced by the tissue tropism of the virus and its resistance to environmental factors like temperature or water.

One of the main protections against virus invasion is healthy skin; the upper layer of the keratinized epidermis effectively prevents entry of viruses. Mucosal surfaces,

although they are commonly bathed in antiviral proteins present in saliva and tears, in acids found in the female outer genital tract or the stomach, or in destructive enzymes such as those present in the upper intestinal tract, provide a more permissive port of entry for viruses

and, consequently, most viruses are transmitted through the mucosal surfaces of either the airways, the intestines, the genital tract, or the eye. Influenza viruses, parainfluenza viruses, some types of adenoviruses, and rhinoviruses spread through the airways and are transmitted by aerosolized droplets expelled by coughing or sneezing.

TABLE 39.1 Taxonomy of Viruses

Order	Family	Subfamily	Genus	Species (Alternative Names)	Abbreviation
Herpes virales	Herpes virideae	Alphaherpes virinae	Simplex virus	Human herpesvirus 1 (Herpes simplex virus-1)	HHV1
				Human herpesvirus 2 (Herpes simplex virus-2)	HHV2
				Saimiriine herpes virus 1	SHV1
			Varicellovirus	Human herpesvirus 3	HHV3
		Betaherpes virinae	Cytomegalovirus	Human herpesvirus 5	HHV5
			Roseolavirus	Human herpesvirus 6	HHV6
				Human herpes virus 7	HHV7
		Gamma-herpes virinae	Lymphocrypti virus	Human herpesvirus 4 Epstein-Barr virus	HHV4 EBV
			Rhadinovirus	Human herpesvirus 8	HHV8
				Saimiriine herpes virus 2	
Mononega virales	Filoviridae		Ebolavirus	Zaire ebolavirus	
	Paramyxo	Paramyxo	Morbillivirus	Measles virus	

	viridae	virinae	Respirovirus	Human parainfluenza virus 1 Sendai virus	
			Rubulavirus	Mumps virus	
		Pneumo virinae	Pneumovirus	Human respiratory syncytial virus	RSV
	Rhabdo viridae		Lyssavirus	Rabies virus	
			Vesiculovirus	Vesicular stomatitis New Jersey virus	VSV
Nidovirales	Corona viridae	Corona virinae	Betacoronavirus	Severe acute respiratory syndrome-related coronavirus	SARS-CoV
Picorna virales	Picorna viridae		Enterovirus Hepatovirus	Human rhinovirus A Hepatitis A virus	 HAV
Unassigned	Adeno viridae		Mastadenovirus	Human adenovirus C	Ad virus
	Arena viridae		Arenavirus	Lymphocytic choriomeningitis virus	LVMV
	Flaviviridae		Flavivirus	Dengue virus Japanese encephalitis virus Tick-borne encephalitis virus West Nile virus Yellow fever virus	WNV
			Hepacivirus	Hepatitis C virus	HCV
	Hepadna viridae		Orthohepadna virus	Hepatitis B virus	HBV
	Orthomyxo viridae		Influenzavirus A	Influenza A virus	Flu
	Papilloma		Alphapapilloma	Human	HPV16

viridae		virus	papillomavirus 16	
			Human papillomavirus 18	HPV18
Parvo viridae	Parvo virinae	Dependovirus	Adeno-associated virus-2	AAV2
Poxviridae	Chordopox virinae	Avipoxvirus	Fowlpox virus	
		Leporipoxvirus	Myxoma virus	
		Molluscipoxvirus	Molluscum contagiosum virus	MCV
		Orthopoxvirus	Cowpox virus	
			Ectromelia virus	
			Vaccinia virus	VV
			Variola virus	
Reoviridae	Sedoreovirinae	Rotavirus	Rotavirus A	
Retroviridae	Orthoretrovirinae	Deltaretrovirus	Primate T-lymphotropic virus 1	HTLV-1
		Lentivirus	Human immunodeficiency virus 1	HIV-1
			Simian immunodeficiency virus	
Togaviridae		Alphavirus	Sindbis virus	
			Venezuelan equine encephalitis virus	VEE virus
		Rubivirus	Rubella virus	

TABLE 39.2 Characteristics of Viruses

Family	Genome	Virion	Capsid
Herpesvirideae	dsDNA	Enveloped	Isocahedral
Filoviridae	Negative-sense ssRNA	Enveloped	Helical
Paramyxoviridae	Negative-sense ssRNA	Enveloped	Helical
Coronaviridae	Positive-sense ssRNA	Enveloped	Helical
Adenoviridae	dsDNA	Enveloped	Isocahedral
Arenaviridae	Negative-sense ssRNA	Enveloped	Complex
Flaviviridae	Positive-sense ssRNA	Enveloped	Isocahedral
Hepadnaviridae	ds-DNA-RT	Enveloped	Isocahedral
Orthomyxoviridae	Negative-sense ssRNA	Enveloped	Isocahedral
Papillomaviridae	dsDNA, circular	Naked	Isocahedral
Parvoviridae	ssDNA	Naked	Isocahedral
Poxviridae	dsDNA	Complex	Complex
Reoviridae	dsRNA	Naked	Isocahedral
Retroviridae	RT-positive-sense ssRNA	Enveloped	Isocahedral
Togaviridae	Positive-sense ssRNA	Enveloped	Isocahedral

DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

Viruses that spread through aerosols tend to be highly contagious, such as influenza viruses, which cause annual epidemics and occasional pandemics that within a few months can spread throughout the world as was shown in the 2009 swine flu pandemic. The new pandemic influenza virus was first identified in Mexico on March 18th, 2009; reached California by March 28th; was detected in Canada, New Zealand, the United Kingdom, Israel, and Spain by April 28th; in Germany by the 29th; in Austria, Switzerland, and the Netherlands by April 30th; in other European countries, as well as in Asia, by May 2nd; in South America by May 5th; and on June 11th was officially declared as a pandemic virus by the World Health Organization. The total death toll of this pandemic was rather modest with approximately 5700 reported deaths by August 10th, 2010, when the World Health Organization announced the official end of the pandemic. Another highly contagious virus is varicella virus, which causes chickenpox. This virus is also spread by droplets from person to person, but, unlike influenza virus, which is fairly stable and can thus infect individuals that touch an infected surface and then their nose, varicella virus is very heat labile and, as a rule, requires direct person-toperson contact.

Other viruses are transmitted by oral ingestion and are then spread by shedding into feces.

These viruses are generally stable, allowing them to resist the acidic environment of the stomach or the digestive enzymes of the intestinal tract. Many of them, such as influenza viruses that predominantly infect aquatic birds through the oral route or rotaviruses that cause severe diarrheal disease in children, can also survive for a prolonged time in water. Improperly treated drinking water can spread a number of other viruses, such as enteric adenovirus, calicivirus, astrovirus, poliovirus, or hepatitis A virus.

Sexually transmitted viral infections include herpes simplex virus (HSV) type 2, HIV-1, and several types of human papilloma viruses (HPV). Interestingly, all of these viruses establish sustained infections. HSV-2, after a replicative phase, persists latently in root ganglia from where it is periodically reactivated causing local sores that shed virus. HIV-1 first causes an acute flu-like infection and then persists mainly in CD4+ T cells while constantly dodging a vigorous antiviral immune response through mutations and immune evasion strategies. Although oncogenic types of HPV such as HPV-16 or -18 are commonly eliminated after genital infection, their persistence can over time cause transformation of the infected cells due to the activity of the two viral oncoproteins E6 and E7, which disrupt key cell cycle checkpoints and then lead to cervical cancer in women or penile or anal cancer in men. Although some of the sexually transmitted viruses (eg, HSV-2) are highly contagious, others (eg, HIV-1) transmit poorly and the average rate of HIV transmission has been estimated at 0.0082 per coital act in humans without comorbidities.[6]

Some viruses literally need to be injected into the body to cause an infection. These viruses are either transmitted by blood sucking insects or animal bites. Three flaviviruses, Dengue virus, West Nile virus, and Japanese encephalitis virus, are spread by mosquitoes, whereas Kyasanur forest disease virus, another flavivirus, is spread by ticks. Rabies virus, another vector-borne virus, is generally transmitted by the bite of an infected animal, most often a dog. The virus replicates in the central nervous system and is then transported to peripheral organs such as the salivary glands from where it is secreted into the saliva ready to spread to its

next victim. Although the vast majority of rabies infections are caused by bites, mucosal transmission[7] and transmission by transplantation of tissues from an infected individual have been reported.[8]

Virus Cell Entry and Replication

Most viruses enter cells upon binding to a receptor, some of which are broadly expressed while others are specific for a certain cell type. In some instances, viruses bind with high avidity to one receptor but are also capable of infecting cells that lack expression of the high-affinity receptor through low avidity binding to an alternative molecule. Other viruses require binding to a receptor and a coreceptor. Receptor usage determines tissue tropism of many viruses and in some cases it also influences their host range.

For example, the hemagglutinin (HA) of influenza A viruses that can spread in humans binds to sialyated glycan receptors with a terminal α2-6 linked N-acetylneuraminic acid. In contrast, α2-3 linked sugar residues are used as receptors for influenza A viruses that circulate in birds. Once the HA has bound to its receptor, it is cleaved. A trypsin-like enzyme present only in the lung cleaves HA into two subunits, which allows the virus envelope to fuse into the cell membrane. Some of the more pathogenic strains, such as the 1918 H1N1 virus or pathogenic 2006 H5N1 viruses, activate HA through a trypsin-independent mechanism.[9] These strains have a multibasic cleavage site that can be digested by furin and furin-like proteases, which are more ubiquitously present in human tissues than the trypsin-like enzymes, allowing these viruses to infect tissues other than lung.

The fiber knob of adenoviruses preferentially binds the coxsackie adenovirus receptor, which is expressed on epithelial cells. In addition, an Arg-Gly-Asp (RGD) motif present within the viral penton can bind α(v)-integrins with lower avidity. The fiber of adenoviruses of subfamily

B2, on the other hand, binds CD46, a ubiquitously expressed complement component that also facilitates entry of measles virus and human herpesvirus (HHV)6. The herpes virus mediator (HVEM) is a bimodal switch that can provide both immunostimulatory and immunoinhibitory signals to the immune system. Upon binding to LIGHT (lymphotoxin [LT]-like, exhibits inducible expression and competes with HSV-1 glycoprotein D [gD] for HVEM), HVEM submits stimulatory signals. Upon binding to the B- and T-lymphocyte attenuator (BTLA), it acts as an immunoinhibitor. HVEM also binds HSV-1 gD, thus facilitating entry of this virus. Binding of gD to HVEM takes place on a site that overlaps with the BTLA binding site; therefore, gD can be used to inhibit an immunoinhibitory pathway. HSV-1 may have evolved to block such a pathway, as activation of NF-κB promotes viral replication and assists in transcription of some of the early viral genes.[10]

The envelope protein of HIV-1 binds cluster of differentiation (CD)4 expressed mainly on T-helper cells. Upon binding, the protein undergoes structural changes that allow for its binding to a coreceptor, which for transmitting virions is CCR5, but following mutations, viruses circulating in an organism can also use CXCR4.[11] A mutant allele of CCR5 termed CCR5d32, which results in lack of CCR5 expression on the cell surface and which is found in 10% of Caucasians of European descent, provides resistance to infections with HIV-1.[12] Other viral receptors include the nicotinic acetylcholine receptor for rabies virus, heparan sulfate for dengue virus, adeno-associated viruses, and some of the herpes viruses, CD155 for poliovirus, CD81 for hepatitis C virus, CD21 for Epstein-Barr virus (EBV), C-type lectins, such as dendritic cell (DC)-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) and liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin for Ebola virus, integrin-β3 for Hantan virus or intercellular adhesion molecule (ICAM)-1 for rhinoviruses. Examples for viral receptors including their physiologic functions are listed in Table 39.3.

Upon binding to a receptor, viruses not only need to gain access into the cell, but most of them then have to traverse to the nucleus to initiate their replication. Viruses enter the cell either through endocytosis[31] or fusion.[32] Clathrin-mediated endocytosis is used by enveloped as well as nonenveloped viruses including adenoviruses, influenza viruses, poxviruses, or rabies virus. In cadherin-mediated endocytosis, the virus-receptor complexes cluster into a cadherin-coated pit on the cell membrane that becomes invaginated, eventually closes, and detaches from the cell membrane. The clathrin-coated vesicles then deliver their cargo to early endosomes from where it travels to late endosomes. Other viruses such as coxsackie B virus, respiratory syncytial virus (RSV), and others enter cells by caveolar endocytosis. Caveolae are invaginations in the plasma membrane that are rich in cholesterol, glycosphingolipids, and claveolin, which are used for uptake of macromolecules into endosomes. In addition, caveolar endocytosis allows for transcytosis of molecules from the basal to the apical side of a cell or vice versa. Human enterovirus has been described to enter cells through a lipid raft dependent pathway, rotavirus infects through a cholesterol- and dynamin-dependent but clathrin-and caveolae-independent pathway, while other viruses enter cells by micropinocytosis or phagocytosis.

Enveloped viruses such as paramyxoviruses, some herpes viruses, or HIV-1 invade cells by direct fusion of the virus envelope with the cell membrane. Fusion is promoted by hydrophobic sequences within a viral surface protein and causes release of the viral genome into the cytoplasm.

Viruses that enter cells through endocytosis end up in endosomes. Mechanisms of escape from endosomes differ for enveloped and nonenveloped viruses. The decrease in pH between early and late endosomes favors conformational changes of viral surface proteins by exposing their hydrophobic residues, which allow for fusion of the viral envelope with the endosomal membrane. This in turn permits escape of the viral core or the genome into the cytoplasm. Nonenveloped viruses disrupt the endosomal membrane either by a pathway called carpet mechanism or by forming pores. In carpet-like disruption of endosomal

membranes, viral peptides act like a detergent and thus interrupt the hydrophobic interactions between membrane lipids allowing for the development of micelles and for transient formation

of holes within the membrane.[33] Other viruses carry proteins, which form amphipathic α-helices that assemble into a pore within the lipid membrane of the endosome where the hydrophobic parts interact with the lipid bilayer while the hydrophilic parts of the coils form the inner wall of the pore.[34] Picornaviruses, parvoviruses, and reoviruses utilize this strategy.

TABLE 39.3 Viral Receptors

Receptor	Virus	Viral Antigen	Physiological Function	References
Herpes virus entry mediator	Herpes simplex virus-1	Glycoprotein D	Receptor for costimualtors/coinhibitors	13,14
Coxsackie adenovirus receptor	Adenovirus C	Fiber	Cell adhesion molecxule	15
	Coxsackie B virus			
CD46	Adenovirus B2	Fiber	Complement regulatory protein	16
	Measles virus	Hemagglutinin		17
CD55	Enteroviruses	Hemagglutinin	Complement regulatory protein	18
CD155	Poliovirus	Virus proteins 1-3	Cell adhesion molecule	19
CD4	Human immunodeficiency virus-1	Glycoprotein 160	T-cell receptor coreceptor	20
Nicotinic acetylcholine receptor	Rabies virus	Glycoprotein	Forms ion channels in neuronal membranes	21
CD81	Hepatitis C virus	Glycoprotein E2	Signal transduction	22
ICAM-1	rhinovirus	Viral proteins 1-4	Cell adhesion molecule	23
CD21	Epstein-Barr virus	Glycoprotein 350/220	Complement component	24

DC-SIGN	Ebola virus	Glycoprotein	C-type lectin, adhesion molecule	25
	Dengue virus	Glycoprotein E		26
	Hepatitis C virus	Glycoprotein E2		27
P-selectin glycoprotein ligand-1	Enterovirus 71		Selectin receptor; mediates leukocyte rolling	28
SLAM/CD150	Measles virus	Hemagglutinin	Signal transduction	29
Transferrin receptor 1	Lassa fever virus	Glycoprotein	Import of iron	30

CD, cluster of differentiation; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; ICAM, intercellular adhesion molecule; SLAM, signaling lymphocytic activation molecule.

Once within the cell, viruses may be broken down by autophagy,[35] a catabolic process involving the degradation of a cell's own components to convert unneeded pieces into nutrients. During this process, so-called autophagosomes form from membrane structures containing autophagiarelated gene products (Atg), such as the ubiquitin-like Atg8, the Atg4 protease, and the Atg12-Atg5-Atg16 complex. The outer membrane of the autophagosome fuses with a lysosome to allow for degradation of its contents. Formation of autophagosomes is initiated by PI3K and Beclin-1. Most viruses block this pathway by inhibiting PI3K activation, but rhinoviruses and poliovirus sponsor formation of early autophagosomes but block their fusion with lysosomes and then use the structures to egress the cells.[36]

Once a virus has reached the cytoplasm, it must deliver its genome to the nucleus. Many viruses such as herpesviruses and adenoviruses use microtubules to reach nuclear pores. Very small genomes can diffuse passively though pores into the nucleus, while larger genomes or particles require an energy-dependent process. Some viruses use viral proteins to facilitate nuclear entry. For example, cytomegalovirus (CMV) encodes two proteins, pUL69 and pUL84, that facilitate the transport of its genome to the nucleus. pUL69 binds to UAP56, which facilitates nuclear export of unspliced RNA; pUL84 binds to importin-alpha proteins,[37] which can dock to nuclear pores and then be transported through it.

Most viruses initiate their replication in the nucleus as they depend on nuclear enzymes for transcription. Poxviruses and some of the RNA viruses are independent of such enzymes and can replicate their genome in the cytoplasm. HIV-1 replicates in the nucleus after it reverse transcribes its RNA in the cytoplasm.

Replication of different types of virus can be exemplified using the following viruses: adenovirus, a dsDNA virus; adeno-associated virus, an ssDNA virus; reovirus, a dsRNA virus; poliovirus, a positive-sense ssRNA viruses; influenza A virus, a negative-sense ssRNA virus; HIV-1, a positive-sense ssRNA-RT virus; and hepatitis B virus, a dsDNA-RT virus.

Adenovirus transcription is typical for that of some of the larger DNA viruses as it proceeds in stages. Initially, the immediate early gene is transcribed. The resulting gene products alter the host cell to provide a more favorable environment for viral replication and initiate transcription of early viral genes, which have regulatory functions and serve to modify host cell functions

or subvert immune responses. Thereafter, the viral genome replicates concomitantly with transcription of the late viral genes that encode

structural proteins. Specifically, the replication cycle of adenovirus starts with expression of E1A, which encodes two polypeptides that bind to cellular proteins, including cellular transcription factors, which in turn changes the cell's gene expression profile and allows for transcription of the other early viral genes E1B, E2, E3, and E4. E1A promotes apoptosis, while E1B proteins are antiapoptotic. E1B polypeptides turn off host cell protein synthesis and help to stabilize, transport, and selectively translate viral RNA. E2 encodes DNA-binding proteins and a polymerase. E3 gene products are nonessential for virus replication but serve to evade immune responses. E4 encodes seven polypeptides, which collaborate with E1 gene products in promoting viral transcription and modulating host cell functions. E4 gene products are also essential for nuclear export of viral RNA. Adenoviruses also encode one or two virus-associated (VA)-RNA species, which form short hairpin loop structure of approximately 200 bases, are transcribed by polymerase III and stimulate translation of viral genes. VA-RNA can be processed into shorter RNAs and act as micro RNA,[38] inhibiting activation of protein kinase R, which inhibits further messenger RNA (mRNA) synthesis through phosphorylation of the translation initiation factor EIF2A. Transcription and translation of early gene products is followed by DNA replication, which is initiated by a terminal protein that is covalently bound to the 5′ ends of the long terminal repeats. Once DNA replication is initiated, the late gene products, which form the viral capsid, are produced from the L1-L4 domains. Viral assembly begins in the cytoplasm and is completed in the nucleus.

Adeno-associated viruses are ssDNA viruses that cause no known disease in humans. They are dependoviruses and require coinfection with another virus, most commonly an adenovirus, to complete their lifecycle. The approximately 4.7 bp genome is flanked by terminal repeats that contain a multipalindromic terminus that forms a loop and thereby promotes priming for DNA replication. The genome contains only two genes; one, the *rep* gene, encodes four regulatory proteins needed for DNA replication and conversion of the dsDNA intermediate into the final ssDNA. The viral capsid is composed of three virus proteins derived from the *cap* gene by transcript splicing. Initiation of transcription of the rep gene requires proteins from a helper virus such as gene products from E1, E2, E4, as well as VA-RNA from adenovirus. Final assembly of the adeno-associated virus takes place in the nucleolus.

Reovirus infections are asymptomatic in humans but cause disease in newborn mice. This virus, which contains 10 to 12 segments of dsRNA, replicates in the cytoplasm of infected cells without completely uncoating. RNA is transcribed from the negative strand of the genomic RNA and leaves the capsid to be translated. Secondary transcription occurs later followed by assembly within the cytoplasm.

The viral genome of positive-sense ssRNA viruses, such as poliovirus, a picornavirus, can directly serve as mRNA. Poliovirus RNA lacks the methylated cap structure that is typical for mammalian mRNA but rather has an internal ribosomal entry site. To avoid competition with translation of mammalian mRNA, poliovirus interferes with recognition of the host's methylated cap, thus inhibiting host cell protein synthesis.[39] The poliovirus RNA is translated into a single polypeptide that is cleaved into a replicase, proteases, and structural proteins. The polymerase transcribes the positive-stranded RNA into a minus-sense RNA to serve as template for new positive-stranded RNA. The latter can either be translated, serve as template for minus stranded RNA, or be packaged into new virions. Replication as well as assembly occurs in the cytoplasm.

Influenza viruses are segmented negative-sense ssRNA viruses, which replicate in the nucleus. The RNA-dependent RNA polymerase transcribes positive-stranded RNA segments that are either transported to the cytoplasm for translation or remain in the nucleus to serve as templates for negative-stranded RNA synthesis. Newly produced internal proteins are

transported into the nucleus where they, together with RNA segments, form new virus particles. The two viral surface proteins, the HA and the neuraminidase are secreted through the Golgi apparatus to the cell surface where they are then picked up by the envelope once the virus leaves the cell.

HIV-1 is initially reversed transcribed in the cytoplasm by the viral reverse transcriptase into an RNA/negativestranded DNA hybrid. This process is error prone and contributes to the high mutation rate of HIV-1. The RNA is degraded, and a positive-stranded DNA is synthesized allowing for the formation of a dsDNA, which, together with some enzymes, enters the nucleus; there the viral genomes integrates with the help of the viral integrase and serves as a template for synthesis of viral transcripts. Two newly produced viral proteins, Tat and Rev, are essential for efficient protein production: Tat by enhancing transcription and Rev by supporting export of unspliced mRNA from the nucleus, which allows for production of the structural proteins Gag and Env. The full-length viral RNA binds initially to Gag and is then packaged into new virus particles. Env is transported to the cell surface after it is cleaved into two subunits and, with the help of cellular chaperone proteins, folded into a trimer. Assembly of mature virions takes place at the plasma membrane.

Hepatitis B virus (HBV) carries a circular partially dsDNA genome that encodes four structural and two nonstructural proteins through overlapping open reading frames (ORFs). Within the nucleus of an infected cell, the genome is converted into a full dsDNA, which serves as a template for the viral transcripts. The largest mRNA, which is longer than the viral genome, is called the RNA pregenome and is packaged into core particles within the cytoplasm. Within these particles, the pregenomic RNA is reverse transcribed into viral DNA genomes. Upon synthesis, the viral surface protein is transported to the cell membrane and complete assembly of the virion takes place during budding of the virus.

Once replication is completed and full virions have been assembled, viruses need to leave the cells. This again can occur through several pathways. Some viruses, such as HIV-1 or influenza virus, assemble their newly synthesized viral surface proteins on the cell surface and then bud through this part of the cell membrane, picking up not only their own surface proteins but also membrane proteins belonging to

the host cell. Budding eventually destroys the cell membrane and leads to cell death. Other viruses, especially those that are nonenveloped, instruct the infected cell to undergo apoptosis and virus released from dying cells is encapsidated into apoptotic bodies, which are taken up by neighboring cells, thus facilitating infection of new cells. Some viruses are released by exocytosis, a process that resembles reversed pinocytosis in which virus particles are encapsidated into small vesicles that enter the secretory pathways. This form of exit does not kill the cells and is used by so-called nonlytic viruses.

Viral Persistence

Some viruses such as poxviruses or influenza viruses are lytic, which means they inevitably kill the cells they infect. Such viruses cause acute infections in immunocompetent hosts. Other viruses can replicate within a cell without causing its demise, or they can switch between a lytic and a nonlytic infection. These viruses can persist and cause chronic or latent infections, which pose unique challenges to the immune system. In chronic infections, some viruses, such as HIV-1 or hepatitis C virus (HCV), replicate constantly, dodging destruction by the immune system. Other viruses replicate and then persist by turning off synthesis of most of their viral proteins, causing so-called latent infections. Herpesviruses can switch to a latent phase from which they are periodically reactivated to undergo renewed lytic cycles of replication. Adenoviruses persist at low levels in activated T cells, presumably as episomes that remain transcriptionally active.[40] Yet other viruses, such as HIV-1 or HPVs, integrate into the host cell genome and thus become an integral part of the cell. In general, DNA viruses with a nuclear replication cycle are able to persist, which may be favored due to the complete lack of DNA degrading enzymes within the nucleus. Rabies virus, a negative-

sense ssRNA virus, does not cause chronic infections but kills within days after causing symptoms. Nevertheless, in some individuals years pass between viral transmission and onset of symptoms,[41] and it is unknown where and how the virus persists during this long incubation time. Measles virus, another negative-sense ssRNA virus of the paramyxovirus family can cause subacute sclerosing panencephalitis in about 1 out of 100,000 infected individuals within 5 to 15 years after primary infection. Subacute sclerosing panencephalitis is most common in children who are infected early in life, and it has been speculated that the relative immaturity of their immune system allows for the development of a chronic central nervous system infection.[42]

INNATE ANTIVIRAL IMMUNE RESPONSES

Both innate and adaptive immune responses are essential to wards off pathogens, and individuals with inherited or acquired immunodeficiencies rapidly succumb to virus infections. Even individuals with weakened immunity, such as the very young whose immune system is still immature, the elderly undergoing immunosenescence, or pregnant women whose immune system is transiently suppressed, show markedly increased susceptibility to many viruses.

Immune responses to viruses can roughly be divided into four stages. At first, the immune system has to recognize the threat, then an immediate early response is mounted by cells of the innate immune system, which is followed a few days later by a response from the adaptive immune system. Once the virus is eliminated, the immune response contracts and adaptive immunity enters a stage of immunologic memory. Memory T and B cells, upon reexposure to the same pathogen, mount a response that is more potent and comes up faster than a primary response. In cases where virus persists, the acute phase of the immune response is followed by a chronic immune response.

Early Recognition of a Virus

Viruses like other microbes carry so-called pathogen-associated molecular patterns (PAMPs) that are recognized by pattern recognition receptors (PRRs) expressed on many cell types including cells of the innate and adaptive immune system.[43] This recognition system is not as specific as the antigen-recognition receptors of T and B cells, but rather it responds to motifs that are commonly found on pathogens but not within mammals or it identifies molecules present in the wrong compartment within the cell. PRRs can be subdivided into four main types (ie, toll-like receptors [TLRs], retinoic acid inducible gene [RIG]-I-like receptors, nucleotide oligomerization domain [NOD]-like receptors [NLRs], and the interferon-inducible p200 family member absent in melanoma 2).

Ten TLRs (TLR1-10) have been defined in humans and 9 in mice.[44] Some TLRs are widely expressed on many different cell types such as TLR1 or 4, while others are expressed mainly on cells of the immune system such as TLR5, antigen-presenting cells (TLR8), or subsets of antigen-presenting cells, such as TLR7 and TLR9, which are primarily expressed in plasmacytoid DCs. Expression patterns of TLRs in humans do not always mirror those in mice. Expression levels of most of the virus-sensing TLRs are upregulated by inflammatory cytokines mainly interferon (IFN)-γ but for TLR3 that is modulated upon cell differentiation. Viruses are sensed by five TLRs. TLR4, which is best known for its response to lipopolysaccharide, also reacts to the fusion protein of RSV[45] and to a surface glycoprotein of Ebola virus.[46] TLR3 senses double-stranded RNA, TLR9 senses viral and bacterial CpG sequences, and TLR7 (in humans only) and TLR8 (in both humans and mice) reacts to ssRNA. It was initially debated if indeed TLRs directly recognized their PAMP or became instead activated by an intermediate host cell protein. More recent evidence has shown direct binding between TRLs and their ligands. TLR4, which can recognize viral surface proteins, is expressed on the cell membrane where an encounter with such an antigen is most likely. TLR3, 7/8, and 9 are within endosomes where many viruses uncoat, which in turn leads to

exposure of their genomes.

Viruses such as herpesviruses,[47] West Nile virus,[48] or influenza virus[49] signal through TLR3; RSV[50] and Ebola virus[46] can signal through TLR4; influenza virus,[51] HIV-1,[52]

and herpesviruses[53] can signal through TLR7 or 8; DNA viruses such as herpesviruses signal through TLR9.[54]

All TLRs carry an intracellular toll-IL-1 receptor-resistance (TIR) domain, which interacts with TIR domains on intracellular adaptor molecules. TLRs, with the exception of TLR3, signal through myeloid differentiation primary response gene (MyD)88, which in turn interacts with interleukin-1 receptor associated kinases (IRAKs)1, 2, and 4, leading to activation of tumor necrosis factor receptor -associated factor (TRAF)6 and upon additional steps to activation of NF-κB, mitogen-activated protein (MAP) kinases, and Jun-terminal kinases (JNKs). TLR3 signals through TIR-domain-containing adapter-inducing interferon-β (TRIF), which binds to TANK-binding kinase 1 (TBK1), thus activating interferon regulatory factor (IRF)3. In addition, TRIF interacts with receptor-interacting protein 1, which can activate NF-κB. TLR4, in addition to signaling through MyD88, can also bind the TRIF-related adaptor molecule, which recruits TRIF, allowing for signaling to IRF3. Activation of IRF3 results in production of type I IFN, whereas activation of NF-κB induces production of a number of proinflammatory cytokines, such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, and IL-12 (Fig. 39.1).

Deficiencies in TLRs can change an individual's susceptibility to viral infections. For example, TLR3 deficiency in humans is associated with increased susceptibility to HSV-1 infections,[47] whereas mice that lack TLR3 are more resistant to West Nile virus.[55] West Nile virus triggers TLR3, and the resulting cytokine response opens the blood-brain barrier, which allows the virus to establish an infection within the central nervous system; in absence of TLR3, the virus remains excluded from the brain.

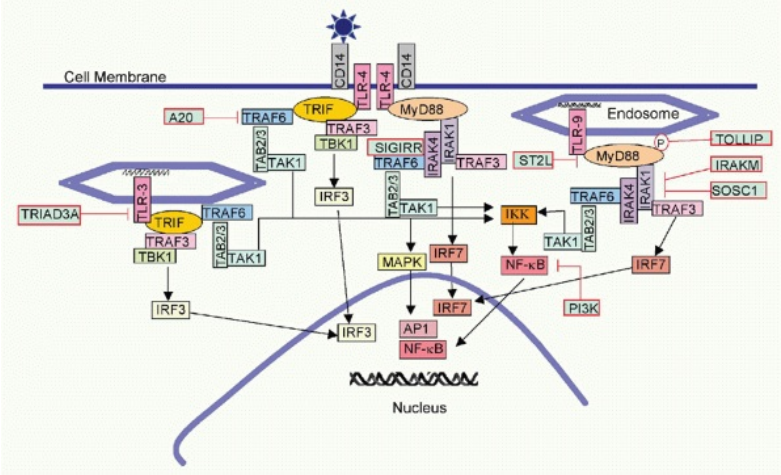

FIG. 39.1. Toll-like Receptor Signaling Pathways.

TLR signaling and the resulting production of cytokines can be extremely toxic. TLR signaling is therefore tightly regulated.[56] Some negative regulators target specific TLRs while others affect common downstream adaptor molecules. Regulators that affect TLRs, which react to viruses, include IRAKM, an IRAK homolog that inhibits IRAK1 and blocks TLR4 and 9, suppressor of cytokine signaling 1, which also suppresses IRAK. Others include phosphoinositide 3-kinase (PI3K), a key regulator of T-cell differentiation, which inhibits JNK and NF-κB functions, toll-interacting protein, which phosphorylates IRAK1, A20 which deubiquitylates TRAF6 and thus affects TLR3, 4, and 9, ST2L which sequesters MyD88,

single immunoglobulin (Ig) IL-1R-related molecule, which binds to TRAF6 and IRAK and TRIAD3A, and E3 ubiquitin-protein ligase, which initiates degradation of TLRs (see Fig. 39.1).

Some viruses neither carry PAMPs on their surface for recognition by membrane-bound TLRs nor enter cells through endosomes, and their genomes thus fail to become accessible for recognition by TLRs. Such viruses can be recognized by RIG-I-like receptors, which are located in the cytoplasm.[57,58] RIG-I-like receptors are RNA helicases, which respond to ss or dsRNA. Three RIG-I-like receptors

have been identified to data (ie, RIG-I, melanoma differentiation-associated gene 5 [Mda5], and LGP2). RIG-I and Mda5 carry N-terminal caspase activation and recruitment domain (CARD)-like regions, which are involved in downstream signaling and C-terminal RNA helicase domains, which bind RNA and can distinguish between viral and cellular RNA species. LPG2 lacks the CARD domain and is assumed to negatively regulate RNA virus-induced inflammatory responses by blocking binding of RNA to RIG-I. RIG-I recognizes a number of ssRNA viruses such as para- and orthomyxoviruses,[59] rotavirus,[60] filoviruses,[61] and flaviviruses,[62] while Mda5 recognizes picornaviruses.[63] RIG-I and Mda5 signaling involves binding of their C-terminal CARD domains to the CARD domain on IPS-1, which then through kinases receptor interacting protein 1 or Fasassociated protein with death domain activates NF-κB; they also activate IRF3 and IRF7 through TRAF3/TBK1, which results in production of IFNs and other proinflammatory cytokines (Fig. 39.2).

NLRs are a very large family of PRRs that respond to viral RNA in the cytoplasm.[64,65] They contain N-terminal domains for protein-protein interactions such as CARD, pyrin or inhibitor of apoptosis domains, NOD domains for nucleotide binding, and C-terminal leucin-rich repeat domains. NLRs are subdivided according to their N-termini into CARD (CIITA, NOD1, 2, NLRC3-5), pyrin domain (PYD, NLRO1-14), or pyrin or inhibitor of apoptosis domain (NAIP) members. Most NLRs activate cytokine responses, although some are inhibitory and dampen innate immune responses. To give some examples of the specificity of NLRs, NOD-2 senses ssRNA and interacts with paramyxoviruses and myxoviruses.[66] NLRC5 interacts with Poly(I:C) and responds to some paramyxoviruses and herpesviruses.[67,68]NLRX1, an inhibitory NLR, signals upon recognition of Sendai or Sindbis virus and blocks activating signals through RIG-1 like helicases.[69] Signaling pathways have not yet been fully characterized for NLRs. NOD-1 and -2, the best known NLRs, bind receptor-interacting serine-threonine kinase 2 resulting in NF-κB and MAP kinase signaling. They also induce autophagy and activate the mitochondrial antiviral signaling protein for induction of type I IFN. Several NLR members containing CARD or PYD domains can assemble into inflammasomes.

Inflammasomes are multiprotein complexes that recruit and activate inflammatory caspases.[70] Inflammasomes include PRRs and, as such, are divided into NLRP3 inflammasomes, RIG-I inflammasomes, and absent in melanoma 2 inflammasomes. In these complexes, the PRR activates caspase I through an adaptor, which cleaves the immature forms of IL-1β and IL-18 resulting in biologically active cytokines. NLRP3 inflammasomes were shown to react with influenza virus, rhabdoviruses, and picornaviruses.[71,72] Viral RNA can directly activate the caspase activities. The M2 protein of influenza virus, which has ion channel activity, localizes to the trans-Golgi network and reduces the H+ content; the acidity of the Golgi then results in NLRP3 activation.[73] Absent in melanoma 2 inflammasomes recognize DNA within the cytoplasm and, as such, play a role in responses to DNA viruses (eg, poxviruses and herpesviruses).[74]

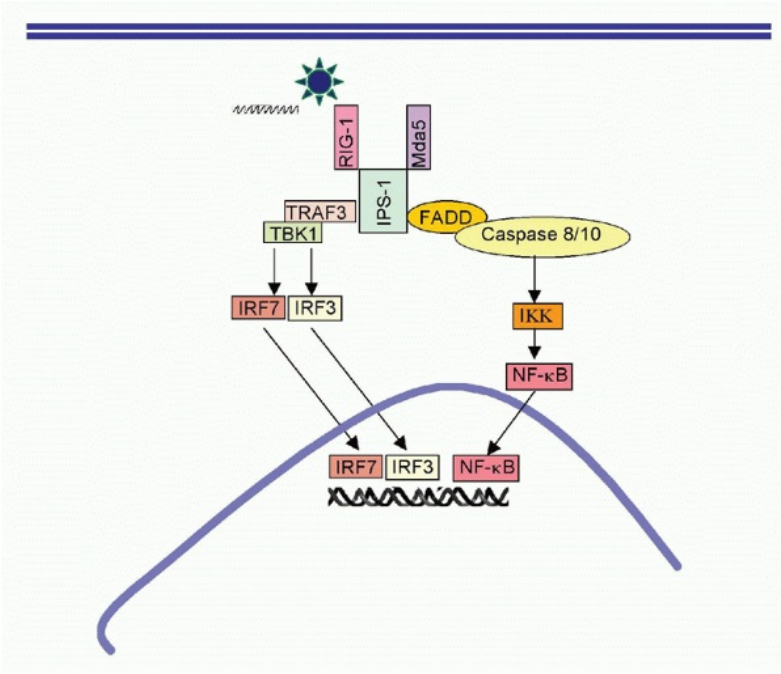

FIG. 39.2. Signaling through Retinoic Acid-Inducible Gene I Receptors.

The Early Inflammatory Response: Cytokines and Chemokines

Interaction of a PRR with a PAMP initiates a cellular response. Signaling through most of the PRRs results in activation of NF-κB. In resting cells, NF-κB is retained in the cytoplasm through binding to IκB. Upon activation of the IκB kinase (IKK), IκB becomes phosphorylated, then ubiquinated and finally degraded. This in turn releases NF-κB and allows for its entry into the nucleus where it initiates gene expression. NF-κB thus does not require de novo synthesis, which accelerates its activity. A very large number of genes have NF-κB binding sites including genes involved in antigen processing and presentation, lymphocyte effector functions and motility, and cell metabolism. Cytokine genes induced by NF-κB include those for type I IFN, IL-1A and B, IL-2, IL-6, IL-9, IL-11, IL-15, TNF-α, colony stimulating factor (CSF)1 to 3, lymphotoxin B, and the chemokine genes IL-8, CCL2, 5, 11, 15, and CXCL5. In addition, several cytokine-inducing transcription factors, such as IRF1, 2, and 4, are produced in response to NF-κB. IRF3 activated by TLR3, TLR4, or cytosolic PRR signaling is a transcription factor that promotes production of type I IFN and the chemokine RANTES. The MAP kinase activated AP-1 transcription factors also have binding sites specific for regulatory sequences of multiple genes. Most pertinent for early immune responses is probably induction of signal transducers and activators of transcription (Stat)1 and 3, which upon formation of homo- or heterodimers bind to the IFN-gamma activated sequences. Thousands of such motifs can be found in the human genome, and their products affect most cell functions.

Most cells can produce cytokines and chemokines, although, during the initial phase of an immune response, many are synthesized by cells of the innate immune system. The early cytokines have a multitude of functions, which are in part antiviral and in part designed to promote further activation of immune responses. Type I IFNs, which can be produced at capacious amounts by plasmacytoid DCs, bind to the IFN-α receptor. This causes activation of tyrosine kinase (Tyk)2 and Janus kinase (Jak)1, which in turn causes tyrosine phosphorylation and then nuclear translocation of Stat1 and Stat2 proteins. In addition, type I IFN has strong antiviral activity[75] and has been licensed for treatment of chronic infections with HBV[76] and HCV[77] and for treatment of HPV-associated genital warts (*Condyloma*

acuminata).[78]

IFN-1s downregulate viral promoters. They dampen expression of viral receptors and thus reduce viral entry. They induce expression of the dsRNA-activated protein kinase PKR, which phosphorylates translation initiation factor 2α (eIF2α) causing inhibition of translation of both viral and cellular transcripts. IFN-1s also trigger expression of 2′5′-oligoadenylsynthases that upon binding dsRNA generate AMP-oligomers, which activate RNAseL to cleave both cellular and viral RNAs. IFN-1s induce dsRNA kinase, which inhibits production of viral progeny. IFN-1s lead to synthesis of MxA.[79] This protein binds to the nuclear membrane and inhibits trafficking of viral nuclear capsids. In addition, MxA can bind and inhibit the RNA polymerase of influenza virus. APO-Bec3G and F are IFN-induced deoxycytidine deaminases that interfere with the replication of retroviruses.[80]

In addition to their antiviral activity, IFNs and other early cytokines play a dominant role in initiating both innate and adaptive immunity by causing activation of macrophages, natural killer cells, and DCs. The early cytokines promote proliferation and differentiation of lymphocytes, granulocytes, and antigen-presenting cells, whereas chemokines recruit such cells. IL-6 should be noted, as this cytokine, due to its immunostimulatory effects on B cells, has been implicated in the pathobiology of EBV-associated lymphoproliferative disorders and HHV-8-associated lymphomas in patients with acquired immunodeficiency syndrome.[81,82]

The apparent redundancy of some of the molecules that contribute to the initial inflammatory response is remarkable. Viruses commonly trigger multiple PRRs, some of which are expressed on or in the same cells, while other are carried by different subsets of cells. For example, influenza viruses thus far are known to signal through TLR3, 7/8, NOD-2, RIG-1, and NLPR3 inflammasomes. Most of the PRRs in the end initiate transcription through NF-κB or through members of the IRF family. Nevertheless, the immune response that is very much guided by the initial inflammatory reaction is unique for each virus, suggesting a finely orchestrated series of events during which the type, strength, and location of PRR signaling elicits for each virus a special mixture of cytokines and chemokines that results in signature immune responses. This is exemplified by recent studies with a vaccine, in which antigen-containing nanoparticles were mixed with ligands for TLR4 and 7.[83] This vaccine did not induce a strong effector cell response but drove differentiation of T cells toward memory and B cells toward long-lived plasma or memory cells. Although the slow release of antigens from the nanoparticles may have contributed to this, both TLR ligands were needed to maximize the vaccine's immunogenicty.

Cells of the Innate Immune System

Cells of the innate immune system, which include DCs, neutrophils, eosinophils, basophils, mast cells, γ/δ T cells, macrophages, and natural killer cells, provide a first layer of defense against virus infections and promote activation of adaptive immunity. Unlike T and B cells, which carry antigen-specific receptors, cells of the innate immune system are, as described previously, activated by PRRs. They act rapidly without undergoing the massive proliferation of T and B cells and then, in general, with the potential exception of natural killer cells,[84] fail to establish long-lasting memory.

Granulocytes: Neutrophils, Basophils, Eosinophils, and Mast Cells

Granulocytes play major roles in controlling bacterial and parasitic infections but also influence viral infections. Neutrophils, which are abundant and contribute to more than 50% of circulating leukocytes, release hydrogen peroxide, free oxygen radicals, and hypochlorite, and have been described to limit replication of HSV-1.[85] They may also

contribute to the immunopathology, such as of pulmonary RSV infections.[86]

Basophils release histamine, which increases blood vessel permeability and thus allows for

lymphocyte trafficking into inflamed tissues. Basophils are recruited and activated by a number of viruses, such as influenza viruses or RSV.[87]

Eosinophils, which accumulate in lungs during RSV infection,[86] release enzymes, such as ribonuclease, deoxyribonucleases, lipase, as well as plasminogen and peroxidase.

Mast cells release heparin, histamine, and chemokines, and have been reported to respond to Dengue virus infections.[88]

Gamma/delta T cells

T cells that carry the γ/δ T-cell receptor (TCR) exhibit characteristics of both innate and adaptive immune cells. They are mainly located in the gut and skin but can also be found at low frequencies in blood and lymphatic tissues. Although they are educated in the thymus, their receptor recognizes pathogen patterns or cellular stress molecules independent of major histocompatibility complex (MHC) molecules, and they can expand without the intricate antigen presentation pathways that dictate differentiation of α/β T cells. γ/δ T cells, which evolutionary predate α/β T cells, can secrete cytokines such as IFN-γ, IL-4, or IL-17,[89] chemokines such as macrophage inflammatory protein (MIP)-1α; MIP-1β; regulated upon activation, normal T cell expressed and secreted; lymphotoxin (LT)[90]; and lytic enzymes (ie, perforin and serine esterases for target cell lysis). These T cells play diverse roles in virus infections. To name a few, they promote Th1 responses following infection with coxsackievirus B3[91]; they can kill cells infected with HSV-1[92]; they have been described to lyse cells infected with influenza virus[93]; and they provide resistance to humans against HIV-1 infections[94] and to mice against infections with West Nile virus[95] or vaccinia virus.[96]

Macrophages

Monocytes upon activation by proinflammatory cytokines, specifically IL-6 and macrophage-CSF (M-CSF), differentiate into macrophages. These cells, as their name “large eaters” implies, are phagocytic. Phagocytosis is a specialized form of endocytosis during which the engulfed particles are transported to lysosomes where they are degraded by a toxic and acidic soup composed of oxygen radicals, nitric oxide, proteases, and defensins. Macrophages, in addition, release cytokines, such as IL-1, 6, 10, and 12, type I IFN, and TNF-α, as well as chemokines, such as MIP-2, IL-8, and cytokine-induced neutrophil chemoattractant-1. Some viruses replicate in macrophages (eg, HIV-1, influenza viruses, rhinoviruses, and Ebola viruses).

Dendritic Cells

A crucial role of DCs is their ability to present antigen to naïve T cells. A few years ago, DCs were divided into myeloid DCs, plasmacytoid DCs, and lymphoid DCs, although it is now accepted that lymphoid DCs do not belong to a separate lineage.

Viral antigens are mainly presented by myeloid DCs. Myeloid DCs, which differentiate from Lin-CD115+Flt3+ $CD117^{lo}$ precursors, can further be divided into subsets according to phenotypic markers, functions, and anatomic site of origin.[97]

Three subsets of myeloid DCs have been identified in human skin. Langerhans cells, which are $CD1^{hi}$ CD14- CD207+, are located in the epidermis. They look and act like DCs, and they are originally derived from bone marrow precursors but unlike other DC subsets, numbers of Langerhans cells are maintained by local proliferation. They only express discreet amounts of TLRs, induce CD8+ T cell responses in vitro and trigger proliferation of naïve CD4+ T cells. Interestingly, CD4+ T cells induced by Langerhans cells do not produce the typical Th1, Th2, or Th17 cytokines but rather produce IL-22,[98] which causes skin inflammation as well as wound healing through induction of keratinocyte proliferation.

The dermal layer of skin contains CD14+ CD11b^{hi} DCs, which differentiate from CD34+ precursors and express TLR 1, 2, 4, 5, 6, 8, and 10. They produce a multitude of proinflammatory cytokines including IL-6 and IL-12, which allows them to induce differentiation of naïve B cells into plasma cells. They also prime CD4+ T cells that can promote B-cell differentiation. The other DC subset in the dermis is CD103+CD11b^{low}, which can cross-present antigen and drive proliferation and differentiation of CD4+ T cells into Th1 cells.

Mouse spleen contains two subsets: CD8+CD205+ DCs and CD8+33D1+CD11b+ DCs; whereas the former can cross-present antigen in association with MHC class I and thus drive activation of CD8+ T cells, the latter is more efficient at processing antigen for MHC class II presentation for CD4+ T cell activation.

Lymph nodes also contain these two subsets and in addition migratory DCs such as Langerhans cells, which transport antigen from other locations to lymph nodes. Lymphatic tissues in addition contain follicular DCs.[99] These cells are not derived from hematopoietic progenitors but are of mesenchymal origin. They are an integral part of B-cell follicles, where they present antigen to naïve B cells and maintain long-lived plasma cell responses by initially capturing and then slowly release antigen-antibody complexes.

The intestine has three populations of DCs. CD103+ CD1b^{low} DCs can be found in Peyer patches and CD103+ CD11b^{hi} and CD103-CD11b^{hi} DCs in the lamina propria. The latter subset is known to transport antigen from the intestine to draining lymph nodes. Other tissues such as lung, kidney, and spleen contain similar subsets.

It was initially thought the DCs are terminally differentiated, nondividing cells. Recent studies have shown that DCs can divide and that this division is driven by fms-related tyrosine kinase (Flt)-3, which is also a key factor for DCs differentiation from bone marrow precursors.[97]

Plasmacytoid DCs were originally though to originate from lymphoid progenitors as they share many of the characteristics of lymphocytes: they lack the typical dendrites of other DC subsets, but look like lymphocytes; they express B-cell markers, such as B220 and T cell markers (eg, CD4), as well as transcription factors involved in lymphocyte development, such as terminal deoxynucleotidyltransferase, recombination activating genes 1 and 2, Ig supergene family members, and the pre-T-cell antigen receptor alpha chain. But plasmacytoid DCs also have characteristics of myeloid

DCs and can even differentiate into myeloid DCs.[100] It is assumed that plasmacytoid DCs can arise from both lymphoid and myeloid bone marrow precursors, although the latter probably provide the dominant source. Plasmacytoid DCs, which can be distinguished from myeloid DCs by expression of plasmacytoid DC antigen-1 (CD317) in mouse or blood DC antigen-2 (CD303) or -4 (CD304) in human, circulate in blood and are present in lymphatic tissues. Unlike myeloid DCs, they express CD62L and CCR7, which allows them to cross high-endothelial venules to enter T-cell-rich areas in lymphatic tissues. While myeloid DCs take up antigen to present it to cells of the adaptive immune system, plasmacytoid DCs do not phagocytose antigen. Their primary role might be regulatory by producing cytokines most notably type I IFNs, which assist maturation of myeloid DCs.

DCs present in tissues are immature. They take up antigen, but they do not produce cytokines, nor are they able to present antigen efficiently to naïve lymphocytes. Their maturation starts either through interactions of a PRR with a PAMP, through cytokines, ligation of CD40, receptor activator of NF-κB (RANK) or even, as has been observed in vitro, vigorous shaking.[101] DC maturation is associated with marked changes in their gene expression profiles[102] and their biological functions. Maturing DCs stop phagocytosis and endocytosis. They start secreting chemokines and thus initiate an inflammatory response at the site of infection. They upregulate expression of CCR7 and migrate from tissue to the T-

cell-rich zones of draining lymph nodes. They increase synthesis of molecules that are involved in antigen processing and presentation, and translocate MHC class II molecules that are preformed and stored within intracellular vesicles to the cell surface. They also, in a presumably orchestrated but yet ill-defined pattern, start to increase expression of cell surface molecules that regulate immune responses (Fig. 39.3). Such molecules include CD80, CD86, CD40, inducible T-cell costimulator (ICOS) ligand, ligands 1 and 2 for programmed death 1 (PDL1, PDL2), HVEM, and others that are less well defined thus far. Interactions of T and B cells not just with their cognate antigen, but also with these ligands, is crucial for the induction of an adaptive immune response that is of sufficient magnitude to combat the threat without being overwhelming and thus causing undue damage. These interactions are also important to determine the ultimate differentiation fate of responding T and B cells.

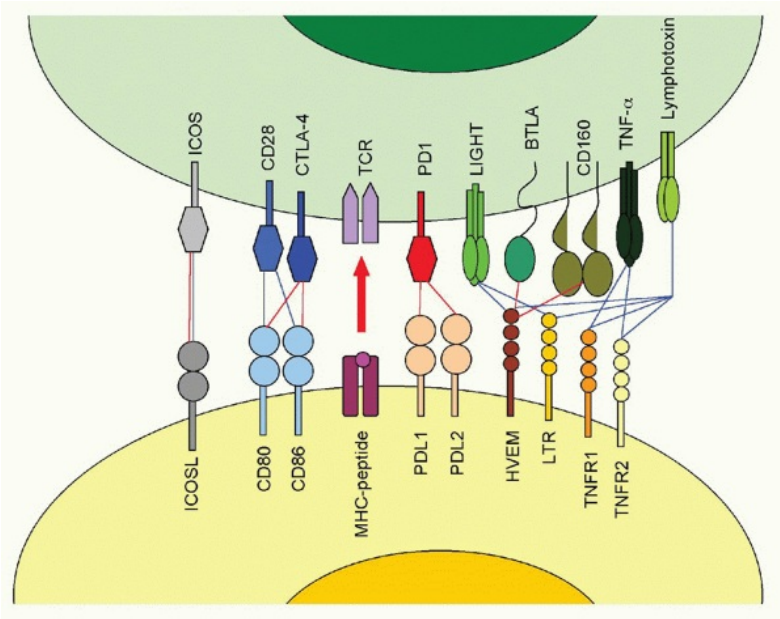

FIG. 39.3. Costimulatory (*blue lines*) and coinhibitory (*red lines*) T cell (*green*)-dendritic cell (*yellow*) interactions.

Signaling through CD40, which is expressed at low levels on immature DCs, facilitates full maturation of DCs; its expression increases upon activation of TLR pathways. CD40 is upregulated on myeloid DCs upon interactions with TLR4 agonists[103] and on both plasmacytoid and myeloid DCs upon TLR7 and 9 signaling.[104] The CD40 ligand (CD40L) is induced upon CD40 stimulation.[105] Ligation of CD40 causes signaling through TRAF6, which results in activation of MAP and Jun kinases and in production of CD86 and cytokines, such as IL-6 and IL-12. CD40 ligation also results in activation of NF-κB and in many aspects seems to complement signaling through TLRs.[106]

Ligation of CD40L upregulates expression of RANK (also called TNF-related activation-induced cytokine [TRANCE]

receptor), which is a member of the TNF-receptor family. RANK interacts with RANK ligand (also called TRANCE), which is induced on T cells upon their TCR ligation. Signaling through TRANCE induces B-cell lymphoma-extra large expression, which in turn promotes DC survival.[107]

Signaling between DCs and cells of the adaptive immune system involves a number of B7

family members of costimulatory molecules that are expressed by mature DCs.[108] CD80 and CD86 on DCs interact with the activating CD28 and inhibitory cytotoxic T-lymphocyte antigen (CTLA)-4 molecules on T cells. Ligation of CD28 activates Akt/PI3K and thus mammalian target of rapamycin (mTOR) while CTLA-4 inhibits this pathway. Activation of these pathways is needed to adjust the increased bioenergetic needs of differentiating lymphocytes by augmenting glucose uptake and glycolysis through the Krebs cycle and inhibiting forkhead box O, thus allowing for cell cycle entry. Another means by which CD80 and CD86 may influence immune responses is through reverse signaling into B7-expressing DCs.[109]

The ICOS ligand pathway has some overlapping functions with CD28. Ligation of ICOS on T and B cells upregulates PI3K and thereby influences cellular metabolism.[110] ICOS through PI3k/Akt and Rho family members also affects lymphocyte polarization and migration.[111] ICOS also plays a role in controlling T-helper-cell development and function.[112] ICOS also plays a critical role in the development of both Th17 cells and follicular T-helper cells by inducing the transcription factor c-Maf and the cytokine IL-21.[113,114] Patients with a defect in ICOS expression exhibit a profound defect in B-cell maturation and Ig isotype switching.[115]

PDL1 and PDL2 are coinhibitors expressed on DCs and some other cells that interact with PD1 expressed on T cells, B cells, macrophages, and some types of DCs. PD1 ligation induces cell cycle arrest.[116] PDL1 regulates development, maintenance, and function of regulatory T cells (T_{reg}s) through downregulation of Akt and mTOR, and upregulation of phosphatase and tensin homolog.[117] PD1 expressed on CD4+ T cells regulates selection and survival of PDL2+ B cells in germinal centers, and affects the magnitude and the quality of the plasma cell response.[118]

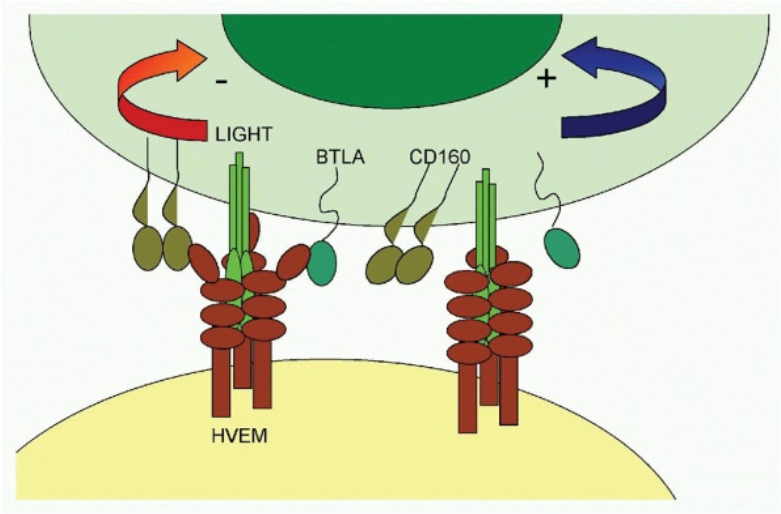

FIG. 39.4. Stimulatory (+) and Inhibitory (−) Signals through Herpes Virus Mediator Interactions.

Another inhibitory pathway involves the BTLA[13] expressed by B and T cells. BTLA provides inhibitory signals upon binding to HVEM expressed on DCs. HVEM also interacts with glycoprotein gD of herpes virus and with the immunoinhibitory receptor CD160.[13,119] Distinct regions of HVEM interact with two immunoactivators of the TNF family members (ie, LIGHT and lymphotoxin-A) (Fig. 39.4). BTLA, unlike other immunoinhibitory molecules, is expressed on naïve T cells, whereas CD160 is induced upon their activation. Blockade of the HVEM-BTLA/CD160 pathways during T-cell stimulation results in enhanced primary T-cell

responses both in young and aged mice.[120,121,122] Signaling pathways initiated by BTLA ligation remain poorly understood. It is known thus far that the cytoplasmic tail of BTLA binds growth factor receptor-bound protein (Grb)-2, which in turn recruits p85 of PI3K.[123] HVEM is also expressed on T_{reg}s, which through ligation of BTLA suppress effector T cells.[124] Another inhibitory molecule on DCs is CD48, which interacts with 2B4 on T and natural killer cells.[125] A number of additional inhibitory molecules have been identified on T and B cells and include LAG3, PIR-B, GP49, KLRG1, NKG2A, and NKG2D,[125,126,127,128] but their matching ligands remain elusive.

Through the expression of costimulatory and coinhibitory receptors, DCs not only activate but also terminate adaptive immune responses. DCs thus play a dominant role in maintaining tolerance through a number of pathways. Thymic DCs negatively select for T cells with receptors that have high reactivity to self-proteins, and they promote thymic T_{reg} selection. Presentation of antigen by immature DCs induces T-cell anergy and promotes the activity of T_{reg}. Immature as well as mature CD123+ DCs can express indoleamine dioxygenase (IDO),[129] which catalyzes the degradation of the L-tryptophan, an amino acid that is

essential for T cells, into N-formylkynurenine. Production of IDO is initiated by CD80/CTLA-4 interactions, TGF-β, or IL-10. IDO inhibits generation of effector T cells but instead promotes activation of T_{reg}s.

DCs influence the homing behavior of T cells. DCs within the intestine activate T cells that preferentially home to gut-associated lymphoid tissues. Such homing is mediated by expression of CCR9 and α4β7 on T cells. Skin or lamina propria-derived DCs, unlike DCs in the spleen or in central lymph nodes, express the integrin chain of CD103, which induces T cells with gut or skin homing preference.[130]

Natural Killer Cells

Although it was initially assumed that natural killer cells originate from bone-marrow precursors, it is now understood that most of them develop in lymph nodes and tonsils. Natural killer cells express TLR2, 3, 4, and 9, but not 7 or 8, and can be activated by PAMPs. They can also be activated by proinflammatory cytokines and chemokines such as IFNs, IL-12, IL-15, IL-18, IL-2, and CCL5.

Numerous activating and inhibiting receptors, some of which are constitutively expressed, while others are induced, regulate effector functions of natural killer cells. Natural killer cell receptors belong to the family of killer cell Iglike receptors in humans or C-type lectin receptors, such as CD94/NKGD2 and Ly49, in mice.[127,128,129] CD244, also known as 2B4, can also serve as a natural killer receptor. Receptors encoded by the same gene families can be activating or inhibitory (Table 39.4).

TABLE 39.4 Natural Killer Cell Receptors

Gene	Protein	Superfamily	Ligand	Function
KIR2DL1	CD158a	Ig superfamily	HLA-A, -Bw, Cw, G	Inhibitory
KIR2DL2,	CD158b1			
KIR2DL3,	CD158b2			

KIR2DL4,	CD158d			
KIR2DL5A,				
KIR2DL5B				
KIR3DL1,	CD158e1			
KIR3DL3	CD158z			
LILRB1	LIR1	Ig superfamily	HLA-G	Inhibitory
LILRB1	LIR2			
PILRalpha		Ig superfamily	Herpes virus, CD99-like protein	
KLRD1/KLRC	CD94/NKG2	C-type lection	HLA-E	Inhibitory
KLRG1	Mafa	C-type lectin	MIC and MHC class I-like proteins	Inhibitory
KIR2DS1,	CD158e2	Ig superfamily	HLA-A, -Bw, Cw, G	Activating
KIR2DS2	CD158j			
KIR2DS4	CD158i			
KIR3DS1	CD158e2			
PILRbeta		Ig superfamily	CD99-like protein	Activating
NCR1	NKp46, CD335	Ig superfamily	Pathogens	Activating
NCR2	NKp44, CD336			
NCR3	NKp40, CD337			
KLRK1	NKG2D	C-type lectin	MIC and MHC class I-like proteins	Activating
KLRC4	NKG2F			
SLAM6	Ly108,	CD2-like	NTBA	Activating

	NTBA		
SLAM7	CD319	molecule	CD319
SLAMF4	CD244		CD48

CD, cluster of differentiation; HLA, human leukocyte antigen; Ig, immunoglobulin; MHC, major histocompatibility complex; MIC, monolayer immune complex; NTBA, NK-T-B antigen; SLAM, signaling lymphocytic activation molecule.

All of the inhibitory natural killer cell receptors carry an immunoreceptor tyrosine-based inhibitory motif in the cytoplasm, which upon phosphorylation recruits the lipid phosphatase SHIP-1 or the tyrosine phosphatases SHP-1 or SHP-2. This in turn results in dephosphorylation of proteins bound to activating natural killer cell receptors. Activating receptors thus largely function through lack of signaling through inhibitory receptors. Activating receptors belong to the same families. The cytoplasmic tails of activating receptors are shorter than those of inhibitory receptors, and they do not carry an immunoreceptor tyrosine-based inhibitory motif. Signaling through combinations of activating receptors is needed to elicit natural killer cell functions. The inhibitory natural killer cell receptors respond to a variety of ligands, including classical MHC class I molecules, nonclassical MHC molecules (Qa-1 in mouse and HLA-E in human), adhesion molecules, cadherins, lectins, CD markers (CD44, 99, and 66), and sugars such as a-2,8-linked disialic acid. Activating receptors respond to some of the same ligands as inhibitory receptors, such as classical and nonclassical MHC determinants. Some also respond to Igs, CD112, CD155, themselves (ie, NTB-A and CD319 encoded by Slam6/7),[130] or C-type lectins, such as AICL. They also recognize viral proteins or stress proteins released by cells in response to an infection. Ly49H, an activating receptor encoded by Kira8,

recognizes the m157 gene product of mouse CMV, while NKp46 recognizes influenza virus HA in humans.[131] NKG2D recognizes the stress-induced MHC class I-related MICA and MICB proteins released in response to CMV infection.[132]

Some activating receptors contain immunoreceptor tyrosine-based activation motifs, which through Syk result in activation of the Ras/Raf/Erk and PI3K/Akt pathways leading to cytokine production and degranulation.[133] Others, such as NKG2D, signal through DAP10, a PI3K adaptor protein that contains a small Tyr-Ile-Asn-Met (YINM) motif, which binds either PI3K or Grb2.[134] Ligation of both is needed for cytolysis of NKG2D targets. Interestingly, IL-15 also promotes NKG2D-dependent lysis by causing Jak3-mediated phosphorylation of DAP10.[135] Signaling through CD244, a signaling lymphocyte activation molecule (SLAM) family member, which depending on circumstances can be activating or inhibitory, involves an immunoreceptor tyrosine-based switch motif, which upon recruitment of SLAM-associated protein causes activation through PI3K, while recruitment of EAT2 or ERT may cause inhibition.[136]

Natural killer cells contribute to early viral control through direct lysis of virus-infected cells and through the production of cytokines such as IFN-γ, IL-10, IL-18, TNF-α, MIP-1α, and MIP-β. Interestingly, activated natural killer cells can not only lyse virus-infected cells, but they can also kill immature DCs.[137] It is assumes that this type of natural killer cell-mediated DC editing prevents presentation of viral antigens by not yet fully matured and hence tolerogenic DCs to T cell. Natural killer cells are clearly crucial for the early defense again viral infections and their lack has been shown to result in enhanced susceptibility to infections with a number of different viruses, such as Sendai virus, influenza virus, or CMV, a finding that was confirmed in human patients with natural killer cell deficiency.[138,139]

Recent publications have shown that natural killer cells upon stimulation with antigens such a murine CMV undergo expansion, persist for months, and mount a recall response upon reexposure with the same antigen, suggesting that they cells can differentiate into memory cells, a function that thus far had been reserved for cells of the adaptive immune system.[140,141]

Natural Killer T Cells

Natural killer T (NKT) cells are a subset of natural killer cells that share characteristics of T and natural killer cells. NKT cells referred to as type 1 or iNKT cells express an invariant TCR that recognizes glycolipids presented by CD1d.[142] Other NKT cells, referred to as type 2 NKT cells express a more variant TCR that does not recognize CD1d-associated ligands. NKT cells express T-cell lineage markers and, for example, in humans, iNKT cells can be CD4+, CD8αβ+, or CD8αα+, but they also express natural killer cell receptors. NKT cells release cytokines, mainly IL-4 and IFN-γ, that affect the differentiation fate of CD4+ T cells, and they can be lytic through secretion of perforin and granzyme or through the Fas/FasL pathway. Upon activation, NKT cells upregulate CD40L and can thus contribute to DC maturation through CD40. In turn, IL-12 secreted by DCs can drive activation of NKT cells. NKT cells are involved in resistance to a number of viruses including HSV-1 and -2, CMV, HIV-1, HBV, HCV, lymphocytic choriomeningitis virus (LCMV), and encephalomyocarditis virus,[142] but they may also contribute to virus-associated immunopathology, such as upon infections with RSV.[143]

ANTIGEN-SPECIFIC IMMUNE RESPONSES

The innate immune system, which can cause extensive symptoms or even death through massive release of cytokines, is our main defense system during the early phase of a viral infection until cells of the adaptive immune system (ie, T and B cells) differentiate into effector cells. This takes time and in a primary infection requires at least 4 to 5 days. Antigen-driven activation of T and B cells depends on signals from the innate immune system and is very much shaped in its flavor by characteristics of the initial inflammatory reaction and the antigen-presenting cells. Although cells of the adaptive immune system can express PRRs, their activation is driven by recognition of antigens with exquisitely specific, clonally expressed receptors, which in the case of B cells have a virtually unlimited repertoire. Prior to an infection, cells with a specific receptor for a given pathogen are only present at very low numbers. Therefore, upon activation, B and T cells first proliferate extensively with an approximate doubling time of 4 to 6 hours to accumulate to numbers suited to eliminate viruses that, if unchecked, can replicate by far more efficiently than lymphocytes.

T Cells

T cells based on surface markers are divided into CD4+ T cells, which have primarily regulatory functions, and CD8+ T cells, which have mainly effector functions. T cells express an antigen-specific receptor that unlike B-cell receptors (BCRs) does not recognize soluble antigen, but rather peptides derived from degraded viral proteins that associate with MHC molecules. This ensures that T cells only respond to cell-associated antigens, which makes them uniquely suited to respond to intracellular parasites such as viruses.

Cluster of Differentiation 4+ T Cells

CD4+ T cells can be divided into Th cells, which promote activation of immune responses, and T_{reg} cells, which have inhibitory functions.

Cluster of Differentation 4 + T-Helper Cells: Subsets. CD4+ Th cells are divided into Th1, Th2, Th17, and follicular Th (Tfh) cells[144,145,146] (Fig. 39.5). Th cells originate from a common precursor in the periphery, the naïve CD4 T cell. The inflammatory cytokine milieu during antigen-driven activation dictates its differentiation into any of these subsets. Th1 cells

are induced when antigen activation occurs in presence of IL-12 or IL-18. Naive cells differentiate into Th2 cells in a milieu that contains IL-4. Th17 cells were discovered more recently. Naive cells develop into Th17 cells in presence of transforming growth factor (TGF)-β and IL-6. IL-1β and IL-23 enhance/stabilize their differentiation. Once activated, Th subsets block differentiation of uncommitted naïve cells into other subsets. Th1 cells

prevent Th2 differentiation through IL-12, Th2 cells block Th1 development through IL-4, and Th1 derived IFN-γ and Th2 derived IL-4 both inhibit Th17 formation. Tfh cells are only found in B-cell follicles in spleens, lymph nodes, and Peyer patches, where they are instrumental in germinal center formation by secretion of IL-21 and IL-4.

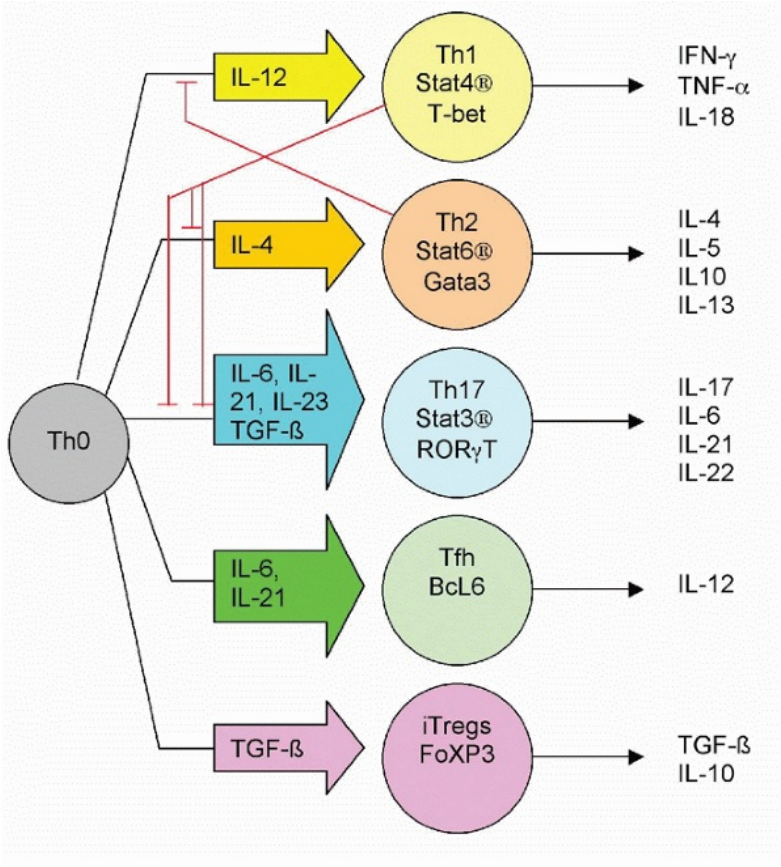

FIG. 39.5. Cluster of differenation 4+ T-helper cells subsets: cytokines driving their induction. Lineage defining transcription factors and effector functions.

Th cell subsets are characterized by lineage defining transcription factors.[147] Th1 cells express T-bet, a T-box transcription factor that controls expression of IFN-γ. Th2 cells express trans-acting T-cell-specific transcription factor (GATA3), which promotes production of IL-4, IL-5, and IL-13. The transcription factors Stat3, the orphan nuclear receptors (ROR) gamma-t, and RORalpha are involved in Th17 differentiation. B-cell lymphoma (Bcl)-6 is required for differentiation of Tfh cells, which are distinguished from other subsets by the expression of CXCR5 that enforces their homing to lymph node follicles. Other Th cell subsets have been suggested, such as Th3 and Th9 cells, but as unique transcription factor signatures have not yet been defined for these proposed subsets, it remains unclear if they indeed represent distinct lineages. It should also be noted that there is a certain degree of plasticity between defined Th subsets; Th1 cells can differentiate into Th2 cells and vice versa, Th17 cells can differentiate into Th1 or Th2 cells and Th1, and Th2 and Th17 cells can turn into T_{reg}s.

Most crucial for control of viral infections are Th1 cells, which are induced by antigen-presenting DCs that upon virus-driven maturation typically produce IL-12. Some viruses produce IL-10 homologs[148] or induce cellular IL-10 mimetics,[149] which support the development of Th2 cells, while suppressing Th1 cell differentiation. Induction of Th17 cells together with Th1 cells was reported in severe influenza A virus infections.[150] Th17 cells are also generated during viral infections and have been reported to promote coxsackie virus-associated myocarditis[151] and persistent infections with Theiler murine encephalomyelitis virus.[152] They have been implicated in promoting liver injury in chronic HBV infections in humans.[153] Tfh cells are presumably crucial for effective antiviral antibody responses, but, thus far rather little is known about their fate during viral infections. It is known that exceptionally strong TCR signaling through high-avidity interaction with MHC class II peptide complexes supports differentiation of naive cells into Thf cells.[154]

Cluster of Differentiation 4 + T-Helper Cells: Antigen Processing and Presentation. CD4+ T cells recognize MHC class II-bound peptides, which originate from pathogens that are broken down by proteolysis in the endosomes.[155] Expression of MHC class II molecules is restricted to macrophages, B cells, and DCs in mice, and in humans is also expressed on CD4+ T cells. Expression of MHC class II on the cell surface increases upon cell activation, in part through translocation of preformed molecules from intracellular compartments to the cell surface, and in part through increased synthesis. MHC class II molecules are heterodimers composed of α and β chains. A number of chaperone proteins ensure transport of MHC class II molecules

to the endosomes. First upon their synthesis, the two MHC class II chains assemble in the endoplasmic reticulum (ER) into a nonameric complex composed of three chains of the α and β molecules and three invariant molecules. The invariant molecules contain a peptide sequence, termed class II linked invariant peptide, that promiscuously binds to the groove of MHC class II α/β chain dimers, thus preventing binding of other peptides. It also contains an ectodomain, which mediates trimerization and interferes with DM/MHC class II binding. The MHC class II-invariant chain complexes are translocated to endosomes/lysosomes, where the invariant chain is degraded by cathepsin S and dissociates from MHC class II molecules, which then bind the DM accessory protein. DM binds close to the peptide groove inducing conformational changes that allow for loading of antigenic polypeptides. Polypeptides that fit poorly are forced to disconnect again through DM-mediated conformational changes, while peptides that fit well are insensitive to DM-mediated dissociation. Long peptides that bind the MHC class II groove are clipped at this stage; the portions of the polypeptide that overhangs the groove are cleaved off by peptidases. The MHC class II-peptide complexes are then transported to the cell surface, where they can be recognized by the TCR of CD4+ T cells.

Cluster of Differentiation 4 + T-Helper Cells: Activation. Naïve CD4+ T cells circulate between blood and secondary lymphoid tissues. Their activation requires presentation of antigen by MHC class II molecules carried by professional antigen-presenting cells, such as mature DCs. Once a CD4+ T cell recognizes its specific antigen, the DC-CD4+ T cell interaction is stabilized through integrins such as ICAM-1 or DC-SIGN on DCs, which bind lymphocyte function-associated antigen (LFA)-1 or ICAM-3 on CD4+ T cells, respectively.[156] Full activation of CD4+ T cells requires then a second signal in form of interactions with costimulatory molecules.[157] In the initial activation of CD4+ T cells, CD80 and CD86, which are both expressed on mature DCs and signal through CD28, provide costimulation. Although costimulation through CD28 is dominant, other molecules, such as CD2, Ox40, ICOS, CD40, or SLAM (CD150) can provide or complement this function. CD2 upon interaction with LFA-3 induces Jun kinases, which in turn results in IL-2 production.[158] Ligation of Ox40, which is expressed 2 to 3 days after T-cell activation, with Ox40L recruits TRAF2 and causes activation of NF-κB, PI3K, and Erk pathways and augments CD4+ T-cell functions, such as

cytokine production, proliferation, and survival at a later stage than CD28-mediated activation and may involve cells other than antigen-presenting DCs.[159] Ox40 has also been implicated to be essential for Tfh development.[160] ICOS has overlapping functions with CD28 and signals through PI3K.[161] It has been reported that myeloid DCs mainly induce CD4+ T cell activation through CD28, while plasmacytoid DCs, which express high levels of ICOS-L, may costimulate through ICOS. CD40L is induced upon CD4+ T-cell activation and upon interaction with CD40 on DCs or B cells promotes CD4+ T-cell proliferation. SLAM, which is also induced upon CD4+ T-cell activation, results in a SLAM-associated protein/proto-oncogene tyrosine-protein kinase (Fyn) signaling cascade, which in turn enhances CD4+ T-cell proliferation and cytokine production.[162]

Upon activation, CD4+ T cells start to produce IL-2. They also increase cell surface expression of CD25, the receptor for IL-2, which promotes their own proliferation. CD4+ T cells do not expand as extensively as CD8+ T cells but they do undergo approximately 8 to 10 cycles following activation.

Cluster of Differentiation 4 + T-Helper Cells: Effector Functions. CD4+ effector cells provide helper functions mainly through the secretion of cytokines and chemokines.[163] All Th cells can produce IL-2, which promotes their own proliferation as well as proliferation of CD8+ T cells and T_{reg}s. The Th1-derived cytokines TNF-α and IFN-γ activate natural killer cells, macrophages, and B cells. They are also essential for activation of CD8+ T cells, although some viruses, such as LCMV, sendai virus, influenza virus, or ectromelia virus (a mouse poxvirus) can induce CD8+ effector T cells without CD4+ T help. Nevertheless, T helper-independent CD8+ T cells are fundamentally defective and fail to differentiate efficiently into memory CD8+ T cells.[164] Th cells can lyse virus-infected target cells that express MHC class II molecules, such as EBV-infected B cells[165] or HSV-1-infected macrophages.[166] Th2 cell secrete cytokines, such as IL-3, IL-4, IL-5, and IL-10, that drive B-cell proliferation and differentiation and activate mast cells and eosinophils. Th17 cells produce IL-17, IL-21, and IL-22 and induce strong inflammatory responses mainly through IL-17-mediated recruitment and activation of neutrophils. Tfh cells, upon initial stimulation through the TCR and CD28 followed by interactions with Ox40, upregulate CXCR5 and home to B-cell follicles, where they assist in B-cell proliferation and differentiation through CD40L interactions and production of cytokines, such as CD21 and IL-4, which promote germinal center formation, class switching, and plasma cell differentiation.

Cluster of Differentiation 4 + T-Helper Cells: Memory Differentiation. Upon removal of antigen, effector CD4+ Th-cell numbers contract over a period of weeks and the remaining antigen-experienced CD4+ T cells differentiate intro long-lived memory cells, which preferentially home to the bone marrow. The overall size of the CD4+ T-cell memory pool depends on signals present during activation such as antigenic load, duration of antigen presentation, and presence and type of costimulators. Transition of CD4+ effector T cells into long-lived CD4+ memory Th cells depends on HVEM-LIGHT interactions.[167] Such interactions do not require classical antigen-presenting cells but can happen between adjacent T cells, which express both HVEM and LIGHT. Memory CD4+ T cells, upon reencounter of their antigen, differentiate into effector Th cells and they assume effector functions more rapidly than naïve T cells, in part through storage of RNA for key cytokines and chemokines, which are available for immediate translation once the TCR has been engaged. Although this reactivation is less dependent on costimulation compared to activation of naive CD4+

T cells, it is nevertheless optimized by interactions between CD28-CD80/86, OX40-OX40L, or ICOS-ICOSL.[168] As a rule, the Th cell's fate decision during primary activation remains unchallenged during recall responses (ie, memory Th1 cells will become effector Th1 cells, memory Th2 cells will become effector Th2 cells, etc.).

Regulatory Cluster of Differentiation 4 + T Cells. Two subsets of CD4+ T cells have immunoinhibitory functions[169]; one subset is constitutively present and is termed natural T_{reg}s (nT_{reg}s) while the other is induced by antigen (iT_{reg}s). CD4+ T_{reg} subsets are characterized by expression of the lineage defining transcription factor forkhead box P (FoxP)3 in mice, although in humans other T-cell subsets can transiently become positive for FoxP3. T_{reg}s constitutively carry surface markers, such as CD25, CTLA-4, GITR, and Lag-3, that are also transiently expressed by recently activated CD4+ Th cells. T_{reg}s suppress immune responses and are essential to maintain tolerance and limit immunopathology following infections. Lack of T_{reg}s, such as in scurfy mice, which have a mutation in the *foxP3* gene, rapidly succumb to a fatal lymphoproliferative disease.[170]

nT_{reg}s, which comprise approximately 5% to 10% of the circulating pool of CD4+ T cells, are educated in the thymus and recognize self-peptides, while iT_{reg}s develop in the periphery from FoxP3-CD4+ T cells. This conversion seems to by driven by DCs that provide a metabolic microenvironment that does not meet the high energetic demands of activating effector T cells. Specifically, DCs that produce enzymes that cannibalize essential amino acids such as IDO, which targets tryptophane; IL-4il, which converts aromatic amino acids; arginase, which depletes arginine; or histidine ammonia lyase, which destroys histidine and promotes iT_{reg} development.[171] Central to this conversion is the mTOR pathway, which is activated by signals from nutrient receptors, growth factor receptors, and presumably branched amino acids, and then initiates translation and mitochondrial energy production. TCR signaling in absence of mTOR activation or under conditions of artificial mTOR inhibition, such as through rapamycin, induces FoxP3 and conversion of CD4+ T cells into iT_{reg}s.[172]

T_{reg}s suppress effector immune responses through several mechanisms. They inhibit IL-2 production and through expression of high levels of CD25 consume large amounts of IL-2, thus causing depletion of this cytokine, which is essential for T-cell proliferation. They produce IL-10, which downregulates the expression of Th1 cytokines, MHC class II, and costimulatory molecule expression by inhibiting NF-κB and Jak/Stat signaling pathways. T_{reg}s produce TGF-β, which upon signaling through similar to mothers against decapentaplegic 2 and 3 modulates gene expression. They also produce IL-35, a heterodimeric protein composed of IL-12α and IL-27β chains, which promotes proliferation of T_{reg}s and inhibits Th17 development.[173] T_{reg}s can form very stable clusters with DCs, and it is assumed that this limits access of other T-cell subsets. Through CTLA-4 or PDL1, T_{reg}s downregulate expression of CD80 and CD86 on DCs, thus reducing their ability to provide costimulatory signals.[174] T_{reg}s can directly bind B and T cells, such as through interactions between HVEM on T_{reg}s and BTLA on T and B cells,[175] and cause lysis of cells through the release of granzyme and perforin.[176]

T_{reg}s play a role in infectious diseases by reducing the magnitude of primary immune responses, by limiting effector T-cell functions, and by reducing secondary immune responses. Mice that lack T_{reg}s are better able to control viral infections, but they are also prone to more damaging immune responses.[169] This is exemplified by ocular infections of mice with HSV, which causes severe blinding infections upon depletion of T_{reg}s. Dampening of potentially pathologic immune responses upon viral infections not only benefits the host but also the virus by allowing for its persistence. For example, in chronic HCV infections, the severity of liver damage as well as viral loads are inversely correlated with numbers of T_{reg}s in liver and blood.[177] CD8+ T-cell responses to HIV-1/simian immunodeficiency virus (SIV) are dampened by T_{reg}s.[178] The chronic phase of such infections is associated with a loss of T_{reg}s, which may contribute to T-cell hyperactivation in response to microbial translocation

from the gut.[179] Chronic infection of mice with Friend leukemia virus is associated with an increase in T_{reg}s and a suppression of CD8+ T-cell function.[180] Depletion of T_{reg}s in this model rescues cytokine production and lysis by CD8+ T cells resulting in sustained lowering of viral loads.

Cluster of Differentiation 8+ T Cells

CD8+ T cells are crucial to resolve virus infections through direct lysis of virus-producing cells and through the release of antiviral cytokines.

Cluster of Differentiation 8 + T Cells: Antigen Processing and Presentation. CD8+ T cells respond to peptides displayed by MHC class I determinants.[181] The pathway for peptide-MHC class I loading differs from that of MHC class II loading. While MHC class II molecules associate with peptides derived from degraded proteins in endosomes, MHC class I peptides largely originate from misfolded newly synthesized proteins. MHC class I molecules, which are expressed on nearly all cells are thus uniquely suited to detect cells that upon infection are producing new viral progeny. Proteins that are not faithfully translated or that are incorrectly folded are degraded in the cytoplasm into peptides by proteolytic enzymes that form the proteasome complex.[182] The resulting peptides are then transported into the ER though the transporter associated with antigen presentation (TAP).[183] Newly synthesized MHC class I heavy chains are also transported into the ER, where they bind to calnexin, which promotes appropriate folding of the protein. The correct folding of the MHC class I heavy chain is further facilitated by calreticulin and the oxidoreductase ERp57, which breaks up incorrectly folded MHC molecules. Once the heavy chains are folded, calnexin is released and the heavy chains bind β2 microglobulin. The resulting complex is linked to TAP by interactions with tapasin. Peptides, once they reach the ER, may be digested further by peptidases, until they get to a size that permits their association with the MHC class I groove, which can accommodate peptides of 8 to 10 amino acids in length. Binding of peptides to MHC

class I molecules causes release of calreticulin and tapasin, and is followed by transport of the MHC-peptide complex through the Golgi apparatus to the cell surface (Fig. 39.6). Synthesis of molecules needed for MHC class I-mediated antigen presentation is upregulated by proinflammatory cytokines, such as IFNs.

Naïve CD8+ T cells generally require antigen presentation by DCs in presence of costimulatory molecules. Proteins from viruses that directly infect DCs readily provide peptides that enter the MHC class I presentation pathways. Antigens from viruses that do not infect DCs are processed and presented by alternative mechanisms, called cross-presentation or cross-priming.[184] In cross-priming, DCs can gather antigen from the outside by a number of pathways (see Fig. 39.6). DCs can pinocytose or phagocytose antigen released by other cells. They can actively chop off parts of viral surface proteins displayed on membranes of infected cells, or they can acquire preformed MHC class I antigen by trogocytosis, which involves conjugation of DCs with antigen-carrying cells through immunologic synapses, followed by ingestion of membrane fragments of one cell by the other. DCs that express connexin-34 can form gap junctions with other connexin-34+ cells, which allows for influx of small molecules, such as peptides, from the cytoplasm. A number of receptors facilitate antigen uptake by DCs, such as mannose receptors, DC-SIGN, DEC205, and Fc receptors, and contribute to cross-presentation. MHC class I molecules are normally formed in the ER, but they can be recycled from the cell surface into early endosomes. TLR signaling increases MHC class I recycling and recruits TAP to endosomes. Proteins that are internalized into endosomes initially exit into the cytoplasm, where, still in close proximity to endosomes, they are degraded by proteasomes. The resulting peptides then reenter the endosomes through TAP and now can bind to empty, recycled MHC class I molecules.

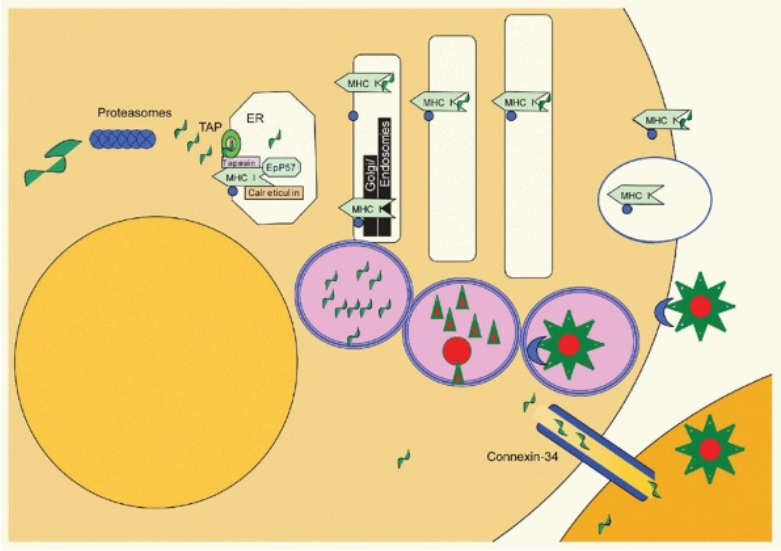

FIG. 39.6. Major histocompatibility complex class I processing and presentation pathways: endogenous pathway (*green*) and cross-presentation (*pink*).

Although MHC class I molecules can, in theory, bind thousands of different peptides that have fitting anchoring residues and, vice versa, viruses, especially those that are large, carry proteins with hundreds of putative MHC class I-binding motifs, antiviral immune responses are commonly dominated by CD8+ T-cell responses to one or two epitopes, with rapidly mutating viruses allowing for their escape.[185] Immunodominance of some peptides and immunosubdominance of others is dictated mainly by affinity between peptide and the MHC class I molecules, and most of the defined immunodominant peptides have an

affinity constant of 500 nm or higher. It is also affected by processing as epitopes can be lost through proteolytic cleavage or they are not generated by lack of flanking sequences that permit such cleavage. Immunodominance is further affected by location of the peptide within the viral protein. Translation is initiated at the 5′ end of an mRNA, which for surface proteins encodes the signal sequence. Incomplete protein synthesis is common, especially for larger proteins or mRNAs with mutations that incorporate a stop codon. Truncated proteins do not fold correctly and are degraded in the cytoplasm. Peptides encoded by the 5′ part of gene and thus located toward the N-terminus of a protein are more likely to be present in incomplete proteins, and as such more likely to be immunodominant compared to epitopic peptides within the C-terminus.

The timing of viral protein synthesis affects which epitopes are predominantly recognized. CD8+ T cells often respond preferentially to early viral proteins, which is highly advantageous for the host, as such proteins are produced well ahead of viral replication, so that destruction of cells expressing early viral protein proceeds assembly of infectious viral progeny.[186] As viruses commonly interfere with cellular protein synthesis, the favored recognition of early viral proteins may in part be a default pathway, as such proteins are produced at a time when the cell is still relatively intact and thus able to process and present the antigen efficiently. Viral antigens are not produced in equal quantities. The most abundant structural protein of HIV-1 is gag, which is also the major target antigen for CD8+ T cells. Enzymes such as the polymerase of rhabdoviruses are produced at minute quantities that are unlikely to be immunogenic. Some viral proteins are extremely stable. For example, EBNA1, which is produced in cells latently infected with EBV, carries a glycine-alanine repeat sequence that is resistant to proteolytic degradation.[187] Last but not least, the TCR repertoire, which has holes to ensure tolerance, influences immunodominance; epitopes that can bind with very high affinity to MHC class I molecules will not elicit a response if they

resemble self peptides.

The TCR of CD8+ T cells only needs a few MHC class I peptide complexes to mount an immune response. Although some viral infection can result in the display of hundreds of peptide-MHC class I molecules on the surface of infected cells, others only express a dozen or so, which still suffices for CD8+ T-cell activation and effector functions.

Cluster of Differentiation 8 + T Cells: Activation. Naive CD8+ T cells are activated within the T-cell-rich zones of lymphatic tissue, which provide a sufficient density of antigen-presenting DCs and Th cells. During activation, T cells interact repeatedly with DCs until they form stable connections, which are sustained by adhesion molecules, such as LFA-1 and CD2 on T cells and ICAM-1 and CD48/CD59 on DCs, respectively, and by costimulatory molecules that can function as adhesion molecules. Binding between T cells and DCs leads to the formation of an immunologic synapse,[188] in which the TCR-MHC-peptide complexes are central, surrounded by interacting costimulators and their ligands. Interacting adhesion molecules form the next ring and the outer ring contains CD43, CD44, and CD45 molecules.[189] Formation of synapses presumably allows for activation of a CD8+ T cell by low density of its cognate antigen. Costimulation is essential for CD8+ T-cell activation and the predominant pathway signals through the constitutively expressed CD28 upon its interaction with CD80 or CD86. Ligation of CD28 initiates a number of signaling cascades that result in activation of NF-κB and Jun/Fos transcription factors. It also activates the PI3K/Akt/mTor pathway, which augments energy production and protein synthesis by increasing amino acid transport, ribosome functions, and translation.[190] In addition, mTOR affects expression of the transcription factors T-bet and eomesodermin, which are crucial for lineage decisions in T cells.[191] Later during CD8+ T effector cell differentiation, CTLA-4 expression is induced, which binds to the same molecules that CD28 recognizes, but serves as a coinhibitor, by for example blocking CD28-induced Akt activation.[192] As CTLA-4 has higher affinity than CD28 to CD80 and CD86, coinhibition prevails if both are expressed. Other costimulators and conihibitors play a role in initiating, sustaining, or dampening CD8+ T-cell activation. Interactions between HVEM-lymphotoxin/LIGHT, Ox40-Ox40L, or 4-1BB-4-1BBL provide costimulatory signals by activation of NF-κB, Jun kinase, and Ap1. Costimulation through different pathways is functionally, spatially, and temporarily segregated. T cells during or shortly after activation also receive inhibitory signals through PD1, BTLA, CD160, or LAG-3, which are either induced or upregulated during T-cell activation. While such inhibitory signals dampen primary immune responses, they may be crucial to allow for fate-decisions of antigen-experienced CD8+ T cells.[193]

As a third signal, CD8+ T-cell activation requires cytokines such as type I IFN or IL-12. Activating CD8+ T cells requires T help in form of IL-2 for sustained proliferation.

Upon activation, CD8+ T cells proliferate extensively in a 4- to 6-hour cycle. Naïve cells, which are present at frequencies below 0.00001% of all CD8+ T cells prior to the infection, can reach frequencies of 10% or more within 5 to 7 days. Early after activation, CD8+ T cells transiently express CD25 and CD69. In addition, they start to express CD44, an adhesion molecule, and CD27, a TNF-receptor family member. T cells destined to become central memory cells increase expression of CD127. Upon activation and proliferation, CD8+ T cells downregulate expression of CCR7, the receptor for CCL19, which is primarily produced in lymphatic tissues and CD62L, an adhesion molecule, which facilitates entry into lymph nodes and then they can enter the bloodstream to migrate to sites of infection following gradients of chemokines, such as CXCL9 or 10. Proinflammatory cytokines produced in response to a virus increase expression of the adhesion molecules P- and E-selectins on adjacent blood vessels, which slows down the flow of CD8+ T cells; they first attach loosely to the blood vessel wall and then assume a rolling motion, which is replace by tight adhesions of T cells allowing them to transmigrate between the endothelial cells to the infected tissue,[194] where, upon encounter of their antigen, they commence effector function.

Cluster of Differentiation 8 + T Cells: Effector Functions. CD8+ T cells can release a number of cytokines and chemokines. Antiviral CD8+ T cells predominantly produce IFN-γ, IL-2, TNF-α, MIP-1-α and MIP-1-β, or regulated upon activation, normal T cell expressed and secreted. CD8+ T cells can secrete single or several cytokines/chemokines, and in infected individuals multiple subsets, defined by their cytokine/chemokine production profile, arise.[195] Although the profile of cytokine/chemokine may be predictive for effectiveness of the CD8+ T cells in limiting viral spread, the highly dynamic patterns that evolve during the course of an infection suggest that they may readily assume new functions or let go of others. IFN-γ upregulates the antigen processing machinery and MHC class I expression, thus facilitating interactions between infected cells and activated CD8+ T cells. IFN-γ also activates natural killer cells and macrophages, and blocks viral spread, similar to IFN-1s. TNF-α can inhibit replication of the viral genome or assembly of viral capsids.[196,197] Another important function of activated CD8+ T cells is their ability to kill infected target cells. CD8+ T cells predominantly lyse through the release of two lytic enzymes, granzyme, and perforin.[198] These two enzymes are present in granules that, once CD8+ T cells encounter their target, are released by exocytosis. Perforin molecules insert themselves into the plasma membrane of target cells and form a pore. Granzyme molecules enter through the pore into the cells where granzyme A activates DNase and inhibits the DNA repair machinery. Granzyme B activates caspase 3, which in turn activates caspase 7, while cleaving the proapoptotic molecule BID, thus causing cells to apoptose. Alternatively, CD8+ T cells can lyse target cells through interactions between CD95 expressed by all activated T cells and Fas expressed by some target cells. Interactions between CD95 and Fas trigger activation of caspase 8 and apoptotic cell death. CD8 + T-cell-mediated lysis is rather inefficient, as each CD8+ T cells can only lyse a limited number of targets; nevertheless, it is crucial for rapid viral clearance as mice lacking lytic enzymes showed increased susceptibility to a number of viruses.[199,200]

Cluster of Differentiation 8 + T Cells: Memory Differentiation. Four transcription factors play a role in determining the fate of an activating CD8+ T cell: T-bet and eomesodermin (Eomes), which are both required for effector and memory formation, the transcriptional repressor B-lymphocyte-induced maturation protein (Blimp)-1 which dampens and Bcl-6 which sponsors differentiation into memory.[201] The differentiation fate of CD8+ T cells is at least in part dictated by the conditions of their stimulation. CD8+ T cells can develop into short-lived effector cells, and this is supported by expression of the transcriptional repressor Blimp-1.[202] Alternatively, CD8+ T cells can transition into the memory T cells' pool. Strong TCR signaling in presence of excessive amounts of antigen seems to favor effector cell differentiation, while limited amounts of antigen promote development of memory T cells.[203] IL-2 through binding to CD25 similar to CD28 activates PI3K, which in turn initiates downstream phosphorylation of Akt/PKB. Activated Akt/PKB inhibits forkhead box O and thus expression of the IL7 receptor CD127, which favors memory formation.[204] Signaling through CD25 downregulates CD127 expression,[205] inhibits expression of Bcl-6, a memory marker,[206] and induces T-bet, which, if present at high levels, induces generation of CD8+ effector T cells. Memory CD8+ T-cell development is promoted by low levels of T-bet and by proinflammatory cytokines such as IFN-1s, IL-12, and IL-15.[207]

In acute viral infections, the immune system eliminates the infected cells within 1 or 2 weeks. Once their cognate antigen is gone, most of the activated CD8+ effector T cells undergo apoptotic cell death and only 5% to 10% survive and differentiate into memory cells, leaving the organism with a 10- to 1000-fold increase in numbers of CD8+ T cells able to react against a given virus. Two major types of memory cells have been identified: effector memory T cells, which remain comparatively active and circulate in the periphery, where upon reencounter of their antigen then can assume immediate effector functions; and central

memory T cells, which express increased levels of CD62L and CCR7 and return to lymphatic tissues, where they become reactivated upon reinfection.[208,209] Effector memory T cells, although longer lived than effector T cells, decline in numbers over time, while numbers of central memory T cells remain at constant levels through homeostatic antigen-independent proliferation driven by IL-7, which provides survival signals, and IL-15, which promotes proliferation.[210] There is ample evidence to suggest that during activation T-cell differentiation is rapidly imprinted, potentially already during the first division of activating T cells.[211] Within a week after the initial activation, T cells that are terminally differentiated can be distinguished from those that will evolve into memory T cells; those that express low levels of CD127 and high levels of KLRG1 die once the effector phase is terminated, while those that are $CD127^{high}$ and $KLRG1^{low}$ become central memory T cells.[212] Lineage relationships between effector, effector memory, and central memory CD8+ T cells remain debated; some studies suggest a linear relationship, in which effector T cells differentiate into effector memory CD8+ T cells and then central memory CD8+ T cells,[213] while others argue for independent development of these populations.[214]

The importance of effector/effector memory CD8+ T cells versus central memory CD8+ T cells in providing protection to viral infections remains debated as well. While some argue that the more activated effector-like CD8+ T cells, which are present at the port of viral entry, are crucial,[215,216] others provide evidence that central memory CD8+ T cells with their higher proliferative potential are essential.[213] For example, in a low-dose SIV infection model of rhesus macaques, effector memory CD8+ T cells were shown to correlate with protection.[217] Similarly, in an influenza virus challenge model of mice, activated CD8+ T cells present in the lung at the time of challenge were required for protection.[218] In contrast, central memory CD8+ T cells were shown to provide superior protection to LCMV.[213] One could argue that mode of infection dictates which T-cell subset is indeed more suited to eliminate virus-infected cells before extensive viral replication takes place. Viruses such as HIV-1 and SIV invade through mucosal surfaces

and a productive infection is established by very small numbers of founder viruses. Activated effector memory CD8+ T cells present within the mucosa might be superior to eliminate the few cells that initially become infected compared to central memory CD8+ T cells, which reside in far away lymph nodes, where they most likely remain ignorant of the incoming few viral particles. In an infection where substantial amounts of virus are implanted directly into a body cavity, central memory CD8+ T cells with their higher proliferative capacity may be better suited to eliminate the virus.

Cluster of Differentiation 8+ T Cells: The Effect of Persistent or Repeated Infections on Cluster of Differentiation 8+ T Cells. Many viruses, especially DNA viruses, are not completely cleared from an organism, but rather establish long-lasting infections. Such infections can be chronic when the virus continues to replicate extensively, such as HIV-1 or HCV in humans or some strains of LCMV in mice. Herpesviruses establish latent infections by shutting off most viral protein synthesis; they periodically become reactivated and resume replication. Other viruses, such as adenoviruses, persist at very low levels and apparently remain transcriptionally active. In this case, the immune system is continuously stimulated by low amounts of antigen, which maintain high frequencies of activated effector-like CD8+ T cells. T cells may encounter the same viral antigen repeatedly due to repeated infections. Although neutralizing antibodies induced by a viral infection may prevent a subsequent infection with this particular virus, they are not effective against other viruses from the same family, which may not share neutralizing antibody binding sites, but, nevertheless, carry conserved T cell epitopes, such as different serotypes of adenoviruses. T cells may thus encounter the same epitope presented by related viruses over and over again. Repeated stimulation in turn affects the quality of memory CD8+ T-cell responses.

Chronic infections overwhelm the immune system. The continued high presence of antigen prevents differentiation of effector CD8+ T cells into central memory CD8+ T cells and instead induces, in a hierarchical fashion, loss of effector cell functions[217]; first, T cells lose the ability to proliferate and then they cease to produce cytokines or lyse target cells. Loss of function is accompanied by upregulation of coinhibitory receptors such as PD1, LAG-3, CD160, 2B4, CTLA-4, PIR-B, and Gp49 in a process that is controlled by Blimp-1.[218] Signaling through coinhibitors such as PD1 inhibits the PI3K/Akt/mTor pathway and thus the T cell's ability to meet its demand for energy. The degree of exhaustion is correlated with the numbers of coinhibitory receptors on the CD8+ T cell's surface, as numbers of upregulated coinhibitors correlate with the severity of the T cell's impairment.[219] Partially, but not fully, exhausted CD8+ T cells can be rescued by treatment with antagonists to immunoinhibitory pathways.[220]

Reactivation of latent viruses causes expansion of specific memory CD8+ T cells, which once activated rapidly control the infection. The profile of memory CD8+ T cell changes upon repeated recall; they become more activated and their numbers increase.[221] This can become rather extreme, and in elderly humans up to 30% of peripheral CD8+ T cells show specificity to antigens of herpesviruses, most notably (HCMV).[222] High frequencies of Hhuman CMV CMV-specific CD8+ T cells are inversely correlated with life expectancy,[223] presumably as a consequence of increased disease susceptibility due to irreversible loss of immunologic space and repertoire.

Frequencies of circulating activated CD8+ T cells to adenoviruses can also be high.[224] It is unknown if this relates to the ability of the virus to persist at low levels causing continuous activation of CD8+ T cells or if it rather reflects repeated infections with serologically distinct adenoviruses. The former pathways seems more likely as other viruses, such as influenza A viruses, which also infect repeatedly, fail to establish high frequencies of specific CD8+ T cells.

Regulatory Cluster of Differentiation 8 + T cells. CD8+ T cells that express FoxP3 and exert immunosuppression through the release of IL-10 or TGF-β have been identified in acute SIV infection of nonhuman primates, where they correlate with viral load and inversely correlate with effector CD8+ T-cell responses.[225] They also may play a role in limiting liver damage during chronic HCV infection.[226]

B Cells

The primary task of B cells is to produce antibodies that can limit viral spread. In addition, they serve as antigen-presenting cells and produce cytokines.

B Cells: Subsets

In mice, B cells are divided into three distinct subsets: marginal zone (MZ) B cells, follicular B cells, and B1-B cells, which are further divided into B1a- and B1b-B cells.[227] B1-B cells, which have not yet been identified in humans, reside mainly in the pleural and peritoneal cavities, although, upon activation, they can migrate to lymph nodes. B1-B cells are activated by PAMPs and can mount early IgM or IgA responses without requiring T-cell help. MZ B cells, which, as their name indicates, reside mainly in the MZ of the spleen, can also rapidly, without T-cell help, differentiate into shortlived plasma cells that produce IgM. MZ B cells, which express high levels of MHC class II antigens and costimulatory molecules, can also present antigen to Th cells. Unlike MZ or B1-B cells, follicular B cells, which circulate between blood and B-cell follicles in lymphatic tissues, are not directly activated by antigen ligation of the BCR and concomitant PRR stimulation, but, in addition, require T-cell help through CD40-CD40L interactions.

B Cells: Activation and Differentiation. Mature naïve B cells derive from bone marrow

precursors. They express IgD and IgM molecules as antigen receptors. B cells with high affinity to self are deleted or edited first in the bone marrow and then again in the periphery. B cells that pass these checkpoints then circulate from blood through lymphatic tissues where they are guided toward follicles by a network of reticular fibers and follicular DC dendrites. Small antigens, such as peptides, reach lymph nodes rapidly by passive diffusion;

once there, they are taken up by macrophages that line the lymph node sinuses or complexed onto follicular DCs through complement and Ig Fc receptors. Larger antigens, such as virions, are engulfed by peripheral DCs, which then migrate to lymph nodes, a process that takes about 12 to 18 hours. B cells through their Ig receptors can acquire antigen from the surface of follicular DCs or macrophages.[228] Once the BCRs are cross-linked by complex antigens, which carry repeat sequences of the same antigen, or are oligomerized by soluble antigen, a conformational change occurs in the cytoplasmic domain of the BCR. This first activates Lyn. Lyn in turn phosphorylates the Ig chains leading to the attachment of Syk and recruitment of CD19.[229] These initial signals promote the formation of larger clusters, which form an immunologic synapse with the BCRs in the center surrounded by LFA-1. Formation of BCR clusters, which are needed to initiate a signaling cascade, is favored by high-affinity BCR-antigen interactions and may thus serve as an early selection process for the best fitting antibodies. Full BCR-CD19 signaling activates a number of cascades that result in activation of the NF-κB, Pi3K/Akt, Erk, and JNK/p38 pathways. This in turn initiates proliferation and maturation of B cells, which at this stage can differentiate into short-lived plasma cells or enter follicles to form germinal centers and differentiate into long-lived plasma cells and memory B cells. Shortlived plasma cells produce antibodies rapidly, although such antibodies are of low affinity compared to those produced by long-lived plasma cells. The fate decision between shortlived versus long-lived plasma cells or memory cells is in part governed by cytokines such as IL-12, which favors the development of short-lived plasma cells, as well as by the affinity between antigen and the BCR with very-high-affinity interactions apparently also favoring the development of shortlived plasma cells. It may also be influenced by local PAMPs and the resulting PRR signaling cascades.[230] For example, vaccines providing antigen together with TLR4 and TLR7 ligands favor the development of long-lived plasma cells and memory B cells.[83]

Germinal center formation requires Tfh cells, which promote B-cell responses through cytokines, such as IL-4, IL-6, IL-10, and IL-21, through direct cell-to-cell interactions and through costimulatory signals, such as CD40, ICOS, or lymphotoxin.[230] Coinhibitory signals also shape the fate of activated B cells. For example, upon activation, B cells rapidly adjust their metabolic needs by increasing glucose uptake and glycolysis in an Akt/PI3K dependant fashion. This in turn is inhibited by signaling through FcγRIIB.[231] PD1 on Tfh cells through interactions with PDL2 and to a lesser extend PDL1 reduce rates of cell death and thus increases numbers of surviving cells with lower-affinity BCR.[118]

During proliferation, B cells undergo hypermutation leading to the development of plasma cells producing antibodies with increased affinity. They also undergo isotype switching through recombination, and this process is influenced by the surrounding cytokine milieu; in mice, IL-4 favors switching to IgG1 and IgE, IL-5 promotes switching to IgA, and IFN-γ supports switching to IgG2a. Competition for T-cell help and T-cell-derived cytokines favors continued proliferation of B cells with the highest affinity receptors.

Differentiation of naïve or memory B cells into plasma cells causes changes in key transcription factors with a reduction of paired box protein-5 and Bcl-6 and an induction of Blimp-1, which turns off germinal cell functions and allows for a switch to antibody secretion.[232] It is not yet fully understood what drives B cells within the germinal center to differentiate into resting memory B cells or longlived plasma cells. B cells with lower-affinity receptors may be outcompeted by those that have higher-affinity receptors and thus be forced to differentiate into memory cells, whereas B cells with the highest-affinity receptors

continue to proliferate and eventually form the pool of long-lived plasma cells. The finding that the memory B-cell pool forms earlier than the pool of long-lived plasma cells supports this idea.[230] Plasma cells home preferentially to bone marrow, while memory B cells home to spleen or bone marrow. Upon reexposure to antigen, memory B cells differentiate into antibody-secreting plasma cells, which requires proliferation accompanied by another round of hypermutations and affinity maturation.

Chronic viral infections can lead to B-cell exhaustion.[233] During this process, B cells express increasing levels of the inhibitory Fc-receptor-like-4 molecule, which is accompanied by a loss of their proliferative capacity. Chronic infection can also cause a pathologic accumulation on antigen-antibody complexes causing vasculitis or glomerulonephritis.

B Cells: Effector Functions. B cells protect against pathogens primarily through the secretion of antibodies. The rapid production of low-affinity antibodies by B1-B cells and MZ B cells can reduce the spread of viral pathogens by forming complexes that are retained within lymphatic tissues, where they may aid activation of other adaptive immune responses. Plasma cells derived from B2-B cells can produce IgM, IgD, IgG, IgA, and IgE. Of those, IgA, IgG, and IgM are important to control viral infections. IgM is produced first and is then over time replaced by IgG or IgA upon isotype switching. IgGs, which are further divided into IgG1 to 4 circulate in blood and tissues. IgA can be transported actively across epithelial surfaces, allowing for its secretion at mucosal surfaces. IgA is thus crucial for protection against pathogens that invade through the mucosal epithelium.

All antibody isotypes can block viral infections by neutralization. Virus-neutralizing antibodies are directed against parts of viral surface proteins that are exposed and commonly nonessential for viral fitness. For example, humans can be infected with more than 40 different serotypes of adenoviruses that belong to six different families; chimpanzees are infected with viruses that are phylogenetically related. The hexon of adenoviruses, which is the main target of neutralizing antibodies, forms trimers on the surface of virions. The stalk of the trimers, which is poorly accessible by antibodies, is highly conserved. The tower region contains a number of flexible loops that are highly variable. Neutralizing antibodies bind to these loops.[234] As these loops do not contribute to the structural integrity of the protein, they can readily be mutated, thus allowing the virus to escape neutralization.

Antibodies of the IgG or IgM isotypes can lyse viruses or virus-infected cells through activation of complement.

They can complex viruses, thus facilitating phagocytosis by macrophages. They can lyse virus-infected cells through antibody-dependent cell-mediated cytotoxicity, during which natural killer cells through Fc-receptors bind to an IgG molecule on an infected cell and then release perforin and granzyme.

Although preexisting natural antibodies provide an immediate layer of defense, which is rapidly joined by antibodies secreted by B1 and MZ B cells, production of affinity-matured antibodies takes up to 2 weeks and, by then, an acute viral infection has been resolved, while in chronic infections the virus, such as HIV-1 or HCV, has mutated extensively, rendering antibodies directed against the incoming virus rather useless. Affinity-matured antibodies are thus unlikely to play a dominant role in limiting a primary infection, but they are crucial to ward off secondary infections. Neutralizing antibodies can completely prevent a secondary infection or they can reduce the amount of incoming virus to levels that can rapidly be controlled. Protection by neutralizing antibodies against reinfection has been documented for numerous viruses such as rabies virus, influenza A virus, poliovirus, HPV, HIV-1, and many others, and is the main correlate of protection for most antiviral vaccines. Nonneutralizing antibodies can also dampen infections without necessarily providing sterilizing immunity. For examples, antibodies to the ectodomain of the matrix protein of influenza A virus lack neutralizing activity, but nevertheless can prevent fatal infections with influenza A viruses in animal models.[235]

In some infections, preexisting antibodies can exacerbate disease as exemplified by Dengue virus. Dengue virus replicates preferentially in macrophages, monocytes, and DCs. A first infection causes relatively mild symptoms and induces antibodies that cross-react between any of the four strains of Dengue virus. Following a second infection with a serologically distinct virus, circulating nonneutralizing antibodies form complexes with the virus. Such complexes facilitate uptake by macrophages, which in response produce IL-10 rather than IFN-γ. This allows for more vigorous replication of the virus, a skewing toward a Th2 immune response leading to hemorrhagic fever, which is characterized by impaired clotting and vascular leakage.[236]

Antibodies play an additional altruistic role. Children are born with an immature immune system and are thus potentially hypersusceptible to viral infections. Antibodies of the IgG isotype are transferred from mothers to their offspring across the placenta and through breast milk. Maternal antibodies are crucial to protect an infant against the onslaught of pathogens that will attack as soon as it has left its mother's womb. Maternal antibodies do not necessarily provide sterilizing immunity, but may attenuate inflections and thus allow for active immunization under conditions that do not threaten the life of the infant. The argument has been made that vaccination reduces transfer of maternal antibodies as natural infections of the mother as a rule elicit higher and more sustained levels of antibodies than most vaccines. This, combined with improved hygienic standards, which delays exposure to common pathogens, may in the end harm the offspring by delaying their first exposure to pathogens until such a time when maternal antibodies have declined from their circulation, which may happen before their own immune system is fully developed. Exposure to viruses at this susceptible stage, in turn, may favor the induction of pathological or self-reactive immune responses.[237] Although there is no direct proof for this ominous side effect of mass vaccination, it might be supported by recent increases in the incidence of allergies and autoimmune diseases.

THE MUCOSAL IMMUNE SYSTEM

The mucosa of the airways, the intestines, and the urogenital tract, which in adult humans covers a total surface area of approximately 400 m^2 are the most common ports of viral entry. Mucosal surfaces are also bathed in antigens that are harmless or useful such as the 10^{14} commensal bacteria that live in the intestine, where they are essential to outcompete pathogenic bacteria and promote normal intestinal functions. The immune system that controls mucosal surfaces, the so-called mucosal immune system, must be able to distinguish between dangerous and nondangerous antigens and has thus unique characteristics.

The mucosal immune system consists anatomically of local inductive sites, called organized mucosa associated lymphoid tissue, such as tonsils in the oral cavity, Peyer patches along the wall of the small intestine, the appendix close to the junction between small and large interesting, and bronchus associated-lymphoid tissues in the respiratory tract. Although these sites resemble lymph nodes, they are not encapsidated nor are they connected by lymphatic vessels.[238,239]

The mucosa of the intestine is covered by the lamina propria (LP) and a single layer glanular epithelium. Both activated and resting B- and T-lymphocytes can be found within the LP, while the mucosal intestine is densely seeded with highly activated T cells. The vaginal surface is covered by a multilayered squamous epithelium that contains both B and T cells originating from iliac lymph nodes. T and B cells can also be found in the epithelium of the airways. B cells in the intestine and airways are skewed toward those that produce IgA. Most T cells in the mucosa of the genital tract and the airways carry the α/β TCR, unlike T cells in the intestinal part, many of which carry a γ/δ TCR. In newborn mice, nearly all if the intraepithelial lymphocytes that initially populate the intestine express the γ/δ receptor, which has a less diverse repertoire than the α/β receptor. T cells with a γδ receptor develop independently of the thymus and do not recognize MHC class I peptide complexes but rather

respond to self-antigens and microbial phosphorylated metabolites. Their response does not involve extensive proliferation and differentiation; upon encounter of antigen, they commence production of cytokines within a few hours.[240] T cells expressing the α/β TCR appear later in the intestine under the influence of antigenic stimulation. T cells within the intestine can also be double positive for CD4 and CD8, or they can carry two CD8α chains rather than heterodimers of CD8α and CD8β.

DCs acquire antigen in the intestine with the help of M cells, which sample luminal antigen and then pass it on to

the underlying DCs.[241] DCs in the mucosa predominantly secrete IL-6, IL-21, and TGF-β, thus favoring induction of Th17 cells, the latter in turn produce IL-17 and IL-22.[242] IL-17 increases tight junction formation in the gut thus reducing permeability, while IL-17 and IL-22 induce granulopoiesis through granulocyte-CSF and recruit neutrophils through CXC chemokines. Infections with HIV-1 or SIV cause rapid depletion of mucosal Th17 cells, which in turn increases gut permeability and causes microbial dissemination and chronic immune activation.[243]

Viral infections induce CD8+ T cells that can be isolated from the LP and the gut epithelium. These CD8+ T cells can produce IFN-γ and kill infected target cells. Antigeninduced CD8+ T cells in the epithelium and the LP were found to contract more rapidly than antigen-specific CD8+ T cell in spleen. Interestingly, antigen-specific CD8+ T cells from the LP and the overlaying mucosa were shown to be distinct in their TCR usage; T cells from epithelium were more oligoclonal and had lower affinity to their antigen, suggesting limited exchange of T cells between these two adjacent compartments.[244] Effector T cells of the central immune system can migrate to the intestinal mucosa, and this process requires expression of α4β7. Strength of the initial activation seems to control expression of α4β7. For example, CD8+ T cells induced by LCMV, which proliferates extensively in mice, express α4β7,[245] while those induce by an E1-deleted adenoviral vector only express this homing marker very transiently and consequently fail to migrate to the intestinal mucosa.[246] Homing of activated T cells is also influenced by characteristics of the antigenpresenting cells; CD103+ DCs derived from skin, lung, and intestine drive T cells to increase expression of $\alpha_4\beta_7$ integrin.[247]

Within the female genital tract, primary immune responses are induced in draining lymph nodes. Upon clearance of a virus, as was shown in mice and humans infected with HSV-2 or HIV-1, respectively, clusters of memory T cells, B cells, and DCs remain underneath the epithelium of the vagina and the cervix, potentially providing an immediate barrier against infection.[248,249] Systemically induced effector CD8+ T cell can readily migrate to the mucosa of the genital tract[250]; it remains unknown if this requires a specialized homing receptor.

Within the airways, an infection, such as with influenza virus, can result in organized lymphoid structures within the lungs composed of B-cell follicles and surrounded by T cells. These structures called inducible bronchus associated-lymphoid tissue can recruit naïve lymphocytes and provide a first-line defense against subsequent pulmonary infections.[251]

THE AGED IMMUNE SYSTEM

Immune responses become impaired during aging, resulting in an increased susceptibility to infectious agents, as exemplified by influenza virus that mainly kills the very young or the aged. Immunosenescence affects multiple aspects of the immune system. DCs derived from blood or bone marrow from the elderly develop defects in their responses to inflammatory cytokines and their ability to present antigen and activate adaptive immune responses.[252] B-cell lymphopoiesis is reduced with aging, leading to a decline of naïve B cells and an increase of antigen-experienced B cells with an extended lifespan. Primary B-cell responses

in the elderly are commonly both low and short-lived, resulting in antibodies with low affinity.[253] Formation of germinal centers decreases, antigen transport is impaired, and follicular DCs have reduced capacity to form antigen depots.[254,255] Autoantibodies are more common, and the B-cell repertoire becomes more restricted. The E2A-encoded transcription factor E47 is downregulated in old splenic B cells, which causes a reduction in the activation-induced cytidine deaminase, needed for class switch recombination and Ig somatic hypermutation.[256] Some of the defects of B-cell responses are secondary to an age-related decline of helper functions from CD4+ T cells, which show reduced expression of critical costimulatory receptors that are essential for activation of B cells, germinal center formation and rearrangement, and hypermutation of Ig genes.[257,258,259] T cells show clonal senescence, their potential for expansion decreases, and their ability to produce certain cytokines or to respond to cytokines may become impaired. The proportion of T cells with a memory cell phenotype increases, while numbers of naïve T cells decrease, in part reflecting loss of thymic output following thymic involution, and in part resulting from chronic stimulation. Expression of activating molecules, such as CD40L and CD28 decreases,[260,261] while that of inhibitory pathways increase.[262,263] Stimulations with new antigens or previously encountered antigens result in CD8+ T-cell responses that are delayed and show defects in transition into memory. The delay in T-cell expansion reflects an intrinsic defect of aged T cells and may well be one of the reasons for increased susceptibility of the aged to rapidly replicating pathogens. Upon aging, the T-cell repertoire loses diversity.[264,265] Chronic antigenic stimulation leads to continued clonal expansion of some T cells. The effect of aging on cytokine production remains controversial.[264,265,266,267] The number of T_{reg} cells appears to increase with age,[268] while autoimmunity is more common, suggesting that inhibitory pathways may become dysregulated during aging.

AUTOIMMUNITY AND DEGENERATIVE DISEASE

Viral infection can lead to the development of autoimmunity. Some viruses carry antigens with superantigen activity, such as the nucleoprotein of rabies virus or nef of HIV-1.[269,270] Superantigens trigger polyclonal activation of T cells and can thus lead to stimulation of self-reactive T cells.

Some viruses carry sequences that resemble those of their host. For example, a sequence in coxsackie B virus is homologous to one in glutamic acid decarboxylase, a protein expressed by insulin-producing pancreatic islet cells. Coxsackie B virus infections can trigger in animals antibodies to glutamic acid decarboxylase, which can cause diabetes.[271] The polymerase of HBV mimics myelin basic protein,[272] and may thus, in theory, stimulate an immune response that could cause multiple sclerosis.

Nevertheless, thus far antigenic mimicry of a self-protein has not been confirmed to be causative for human autoimmune diseases.

Strong inflammatory responses elicited by a virus can cause concomitant activation of immune responses to selfproteins. For example, infections of mice with Theiler virus can elicit T cells recognizing myelin basic protein or proteolipid protein causing neurologic damage.[273] Coxsackievirus B3 can infect the heart muscle and inflict initial damage; this can induce autoreactive lymphocytes against cardiac myosin,[274] which cause chronic myocarditis resulting in permanent heart damage and even death.

Herpesviruses have been implicated as etiologic factors in the pathogenesis of human arteriosclerosis by accumulating saturated cholesteryl esters and triacylglycerols to the endothelial walls of blood vessels.[275] This can be prevented by cytokines such as TNF-α or IL-1.[275]

CANCER

Viruses can cause cancer. Oncogenic types of HPV such as HPV-16 or -18 are causative for cervical[276] and head and neck cancer through the activity of two oncoproteins, E7 and E6, which disrupt the retinoblastoma (Rb) and p53 pathways, respectively. Chronic infections with HCV and HBV can lead to hepatocellular carcinoma, primate T-lymphotropic virus 1 can cause T-cell leukemia, EBV can result in lymphoma and nasopharyngeal carcinoma, Merkel cell polyoma virus is associated with Merkel cell carcinoma, and HHV-8 is associated with with Kaposi sarcoma.[277] Some types of viral cancers are common in the general population, while others arise primarily in immunocompromised individuals. The immune system, although able to prevent the establishment of some types of viral cancers, is in general ill suited to halt progression of an already established advanced cancer even if its cells express viral antigen, as tumors establish an immunosuppressive microenvironment that is effective at suppressing immune responses.[278]

Virus-specific T cells have found utility in treatment of cancer. EBV-specific T cells isolated from children with neuroblastoma were expanded in vitro and transduced with an artificial TCR composed of an antibody variable region with specificity to a surface antigen on the cancer linked to the intracellular signaling domain of the TCR. Upon transfer of these modified T cells back into the children, remission of some of the advanced cancers was achieved.[279]

IMMUNOEVASION BY VIRUSES

While cellular organisms evolved over the millennia to combat virus infections, first with a primitive innate immune system and then, about 410 million years ago, once jawed vertebrates developed with the more sophisticated adaptive immune system, viruses evolved in tandem to subvert their hosts' defense mechanisms. While small RNA viruses, which replicate and spread rapidly and, as a rule, do not establish chronic infections, attack mainly the early defense mechanisms, the more complex DNA viruses, especially those with large genomes, also subvert adaptive immune responses.

Escape by Hiding

The central nervous system is an immunologically privileged site hidden behind the blood-brain barrier that cannot be penetrated by antibodies or resting lymphocytes. It also contains cells such as neurons that lack MHC class I expression and can thus not be attacked by CD8+ T cells. Viruses, exemplified by rabies virus, take advantage of this safe haven. Herpesviruses after an acute lytic infection assume a stage of latency where most viral synthesis is shut off. HSV-1 establishes latent infections in neurons, where it is sheltered from attack by CD8+ T cells, while EBV remains more exposed in B cells. The only protein of EBV that is expressed during deep latency is EBNA-1, which contains a glycine-alanine repeat domain that appears to protect the antigen from proteasomal breakdown and thus from the display of antigenic peptides on MHC molecules.[280]

Escape through Mutation

Viruses can evade by rapid mutations caused by the infidelity of their replication machinery that lacks the proofreading capacity of mammalian polymerases. Although lack of faithful genome replication is costly and many of the resulting viruses lack the fitness of the parent virus, it serves the virus population as a whole, which produces thousands of new infectious viruses from each original virus and can thus afford to lose some due to defective genomes. Mutations are primarily driven by neutralizing antibodies through selection of variants with mutations of their surface molecules. Most binding sites for neutralizing antibodies are on exposed areas on the very top of viral surface proteins, which do not contribute to the assembly of the virus and as such are not essential for the virus. Nevertheless, such mutations can affect viral fitness. Mutations of the HA of influenza virus can increase the

affinity to its receptor, which makes it difficult for the virus to gain release from the cell where it replicated. Influenza viruses compensate by additional mutations that readjust the affinity between HA and its receptor.[281] Some neutralization sites are located in domains that are essential for the virus and that cannot be mutated without devastating consequences. Neutralizing antibodies can bind to the stalk of the HA of influenza virus.[282] This antibody-binding site is highly conserved in several strains, indicating that it is crucial for some aspect of the life cycle of the virus. Nevertheless, this site is so well hidden that most humans fail to produce antibodies against it. HIV-1 is a master in escaping neutralizing antibodies. HIV-1 originated from SIVcpz, which is endemic in common chimpanzees, *Pan troglodytes troglodytes*, although it can spill over into gorillas. It has been calculated that humans acquired this virus sometimes at the beginning of the last century around 1908 on three occasions from chimpanzees and in one event potentially from a gorilla, resulting in infections with three groups of HIV-1 (ie, M, N, and O).[283] Group M was the most

successful and within a mere hundred years evolved into several clades and multiple subspecies. Even in a new host, where a few founder viruses establish an infection, viral variants evolve so rapidly that the humeral immune system is hard pressed to keep up.

T cells can select for mutations in the generally more conserved internal proteins. This is typically found during choric infections such as with HIV-1 or HCV,[283,284] but can also happen in acute infection, as exemplified by influenza virus.[285]

Antiviral cytokines, such as IFNs, can select for mutants. The only approved treatment for chronic infections of HCV is IFN-α, which results in complete viral clearance in only a fraction of patients. In many patients, the virus escapes through mutations that increase its resistance to IFN-α.[286]

Escape by Destruction of Immune Cells

An efficient way to stage a hostile takeover is to eliminate a crucial component of the opponent's armor. HIV-1 infects and eventually kills CD4+ T cells, which are essential to drive B and CD8+ T cell responses. HIV-1 preferentially replicates in activated CD4+ T cells as resting CD4+ T cells express the antiretroviral deoxycytidine deaminase APOBEC3G,[287] which through editing C to U nucleotide base exchanges inhibits elongation of the HIV-1 genome. To ensure an ample supply of activated CD4+ T cells, HIV-1 rapidly depletes gut-residing CD4+ T cells, causing intestinal hyperpermeability and leakage of bacterial products into the organism, which in turn nonspecifically activates more CD4+ T cells.

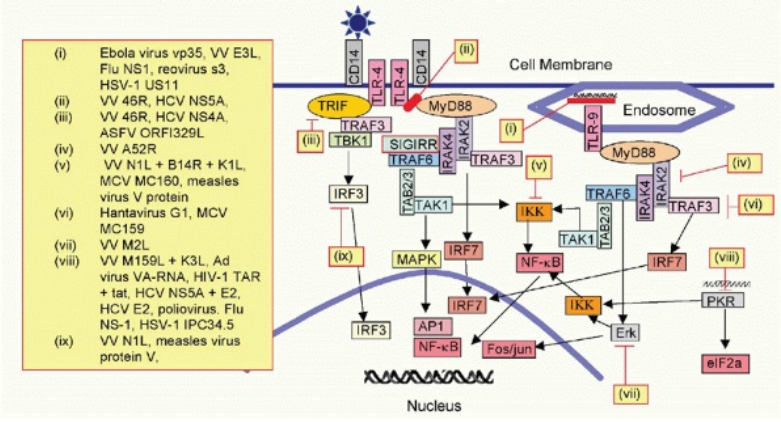

FIG. 39.7. Viral evasion of toll-like receptor signaling: (*i*) to (*ix*) indicate points of interactions between viral antigens and components of the pathways.

Escape from Early Innate Immune Responses

Inhibition of Pattern Recognition Receptor Signaling

The invention of stealth technology should be credited to viruses, which long ago figured out that flying under the radar (ie, dodging initial recognition by PRRs) promotes their own ability to propagate. Many viruses antagonize recognition by PRRs either by sheltering products that can be sensed or by directly interfering with components of PRR signaling cascades.

An example of sheltering recognizable patterns is the Ebola virus vp35. It binds viral dsRNA in a rather peculiar manner; one molecule binds the backbone of the RNA, whereas the other caps the terminus, thus effectively blocking dsRNA binding to TLR3, MDA5, RIG-I, or PKR.[288] Similar dsRNA hiding mechanisms are employed by vaccinia virus E3L, influenza virus NS1, reovirus σ protein, and HSV-1 US11.[289]

Several viruses interfere with TLR signaling. Vaccinia virus A46R and HCV NS5A bind to the TIR domain of MyD88, thus preventing activation of most TLRs. Vaccinia virus A46R, HCV NS4A, and ORFI329L of African swine fever virus[290] bind TRIF thus blocking stimulation through the alternative TLR4 pathway. Hantavirus G1 and MC159 from molluscum contagiosum virus (MCV) block TRAF3.[291] Vaccinia virus A52R binds IRAK2 thereby inhibiting signaling to TRAF6. Of note, IRAK2 operates redundantly with IRAK1 (see Fig. 39.1) in the initial response but continues to function after IRAK1 disappears (Fig. 39.7).

A number of viral proteins target sensors in the cytoplasm (Fig. 39.8). Influenza virus NS1 binds and blocks RIG-I.

Protein V of paramyxoviruses binds MDA5 and inhibits access of ssRNA. Picornaviruses, such as poliovirus, cause proteasome-mediated degradation of MDA5. IPS1, which is downstream of RIG-I or MDA5, is cleaved by HCV NS3-4A or hepatitis A virus 3ABC. The ATP-dependent DEAD box RNA helicase, DDX3, is part of the RIG-I signaling complex. By binding to RIG-I-associated IPS-1, DDX3 can bind viral RNA and as such enhance its recognition by RIG-I. Vaccinia virus K7, HCV core protein, or HBV polymerase can bind DDX3 and inhibit its association with cellular proteins.[289] Kaposi sarcoma-associated herpesvirus (KSHV) Orf63 is an inhibitory homolog of NLRP1.[291]

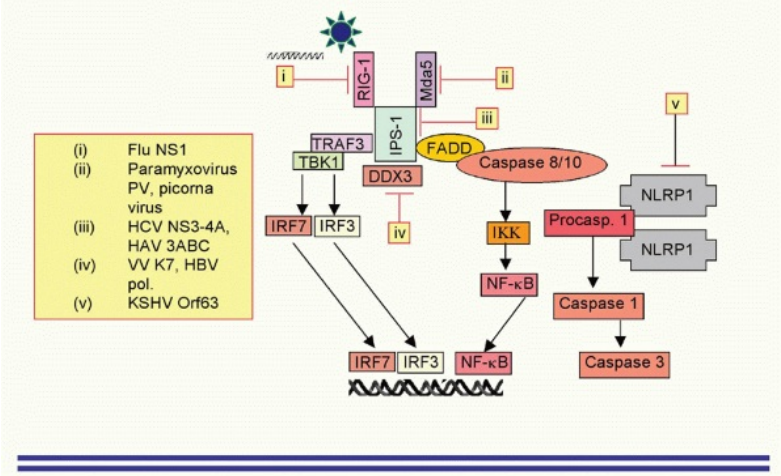

FIG. 39.8. Inhibition of retinoic acid-inducible gene I signaling by viral antigens: (*i*) to (*v*) indicate points of interactions between viral antigens and components of the pathways.

Multiple proteins of vaccinia virus interfere with NF-κB or IRF3 activation. Vaccinia virus N1L

associates with several components of the multisubunit IKK-B kinase complex, including the TBK1 and thus inhibits activation of both NF-κB and IRF3 pathways.[292] B14R of vaccinia virus binds the IKK complex and prevents phosphorylation of Iκκβ,[293] K1L inhibits NF-κB activation by blocking degradation of IκBα,[294] and M2L downregulates ERK-mediated NF-κB induction.[295] Another poxvirus, MCV, encodes MC160, which induces IKK1 degradation[296] and M159L, which inhibits PKR signaling.[297] Measles virus V protein can bind Iκκα and thus prevent phosphorylation of IRF7. It can also prevent phosphorylation of IRF3 by acting as an IRF3 mimetic[298] (see Fig. 39.7).

KSHV encodes an immediate-early nuclear transcription factor that promotes the ubiquitination and degradation of IRF7[299] and another protein K-bZIP, which binds DNA and competes with IRF3 binding.[300] ICP10 from bovine herpesvirus, protease N from flaviviruses, E6 of HPV-16, HIV-1 Vpr, and Vif cause degradation of IRF3. NSP1 of rotavirus causes destruction of IRF5 and 7.[289] Rabies virus protein P prevents phosphorylation of IRF3.[301] Papain-like protease of SARS-CoV blocks the nuclear transport of IRF3.[302]

PKR, once activated by dsRNA, inhibits viral protein synthesis and as such is another favorite target for viruses in their zest to optimize their chance for replication. The VA-RNA of adenoviruses can serve as a decoy; it binds PKR but does not trigger signaling.[303] Similarly, the TAR RNA of HIV-1's long terminal repeat, which initiates tat-mediated activation of transcription, can block PKR activation.[304] Tat is phosphorylated by PKR and outcompetes phosphorylation of eIF2α. HCV NS5A prevents dimerization of PKR.[305] Vaccinia virus K3L[306] and HCV E2[307] prevent interactions between PKR and eIF2α. Poliovirus activates a protease that destroys PKR,[308] while influenza virus NS-1 activates the cellular PKR inhibitor p38 to prevent PKR dimerization.[309] HSV-1 protein IPC34.5 activates a cellular phosphatase that dephosphorylates PKR and eIF2α.[310]

Some viruses manipulate PRR signaling pathway to create a more favorable environment. Vaccinia virus A52R blocks IRAK2 but allows signaling through TRAF6, which activates MAPKp38 and JNK leading to the induction of IL-10, which in turn suppresses Th1-type immune

responses.[311] The ATP-dependent DEAD box RNA helicase DDX3 interacts with rev of HIV-1 and enhances nuclear export of viral RNA. HCV utilizes DDX3 for its replication possibly by taking advantage of DDX3's ability to unwind dsRNA.[312]

Inhibition of Apoptosis

Inhibition of NF-κB, activation of PKR, or signaling through TNF receptors can induce apoptotic pathways. Apoptosis can also be initiated in response to cell damage. Although many viruses eventually cause death of the infected cells, premature apoptosis before the virus replication has been completed would be counterproductive and viruses therefore devised mechanisms to block apoptosis pathways (Fig. 39.9).

Apoptosis can be triggered through members of the TNF-receptor family. TNF receptors carry an intracellular receptor domain that contains a death effector domain (DED), which binds to corresponding DEDs on adaptor proteins such as Fas-associated protein with death domain. Upon ligation of the extracellular part of the receptor, the cytoplasmic tail binds to the death inducing signal complex containing procaspase 8, which is then cleaved into caspase 8. Caspase 8 activates caspase 3, which induce cells death. Cellular FLICE-like inhibitors can inhibit caspase 8 activation by competitive binding to the death inducing signal complex. Inhibition of the AKT/PI3K pathways or p53 can lead to dephosphorylation of the Bcl-2-associated death promoter, which forms a heterodimer with Bcl-2-associated X protein. This

complex can increase the permeability of mitochondrial walls. Consequently, cytochrome C is released from mitochondria, which forms a complex with apoptotic protease activating factor 1 (APAF1) and procaspase 9. Procaspase 9 is cleaved into an active form and then activates caspase 3. This apoptosis pathway can be blocked by Bcl-2.

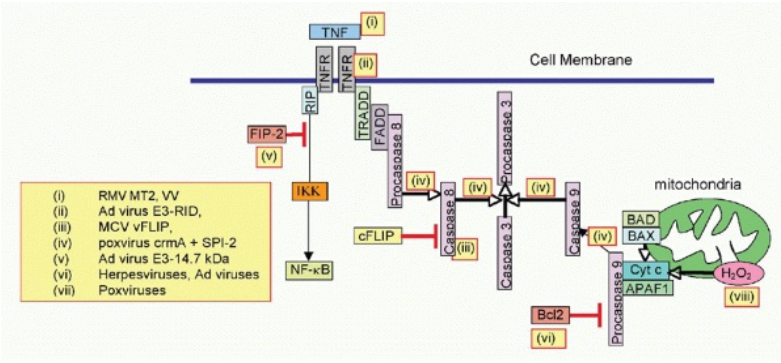

FIG. 39.9. Viral interference with apoptotic pathways: (*i*) to (*vii*) indicate points of interactions between viral antigens and components of the pathways. *Cyt c*, cytochrome C.

Poxviruses encode proteins that subvert TNF-receptor signaling. MT2 of rabbit myxomavirus sequesters TNF-α, and vaccinia virus encodes a number of TNF-receptor homologs, which can be secreted or expressed on cell surfaces.[313] The adenovirus E3 gene product RID induces internalization and degradation of TNF-receptor family members.[314]

Several herpesviruses and MCV prevent caspase 8 activation through a viral FLICE inhibitory protein.[315,316] Some poxviruses produce serine protease inhibitor-like molecules called crmA (cowpox) or SPI-2 (ectromelia virus), which contain DED domains and inhibit activation of caspases.[316,317] The adenovirus E3 14.7 kDa protein inhibits caspase 8 through a yet-to-be-identified pathway upon binding to the host protein FIP-2.[318] A number of herpesviruses and adenoviruses increase antiapoptotic pathways by encoding a Bcl-2 homolog.[319]

Cells infected with viruses can undergo a stress response with accumulation of reactive oxygen species, which in turn increase mitochondrial permeability and induce apoptotic cell death. Cellular enzymes, such as glutathione peroxidase, a selenocysteine containing protein, can reduce H_2O_2. Poxviruses encode a homologous protein.[320] The p28 of ectromelia virus (mousepox) and the N1R of Shope fibroma virus have a RING finger motif that inhibits apoptosis induced by UV light.[321]

Inhibition of Cytokines/Chemokines

Manipulation of PRR signaling reduces production of proinflammatory cytokines. Viruses have developed additional strategies to inhibit production and effector activities of cytokines and chemokines, which may interfere with their replication.

Interferons. IFNs and IFN-induced proteins have potent antiviral activity and are thus targeted by numerous viruses. IFN-1, upon binding to its receptor, activates Jak1 and Tyk2. This causes phosphorylation of Stat1 and Stat2, which form heterodimers, translocate to the nucleus, and associate with IRF9 to form the transcription factor complex ISGF3. This complex initiates transcription of IFN-stimulated genes (ISGs). The binding of IFN-γ to its receptor causes complex-formation between Jak1 and Jak2. This causes phosphorylation of Stat1, which forms homodimers, which then translocates to the nucleus to interact with

gamma activated sequence elements in promoters.

Poxviruses encode proteins that are secreted from infected cells and bind with high-affinity type I or II IFN receptors, thus preventing those neighboring, not-yet-infected cells from becoming resistant. They also produce molecules that mimic the receptors for IFNs and thus soak up IFN molecules to prevent their binding to cellular receptors.[322]

Rabies virus P protein blocks translocation of phosphorylated Stat1 to the nucleus, and in the nucleus binds to Stat1/2-IRF9 complexes thus preventing activation of transcription.[323] SARS-CoV ORF6 binds to nuclear transport proteins thus blocking transport of Stat1.[324] Stat2 is targeted for destruction by Dengue virus NS5[325] or RSV NS1.[326] Stat1 can be degraded by a cellular ubiquinase induced by paramyxoviruses.[327]

IFN induces synthesis of over 300 proteins, many of which have antiviral or immunomodulatory functions. One of those is tetherin, which can prevent the cellular egress of viruses such as lyssaviruses, KSHV, HIV-1, or Ebola virus. KSHV K5 or HIV-2 Vpu ubiquinate tetherin and thereby initiate its degradation.[328] ISG15 is an IFN-induced ubiquitin-like protein, which upon binding to proteins causes their degradation through an ubiquitin-independent pathway in a process called ISGylation. As ISG15 has broad antiviral activity and increases resistance to numerous viruses, many have devised avenues to subvert its activity (341). Influenza B virus NS1 sequesters ISG15,[329] Crimean Congo hemorrhagic fever virus, a member of the *Bunyaviridae* family, encodes proteins with de-ISGylating activity, which removes ISG15 from its target.[330]

MX-1 is another IFN-induced protein that in mice is critical for recovery from influenza A virus infection. A human homolog MxA has broad antiviral activity against RNA viruses and DNA viruses with an RNA intermediate.[331] The functions of Mx1 or MxA are not yet fully understood, but the proteins seem to bind capsid or nucleoproteins early after infection, thus somehow blocking virus replication. A rather old-fashioned mechanism allows some influenza A viruses to escape control by Mx1; they grow so fast that they can outrun the IFN-induced production of Mx1.

Other Cytokines. Parapoxvirus, which infects sheep and goats, as well as EBV, encodes an IL-10-like protein that inhibits synthesis of IL-12 and thus generation of Th1 immune responses.[332,333] Similarly, the UL111A ORF of HCMV has sequence homology with IL-10.[334] IL-18-binding proteins are encoded by poxviruses, such as MCV, ectromelia virus, cowpox, or vaccinia viruses.[335] KSHV encodes an IL-6-like protein.[336] The poxvirus-encoded GIF binds to both granulocyte M-CSF and IL-2.[337] EBV encodes BARF1, a M-CSF-binding protein.[338] Vaccinia virus encodes two proteins (ie, B15R and B18R), which bind and inhibit IL-1β.[339] The C10L protein of vaccinia virus encodes a protein that blocks the IL-1 receptor.[340] IL-1β is synthesized as a precursor that only gains functional activity upon cleavage by caspase 1. The same caspase also cleaves the precursor molecule of IL-18. Cowpoxvirus encodes a serine protease inhibitor called crmA that inhibits the function of caspase 1.[341]

Several viruses encode molecules that resemble those of the mammalian TNF-receptor family. HCMV encodes UL144, which is related to HVEM.[342] Shope fibroma virus and myxoma virus encode a single TNF-receptor homolog called T2,[343,344] whereas orthopoxviruses encode one to three different TNF-receptor homologues called crmB, crmC, crmD, and CrmE.[345] T2, crmB, and cmrD bind TNF-α and lymphotoxin A, while crmC binds only TNF-α. T2, crmC, and crmD TNF-α-mediated cell lysis. The E13 of ectromelia virus resembles CD30, another TNF-receptor family member, and can bind and inhibit CD30L signaling, which under physiologic conditions can support DC maturation.[346]

Chemokines Chemokines direct trafficking of leukocytes and are essential to initiate an inflammatory response by attracting a cellular infiltrate. Chemokines are divided into four classes depending on the spacing of their first two cysteine residues (ie, C CC, CXC, and CX3C). Chemokines are referred to as ligands (L), which bind to correspondingly named receptors (R). There are a total of 27 CC chemokines, 17 CXC chemokines, 2 C chemokines, and 1 CX3C chemokine. Viruses manipulate chemokine functions by producing decoy chemokines or chemokine receptors or by secreting chemokine-binding proteins.

Viral chemokines can be agonistic or antagonistic. Examples of agonists are tat of retroviruses, which binds CCR2/3,[347] herpesvirus UL146, a CXCR2 binder,[348] and murine CMV m131. MC148R of molluscipox virus and K4 of HHV8 are CCR antagonists.[349,350] Marek disease virus produces a viral IL-8, which recruits granulocytes.[351] UL146 and 147 of HCMV also encode an IL-8 like molecule.[352]

Herpes and poxviruses encode numerous members of the chemokine receptor family that serve as decoys for chemokines. UL33, UL78, US27, and US28 of HCMV,[353] U12 and U51 of HHV6/7,[354] and M78 and M33 of murine CMV show homology with CCR receptors.[355] ORF74 of several herpesvirus strains mimics CXCR2.[356] K2R of is a homolog of a CXCR[357] whereas MC148 protein of molluscipox virus shows similarity with CCR8.[358]

Poxviruses and herpesviruses produce secreted viral chemokine proteins, which prevent binding of chemokines to their receptors. MT-1 of myxoma, B29R of vaccinia virus, and a 35 kDa protein of Shope fibroma virus inhibit CC chemokines.[359,360,361] M3 of murine gamma-herpesvirus 68 inhibits all four classes of chemokines.[362]

Viruses, by enhancing the effects of some chemokines while inhibiting others, presumably create a microenvironment that is best suited for their propagation. They bias the immune response so that it is only marginally harmful to

virus-producing cells, by, for example, recruiting eosinophiles and Th2 cells and excluding Th1 cells, or by secreting IL-8, which can block the activity of type I IFN. They may also selectively recruit cells that are permissive for viral replication, such as CMV, which through production of viral IL-8 recruits neutrophils so that they can then become infected.[363]

Inhibition of Complement Activation

The complement system is composed of more than 25 proteins that are sequentially activated through three distinct pathways called the classical, alternative, and lectin pathways. The classical pathway is activated once a pathogen or an antigen-antibody complex binds to C1q, which forms the C1 complex with C1r and C1s. Conformational changes of C1q activate C1r, which cleaves C1s into a protease, which in turn cleaves C2 and C4 into a and b subunits. C4b and C2a bind and form C3 convertase, which cleaves C3 into a and b. C3b binds the C3 convertase forming C5 convertase, which cleaves C5. C5b, C6, C7, C8, and C9 form the membrane attack complex, which initiates cell lysis. In the alternative complement pathway, C3 is spontaneously split into C3a and C3b. C3b can bind to a cell surface-expressed antigen from a pathogen and then also to factor B, which, in presence of factor D, is cleaved into Ba and Bb. The C3bBb complex can act as a C3 convertase. In the lectin pathway, mannan-binding lectin on the cell surface binds mannose on the surface of pathogens. This activates mannan-binding lectin-associated serine protease-1 and -2, which can cleave C4 and C2 thus allowing formation of C3 convertase. The complement pathways, which can be very damaging once fully activated, are tightly controlled by regulators of complement activation, which acts at three key points. C1 inhibitors block spontaneous activation of C1 and bind C1 in serum. Decay accelerating factor (CD55) and CR1 destroy C3 convertase. C4 binding protein and factor H promote dissociation of C4 or C3 subunits from complement complexes. They can also activate factor I, which together with its cofactor CD46 cleaves

C3b and C4b. Polymerization of C9 into the final membrane attack complex is blocked CD59 and homologous restriction factor.

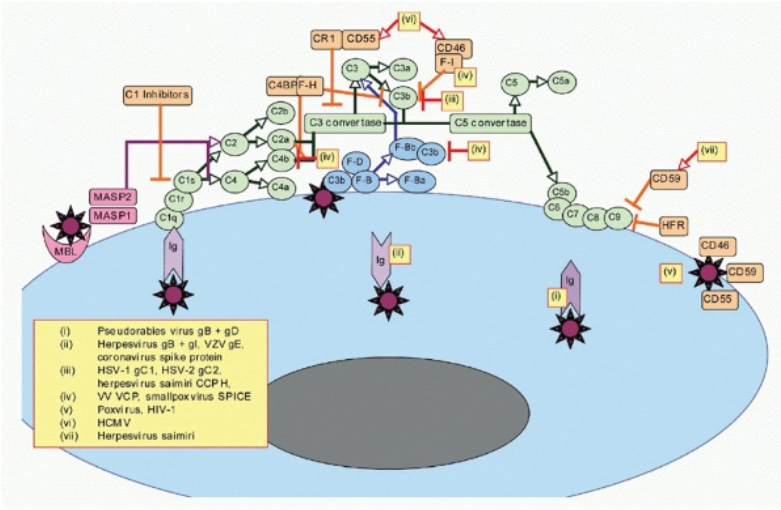

FIG. 39.10. Virus-Mediated Inhibition of Complement Pathways. The classical complement pathway is shown in *green*, the alternative complement pathway is shown in *blue*, and the lectin pathway is shown in *pink*; physiological inhibitors are shown in *orange*; *Ig*, virus-specific antibody. (*i*) to (*vii*) indicate compenents of the pathway that are inhibited or enhanced by viral antigens.

Complement can alter the structure of viruses, together with antibodies lyse viruses or virus-infected cells, and promote phagocytosis. Viruses have evolved three strategies to avoid destruction by complement: 1) they can interfere with activation of the classical pathway by shedding antigenantibody complexes from the surface of infected cells or by expressing Fc-receptor-like structures that complex antibodies that are bound to viral surface proteins; 2) they can encode complement inhibitors; and 3) they can incorporate host-derived complement inhibitors into their envelope (Fig. 39.10).

Herpesviruses and coronaviruses avoid recognition of antigen-antibody complexes by shedding such complexes from the surface of infected cells, by internalizing them, or by expressing Fc-receptor-like molecules. More specifically, gB and gD of pseudorabies virus are internalized once they bind antibodies.[364] The gE and gI of a number of herpesviruses,[365] the gE of varicella zoster virrs,[366] and the spike protein of some coronaviruses[367] resemble mammalian Fc receptors that nonspecifically bind Igs, thus sterically hindering binding of antibodies to their antigen on infected cells. Fc receptors present on an infected cell force antibodies bound with their variable region to an antigen to loop over and bind with their constant region to the Fc receptors, which prevents complement activation.

Viral complement inhibitors interfere with complement activation. HSV-1, HSV-2,[368] and herpesvirus saimiri[369] encode gC1, gC2, or CCPH, respectively, which bind C3b and inhibits formation or accelerates decay of C3 convertases. VCP of vaccinia virus[370] and SPICE of smallpoxvirus[371] bind C4b and C3b and serve as cofactors for factor I.

Enveloped viruses bud through the cell membrane at lipid rafts that are rich in cholesterol and other lipids and also contain glycosyl phosphatidyl inositol-anchored complement control proteins such as CD46, CD55, and CD59, which are incorporated into the membrane of the extracellular enveloped form of poxviruses[372] and HIV-1.[373] Furthermore, some viruses

directly increase numbers of complement regulatory proteins; HCMV augments cellular expression of CD55 and CD46,[374] while herpesvirus saimiri encodes a CD59 homolog.[375]

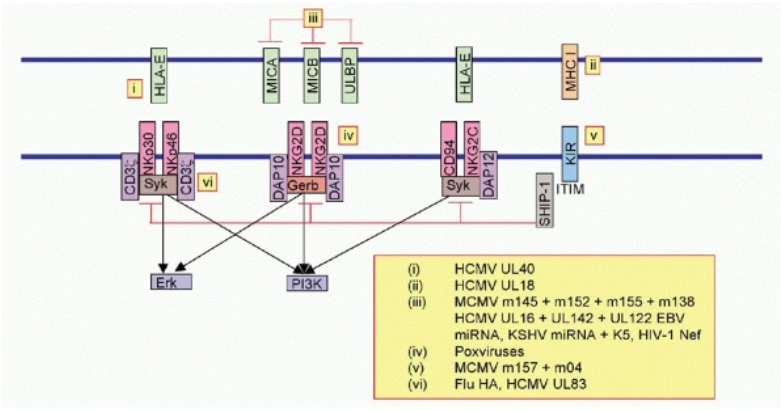

FIG. 39.11. Subversion of Natural Killer Cell Responses by Viruses. Activating receptors are shown in *pink*, inhibitory receptors in *blue*, and ligands in *green* and *orange*, respectively. (*i*) to (*vi*) indicate interactions with viral antigens and components of natural killer cells or their ligands.

Modulation of Natural Killer Cell Activity

Natural killer cells have antiviral functions through secretion IFN, which also promotes adaptive immune responses, and they can lyse virus-infected cells directly, especially if these cells downregulate MHC class I expression or if they bind antibody-dependent cellular cytotoxicity-inducing antibodies. Viruses thus evolved to kill natural killer cells, strengthen signals through the natural killer cell's inhibitory receptors, or weaken those through activating receptors (Fig. 39.11).

Influenza virus can infect natural killer cells and induce their apoptosis.[376] HCV E2 uses CD81, which is expressed on natural killer cells, as a receptor and attenuates its signaling.[377]

Herpesviruses reduce MHC class I expression, which would allow for activation of natural killer cells through loss of inhibitory signals. Herpesviruses solve this problem by ensuring expression of HLA-E, an MHC class I-like molecule that only presents the leader sequence of other MHC class I molecules and as such is not recognized by traditional CD8+ T cells, but by inhibitory receptors on natural killer cells. The signal sequence of UL40, an HCMV-encoded protein, carries a sequence that associates with HLA-E, thus allowing its translocation to the cell surface.[378] In addition, UL18 of HCMV is an MHC class I analog and binds inhibitory ILT2 (LIR1), but not the TCR of CD8+ T cells.[379] Flaviviruses take the opposite approach and augment MHC class I expression.[380]

Cellular stress responses can lead to expression of MHC class I chain-related genes, such as MICA/MICB and ULBP proteins in humans, and Rae-1, a likely RNA export protein,

H60, a minor histocompatibility antigen, and mouse UL16-binding protein-like transcript 1, an MHC class I-like molecule, which can be spotted by activating natural killer cell receptors, especially NKG2D.[381,382] Both HCMV and murine CMV encode proteins that downregulate the cellular ligands for NKG2D. Murine CMV proteins m145, m152, and m155 interfere with the expression of all known NKG2D ligands, whereas m138 affects the expression of mouse UL16-binding protein-like transcript 1 and H60. HCMV UL16 causes intracellular retention of MICB and ULBP, UL142 downregulates MICA, micro-RNA UL122 interferes with the

translation of MICB, as does the EBV encoded micro-RNA BART2-5b, and the KSHV encoded micro-RNA K12-7. KSHV also produces K5, which causes ubiquitination of MICA and MICB. HIV-1 nef down-regulates expression of MICA and ULBP.[383] Orthopoxviruses produce a secreted NKG2D ligand thus saturating the receptor.[384]

Ly49s are inhibitory receptors that recognize MHC class I molecules. MCMV encodes two proteins, m157 and m04, that can serve as ligands for inhibitory Ly49 receptors, specifically in murine CMV-resistant strains of mice; m157 is a ligand for the natural killer-activating Ly49H receptor; in susceptible strains of mice it binds the inhibitory Ly49A receptor.[385] NCR1-encoded NKp46 recognizes the HA of influenza virus. NKp46 and NKp30 receptors associate with the CD3 ζ-chain for signaling. Free HA can induce lysosomal ζ chain degradation thus blocking signaling.[386] HCMV UL83 uses a similar pathway and dissociates the ζ chain to block its recognition by NKp30.[387]

Viruses not only increase the strength of inhibitory signals to natural killer cells, but also interfere with activating receptors. K5 of KSHV downregulates AICL, a target for NKp80.[388] UL141 of HCMV sequesters CD155, the poliovirus receptor that is a target for DNAM-1.[389] UL16 of HCMV reduces expression of LFA-3, an adhesion molecule that binds to CD2 on natural killer cells and by unknown mechanisms increases the infected cells's resistance to natural killer cell-mediated lysis.[390]

Escape from Adaptive Immune Responses

Subversion of Antigen Presentation by Major Histocompatibility Complex Class II

Stimulation of CD4+ T cells, which requires presentation of epitopes on MHC class II, can be reduced by limiting the amount of antigen available for processing and presentation of by decreasing expression of MHC class II molecules on the cell surface.

Autophagy serves the cells to eliminate unwanted proteins from the cytoplasm by delivering them to lysosomes for degradation. This pathway is also used to destroy viruses and deliver viral peptides to compartments, where they associate with MHC class II molecules. The importance of autophagy in antiviral defense is underscored by its induction through signals from viral sensors, such as TLR4. Viruses, as described previously, interfere with such signals. Alpha- and gamma-herpesviruses through ICP34.5 and viral BcL-2, respectively, can directly block Beclin-1, a molecule needed for formation of autophagosomes.[391,392]

Herpesviruses, poxviruses, and HCV interfere with MHC class II expression. For example, gB of HSV-1 binds HLA-DR complexes in a post-Golgi department and then shuttles them into exosomes rather than allowing for their transfer to the cell surface.[393] A mutation of the precore protein of HBV has been linked to decreased levels of MHC class II on antigen-presenting cells, suggesting a potential evasion pathway,[394] and a variant in the HCV NS3 proteins has been shown to serve as an epitope antagonist by binding MHC class II molecules and blocking binding of the CD4+ T-cell epitope.[395] HCMV can impair MHC class II expression through two pathways. MHC class II transcription is tightly regulated, mainly through the class II transactivator (CIITA), a non-DNA-binding protein that interacts with transcription factors, which then associate with the MHC class II promoter. CIITA is rate-limiting factor for MHC class II production and thus determines the expression pattern of these molecules. Four promoters, two of which sponsor constitutive expression of CIITA in antigen-presenting cells, regulate CIITA production. One is activated by IFN-γ through the Jak/Stat1 pathway. Proteins of HCMV inhibit induction of CIITA by disrupting this pathway.[396]

Subversion of Antigen Presentation by Major Histocompatibility Complex Class I

Many viruses subvert presentation of their antigens by the MHC class I pathway, thus

reducing activation and effector functions of antigen-specific CD8+ T cells, again stressing the importance of this cell subset for antiviral defense. Nearly every step of the MHC class I presentation pathway can be affected, and some viruses encode multiple proteins that act at different levels of the MHC class I processing/presentation pathway (see Fig. 39.12).

Major Histocompatibility Complex Class I Synthesis. Viral gene products such as tat of HIV-1 can suppress transcription of MHC class I genes.[397]

Protein Degradation. EBNA-1 of EBV and LANA1 of KSHV carry leucine-alanine repeats that appear to block their degradation by proteasomes.[398] Once proteins are degraded, the aminopeptidases CD10 and CD13 trim them to a size that allows their binding to the groove of MHC class I molecules. HCMV blocks synthesis of CD10 and retains CD13 within the ER.[399]

Peptide Transport. Loading of MHC class I molecules depends of active transport of peptides from the cytoplasm to the ER with the help of TAP. The E7 of HPV-18 and the E1A protein of adenovirus human serotype 12 inhibit the promoter controlling TAP production. EBV encodes a viral IL-10 receptor homolog that reduces expression of TAP. The mK3 protein of mouse gamma-herpesviruses binds to TAP and tapasin resulting in their ubiquination and degradation. The 19K polypeptide encoded by the E3 domain of adenoviruses binds to TAP and prevents association between TAP and tapasin. US6, a protein encoded by HCMV, binds to TAP and prevents binding of ATP, which is needed for peptide transport. The ICP47 polypeptide from HSV inhibits TAP by blocking its peptide binding. EBV encodes

a protein that weakly binds the IL-10 receptor. Similar to cellular IL-10, the EBV protein reduces expression of TAP.[400] BNLF2a, a lytic phase protein of EBV is anchored by its C-terminal tail to the ER membrane, while its cytosolic N terminus inhibits TAP.[401]

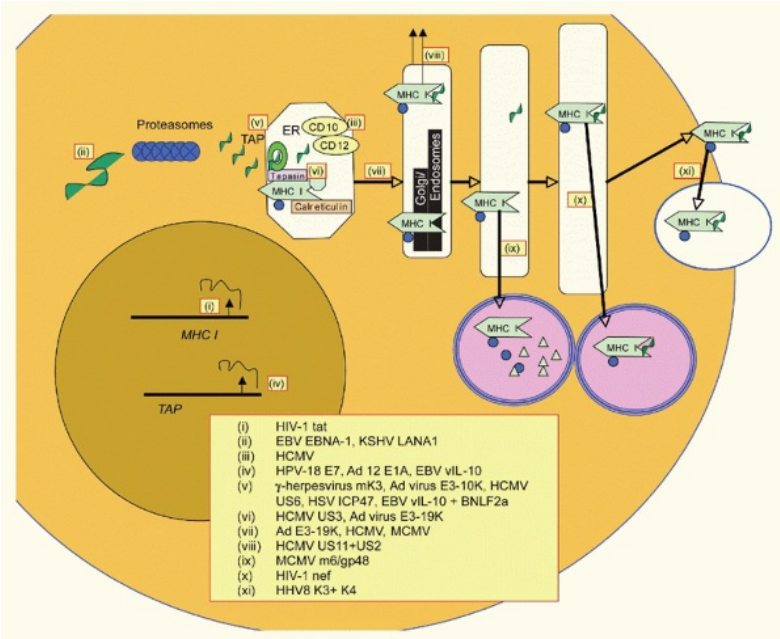

FIG. 39.12. Inhibition of Major Histocompatibility Complex Class I Processing and Presentation of Endogenously Produced Proteins by Viral Antigens. (*i*) to (*xi*) indicate at what points the pathway is attacked by viral antigens.

Major Histocompatibility Complex Class I Peptide Loading. Loading of peptides to MHC class I is supported by tapasin, which stabilizes the molecule and prevents binding of self-

peptides. US3 of HCMV binds and inhibits tapasin.[401] E3-19K of adenovirus blocks the formation of the TAP-tapasin complex.[402]

Major Histocompatibility Complex Class I Transport. Once MHC class I molecules have bound peptides, they are transported to the cell surface. A number of viral products interfere with this transport by either retaining MHC class I molecules within the ER, by inducing their export into the cytoplasm, or by interfering with passage through the Golgi apparatus. The E3-19K protein of adenovirus[402] and proteins of HCMV and murine CMV prevent the exit of MHC class I from the ER and from pre-Golgi compartments.[403] US11 and US2 of HCMV dislocate the MHC class I molecules from the ER to the cytoplasm, where they are rapidly degraded. The m6/gp48 protein of MCMV binds to MHC class I-β2-microglobulin in the ER and during transport to the cell surface, targets the MHC class I complexes to lysosomes, where they are proteolytically digested.[404] Nef of HIV-1 reduces cell-surface expression of MHC class I by redirecting the molecules from the trans-Golgi network to the endosomes.[405] The K3 and K4 proteins of HHV8 lower expression of cell surface MHC class I molecules by enhancing their endocytosis into clathrin-coated pits.[406]

Escape from Antibodies

To summarize, viruses escape antibody-mediated neutralization mainly by mutations; they evade destruction by antibody and complement by interfering with activation of complement, and they dodge antibody-dependent cellular cytotoxicity by masking antigen display on cell surfaces and by inhibiting natural killer cell functions. All of these pathways have been described previously. EBV directly subverts B cells, where it resides during latency and where it can be transformed through the viral oncoprotein LMP1, a molecule that has functional homology with CD40 and can activate the NF-κB pathway. Unlike CD40 signaling, which requires ligation with CD40L, LMP1 is constitutively active, although one would assume that it is negatively regulated by yet unknown pathways as it would otherwise inevitably lead to B-cell transformation.

CONCLUSION

Viruses continue to raise havoc. Each year, millions of humans die prematurely due to viral infections and many more become temporarily incapacitated. Vaccines licensed for a handful of viruses and antiviral drugs available for even fewer have lessened the impact of some viral infections, but for most the immune system remains our only defense. Our knowledge in basic immunology has exploded over that last century. Although early vaccines were developed without knowing anything about immunology, but as it is stated in the “Míshlê Shlomoh,” the Book of Proverbs: *‘scientia potentia est*,” knowledge is power. A better understanding of the intricate pathways that regulate the interactions between viruses and their hosts' immune system should empower us to eventually win this eternal battle and lessen human suffering caused by something as small but as deadly as a bit of invasive genome with a few proteins around it.

REFERENCES

1. Woolhouse ME, Howey R, Gaunt E, et al. Temporal trends in the discovery of human viruses. *Proc Biol Sci.* 2008;275:2111-2115.

2. Mahy BWJ, van Regenmortel MHV, eds. *Desk Encyclopedia of General Virology* . Oxford, United Kingdom: Academic Press; 2009.

3. Koonin EV, Senkevich TG, Evolution of thymidine and thymidylate kinases: the possibility of independent capture of TK genes by different groups of viruses. *Virus*

Genes. 1992;6:187-196.

4. Poon LL, Guan Y, Nicholls JM, et al. The aetiology, origins, and diagnosis of severe acute respiratory syndrome. *Lancet Infect Dis.* 2004;4:663-671.

5. *ICTV Virus Taxonomy.* 2009. www.ictvonline.org/virusTaxonomy.asp?version=2009. Accessed January 31, 2011.

6. Wawer MJ, Gray RH, Sewankambo NK, et al. Rates of HIV-1 transmission per coital act, by stage of HIV-1 infection, in Rakai, Uganda. *J Infect Dis.* 2005;191:1403-1409.

7. Langley R, Morris T. That horse bit me: zoonotic infections of equines to consider after exposure through the bite or the oral/nasal secretions. *J Agromedicine.* 2009;14:370-381.

8. Maier T, Schwarting A, Mauer D, et al. Management and outcomes after multiple corneal and solid organ transplantations from a donor infected with rabies virus. *Clin Infect Dis.* 2011;50:1112-1119.

9. Zambon MC. The pathogenesis of influenza in humans. *Rev Med Virol.* 2001;11:227-241.

10. Liu X, Fitzgerald K, Kurt-Jones E, et al. Herpesvirus tegument protein activates NF-kappaB signaling through the TRAF6 adaptor protein. *Proc Natl Acad Sci U S A.* 2008;105:11335-11339.

11. Cicala C, Arthos J, Fauci AS. HIV-1 envelope, integrins and co-receptor use in mucosal transmission of HIV. *J Transl Med.* 2010;9(suppl 1):S2.

12. Benkirane M, Jin DY, Chun RF, et al. Mechanism of transdominant inhibition of CCR5-mediated HIV-1 infection by ccr5delta32. *J Biol Chem.* 1997;272:30603-30606.

13. Cai G, Freeman GJ. The CD160, BTLA, LIGHT/HVEM pathway: a bidirectional switch regulating T-cell activation. *Immunol Rev.* 2009; 229:244-258.

14. Whitbeck JC, Peng C, Lou H, et al. Glycoprotein D of herpes simplex virus (HSV) binds directly to HVEM, a member of the tumor necrosis factor receptor superfamily and a mediator of HSV entry. *J Virol.* 1997;71:6083-6093.

15. Bergelson JM, Cunningham JA, Droguett G, et al. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science.* 1997;275:1320-1323.

16. Segerman A, Atkinson JP, Marttila M, et al. Adenovirus type 11 uses CD46 as a cellular receptor. *J Virol.* 2003;77:9183-9191.

17. Naniche D, Varior-Krishnan G, Cervoni F, et al. Human membrane cofactor protein (CD46) acts as a cellular receptor for measles virus. *J Virol.* 1993;67:6025-6032.

18. Bergelson JM, Chan M, Solomon KR, et al. Decay-accelerating factor (CD55), a glycosylphosphatidylinositol-anchored complement regulatory protein, is a receptor for several echoviruses. *Proc Natl Acad Sci U S A.* 1994;91:6245-6248.

19. He Y, Bowman VD, Mueller S, et al. Interaction of the poliovirus receptor with

poliovirus. *Proc Natl Acad Sci U S A.* 2000;97:79-84.

20. Kowalski M, Potz J, Basiripour L, et al. Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. *Science.* 1987;237:1351-1355.

21. Gastka M, Horvath J, Lentz TL. Rabies virus binding to the nicotinic acetylcholine receptor alpha subunit demonstrated by virus overlay protein binding assay. *J Gen Virol.* 1996;77(pt 10):2437-2440.

22. Pileri P, Uematsu Y, Campagnoli S, et al. Binding of hepatitis C virus to CD81. *Science.* 1998;282:938-941.

23. Greve JM, Davis G, Meyer AM, et al. The major human rhinovirus receptor is ICAM-1. *Cell.* 1989;56:839-847.

24. Boyd AW, Fecondo J. Role of the CD21 antigen in lymphocyte transformation by Epstein-Barr virus. *Immunol Cell Biol.* 1988;66(pt 2):159-165.

25. Alvarez CP, Lasala F, Carrillo J, et al. C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in trans. *J Virol.* 2002;76:6841-6844.

26. Tassaneetrithep B, Burgess TH, Granelli-Piperno A, et al. DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J Exp Med.* 2003;197:823-829.

27. Pöhlmann S, Zhang J, Baribaud F, et al. Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. *J Virol.* 2003;77:4070-4080.

28. Nishimura Y, Shimojima M, Tano Y, et al. Human P-selectin glycoprotein ligand-1 is a functional receptor for enterovirus 71. *Nat Med.* 2009;15:794-797.

29. Tatsuo H, Ono N, Tanaka K, et al. SLAM (CDw150) is a cellular receptor for measles virus. *Nature.* 2000;406:893-897.

30. Radoshitzky SR, Abraham J, Spiropoulou CF, et al. Transferrin receptor 1 is a cellular receptor for New World haemorrhagic fever arenaviruses. *Nature.* 2007;446:92-96.

31. Mercer J, Schelhaas M, Helenius A. Virus entry by endocytosis. *Annu Rev Biochem.* 2010;79:803-833.

32. Poste G. Mechanisms of virus-induced cell fusion. *Int Rev Cytol.* 1972;33: 157-252.

33. Hinz A, Galla HJ. Viral membrane penetration: lytic activity of a nodaviral fusion peptide. *Eur Biophys J.* 2005;34:285-293.

34. Brabec M, Schober D, Wagner E, et al. Opening of size-selective pores in endosomes during human rhinovirus serotype 2 in vivo uncoating monitored by single-organelle flow analysis. *J Virol.* 2005;79: 1008-1016.

35. Tanida I. Autophagy basics. *Microbiol Immunol.* 2011;55:1-11.

36. Taylor MP, Kirkegaard K. Potential subversion of autophagosomal pathway by picornaviruses. *Autophagy.* 2008;4:286-289.

37. Stamminger T. Interactions of human cytomegalovirus proteins with the nuclear transport machinery. *Curr Top Microbiol Immunol.* 2008; 325:167-185.

38. Aparicio O, Carnero E, Abad X, et al. Adenovirus VA RNA-derived miRNAs target cellular genes involved in cell growth, gene expression and DNA repair. *Nucleic Acids Res.* 2010;38:750-763.

39. Kaariainen L, Ranki M. Inhibition of cell functions by RNA-virus infections. *Annu Rev Microbiol.* 1984;38:91-109.

40. Snejdarova V, Vonka V, Kutinova L, et al. The nature of adenovirus persistence in human adenoid vegetations. *Arch Virol.* 1975;48:347-357.

41. Johnson N, Fooks A, McColl K. Human rabies case with long incubation, Australia. *Emerg Infect Dis.* 2008;14:1950-1951.

42. ter Meulen V, Katz M, Muller D. Subacute sclerosing panencephalitis: a review. *Curr Top Microbiol Immunol.* 1972;57:1-38.

43. Janeway CA Jr. How the immune system protects the host from infection. *Microbes Infect.* 2001;3:1167-1171.

44. Kumar H, Kawai T, Akira S. Toll-like receptors and innate immunity. *Biochem Biophys Res Commun.* 2009;388:621-625.

45. Kurt-Jones EA, Popova L, Kwinn L, et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol.* 2000;1:398-401.

46. Okumura A, Pitha PM, Yoshimura A, et al. Interaction between Ebola virus glycoprotein and host toll-like receptor 4 leads to induction of proinflammatory cytokines and SOCS1. *J Virol.* 2011;84:27-33.

47. Zhang SY, Jouanguy E, Ugolini S, et al. TLR3 deficiency in patients with herpes simplex encephalitis. *Science.* 2007;317:1522-1527.

48. Wang T, Town T, Alexopoulou L, et al. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat Med.* 2004;10:1366-1373.

49. Guillot L, Le Goffic R, Bloch S, et al. Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J Biol Chem.* 2005;280:5571-5580.

50. Haynes LM, Moore DD, Kurt-Jones EA, et al. Involvement of toll-like receptor 4 in innate immunity to respiratory syncytial virus. *J Virol.* 2001;75:10730-10737.

51. Diebold SS, Kaisho T, Hemmi H, et al. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science.* 2004;303:1529-1531.

52. Schlaepfer E, Audige A, Joller H, et al. TLR7/8 triggering exerts opposing effects in acute versus latent HIV infection. *J Immunol.* 2006;176: 2888-2895.

53. Li H, Zhang J, Kumar A, et al. Herpes simplex virus 1 infection induces the expression

of proinflammatory cytokines, interferons and TLR7 in human corneal epithelial cells. *Immunology* 2006;117:167-176.

54. Delale T, Paquin A, Asselin-Paturel C, et al. MyD88-dependent and -independent murine cytomegalovirus sensing for IFN-αlpha release and initiation of immune responses in vivo. *J Immunol.* 2005;175: 6723-6732.

55. Daffis S, Samuel MA, Suthar MS, et al. Toll-like receptor 3 has a protective role against West Nile virus infection. *J Virol.* 2008;82:10349-10358.

56. Wang J, Hu Y, Deng WW, et al. Negative regulation of toll-like receptor signaling pathway. *Microbes Infect.* 2009;11:321-327.

57. Matsumiya T, Stafforini DM. Function and regulation of retinoic acidinducible gene-I. *Crit Rev Immunol.* 2010;30:489-513.

58. Brennan K, Bowie AG. Activation of host pattern recognition receptors by viruses. *Curr Opin Microbiol.* 2010;13:503-507.

59. Rehwinkel J, Tan CP, Goubau D, et al. RIG-I detects viral genomic RNA during negative-strand RNA virus infection. *Cell.* 2011;140:397-408.

60. Broquet AH, Hirata Y, McAllister CS, et al. RIG-I/MDA5/MAVS are required to signal a protective IFN response in rotavirus-infected intestinal epithelium. *J Immunol.* 2011;186:1618-1626.

61. Cárdenas WB. Evasion of the interferon-mediated antiviral response by filoviruses. *Viruses.* 2010;2:262-282.

62. Chang TH, Liao CL, Lin YL. Flavivirus induces interferon-beta gene expression through a pathway involving RIG-I-dependent IRF-3 and PI3K-dependent NF-kappaB activation. *Microbes Infect.* 2006;8: 157-171.

63. Barral PM, Sarkar D, Fisher PB, et al. RIG-I is cleaved during picornavirus infection. *Virology.* 2009;391:171-176.

64. Athman R, Philpott D. Innate immunity via toll-like receptors and NOD proteins. *Curr Opin Microbiol.* 2004;7:25-32.

65. Philpott DJ, Girardin SE. Nod-like receptors: sentinels at host membranes. *Curr Opin Immunol.* 2010;22(4):428-434.

66. Sabbah A, Chang TH, Harnack R, et al. Activation of innate immune antiviral responses by Nod2. *Nat Immunol.* 2009;10:1073-1080.

67. Kuenzel S, Till A, Winkler M, et al. The nucleotide-binding oligomerization domain-like receptor NLRC5 is involved in IFN-dependent antiviral immune responses. *J Immunol.* 2011;184:1990-2000.

68. Neerincx A, Lautz K, Menning M, et al. A role for the human nucleotide-binding domain, leucine-rich repeat-containing family member NLRC5 in antiviral responses. *J Biol Chem.* 2010;285:26223-26232.

69. Moore CB, Bergstralh DT, Duncan JA, et al. NLRX1 is a regulator of mitochondrial antiviral immunity. *Nature.* 2008;451:573-577.

70. Lamkanfi M. Emerging inflammasome effector mechanisms. *Nat Rev Immunol.* 2011;11:213-220.

71. Ichinohe T, Lee HK, Ogura Y, et al. Inflammasome recognition of influenza virus is essential for adaptive immune responses. *J Exp Med.* 2009;206:79-87.

72. Rajan JV, Rodriguez D, Miao EA, et al. The NLRP3 inflammasome detects EMCV and VSV infection. *J Virol.* 2011;85(9):4167-4172.

73. Ichinohe T, Pang IK, Iwasaki A. Influenza virus activates inflammasomes via its intracellular M2 ion channel. *Nat Immunol.* 2010;11:404-410.

74. Rathinam VA, Jiang Z, Waggoner SN, et al. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat Immunol.* 2010;11:395-402.

75. Samuel CE. Antiviral actions of interferons. *Clin Microbiol Rev.* 2001;14: 778-809.

76. Hadziyannis SJ. Milestones and perspectives in viral hepatitis B. *Liver Int.* 2011;31(suppl 1):129-134.

77. Munir S, Saleem S, Idrees M, et al. Hepatitis C treatment: current and future perspectives. *Virol J.* 2011;7:296.

78. Rockley PF, Tyring SK. Interferons alpha, beta and gamma therapy of anogenital human papillomavirus infections. *Pharmacol Ther.* 1995;65: 265-287.

79. Haller O, Staeheli P, Kochs G. Protective role of interferon-induced Mx GTPases against influenza viruses. *Rev Sci Tech.* 2009;28:219-231.

80. Goncalves J, Santa-Marta M. HIV-1 Vif and APOBEC3G: multiple roads to one goal. *Retrovirology.* 2004;1:28.

81. Tanner JE, Alfieri C. Interactions involving cyclosporine A, inter leukin-6, and Epstein-Barr virus lead to the promotion of B-cell lymphoproliferative disease. *Leuk Lymphoma.* 1996;21:379-390.

82. Fassone L, Gaidano G, Ariatti C, et al. The role of cytokines in the pathogenesis and management of AIDS-related lymphomas. *Leuk Lymphoma.* 2000;38:481-488.

83. Kasturi SP, Skountzou I, Albrecht RA, et al. Programming the magnitude and persistence of antibody responses with innate immunity. *Nature.* 2011;470:543-547.

84. Vivier E, Raulet DH, Moretta A, et al. Innate or adaptive immunity? The example of natural killer cells. *Science.* 2011;331:44-49.

85. Tumpey TM, Chen SH, Oakes JE, et al. Neutrophil-mediated suppression of virus replication after herpes simplex virus type 1 infection of the murine cornea. *J Virol.* 1996;70:898-904.

86. Jaovisidha P, Peeples ME, Brees AA, et al. Respiratory syncytial virus stimulates neutrophil degranulation and chemokine release. *J Immunol.* 1999;163:2816-2820.

87. Saito T, Deskin RW, Casola A, et al. Respiratory syncytial virus induces selective production of the chemokine RANTES by upper airway epithelial cells. *J Infect Dis.* 1997;175:497-504.

88. King CA, Anderson R, Marshall JS. Dengue virus selectively induces human mast cell chemokine production. *J Virol.* 2002;76:8408-8419.

89. Chomarat P, Kjeldsen-Kragh J, Quayle AJ, et al. Different cytokine production profiles of gamma delta T cell clones: relation to inflammatory arthritis. *Eur J Immunol.* 1994;24:2087-2091.

90. Boismenu R, Feng L, Xia YY, et al. Chemokine expression by intraepithelial gamma delta T cells. Implications for the recruitment of inflammatory cells to damaged epithelia. *J Immunol.* 1996;157:985-992.

91. Huber S, Shi C, Budd RC. Gammadelta T cells promote a Th1 response during coxsackievirus B3 infection in vivo: role of Fas and Fas ligand. *J Virol.* 2002;76:6487-6494.

92. Bukowski JF, Morita CT, Brenner MB. Recognition and destruction of virus-infected cells by human gamma delta CTL. *J Immunol.* 1994;153:5133-5140.

93. Hoq MM, Suzutani T, Toyoda T, et al. Role of gamma delta TCR +lymphocytes in the augmented resistance of trehalose 6,6′-dimycolate-treated mice to influenza virus infection. *J Gen Virol.* 1997;78(pt 7):1597-1603.

94. Lopez RD. Human gammadelta-T cells in adoptive immunotherapy of malignant and infectious diseases. *Immunol Res.* 2002;26:207-221.

95. Wang T, Gao Y, Scully E, et al. Gamma delta T cells facilitate adaptive immunity against West Nile virus infection in mice. *J Immunol.* 2006;177:1825-1832.

96. Selin LK, Santolucito PA, Pinto AK, et al. Innate immunity to viruses: control of vaccinia virus infection by gamma delta T cells. *J Immunol.* 2001;166:6784-6794.

97. Liu K, Nussenzweig MC. Origin and development of dendritic cells. *Immunol Rev.* 2010;234:45-54.

98. Fujita H, Nograles KE, Kikuchi T, et al. Human Langerhans cells induce distinct IL-22-producing CD4+ T cells lacking IL-17 production. *Proc Natl Acad Sci U S A.* 2009;106:21795-21800.

99. Klaus GG, Humphrey JH, Kunkl A, et al. The follicular dendritic cell: its role in antigen presentation in the generation of immunological memory. *Immunol Rev.* 1980;53:3-28.

100. Colonna M, Trinchieri G, Liu YJ. Plasmacytoid dendritic cells in immunity. *Nat Immunol.* 2004;5:1219-1226.

101. van Vliet SJ, den Dunnen J, Gringhuis SI, et al. Innate signaling and regulation of dendritic cell immunity. *Curr Opin Immunol.* 2007;19: 435-440.

102. Dietz AB, Bulur PA, Knutson GJ, et al. Maturation of human monocyte-derived dendritic cells studied by microarray hybridization. *Biochem Biophys Res Commun.* 2000;275:731-738.

103. Kaisho T, Takeuchi O, Kawai T, et al. Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J Immunol*. 2001;166:5688-5694.

104. Larange A, Antonios D, Pallardy M, et al. TLR7 and TLR8 agonists trigger different signaling pathways for human dendritic cell maturation. *J Leukoc Biol.* 2009;85:673-683.

105. Zou GM, Tam YK. Cytokines in the generation and maturation of dendritic cells: recent advances. *Eur Cytokine Netw.* 2002;13:186-199.

106. Hostager BS. Roles of TRAF6 in CD40 signaling. *Immunol Res.* 2007;39: 105-114.

107. Wong BR, Josien R, Lee SY, et al. TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J Exp Med.* 1997;186:2075-2080.

108. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol.* 2005;23:515-548.

109. Alegre ML, Fallarino F. Mechanisms of CTLA-4-Ig in tolerance induction. *Curr Pharm Des.* 2006;12:149-160.

110. Fos C, Salles A, Lang V, et al. ICOS ligation recruits the p50alpha PI3K regulatory subunit to the immunological synapse. *J Immunol.* 2008;181:1969-1977.

111. Nukada Y, Okamoto N, Konakahara S, et al. AILIM/ICOS-mediated elongation of activated T cells is regulated by both the PI3-kinase/Akt and Rho family cascade. *Int Immunol.* 2006;18:1815-1824.

112. McAdam AJ, Chang TT, Lumelsky AE, et al. Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of CD4+ T cells. *J Immunol.* 2000;165:5035-5040.

113. Paulos CM, Carpenito C, Plesa G, et al. The inducible costimulator (ICOS) is critical for the development of human T(H)17 cells. *Sci Transl Med.* 2010;2:55ra78.

114. Pot C, Jin H, Awasthi A, et al. Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. *J Immunol.* 2009;183:797-801.

115. Yong PF, Salzer U, Grimbacher B. The role of costimulation in antibody deficiencies: ICOS and common variable immunodeficiency. *Immunol Rev.* 2009;229:101-113.

116. Keir ME, Francisco LM, Sharpe AH. PD-1 and its ligands in T-cell immunity. *Curr Opin Immunol*. 2007;19:309-314.

117. Francisco LM, Salinas VH, Brown KE, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med.* 2009;206:3015-

3029.

118. Good-Jacobson KL, Szumilas CG, Chen L, et al. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol.* 2010;11:535-542.

119. Cai G, Freeman GJ. The CD160, BTLA, LIGHT/HVEM pathway: a bidirectional switch regulating T-cell activation. *Immunol Rev.* 2009;229: 244-258.

120. Lasaro MO, Ertl HC. Targeting inhibitory pathways in cancer immunotherapy. *Curr Opin Immunol.* 2010;22:385-390.

121. Lasaro MO, Tatsis N, Hensley SE, et al. Targeting of antigen to the herpesvirus entry mediator augments primary adaptive immune responses. *Nat Med.* 2008;14:205-212.

122. DiMenna L, Latimer B, Parzych E, et al. Augmentation of primary influenza A virus-specific CD8+ T cell responses in aged mice through blockade of an immunoinhibitory pathway. *J Immunol.* 2010;184: 5475-5484.

123. Gavrieli M, Murphy KM. Association of Grb-2 and PI3K p85 with phosphotyrosile peptides derived from BTLA. *Biochem Biophys Res Commun.* 2006;345:1440-1445.

124. Tao R, Wang L, Murphy KM, et al. Regulatory T cell expression of herpesvirus entry mediator suppresses the function of B and T lymphocyte attenuator-positive effector T cells. *J Immunol.* 2008;180: 6649-6655.

125. Kubin MZ, Parshley DL, Din W, et al. Molecular cloning and biological characterization of NK cell activation-inducing ligand, a counterstructure for CD48. *Eur J Immunol.* 1999;29:3466-3477.

126. McNerney ME, Lee KM, Kumar V. 2B4 (CD244) is a non-MHC binding receptor with multiple functions on natural killer cells and CD8+ T cells. *Mol Immunol.* 2005;42:489-494.

127. Workman CJ, Vignali DA. Negative regulation of T cell homeostasis by lymphocyte activation gene-3 (CD223). *J Immunol.* 2005;174: 688-695.

128. Takai T, Ono M. Activating and inhibitory nature of the murine paired immunoglobulin-like receptor family. *Immunol Rev.* 2001;181: 215-222.

129. Munn DH, Sharma MD, Lee JR, et al. Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. *Science.* 2002;297:1867-1870.

130. Ng SC, Kamm MA, Stagg AJ, et al. Intestinal dendritic cells: their role in bacterial recognition, lymphocyte homing, and intestinal inflammation. *Inflamm Bowel Dis.* 2010;16:1787-1807.

131. Lopez-Botet M, Llano M, Navarro F, et al. NK cell recognition of nonclassical HLA class I molecules. *Semin Immunol.* 2000;12:109-119.

132. Cerwenka A, Lanier LL. Ligands for natural killer cell receptors: redundancy or specificity. *Immunol Rev.* 2001;181:158-169.

133. Lanier LL. NK cell recognition. *Annu Rev Immunol.* 2005;23:225-274.

134. Flaig RM, Stark S, Watzl C. Cutting edge: NTB-A activates NK cells via homophilic interaction. *J Immunol.* 2004;172:6524-6527.

135. Tripathy SK, Smith HR, Holroyd EA, et al. Expression of m157, a murine cytomegalovirus-encoded putative major histocompatibility class I (MHC-I)-like protein, is independent of viral regulation of host MHC-I. *J Virol.* 2006;80:545-550.

136. Mandelboim O, Lieberman N, Lev M, et al. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature.* 2001;409:1055-1060.

137. Chalupny NJ, Rein-Weston A, Dosch S, et al. Down-regulation of the NKG2D ligand MICA by the human cytomegalovirus glycoprotein UL142. *Biochem Biophys Res Commun.* 2006;346:175-181.

138. Vivier E, Nunes JA, Vely F. Natural killer cell signaling pathways. *Science.* 2004;306:1517-1519.

139. Lopez-Larrea C, Suarez-Alvarez B, Lopez-Soto A, et al. The NKG2D receptor: sensing stressed cells. *Trends Mol Med.* 2008;14:179-189.

140. Horng T, Bezbradica JS, Medzhitov R. NKG2D signaling is coupled to the interleukin 15 receptor signaling pathway. *Nat Immunol.* 2007;8: 1345-1352.

141. Roncagalli R, Taylor JE, Zhang S, et al. Negative regulation of natural killer cell function by EAT-2, a SAP-related adaptor. *Nat Immunol.* 2005;6:1002-1010.

142. Pietra G, Romagnani C, Mazzarino P, et al. Comparative analysis of NK- or NK-CTL-mediated lysis of immature or mature autologous dendritic cells. *Eur J Immunol.* 2003;33:3427-3432.

143. Orange JS. Human natural killer cell deficiencies and susceptibility to infection. *Microbes Infect.* 2002;4:1545-1558.

144. Latour S. Natural killer T cells and X-linked lymphoproliferative syndrome. *Curr Opin Allergy Clin Immunol.* 2007;7:510-514.

145. Kronenberg M, Gapin L. The unconventional lifestyle of NKT cells. *Nat Rev Immunol.* 2002;2:557-568.

146. O'Leary JG, Goodarzi M, Drayton DL, et al. T cell- and B cellindependent adaptive immunity mediated by natural killer cells. *Nat Immunol.* 2006;7:507-516.

147. Sun JC, Beilke JN, Lanier LL. Adaptive immune features of natural killer cells. *Nature.* 2009;457:557-561.

148. Biron CA, Brossay L. NK cells and NKT cells in innate defense against viral infections. *Curr Opin Immunol.* 2001;13:458-464.

149. Moore ML, Newcomb DC, Parekh VV, et al. STAT1 negatively regulates lung

basophil IL-4 expression induced by respiratory syncytial virus infection. *J Immunol.* 2009;183:2016-2026.

150. Zhou L, Chong MM, Littman DR. Plasticity of CD4+ T cell lineage differentiation. *Immunity.* 2009;30:646-655.

151. Crotty S. Follicular helper CD4 T cells (T(FH)). *Annu Rev Immunol.* 2010;29:621-663.

152. O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science.* 2010;327:1098-1102.

153. Zhu J, Paul WE. Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors. *Immunol Rev.* 2010;238:247-262.

154. Kurilla MG, Swaminathan S, Welsh RM, et al. Effects of virally expressed interleukin-10 on vaccinia virus infection in mice. *J Virol.* 1993;67:7623-7628.

155. Endoh Y, Chung YM, Clark IA, et al. IL-10-dependent S100A8 gene induction in monocytes/macrophages by double-stranded RNA. *J Immunol.* 2009;182:2258-2268.

156. Bermejo-Martin JF, Ortiz de Lejarazu R, Pumarola T, et al. Th1 and Th17 hypercytokinemia as early host response signature in severe pandemic influenza. *Crit Care.* 2009;13:R201.

157. Noutsias M, Rohde M, Goldner K, et al. Expression of functional T-cell markers and T-cell receptor Vbeta repertoire in endomyocardial biopsies from patients presenting with acute myocarditis and dilated cardiomyopathy. *Eur J Heart Fail.* 2011;13(6):611-618.

158. Hou W, Kang HS, Kim BS. Th17 cells enhance viral persistence and inhibit T cell cytotoxicity in a model of chronic virus infection. *J Exp Med.* 2009;206:313-328.

159. Kawamata S, Hori T, Imura A, et al. Activation of OX40 signal transduction pathways leads to tumor necrosis factor receptor-associated factor (TRAF) 2- and TRAF5-mediated NF-kappaB activation. *J Biol Chem.* 1998;273:5808-5814.

160. Platt AM, Gibson VB, Patakas A, et al. Abatacept limits breach of selftolerance in a murine model of arthritis via effects on the generation of T follicular helper cells. *J Immunol.* 2010;185:1558-1567.

161. Gigoux M, Shang J, Pak Y, et al. Inducible costimulator promotes helper T-cell differentiation through phosphoinositide 3-kinase. *Proc Natl Acad Sci U S A.* 2009;106:20371-20376.

162. Cannons JL, Yu LJ, Jankovic D, et al. SAP regulates T cell-mediated help for humoral immunity by a mechanism distinct from cytokine regulation. *J Exp Med.* 2006;203:1551-1565.

163. Swain SL, Agrewala JN, Brown DM, et al. CD4+ T-cell memory: generation and multi-faceted roles for CD4+ T cells in protective immunity to influenza. *Immunol Rev.* 2006;211:8-22.

164. Janssen EM, Droin NM, Lemmens EE, et al. CD4+ T-cell help controls CD8+ T-cell

memory via TRAIL-mediated activation-induced cell death. *Nature.* 2005;434:88-93.

165. Heller KN, Gurer C, Munz C. Virus-specific CD4+ T cells: ready for direct attack. *J Exp Med.* 2006;203:805-808.

166. Torpey DJ III, Lindsley MD, Rinaldo CR Jr. HLA-restricted lysis of herpes simplex virus-infected monocytes and macrophages mediated by CD4+ and CD8+ T lymphocytes. *J Immunol.* 1989;142:1325-1332.

167. Soroosh P, Doherty TA, So T, et al. Herpesvirus entry mediator (TNFRSF14) regulates the persistence of T helper memory cell populations. *J Exp Med*; 2010208(4):797-809.

168. Carter LL, Zhang X, Dubey C, et al. Regulation of T cell subsets from naive to memory. *J Immunother.* 1998;21:181-187.

169. Rouse BT, Sarangi PP, Suvas S. Regulatory T cells in virus infections. *Immunol Rev.* 2006;212:272-286.

170. Humblet-Baron S, Sather B, Anover S, et al. Wiskott-Aldrich syndrome protein is required for regulatory T cell homeostasis. *J Clin Invest.* 2007;117:407-418.

171. Cobbold SP, Adams E, Nolan KF, et al. Connecting the mechanisms of T-cell regulation: dendritic cells as the missing link. *Immunol Rev.* 2010;236:203-218.

172. Khattar M, Chen W, Stepkowski SM. Expanding and converting regulatory T cells: a horizon for immunotherapy. *Arch Immunol Ther Exp (Warsz).* 2009;57:199-204.

173. Niedbala W, Wei XQ, Cai B, et al. IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *Eur J Immunol.* 2007;37:3021-3029.

174. Bour-Jordan H, Bluestone JA. Regulating the regulators: costimulatory signals control the homeostasis and function of regulatory T cells. *Immunol Rev.* 2009;229:41-66.

175. Bour-Jordan H, Blueston JA. CD28 function: a balance of costimulatory and regulatory signals. *J Clin Immunol.* 2002;22(1):1-7.

176. Shevach EM, DiPaolo RA, Andersson J, et al. The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. *Immunol Rev.* 2006;212:60-73.

177. Claassen MA, de Knegt RJ, Tilanus HW, et al. Abundant numbers of regulatory T cells localize to the liver of chronic hepatitis C infected patients and limit the extent of fibrosis. *J Hepatol.* 2010;52: 315-321.

178. Kinter AL, Horak R, Sion M, et al. CD25+ regulatory T cells isolated from HIV-infected individuals suppress the cytolytic and nonlytic antiviral activity of HIV-specific CD8+ T cells in vitro. *AIDS Res Hum Retroviruses.* 2007;23:438-450.

179. Terzieva V, Popova D, Kicheva M, et al. Correlation between the degree of immune activation, production of IL-2 and FOXP3 expression in CD4+ CD25+ T regulatory cells in

HIV-1 infected persons under HAART. *Int Immunopharmacol.* 2009;9:831-836.

180. Zelinskyy G, Dietze K, Sparwasser T, et al. Regulatory T cells suppress antiviral immune responses and increase viral loads during acute infection with a lymphotropic retrovirus. *PLoS Pathog.* 2009;5:e1000406.

181. Yewdell J, Anton LC, Bacik I, et al. Generating MHC class I ligands from viral gene products. *Immunol Rev.* 1999;172:97-108.

182. Pamer E, Cresswell P. Mechanisms of MHC class I—restricted antigen processing. *Annu Rev Immunol.* 1998;16:323-358.

183. McCluskey J, Rossjohn J, Purcell AW. TAP genes and immunity. *Curr Opin Immunol.* 2004;16:651-659.

184. Yewdell JW. Designing CD8+ T cell vaccines: it's not rocket science (yet). *Curr Opin Immunol.* 2010;22:402-410.

185. Yewdell JW, Bennink JR. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu Rev Immunol.* 1999;17:51-88.

186. Koszinowski U, Ertl H. Role of early viral surface antigens in cellular immune response to vaccinia virus. *Eur J Immunol.* 1976;6:679-683.

187. Mautner J, Pich D, Nimmerjahn F, et al. Epstein-Barr virus nuclear antigen 1 evades direct immune recognition by CD4+ T helper cells. *Eur J Immunol.* 2004;34:2500-2509.

188. Revy P, Sospedra M, Barbour B, et al. Functional antigen-independent synapses formed between T cells and dendritic cells. *Nat Immunol.* 2001;2:925-931.

189. Dustin ML, Long EO. Cytotoxic immunological synapses. *Immunol Rev.* 2010;235:24-34.

190. Mondino A, Mueller DL. mTOR at the crossroads of T cell proliferation and tolerance. *Semin Immunol.* 2007;19:162-172.

191. Rao RR, Li Q, Odunsi K, et al. The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin. *Immunity.* 2010;32:67-78.

192. Parry RV, Chemnitz JM, Frauwirth KA, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol.* 2005;25:9543-9553.

193. Krieg C, Boyman O, Fu YX, et al. B and T lymphocyte attenuator regulates CD8+ T cell-intrinsic homeostasis and memory cell generation. *Nat Immunol.* 2007;8:162-171.

194. Oppenheimer-Marks N, Davis LS, Bogue DT, et al. Differential utilization of ICAM-1 and VCAM-1 during the adhesion and transendothelial migration of human T lymphocytes. *J Immunol.* 1991;147: 2913-2921.

195. Betts MR, Nason MC, West SM, et al. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood.* 2006;107:4781-4789.

196. Vilcek J, Palombella VJ, Zhang Y, et al. Mechanisms and significance of the mitogenic and antiviral actions of TNF. *Ann Inst Pasteur Immunol.* 1988;139:307-311.

197. Mayer A, Gelderblom H, Kumel G, et al. Interferon-gamma-induced assembly block in the replication cycle of adenovirus 2: augmentation by tumour necrosis factor-alpha. *Virology.* 1992;187:372-376.

198. Liu CC, Persechini PM, Young JD. Perforin and lymphocyte-mediated cytolysis. *Immunol Rev.* 1995;146:145-175.

199. Dix RD, Podack ER, Cousins SW. Loss of the perforin cytotoxic pathway predisposes mice to experimental cytomegalovirus retinitis. *J Virol.* 2003;77:3402-3408.

200. Mullbacher A, Ebnet K, Blanden RV, et al. Granzyme A is critical for recovery of mice from infection with the natural cytopathic viral pathogen, ectromelia. *Proc Natl Acad Sci U S A.* 1996;93:5783-5787.

201. D'Cruz LM, Rubinstein MP, Goldrath AW. Surviving the crash: transitioning from effector to memory CD8+ T cell. *Semin Immunol.* 2009;21:92-98.

202. Rutishauser RL, Martins GA, Kalachikov S, et al. Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity.* 2009;31:296-308.

203. Prlic M, Hernandez-Hoyos G, Bevan MJ. Duration of the initial TCR stimulus controls the magnitude but not functionality of the CD8+ T cell response. *J Exp Med.* 2006;203:2135-2143.

204. Kerdiles YM, Beisner DR, Tinoco R, et al. Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. *Nat Immunol.* 2009;10:176-184.

205. Yeo CJ, Fearon DT. T-bet-mediated differentiation of the activated CD8+ T cell. *Eur J Immunol.* 2010;41:60-66.

206. Pipkin ME, Sacks JA, Cruz-Guilloty F, et al. Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity.* 2011;32:79-90.

207. Joshi NS, Cui W, Chandele A, et al. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity.* 2007;27: 281-295.

208. Wherry EJ, Ahmed R. Memory CD8 T-cell differentiation during viral infection. *J Virol.* 2004;78:5535-5545.

209. Masopust D, Vezys V, Marzo AL, et al. Preferential localization of effector memory cells in nonlymphoid tissue. *Science.* 2001;291:2413-2417.

210. Williams MA, Holmes BJ, Sun JC, et al. Developing and maintaining protective CD8+ memory T cells. *Immunol Rev.* 2006;211:146-153.

211. Reiner SL, Sallusto F, Lanzavecchia A. Division of labor with a workforce of one: challenges in specifying effector and memory T cell fate. *Science.* 2007;317:622-625.

212. Sarkar S, Kalia V, Haining WN, et al. Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *J Exp Med.* 2008;205:625-640.

213. Wherry EJ, Teichgraber V, Becker TC, et al. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol.* 2003;4:225-234.

214. Huster KM, Koffler M, Stemberger C, et al. Unidirectional development of CD8+ central memory T cells into protective Listeria-specific effector memory T cells. *Eur J Immunol.* 2006;36:1453-1464.

215. Hansen SG, Vieville C, Whizin N, et al. Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. *Nat Med.* 2009;15: 293-299.

216. Cerwenka A, Morgan TM, Dutton RW. Naive, effector, and memory CD8 T cells in protection against pulmonary influenza virus infection: homing properties rather than initial frequencies are crucial. *J Immunol.* 1999;163:5535-5543.

217. Kalia V, Sarkar S, Ahmed R. CD8 T-cell memory differentiation during acute and chronic viral infections. *Adv Exp Med Biol.* 2010; 684:79-95.

218. Shin H, Blackburn SD, Intlekofer AM, et al. A role for the transcriptional repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection. *Immunity.* 2009;31:309-320.

219. Blackburn SD, Shin H, Haining WN, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol.* 2009;10:29-37.

220. Barber DL, Wherry EJ, Masopust D, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature.* 2006;439:682-687.

221. Tatsis N, Lin SW, Harris-McCoy K, et al. Multiple immunizations with adenovirus and MVA vectors improve CD8+ T cell functionality and mucosal homing. *Virology.* 2007;367:156-167.

222. Pawelec G, Koch S, Franceschi C, et al. Human immunosenescence: does it have an infectious component? *Ann N Y Acad Sci.* 2006;1067:56-65.

223. De Martinis M, Franceschi C, Monti D, et al. Inflamm-ageing and lifelong antigenic load as major determinants of ageing rate and longevity. *FEBS Lett.* 2005;579:2035-2039.

224. Hutnick NA, Carnathan D, Demers K, et al. Adenovirus-specific human T cells are pervasive, polyfunctional, and cross-reactive. *Vaccine.* 2010; 28:1932-1941.

225. Billerbeck E, Thimme R. CD8+ regulatory T cells in persistent human viral infections. *Hum Immunol.* 2008;69:771-775.

226. Accapezzato D, Francavilla V, Paroli M, et al. Hepatic expansion of a virus-specific

regulatory CD8(+) T cell population in chronic hepatitis C virus infection. *J Clin Invest.* 2004;113:963-972.

227. Martin F, Kearney JF. B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a “natural immune memory.” *Immunol Rev.* 2000;175:70-79.

228. Gonzalez SF, Pitcher LA, Mempel T, et al. B cell acquisition of antigen in vivo. *Curr Opin Immunol.* 2009;21:251-257.

229. Tolar P, Pierce SK. A conformation-induced oligomerization model for B cell receptor microclustering and signaling. *Curr Top Microbiol Immunol.* 2010;340:155-169.

230. Good-Jacobson KL, Shlomchik MJ. Plasticity and heterogeneity in the generation of memory B cells and long-lived plasma cells: the influence of germinal center interactions and dynamics. *J Immunol.* 2010;185:3117-3125.

231. McGaha TL, Sorrentino B, Ravetch JV. Restoration of tolerance in lupus by targeted inhibitory receptor expression. *Science.* 2005;307:590-593.

232. Shapiro-Shelef M, Calame K. Regulation of plasma-cell development. *Nat Rev Immunol.* 2005;5:230-242.

233. Moir S, Ho J, Malaspina A, et al. Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. *J Exp Med.* 2008;205:1797-1805.

234. Pichla-Gollon SL, Drinker M, Zhou X, et al. Structure-based identification of a major neutralizing site in an adenovirus hexon. *J Virol.* 2007;81:1680-1689.

235. Mozdzanowska K, Feng J, Eid M, et al. Induction of influenza type A virus-specific resistance by immunization of mice with a synthetic multiple antigenic peptide vaccine that contains ectodomains of matrix protein 2. *Vaccine.* 2003;21:2616-2626.

236. Halstead SB, Mahalingam S, Marovich MA, et al. Intrinsic antibodydependent enhancement of microbial infection in macrophages: disease regulation by immune complexes. *Lancet Infect Dis.* 2011;10: 712-722.

237. Zinkernagel RM. Maternal antibodies, childhood infections, and autoimmune diseases. *N Engl J Med.* 2001;345:1331-1335.

238. Pabst R. The anatomical basis for the immune function of the gut. *Anat Embryol (Berl).* 1987;176:135-144.

239. Mestecky J, Fultz PN. Mucosal immune system of the human genital tract. *J Infect Dis.* 1999;179(suppl 3):S470-S474.

240. Kagnoff MF. Current concepts in mucosal immunity. III. Ontogeny and function of gamma delta T cells in the intestine. *Am J Physiol.* 1998;274:G455-G458.

241. Niedergang F, Kweon MN. New trends in antigen uptake in the gut mucosa. *Trends Microbiol.* 2005;13:485-490.

242. Mucida D, Salek-Ardakani S. Regulation of TH17 cells in the mucosal surfaces. *J Allergy Clin Immunol*. 2009;123:997-1003.

243. Blaschitz C, Raffatellu M. Th17 cytokines and the gut mucosal barrier. *J Clin Immunol*. 2010;30:196-203.

244. Isakov D, Dzutsev A, Belyakov IM, et al. Non-equilibrium and differential function between intraepithelial and lamina propria virus-specific TCRalphabeta(+) CD8alphabeta(+) T cells in the small intestinal mucosa. *Mucosal Immunol*. 2009;2:450-461.

245. Masopust D, Choo D, Vezys V, et al. Dynamic T cell migration program provides resident memory within intestinal epithelium. *J Exp Med.* 2010;207:553-564.

246. Lin SW, Cun AS, Harris-McCoy K, et al. Intramuscular rather than oral administration of replication-defective adenoviral vaccine vector induces specific CD8+ T cell responses in the gut. *Vaccine.* 2007;25:2187-2193.

247. Johansson-Lindbom B, Agace WW. Generation of gut-homing T cells and their localization to the small intestinal mucosa. *Immunol Rev.* 2007;215:226-242.

248. Tang VA, Rosenthal KL. Intravaginal infection with herpes simplex virus type-2 (HSV-2) generates a functional effector memory T cell population that persists in the murine genital tract. *J Reprod Immunol*. 2010;87:39-44.

249. Kaul R, Thottingal P, Kimani J, et al. Quantitative ex vivo analysis of functional virus-specific CD8 T lymphocytes in the blood and genital tract of HIV-infected women. *AIDS.* 2003;17:1139-1144.

250. Haut LH, Lin SW, Tatsis N, et al. Robust genital gag-specific CD8+ T-cell responses in mice upon intramuscular immunization with simian adenoviral vectors expressing HIV-1-gag. *Eur J Immunol*. 2011;40: 3426-3438.

251. Randall TD. Bronchus-associated lymphoid tissue (BALT) structure and function. *Adv Immunol*. 2010;107:187-241.

252. Agrawal A, Agrawal S, Cao JN, et al. Altered innate immune functioning of dendritic cells in elderly humans: a role of phosphoinositide 3-kinase-signaling pathway. *J Immunol*. 2007;178:6912-6922.

253. Ginaldi L, De Martinis M, D'Ostilio A, et al. The immune system in the elderly: I. Specific humoral immunity. *Immunol Res.* 1999;20:101-108.

254. Aydar Y, Balogh P, Tew JG, et al. Follicular dendritic cells in aging, a “bottle-neck” in the humoral immune response. *Ageing Res Rev.* 2004;3:15-29.

255. Zheng B, Han S, Takahashi Y, et al. Immunosenescence and germinal center reaction. *Immunol Rev.* 1997;160:63-77.

256. Frasca D, Landin AM, Lechner SC, et al. Aging down-regulates the transcription factor E2A, activation-induced cytidine deaminase, and Ig class switch in human B cells. *J Immunol*. 2008;180:5283-5290.

257. Eaton SM, Burns EM, Kusser K, et al. Age-related defects in CD4 T cell cognate helper function lead to reductions in humoral responses. *J Exp Med.* 2004;200:1613-1622.

258. Lazuardi L, Jenewein B, Wolf AM, et al. Age-related loss of naive T cells and dysregulation of T-cell/B-cell interactions in human lymph nodes. *Immunology.* 2005;114:37-43.

259. Yang X, Stedra J, Cerny J. Relative contribution of T and B cells to hypermutation and selection of the antibody repertoire in germinal centers of aged mice. *J Exp Med.* 1996;183:959-970.

260. Maue AC, Yager EJ, Swain SL, et al. T-cell immunosenescence: lessons learned from mouse models of aging. *Trends Immunol*. 2009;30: 301-305.

261. Larbi A, Pawelec G, Wong SC, Goldeck D, Tai JJ, Fulop T. Impact of age on T cell signaling: a general defect or specific alterations? *Ageing Res Rev.* 2010;10(3):370-378.

262. Decman V, Laidlaw BJ, Dimenna LJ, et al. Cell-intrinsic defects in the proliferative response of antiviral memory CD8 T cells in aged mice upon secondary infection. *J Immunol.* 2010;184:5151-5159.

263. Channappanavar R, Twardy BS, Krishna P, Suvas S. Advancing age leads to predominance of inhibitory receptor expressing CD4 T cells. *Mech Ageing Dev.* 2009;130:709-712.

264. Franceschi C, Bonafe M, Valensin S. Human immunosenescence: the prevailing of innate immunity, the failing of clonotypic immunity, and the filling of immunological space. *Vaccine.* 2000;18:1717-1720.

265. Yager EJ, Ahmed M, Lanzer K, et al. Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus. *J Exp Med.* 2008;205:711-723.

266. Effros RB, Walford RL. The immune response of aged mice to influenza: diminished T-cell proliferation, interleukin 2 production and cytotoxicity. *Cell Immunol.* 1983;81:298-305.

267. Elrefaei M, Blank KJ, Murasko DM. Decreased IL-2, IFN-gamma, and IL-10 production by aged mice during the acute phase of E55 + retrovirus infection. *Virology.* 2002;299:8-19.

268. Gregg R, Smith CM, Clark FJ, et al. The number of human peripheral blood CD4+ CD25high regulatory T cells increases with age. *Clin Exp Immunol*. 2005;140:540-546.

269. Lafon M. Rabies virus superantigen. *Res Immunol.* 1993;144:209-213.

270. Lapatschek MS, Durr S, Sutter G, et al. Functional evaluation of HIV/SIV Nef as superantigen. *Virology.* 2001;282:329-337.

271. Atkinson MA, Bowman MA, Campbell L, et al. Cellular immunity to a determinant common to glutamate decarboxylase and coxsackie virus in insulin-dependent diabetes. *J*

Clin Invest. 1994;94:2125-2129.

272. Faure E. Multiple sclerosis and hepatitis B vaccination: could minute contamination of the vaccine by partial hepatitis B virus polymerase play a role through molecular mimicry? *Med Hypotheses.* 2005;65:509-520.

273. Tompkins SM, Fuller KG, Miller SD. Theiler's virus-mediated autoimmunity: local presentation of CNS antigens and epitope spreading. *Ann N Y Acad Sci.* 2002;958:26-38.

274. Rose NR. Autoimmunity in coxsackievirus infection. *Curr Top Microbiol Immunol.* 2008;323:293-314.

275. Etingin OR, Hajjar DP. Evidence for cytokine regulation of cholesterol metabolism in herpesvirus-infected arterial cells by the lipoxygenase pathway. *J Lipid Res.* 1990;31:299-305.

276. Durst M, Gissmann L, Ikenberg H, et al. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc Natl Acad Sci U S A.* 1983;80:3812-3815.

277. Dalgleish AG. Viruses and cancer. *Br Med Bull.* 1991;47:21-46.

278. Ferrone S, Whiteside TL. Tumor microenvironment and immune escape. *Surg Oncol Clin N Am.* 2007;16:755-774, viii.

279. Pule MA, Savoldo B, Myers GD, et al. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med.* 2008;14:1264-1270.

280. Dantuma NP, Sharipo A, Masucci MG. Avoiding proteasomal processing: the case of EBNA1. *Curr Top Microbiol Immunol.* 2002;269:23-36.

281. Hensley SE, Das SR, Bailey AL, et al.. Hemagglutinin receptor binding avidity drives influenza A virus antigenic drift. *Science.* 2009;326:734-736.

282. Sui J, Hwang WC, Perez S, et al. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat Struct Mol Biol.* 2009;16:265-273.

283. Uebelhoer L, Han JH, Callendret B, et al. Stable cytotoxic T cell escape mutation in hepatitis C virus is linked to maintenance of viral fitness. *PLoS Pathog.* 2008;4:e1000143.

284. Lopez M, Soriano V, Benito JM. Escape mutations in HIV infection and its impact on CD8+ T cell responses. *Curr Mol Med.* 2007;7:446-458.

285. Rimmelzwaan GF, Boon AC, Voeten JT, et al. Sequence variation in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. *Virus Res.* 2004;103:97-100.

286. Zeuzem S. Hepatitis C virus: kinetics and quasispecies evolution during anti-viral therapy. *Forum (Genova).* 2000;10:32-42.

287. Chiu YL, Soros VB, Kreisberg JF, et al. Cellular APOBEC3G restricts HIV-1 infection in resting CD4+ T cells. *Nature.* 2005;435:108-114.

288. Leung DW, Prins KC, Borek DM, et al. Structural basis for dsRNA recognition and interferon antagonism by Ebola VP35. *Nat Struct Mol Biol.* 2010;17:165-172.

289. Bowie AG, Unterholzner L. Viral evasion and subversion of patternrecognition receptor signalling. *Nat Rev Immunol.* 2008;8:911-922.

290. Gregory SM, Davis BK, West JA, et al. Discovery of a viral NLR homolog that inhibits the inflammasome. *Science.* 2011;331:330-334.

291. de Oliveira VL, Almeida SC, Soares HR, et al. A novel TLR3 inhibitor encoded by African swine fever virus (ASFV). *Arch Virol.* 2010;156:597-609.

292. DiPerna G, Stack J, Bowie AG, et al. Poxvirus protein N1L targets the I-kappaB kinase complex, inhibits signaling to NF-kappaB by the tumor necrosis factor superfamily of receptors, and inhibits NF-kappaB and IRF3 signaling by toll-like receptors. *J Biol Chem.* 2004;279:36570-36578.

293. Chen RA, Ryzhakov G, Cooray S, et al. Inhibition of IkappaB kinase by vaccinia virus virulence factor B14. *PLoS Pathog.* 2008;4:e22.

294. Shisler JL, Jin XL. The vaccinia virus K1L gene product inhibits host NF-kappaB activation by preventing IkappaBalpha degradation. *J Virol.* 2004;78:3553-3560.

295. Gedey R, Jin XL, Hinthong O, Shisler JL. Poxviral regulation of the host NF-kappaB response: the vaccinia virus M2L protein inhibits induction of NF-kappaB activation via an ERK2 pathway in virus-infected human embryonic kidney cells. *J Virol.* 2006;80:8676-8685.

296. Thurau M, Everett H, Tapernoux M, et al. The TRAF3-binding site of human molluscipox virus FLIP molecule MC159 is critical for its capacity to inhibit Fas-induced apoptosis. *Cell Death Differ.* 2006;13:1577-1585.

297. Gil J, Rullas J, Alcami J, et al. MC159L protein from the poxvirus molluscum contagiosum virus inhibits NF-kappaB activation and apoptosis induced by PKR. *J Gen Virol.* 2001;82:3027-3034.

298. Pfaller CK, Conzelmann KK. Measles virus V protein is a decoy substrate for IkappaB kinase alpha and prevents toll-like receptor 7/9-mediated interferon induction. *J Virol.* 2008;82:12365-12373.

299. Joo CH, Shin YC, Gack M, et al. Inhibition of interferon regulatory factor 7 (IRF7)-mediated interferon signal transduction by the Kaposi's sarcoma-associated herpesvirus viral IRF homolog vIRF3. *J Virol.* 2007; 81:8282-8292.

300. Lefort S, Gravel A, Flamand L. Repression of interferon-alpha stimulated genes expression by Kaposi's sarcoma-associated herpesvirus K-bZIP protein. *Virology.* 2010;408:14-30.

301. Brzozka K, Finke S, Conzelmann KK. Identification of the rabies virus alpha/beta

interferon antagonist: phosphoprotein P interferes with phosphorylation of interferon regulatory factor 3. *J Virol.* 2005;79:7673-7681.

302. Devaraj SG, Wang N, Chen Z, et al. Regulation of IRF-3-dependent innate immunity by the papain-like protease domain of the severe acute respiratory syndrome coronavirus. *J Biol Chem.* 2007;282:32208-32221.

303. Ghadge GD, Malhotra P, Furtado MR, et al. In vitro analysis of virusassociated RNA I (VAI RNA): inhibition of the double-stranded RNA-activated protein kinase PKR by VAI RNA mutants correlates with the in vivo phenotype and the structural integrity of the central domain. *J Virol.* 1994;68:4137-4151.

304. Kim I, Liu CW, Puglisi JD. Specific recognition of HIV TAR RNA by the dsRNA binding domains (dsRBD1-dsRBD2) of PKR. *J Mol Biol.* 2006;358:430-442.

305. Gale MJ Jr, Korth MJ, Katze MG. Repression of the PKR protein kinase by the hepatitis C virus NS5A protein: a potential mechanism of interferon resistance. *Clin Diagn Virol.* 1998;10:157-162.

306. Gale M Jr, Tan SL, Wambach M, et al. Interaction of the interferoninduced PKR protein kinase with inhibitory proteins P58IPK and vaccinia virus K3L is mediated by unique domains: implications for kinase regulation. *Mol Cell Biol.* 1996;16:4172-4181.

307. Pavio N, Taylor DR, Lai MM. Detection of a novel unglycosylated form of hepatitis C virus E2 envelope protein that is located in the cytosol and interacts with PKR. *J Virol.* 2002;76:1265-1272.

308. Gil LH, van Olphen AL, Mittal SK, et al. Modulation of PKR activity in cells infected by bovine viral diarrhea virus. *Virus Res.* 2006;116:69-77.

309. Li S, Min JY, Krug RM, et al. Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. *Virology.* 2006;349:13-21.

310. Cheng G, Yang K, He B. Dephosphorylation of eIF-2alpha mediated by the gamma(1)34.5 protein of herpes simplex virus type 1 is required for viral response to interferon but is not sufficient for efficient viral replication. *J Virol.* 2003;77:10154-10161.

311. Maloney G, Schroder M, Bowie AG. Vaccinia virus protein A52R activates p38 mitogen-activated protein kinase and potentiates lipopolysaccharide-induced interleukin-10. *J Biol Chem.* 2005;280: 30838-30844.

312. Schroder M. Viruses and the human DEAD-box helicase DDX3: inhibition or exploitation? *Biochem Soc Trans.* 2010;39:679-683.

313. McFadden G, Schreiber M, Sedger L. Myxoma T2 protein as a model for poxvirus TNF receptor homologs. *J Neuroimmunol.* 1997;72: 119-126.

314. Fessler SP, Chin YR, Horwitz MS. Inhibition of tumor necrosis factor (TNF) signal transduction by the adenovirus group C RID complex involves downregulation of surface levels of TNF receptor 1. *J Virol.* 2004;78:13113-13121.

315. Shisler JL, Moss B. Molluscum contagiosum virus inhibitors of apoptosis: the MC159

v-FLIP protein blocks Fas-induced activation of procaspases and degradation of the related MC160 protein. *Virology.* 2001;282:14-25.

316. Grundhoff A, Ganem D. Mechanisms governing expression of the v-FLIP gene of Kaposi's sarcoma-associated herpesvirus. *J Virol.* 2001;75:1857-1863.

317. Wasilenko ST, Meyers AF, Vander Helm K, et al. Vaccinia virus infection disarms the mitochondrion-mediated pathway of the apoptotic cascade by modulating the permeability transition pore. *J Virol.* 2001;75:11437-11448.

318. Schneider-Brachert W, Tchikov V, Merkel O, et al. Inhibition of TNF receptor 1 internalization by adenovirus 14.7K as a novel immune escape mechanism. *J Clin Invest.* 2006;116:2901-2913.

319. Polster BM, Pevsner J, Hardwick JM. Viral Bcl-2 homologs and their role in virus replication and associated diseases. *Biochim Biophys Acta.* 2004;1644:211-227.

320. Shisler JL, Senkevich TG, Berry MJ, et al. Ultraviolet-induced cell death blocked by a selenoprotein from a human dermatotropic poxvirus. *Science.* 1998;279:102-105.

321. Brick DJ, Burke RD, Minkley AA, et al. Ectromelia virus virulence factor p28 acts upstream of caspase-3 in response to UV light-induced apoptosis. *J Gen Virol.* 2000;81:1087-1097.

322. Perdiguero B, Esteban M. The interferon system and vaccinia virus evasion mechanisms. *J Interferon Cytokine Res.* 2009;29:581-598.

323. Ito N, Moseley GW, Blondel D, et al. Role of interferon antagonist activity of rabies virus phosphoprotein in viral pathogenicity. *J Virol.* 2010;84:6699-6710.

324. Frieman M, Yount B, Heise M, et al. Severe acute respiratory syndrome coronavirus ORF6 antagonizes STAT1 function by sequestering nuclear import factors on the rough endoplasmic reticulum/Golgi membrane. *J Virol.* 2007;81:9812-9824.

325. Ashour J, Laurent-Rolle M, Shi PY, et al. NS5 of dengue virus mediates STAT2 binding and degradation. *J Virol.* 2009;83:5408-5418.

326. Elliott J, Lynch OT, Suessmuth Y, et al. Respiratory syncytial virus NS1 protein degrades STAT2 by using the Elongin-Cullin E3 ligase. *J Virol.* 2007;81:3428-3436.

327. Nishio M, Tsurudome M, Ito M, et al. Identification of paramyxovirus V protein residues essential for STAT protein degradation and promotion of virus replication. *J Virol.* 2005;79:8591-8601.

328. Douglas JL, Gustin JK, Viswanathan K, et al. The great escape: viral strategies to counter BST-2/tetherin. *PLoS Pathog.* 2010;6:e1000913.

329. Sridharan H, Zhao C, Krug RM. Species specificity of the NS1 protein of influenza B virus: NS1 binds only human and non-human primate ubiquitin-like ISG15 proteins. *J Biol Chem.* 2011;285:7852-7856.

330. Capodagli GC, McKercher MA, Baker EA, et al. Structural analysis of a viral ovarian tumor domain protease from the crimean-congo hemorrhagic fever virus in complex with

covalently bonded ubiquitin. *J Virol.* 2010;85:3621-3630.

331. Haller O, Weber F. The interferon response circuit in antiviral host defense. *Verh K Acad Geneeskd Belg.* 2009;71:73-86.327.

332. Fleming SB, Haig DM, Nettleton P, et al. Sequence and functional analysis of a homolog of interleukin-10 encoded by the parapoxvirus orf virus. *Virus Genes.* 2000;21:85-95.

333. Stewart JP, Rooney CM. The interleukin-10 homolog encoded by Epstein-Barr virus enhances the reactivation of virus-specific cytotoxic T cell and HLA-unrestricted killer cell responses. *Virology.* 1992;191: 773-782.

334. Jenkins C, Garcia W, Abendroth A, et al. Expression of a human cytomegalovirus latency-associated homolog of interleukin-10 during the productive phase of infection. *Virology.* 2008;370:285-294.

335. Smith VP, Bryant NA, Alcami A. Ectromelia, vaccinia and cowpox viruses encode secreted interleukin-18-binding proteins. *J Gen Virol.* 2000;81:1223-1230.

336. Li H, Wang H, Nicholas J. Detection of direct binding of human herpesvirus 8-encoded interleukin-6 (vIL-6) to both gp130 and IL-6 receptor (IL-6R) and identification of amino acid residues of vIL-6 important for IL-6R-dependent and -independent signaling. *J Virol.* 2001;75:3325-3334.

337. Deane D, Ueda N, Wise LM, et al. Conservation and variation of the parapoxvirus GM-CSF-inhibitory factor (GIF) proteins. *J Gen Virol.* 2009;90:970-977.

338. Strockbine LD, Cohen JI, Farrah T, et al. The Epstein-Barr virus BARF1 gene encodes a novel, soluble colony-stimulating factor-1 receptor. *J Virol.* 1998;72:4015-4021.

339. Smith GL, Chan YS. Two vaccinia virus proteins structurally related to the interleukin-1 receptor and the immunoglobulin superfamily. *J Gen Virol.* 1991;72(pt 3):511-518.

340. Alcami A, Smith GL. A soluble receptor for interleukin-1 encoded by vaccinia virus: a novel mechanism of virus modulation of the host response to infection. *Cell.* 1992;71:155-167.

341. Ray CA, Black RA, Kronheim SR, et al. Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1 beta converting enzyme. *Cell.* 1992;69:597-604.

342. Poole E, King CA, Sinclair JH, et al. The UL144 gene product of human cytomegalovirus activates NFkappaB via a TRAF6-dependent mechanism. *EMBO J.* 2006;25:4390-4399.

343. Smith CA, Davis T, Wignall JM, et al. T2 open reading frame from the Shope fibroma virus encodes a soluble form of the TNF receptor. *Biochem Biophys Res Commun.* 1991;176:335-342.

344. Upton C, Macen JL, Schreiber M, et al. Myxoma virus expresses a secreted protein

with homology to the tumor necrosis factor receptor gene family that contributes to viral virulence. *Virology.* 1991;184:370-382.

345. Gileva IP, Nepomnyashchikh TS, Antonets DV, et al. Properties of the recombinant TNF-binding proteins from variola, monkeypox, and cowpox viruses are different. *Biochim Biophys Acta.* 2006;1764:1710-1718.

346. Saraiva M, Smith P, Fallon PG, et al. Inhibition of type 1 cytokinemediated inflammation by a soluble CD30 homologue encoded by ectromelia (mousepox) virus. *J Exp Med.* 2002;196:829-839.

347. Albini A, Ferrini S, Benelli R, et al. HIV-1 Tat protein mimicry of chemokines. *Proc Natl Acad Sci U S A.* 1998;95:13153-13158.

348. Penfold ME, Dairaghi DJ, Duke GM, et al. Cytomegalovirus encodes a potent alpha chemokine. *Proc Natl Acad Sci U S A.* 1999;96:9839-9844.

349. Bugert JJ, Lohmuller C, Damon I, et al. Chemokine homolog of molluscum contagiosum virus: sequence conservation and expression. *Virology.* 1998;242:51-59.

350. Kledal TN, Rosenkilde MM, Coulin F, et al. A broad-spectrum chemokine antagonist encoded by Kaposi's sarcoma-associated herpesvirus. *Science.* 1997;277:1656-1659.

351. Parcells MS, Lin SF, Dienglewicz RL, et al. Marek's disease virus (MDV) encodes an interleukin-8 homolog (vIL-8): characterization of the vIL-8 protein and a vIL-8 deletion mutant MDV. *J Virol.* 2001;75:5159-5173.

352. Beisser PS, Lavreysen H, Bruggeman CA, Vink C. Chemokines and chemokine receptors encoded by cytomegaloviruses. *Curr Top Microbiol Immunol.* 2008;325:221-242.

353. Vischer HF, Leurs R, Smit MJ. HCMV-encoded G-protein-coupled receptors as constitutively active modulators of cellular signaling networks. *Trends Pharmacol Sci.* 2006;27:56-63.

354. Tadagaki K, Nakano K, Yamanishi K. Human herpesvirus 7 open reading frames U12 and U51 encode functional beta-chemokine receptors. *J Virol.* 2005;79:7068-7076.

355. Tadagaki K, Yamanishi K, Mori Y. Reciprocal roles of cellular chemokine receptors and human herpesvirus 7-encoded chemokine receptors, U12 and U51. *J Gen Virol.* 2007;88:1423-1428.

356. Guo HG, Browning P, Nicholas J, et al. Characterization of a chemokine receptor-related gene in human herpesvirus 8 and its expression in Kaposi's sarcoma. *Virology.* 1997;228:371-378.

357. Cao JX, Gershon PD, Black DN. Sequence analysis of HindIII Q2 fragment of capripoxvirus reveals a putative gene encoding a G-proteincoupled chemokine receptor homologue. *Virology.* 1995;209:207-212.

358. Luttichau HR, Stine J, Boesen TP, et al. A highly selective CC chemokine receptor (CCR)8 antagonist encoded by the poxvirus molluscum contagiosum. *J Exp Med.* 2000;191:171-180.

359. Lalani AS, Masters J, Graham K, et al. Role of the myxoma virus soluble CC-chemokine inhibitor glycoprotein, M-T1, during myxoma virus pathogenesis. *Virology.* 1999;256:233-245.

360. Reading PC, Symons JA, Smith GL. A soluble chemokine-binding protein from vaccinia virus reduces virus virulence and the inflammatory response to infection. *J Immunol.* 2003;170:1435-1442.

361. Smith CA, Smith TD, Smolak PJ, et al. Poxvirus genomes encode a secreted, soluble protein that preferentially inhibits beta chemokine activity yet lacks sequence homology to known chemokine receptors. *Virology.* 1997;236:316-327.

362. Van Berkel V, Barrett J, Tiffany HL, et al. Identification of a gammaherpesvirus selective chemokine binding protein that inhibits chemokine action. *J Virol.* 2000;74:6741-6747.

363. Saederup N, Aguirre SA, Sparer TE, et al. Murine cytomegalovirus CC chemokine homolog MCK-2 (m131-129) is a determinant of dissemination that increases inflammation at initial sites of infection. *J Virol.* 2001;75:9966-9976.

364. Van de Walle GR, Favoreel HW, Nauwynck HJ, et al. Antibodyinduced internalization of viral glycoproteins and gE-gI Fc receptor activity protect pseudorabies virus-infected monocytes from efficient complement-mediated lysis. *J Gen Virol.* 2003;84:939-948.

365. Nagashunmugam T, Lubinski J, Wang L, et al. In vivo immune evasion mediated by the herpes simplex virus type 1 immunoglobulin G Fc receptor. *J Virol.* 1998;72:5351-5359.

366. Litwin V, Jackson W, Grose C. Receptor properties of two varicellazoster virus glycoproteins, gpI and gpIV, homologous to herpes simplex virus gE and gI. *J Virol.* 1992;66:3643-3651.

367. Oleszak EL, Kuzmak J, Hogue B, et al. Molecular mimicry between Fc receptor and S peplomer protein of mouse hepatitis virus, bovine corona virus, and transmissible gastroenteritis virus. *Hybridoma.* 1995;14:1-8.

368. Kostavasili I, Sahu A, Friedman HM, et al. Mechanism of complement inactivation by glycoprotein C of herpes simplex virus. *J Immunol.* 1997;158:1763-1771.

369. Fodor WL, Rollins SA, Bianco-Caron S, et al. The complement control protein homolog of herpesvirus saimiri regulates serum complement by inhibiting C3 convertase activity. *J Virol.* 1995;69:3889-3892.

370. Sahu A, Isaacs SN, Soulika AM, et al. Interaction of vaccinia virus complement control protein with human complement proteins: factor I-mediated degradation of C3b to iC3b1 inactivates the alternative complement pathway. *J Immunol.* 1998;160:5596-5604.

371. Liszewski MK, Leung MK, Hauhart R, et al. Smallpox inhibitor of complement enzymes (SPICE): dissecting functional sites and abrogating activity. *J Immunol.* 2009;183:3150-3159.

372. Vanderplasschen A, Mathew E, Hollinshead M, et al. Extracellular enveloped

vaccinia virus is resistant to complement because of incorporation of host complement control proteins into its envelope. *Proc Natl Acad Sci U S A.* 1998;95:7544-7549.

373. Marschang P, Gurtler L, Totsch M, et al. HIV-1 and HIV-2 isolates differ in their ability to activate the complement system on the surface of infected cells. *AIDS.* 1993;7:903-910.

374. Spiller OB, Morgan BP, Tufaro F, et al. Altered expression of hostencoded complement regulators on human cytomegalovirus-infected cells. *Eur J Immunol.* 1996;26:1532-1538.

375. Albrecht JC, Nicholas J, Cameron KR, et al. Herpesvirus saimiri has a gene specifying a homologue of the cellular membrane glycoprotein CD59. *Virology.* 1992;190:527-530.

376. Mao H, Tu W, Qin G, et al. Influenza virus directly infects human natural killer cells and induces cell apoptosis. *J Virol.* 2009;83:9215-9222.

377. Tseng CT, Klimpel GR. Binding of the hepatitis C virus envelope protein E2 to CD81 inhibits natural killer cell functions. *J Exp Med.* 2002;195:43-49.

378. Ulbrecht M, Martinozzi S, Grzeschik M, et al. Cutting edge: the human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis. *J Immunol.* 2000;164:5019-5022.

379. Park B, Oh H, Lee S, et al. The MHC class I homolog of human cytomegalovirus is resistant to down-regulation mediated by the unique short region protein (US)2, US3, US6, and US11 gene products. *J Immunol.* 2002;168:3464-3469.

380. Hershkovitz O, Zilka A, Bar-Ilan A, et al. Dengue virus replicon expressing the nonstructural proteins suffices to enhance membrane expression of HLA class I and inhibit lysis by human NK cells. *J Virol.* 2008;82:7666-7676.

381. Lenac T, Arapovic J, Traven L, et al. Murine cytomegalovirus regulation of NKG2D ligands. *Med Microbiol Immunol.* 2008;197:159-166.

382. Wilkinson GW, Tomasec P, Stanton RJ, et al. Modulation of natural killer cells by human cytomegalovirus. *J Clin Virol.* 2008;41:206-212.

383. Cerboni C, Neri F, Casartelli N, et al. Human immunodeficiency virus 1 Nef protein downmodulates the ligands of the activating receptor NKG2D and inhibits natural killer cell-mediated cytotoxicity. *J Gen Virol.* 2007;88:242-250.

384. Campbell JA, Trossman DS, Yokoyama WM, et al. Zoonotic orthopoxviruses encode a high-affinity antagonist of NKG2D. *J Exp Med.* 2007;204:1311-1317.

385. Cheng TP, French AR, Plougastel BF, et al. Ly49h is necessary for genetic resistance to murine cytomegalovirus. *Immunogenetics.* 2008;60:565-573.

386. Mao H, Tu W, Liu Y, et al. Inhibition of human natural killer cell activity by influenza virions and hemagglutinin. *J Virol.* 2011;84:4148-4157.

387. Arnon TI, Achdout H, Levi O, et al. Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus. *Nat Immunol.* 2005;6:515-523.

388. Thomas M, Boname JM, Field S, et al. Down-regulation of NKG2D and NKp80 ligands by Kaposi's sarcoma-associated herpesvirus K5 protects against NK cell cytotoxicity. *Proc Natl Acad Sci U S A.* 2008;105:1656-1661.

389. Tomasec P, Wang EC, Davison AJ, et al. Downregulation of natural killer cell-activating ligand CD155 by human cytomegalovirus UL141. *Nat Immunol.* 2005;6:181-188.

390. Odeberg J, Browne H, Metkar S, et al. The human cytomegalovirus protein UL16 mediates increased resistance to natural killer cell cytotoxicity through resistance to cytolytic proteins. *J Virol.* 2003;77: 4539-4545.

391. Orvedahl A, Alexander D, Talloczy Z, et al. HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. *Cell Host Microbe.* 2007;1:23-35.

392. Temme S, Eis-Hubinger AM, McLellan AD, et al. The herpes simplex virus-1 encoded glycoprotein B diverts HLA-DR into the exosome pathway. *J Immunol.* 2010;184:236-243.

393. Mohamadkhani A, Sotoudeh M, Bowden S, et al. Down regulation of HLA class II molecules by G1896A pre-core mutation in chronic hepatitis B virus infection. *Viral Immunol.* 2009;22:295-300.

394. Cusick MF, Yang M, Gill JC, et al. Naturally occurring CD4(+) T-cell epitope variants act as altered peptide ligands leading to impaired helper T-cell responses in hepatitis C virus infection. *Hum Immunol.* 2010;72:379-385.

395. Miller DM, Rahill BM, Boss JM, et al. Human cytomegalovirus inhibits major histocompatibility complex class II expression by disruption of the Jak/Stat pathway. *J Exp Med.* 1998;187:675-683.

396. Weissman JD, Brown JA, Howcroft TK, et al. HIV-1 tat binds TAFII250 and represses TAFII250-dependent transcription of major histocompatibility class I genes. *Proc Natl Acad Sci U S A.* 1998;95: 11601-11606.

397. Daskalogianni C, Apcher S, Candeias MM, et al. Gly-Ala repeats induce position- and substrate-specific regulation of 26 S proteasomedependent partial processing. *J Biol Chem.* 2008;283:30090-30100.

398. Phillips AJ, Tomasec P, Wang EC, et al. Human cytomegalovirus infection downregulates expression of the cellular aminopeptidases CD10 and CD13. *Virology.* 1998;250:350-358.

399. Scholz C, Tampe R. The intracellular antigen transport machinery TAP in adaptive immunity and virus escape mechanisms. *J Bioenerg Biomembr.* 2005;37:509-515.

400. Horst D, Favaloro V, Vilardi F, et al. EBV protein BNLF2a exploits host tail-anchored protein integration machinery to inhibit TAP. *J Immunol.* 2011;186:3594-3605.

401. Park B, Kim Y, Shin J, et al. Human cytomegalovirus inhibits tapasindependent peptide loading and optimization of the MHC class I peptide cargo for immune evasion.

Immunity. 2004;20:71-85.

402. Bennett EM, Bennink JR, Yewdell JW, et al. Cutting edge: adenovirus E19 has two mechanisms for affecting class I MHC expression. *J Immunol.* 1999;162:5049-5052.

403. del Val M, Hengel H, Hacker H, et al. Cytomegalovirus prevents antigen presentation by blocking the transport of peptide-loaded major histocompatibility complex class I molecules into the medial-Golgi compartment. *J Exp Med.* 1992;176:729-738.

404. van der Wal FJ, Kikkert M, Wiertz E. The HCMV gene products US2 and US11 target MHC class I molecules for degradation in the cytosol. *Curr Top Microbiol Immunol.* 2002;269:37-55.

405. Lubben NB, Sahlender DA, Motley AM, Lehner PJ, Benaroch P, Robinson MS. HIV-1 Nef-induced down-regulation of MHC class I requires AP-1 and clathrin but not PACS-1 and is impeded by AP-2. *Mol Biol Cell.* 2007;18:3351-3365.

406. Lorenzo ME, Jung JU, Ploegh HL. Kaposi's sarcoma-associated herpesvirus K3 utilizes the ubiquitin-proteasome system in routing class major histocompatibility complexes to late endocytic compartments. *J Virol.* 2002;76:5522-5531.

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Chapter 40

Immunity to Intracellular Bacteria

Anca Dorhoi

Stephen T. Reece

Stefan H. E. Kaufmann

INTRODUCTION

This chapter focuses on infections by intracellular bacteria with emphasis on both pathogenicity and general immune mechanisms underlying protection. Intracellular bacteria comprise numerous pathogens, some of which are of utmost medical importance. Ancient (but still existent), as well as newly emerging, diseases are caused by intracellular bacteria, with tuberculosis (TB), typhoid, leprosy, and trachoma as the most relevant etiologically associated infections.

Intracellular bacteria live inside host cells for most of their lives. This coexistence must allow survival of the infected cell; therefore, intracellular bacteria generally lack toxicity. Instead, they have evolved multiple strategies to interfere with key host cell biologic processes to promote replication or persistence. This has direct consequences for the immune response evoked by the host attempting to resolve intracellular infection. In fact, acquired resistance against intracellular bacterial infections depends on activation of multiple arms of the immune system. In this chapter, we start by examining the complex molecular crosstalk between pathogen and host. We then consider how these molecular events translate into the development of adaptive immunity. Finally, we examine effects of both adaptive and innate immune mechanisms on long-term effects of intracellular bacterial infection. Such long-term effects include tissue remodelling, nonresolved inflammation, and chronic immune responses, which for the most part are tolerated by the host. We hope this chapter will stimulate an interest in intracellular infection and the unique immunologic insights it can provide.

FEATURES OF INFECTIONS WITH INTRACELLULAR BACTERIA

Intracellular Bacteria/Public Health Relevance

Bacterial pathogens are prokaryotic microorganisms that cause disease in a given host species. They are single cells, typically micrometers in length, and present a specific spectrum of interactions with their human host. Infectious disease is the direct and invariable consequence of an encounter between host and pathogen. Often, it is the eventual outcome of complex interactions between them. There is a marked degree of diversity among bacterial species that are able to induce intracellular infections. This encompasses structural organization of the cell wall (ie, gram-negative versus -positive, salmonellae versus listeriae), presence of motility organelles (eg, fimbriae and flagella, of salmonellae and listeriae[1,2]) or secretion apparatus (type III secretion system [T3SS], type IV secretion system (T4SS), type VII secretion system [T7SS] in *Salmonella*,[3] *Legionella*,[4] and *Mycobacteria*,[5] respectively), and metabolic characteristics that dictate replication time (fast- versus slow-growing,

Salmonella versus *Mycobacterium*). This diversity is reflected most often in differences related to cell biology of the pathogen. However, major patterns of the immune response are shared, as detailed in the following.

Of paramount significance for humans are *Mycobacterium tuberculosis*, *Mycobacterium leprae, Salmonella enterica* serovar Typhi, *Chlamydia trachomatis*, and the etiologic agents of TB, leprosy, typhoid, and trachoma, respectively, which together afflict more than 200 million people globally. Some opportunistic pathogens such as *Mycobacterium avium/Mycobacterium intracellulare* are gaining increasing significance particularly for immunodeficient patients, such as acquired immunodeficiency syndrome (AIDS) sufferers. Many zoonotic agents are intracellular bacteria and include *Chlamydia psittaci* (psittacosis), *Brucella* (brucellosis, Malta fever/Bang disease), *Coxiella burnetii* (Q fever), *Rickettsia* (Rocky Mountain/Mediterranean spotted fever), *Francisella tularensis* (tularemia), *Burkholderia mallei* (glanders), *Yersinia pseudotuberculosis* (yersiniosis), *Yersinia pestis* (plague), *Bartonella* (cat scratch disease), and *Listeria monocytogenes* (listeriosis). Other opportunistic pathogens with an intracellular lifestyle that are relevant for human health include *Legionella pneumophila* (Legionnaires disease) and *Ehrlichia* (ehrlichiosis).

Epidemiology and Pathogenesis

Some intracellular bacteria, in particular *Rickettsia*, are introduced directly into the bloodstream by insect bites from where they have ready access to internal tissues. Most intracellular bacteria, however, enter the host through the mucosa.[6] Major ports of entry are the lung for airborne pathogens, such as *M. tuberculosis* and *L. pneumophila*, and the intestine for foodborne pathogens, such as *S. enterica* and *L. monocytogenes.* Subsequently, intracellular bacteria pass through the epithelial layers. Either they actively induce transcytosis (ie, endo- and exocytosis) through the epithelial cells or they are passively translocated within phagocytes. Bacteria may be removed by nonspecific defense mechanisms such as mucociliary movements and gut peristalsis, or they may be destroyed by professional phagocytes without necessitating the specific attention of the immune system. Cells that survive these nonspecific defense reactions

colonize deeper tissue sites and stably infect a suitable niche. At this stage, the host generally develops a specific acquired immune response.

BOX 40.1. CENTRAL FEATURES OF INFECTIONS WITH INTRACELLULAR BACTERIA

- Infection is separated from disease, and the immune response is already induced at the stage of infection.
- Infection persists latently in the face of dynamic interactions between pathogen and immune mechanisms.
- The host-pathogen relationship represents a highly sophisticated form of parasitism that does not necessarily lead to disease but rather allows for long-lasting coexistence.
- Infection includes the potential to harm the host severely at a later stage, and pathogenesis is strongly influenced by the immune response.

Infection is unsuccessful when the immune system succeeds in eliminating the pathogen before overt clinical disease develops. Alternatively, tissue damage increases to a significant level before the immune system succeeds in controlling the pathogen effectively and clinical disease develops. This is the case with many extracellular bacteria and is due to their cell lytic strategies (toxins) that cause diseases of acute type, but is less common for intracellular bacteria. Finally, it is possible that the immune response restrains the infectious agent but fails to completely eradicate it. Under these conditions, a long-lasting equilibrium between

microbial persistence and the immune response unfolds. This balance, however, remains unstable and can be tipped in favor of the pathogen at a later time, converting infection into disease.

The time lapse between host entry and expression of clinical disease is termed incubation time and from what has been said previously, it follows that in many intracellular bacterial infections the incubation times are long-lasting to lifelong. By improving the immune response or by impairing bacterial growth (typically accomplished by chemotherapy), or both, disease can be overcome. Ideally, bacterial eradication is achieved; alternatively, some dormant bacteria continue to persist in niches poorly accessible to the immune response (Box 40.1).

There is a close correlation between the cell biology and cell tropism of the pathogen and disease signs and pathology in, for instance, enteric versus lung conditions. However, even bacteria that use the same niches as habitat show major clinical differences. This is due to pathogenintrinsic biology (ie, metabolism) and peculiarities of each species to subvert host elimination. For instance, *M. tuberculosis*, *L. pneumophila*, *F. tularensis*, and *C. burnetii* cause aerogenic infections with *Coxiella* being the most successful in establishing infection (one bacterium is sufficient to induce disease, probably the most infectious bacterium). These pathogens successfully infect lung macrophages and parenchymal cells to establish infection. However, TB has a protracted course and insidious clinical signs while francisellae and coxiellae induce acute and subacute pneumonias with tissue changes substantially different from TB. Disease pathogenesis is principally the outcome of the crosstalk between host and pathogen at the cellular level with strong influences from bacteria-specific behavior, while clinical signs and pathology are often induced by host responses.

"Idealized" Intracellular Bacterium

Although this chapter focuses on general mechanisms underlying immunity to intracellular bacteria, it is important to emphasize that this group is extremely heterogeneous. Therefore, the major hallmarks of intracellular bacterial infections will first be described for a nonexistent "idealized" intracellular bacterium (Table 40.1). Subsequently, characteristics of selected intracellular bacteria will be specified.

Facultative and Obligate Intracellular Bacteria

With respect to preferred habitat, intracellular bacteria can be divided into two groups: First, those pathogens that do not essentially depend on the intracellular habitat, including *M. tuberculosis*, *Mycobacterium bovis*, *M. leprae*, *S. enterica*, *Brucella*, *L. pneumophila*, *L. monocytogenes*, and *F. tularensis* (Table 40.2).[7,8,9,10,11,12,13,14,15] Although these pathogens

favor mononuclear phagocytes (MPs) as their habitat, other types of host cells are infected as well. *M. leprae*, for example, lives in numerous host cell types, notably in Schwann cells and hepatocytes serve as an important reservoir for *L. monocytogenes.* Although *M. tuberculosis* can infect a variety of mammalian cells in vitro, in vivo it seems to restrict itself to phagocytes and perhaps epithelial cells.

TABLE 40.1 Hallmarks of Intracellular Bacterial Infections

Hallmark 1	The intracellular lifestyle represents the distinguishing feature of intracellular bacteria.

Hallmark 2	T cells are the central mediators of protection against intracellular bacterial infections. In contrast, antibodies play a facilitating role.
Hallmark 3	Infections with intracellular bacteria are accompanied by delayed-type hypersensitivity, which expresses itself after local administration of soluble antigens as a delayed tissue reaction mediated by T cells and effected by macrophages.
Hallmark 4	Tissue reactions against intracellular bacteria are granulomatous. Protection against, as well as pathology caused by, intracellular bacteria are centered on these lesions. Rupture of a granuloma promotes bacterial dissemination.
Hallmark 5	Intracellular bacteria express little or no toxicity for host cells by themselves, and pathology is primarily a result of immune reactions, particularly those mediated by T-lymphocytes.
Hallmark 6	Intracellular bacteria coexist with their cellular habitat for long periods of time. A labile balance develops between persistent infection and protective immunity, resulting in long incubation time and in chronic disease. Accordingly, infection is dissociated from disease.

Hallmarks 1 to 4 should be considered essential, and Hallmarks 5 and 6 conditional, criteria for defining intracellular bacteria.

TABLE 40.2 Major Infections of Humans Caused by Facultative Intracellular Bacteria

Pathogen	Disease	Preferred Target Cell	Preferred Location in Host Cell	Preferred Port of Entry
Mycobacterium tuberculosis	Tuberculosis	Macrophages	Early phagosome	Lung
Mycobacterium leprae	Leprosy	Macrophages, Schwann cells, other cells	Phagolysosome, cytosol	Nasopharyngeal mucosa
Yersinia pestis	Plague	Macrophages	Autophagosome	Gut, skin
Salmonella enterica serovar Typhi	Typhoid fever	Macrophages	Late phagosome, spacious phagosome	Gut

Shigella spp.	Shigellosis	Macrophages	Cytosol	Gut
Brucella spp.	Brucellosis	Macrophages	Early phagosome	Mucosa
Legionella spp.	Legionnaires' disease	Macrophages	Endoplasmic reticulum-derived phagosome	Lung
Listeria monocytogenes	Listeriosis	Macrophages, hepatocytes	Cytosol	Gut
Francisella tularensis	Tularemia	Macrophages	Late endosome, cytosol	Skin, lung, mucosa

The second group includes so-called obligate intracellular bacteria, which fail to survive outside host cells. Most of these bacteria prefer nonprofessional phagocytes as their habitat —for example, endothelial and epithelial cells. Rickettsiae, chlamydiae, and ehrlichiae are representatives of this group. They include *Rickettsia prowazekii*, *Rickettsia rickettsii*, *Rickettsia typhi*, and *Orientia tsutsugamushi* (*Rickettsia tsutsugamushi* until 1995), the etiologic agents of louse-borne typhus, Rocky Mountain spotted fever, typhus, and scrub typhus, respectively.[16,17,18,19] Various biovars of *Chlamydia trachomatis*, which are responsible for trachoma,[20] conjunctivitis, urogenital infections, and lymphogranuloma venerum,[21] as well as *C. psittaci* and *Chlamydia pneumoniae*, causative agents of psittacosis or rare types of pneumonia,[22] respectively, also belong to this group (Table 40.3). Certain obligate intracellular bacteria, such as *Ehrlichia* and *Anaplasma phagocytophilum*, parasitize blood cells.[23,24] *C. burnetii*, the causative agent of Q fever, resides in macrophages and lung parenchymal cells.[25,26]

TABLE 40.3 Major Infections of Humans Caused by Obligate Intracellular Bacteria

Pathogen	Disease	Preferred Target Cell	Preferred Location in Host Cell	Preferred Port of Entry
Rickettsia rickettsii	Rocky Mountain spotted fever	Endothelial cells, smooth muscle cells	Cytosol	Blood vessel (tick bite)
Rickettsia prowazekii	Endemic typhus	Endothelial cells	Cytosol	Broken skin, mucosa
Rickettsia typhi	Typhus	Endothelial cells	Cytosol	Blood vessel

				(flea bite)
Rickettsia tsutsugamushi	Scrub typhus	Endothelial cells	Cytosol	Blood vessel (mite bite)
Coxiella burnetii	Q fever	Macrophages, lung parenchymal cells	Late phagosome	Lung
Chlamydia trachomatis	Urogenital infection, conjunctivitis, trachoma, lymphogranuloma, venereum (different serovars)	Endothelial cells	Phagosome/nonacidified inclusion	Eye, urogenital mucosa
Chlamydia psittaci	Psittacosis	Macrophages, lung parenchymal cells	Phagosome/nonacidified inclusion	Lung
Chlamydia pneumoniae	Pneumonia	Lung parenchymal cells	Phagosome	Lung
Ehrlichia ewigii	Ehrlichiosis	Granulocytes	Cytosol	Blood vessel (tick bite)
Ehrlichia chaffeensis	Ehrlichiosis	Monocytes, macrophages	Cytosol	Blood vessel (tick bite)
Anaplasma phygocytophilum	Anaplasmosis	Granulocytes	Cytosol	Blood vessel (tick bite)

Preferential living in macrophages does not depend on specific invasion mechanisms but rather on highly sophisticated intracellular survival strategies. Yet, most facultative intracellular bacteria express unique invasion factors, if only to cross epithelial layers. Selection of nonprofessional phagocytes as habitat essentially depends on invasion molecules whereas survival inside these cells is generally less hazardous.

CELL BIOLOGY

For intracellular bacteria, entry into host cells represents the central requirement for survival in, as well as elimination by, the host. Host cell-directed uptake, called phagocytosis, is a feature of the so-called professional phagocytes that comprise polymorphonuclear granulocytes (PNGs) and MPs. Examples of bacteria that are engulfed by phagocytosis include *M. tuberculosis*, *L. pneumophila*, and *C. burnetii.* Entry induced by the pathogen is termed invasion, which allows entry into nonphagocytic cells (nonprofessional phagocytes). Salmonellae, shigellae, and listeriae are paradigms of enteroinvasive pathogens.[27] Contact between host cells and pathogens proceeds either directly via receptor-ligand interactions or indirectly via deposition on the surface of the pathogen of host molecules for which physiologic receptors exist on the target cell.

Depending on the cellular target, the final outcome of host cell entry varies markedly.

1. Nonprofessional phagocytes are nonphagocytic, and hence entry depends on expression of surface receptors that can be exploited for invasion. Because of their low antibacterial activities, they primarily serve as a habitat.
2. PNGs are short-lived. Because they are highly phagocytic and express potent antibacterial activities constitutively, uptake by PNGs is often fatal for the pathogen.
3. MPs are phagocytic and express medium to high antibacterial activities depending on their activation status. Accordingly, they serve both as habitat and as effector cell.

Following entry, bacterial pathogens begin intravacuolar life.[28] Two main strategies are followed to avoid killing: 1) avoidance of delivery to degradative lysosomes, either by blocking phagosome maturation, divergence from the endocytic pathway to establish a vacuole with unique features, or by escape into the cytosol; and 2) development of strategies to survive within acidic degradative organelles. Certain bacteria have developed mechanisms that allow them to impede nutrient flow inside the infected cell for their own benefit, to modulate generation of antimicrobial molecules, or to alter cell death pathways.[29,30] In the following, the major steps from uptake to bacterial elimination by, or survival in, host cells will be described (Fig. 40.1).

Adhesion and Invasion

Adhesion to mammalian cells is a prerequisite for extracellular colonization and for host cell invasion. Bacterial adhesins that solely expedite adhesion to host cells are expressed by numerous extracellular bacteria. In contrast, invasion-inducing molecules are a feature of bacteria that permanently or transiently enter host cells. Adherence to the cell membrane is based on protein-protein interactions mediated by adhesins, such as internalins (bind E-cadherin) of *L. monocytogens* or invasins (bind β1 integrins) from *Yersinia.* Adhesins may be located on the bacterial surface or on pili. In addition, adhesion may be induced by bacterial virulence factors, which recruit fibronectin to attach to host cells by binding to integrins.[29,31]

Although induced by the bacterium, invasion is ultimately a function of the host cell. Following adhesion, invasion can be induced in two ways. First, cell signaling by host cell receptors that serve as targets of adhesion induces uptake; second, uptake is induced independently from the molecules that mediate adhesion.[31,32] The term “zipper mechanism” refers to the first process. Bacterial proteins interact with host cell surface proteins to mediate internalization. This term has been suggested for the highly selective receptor-mediated bacterial entry, whereas the term “trigger mechanism” has been proposed for indiscriminate, apparently adhesion-independent uptake. Bacterial effectors are delivered to the host cytosol via a secretion system to induce bacterial entry.[27]

Entry by Zipper Mechanisms

Host cell invasion by *Yersinia* and *L. monocytogenes* are examples of invasion via the "zipper mechanism." Receptor binding induces phagocytic mechanisms in nonprofessional phagocytes similar to those that are constitutively operative in MPs. The eukaryotic cell membrane tightly enwraps the bacterium and a cascade of events, including protein phosphorylation, ubiquitination, and phospholipid modifications then contribute to vacuole genesis.[28] Host entry of *L. monocytogenes* through the intestinal epithelia is mediated by internalin on the surface of this pathogen and E-cadherin on human epithelial cells.[33] Murine E-cadherin does not serve as a receptor for internalin due to an amino acid substitution in position 16.[34] Schwann cells, a major target of *M. leprae*, are shielded by a basal lamina composed of laminin, collagen, and proteoglycans. The unique tropism of *M. leprae* for peripheral nerves appears to be due to bacterial binding to laminin. This molecule, which serves as natural ligand for integrins, thus provides a link between pathogen and Schwann cell.[35] Caveolin and lipid rafts serve as entry portals for *Brucella* and certain strains of *Chlamydia.* More recently, evidence was provided that clathrin-mediated endocytosis contributes to entry of *L. monocytogenes* and *Rickettsia* in a similar zipper mechanism.[36,37] Septins, which are small guanine triphosphatases (GTPases), are able to form filaments and interact with actin to facilitate bacterial entry as well.[38]

Entry by Trigger Mechanisms

Different molecules and mechanisms participate in host cell entry by *S. enterica.* Interactions between *S. enterica* and host cells causes large "membrane ruffling" at the site of attachment followed by bacterial entry. Ruffling induces indiscriminate uptake even of other particles in the vicinity of *S. enterica*. This process has been termed macropinocytosis. *S. enterica* triggers its uptake by exploiting the signaling machinery of

the host cell, thus inducing cytoskeletal rearrangements. In certain mouse cells, *S. enterica* induces phosphorylation of the receptor for the epidermal growth factor.[39] Yet, *S. enterica* can also enter cells that do not express the epidermal growth factor receptor. This pathogen possesses two T3SS that allow it to directly manipulate intracellular molecules within host cells.[40] *Salmonella* outer proteins are secreted into the host cells rapidly after contact. *Salmonella* outer proteins activate the small GTP-binding protein cell division control (CDC)42 of the Ras superfamily, which, in turn, induces the reorganization of the actin cytoskeleton, thus promoting bacterial invasion through membrane ruffling. A homolog of *Salmonella* outer protein (termed *Salmonella* outer protein 2) performs similar functions, and hence the two molecules may partly compensate each other's functions. The transiently intracellular pathogen *Shigella* utilizes similar mechanisms for uptake via membrane ruffling.[41,42] More recently, filopodia were shown to trap and direct shigellae to target cells in a process that involves bacterial T3SS.[43] Generally, modulators of the eukaryotic cell cytoskeleton are T3SS or T4SS products, which modulate GTPase cycling of proteins of the Rho, Rab, and Arf families.[44]

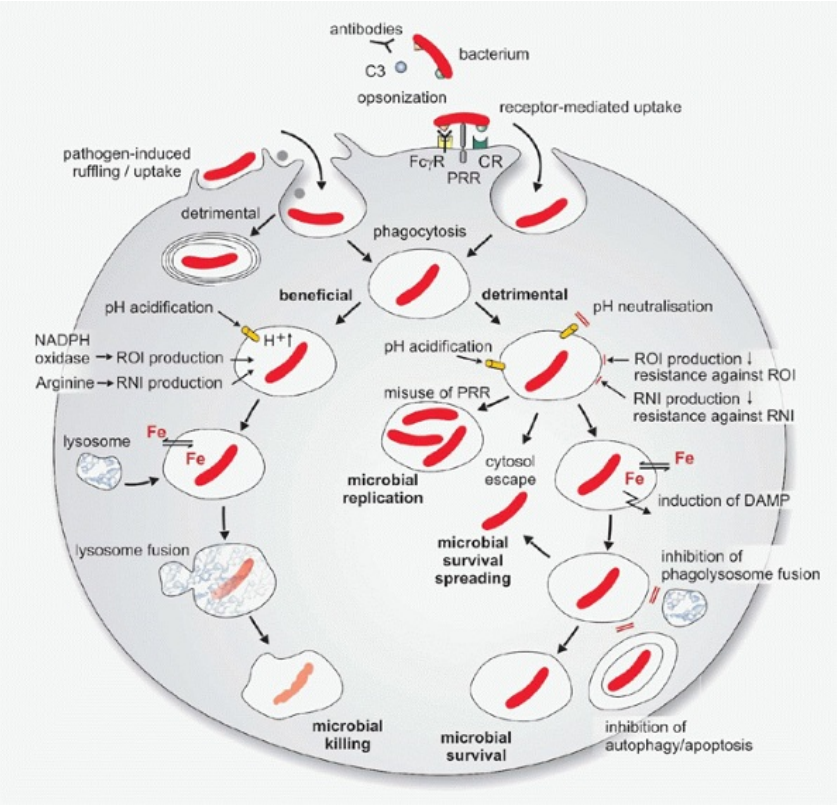

FIG. 40.1. The Multiple Encounters Between Phagocytes and Intracellular Bacteria. Bacterial uptake is both pathogen-induced and receptormediated. Multiple opsonic receptors, including complement receptor (CR) and receptors for immunoglobulins (FcγR), facilitate phagocytosis. In addition, certain pattern recognition receptors (PRRs) may contribute to bacterial internalization. Macrophages and neutrophils direct their bacterial cargo to the endosomal pathway, endosomes become acidic and progressively accumulate reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs). The phagosome maturation process culminates in fusion between late endosomes and lysosomes, which leads to bacterial killing but intracellular bacteria can interfere with phagosomal killing. Certain bacteria modulate early uptake events to prevent endosomal maturation. Other species prevent acidification of the phagosome or nullify ROIs and RNIs. Escape from the phagosome into the host cytosol is a strategy used by pathogenic bacteria and can occur at multiple stages of phagosome maturation. Certain bacteria exploit PRRs to enable self-replication in modified endosomes or modulate iron (Fe) abundance. Inhibition of phagolysosome formation, apoptosis, and autophagy also contribute to establishment of infection with intracellular bacteria. DAMP, danger-associated molecular pattern; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase.

Invasion of Nonprofessional Phagocytes

Microbe-directed uptake allows entry into nonphagocytic cells and hence can be seen as an evasion mechanism of phagocytosis by professional phagocytes. The target spectrum of intracellular bacteria ranges from very broad to

highly specific. *M. leprae* is found in a large variety of host cells and hence shows a broad target cell spectrum. *L. monocytogenes* enters the host through the gut epithelium and its major target besides MPs is the hepatocyte; *M. tuberculosis* is almost, if not exclusively,

restricted to MPs, although pneumocytes have been proposed as a safe niche in the lung. It is noteworthy that intracellular bacteria are often capable of entering a variety of cell lines in vitro. These in vitro experiments do not necessarily reflect an in vivo situation, and care should be taken in extrapolating conclusions from them. For obligate intracellular bacteria, nonprofessional phagocytes rather than MPs represent the preferred habitat. These bacteria are primarily found in endothelial and epithelial cells.[17,21]

Recognition and Downstream Events

Microbes are composed of various molecules that are structurally different from host cell/tissue composition (ie, lipopolysaccharide [LPS], peptidoglycan [PGN], cytosinphosphatidyl-guanosin [CpG] deoxyribonucleic acid [DNA]). These microbe-specific molecules encompass various biochemical entities and have been named pathogenassociated molecular patterns (PAMPs).[45] Once pathogens assault host tissues, PAMPs are recognized by pattern recognition receptors (PRRs). PRRs are nonclonally distributed on various cell types; they are germline encoded and their activation, following PAMP ligation, is an essential event for initiation of the immune response and disposal of the intruder.[46,47,48,49] Microbial ligand diversity parallels the receptor repertoire, and currently there are several classes of PRRs known (Fig. 40.2), which may be classified based on chemical structure as detailed in the following:

- Toll-like receptors (TLRs) are type-I transmembrane proteins. Currently, 10 TLR members in humans and 12 in mice have been described.[50] TLRs 1, 2, 4, 5, and 6 are cell surface-associated, and TLRs 3, 7, 8, and 9 are associated with vesicles of the endoplasmic reticulum (ER), endosomes, and lysosomes (see Fig. 40.2).[51] TLRs are signaling receptors and recruit single or a combination of toll-interleukin (IL)-1 receptor-resistance (TIR)-containing adaptors, including myeloid differentiation primary response gene 88 (MyD88), TIR domain-conining adaptor protein, TIR domain-containing adaptor-inducing interferon-beta (IFN-β, and TIR domain-containing adaptor-inducing IFN-β-related adaptor molecules.[46] Timing (sequential versus simultaneously) and space (vacuole characteristics, early versus late phagosome) are essential for TLR downstream effects. TLR-4 senses LPS and thus detects extracellular gram-negative bacteria. TLR-4 first initiates nuclear factor kappa-beta (NF-κB) and secondly IFN I pathway activation via MyD88 and TIR domain-containing adaptor-inducing IFN-β, respectively. Once bacteria are phagocytosed, TLR-9 senses CpG DNA and signals via MyD88 to NF-κB in early endosomes while in lysosomes Traf3/IRF3 is assembled to enable IFN I responses.[50] TLR activation may impact the microbicidal capacity of the infected cell.[52]
- C-type lectin receptors (CLRs) belong to a large superfamily of membrane or soluble proteins, which have one or more calcium-dependent carbohydrate-binding lectin domains (see Fig. 40.2).[53,54] CLRs have high avidity for carbohydrates/glycans. CLRs mediate endocytic uptake, a feature distinct from that of TLRs. Signaling is mediated by immune-receptor tyrosine-based activation motif/spleen tyrosine kinase (Dectins; macrophage-inducible C-type lectin, Mincle), hemITAM (Dectin-1), immune-receptor tyrosine-based inhibition motif, and the kinase Raf1 (Dectin-1, dendritic cell [DC]-specific intercellular adhesion molecule-3-grabbing nonintegrin [SIGN]) to activate mitogenactivated protein kinase (MAPK) and NF-κB. CLRs are involved in generation of reactive oxygen species via the syk pathway in myeloid cells, modulate adaptive immune responses, and seem to be indispensable elements for T helper (Th)17 responses. Other CLR, notably DC-SIGN, induce inhibitory signals.

- Nucleotide oligomerization domain (NOD)-like receptors (NLRs) are cytosolic proteins (see Fig. 40.2). This family comprises more than 20 members, which are structurally diverse and are involved in pathogen sensing (NOD1, NOD2) and initiation of the inflammatory response (nucleotide-binding domain and leucine-rich repeat with pyrin domain-containing [NLRP]1, NLRP3, nucleotide-binding domain with leucine rich repeat and caspase recruitment domain [NLRC]4; NLRC5).[55,56,57] NOD1 and 2 use the receptorinteracting protein 2 adaptor to signal and are involved in PGN recognition. Thus, they are essential for sensing cytosolic bacteria in professional and nonprofessional phagocytes. NRLP and NLRC members, as well as NOD2, are essential components of inflammasomes, which are cytosolic platforms responsible for secretion of IL-1β, as detailed later.
- Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are cytosolic ribonucleic acid helicases (RIG-I, MDA5, and LGP2) involved in antiviral immunity.[58]
- Scavenger receptors (SRs) are cell membrane proteins that recognize modified host molecules, as well as microbial structures.[59] These receptors are mainly involved in uptake of particles including intracellular bacteria (eg, macrophage receptor with collagenous structure, MARCO; SR-A, cluster of differentiation [CD]36) (see Fig. 40.2).
- Pyrin- and HIN-containing proteins (PYHINs) have been recently associated with pathogen sensing (see Fig. 40.2). Absent in melanoma 2 (AIM2) and gamma-IFN-inducible protein 16 are the most prominent receptors involved in DNA sensing and consequently modulation of IFN secretion and inflammation.[60] AIM2 is associated with inflammasome activation and senses host and bacterial cytosolic DNA.

A key issue for host defense is the receptor profile at particular time and tissue site of infection. PRR expression in phagocytes is influenced by cell type (resident tissue macrophage versus inflammatory monocytes), tissue (lung versus

gut), milieu, and activation status (certain cytokines up- or downregulate receptors).[61] Importantly, multiple PAMPs engage multiple PRRs simultaneously or sequentially and therefore, a given pathogen by means of specific combinations of PAMPs can tailor the host response in a highly specific way. TLRs can form dimers (eg, TRL-1/2 or TLR-2/6), and may collaborate with distinct CLRs (TLR-2/Dectin-1; TLR-2/MARCO; TLR-4/CD14). On the other hand, a given PAMP may be sensed by multiple PRRs (glucans sensed by Dectin-1; complement receptor [CR]; CD36). Thus, PRRs orchestrate pathogen- and cell type-specific host immune responses. Pathogens able to alter molecules or modulate host cell death add an additional layer to this recognition process by inducing activation of PRRs via recognition of pathogens in combination with sensing of danger signals. Certain PRRs are involved in sensing both microbial components and danger signals. For instance, Mincle recognizes mycobacterial trehalose dimycolate (TDM) and the ribonucleoprotein spliceosome-associated protein 130, a protein associated with eukaryotic cell death. Despite their name, PAMPs are not restricted to pathogens but are also expressed by nonpathogenic microbes. Hence, the host probably needs to sense pathogen-specific signals in addition. In the case of intracellular bacteria, such signals could emanate from bacterial persistence, that is, from duration of PAMP expression. More recently, bacterial messenger ribonucleic acid was suggested as a signal from viable bacteria (vitaPAMP), which alerts the immune system.[62]

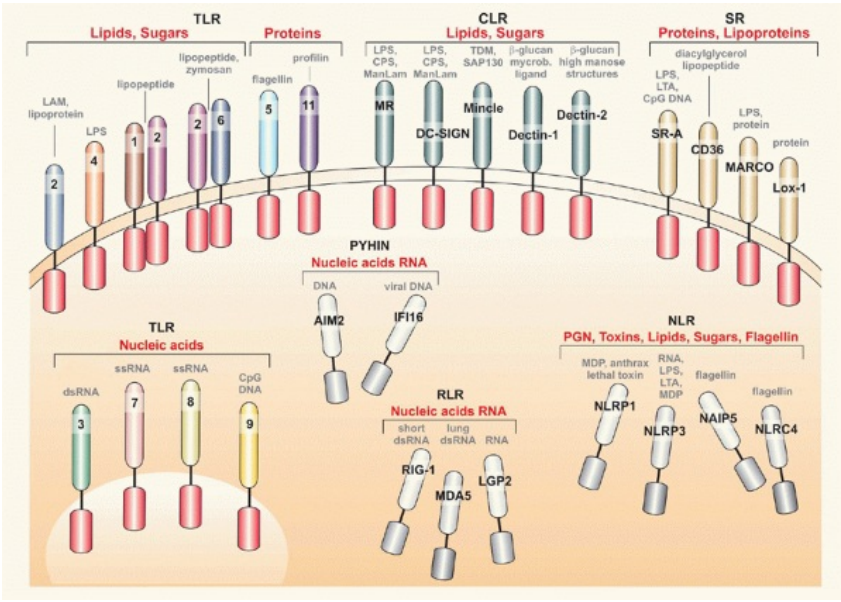

FIG. 40.2. The Pattern Recognition Receptors. Bacterial recognition is paramount in the crosstalk between host and pathogen. Several classes of receptors contribute to this process. Toll-like receptors are present at the surface membrane or within ensodomal compartments and recognize lipids, carbohydrates or proteins. C-type lectin receptors and scavenger receptors are expressed at the cell surface and sense glycolipids and lipoproteins, respectively. Surveillance of the cytosol is mainly performed by nucleotide oligomerization domain-like receptors (NLRs), RIG I-helicase receptors (RLRs), and pyrin- and HIN-containing proteins (PYHINs). RLRs are exclusively involved in viral detection, whereas NLRs recognize a wide range of structures. PYHIN detect deoxyribonucleic acid from various sources. AIM2, absent in melanoma 2; CpG, cytosinphosphatidyl-guanosin; CPS, capsular polysaccharide; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin; ds, double stranded; FI16, gamma-interferon-inducible protein 16; LAM, lipoarabinomannan; LGP2, laboratory of genetics and physiology 2; Lox-1, lectin-type oxidized LDL receptor 1; LPS, lipopolysaccharide; LTA, lipoteichoic acid; ManLAM, mannosylated lipoarabinomannan; MARCO, macrophage receptor with collagenous structure; MDA5, melanoma differentiation-associated gene 5; MDP, muramyl dipeptide; Mincle, macrophage-inducible C-type lectin; MR, mannose receptor; NAIP, baculoviral IAP repeat-containing protein; NLRP, NACHT, LRR and PYD domaincontaining protein; NLRC, NLR family CARD domain-containing protein 4; PGN, peptidoglycan; RIG-I, retinoic acid-inducible gene I; ss, singlestranded; TDM, trehalosedimycolate; SAP130, spliceosome-associated protein 130.

PRRs may be classified according to pathogen-binding propensity and impact on uptake as:

- Opsonic receptors: Fc receptors (FcRs), CRs, pentraxins, surfactant proteins, ficollins
- Nonopsonic receptors: TLRs, NLRs, RLRs

Based on their effects on pathogen internalization, PRRs may be classified as:

- Phagocytic receptors: CRs, FcRs, mannose receptors (MRs), and DC-SIGN (CLRs), CD36, MARCO, and SR-A (SRs)
- Signaling receptors: TLRs, NLRs, RLRs, CLRs

Bacterial cell wall composition, secretion products, as well as intracellular location are fundamental aspects for recognition of intracellular bacteria. *M. tuberculosis* is rich in several classes of PAMPs. This pathogen interferes with the endocytic pathway and resides in an early phagosome. Phagocytosis is mediated by CLRs (MRs, DC-SIGN) and SRs (CD36), which primarily recognize mannosylated cell wall mannans and by opsonic receptors, FcRs, and CRs. Another CLR, namely Langerin, seems to have a role in *M. leprae* recognition. Lung surfactant proteins mediate the recognition of tubercle bacilli in the alveolar space. Sensing of the lipopeptides and phosphatidylinositol mannans is performed by TLR-2, while TLR-4 involvement is still debated. Multiple CLRs sense mycobacterial glycans or lipoglycans. TDM, a specific component of mycobacteria, is recognized by Mincle, while DC-SIGN senses mannosylated lipoarabinomannan. SIGNR3 and Dectin-1 and -2 are also involved in recognition. However, to date no specific structure from mycobacteria has been identified as a ligand for these lectins. Once in the phagosome, bacteria may be sensed by TLR-9. Bacterial PGN, which accesses the cytosol, is recognized by NOD2. Notably, there is a degree of redundancy between receptors belonging to different classes, as revealed by studies with mice with single or multiple deficiencies in TLRs, NLRs, and SRs.[63] It appears that adaptors able to gather signals from various PRRs, such as MyD88[64] and caspase recruitment domain-containing protein 9 (Card9)[65] are key in controlling susceptibility to TB.

Recognition of *Salmonella*, which lives in a late phagosome, is dominated by TLR members. LPS and the lipopeptides from the cell wall are sensed by TLR-2 and -4 before internalization. Ablation of these TLR renders mice highly susceptible to disease.[66] Vacuolar bacteria are recognized by TLR-9 via CpG DNA. Interestingly, triple-deficient (TLR- 2, -4, -9) animals are resistant as simultaneous TLR activation is necessary for acidification of the vacuole, which in turn induces *Salmonella* pathogenicity island 2 (SPI2) genes. Virulence effectors are translocated into the cytosol transforming the phagosome into a replicative niche for *Salmonella.*[67] Flagella are sensed by TLR-5 and NLRC4.

Recognition of *L. monocytogenes*, which escapes from phagosome into cytosol, is complex. Cell wall lipopeptides are sensed by TLR-2, while PGN is recognized by NOD1 and NOD2. MyD88 seems to be essential for defense against listeriae.[68] Bacterial DNA is monitored by multiple sensors in the cytosol, including AIM2[69,70,71,72,73] and leucine-rich repeat flightless-interacting protein 1.[74] *Francisella*, which also egresses into the cytosol, is recognized in a similar way by multiple receptors, including AIM2[70,75] and NLRC4.

By sensing the broad molecular spectrum of pathogens, PRRs induce cellular programs as the first line of defense, including antimicrobial effector functions and maturation of DCs for instruction of the adaptive immune response. However, PRRs and their adaptors may be exploited by intracellular pathogens to escape killing and establish stable infection. Mycobacteria use CRs to ensure a safe entry into macrophages and limit maturation of the phagosome. They also use the TLR/MyD88 pathway to induce cytokines (IL-6 and IL-10), which through signal transducer and activator of transcription (STAT)3 signalling bias arginine metabolism to arginase-1 over nitric oxide production by inducible nitric oxide synthase (iNOS).[76,77] In addition, polymorphonuclear leukocyte encounters with mycobacteria results in MyD88/Card9-dependent IL-10 release to dampen immune responses.[65,78] Similarly *Yersinia* uses TLR-2 to induce IL-10.[79]

Phagocytosis and Phagosome Dynamics

Phagocytosis of inert particles initiates a series of events that ultimately lead to the formation

of a phagolysosome (see Fig. 40.1).[80,81] Phagosome maturation is a strictly coordinated sequence of fusion and fission events, which involves defined compartments of the endocytic pathway.[81,82] Immediately after or even during phagosome sealing, phagosome maturation proceeds.

- The early phagosome is characterized by a slightly acidic to neutral pH (6.0 to 6.5) and membrane markers, such as MR, the tryptophane aspartate-containing coat protein, and the transferrin receptor (TfR) with its ligand transferrin, small GTPase (Rab5), early endosomal antigen 1, phosphatidylinositol 3-phosphate, and Syntaxin13.
- The late phagosome is characterized by a pH between 5.0 and 6.0 and the acquisition of the vacuolar adenosine triphosphatase (ATPase) proton pump (V-H+ATPase), mannose-6 phosphate receptor, Rab-interacting lysosomal protein, and Rab7.
- The phagolysosome results from the fusion between phagosomes and lysosomes, characterized by a pH between 4.0 and 5.5, high density of lysosome-associated membrane proteins, and typical lysosomal enzymes (such as cathepsins).

The three stages form a continuum involving the sorting of membrane proteins, as well as budding of, and fusion with, other vesicles. During this dynamic process, the phagosomes successively interact with the corresponding endosomes and subsequently with lysosomes.[81] Characterization of the Rab family of GTPases on vacuoles harboring pathogens facilitates identification of the host membrane transport pathways, which are turned on during infection.[83] *M. tuberculosis* and *S. enterica* interfere with the endocytic pathway by retaining Rab5 and Rab7, respectively, on their vacuoles.[84] *L. pneumophila*, *Brucella abortus*, and *C. trachomatis* interact with the secretory pathway as revealed

by the presence of Rab1, Rab2, and Rab6, respectively, on their phagosomes.[85,86]

Acquisition of a vacuolar ATPase proton pump plays a central role in acidification of the phagosome.[81,87] Immediately after phagocytosis, the phagosome becomes alkaline for a short time before acidification is initiated. The basic milieu is optimal for the activity of defensins and basic proteins, whereas the acidic pH is optimal for lysosomal enzymes. Defensins are small (3.5 to 4.0 kD) peptides rich in arginine and cysteine.[88,89] They are abundant in PNG and present in some, though not all, MPs (depending on species and tissue location). Purified defensins are microbicidal for certain intracellular bacteria, such as *S. enterica* and *L. monocytogenes.* The contribution of lysosomal enzymes to bacterial killing is likely minor. Their major task is the degradation of already killed bacteria. These enzymes reside in the lysosome and are delivered into the phagosome during maturation through several independent waves, and they reach their optimum activity during later stages, that is, in the phagolysosome.

Most intracellular bacteria interfere with phagosome maturation and alter the phagosome in order to facilitate their own survival (see Fig. 40.1).[80,90,91] These include *L. pneumophila*, *M. tuberculosis*, *S. enterica*, *C. burnetii*, and *Chlamydia.* Although the specific mechanisms are incompletely understood, mycobacterial sulfatides and some mycobacterial glycolipids, most notably mannosylated lipoarabinomannans, impede phagolysosome fusion. Mycobacterial products, such as SapM and MptpB, contribute as well to the arrest of maturation of the early endosome. Antibody-coated *M. tuberculosis* organisms lose their capacity to block discharge of lysosomal enzymes, suggesting an auxiliary function of antibodies in cell-mediated protection against TB.[92] Finally, the robust, lipid-rich cell wall of mycobacteria renders them highly resistant against enzymatic attack. *M. tuberculosis*, as well

as *M. avium*, arrest phagosome maturation at an early stage. They restrict phagosome acidification via the exclusion of the V-H+ATPase proton pump from the phagosome. Additional mechanisms may contribute to this event, such as NH_4+ production by *M. tuberculosis.* Consistent with intraphagosomal NH_4+ production, the urease of *M. tuberculosis* is active at low pH. It has been known for some time that NH_4+ also interferes with phagosome-lysosome fusion. Exogenous adenosine triphosphate (ATP) has been shown to promote phagolysosome fusion resulting in concomitant death of macrophages and killing of *M. bovis* bacillus Calmette-Guérin (BCG).[93,94]

Phagosome maturation is arrested somewhere between the early and late stages by *M. tuberculosis*, *M. bovis* BCG, *L. pneumophila*, *S. enterica*, and *C. trachomatis*, all of which replicate in nonacidified vacuoles. Phagosomes containing *S. enterica*, *M. bovis* BCG, or *C. trachomatis* appear uncoupled from the maturation process through which phagosomes containing inert particles proceed.[80,90] *S. enterica* remains in the spacious membrane-bound phagosome, which is formed after uptake by the trigger mechanism. Moreover, the bacteria manipulate the cytoskeleton via kinesin and tether the vacuole to membranes of the Golgi apparatus.[95] The vacuole containing *C. trachomatis*, which lacks any specific phagosome markers, is loaded with ATP, which is required by the pathogen, by an unknown mechanism. In addition, elements of the cytoskeleton (actin and filamin) are used to stabilize the vacuole.[96] *L. pneumophila* prevents fusion of the vacuole with the endosomal compartment and recruits vesicles derived from Golgi and ER[97] by use of the T4SS. Moreover, *Chlamydia* employ mimics of soluble NSF attachment protein receptor to modulate membrane transport. *Legionella* uses an array of proteins to control Rab1 activity in the vacuole.[98,99] Bacterial DrrA (SidM protein) is a highly efficient guanine nucleotide exchange factor for Rab1A.[44] *C. burnetii* have evolved to live in the acidic late phagosome.[82,100] Recent in vitro studies confirmed *Coxiella*'s requirement for low pH and oxygen tension. The fusogenic properties of the vacuole are tightly regulated by the T4SS apparatus, through ankirin proteins.[101]

Egression into the Cytoplasm

Egress from the phagosomal into the cytoplasmic compartment represents a highly successful microbial survival strategy because bacterial killing is focused on the phagolysosome to limit self-damage of MPs. This egression has been extensively studied in *L. monocytogenes*, but is known to be utilized by other intracellular pathogens, including shigellae, rickettsiae, francisellae, and mycobacteria (see Fig. 40.1).[102]

Cytoplasmic invasion by *L. monocytogenes* depends on listeriolysin (LLO), an SH-activated cholesterol-dependent cytolysin. LLO requires activation by a host factor (gamma-IFN-inducible lysosomal thiol reductase), a thiol reductase.[103] In the cytosol, LLO is degraded, thus avoiding killing of host cells.[104] Deletion of the LLO gene (*hly*) renders *L. monocytogenes* avirulent. LLO is also required for replication of *L. monocytogenes* in spacious listeria-containing phagosomes. These compartments are nonacidic and allow slow replication of the pathogen.[105] Other molecules, such as phospholipase and lecithinase, are likely involved in membrane transition but are insufficient on their own. Invasion of *L. monocytogenes* into the cytoplasmic compartment is markedly reduced in IFNγ-activated macrophages in which the microbe, entrapped in the phagosome, rapidly succumbs to attack by toxic oxygen and nitrogen species and/or defensins. Cytosol evasion of shigellae is mediated by factors, which are also involved in bacterial entry (eg, IpaB, product of T3SS).

Listeria simultaneously activates caspase-1 and consequently modulates death of infected cells via danger signals represented by remnant vacuolar membranes.[106] *M. tuberculosis* uses proteins encoded by the T7SS, located in the region of difference 1 gene region, to successfully translocate to the cytosol.[5,107]

Cell-to-Cell Spreading

L. monocytogenes is cleared from the blood by Kupffer cells and then spreads to adjacent hepatocytes without reentering the extracellular milieu. This mechanism of cell-to-cell spreading has been carefully studied in vitro.[108] Having entered the cytoplasm, *L. monocytogenes* induces a tail of host

actin filaments, which push the bacterium forward to the outer region of the cell, where it induces pseudopod formation. Intracellular movement is achieved by coordinated actin polymerization at, and polarized release from, the bacterial surface. The ActA gene encodes a 90-kDa protein located on the bacterial surface, which is responsible for these actin-based movements.[109] A host cytosolic complex composed of eight polypeptides has been identified which, on binding Act A, induces actin polymerization.[110] The pseudopod-containing *L. monocytogenes* is engulfed by the adjacent cell, and the microbe reaches the phagosome of the recipient cell, which is still enclosed by cytoskeletal material from the donor cell. The two plasma membranes of the host and recipient cells apparently fuse, thereby allowing the introduction of *L. monocytogenes* into the cytoplasm of the recipient cell. Thus, *L. monocytogenes* can infect numerous cells without contacting extracellular defense mechanisms. *Shigella* use similar mechanisms for evasion and intracellular movement, and a similar spreading mechanism seems to be employed by *S. enterica* and by *R. rickettsii*, but not by *R. prowazekii* and *R. typhi*. A role for motility and manipulation of host actin-based structures was recently demonstrated for virulent mycobacteria.[111] Bacterial T7SS mediates ejection from the infected host cell and facilitates spreading through actin structures coined “ejectosomes.”

Cell Death Patterns

Death of mammalian cells occurs by accidental or programmed cell death, which were once thought to be associated exclusively with necrosis or apoptosis, respectively.[112] Increasing evidence, however, suggests that necrosis can progress in a programmed sequence. Moreover, in addition to necrosis and apoptosis, additional modes of cell death have been described recently, namely autophagic cell death, pyroptosis, pyronecrosis, necroptosis, mitotic catastrophe, NETosis, and lysosomal membrane permeability.[113,114,115] Although autophagy is a complex process aimed at preventing cell death, it has emerged as relevant mechanism to control infection and hence will be described in this section. Generally, intracellular pathogens often counteract host cell death in order to maintain their habitat. Thus, ability of microbes to modulate eukaryotic cell survival evolved as an essential pathogenicity feature.

Apoptosis and Necrosis

Apoptosis is a tightly controlled process that is initiated by intrinsic mechanisms within the dying cell. It involves a series of tightly controlled enzymatic events, notably intracellular caspases. Necrosis is the result of cell destruction caused by various exogenous effector mechanisms, including those mediated by complement and cytolytic T-lymphocytes. In contrast to necrosis, apoptosis is generally noninflammatory and thus associated with tissue

repair rather than destruction. However, cell death associated with bacterial infection frequently results in release of microbial PAMPs, which serve as inflammatory signals. Intracellular bacteria interfere with apoptosis in various ways to delay or even block this process and thus sustain their preferred habitat. *C. burnetii* antagonizes the intrinsic apoptotic death of macrophages by means of ankirin proteins related to T4SS.[116,117] *A. phagocytophilum* blocks PNG apoptosis by secreting the Ats1 protein via T4SS across the phagosomal membrane. Ats1 enters the mitochondria and subverts apoptotic signaling.[118] *Chlamydia* prevents immature cell death and uses autophagy in addition.[113] *L. pneumophila* induces rapid apoptosis in DCs,[119] but prevents programmed cell death in other cell types.[120] Moreover, this pathogen limits multiple pathways leading to cell death, including necrosis in macrophages.[121] Mycobacteria offer a good example of how virulence is associated with modulation of death of infected cells. Avirulent mycobacteria induce apoptosis, while *M. tuberculosis* preferentially causes necrosis.[122] Tubercle bacilli interfere with eicosanoid-regulated cell death to facilitate necrosis. Generally, death of infected cells impacts on the acquired immune responses, pathology, and ultimately disease manifestation.

Autophagy

Autophagy is a catabolic process that controls the integrity of eukaryotic cells.[123] This mode of "self-digestion" is paramount for the disposal of protein complexes that cannot be degraded via the proteasomal route and for the elimination of damaged organelles. Three different autophagic processes have been described: chaperone-mediated autophagy, microautophagy, and macroautophagy (canonical autophagy).[115] A unique feature of autophagy is the direct sequestration of the cargo into autophagosomes, which are surrounded by a double membrane and delivered to the lysosomal compartment. Autophagy is tightly orchestrated by over 30 autophagic components. This cell-autonomous housekeeping process is also an efficient system for the elimination of intracellular pathogens. Direct autophagy of intracellular pathogens occurs in any cell type and has been named xenophagy.[124] As a process of cytosol surveillance, autophagy is directed primarily against pathogens that egress into the cytosol. However, most bacteria, which successfully adapted to the intracellular milieu, have developed mechanisms to protect themselves against autophagy. Some species even harness the autophagic machinery to their advantage.

Autophagosome-like structures were first described in PNG infected with *Rickettsia*,[125] and a role for autophagy in control of intracellular bacteria was first demonstrated for *M. tuberculosis.*[126] Subsequently, the protective functions of autophagy were established in infections with *M. tuberculosis*,[127,128] *S. enterica*,[129] and *L. monocytogenes.*[130,131,132] Microbe-derived PAMPs stimulate autophagy[131,133] as do cytokines like IFNγ, most likely through induction of GTP/guanylate-binding protein (GBP) molecules.[134,135] Lipids were also found critical for autophagy in intracellular bacterial infections. Thus, the signaling lipid diacylglycerol is required for autophagy induced by *S. typhimurium.*[136] Recently the term sequestosome-like receptors was coined to define cytosolic innate receptors (p62, NBR1, NDP52), which target intracellular pathogens to the autophagic machinery[115] including salmonellae,[137,138] shigellae,[106] listeriae,[139] and mycobacteria.[140]

Pathogens interfere with autophagy through multiple strategies: by blocking induction or maturation of autophagosomes into autolysosomes, by evading recognition by the autophagic

machinery, and by misusing autophagy for their own benefit. Microarray studies revealed that *F. tularensis* downregulates autophagy-related genes.[141] Similarly, whole genome-wide profiling of macrophages infected with *M. tuberculosis* suggests that host factors that regulate bacterial replication are regulators of autophagy.[142] *S. enterica* blocks autophagy by interference with the ubiquitination machinery,[137,138] and *L. pneumophila* induces autophagy, but delays fusion with lysosomes.[143] A similar scenario was reported for *C. burnetii*[144] and *A. phagocytophilum.*[145] A different strategy is employed by bacteria that egress into the cytosol. The shigella protein VirG targets bacteria to the autophagic pathway by binding to the autophagic protein Atg5. However, once in the cytosol, the pathogen uses the T3SS IcsB to competitively bind to Atg5 and thus renders it unavailable for VirG.[146] Similarly, ActA impedes autophagic targeting of listeriae by recruiting actin and protecting it.[139,147] Some intracellular bacteria not only affect autophagy but may also exploit it. Thus, chlamydiae gain access to nutrients by stimulating autophagy,[148] and listeriae exploit this process for establishing chronic infection.[105] *F. tularensis* reenters the endocytic pathway following its egression into the cytosol through autophagosomes.[149] Autophagy is implicated in the biogenesis of vacuoles and persistence of *C. burnetii* therein.[150]

Pyroptosis

Pyroptosis is a form of programmed cell death that is controlled by caspase-1. It is an inflammatory process that results in rapid lysis of infected cells, mostly macrophages.[114] This process was first described for shigellae[151] and subsequently for salmonellae,[152,153] legionellae, and burkholderiae.[154] Mouse studies showed that pyroptosis is an innate immune effector mechanism during infection with *S. typhimurium*.[154] It still needs to be clarified how intracellular bacteria escape or modulate pyroptosis.

Intracellular Iron

Iron is required by most organisms and is a cofactor for enzymes involved in many essential biologic processes. The same divalent cation is toxic at high concentration and therefore is tightly controlled by multiple elements.[155] As both host and intracellular pathogens require iron, microbes have evolved ways to interfere with iron homeostasis. Generally, the strategy of the mammalian host is to deprive the pathogen of iron. However, depending on microbial habitat (extracellular versus intracellular bacteria), a given mechanism may be beneficial or detrimental. While systemic iron withdrawal and hypoferremia contribute to the control of extracellular bacteria, these strategies are counterproductive for intracellular microbes. Rather, down regulation of iron uptake and augmentation of iron export are advantageous for the host in defense against intracellular pathogens.[156,157]

Intracellular bacteria require iron, and production of reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) also depends on iron. Thus, competition for the intracellular iron pool between the intracellular pathogen and the host cell markedly influences the outcome of their relationship.[158] To improve their iron supply, mammalian cells utilize specific molecules. In the extracellular host milieu, iron is tightly bound to transferrin and lactoferrin, and the transferrin-iron complex is taken up by host cells via TfRs. The lactoferrin-iron complex does not enter the cell. Iron is released from the transferrin-TfR complex under the reducing conditions of the early phagosome. This event is controlled by Hfe (the product of the hereditary hemachromatosis gene).[155] Hfe reduces iron uptake either

by inhibiting TfR internalization or by blocking iron release from transferrin in the early phagosome. Macrophages can also acquire iron via the divalent metal transporter-1. Iron may also bind to haptoglobin, haemopexin, and lipocalin-1 and -2 in the extracellular space. Within the endocytic pathway, the Nramp system is involved in iron transport from the phagosome to the cytosol, where iron is bound to ferritin. Ferroportin (FPN) is one of the best-characterized iron export molecules in eukaryotic cells. Hepcidin, a product not only of liver cells but also macrophages, regulates FPN activity by inducing FPN internalization and proteasomal degradation. Accordingly, iron availability is controlled in multiple ways, including lactoferrin concentration in the extracellular space, intracytosolic ferritin concentration, hepcidin levels and abundance of TfRs, divalent metal transporter-1, and FPN on the cell surface. Many intracellular bacteria, including *M. tuberculosis*, *L. pneumophila*, and *S. enterica*, accommodate themselves in the early phagosome, where the abundance of iron-loaded transferrin guarantees a high availability of iron. Moreover, these bacteria, as well as *Chlamydia* and *F. tularensis*, induce divalent metal transporter-1 expression to support their replication.[159] Hepcidin is induced in mycobacterial phagosomes[160] and limits iron export by degrading FPN. Hepcidin also promotes *Salmonella*'s growth.[161] Similar observations, suggesting that iron efflux is detrimental for intracellular pathogens, were reported for *C. psittaci* and *L. pneumophila*.[162] To successfully compete for iron, bacteria possess a variety of iron-binding proteins. These include iron chelators (siderophores), transferrin-binding proteins, hemelike proteins, and ATP-binding cassette transporters.[163,164,165] Expression of genes involved in iron uptake is controlled by a conserved mechanism involving the Fur protein. However, lipocalins, which are host proteins produced by professional phagocytes and epithelial cells, bind siderophores from mycobacteria and salmonellae and thus minimize iron usage by microbes. Moreover, deletion of lipocalin-2 impacts on invasion of epithelial cells by tubercle bacilli.[161,166]

Importantly, there are many crossregulatory interactions linking iron homeostasis and immune responses. Th1 immunity and iron availability are interconnected. IFNγ-activated MPs downmodulate TfR expression and intracellular ferritin, resulting in reduced iron availability within the phagosome. Overall, IFNγ induces iron sequestration by downregulating FPN, while IL-4 favors iron release.[156,167] IFNγ, on the other hand, induces Nramp1, whose relevance for protection against salmonellae and mycobacteria has

been established.[168] The iron content of the phagosome in the resting MP seems to be sufficient for *L. pneumophila.* However, available iron is markedly reduced in IFNγ-activated MPs and, as a consequence, *L. pneumophila*, which lacks efficient iron uptake mechanisms, starves from iron deprivation in activated macrophages. In contrast, *M. tuberculosis* possesses a potent iron acquisition system comprising exochelins and mycobactins. The exochelins successfully compete for iron under limiting conditions and transfer it to mycobactins in the cell wall.[169,170] Similarly, SPI2 are upregulated as a response to Nramp-1-induced iron starvation and modify the vacuole to ensure bacterial replication.

Toxic Effector Molecules

Killing of intracellular bacteria by MPs and/or PNGs is primarily accomplished by highly reactive toxic molecules, notably ROIs and RNIs.[171,172,173,174] Within infected cells, these molecules are bactericidal. However, both ROIs and RNIs have a broader functional spectrum. They are also involved in signaling, regulation of vascular tone, tissue injury, and control of inflammation. Thus, ROIs and RNIs can both alleviate and promote tissue damage.[171]

Most if not all bacteria are susceptible to ROIs in vitro. Yet, contribution of ROIs to killing of intracellular bacteria by MPs is less clear; in murine macrophages, RNIs are more important. On the contrary, in PNGs, the role of ROIs seems to prevail.[175] In the mouse, ROIs and RNIs act consecutively in defence against *S. enterica* infection.[176,177] Production of RNIs by human MPs at concentrations sufficiently high for bacterial killing remains controversial.[178,179,180] However, evidence is accumulating that human MPs from sites of intracellular bacterial infection produce adequate concentrations of RNIs. Thus, using antibodies with exquisite specificity for human iNOS, this enzyme could be detected in a large proportion of lung macrophages from patients with TB.[181,182,183,184] iNOS can be induced by different mechanisms in different species as suggested, for example, by divergence of the iNOS promoter in mouse and human.[171,185,186]

ROI production is initiated by a membrane-bound nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) complex composed of six enzyme units (Rho guanosine triphosphatase, gp91PHOX also named NOX2, p22phox, p40phox, p47phox, and p67phox). NADPH oxidase is activated by IFNγ and by immunoglobulin (Ig)G-FcR binding:

$$O_2 + NADPH \xrightarrow{\text{NADPH oxidase}} NADP + O_2^- H^+$$

O_2^- is further metabolized by superoxide dismutase:

$$2O_2^- + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

In the presence of appropriate iron catalysts, the Haber-Weiss reaction takes place:

$$O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \longrightarrow {}^{\bullet}OH + OH^- + Fe^{3+} \text{ (Fenton reaction)}$$

$$\text{Net reaction: } O_2^- + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^- + O_2$$

In addition, O_2^- is transformed into $^1O_2$. The $^1O_2$ and $^{\bullet}OH$ radicals are short-lived powerful oxidants with high bactericidal activity causing damage to DNA, membrane lipids, and proteins. (Note: O_2^-, hyperoxide anion; $^{*}OH$, hydroxyl radical containing a free electron; $^1O_2$, singlet oxygen, a highly reactive form of O_2.)

Granulocytes, blood monocytes, and certain populations of tissue macrophages possess myeloperoxidase, thus allowing halogenation of microbial proteins[172]:

$$H_2O_2 + Cl^- \xrightarrow{MPO} OCl^- + H_2O$$

In addition to hypochlorous acid, chloramines are formed and both agents further increase the bactericidal power of the ROI system by destroying biologically important proteins through chlorination.

Nitric oxide is exclusively derived from the terminal guanidino nitrogen atom of L-arginine (Fig. 40.3). This reaction is catalyzed by the iNOS, which leads to the formation of L-citrulline and $NO^{\bullet}$.

$NO^{\bullet}$ can act as oxidizing agent alone or interact with O_2^- to form the unstable peroxynitrite ($ONOO^-$). This may then be transformed to the more stable anions, NO_2^- and NO_3^-, or

decomposed to $NO^{\bullet}$:

$$O_2^- + NO^{\bullet} \longrightarrow ONOO^-$$

$$ONOO^- + H^+ \longrightarrow NO_2^- + {}^{\bullet}OH$$

$$NO_2^- + {}^{\bullet}OH \longrightarrow NO_3^- + H^+$$

$$ONOO^- + H^+ \longrightarrow {}^{\bullet}OH + NO^{\bullet}$$

$NO^{\bullet}$ and $ONOO^-$ are highly reactive antimicrobial agents. $NO^{\bullet}$ may be transformed to nitrosothiol, which expresses the most potent antimicrobial activity. In contrast, NO_2^- and NO_3^- are without notable effects on microorganisms.

Three distinct nitric oxide synthase isoenzymes are known. The two constitutive nitric oxide synthases (neuronal nitric oxide synthase and endothelial nitric oxide synthase) exist in various host cells and account for basal nitric oxide synthesis, whereas iNOS is primarily found in professional phagocytes and is responsible for microbial killing. Its induction is controlled by exogenous stimuli such as IFNγ, agonist of PRRs, and inflammatory cytokines. This iNOS stimulation results in a burst of high RNI concentrations required for microbial killing, whereas the low nitric oxide levels produced by neuronal nitric oxide synthase

and endothelial nitric oxide synthase perform physiologic functions. The RNIs exert their bactericidal activities by directly inactivating iron-sulfur-containing enzymes, by S-nitrosylating proteins, by damaging DNA, or by synergizing with ROIs. Both ROIs and RNIs are relevant for killing of *Salmonella*, while most other intracellular pathogens are primarily controlled by iNOS products. In chlamydial infections, insufficient as well as excessive production of nitric oxide can be immunosuppressive.[187] RNIs prevail as antimicrobial molecules against *L. monocytogenes*,[188] *C. burnetii*,[189] *C. trachomatis*, *F. tularensis*, *Brucella*, and *L. pneumophila.*[190] The pivotal role of iNOS in control of *M. tuberculosis* infection has been demonstrated in the mouse system.[191] Despite resistance of NOX2-ablated mice against TB, recent data supporting a role of macrophage ROIs in humans have been reported.[192] Thus, nitrosative and oxidative processes under the control of iNOS and NADPH oxidase together are paramount for protection against intracellular bacteria.

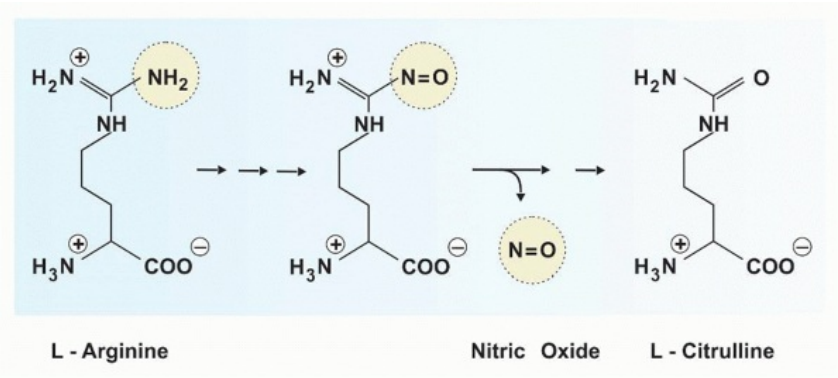

FIG. 40.3. Generation of Nitric Oxide from L-Arginine. L-Arginine is metabolized to the toxic molecules nitric oxide and L-citrulline by inducible nitric oxide synthase.

Evasion of Killing by Reactive Oxygen Intermediates and Reactive Nitrogen Intermediates

Microbes use multiple strategies to nullify ROI and RNI attack: evasion, inhibition, enzymatic inactivation, generation of scavenger molecules, as well as stress and repair mechanisms (see Fig. 40.1).[171] SPI2 enables *S. enterica* to exclude NADPH oxidase from the phagosomal membrane, thus interfering with ROI release into the *Salmonella* phagosome. *S. enterica* mutants deficient in SPI2 are susceptible to ROIs.[193] Similarly, proteins translocated into the cytosol interfere with iNOS activity.[194] *F. tularensis* undergoes phase variation to switch to a phenotype that is less stimulatory for iNOS.[195] *A. phagocytophilum* inhibits ROI production in PNGs after an initial ROI induction process.[196] Many intracellular bacteria produce superoxide dismutase and superoxide catalase that detoxify O_2 and H_2O_2, respectively.[171,197] Production of ROI-detoxifying molecules by intracellular bacteria is not constitutive; rather, expression of these enzymes is controlled by regulators such as soxR or oxyR that sense for concentrations of O_2 or H_2O_2, respectively. Accordingly, transposon mutants of *S. enterica* that fail to survive inside murine MPs are highly sensitive to ROI in vitro. Although less is known about specific mechanisms by which intracellular bacteria interfere with killing by RNIs, catalase and other antioxidative enzymes may indirectly inhibit RNI functions. ROI- and RNI-detoxifying gene products have been identified in *M. tuberculosis.*[198] KatG,[199] SodA, and SodC are involved in these processes. Recently, mycobacterial nicotinamide adenine dinucleotide dehydrogenase and enhanced intracellular survival (*eis*) gene products were reported to be relevant for inflammatory responses by controlling infected cell death as a consequence of ROI generation.[200,201] These mechanisms are separated from the direct antimicrobial effects of toxic radicals and will be discussed in the following. Intracellular pathogens may generate scavenger molecules to dispose of toxic radicals. Low molecular mass thiols, such as mycothiols from *M. tuberculosis*[197] or homocysteine in *Salmonella*,[202] are examples of ROI/RNI scavengers. In addition, both oxidative and nitrosative stresses are reflected in transcriptional changes in bacteria and initiation of DNA repair processes. Proteasome involvement in RNI resistance was reported for *M. tuberculosis.*[203] Further, certain macrophage receptors interfere with NADPH oxidase function. Binding to CR1/CR3 does not induce respiratory burst and ROI production.[204] The CRs, therefore, provide a relatively safe way of entry for intracellular bacteria.

Antimicrobial Peptides

Antimicrobial peptides (AMPs) encompass diverse groups of molecules that can be divided in subgroups based on their amino acid composition and structure. They are present systemically, at mucosal sites, or packed in granules of phagocytes.[205] AMPs active against intracellular microbes belong to different classes. Cathelicidin (LL-37) is a cationic peptide with bactericidal functions against *M. tuberculosis.*[206] Cationic and anionic peptides, including human neutrophilic peptides and defensins, kill microbes inside PNGs. PR-39 promotes filamentation of *S. typhymurium.* Most AMPs need to attach to the bacterial cell wall. Next, the peptides are inserted into the bacterial membrane rendering it permeable. AMPs are also generated upon infection-induced autophagy. During mycobacterial infection, ubiquitin generates peptides with lytic activity within autophagosomes.[207] Importantly, microbes develop mechanisms of resistance against AMPs.

Cell Autonomous Defense: Guanine Triphosphatases

The dominant role of IFNγ in defense against intracellular pathogens is beyond doubt. Besides ROI and RNI generation (detailed previously), a role for small GTPase family cognates was recently established. Three families of GT Pases are regulated by IFN: 65kDa GBPs, Mx family, and p47 kDa GTPases or immune-related GTPases.[208] Mx proteins are critical for antiviral responses and are mostly induced by type I IFN. In their promoters, GBPs and immune-related GTPases possess both gamma-activated site and IFN-stimulated response element binding sites. Accordingly, although the main inducer is IFNγ, type I IFN may also initiate their transcription.[209] Studies with mice ablated of proteins from these GTPases revealed that distinct members are paramount for protection against a range of intracellular bacteria, while other GTPases are redundant. Irgm1 (formerly Lrg-47) is essential for resistance against infection with *M. tuberculosis*, *M. avium*, *L. monocytogenes*, *S. typhymurium*, and *C. psittaci.*[210,211,212,213,214,215] Irga6 (formerly Iigp1), on the other hand, restricts *C. thrachomatis* replication only. More recently, GBP7 and GBP1 were found to be involved in control of infection with *L. monocytogenes* and *M. bovis* BCG.[134,135] Interestingly, GBPs and immune-related GTPases seem to trigger multiple pathways, as members of both families are essential for defense against intracellular bacteria residing in different cellular compartments. Members of these GTPase families localize to the ER, Golgi, plasma membrane, or intracellular vesicles. They may be recruited to the bacterial phagosome early upon infection, as demonstrated for Irgm1 in infections with mycobacteria or listeriae. However, the antimicrobial mechanisms are

diverse and encompass enhancement of phagosome fusion with lysosomes, interference with the autophagy machinery, regulation of oxidative responses, or induction of bacteriolytic peptides. Irgm-1 regulates T-cell dynamics and macrophage motility, suggesting cellular responses rather than effector functions are controlled by this and perhaps other GTPases.[216] In contrast to the multitude of p65 and p47 kDa GTPases in mice, only few cognates exist in humans. Accordingly, the relevance of these molecules in human diseases remains to be clarified.

INNATE IMMUNITY: PROFESSIONAL PHAGOCYTES

The innate immune system comprises various cell types of different hematopoietic lineages. Besides phagocytes (macrophages, monocytes, DCs, PNGs) and other cells of myeloid origin (eosinophils, basophils, mast cells), certain lymphoid cells (natural killer [NK], NKT, γδT lymphocytes, B1 lymphocytes), as well as platelets and the nuocytes act in concert to limit replication of the pathogens. Many intracellular bacteria infect professional phagocytes, and this strategy contributes to their evolutionary success. The professional phagocytes are represented by mononuclear cells, including monocytes, macrophages, and DCs, and by polymorphonuclear cells, namely the PNGs.

Mononuclear Phagocytes

Metchnikoff[217] was the first to realize the importance of professional phagocytes in resistance against bacterial infections. He observed that leukocytes accumulated at the site of inflammation and bacterial growth, and were heavily engaged in microbial engulfment and destruction. Metchnikoff distinguished two types of phagocytes: 1) the early appearing and short-lived microphages now referred to as PNGs, and 2) the late-appearing long-lived macrophages still known under the same name. The preferential localization of tubercle bacilli inside macrophages discovered at the time of Koch[218] and Metchnikoff[217] pointed to

the central role of these MPs in defense against intracellular bacteria. Metchnikoff also observed that during infection, macrophages are nonspecifically activated. Macrophage activation as an important factor of acquired resistance against bacterial infections was further substantiated by Lurie[219] and shown to be under the control of lymphocytes by Mackaness.[220] Later, cytokines were identified as the mediator of macrophage activation.[221,222]

Ontology studies and careful characterization of MP populations revealed that they show a tremendous degree of diversity and plasticity.[223] Tissue cues modulate the MP mode of response to bacterial insult (eg, lung versus liver, alveolar macrophages in the lung are mostly antiinflammatory, whereas liver Kupffer cells are mostly proinflammatory). Moreover, developmental branching of the mononuclear progenitor enables differential responses to pathogens. Thus, monocytes are specialized in their response to pathogens: certain populations ($CD14^{dim}$) primarily react to viral infections while others (CD14+) respond to a broad range of microbes.[224]

Many of the antibacterial activities of the MPs are not constitutively expressed. Rather, expression of full antibacterial activities by MPs depends on appropriate stimulation by cytokines, with IFNγ being of paramount importance.[225] Furthermore, significant differences exist among MPs of different maturation states or from different species. For example, higher RNI levels are produced by murine MPs as compared to human MPs. IFNγ activation of MPs coincides with increased phagocytosis, elevated CRs, reduced FcR expression, and a higher overall metabolic rate. Most importantly, during macrophage activation, iNOS and NADPH oxidase, which initiate RNI or ROI production, respectively, are stimulated. Moreover, this type of activation augments autophagy, which in turn contributes to microbial destruction. In other words, activation by cytokines results in transition of MPs from habitat-supporting microbial replication into an effector cell capable of terminating, or at least restricting, microbial survival.[185,226,227,228]

Polymorphonuclear Granulocytes

Although the role of PNGs in intracellular bacterial infections has often been neglected, their high antibacterial potential allows them to kill many intracellular bacteria. Pathogen disposal is the synergistic outcome of toxic molecules (mainly ROIs), PNG granular enzymes, and antimicrobial peptides.[229] PNGs are short-lived, however longer than previously appreciated, and thus may interact not only directly with bacteria, but modulate the function of other immune cells.[230] Generally, intracellular bacteria are sequestered in intracellular niches; hence, the overall contribution of PNGs to defense against chronic infections seems to be hindered to a certain extent due to the location of the pathogen. Nevertheless, PNGs may exclusively harbor certain intracellular bacteria (*A. phagocytophilum*) despite their broad arsenal of antimicrobial factors. *M. tuberculosis*, although it infects primarily MPs, has been found in PNGs in sputum from patients with TB.[231] It is likely that during the early acute inflammatory response, PNGs can help to reduce initial bacterial load. During experimental listeriosis, which is an acute disease, the first day of infection is characterized by extensive PNG infiltration at sites of listerial growth.[232] Elimination of PNGs and inflammatory monocytes/macrophages by monoclonal antibody treatment remarkably exacerbates listeriosis.[233,234] However, recent studies using specific depletion of PNGs have questioned the central role of these cells during systemic listeriosis.[235] Depletion of PNG apparently does not affect experimental TB in resistant mice, despite modulation of T-cell

responsiveness.[236,237] However, susceptible mouse strains can be rendered resistant following PNG depletion.[63,238] The detrimental effect of PNGs is largely due to their tissue-damaging propensity. PNGs are potent secretors of hypochlorous acid as well as of proteolytic enzymes, such as elastase (a serine proteinase), collagenase, and gelatinase.[172,239,240] Such proteases express potent microbicidal activity but can also be deleterious and induce tissue damage. These secretion products of PNGs act as mediators of tissue destruction. At the same time, neutrophil elastase has been shown to specifically destroy the virulence

proteins of *Salmonella* organisms.[239,240] In the extracellular milieu, protease inhibitors are normally present, preventing tissue damage by these proteases. However, the concomitant secretion of hypochlorous acid inactivates these proteinase inhibitors, thus promoting cell lysis. Accordingly, PNGs have been shown to cause inflammatory liver damage by destroying infected hepatocytes during early listeriosis.[232] MPs are less potent secretors of proteinases and fail to produce the major inactivator of proteinase inhibitors, hypochlorous acid. Thus, solid granulomas in chronic TB are dominated by MPs and are characterized by necrosis and fibrosis and lack signs of tissue liquefaction. During reactivation, however, PNGs may eventually be recruited to tuberculous granulomas and then contribute to granuloma caseation and liquefaction.

In PNGs, pathogen sensing or exposure to cytokines or chemokines rapidly causes transcriptional events that result in release of soluble mediators. PNGs are important mediators and regulators of the Th17 pathway. They release proinflammatory cytokines (eg, IL-1α, IL-1β, tumor necrosis factor-α [TNF]-α) and chemokines,[230] thus amplifying inflammation. Recent studies suggest that PNGs may participate in the resolution of inflammatory processes and thus may have a role in nonresolving inflammation. Mycobacteria stimulate production of IL-10 from PNGs via MyD88[78] and Card9[65] pathways. The capacity to manifest diverse and probably context-dependent functions suggests that PNGs can polarize into distinct phenotypes.[230] This concept has been developed for certain cancers and awaits clarification for infectious diseases.

PNGs are able to kill microbes via a unique process coined neutrophil extracellular trap formation.[241] This mechanism is characterized by entrapment of pathogens in fibrillar structures composed of chromatin and specific proteins from PNG granules, NADPH and myeloperoxidase dependence, and by tight coordination.[242,243] Neutrophil extracellular trap killing has thus far been reported for shigellae and salmonellae, and may be relevant for other intracellular microbes.

ACQUIRED IMMUNITY

Acquisition of immunity against intracellular bacteria crucially depends on T-lymphocytes that, ideally, accomplish sterile bacterial eradication. Bacterial clearance is rapidly achieved in the case of bacteria such as *L. monocytogenes.* In the case of persistent pathogens such as *M. tuberculosis*, clearance frequently remains incomplete and is arrested at the stage of bacterial containment. Bacterial containment and eradication occur in granulomatous lesions. The longer the struggle between host and microbial pathogen continues, the more essential the granuloma becomes.

The T-cell requisite is probably best exemplified by the high incidence of TB and other intracellular bacterial infections in patients suffering from T-cell deficiencies, particularly AIDS.[244,245,246,247] At the same time, T-lymphocytes are an unavoidable element of the

pathogenesis of intracellular bacterial infections. First, granulomas impair tissue functions by occupying space and affecting surrounding cells. Second, the physiologic functioning of host cells may be affected by specific T-lymphocytes and their cytokines.

Dendritic Cells

MPs not only serve as major habitat for intracellular bacteria, they are also potent antigen-presenting cells (APCs). However, MPs are not the most efficacious APCs, and their capacity may even be reduced during infection. DCs are the most proficient APCs.[248,249,250] DCs, like MPs, are present in all tissues (resident DCs), and they rapidly accumulate at sites of pathogen entry. PRR composition in DC populations is variable, according to origin and location. The "maturation" status of DCs, which relates to their propensity to stimulate naïve T cells, anticorrelates with their phagocytic potential. Immature DCs, exposed to pathogens for the first time, are phagocytic, while upon microbial encounter they augment their T-cell stimulatory capacities and minimize their phagocytic propensities. Following pathogen encounter in peripheral tissues, DCs migrate to draining lymph nodes (dLNs), where stimulation of T cells and antigen presentation takes places. Microbial killing is limited in DCs. Thus, DCs are specialized in linking innate immunity to adaptive immunity rather than in pathogen elimination.[251] Antigen presentation requires controlled proteolytic processing of microbial antigens. In DCs, phagosomal degradation[252] and acidification[253] is limited compared to MPs, thus conserving antigenic peptides for presentation via major histocompatibility complex (MHC)-class I and -II molecules. In addition, the superior antigen-presenting capacity of DCs is promoted by their copious expression of 1) MHC and CD1 molecules for antigen presentation; 2) PRRs to rapidly sense infection[47]; and 3) costimulatory molecules to regulate T-cell stimulation. Moreover, they produce cytokines that influence T-cell activation and differentiation.

DCs have critical roles in infections with intracellular bacteria. First, DCs can be infected by these bacteria, and can thus readily present accessible antigens from microbes they harbor. For instance, tubercle bacilli infect lung-resident and -recruited DCs.[254] Second, the transfer of microbial antigens from infected MPs to bystander DCs can combine the high phagocytic and degradative capacity of MPs with the high antigen-presenting capacity of DCs ("cross presentation," see following discussion).

Induction and Modulation of T-Cell Responses

The peripheral T-cell system comprises several phenotypically distinct and stable populations. T-lymphocytes expressing the αβ T-cell receptor (TCR) make up >90% of all T cells in secondary lymphoid organs and peripheral blood of humans and experimental mice. They are further subdivided into CD4 αβ T cells that recognize antigenic peptides presented by gene products of the MHC class II, and CD8 αβ T cells that interact with antigenic peptides in the context of MHC class I molecules. Undoubtedly, these conventional αβ T cells are of primary importance for antibacterial resistance, although evidence exists that unconventional T cells also participate in the control of intracellular bacteria.[226,255,256,257]

Generally, antigen presentation and activation of distinct T-cell populations is highly dependent on the route of antigen acquisition. Most intracellular bacteria reside in the phagosomal compartment of APCs and hence pathogenderived peptides have ready access to the MHC-II presentation pathway to stimulate CD4 T cells (Fig. 40.4). Cytosolic antigens, potentially derived from microbes, which access the cytosol, are processed by the proteasomes and loaded to MHC-I to stimulate CD8 T cells. Certain intracellular bacteria are

capable of egressing into the cytosol (eg, listeriae, shigellae, and mycobacteria). Obviously, antigens from cytosolic pathogens can be readily introduced into the MHC-I processing pathway, thus promoting activation of CD8 T cells. Yet, CD8 T cells have also been isolated from mice infected with phagosomal pathogens, such as *S. enterica.* Thus, bacteria unable to egress into cytosol may induce potent CD8 T-cell immunity. This is achieved by a process coined cross presentation, namely presentation of exogenous antigens by MHC-I molecules. Antigen cross presentation may occur directly and multiple pathways likely lead to this phenomenon. Another way of accomplishing cross presentation is indirect and is a consequence of engulfment of vesicles derived from infected cells undergoing infection-induced apoptosis, by bystander APCs (see Fig. 40.4). During mycobacterial infection, the direct and indirect modes of cross presentation coexist. *M. tuberculosis* can access the cytosol[107] and induce death of infected cells.[258,259]

Intracellular bacteria also invade nonprofessional phagocytes, some of which do not express MHC-II molecules constitutively. Consequently, such cells remain unrecognized by CD4 T-lymphocytes and provide a niche for persistent bacteria—a situation that has consequences for the course of disease. Because MHC-I molecules are expressed by almost every cell, CD8 T-lymphocytes have the potential to survey the whole body. This is particularly important for intracellular bacteria that hide in MHC-II-deficient host cells. Obviously, recognition of these cells depends on CD8 T-lymphocytes (and perhaps unconventional T cells). *L. monocytogenes*, on the one hand, resides in nonprofessional phagocytes with low antibacterial potential and, on the other hand, promotes MHC-I presentation of its antigens. This may explain the predominance of MHC-I-restricted CD8 T-lymphocytes in defense against experimental listeriosis both by number and by biologic relevance. In contrast, *S. enterica* is primarily restricted to MPs and remains in the phagosome. This is compatible with its preferential control by MHC-II-restricted CD4 T-lymphocytes.

Intracellular bacteria interfere at various levels with antigen presentation. Tubercle bacilli affect activation of CD4 T cells by interfering with MHC-II expression.[260] Autophagy can contribute to T-cell activation[261] and numerous intracellular bacteria impair autophagy thus reducing adaptive immune responses.

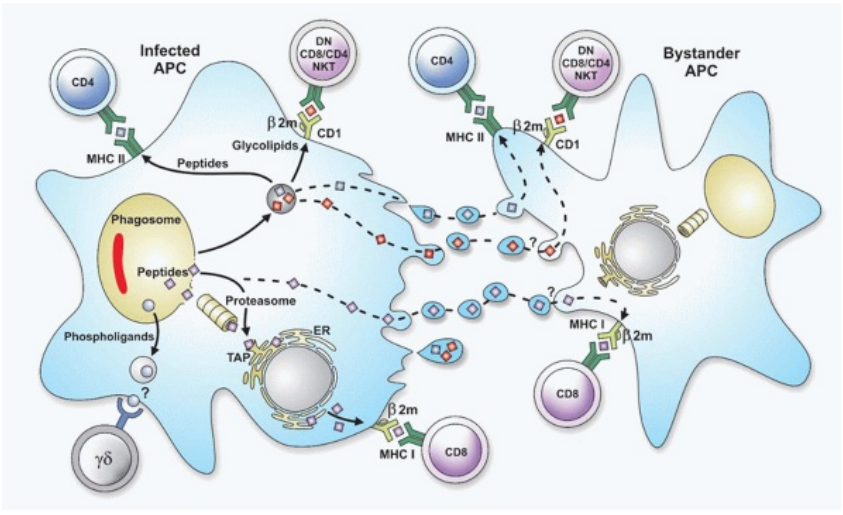

FIG. 40.4. Multiple Antigen-Processing Pathways for Stimulation of T Cells during Bacterial Infections. Infected antigen-presenting cells (APCs) directly present phagocytosed bacterial antigens to naïve cluster of differentiation (CD)4 T cells through the major histocompatibility complex (MHC)-II pathway. In addition, bacterial glycolipids

are processed for presentation by CD1 molecules to stimulate double negative CD4/CD8 lymphocytes or natural killer T (NKT) cells. Antigen cross presentation occurs either following the direct way, as a consequence of cytosolic access of the bacterial antigens in infected APCs, or following a detour pathway. In the latter case, apoptotic bodies from infected cells carry antigens to bystander APCs, which activate MHC-I-restricted CD8 T cells. Recognition of infected cell death also stimulates CD4 and NKT cells. β2m, β2 microglobulin; DN, double negative; ER, endoplasmic reticulum; TAP, transporter associated with antigen processing.

T cells may be polarized to various phenotypes, as discussed in the following, and this has a tremendous impact on the ensuing immune response. Certain factors are pivotal for this process. For instance, microbes rich in CLR ligands (eg, mycobacterial TDM) polarize T cells toward a Th17 phenotype.[262] Abundance and composition of PRRs in various APC populations contribute to this process by modulating cytokine production. Susceptibility of resident tissue (eg, epithelia) to infection and their capacity to release cytokines/chemokines influences T-cell differentiation. Finally, death of infected cells and subsequent recognition of pathogens in context of inflammatory signals from APCs impacts on T-cell polarization.[263]

Cluster of Differentiation 4 T Cells

Overwhelming evidence, including experimental animal studies, and their abundance in "protective" granulomas of patients suffering from bacterial infections clearly demonstrate a critical role of CD4 T cells in immune defense against intracellular bacteria. Further evidence for this is high prevalence of disease caused by intracellular bacteria in patients with AIDS suffering low CD4 T-cell counts.[264] CD4 T cells recognize peptide epitopes in the context of MHC-II molecules, which gain access to peptides present within the endosomal system. Thus, antigens from all intracellular bacteria, even those that evade the phagosome at later stages, are accessible to processing and presentation through the MHC-II pathway. However, cells that do not express the MHC-II machinery consistently, namely endothelial cells, epithelial cells, hepatocytes, and Schwann cells, are invisible for CD4 T cells and thus potential targets of *Rickettsia*, *C. trachomatis*, *L. monocytogenes*, and *M. leprae*, respectively.

The CD4 T-cell population has been subdivided into distinct subsets, according to the pattern of cytokine production. Th1 cells that overwhelmingly produce IFNγ, TNF-α, and IL-2, and Th2 cells which produce IL-4, IL-5, and IL-13 were the first subsets to be defined in both mice and humans. More recently, these subsets have been categorized based on expression of transcription factors, which mediate characteristic patterns of gene expression. The Th1 subset typically expresses the T-bet transcription factor, while the Th2 subset is consistent with expression of the transcription factor GATA-3.[265] More recently, a further distinct Th-cell population, termed Th17, was identified that produces the cytokines IL-17, IL-22, and granulocyte macrophage-colony stimulating factor. In addition, Th17 cells are also characterized by expression of the transcription factor RORγt.[266] Cytokines of the IL-17 family strongly induce mobilization of granulocytes during infection. This occurs by abundant production of proinflammatory mediators such as IL-6, and more specifically by increased secretion of the chemokines CXCL1, CXCL8, and CXCL6. These chemokines both attract neutrophils and eosinophils into infected tissue but also act as prosurvival factors to prolong the activity of these cells.[267] Th17 cells appear to have limited importance for protection in murine models of primary infection with mycobacteria, salmonellae, and listeriae. Despite this,

Th17 cells are instrumental in driving more rapid Th1 responses against pulmonary TB in mice resulting in enhanced protection.[268] Therefore, induction of the Th17 subset could be important in vaccination strategies against TB. Recent work has also demonstrated that IL-17 is required for optimally protective Th1 responses during primary infection of mice with *F. tularensis*.[269] In this model, IL-17A produced by Th17 cells directly induced DCs to produce IL-12, which in turn enhanced Th1 subset differentiation. IL-17A also acted directly on *F. tularensis*-infected MPs to produce both IL-12 and IFNγ, which enhanced killing of intracellular bacteria indicating a prominent role for this T-cell subset in control of intracellular bacterial infection.

Cluster of Differentiation 8 T Cells

In humans, the major function of CD8 T cells is believed to be via cell contact-mediated target cell lysis. CD8 T cells recognize peptide epitopes in the context of MHC-I gene products, which acquire peptides for presentation from the cell cytosol. Intracellular bacteria are under some circumstances able to enter the cytoplasm, where antigen becomes available to MHC-I processing and presentation in a manner similar to newly synthesized host or viral proteins.[107,270] Upon target cell recognition by CD8 T cells, perforin, granzymes, and granulysin are transferred to the infected host cell to allow direct killing of intracellular bacteria in addition to host cell lysis.[271] Perforin oligomerizes to form a pore in the target cell, through which granules containing granzymes and granulysin are conveyed. Granzymes are a family of serine proteases that include granzymes A and B.[272] Granzyme B induces apoptosis of target cells by both caspase-dependent and -independent mechanisms, while granzyme A induces apoptosis by a caspase-independent mechanism. In addition, CD8 T cells also function by producing T_H1 cytokines including IFNγ and TNF-α, and directly activating infected macrophages to control intracellular bacteria in mice, which lack granulysin. Perforin- or granzyme B-deficient mice are able to control *M. tuberculosis* equivalently to wild-type mice.[273,274] Moreover, protection against primary infection in mouse models of *C. pneumonia* and *C. trachomatis* is perforin-independent.[275,276] Despite this, perforin is critical for protection of mice against secondary *L. monocytogenes* infection.[277] Granulysin is a saponin-like protein that has been shown to directly kill intracellular *S. typhimurium*, *M. tuberculosis*, and *L. monocytogenes.*[278]

Conventional CD8 T cells recognize peptide in the context of polymorhphic MHC-I. A group of CD8 T cells also recognize MHC-I of limited polymorphism and present short formylated peptides, which are often characteristic of intracellular bacterial infection in mice.[279] In humans, this group includes human leukocyte antigen (HLA)-E-restricted T cells, which respond to *M. tuberculosis* antigens, and lung mucosal-associated invariant T cells, which recognize antigen in the context of MHC-related protein 1.[280] Protection conferred by CD8 T cells is likely to be complementary to that conferred by CD4 T cells as unlike MHC-II expression, which is restricted to professional phagocytes, virtually all nucleated cells express MHC-I molecules.

Unconventional T Cells

Unconventional T cells express nonpolymorphic receptors for antigen recognition and make an important contribution to protection during the early stages of infection, shaping the response mediated by conventional αβ T cells. These comprise T cells that express the gamma-delta γδ TCRs, named γδ T cells and T cells that express a low-varient form of the αβ

TCRs.[281] Similar to CD8 T cells, γδ T cells both produce inflammatory cytokines and are cytolytic. In mice, γδ T cells recognize heat shock protein-derived peptides presented by nonpolymorphic MHC-I-like molecules.[281] Human γδ T cells can respond to nonpeptidic phosphorylated metabolites, for example, of the isoprenoid pathway of bacterial and host origin.[281] In experimental murine listeriosis, γδ T cells participate in granuloma development in the liver with liver abcesses developing in their absence.[282] Recently, it was also shown that in the early stages of *M. tuberculosis* infection of mice, γδ T cells constitute a major source of IL-17, perhaps until canonical Th17 αβ T cells can take over production of this cytokine during the ensuing adaptive response.[283]

Unconventional T cells that express the αβ TCR also respond to CD1, a group of nonpolymorphic MHC-related molecules. In humans, T cells that respond to CD1a, b, and c (group I CD1) are either CD4-/CD8- or CD8+. These T cells recognize a variety of microbial glycolipids, including lipoarabinomannan, phosphatidylinositol mannans, mycolic acids, sulfatides, sulfoglycolipids, and lipopeptides.[257] Their TCR repertoire shows broad variability, and these group I CD1-restricted T cells appear to perform similar biologic functions as canonical αβ T cells. In contrast, CD1d (group II CD1) is expressed by both humans and mice, and controls development of invariant NKT (iNKT) cells that express both the NK cell marker NK1.1 and an evolutionarily conserved TCR with restricted variability.[284] iNKT cells can recognize phosphatidylinositol mannans from mycobacteria and glycosphingolipids from *Ehrlichia* and *Sphingomonas.*[285] Recent findings suggest that iNKT-cell activation may also be mediated by altered host cell metabolic activity. TLR engagement by bacterial ligands results in a temporary downregulation of the enzyme α-galactosidase A, which in turn leads to accumulation of lipid metabolites as α-galactosidase A processes these lipids during homeostatic metabolism. iNKT cells can respond to these lipids loaded onto CD1d.[286] In addition, an abundant host glycolipid, β-D-glucopyranosylceramide can act as a potent iNKT stimulator, with activity dependent on composition of the N-acyl chain.[287] Upon antigen activation, iNKT cells rapidly produce cytokines and are capable of secreting both IL-4 and IFNγ, and could be critical in early responses that drive later differentiation of canonical antigen-specific T cells during development of adaptive immune responses. iNKT cells can also provide cognate antigen-specific help to B cells that express CD1d, driving primary B-cell responses, but not B-cell memory.[288] Therefore, iNKT not only rapidly produce cytokines to drive T-cell responses, they also stimulate rapid antibody production by both noncognate and antigen-cognate mechanisms. As a result, iNKT cells might play central roles in development of protective immunity against intracellular bacteria.

B Cells

B cells and antibodies clearly play a role in infections with intracellular bacteria. Accumulating evidence suggests that IgG and IgA are important in preventing intracellular bacteria from gaining entry via mucosal surfaces.[289] Furthermore, salmonellae are often present outside cells during infection where antibody is important in neutralizing them. B cells are also potent APCs for soluble antigens including lipids presented by CD1c[257] and secrete many cytokines otherwise associated with T cells, DCs, and MPs. Recently, B cells have been shown to perform regulatory functions during development of host immunity, which may benefit intracellular bacteria. B cell signalling via MyD88 during *S. typhimurium* infection has been associated with B-cell production of IL-10 and mice with B cell-specific MyD88 deficiency were found to be more resistant to infection.[290]

Regulatory T Cells

Th1 responses that are required to control intracellular bacteria ultimately contribute to exacerbated tissue pathology requiring host counter measures. This is achieved largely by production of IL-10 and TGF-β, which limit inflammation and control IFNγ production. Although other cells such as macrophages and DCs can produce these cytokines as well, regulatory T (T_{reg}) cells are the main producers of these cytokines. Natural T_{reg} cells are responsive to IL-2 due to constitutive CD25 expression and are characterized by expression of the transcription factor FOXP3.[291] The inducible T_{reg} are antigen-specific and hence can develop during infection to specifically deaccelerate ongoing immune responses and thus avoid excessive damage.[292] Hence, expansion of T_{reg} cells appears to be both antigen-dependent and -independent. T_{reg} cells also selectively express TLRs and can be activated by TLR ligands. Therefore, T_{reg} cells could act both during innate and adaptive immune responses. Although T_{reg} act by controlling T-cell responses and immunopathology, they can also prevent the complete elimination of bacteria, and benefit intracellular bacteria by promoting the persistent chronic state of infection.

Memory T Cells

Protective immunity against intracellular bacteria is believed to last for decades due to generation of immune memory. This notion remains a touchstone in efforts to develop effacious vaccines against intracellular bacteria, which aim at efficiently driving development of T- and B-cell memory.[293] It is thought that during an ongoing immune response, most T cells and B cells become effector cells and eventually die of exhaustion during a primary immune response.[294] A small proportion of these cells that receive stimulatory signals of intermediate strength change their phenotype and become long-lived memory T or B cells. The memory T cells respond to the homeostatic cytokines IL-7 and IL-15 via expression of the respective cytokine receptors.[295] Central memory T cells and effector memory T cells are defined by differential surface molecules and functions.[295] Effector memory T cells are not able to home to LNs and instead traffic to peripheral

tissues where they secrete cytokines and express cytotoxic functions. Central memory T cells persist in LNs and are able to quickly propagate upon IL-2 stimulation due to IL-2 receptor expression. It is believed that memory T cells survive in the absence of MHC peptide recognition. Little is known about the induction and maintenance of long-lasting T cell immunity during chronic infections where antigen is everpresent.[296] In experimental listeriosis of mice, induction of memory T cells is dependent on duration of infection.[297] A clearer understanding of the requirements for memory T-cell development is essential if novel vaccines are to achieve longlasting T-cell immunity against intracellular bacteria.

BACTERIAL-TRIGGERED INFLAMMATION

Inflammation during infection with intracellular bacteria is initiated by sensing of bacterial components with PRRs. Immune cells are attracted to the site of infection in an attempt to combat the invader. Although direct toxicity of intracellular bacteria is generally low, their propensity to induce release of alarmins as a consequence of necrotic host cell death exacerbates inflammation.[298] Inability to dispose of the intruders and to accomplish tissue healing can result in unresolved inflammation.[299] As a consequence, host factors rather than microbial virulence factors drive pathology. Most of the factors involved in inflammation (ie, cytokines [TNF-α, IFNγ], ROIs/RNIs, and eicosanoids [prostaglandins, leukotrienes]) can act

in both a pro- and anti-inflammatory direction, depending on the context. Thus, a fine-tuned balance of these factors is essential as recently demonstrated for infection with mycobacteria.[300]

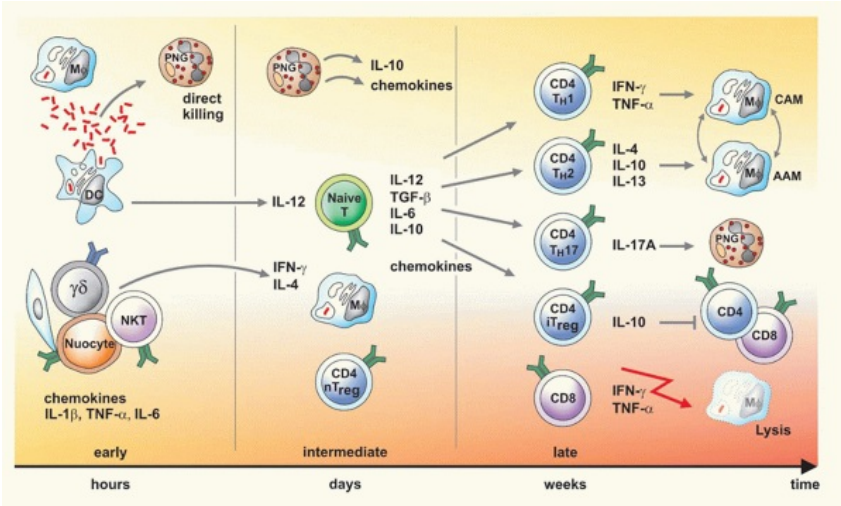

FIG. 40.5. Kinetics of the Immune Response during Infection with Intracellular Bacteria. This figure depicts critical events in the development of immunity to intracellular bacterial infection over a temporal scale. *Early innate responses (hours):* Polymorphonuclear granulocytes (PNGs) phagocytose extracellular bacteria and kill them. Macrophages (MΦ) and dendritic cells (DCs) become infected. DCs secrete interleukin (IL)-12 and present antigen to naïve T cells. Natural killer T cells, nuocytes, and gammadelta (γδ) T cells respond to presented bacterial products, become activated and produce cytokines such as IL-4 and interferon (IFN)γ. The host cell epithelium also responds to infection by producing chemokines and cytokines. These molecules act in concert to trigger inflammation and help shape naïve T-cell differentiation. *Intermediate innate/adaptive responses (days):* Naïve T cells activated by antigen presentation and costimulation respond to a milieu of cytokines including IL-12, IL-6, IL-10, and transforming growth factor-beta (TGF-β). This results in programs of gene transcription causing T cells to embark on distinct pathways of differentiation. PNGs respond to inflammatory signals and produce IL-10. Natural Tregulatory cells also produce IL-10 to dampen inflammation. *Late adaptive responses (weeks):* Cluster of differentiation (CD)4 T-cell differentiation gives rise to T-cell subsets that are able to produce different patterns of cytokines. These cytokines then act in concert to drive different MΦ activation states, such as classically activated macrophages (T helper 1 via IFNγ) and alternatively activated macrophages (T helper 2 via IL-4, IL-10, and IL-13) activation, control unregulated host immunity (inducible regulatory T cell via IL-10). T helper 17 drive inflammation, potentiate T helper 1 and mobilizes PNGs. CD8 T-cell activation and expansion result in cells able to directly lyse infected cells. These combined effects are critical in defining whether infection is completely cleared or remains persistent. TNFα, tumor necrosis factor-alpha.

Kinetics of Infection

The course of infection with intracellular bacteria can be conveniently separated into three stages (Fig. 40.5). At each stage, cytokines are produced that perform two functions.

First, they execute effector functions directed at reducing the microbial burden, and second, they express regulatory functions that influence the subsequent course of infection. The *early stage* is initiated within minutes after microbial entry and dominated by cells of the innate immune system, in particular PNGs and MPs, which are attracted to the site of bacterial replication by chemokines and proinflammatory cytokines. Phagocytosis and intracellular killing of bacterial pathogens by PNGs and MPs probably represents the predominant effector function at this early stage. At the same time, DCs mature and migrate to dLNs, where they produce proinflammatory and immunoregulatory cytokines, notably IL-12 for Th1 priming and IL-6 and IL-23 for Th17 cell stimulation,[301,302,303] and thus influence the subsequent stages by promoting induction of the protective acquired immune response. In addition, during early infection, NK cells, NKT cells, and γδ T cells can produce IFNγ and IL-17. Because NKT cells express a lowvariant TCR, they recognize a limited set of antigens and hence lack the diversity of conventional T cells governing the late stage. Moreover, cells operative at the intermediate stage can be activated via nonclonally distributed receptors such as TLRs.[304,305,306] The intermediate stage links the early (innate) with the late (acquired) immune response. This stage is essential for priming and polarization of conventional T cells. APCs, depending on the context and pathogen, tailor CD4 T-cell responses. Moreover, innate cells harboring the pathogen are activated by cytokines in an autocrine manner or by cytokines produced by innate lymphocytes, nuocytes, and tissue-resident cells. At the late stage, conventional αβ T cells with unique specificity become operative, which mobilize and sustain host defense that results in effective control and ideally sterile eradication of the pathogen. These include both MHC-I/peptide and group I CD1/lipid-specific αβ T cells. Subsequently, reparatory processes are initiated resulting in tissue repair and remodeling and return to homeostasis. In this last stage, the trophic functions of MPs surmount their antimicrobial capacities.[185,307]

The length and importance of each stage are markedly influenced by the type of intracellular pathogen. In experimental listeriosis of mice, the complete sequence of the host response lasts for less than 2 weeks, whereas in human TB it may endure for decades. The early stage is particularly important for control of *L. monocytogenes* organisms that divide rapidly and, at the same time, are highly susceptible to intracellular killing. The more robust and slowly dividing *M. tuberculosis* organisms are less vulnerable to this early stage of response. The relevance of the intermediate stage to microbial control is significantly influenced by the strength of the innate and the acquired immune response. The broader the window between these two stages, the more important the intermediate stage becomes. Upon secondary infection, the conventional T cells are activated more rapidly from the pool of memory T cells. The early and intermediate stages become largely dispensable because invading pathogens are rapidly confronted with the late-stage immune response. Suffice it to say that this is the major principle of vaccination. Deciphering the innate immune factors activated promptly after infection or vaccination, which determine the later ensuing protective immune response, has become a highly active topic of research, termed systems immunology or systems vaccinology.[293]

Some bacteria persist in the host lifelong. This is frequently the case for *M. tuberculosis*, which persists during latent *M. tuberculosis* infection without overt clinical signs. Control of latent TB, however, is T cell-dependent and hence, these T cells need to be fine-tuned to avoid deviation from protective immunity by confounding coinfections, for example, with helminths.[308,309,310] Protective immunity, however, breaks down after coinfection with human immunodeficiency virus frequently causing active TB within months after coinfection.[246]

Cytokines and Chemokines

Cytokines are central to resistance against intracellular bacteria. At all stages, cytokines are produced that perform regulatory and/or effector functions. Although cytokines are essential for control of infection, they can also cause harm to the host. To avoid such harmful consequences, downregulation of the immune response is required at later stages of infection. Neutralization of cytokines with specific antibodies and application of knockout mice lacking defined cytokines or cytokine receptor genes have provided deep insights into the role of single cytokines. The highly intertwined steps of the anti-infectious host response, which are controlled by cytokines, are listed in the following.

- Leukocyte recruitment to the site of bacterial deposition
- Formation of granulomatous lesions
- Activation of antibacterial functions in MPs
- Induction and maintenance of a protective T-cell response
- Downregulation of the antibacterial host response to avoid harmful sequelae of exaggerated immunity

Inflammation is usually a life-preserving process that needs to be tightly controlled. Certain steps are essential for recruitment of leukocytes, including PNGs and MPs, which phagocytose and—following activation—destroy the microbe. The essentiality of these processes is underlined by the high risks of lethal bacterial infection in humans with genetic defects affecting leukocyte motility/adhesion.[311] Accumulation of leukocytes at sites of microbial assault is guided by cytokine/chemokine gradients, and the process has been coined leukocyte recruitment. Tissue influx of leukocytes is a consequence of their extravasation and is mediated by close interactions between circulating leukocytes and the blood vessel endothelia, in close proximity to the area of infection.

Leukocyte Recruitment

Influx of inflammatory phagocytes occurs prior to the appearance of specific T-lymphocytes, and accordingly the relevant cytokines are primarily produced by MPs, as well as by epithelial and endothelial cells in response

to microbial invasion. Early produced cytokines with effector functions include the proinflammatory cytokines, TNF-α, IL-1, IL-6, as well as chemokines.[312,313,314] Four subgroups of chemokines can be distinguished on the basis of a conserved cysteine motif: the CC chemokines with two unseparated terminal cysteine residues, the CXC chemokines with nonconserved amino acids separating the two terminal cysteine residues, the CX3C chemokines with several amino acids separating the cysteine residues, and the C chemokine with only one terminal cysteine (C chemokine). Grouping of the large chemokine family can be further extended on the basis of different chemokine receptors.[315] The CC chemokines preferentially act on MPs, whereas PNGs are primarily activated by CXC chemokines. Other leukocytes, including lymphocytes, eosinophils, and basophils, may also be stimulated by these chemokines. The C and CX3C chemokines primarily recruit NK cells and lymphocytes.

Chemokines as a group play an important role in early mobilization of host defense. Knockout mice lacking the receptor for the CC chemokine MCP-1 are more susceptible to listeriosis than controls.[316] Knockout mice deficient in CC chemokine receptor 2 (CCR2) succumb to TB. In these mutants, the recruitment of macrophages, DCs, and T cells to the lung is impaired.[317] On the other hand, MCP-1-deficient knockout mice control *M. tuberculosis*

infection similarly to wild-type animals. MCP-1 acts via the CCR2 receptor. It therefore appears that these chemokines are essential for antibacterial protection but are mutually compensatory.[318] Experiments in other systems have revealed a central role of chemokines in early inflammation, in particular PNGs and monocyte extravasation. Chemokines, particularly those able to bind CCR2 receptor, are essential for bone marrow emigration of MPs and are therefore essential for infection with intracellular pathogens, as demonstrated for listeriae.[319] In addition, some chemokines activate professional phagocytes and in this way probably promote early reduction of bacterial load. Abundant levels of CC chemokines are on the other hand deleterious, as mice lacking a chemokine decoy receptor (D6) have been shown to be impaired in controlling tissue inflammation during TB.[320] The proinflammatory cytokines, IL-1, IL-6, TNF-α, and migration inhibitory factor are also involved in the early accumulation of inflammatory phagocytes at the site of bacterial growth. The essential role of IL-6 and TNF-α in antibacterial immunity is demonstrated by the exacerbated susceptibility to listeriosis and TB of knockout mice with a deficient IL-6 or TNF-type 1 receptor gene.[321] Similarly knockout mice deficient in migration inhibitory factor suffer from exacerbated *S. enterica* infection.[322] The proinflammatory cytokines, when produced in high amounts, cause acute-phase responses by inducing release of various plasma proteins from hepatocytes. They also serve as endogenous pyrogens that stimulate fever, and TNF-α is responsible for cachexia, the characteristic feature of wasting in infections with many intracellular bacteria, notably TB. Clinical trials showing that detrimental effects of excessive TNF-α production in patients with TB and leprosy can be ameliorated by treatment with thalidomide emphasize the double-sided role of TNF-α in chronic infections.[323,324]

Leukocyte Extravasation

In the early phase of inflammation, extravasation of (and invasion by) PNGs and, subsequently, blood monocytes is induced by proinflammatory signals mediated by bacterial components (N-f-met-containing peptides, such as f-Met-Leu-Phe or ligands for PRRs), complement components (C5a), arachidonic acid metabolites (PGE2), and cytokines. Infected MPs produce numerous proinflammatory cytokines, as well as various chemokines that stimulate local endothelial cells and blood phagocytes. The inflamed endothelium around the primary lesion expresses elevated levels of adhesion molecules, thus promoting extravasation of inflammatory phagocytes.[325] Extravasation is mediated by interactions between leukocytes and endothelial cells by means of adhesion molecules. These include selectins, integrins, and members of the Ig superfamily.

The L-selectins are found on leukocytes, whereas the P- and E-selectins are expressed by endothelial cells. Selectins bind to carbohydrate ligands on the corresponding cell type. The integrins are expressed on many cell types, including leukocytes and endothelial cells. Contact between leukocytes and endothelial cells is initiated when the blood vessel is suddenly broadened in diameter at inflammatory foci.[326] This fast process is controlled by histamines, eicosanoids, and tryptases derived from tissue mast cells and recruited PNGs. Release of platelet activation factor following PRR stimulation in MPs and the subsequent coagulation cascade augment blood vessel permeability changes as well. Activated endothelial cells and leukocytes upregulate surface expression of adhesion molecules and thus promote leukocyte binding to the endothelium. This sets into motion the cascade of adhesion events. Selectin-mediated interactions result in leukocyte tethering and rolling. Subsequently, integrin interactions with Ig superfamily molecules cause tight leukocyte adhesion to endothelial cells. Once leukocytes firmly adhere to the endothelium, transmigration to the inflammatory focus occurs. Upregulation of P- and E-selectin expression

primarily promotes PNG extravasation. In contrast, the L-selectins are constitutively expressed on virtually all leukocytes. Activated and memory T cells, as well as inflammatory phagocytes, however, express higher levels of integrins such as lymphocyte function-associated antigen-1 and very late antigen-4 and activated endothelial cells show elevated expression of Ig superfamily molecules. Intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 upregulation is primarily important for monocyte and T-cell transmigration to inflammatory foci. Chemokine receptors are decisive factors for selective T-cell migration.[314,327] Differential expression of chemokine receptors on human Th1 and Th2 cells, effector versus memory T cells, as well as central memory T cells versus effector memory T cells, direct preferential migration of selected T cells to the site of inflammation.[328,329] Chemokines can bind to glycosaminoglycans bound to the endothelial surface without loss of biologic activity. Interactions between

chemokine receptors on lymphocytes and chemokines bound to endothelial cells further promote selective T-cell migration. The lack of CCR7 (receptor type 7 for CC chemokines) combined with the low expression of CD62L and the high expression of lymphocyte function-associated antigen and of α4β1 integrin seems characteristic for effector memory T cells of Th1 type that migrate to sites of inflammation. In summary, tethering and rolling, which is then succeeded by tight adhesion and subsequent extravasation of leukocytes, results in leukocyte accumulation at the site of microbial colonization. This forms the basis for antimicrobial defense at site of microbial residence.

The Inflammasome

IL-1β is a key inflammatory cytokine in host defense,[330] and the generation of biologically active IL-1β is controlled by the inflammasome. Transcriptional regulation of this cytokine is primarily induced by PRR agonists and distinct cytokines, while cleavage of the proform is executed in the inflammasome. The inflammasome is a multiprotein cytosolic platform consisting of an NLR or PYHIN member, the ASC protein, and the procaspase-1.[331] Depending on the NLR/PYHIN cognate that is recruited to the cytosolic complex, four major inflammasomes have been described: NLRP1, NLRP3, NLRC4, and the AIM2 inflammasome. These inflammasomes detect intracellular cues from microbes, which provide the basis for optimal responses against infections with intracellular bacteria. Certain microbes induce activation of single or multiple inflammasomes.[332,333,334] Thus, *L. monocytogenes* activates NLRP3 by virtue of its virulence factor LLO. Bacterial DNA is recognized through the AIM2 inflammasome and bacteria may also interfere with NLRC4 inflammasome assembly. AIM2 is relevant for recognition of other cytosolic bacteria, particularly for francisellae.[70,75] Mycobacteria stimulate the NLRP3 inflammasome in macrophages, although evidence for inhibitory activity of tubercle bacilli has also been reported.[334] Salmonellae induce activation of NLRC4 inflammasome via T3SS and flagella, and also trigger the NLRP3 inflammasome.[335] Thus, most pathogens are sensed by multiple inflammasomes, suggesting that during coevolution, diverse pathways converge to ensure release of bioactive IL-1β upon infection. In addition, inflammasomes sense danger signals released by dying cells.[332,334] Accordingly, inflammasome activation by intracellular microbes, which induce extensive tissue destruction, represents a double-edged sword: the benefit of IL-1β in the early phase of infection bears risk for the host in the case of prolonged release during chronic infection.

Interferons

Both classes of interferons, IFN-I (comprising multiple members, with IFNα and IFNβ being of

highest relevance) and IFN-II (IFNγ), are closely associated with host defense. IFN-I and IFN-II show partition in defence against viral and bacterial infections, respectively. However, this classification is oversimplified, and it is becoming increasingly clear that both IFN classes participate in infections with intracellular bacteria. IFN-II can be viewed as primarily beneficial, whereas type I IFN often seem to be detrimental.

Activation of antibacterial macrophage properties by IFNγ represents a central step in acquired resistance against intracellular bacteria. IFNγ-activated macrophages rapidly kill susceptible intracellular bacteria, such as *L. monocytogenes*. Although the question as to whether IFNγ-stimulated MPs actually kill *M. tuberculosis* remains a matter of controversy, it is certain that they markedly inhibit growth of this pathogen. Consistent with such a central role of IFNγ in antibacterial immunity, IFNγ- or IFNγR-deficient knockout mice rapidly succumb to infections with *L. monocytogenes*, *M. tuberculosis*, *S. enterica*, and other intracellular pathogens.[336,337,338] Support for the central role of IFNγ in control of intracellular bacterial infections in humans stems from the identification of hereditary IFNγR deficiency in children who suffer from frequent infections with various intracellular bacteria or even from *M. bovis* BCG vaccination.[339,340] Conversely, IFNγ treatment in adjunct to chemotherapy has been used successfully in the treatment of leprosy, TB, and atypical mycobacteriosis.[341]

IFN-I cognates on the contrary are deleterious during infection with listeriae,[342,343,344] mycobacteria,[345,346,347] chlamydiae,[348] and francisellae.[349] These notions emerged from studies with mice lacking the receptor for the cytokine (IFNAR knockout mice), revealing impact of IFN-I on both innate and adaptive immune responses. Recently, crosstalk between type I and II IFN was described during infection with *L. monocytogenes*.[350] The role of IFN-I during infections with other intracellular pathogens as well as detailed mechanistic explanations as to how these cytokines negatively impact on disease control awaits clarification.

Macrophage Heterogeneity and Polarization

The myelomonocytic pathway, which comprises mature and immature cells, is characterized by a tremendous diversity and plasticity.[351] The most prominent member of this pathway, the macrophage, performs a dual role in infections with intracellular bacteria. It may harbor, and at the same time dispose of, the intruder. These binary effects are correlated with the functional plasticity of the MP. From sensing of pathogens and initiation of inflammation to the resolution of infection and tissue repair/remodelling, MPs play diverse functions of critical importance. Similar to the dichotomy of Th1/Th2 cells and closely related to the cytokines produced by these lymphocyte subsets, macrophages may develop into classically activated macrophages (CAMs) or alternatively activated macrophages (AAMs).[352] These two forms should be viewed as extreme poles of a continuum, with intermediate forms occurring in between. In stark contrast to T cells, chromatin reorganization in activated macrophages is not fixed. As a corollary, macrophage activation mirrors functionality of these cells in a particular spatiotemporal context. More recently, epigenetic modifications (Jmjd3-Irf4 axis) were associated with preferential AAM development.[353,354]

CAMs or M1 macrophages are polarized by IFNγ or TLR agonists, such as LPS. Thus, Th1 cells and NK/NKT cells

promote CAM genesis. The polarized CAMs secrete copious amounts of inflammatory cytokines (TNF-α, IL-12/23) and antimicrobial molecules (RNIs, ROIs), modulate iron homeostasis by repressing ferroportin and inducing ferritin, and upregulate costimulatory and

MHC-II molecules on their cell surface. Production of CXCL9 and CXCL10 by CAMs boosts Th1-cell recruitment. Concomitantly, the beneficial antimicrobial effects may cause collateral tissue damage.[355]

AAM or M2 macrophages are polarized by the canonical cytokines IL-4 and IL-13 as well as by the more recently described mediators IL-21,[356] IL-33,[357] and IL-25.[358] Accordingly, Th2 lymphocytes and nuocytes[358,359,360] may induce AAM polarization. AAMs seem to exclusively derive from resident tissue macrophages.[361] Functionally, AAMs are characterized by increased endocytic activity, release of IL-10, upregulation of IL-1RA and IL-1R2, and secretion of chemokines (CCL17, CCL22, CCL24) involved in recruitment of Th2 cells, T_{reg} cells, eosinophils, and basophils. AAMs upregulate ferroportin and express folate receptors. A hallmark of these macrophages is the expression of arginase-1, which not only enables AAMs to perform tissue remodeling, but also induces expression of E-cadherin,[362] involved in homotypic fusion. IL-4 and IL-13 induce homokaryon formation, a cell pathologic event particularly relevant for infections with intracellular bacteria. The metabolic and secretory properties of AAMs favor trophic functions and tissue tolerance.[363] The precise role of AAMs and their cytokines in giant cell formation during chronic bacterial infection awaits further clarification.

Although CAMs and AAMs are phenotypically and functionally different, both undergo a vivid crosstalk with adaptive lymphocytes (αβ T cells) and innate lymphocytes (γδ, NK, and NKT cells). The tremendous plasticity of polarized macrophages has been elucidated in malignancy,[351,364] and should similarly hold true in bacterial infections. During *L. monocytogenes* infection, CAMs ($Gr1^{low}$ patrolling monocytes) remodel to AAMs.[365] *F. tularensis* induces similar transition in macrophage polarization, to the advantage of the bacterium,[366] and *M. tuberculosis* seems to hijack this polarization pattern toward AAMs.[367,368] Blood cell gene transcripts from patients with TB show a combined IFN-I and IFN-II response accompanied by classical CAM gene expression.[369,370] Patients suffering from typhoid fever present enrichment for genes encoding IFNγ-mediated immune responses. However, patients in which a CAM signature has been replaced by an AAM signature and have maintained it are prone to becoming carriers or to manifesting disease relapse.[371] A clear scenario related to CAM/AAM balance has been reported for leprosy.[372]

Remodeling of recruited macrophages from alternative to classical phenotype occurs in patients who have converted from a lepromatous to a tuberculoid form of this disease.

GRANULOMA FORMATION: A PATHOLOGIC HALLMARK OF INTRACELLULAR BACTERIAL INFECTION

A characteristic feature of many infections caused by intracellular bacteria is the need for tissue remodeling by the host at the site of infection when the ensuing inflammatory response is not successfully resolved. This process leads to formation of structures called granulomas[373] (Fig. 40.6). The granuloma is primarily composed of T-lymphocytes and MPs, some of which contain indigestible antigen. In addition, granulomas also contain B-lymphocytes and DCs. MPs in granulomas demonstrate a degree of morphologic plasticity. Major macrophage phenotypes seen in granulomas include epitheloid-like and multinucleated giant cells. Macrophage plasticity may also be reflected in presence of both CAMs and AAMs, although the contribution of different states of macrophage maturation and activation is unclear at present.

Granulomatous lesions are generally initiated by innate inflammatory signals mediated by

microbial products, chemokines, and proinflammatory cytokines that are produced by endothelial cells and MPs at the site of infection. The best-studied granulomatous disease is TB where development of granulomas in the lung eventually leads to transmission of bacteria via cough. During TB, the granuloma is encapsulated by a fibrotic wall and its center becomes necrotic.[374] The combined effects of prolonged macrophage activation, persistence of intracellular bacteria, and tissue hypoxia likely lead to enhanced cell death in the center of granulomas resulting in the formation of caseum. This caseum may favor the local replication of facultative intracellular bacteria in the cellular detritus, as well as microbial dissemination to distant tissue sites and to the environment to transmit infection. T cells are the critical driving forces of granuloma formation and maintenance in most granulomatous diseases, and T cell production of IFNγ and TNF-α is crucial. Experiments utilizing the respective knockout mice emphasize a role of IFNγ, TNF-α, and lymphotoxin-α3 in granuloma formation and maintenance during TB.[337,375,376] Hereditary IFNγR deficiency has been described in humans, and these immunodeficient patients severely suffer and ultimately die of infections with intracellular bacteria.[339,340] That this high susceptibility was accompanied by impaired granuloma formation is suggestive of the central role of granuloma formation in control of bacterial growth. The critical role of TNF-α in the containment of *M. tuberculosis* in humans has been impressively demonstrated by the increased risk of the reactivation of TB in patients with rheumatoid arthritis undergoing treatment with anti-TNF-α monoclonal antibody.[377] Noncontagious diseases also characterized by granuloma formation include berylliosis caused by the undegradable irritant beryllium[378] and sarcoidosis of unclear etiology.[379]

Early Immunologic Events in Granuloma Development

The laboratory mouse model provides a plethora of genetic and immunologic tools to dissect the granulomatous response to infection. Although the classically used mouse model of TB fails to fully reproduce human-like granulomas, it represents a useful model for the generalized early stages of granuloma development during chronic infection. Orchestration of granuloma formation requires induction of *M. tuberculosis*-specific T-cell responses. In this section, we highlight recent findings using the murine model of TB to

give insight into events governing how granulomas develop during the early stages of infection. These responses can be enhanced by early production of the cytokine IL-17 by activated γδ T cells.[283] IL-17 enhances influx of Th1 cells producing IFNγ and TNF-α into the infected lung that are in turn able to control bacterial replication.[380] Excessive IL-17 may also enhance host pathology due to enhanced Th1 responses and require counter measures of control.[381] Such control could be provided by IL-10 as Th1 cell influx into the lung during murine TB is enhanced in its absence.[382] Furthermore, enhanced IL-17 production in the lung of mice after repeated application of *M. bovis* BCG during TB results in exacerbated disease, demonstrating the need to regulate inflammation to maintain a protective immune balance.[383]

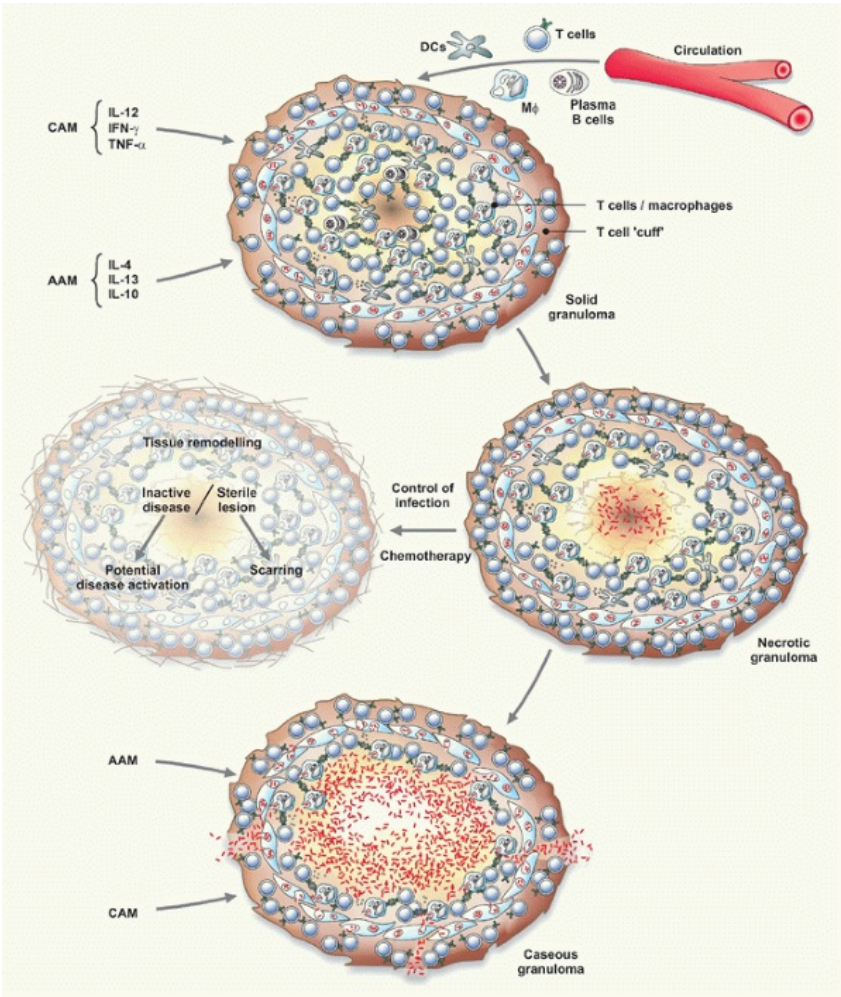

FIG. 40.6. Stages of Granuloma Development during Intracellular Infection. This figure describes a generalized scheme of granuloma development during intracellular infection, using tuberculosis (TB) as a paradigm. The *upper panel* depicts formation of a solid granuloma from bloodborne macrophages (MΦ), T cells, B cells, and dendritic cells (DCs) in response to infection. The center of the solid granuloma is composed of T cells as well as infected and uninfected MΦ which are, in turn, orchestrated by T-cell cytokines. Such signaling leads to classically activated macrophages and alternatively activated macrophages within granulomas. Eventually, the center of the granuloma shows extensive necrosis of MΦ to produce a necrotic granuloma. The necrotic granuloma may spontaneously heal or become sterilized by chemotherapy. Complete inactivation and scarring then ensues or in the event of incomplete clearance of persistent bacteria, reactivation of disease may occur later. In the case of TB, the necrotic granuloma may also further develop to produce a large central caseous mass that eventually liquefies to give rise to a caseous granuloma. Absorption of bronchi by this structure allows egression of bacteria into the airway mediating transmission. IFNγ, interferon gamma; IL, interleukin; TNF-α, tumor necrosis factor-alpha.

In addition, IL-12 signalling of DCs represents a key event initiating Th1 responses, and this IL-12 signaling shows multifunctionality during the early granulomatous response to infection. Signaling of immature DCs via engagement of IL-12 receptor β1 chain by the IL-12p40 homodimer, IL-12p80, mediates DC migration to dLNs, a prerequisite for driving rapid T-cell responses in the lung.[384] DCs can also produce an IL-12 receptor splice variant that not only

drives naïve T-cell proliferation in lymph nodes but also enhances migratory activity of T cells.[385] Chemokines, which play multiple roles in early immune responses, are also involved in granuloma formation. CCR7 is required for murine granuloma formation[386]; CCL19 and CCL21 participate in development of the Th1 responses while another homeostatic chemokine, CXCL13, is involved in the spatial construction of granulomatous lesions.[387] CCL19 and CCL21 are ligands of CCR7 on DCs and also signal their trafficking to dLNs to prime T-cell responses.[254] Therefore, homeostatic chemokine function demonstrates an intimate relationship between the need to drive T-cell responses in dLNs and the orchestration of granuloma formation in the lung during early stages of TB. During later stages of this disease, when granuloma formation has been fully established, T-cell activation can occur in the lung via ectopic or tertiary lymph node structures, such as inducible bronchus-associated lymphoid tissue.[388] This ability to prime T cells directly in the lung may become more relevant in later stages of human disease when cavity formation and extracellular growth of *M. tuberculosis* results in the need to provide protective immunity in the face of increased bacterial loads in the lung.[388,389] In addition, IL-17 produced by T cells enhances CXCL13 expression, which is required for inducible bronchus-associated lymphoid tissue formation, indicating an additional role for this cytokine in protective immunity.[388,390]

Contribution of Granuloma to Disease

In addition to its central role in TB, granuloma formation and development represents a central feature of disease resulting from intracellular infection with brucellae.[391] Brucellosis is a multiorgan disease in which granulomas contain largely epithelioid macrophages occurring in lymphatics, brain, lung, and bone. Hepatic granulomas can develop central necrosis. The structural similarity of these granulomas to those seen in other infections makes a definitive diagnosis of brucellosis complicated.[392]

Melioidosis represents another systemic disease with an important granulomatous component in bone, brain, and lung.[393] The disease in humans is caused by *B. pseudomallei*. Granulomas often show layers of epithelioid macrophages and presence of giant cells. These granulomas are often unable to contain bacteria because systemic spread of bacteria and septicemia can occur.

Lymphogranuloma venereum, a sexually transmitted disease where granuloma formation is central, is caused by the L1, L2, and L3 serotypes of *C. trachomatis.* Trachoma, which results from a granulomatous response of the upper eyelid due to *C. trachomatis* infection, is a leading cause of infectious blindness worldwide.[20] In human infection with *S. enterica* serovars, *Typhi* and *Paratyphi*, bacteria are disseminated within MPs to the liver, spleen, and lymph nodes where granuloma are formed.[394]

The definitive role of granuloma formation in development of active TB has been difficult to establish. A century ago, Anton Ghon defined latent subclinical TB in humans as the Ghon complex, which is composed of single subpleural caseous granulomas accompanied by caseation in the corresponding dLNs.[395] Animal models currently in use are biased toward accelerated disease, where granuloma development consistently accompanies pathology, making understanding the role of the granuloma in protection difficult to discern. Key to initial control of *M. tuberculosis* very early in infection is how the infected cell dies. Should the cell die by apoptosis, bacterial growth is primarily prevented. Should the cell die to necrosis, where the host cell membrane is breached, *M. tuberculosis* can proliferate and

disseminate.[396,397] Central necrosis and caseation in granulomas could in some way reflect the balance between these mechanisms of cell death. Infected macrophages also employ antibacterial mechanisms to control intracellular bacterial growth, such as autophagy[126,142] and production of antimicrobial peptides.[398,399] IFNγ enhances these mechanisms, but intracellular bacteria are often incompletely eradicated requiring consecutive waves of specific T cells and monocytes to arrive in the lung or dLNs, which then arrange into the classic stratified granuloma structure.[374,400,401]

The murine model of mycobacterial disease has addressed the contribution of T-cell immunity to granuloma development using intravital imaging. These studies have shown that antigen presentation to T cells within granulomas leads to production of TNF-α, which is critical to maintenance of granuloma structural integrity.[402] Furthermore, the T-cell compartment within granulomas was shown to be highly dynamic with T cells entering and exiting lesions. T-cell effector function in this model could be potentiated by exogenously introduced antigen, and this strategy might be explored using therapeutic strategies to augment the protective properties of granulomas.[403]

The understanding of granuloma development has benefited remarkably from use of zebrafish infected with *Mycobacterium marinum* to simplify study of the early events of granuloma formation in vivo. In particular, intravital imaging of zebra fish embryos to model early events in granuloma formation in the absence of adaptive T-cell responses showed that infected macrophages traffic readily

between multiple nascent granulomas.[404] This suggests that granuloma formation before the onset of adaptive immunity may in fact assist stable establishment of infection at an early stage. Later, T cells may be required to imprint on the granuloma the ability to contain mycobacterial growth. The zebrafish has been exploited for forward genetic screening revealing a critical function of *lta4h*, whose gene product catalyses leukotriene B4 synthesis. Leukotrienes are eicosanoids derived from essential fatty acid metabolism that demonstrate chemotactic and anti-inflammatory activity. Increased leukotriene B4 synthesis was found to suppress TNF-α production, which in turn abrogated protection in nascent granulomas.[300] This finding highlights the utility of using diverse models to address fundamental issues in granuloma biology.

Other animal models of TB, namely nonhuman primates (NHPs), rabbits, and guinea pigs show some ability to model granuloma formation. In contrast to the murine model, granulomas formed in rabbits and guinea pigs demonstrate central caseation and hypoxia.[405,406]

The NHP represents the closest model to human granuloma formation. Infection of NHPs results in a portion of animals with latent *M. tuberculosis* infection. Unlike other animal models of TB, disease in NHPs transitions through formation of a Ghon complex, suggesting that this initial event may represent the very early response in all cases.[407] The lymphatic lesion then undergoes necrosis and caseation as early as 3 to 4 weeks postinfection. These data and those seen in humans suggest that the Ghon complex indeed represents the site at which disease is either contained or progresses. Data from NHPs indicate that granuloma cellular composition and T-cell function can vary greatly in the same host and even in the same organ, for example, the lung. Additional studies in NHPs have established that early granuloma formation is accompanied by potent inflammatory responses, which are characterized by transcriptional networks controlled by the cytokines IFNγ and TNF-α as well as the intracellular JAK and STAT signaling pathways, while later granulomas show transcriptional networks reflective of tight control of inflammation and chemokine

production.[408] This inhibitory property of the late-stage granuloma could reflect the necessity to limit host tissue damage resulting from a nonresolved inflammatory response.

Cellular Mechanisms Active within Granulomas

In terms of understanding of cellular mechanisms operative in granulomas and how these impact granuloma biology, TB is the most widely studied granulomatous disease. Macrophages within granulomas demonstrate remarkable morphologic plasticity. Different macrophage morphologies in granulomas include epithelioid and foamy cells. In addition, macrophages can fuse to produce multinucleated giant cells.[374] On the tissue scale, one of the most characteristic gross morphologic features of granulomas in TB is their propensity to form a region of central necrosis, which softens in end stages to become caseous or cheese-like. This white mass results from extensive MP cell death.[409] Central necrosis appears at the onset of vigorous T-cell immunity, suggesting that it could represent a sacrifice of tissue to allow efficient killing of intracellular bacteria. At this stage, granulomas can become inert and eventually calcify. However, if intracellular bacteria continue to grow to up to hundreds of billions of microorganisms, granulomas in the lung become enlarged and can erupt into a bronchus with the central caseous mass liquefying. Coughing up the liquefied caseous mass containing bacteria allows transmission of TB. The molecular and cellular events that define these processes enable understanding of how TB is transmitted and offer important targets for therapy. In a model of granuloma caseation in the lung after dermal infection of knockout mice unable to produce RNIs, central granuloma caseation is accompanied by a local increase in serine protease activity. The serine proteases cathepsin G and neutrophil elastase are both active at neutral pH. Although both serine proteases show antibacterial activity,[398,410] cathepsin G enhances necrosis of IFNγ-activated macrophages infected with *M. tuberculosis.*[410] Therefore, deployment of serine protease activity in the lung may represent a double-edged sword, reflecting at a molecular level the need to balance mycobacterial killing with macrophage cell death in the granuloma. Recent studies suggest that products of the *sst1* genetic locus in mice prevent development of necrotic lung lesions during murine TB. A protein product encoded at this locus termed intracellular pathogen resistance 1 shows ability to direct infected macrophages to undergo apoptosis rather than necrosis.[411]

For disease transmission to occur, the lung extracellular matrix must be remodeled. Fibrillar collagens, highly resistant to enzymatic degradation, lend the lung extracellular matrix its extraordinarily tensile strength. In matrix remodelling, matrix metalloproteinases (MMPs), which cleave collagen at neutral pH, appear to be critical players. Patients with TB demonstrate increased levels of MMP-1 activity in sputum, and a transgenic mouse expressing human MMP-1 suffered increased lung tissue destruction during TB, suggesting MMP-1 is a key proponent of lung remodeling.[412]

Tissue containing caseous granulomas shows an increase in host cell lipid metabolism.[413] Foamy macrophages, containing multiple lipid droplets, are also found in regions surrounding central necrosis in granulomas.[413] In addition, human macrophages infected in vitro with *M. tuberculosis* demonstrate upregulated lipid metabolism.[414] *M. tuberculosis* in sputum from patients with TB are contained in lipid droplets and show reduced antibiotic susceptibility.[415] It is therefore possible that persistence of mycobacteria in foamy macrophages my represent a survival strategy in the face of drug therapy.

GENETIC CONTROL OF RESISTANCE AGAINST INTRACELLULAR BACTERIA

Resistance against intracellular bacteria is genetically controlled, and inherited factors are of particular importance in chronic infections with broad clinical spectrum, such as TB and leprosy. Although the impact of host genetic mechanisms on the outcome of infectious disease has been recognized for a long time, our understanding of the underlying

factors remains fragmented.[416,417] First, resistance to infection is highly polygenic. Second, a marked heterogeneity exists within populations. Third, variability in the genome of the pathogen, as well as environmental factors such as the availability of nutrients, further affect the outcome of the host-pathogen relationship. However, technologic advances in recent years have markedly facilitated the progress of immunogenetic studies. Single nucleotide polymorphism arrays and DNA sequencing are frequently applied in such investigations and genome-wide association studies have allowed identification of distinct loci associated with susceptibility to infection.[418,419,420]

The significance of genetic factors is perhaps most dramatically illustrated by the Lübeck disaster in 1927, when 251 babies were accidentally vaccinated with viable *M. tuberculosis* instead of BCG. At the end of the 6-year observation period, six children (2%) still suffered from TB, 129 (51%) had become ill but recovered, 77 (31%) had died, and in 39 children (16%) clinical signs of TB had never developed.[421] The marked influence of ethnic differences on the prevalence of TB further supports the role of genetic factors.[422]

In the 1940s, Lurie studied native resistance to TB in rabbits, and by selective inbreeding he succeeded in establishing strains of rabbits that differed remarkably in their susceptibility to infection with *M. tuberculosis.*[219] Similarly, congenic mouse strains that differ in their susceptibility to experimental infection with several intracellular bacteria have been developed.[423]

At least three levels of the host-pathogen relationship serve as potential targets for genetic control, which are briefly described in the following.

1. Genetic factors decide whether infection becomes abortive or establishes itself in a stable form. Convincing evidence for genetic control mechanisms at this level does not exist.
2. Genetic factors control transition from infection to disease. This control step distinguishes susceptible from resistant individuals. Such inherited influences are well proven in mice and begin to unfold in the human population.
3. Severity and/or form of disease are controlled by genetic factors.

Primary Immunodeficiencies

Numerous single-gene (Mendelian) disorders that perturb immune functions (ie, currently >300 primary immunodeficiencies) have been reported.[424] These monogenic diseases are rare, arise from major functional aberrations at single genes, and confer predisposition to a certain type of infection. The most thoroughly characterized of these syndromes in context of infection with intracellular pathogens is the Mendelian susceptibility to mycobacterial diseases.[423] The IFNγR1 deficiency was described first.[339,425,426,427] Subsequently, deficiencies in other Th1-associated molecules, namely IFNγR2,[428,429] STAT1,[430,431] IL-12B,[432] and IL-12B1[433] were reported. More recently, IRF8 mutations, which affect development of monocytes and DCs, have been associated with susceptibility to mycobacterial diseases.[434] Patients with Mendelian susceptibility to mycobacterial diseases also develop infections with typhoidal and especially nontyphoidal salmonellae with high

frequencies.[435]

Multigenic Predispositions/Major Genes

Linkage studies, and more recently genome-wide association studies, suggest polygenic characteristics of most infectious diseases. Studies in the mouse system have revealed a single dominant autosomal gene on chromosome 1, which is responsible for resistance against *M. bovis* BCG, *M. lepraemurium*, *M. avium/M. intracellulare*, *S. enterica*, and the protozoan pathogen *Leishmania donovani*. In contrast, murine resistance against other intracellular bacteria, most remarkably *M. tuberculosis*, is apparently not controlled by this gene.[436] The responsible gene has been named Nramp1 for natural resistance-associated macrophage protein.[437] The role of Nramp1 in controlling infectious diseases in humans remains controversial.[438,439] Other mouse gene loci affecting susceptibility to mycobacterial disease are Icbp,[440] Trl-1,[441] Trl-4,[442] Tbs1,[443,444] and Ipr1.[411,445] Certain loci impact on resistance against multiple intracellular bacteria. Ipr1 affects susceptibility to listeriae,[411] whereas Icbp affects responses against salmonellae.[446] The contribution of human homologues of the previously mentioned loci to antibacterial resistance needs further in-depth investigations. Genomewide association studies have described susceptibility loci for TB on chromosome 18q11.2[447] and chromosome 8q12.[448] Susceptibility to leprosy is associated with multiple innate immune-relevant genes.[449,450] Legionnaires' disease seems to be differently associated with polymorphisms in TLR-4 and TLR-5.[451,452,453] Single nucleotide polymorphism arrays, to analyze the association between clinical TB and certain PRRs (TLR-1, 2, 4, 6, 8, 9; DC-SIGN; NOD2; mannan-binding lectin) and adaptor molecules (TIR domain-containing adaptor protein), came to divergent results indicating a need for further studies.[423,454] Polygenic predispositions and immune defects in innate elements are currently being investigated and seem to bear an unanticipated role in resistance against infectious diseases.[455]

Major Histocompatibility Complex Control of Severity and Form of Disease

Segregation analyses in various human populations also indicate linkage of HLA types with severity of TB and leprosy. Strong evidence exists suggesting an influence of HLA on the development toward the tuberculoid or the lepromatous pole of leprosy. Although some linkage with MHC-I molecules has been observed in certain populations, MHC-II control appears to be more important.[456] Originally, it was found that HLA-DR2 subtypes are linked with increased incidences of lepromatous leprosy and that HLA-DR3 represents a linkage marker for tuberculoid leprosy. Recent population-based association studies, however, have provided evidence for an association between distinct HLA-DR2 alleles and susceptibility to tuberculoid leprosy. In TB, evidence for association of HLA-DR2 subtypes with pulmonary TB has been found. With more data from various population groups

becoming available, it is increasingly clear that HLA-DR associations with distinct disease forms differ among population groups, thus making it impossible to extrapolate from one population to another. These discrepancies underline the polygenic nature of resistance to infectious diseases.

CONCLUSION AND OUTLOOK

It is hoped that the reader of this chapter has not only become familiar with the principal mechanisms underlying immunity against intracellular bacteria, but also realizes the great

complexity at the interface between prokaryotic and mammalian eukaryotic systems. Understanding intracellular bacterial infections requires knowledge not only of immunology, but also of molecular biology of the infectious agent and biology of the target cell. In vitro analyses can only provide incomplete answers to the questions relevant to antibacterial immunity and must be complemented by in vivo experiments. Despite the high degree of complexity, such interdisciplinary research efforts certainly provide rewards.

First, understanding the performance of the immune system in bacterial infections can provide clues to questions pertinent to basic immunology. Knowledge of the rules underlying the extraordinary plasticity and adaptability of the immune system required for coping with transmutable “viable antigens” that developed during millennia of coexistence will provide deeper insights into the immunoregulation and evolution of the immune system. Second, applied questions will benefit equally well from these approaches. With the increasing inadequacy of chemotherapy in the control of bacterial infections, the need for adjunctive immune measures is gaining importance. Rational strategies toward vaccination and immunotherapy will benefit from the deeper understanding of the immune mechanisms operative in intracellular bacterial infections. With the elucidation of the genomes of major intracellular pathogens, as well as of the human and murine genomes, this type of interdisciplinary research has, in fact, entered a new phase and novel next-generation deep-sequencing technologies promise more rapid and less costly progress. Global analyses of the transcriptome and proteome down to the single-cell level will undoubtedly provide a comprehensive view of this dynamic interplay in the near future. The reader may find it ironic that the spirit of these investigations remains the same as it was at the early beginnings of immunology, which started as an approach to the intervention of bacterial infections.

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REFERENCES

1. Miao EA, Andersen-Nissen E, Warren SE, Aderem A. TLR5 and Ipaf: dual sensors of bacterial flagellin in the innate immune system. *Semin Immunopathol.* 2007;29(3):275-288.

2. Ibarra JA, Steele-Mortimer O. Salmonella—the ultimate insider. Salmonella virulence factors that modulate intracellular survival. *Cell Microbiol.* 2009;11(11):1579-1586.

3. Galan JE, Wolf-Watz H. Protein delivery into eukaryotic cells by type III secretion machines. *Nature.* 2006;444(7119):567-573.

4. Hubber A, Roy CR. Modulation of host cell function by Legionella pneumophila type IV effectors. *Annu Rev Cell Dev Biol.* 2010;26:261-283.

5. Simeone R, Bottai D, Brosch R. ESX/type VII secretion systems and their role in host-

pathogen interaction. *Curr Opin Microbiol.* 2009;12(1):4-10.

6. Neutra MR, Kraehenbuhl JP. Regional immune response to microbial pathogens 3119. In: Kaufmann SHE, Ahmed R, Sher A, eds. *Immunology of Infectious Diseases.* Washington, DC: ASM Press; 2002:191-206.

7. Kaufmann SH. Tuberculosis: back on the immunologists' agenda. *Immunity.* 2006;24(4):351-357.

8. Cooper AM. Cell-mediated immune responses in tuberculosis. *Annu Rev Immunol.* 2009;27:393-422.

9. North RJ, Jung YJ. Immunity to tuberculosis. *Annu Rev Immunol.* 2004;22:599-623.

10. Rodrigues LC, Lockwood DN. Leprosy now: epidemiology, progress, challenges, and research gaps. *Lancet Infect Dis.* 2011;11(6):464-470.

11. Hussain T. Leprosy and tuberculosis: an insight-review. *Crit Rev Microbiol.* 2007;33(1):15-66.

12. Newton HJ, Ang DK, van Driel IR, Hartland EL. Molecular pathogenesis of infections caused by Legionella pneumophila. *Clin Microbiol Rev.* 2010;23(2):274-298.

13. Hamon M, Bierne H, Cossart P. Listeria monocytogenes: a multifaceted model. *Nat Rev Microbiol.* 2006;4(6):423-434.

14. Elkins KL, Cowley SC, Bosio CM. Innate and adaptive immunity to Francisella. *Ann N Y Acad Sci.* 2007;1105:284-324.

15. Atluri VL, Xavier MN, de Jong MF, den Hartigh AB, Tsolis RE. Interactions of the human pathogenic Brucella species with their hosts. *Annu Rev Microbiol.* 2011;65:523-541.

16. Olano JP. Rickettsial infections. *Ann N Y Acad Sci.* 2005;1063:187-196.

17. Walker DH, Ismail N. Emerging and re-emerging rickettsioses: endothelial cell infection and early disease events. *Nat Rev Microbiol.* 2008;6(5):375-386.

18. Balraj P, Renesto P, Raoult D. Advances in rickettsia pathogenicity. *Ann N Y Acad Sci.* 2009;1166:94-105.

19. Hackstadt T. The biology of rickettsiae. *Infect Agents Dis.* 1996;5(3):127-143.

20. Wright HR, Turner A, Taylor HR. Trachoma. *Lancet.* 2008;371(9628): 1945-1954.

21. Darville T, Hiltke TJ. Pathogenesis of genital tract disease due to Chlamydia trachomatis. *J Infect Dis.* 2010;201(suppl 2):S114S125.

22. Stewardson AJ, Grayson ML. Psittacosis. *Infect Dis Clin North Am.* 2010;24(1):7-25.

23. Rikihisa Y. Anaplasma phagocytophilum and Ehrlichia chaffeensis: subversive manipulators of host cells. *Nat Rev Microbiol.* 2010;8(5):328-339.

24. Ismail N, Bloch KC, McBride JW. Human ehrlichiosis and anaplasmosis. *Clin Lab Med.* 2010;30(1):261-292.

25. Ghigo E, Pretat L, Desnues B, Capo C, Raoult D, Mege JL. Intracellular life of Coxiella burnetii in macrophages. *Ann N Y Acad Sci.* 2009;1166:55-66.

26. Parker NR, Barralet JH, Bell AM. Q fever. *Lancet.* 2006;367(9511): 679-688.

27. Cossart P, Sansonetti PJ. Bacterial invasion: the paradigms of enteroinvasive pathogens. *Science.* 2004;304(5668):242-248.

28. Cossart P, Roy CR. Manipulation of host membrane machinery by bacterial pathogens. *Curr Opin Cell Biol.* 2010;22(4):547-554.

29. Bhavsar AP, Guttman JA, Finlay BB. Manipulation of host-cell pathways by bacterial pathogens. *Nature.* 2007;449(7164):827-834.

30. Flannagan RS, Cosio G, Grinstein S. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat Rev Microbiol.* 2009;7(5): 355-366.

31. Pizarro-Cerda J, Cossart P. Bacterial adhesion and entry into host cells. *Cell.* 2006;124(4):715-727.

32. Finlay BB. Interactions of enteric pathogens with human epithelial cells. Bacterial exploitation of host processes. *Adv Exp Med Biol.* 1997;412:289-293.

33. Mengaud J, Ohayon H, Gounon P, Mege R-M, Cossart P. E-cadherin is the receptor for internalin, a surface protein required for entry of L. monocytogenes into epithelial cells. *Cell.* 1996;84(6):923-932.

34. Lecuit M, Vandormael-Pournin S, Lefort J, et al. A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. *Science.* 2001;292(5522):1722-1725.

35. Rambukkana A, Salzer JL, Yurchenco PD, Tuomanen EI. Neural targeting of Mycobacterium leprae mediated by the G domain of the laminin-alpha2 chain. *Cell.* 1997;88(6):811-821.

36. Veiga E, Guttman JA, Bonazzi M, et al. Invasive and adherent bacterial pathogens co-Opt host clathrin for infection. *Cell Host Microbe.* 2007;2(5):340-351.

37. Veiga E, Cossart P. Listeria hijacks the clathrin-dependent endocytic machinery to invade mammalian cells. *Nat Cell Biol.* 2005;7(9):894-900.

38. Mostowy S, Cossart P. Cytoskeleton rearrangements during Listeria infection: clathrin

and septins as new players in the game. *Cell Motil Cytoskeleton.* 2009;66(10):816-823.

39. Galan JE, Pace J, Hayman MJ. Involvement of the epidermal growth factor receptor in the invasion of cultured mammalian cells by Salmonella typhimurium. *Nature.* 1992;357(6379):588-589.

40. Jones BD, Falkow S. Salmonellosis: host immune responses and bacterial virulence determinants. *Annu Rev Immunol.* 1996;14:533-561.

41. Ashida H, Ogawa M, Mimuro H, Sasakawa C. Shigella infection of intestinal epithelium and circumvention of the host innate defense system. *Curr Top Microbiol Immunol.* 2009;337:231-255.

42. Parsot C, Sansonetti PJ. Invasion and the pathogenesis of Shigella infections. *Curr Top Microbiol Immunol.* 1996;209:25-42.

43. Romero S, Grompone G, Carayol N, et al. ATP-mediated Erk1/2 activation stimulates bacterial capture by filopodia, which precedes Shigella invasion of epithelial cells. *Cell Host Microbe.* 2011;9(6):508-519.

44. Aktories K. Bacterial protein toxins that modify host regulatory GTPases. *Nat Rev Microbiol.* 2011;9(7):487-498.

45. Janeway CA, Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol.* 1989;54(pt 1): 1-13.

46. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. *Nat Immunol.* 2010;11(5): 373-384.

47. Medzhitov R. Recognition of microorganisms and activation of the immune response. *Nature.* 2007;449(7164):819-826.

48. Ronald PC, Beutler B. Plant and animal sensors of conserved microbial signatures. *Science.* 2010;330(6007):1061-1064.

49. Ferrandon D, Imler JL, Hetru C, Hoffmann JA. The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections. *Nat Rev Immunol.* 2007;7(11):862-874.

50. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity.* 2011;34(5):637-650.

51. Blasius AL, Beutler B. Intracellular toll-like receptors. *Immunity.* 2010;32(3):305-315.

52. West AP, Brodsky IE, Rahner C, et al. TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. *Nature.* 2011;472(7344):476-480.

53. Osorio F, Reis e Sousa. Myeloid C-type lectin receptors in pathogen recognition and

host defense. *Immunity.* 2011;34(5):651-664.

54. Kerrigan AM, Brown GD. Syk-coupled C-type lectins in immunity. *Trends Immunol.* 2011;32(4):151-156.

55. Elinav E, Strowig T, Henao-Mejia J, Flavell RA. Regulation of the antimicrobial response by NLR proteins. *Immunity.* 2011;34(5):665-679.

56. Ting JP, Duncan JA, Lei Y. How the noninflammasome NLRs function in the innate immune system. *Science.* 2010;327(5963):286-290.

57. Franchi L, Warner N, Viani K, Nunez G. Function of Nod-like receptors in microbial recognition and host defense. *Immunol Rev.* 2009;227(1):106-128.

58. Kato H, Takahasi K, Fujita T. RIG-I-like receptors: cytoplasmic sensors for non-self RNA. *Immunol Rev.* 2011;243(1):91-98.

59. Areschoug T, Gordon S. Scavenger receptors: role in innate immunity and microbial pathogenesis. *Cell Microbiol.* 2009;11(8):1160-1169.

60. Schattgen SA, Fitzgerald KA. The PYHIN protein family as mediators of host defenses. *Immunol Rev.* 2011;243(1):109-118.

61. Pluddemann A, Mukhopadhyay S, Gordon S. Innate immunity to intracellular pathogens: macrophage receptors and responses to microbial entry. *Immunol Rev.* 2011;240(1):11-24.

62. Sander LE, Davis MJ, Boekschoten MV, et al. Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. *Nature.* 2011;474(7351):385-389.

63. Dorhoi A, Reece ST, Kaufmann SH. For better or for worse: the immune response against Mycobacterium tuberculosis balances pathology and protection. *Immunol Rev.* 2011;240(1):235-251.

64. Fremond CM, Yeremeev V, Nicolle DM, Jacobs M, Quesniaux VF, Ryffel B. Fatal Mycobacterium tuberculosis infection despite adaptive immune response in the absence of MyD88. *J Clin Invest.* 2004;114(12): 1790-1799.

65. Dorhoi A, Desel C, Yeremeev V, et al. The adaptor molecule CARD9 is essential for tuberculosis control. *J Exp Med.* 2010;207(4):777-792.

66. Weiss DS, Raupach B, Takeda K, Akira S, Zychlinsky A. Toll-like receptors are temporally involved in host defense. *J Immunol.* 2004;172(7): 4463-4469.

67. Arpaia N, Godec J, Lau L, et al. TLR signaling is required for Salmonella typhimurium virulence. *Cell.* 2011;144(5):675-688.

68. Eitel J, Suttorp N, Opitz B. Innate immune recognition and inflammasome activation in listeria monocytogenes infection. *Front Microbiol.* 2010;1:149.

69. Kim S, Bauernfeind F, Ablasser A, et al. Listeria monocytogenes is sensed by the NLRP3 and AIM2 inflammasome. *Eur J Immunol.* 2010;40(6):1545-1551.

70. Rathinam VA, Jiang Z, Waggoner SN, et al. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat Immunol.* 2010;11(5):395-402.

71. Tsuchiya K, Hara H, Kawamura I, et al. Involvement of absent in melanoma 2 in inflammasome activation in macrophages infected with Listeria monocytogenes. *J Immunol.* 2010;185(2):1186-1195.

72. Warren SE, Armstrong A, Hamilton MK, et al. Cutting edge: Cytosolic bacterial DNA activates the inflammasome via Aim2. *J Immunol.* 2010;185(2):818-821.

73. Wu J, Fernandes-Alnemri T, Alnemri ES. Involvement of the AIM2, NLRC4, and NLRP3 inflammasomes in caspase-1 activation by Listeria monocytogenes. *J Clin Immunol.* 2010;30(5):693-702.

74. Yang P, An H, Liu X, et al. The cytosolic nucleic acid sensor LRRFIP1 mediates the production of type I interferon via a beta-catenin-dependent pathway. *Nat Immunol.* 2010;11(6):487-494.

75. Fernandes-Alnemri T, Yu JW, Juliana C, et al. The AIM2 inflammasome is critical for innate immunity to Francisella tularensis. *Nat Immunol.* 2010;11(5):385-393.

76. El Kasmi KC, Qualls JE, Pesce JT, et al. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. *Nat Immunol.* 2008;9(12):1399-1406.

77. Qualls JE, Neale G, Smith AM, et al. Arginine usage in mycobacteriainfected macrophages depends on autocrine-paracrine cytokine signaling. *Sci Signal.* 2010;3(135):ra62.

78. Zhang X, Majlessi L, Deriaud E, Leclerc C, Lo-Man R. Coactivation of Syk kinase and MyD88 adaptor protein pathways by bacteria promotes regulatory properties of neutrophils. *Immunity.* 2009;31(5):761-771.

79. Sing A, Reithmeier-Rost D, Granfors K, Hill J, Roggenkamp A, Heesemann J. A hypervariable N-terminal region of Yersinia LcrV determines toll-like receptor 2-mediated IL-10 induction and mouse virulence. *Proc Natl Acad Sci U S A.* 2005;102(44):16049-16054.

80. Schaible UE, Collins HL, Kaufmann SH. Confrontation between intracellular bacteria and the immune system. *Adv Immunol.* 1999;71:267-377.

81. Flannagan RS, Jaumouille V, Grinstein S. The cell biology of phagocytosis. *Annu Rev Pathol.* 2012;7:61-98.

82. Barry AO, Mege JL, Ghigo E. Hijacked phagosomes and leukocyte activation: an intimate relationship. *J Leukoc Biol.* 2011;89(3):373-382.

83. Brumell JH, Scidmore MA. Manipulation of rab GTPase function by intracellular bacterial pathogens. *Microbiol Mol Biol Rev.* 2007;71(4): 636-652.

84. Russell DG. Mycobacterium tuberculosis and the intimate discourse of a chronic infection. *Immunol Rev.* 2011;240(1):252-268.

85. Fugier E, Salcedo SP, de CC, et al. The glyceraldehyde-3-phosphate dehydrogenase and the small GTPase Rab 2 are crucial for Brucella replication. *PLoS Pathog.* 2009;5(6):e1000487.

86. Rejman LA, Heymann J, Meissner C, et al. Rab6 and Rab11 regulate Chlamydia trachomatis development and golgin-84-dependent Golgi fragmentation. *PLoS Pathog.* 2009;5(10):e1000615.

87. Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, et al. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. *Science.* 1994;263(5147):678-681.

88. Ganz T, Lehrer RI. Defensins and cathelicidins: antimicrobial peptide effectors of mammalian innate immunity. In: Kaufmann SHE, Ahmed R, Sher A, eds. *Immunology of Infectious Diseases.* Washington, DC: ASM Press, 2002:105-110.

89. Selsted ME, Ouellette AJ. Defensins in granules of phagocytic and nonphagocytic cells. *Trends Cell Biol.* 1995;5(3):114-119.

90. Diacovich L, Gorvel JP. Bacterial manipulation of innate immunity to promote infection. *Nat Rev Microbiol.* 2010;8(2):117-128.

91. Pieters J. Evasion of host cell defense mechanisms by pathogenic bacteria. *Curr Opin Immunol*. 2001;13(1):37-44.

92. Armstrong JA, Hart PD. Response of cultured macrophages to Mycobacterium tuberculosis, with observations on fusion of lysosomes with phagosomes. *J Exp Med.* 1971;134(3, pt 1):713-740.

93. Lammas DA, Stober C, Harvey CJ, Kendrick N, Panchalingam S, Kumararatne DS. ATP-induced killing of mycobacteria by human macrophages is mediated by purinergic P2Z(P2X7) receptors. *Immunity.* 1997;7(3):433-444.

94. Molloy A, Laochumroonvorapong P, Kaplan G. Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guérin. *J Exp Med.* 1994;180(4):1499-1509.

95. Boucrot E, Henry T, Borg JP, Gorvel JP, Meresse S. The intracellular fate of Salmonella depends on the recruitment of kinesin. *Science.* 2005;308(5725):1174-1178.

96. Kumar Y, Valdivia RH. Actin and intermediate filaments stabilize the Chlamydia trachomatis vacuole by forming dynamic structural scaffolds. *Cell Host Microbe.* 2008;4(2):159-169.

97. Isberg RR, O'Connor TJ, Heidtman M. The Legionella pneumophila replication vacuole: making a cosy niche inside host cells. *Nat Rev Microbiol.* 2009;7(1):13-24.

98. Ingmundson A, Delprato A, Lambright DG, Roy CR. Legionella pneumophila proteins that regulate Rab1 membrane cycling. *Nature.* 2007;450(7168):365-369.

99. Machner MP, Isberg RR. Targeting of host Rab GTPase function by the intravacuolar pathogen Legionella pneumophila. *Dev Cell.* 2006;11(1):47-56.

100. Voth DE, Heinzen RA. Lounging in a lysosome: the intracellular lifestyle of Coxiella burnetii. *Cell Microbiol.* 2007;9(4):829-840.

101. Pan X, Luhrmann A, Satoh A, Laskowski-Arce MA, Roy CR. Ankyrin repeat proteins comprise a diverse family of bacterial type IV effectors. *Science.* 2008;320(5883):1651-1654.

102. Ray K, Marteyn B, Sansonetti PJ, Tang CM. Life on the inside: the intracellular lifestyle of cytosolic bacteria. *Nat Rev Microbiol.* 2009;7(5): 333-340.

103. Singh R, Jamieson A, Cresswell P. GILT is a critical host factor for Listeria monocytogenes infection. *Nature.* 2008;455(7217):1244-1247.

104. Decatur AL, Portnoy DA. A PEST-like sequence in listeriolysin O essential for Listeria monocytogenes pathogenicity. *Science.* 2000;290(5493): 992-995.

105. Birmingham CL, Canadien V, Kaniuk NA, Steinberg BE, Higgins DE, Brumell JH. Listeriolysin O allows Listeria monocytogenes replication in macrophage vacuoles. *Nature.* 2008;451(7176):350-354.

106. Dupont N, Lacas-Gervais S, Bertout J, et al. Shigella phagocytic vacuolar membrane remnants participate in the cellular response to pathogen invasion and are regulated by autophagy. *Cell Host Microbe.* 2009;6(2):137-149.

107. van der Wel N, Hava D, Houben D, et al. M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid cells. *Cell.* 2007;129(7):1287-1298.

108. Lasa I, Cossart P. Actin-based bacterial motility: towards a definition of the minimal requirements. *Trends Cell Biol.* 1996;6(3):109-114.

109. Pizarro-Cerda J, Cossart P. Listeria monocytogenes membrane trafficking and lifestyle: the exception or the rule? *Annu Rev Cell Dev Biol.* 2009;25:649-670.

110. Welch MD, Iwamatsu A, Mitchison TJ. Actin polymerization is induced by Arp2/3 protein complex at the surface of Listeria monocytogenes. *Nature.* 1997;385(6613):265-269.

111. Hagedorn M, Rohde KH, Russell DG, Soldati T. Infection by tubercular mycobacteria is spread by nonlytic ejection from their amoeba hosts. *Science.* 2009;323(5922):1729-1733.

112. Majno G, Joris I. Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol.* 1995;146(1):3-15.

113. Rudel T, Kepp O, Kozjak-Pavlovic V. Interactions between bacterial pathogens and mitochondrial cell death pathways. *Nat Rev Microbiol.* 2010;8(10):693-705.

114. Miao EA, Rajan JV, Aderem A. Caspase-1-induced pyroptotic cell death. *Immunol Rev.* 2011;243(1):206-214.

115. Deretic V. Autophagy in immunity and cell-autonomous defense against intracellular microbes. *Immunol Rev.* 2011;240(1):92-104.

116. Luhrmann A, Nogueira CV, Carey KL, Roy CR. Inhibition of pathogen-induced apoptosis by a Coxiella burnetii type IV effector protein. *Proc Natl Acad Sci U S A.* 2010;107(44):18997-19001.

117. Voth DE, Heinzen RA. Sustained activation of Akt and Erk1/2 is required for Coxiella burnetii antiapoptotic activity. *Infect Immun.* 2009;77(1):205-213.

118. Niu H, Kozjak-Pavlovic V, Rudel T, Rikihisa Y. Anaplasma phagocytophilum Ats-1 is imported into host cell mitochondria and interferes with apoptosis induction. *PLoS Pathog.* 2010;6(2):e1000774.

119. Nogueira CV, Lindsten T, Jamieson AM, et al. Rapid pathogen-induced apoptosis: a mechanism used by dendritic cells to limit intracellular replication of Legionella pneumophila. *PLoS Pathog.* 2009;5(6): e1000478.

120. Banga S, Gao P, Shen X, et al. Legionella pneumophila inhibits macrophage apoptosis by targeting pro-death members of the Bcl2 protein family. *Proc Natl Acad Sci U S A.* 2007;104(12):5121-5126.

121. Laguna RK, Creasey EA, Li Z, Valtz N, Isberg RR. A Legionella pneumophila-translocated substrate that is required for growth within macrophages and protection from host cell death. *Proc Natl Acad Sci U S A.* 2006;103(49):18745-18750.

122. Behar SM, Divangahi M, Remold HG. Evasion of innate immunity by Mycobacterium tuberculosis: is death an exit strategy? *Nat Rev Microbiol.* 2010;8(9):668-674.

123. Deretic V, Levine B. Autophagy, immunity, and microbial adaptations. *Cell Host Microbe.* 2009;5(6):527-549.

124. Levine B. Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. *Cell.* 2005;120(2):159-162.

125. Rikihisa Y. Glycogen autophagosomes in polymorphonuclear leukocytes induced by rickettsiae. *Anat Rec.* 1984;208(3):319-327.

126. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell.* 2004;119(6):753-766.

127. Alonso S, Pethe K, Russell DG, Purdy GE. Lysosomal killing of Mycobacterium mediated by ubiquitin-derived peptides is enhanced by autophagy. *Proc Natl Acad Sci U S A.* 2007;104(14):6031-6036.

128. Biswas D, Qureshi OS, Lee WY, Croudace JE, Mura M, Lammas DA. ATP-induced autophagy is associated with rapid killing of intracellular mycobacteria within human monocytes/macrophages. *BMC Immunol.* 2008;9:35.

129. Birmingham CL, Brumell JH. Autophagy recognizes intracellular Salmonella enterica serovar Typhimurium in damaged vacuoles. *Autophagy.* 2006;2(3):156-158.

130. Py BF, Lipinski MM, Yuan J. Autophagy limits Listeria monocytogenes intracellular growth in the early phase of primary infection. *Autophagy.* 2007;3(2):117-125.

131. Yano T, Mita S, Ohmori H, et al. Autophagic control of listeria through intracellular innate immune recognition in drosophila. *Nat Immunol.* 2008;9(8):908-916.

132. Zhao Z, Fux B, Goodwin M, et al. Autophagosome-independent essential function for the autophagy protein Atg5 in cellular immunity to intracellular pathogens. *Cell Host Microbe.* 2008;4(5):458-469.

133. Delgado M, Singh S, De HS, et al. Autophagy and pattern recognition receptors in innate immunity. *Immunol Rev.* 2009;227(1):189-202.

134. Deretic V, Singh S, Master S, et al. Mycobacterium tuberculosis inhibition of phagolysosome biogenesis and autophagy as a host defence mechanism. *Cell Microbiol.* 2006;8(5):719-727.

135. Kim BH, Shenoy AR, Kumar P, Das R, Tiwari S, MacMicking JD. A family of IFN-gamma-inducible 65-kD GTPases protects against bacterial infection. *Science.* 2011;332(6030):717-721.

136. Shahnazari S, Yen WL, Birmingham CL, et al. A diacylglycerol-dependent signaling pathway contributes to regulation of antibacterial autophagy. *Cell Host Microbe.* 2010;8(2):137-146.

137. Thurston TL, Ryzhakov G, Bloor S, von MN, Randow F. The TBK1 adaptor and autophagy receptor NDP52 restricts the proliferation of ubiquitin-coated bacteria. *Nat Immunol.* 2009;10(11):1215-1221.

138. Zheng YT, Shahnazari S, Brech A, Lamark T, Johansen T, Brumell JH. The adaptor protein p62/SQSTM1 targets invading bacteria to the autophagy pathway. *J Immunol.*

2009;183(9):5909-5916.

139. Yoshikawa Y, Ogawa M, Hain T, et al. *Listeria monocytogenes* ActA-mediated escape from autophagic recognition. *Nat Cell Biol.* 2009;11(10):1233-1240.

140. Ponpuak M, Davis AS, Roberts EA, et al. Delivery of cytosolic components by autophagic adaptor protein p62 endows autophagosomes with unique antimicrobial properties. *Immunity.* 2010;32(3):329-341.

141. Butchar JP, Cremer TJ, Clay CD, et al. Microarray analysis of human monocytes infected with Francisella tularensis identifies new targets of host response subversion. *PLoS One.* 2008;3(8):e2924.

142. Kumar D, Nath L, Kamal MA, et al. Genome-wide analysis of the host intracellular network that regulates survival of Mycobacterium tuberculosis. *Cell.* 2010;140(5):731-743.

143. Amer AO, Swanson MS. Autophagy is an immediate macrophage response to Legionella pneumophila. *Cell Microbiol.* 2005;7(6):765-778.

144. Beron W, Gutierrez MG, Rabinovitch M, Colombo MI. Coxiella burnetii localizes in a Rab7-labeled compartment with autophagic characteristics. *Infect Immun.* 2002;70(10):5816-5821.

145. Niu H, Yamaguchi M, Rikihisa Y. Subversion of cellular autophagy by Anaplasma phagocytophilum. *Cell Microbiol.* 2008;10(3):593-605.

146. Ogawa M, Yoshimori T, Suzuki T, Sagara H, Mizushima N, Sasakawa C. Escape of intracellular Shigella from autophagy. *Science.* 2005;307(5710):727-731.

147. Rich KA, Burkett C, Webster P. Cytoplasmic bacteria can be targets for autophagy. *Cell Microbiol.* 2003;5(7):455-468.

148. Al-Younes HM, Brinkmann V, Meyer TF. Interaction of Chlamydia trachomatis serovar L2 with the host autophagic pathway. *Infect Immun.* 2004;72(8):4751-4762.

149. Checroun C, Wehrly TD, Fischer ER, Hayes SF, Celli J. Autophagymediated reentry of Francisella tularensis into the endocytic compartment after cytoplasmic replication. *Proc Natl Acad Sci U S A.* 2006;103(39):14578-14583.

150. Romano PS, Gutierrez MG, Beron W, Rabinovitch M, Colombo MI. The autophagic pathway is actively modulated by phase II Coxiella burnetii to efficiently replicate in the host cell. *Cell Microbiol.* 2007;9(4):891-909.

151. Zychlinsky A, Prevost MC, Sansonetti PJ. Shigella flexneri induces apoptosis in infected macrophages. *Nature.* 1992;358(6382):167-169.

152. Monack DM, Raupach B, Hromockyj AE, Falkow S. Salmonella typhimurium invasion induces apoptosis in infected macrophages. *Proc Natl Acad Sci U S A.*

1996;93(18):9833-9838.

153. Chen LM, Kaniga K, Galan JE. Salmonella spp. are cytotoxic for cultured macrophages. *Mol Microbiol.* 1996;21(5):1101-1115.

154. Miao EA, Leaf IA, Treuting PM, et al. Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nat Immunol.* 2010;11(12):1136-1142.

155. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of Mammalian iron metabolism. *Cell.* 2010;142(1):24-38.

156. Nairz M, Schroll A, Sonnweber T, Weiss G. The struggle for iron— a metal at the host-pathogen interface. *Cell Microbiol.* 2010;12(12): 1691-1702.

157. Ganz T. Iron in innate immunity: starve the invaders. *Curr Opin Immunol.* 2009;21(1):63-67.

158. Schaible UE, Kaufmann SH. Iron and microbial infection. *Nat Rev Microbiol.* 2004;2(12):946-953.

159. Pan X, Tamilselvam B, Hansen EJ, Daefler S. Modulation of iron homeostasis in macrophages by bacterial intracellular pathogens. *BMC Microbiol.* 2010;10:64.

160. Sow FB, Florence WC, Satoskar AR, Schlesinger LS, Zwilling BS, Lafuse WP. Expression and localization of hepcidin in macrophages: a role in host defense against tuberculosis. *J Leukoc Biol.* 2007;82(4):934-945.

161. Cherayil BJ. The role of iron in the immune response to bacterial infection. *Immunol Res.* 2011;50(1):1-9.

162. Paradkar PN, De Domenico I, Durchfort N, Zohn I, Kaplan J, Ward DM. Iron depletion limits intracellular bacterial growth in macrophages. *Blood.* 2008;112(3):866-874.

163. Braun V, Hantke K. Recent insights into iron import by bacteria. *Curr Opin Chem Biol.* 2011;15(2):328-334.

164. Wandersman C, Delepelaire P. Bacterial iron sources: from siderophores to hemophores. *Annu Rev Microbiol.* 2004;58:611-647.

165. Rodriguez GM. Control of iron metabolism in Mycobacterium tuberculosis. *Trends Microbiol.* 2006;14(7):320-327.

166. Saiga H, Nishimura J, Kuwata H, et al. Lipocalin 2-dependent inhibition of mycobacterial growth in alveolar epithelium. *J Immunol.* 2008;181(12):8521-8527.

167. Cairo G, Recalcati S, Mantovani A, Locati M. Iron trafficking and metabolism in macrophages: contribution to the polarized phenotype. *Trends Immunol.* 2011;32(6):241-

247.

168. Cellier MF, Courville P, Campion C. Nramp1 phagocyte intracellular metal withdrawal defense. *Microbes Infect.* 2007;9(14-15):1662-1670.

169. Gobin J, Moore CH, Reeve JR Jr, Wong DK, Gibson BW, Horwitz MA. Iron acquisition by Mycobacterium tuberculosis: isolation and characterization of a family of iron-binding exochelins. *Proc Natl Acad Sci U S A.* 1995;92(11):5189-5193.

170. Gobin J, Horwitz MA. Exochelins of Mycobacterium tuberculosis remove iron from human iron-binding proteins and donate iron to mycobactins in the M. tuberculosis cell wall. *J Exp Med.* 1996;183(4): 1527-1532.

171. Fang FC. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol.* 2004;2(10):820-832.

172. Elsbach P, Weiss J. A reevaluation of the roles of the O2-dependent and O2-independent microbicidal systems of phagocytes. *Rev Infect Dis.* 1983;5(5):843-853.

173. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. *Annu Rev Immunol.* 1997;15:323-350.

174. Bogdan C. Nitric oxide and the immune response. *Nat Immunol.* 2001;2(10):907-916.

175. Nathan C, Shiloh MU. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A.* 2000;97(16):8841-8848.

176. Mastroeni P, Vazquez-Torres A, Fang FC, et al. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival in vivo. *J Exp Med.* 2000;192(2):237-248.

177. Vazquez-Torres A, Jones-Carson J, Mastroeni P, Ischiropoulos H, Fang FC. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. *J Exp Med.* 2000;192(2):227-236.

178. Fang FC, Nathan CF. Man is not a mouse: reply. *J Leukoc Biol*. 2007; 81(3):580.

179. Nathan C. Role of iNOS in human host defense. *Science.* 2006; 312(5782):1874-1875.

180. Chan ED, Chan J, Schluger NW. What is the role of nitric oxide in murine and human host defense against tuberculosis? Current knowledge. *Am J Respir Cell Mol Biol.* 2001;25(5):606-612.

181. Choi HS, Rai PR, Chu HW, Cool C, Chan ED. Analysis of nitric oxide synthase and

nitrotyrosine expression in human pulmonary tuberculosis. *Am J Respir Crit Care Med.* 2002;166(2):178-186.

182. Kaufmann SH, Cole ST, Mizrahi V, Rubin E, Nathan C. Mycobacterium tuberculosis and the host response. *J Exp Med.* 2005;201(11):1693-1697.

183. Schon T, Elmberger G, Negesse Y, Pando RH, Sundqvist T, Britton S. Local production of nitric oxide in patients with tuberculosis. *Int J Tuberc Lung Dis.* 2004;8(9):1134-1137.

184. Nicholson S, Bonecini-Almeida Mda G, Lapa e Silva JR, et al. Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J Exp Med.* 1996;183(5):2293-2302.

185. Murray PJ, Wynn TA. Obstacles and opportunities for understanding macrophage polarization. *J Leukoc Biol.* 2011;89(4):557-563.

186. Weinberg JB. Nitric oxide production and nitric oxide synthase type 2 expression by human mononuclear phagocytes: a review. *Mol Med.* 1998;4(9):557-591.

187. Huang J, DeGraves FJ, Lenz SD, et al. The quantity of nitric oxide released by macrophages regulates Chlamydia-induced disease. *Proc Natl Acad Sci U S A.* 2002;99(6):3914-3919.

188. MacMicking JD, Nathan C, Hom G, et al. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell.* 1995;81(4):641-650.

189. Howe D, Barrows LF, Lindstrom NM, Heinzen RA. Nitric oxide inhibits Coxiella burnetii replication and parasitophorous vacuole maturation. *Infect Immun.* 2002;70(9):5140-5147.

190. Chakravortty D, Hensel M. Inducible nitric oxide synthase and control of intracellular bacterial pathogens. *Microbes Infect.* 2003;5(7):621-627.

191. MacMicking JD, North RJ, LaCourse R, Mudgett JS, Shah SK, Nathan CF. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc Natl Acad Sci U S A.* 1997;94(10):5243-5248.

192. Bustamante J, Arias AA, Vogt G, et al. Germline CYBB mutations that selectively affect macrophages in kindreds with X-linked predisposition to tuberculous mycobacterial disease. *Nat Immunol.* 2011;12(3): 213-221.

193. Vazquez-Torres A, Xu Y, Jones-Carson J, et al. Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. *Science.* 2000;287(5458):1655-1658.

194. Chakravortty D, Hansen-Wester I, Hensel M. Salmonella pathogenicity island 2 mediates protection of intracellular Salmonella from reactive nitrogen intermediates. *J Exp Med.* 2002;195(9):1155-1166.

195. Cowley SC, Myltseva SV, Nano FE. Phase variation in Francisella tularensis affecting intracellular growth, lipopolysaccharide antigenicity and nitric oxide production. *Mol Microbiol.* 1996;20(4):867-874.

196. Choi KS, Dumler JS. Early induction and late abrogation of respiratory burst in A. phagocytophilum-infected neutrophils. *Ann N Y Acad Sci.* 2003;990:488-493.

197. Ehrt S, Schnappinger D. Mycobacterial survival strategies in the phagosome: defence against host stresses. *Cell Microbiol.* 2009;11(8):1170-1178.

198. Ehrt S, Shiloh MU, Ruan J, et al. A novel antioxidant gene from Mycobacterium tuberculosis. *J Exp Med.* 1997;186(11):1885-1896.

199. Ng VH, Cox JS, Sousa AO, MacMicking JD, McKinney JD. Role of KatG catalase-peroxidase in mycobacterial pathogenesis: countering the phagocyte oxidative burst. *Mol Microbiol.* 2004;52(5):1291-1302.

200. Miller JL, Velmurugan K, Cowan MJ, Briken V. The type I NADH dehydrogenase of Mycobacterium tuberculosis counters phagosomal NOX2 activity to inhibit TNF-alpha-mediated host cell apoptosis. *PLoS Pathog.* 2010;6(4):e1000864.

201. Shin DM, Jeon BY, Lee HM, et al. Mycobacterium tuberculosis eis regulates autophagy, inflammation, and cell death through redox-dependent signaling. *PLoS Pathog.* 2010;6(12):e1001230.

202. De Groote MA, Testerman T, Xu Y, Stauffer G, Fang FC. Homocysteine antagonism of nitric oxide-related cytostasis in Salmonella typhimurium. *Science.* 1996;272(5260):414-417.

203. Darwin KH, Ehrt S, Gutierrez-Ramos JC, Weich N, Nathan CF. The proteasome of Mycobacterium tuberculosis is required for resistance to nitric oxide. *Science.* 2003;302(5652):1963-1966.

204. Carroll MC. The role of complement and complement receptors in induction and regulation of immunity. *Annu Rev Immunol.* 1998;16: 545-568.

205. Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol.* 2005;3(3):238-250.

206. Sonawane A, Santos JC, Mishra BB, et al. Cathelicidin is involved in the intracellular killing of mycobacteria in macrophages. *Cell Microbiol.* 2011;13(10):1601-1617.

207. Purdy GE. Taking out TB-lysosomal trafficking and mycobactericidal ubiquitin-derived peptides. *Front Microbiol.* 2011;2:7.

208. Taylor GA, Feng CG, Sher A. p47 GTPases: regulators of immunity to intracellular pathogens. *Nat Rev Immunol.* 2004;4(2):100-109.

209. Shenoy AR, Kim BH, Choi HP, Matsuzawa T, Tiwari S, MacMicking JD. Emerging themes in IFN-gamma-induced macrophage immunity by the p47 and p65 GTPase families. *Immunobiology.* 2007;212(9-10): 771-784.

210. MacMicking JD, Taylor GA, McKinney JD. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. *Science.* 2003;302(5645): 654-659.

211. Coers J, Starnbach MN, Howard JC. Modeling infectious disease in mice: co-adaptation and the role of host-specific IFNgamma responses. *PLoS Pathog.* 2009;5(5):e1000333.

212. Miyairi I, Tatireddigari VR, Mahdi OS, et al. The p47 GTPases ligp2 and Irgb10 regulate innate immunity and inflammation to murine Chlamydia psittaci infection. *J Immunol.* 2007;179(3):1814-1824.

213. Feng CG, Collazo-Custodio CM, Eckhaus M, et al. Mice deficient in LRG-47 display increased susceptibility to mycobacterial infection associated with the induction of lymphopenia. *J Immunol.* 2004;172(2):1163-1168.

214. Collazo CM, Yap GS, Sempowski GD, et al. Inactivation of LRG-47 and IRG-47 reveals a family of interferon gamma-inducible genes with essential, pathogen-specific roles in resistance to infection. *J Exp Med.* 2001;194(2):181-188.

215. Henry SC, Daniell X, Indaram M, et al. Impaired macrophage function underscores susceptibility to Salmonella in mice lacking Irgm1 (LRG-47). *J Immunol.* 2007;179(10):6963-6972.

216. Hunn JP, Feng CG, Sher A, Howard JC. The immunity-related GTPases in mammals: a fast-evolving cell-autonomous resistance system against intracellular pathogens. *Mamm Genome.* 2011;22(1-2):43-54.

217. Metchnikoff M. *Immunity to Infectious Diseases.* London, United Kingdom: Cambridge University Press; 1905.

218. Koch R. Weitere Mittheilungen über ein Heilmittel gegen Tuberculose. *Dt med Wochenschr.* 1890;16:1029-1032.

219. Lurie MB. *Resistance to Tuberculosis.* Cambridge, MA: Harvard University Press, 1964.

220. Mackaness GB. Cellular resistance to infection. *J Exp Med.* 1962;116: 381-406.

221. Bloom BR, Bennett B. Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. *Science.* 1966;153(731):80-82.

222. David JR. Delayed hypersensitivity in vitro: its mediation by cell-free substances formed by lymphoid cell-antigen interaction. *Proc Natl Acad Sci U S A.* 1966;56(1):72-77.

223. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of

monocytes, macrophages, and dendritic cells. *Science.* 2010;327(5966):656-661.

224. Cros J, Cagnard N, Woollard K, et al. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity.* 2010;33(3):375-386.

225. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol.* 2004;75(2):163-189.

226. Kaufmann SH. Immunity to intracellular bacteria. *Annu Rev Immunol.* 1993;11:129-163.

227. Mege JL, Mehraj V, Capo C. Macrophage polarization and bacterial infections. *Curr Opin Infect Dis.* 2011;24(3):230-234.

228. Benoit M, Desnues B, Mege JL. Macrophage polarization in bacterial infections. *J Immunol.* 2008;181(6):3733-3739.

229. Segal AW. How neutrophils kill microbes. *Annu Rev Immunol.* 2005;23:197-223.

230. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol.* 2011;11(8):519-531.

231. Eum SY, Kong JH, Hong MS, et al. Neutrophils are the predominant infected phagocytic cells in the airways of patients with active pulmonary TB. *Chest.* 2010;137(1):122-128.

232. Conlan JW, North RJ. Neutrophil-mediated dissolution of infected host cells as a defense strategy against a facultative intracellular bacterium. *J Exp Med.* 1991;174(3):741-744.

233. Rogers HW, Unanue ER. Neutrophils are involved in acute, nonspecific resistance to Listeria monocytogenes in mice. *Infect Immun.* 1993;61(12):5090-5096.

234. Conlan JW, North RJ. Neutrophils are essential for early anti-Listeria defense in the liver, but not in the spleen or peritoneal cavity, as revealed by a granulocyte-depleting monoclonal antibody. *J Exp Med.* 1994;179(1): 259-268.

235. Shi C, Hohl TM, Leiner I, Equinda MJ, Fan X, Pamer EG. Ly6G + neutrophils are dispensable for defense against systemic Listeria monocytogenes infection. *J Immunol.* 2011;187(10):5293-5298.

236. Kang DD, Lin Y, Moreno JR, Randall TD, Khader SA. Profiling early lung immune responses in the mouse model of tuberculosis. *PLoS One.* 2011;6(1):e16161.

237. Seiler P, Aichele P, Raupach B, Odermatt B, Steinhoff U, Kaufmann SH. Rapid neutrophil response controls fast-replicating intracellular bacteria but not slow-replicating Mycobacterium tuberculosis. *J Infect Dis.* 2000;181(2):671-680.

238. Nandi B, Behar SM. Regulation of neutrophils by interferon-γ limits lung

inflammation during tuberculosis infection. *J Exp Med.* 2011;208(11):2251-2262.

239. Reeves EP, Lu H, Jacobs HL, et al. Killing activity of neutrophils is mediated through activation of proteases by K+ flux. *Nature.* 2002;416(6878):291-297.

240. Weinrauch Y, Drujan D, Shapiro SD, Weiss J, Zychlinsky A. Neutrophil elastase targets virulence factors of enterobacteria. *Nature.* 2002;417(6884):91-94.

241. Brinkmann V, Zychlinsky A. Beneficial suicide: why neutrophils die to make NETs. *Nat Rev Microbiol.* 2007;5(8):577-582.

242. Metzler KD, Fuchs TA, Nauseef WM, et al. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. *Blood.* 2011;117(3):953-959.

243. Papayannopoulos V, Zychlinsky A. NETs: a new strategy for using old weapons. *Trends Immunol.* 2009;30(11):513-521.

244. Douek DC, Picker LJ, Koup RA. T cell dynamics in HIV-1 infection. *Annu Rev Immunol.* 2003;21:265-304.

245. Modjarrad K, Vermund SH. Effect of treating co-infections on HIV-1 viral load: a systematic review. *Lancet Infect Dis.* 2010;10(7):455-463.

246. Kwan CK, Ernst JD. HIV and tuberculosis: a deadly human syndemic. *Clin Microbiol Rev.* 2011;24(2):351-376.

247. Graham SM. Nontyphoidal salmonellosis in Africa. *Curr Opin Infect Dis.* 2010;23(5):409-414.

248. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature.* 1998;392(6673):245-252.

249. Reis e Sousa. Dendritic cells in a mature age. *Nat Rev Immunol.* 2006;6(6):476-483.

250. Guermonprez P, Valladeau J, Zitvogel L, Thery C, Amigorena S. Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol.* 2002;20:621-667.

251. Savina A, Amigorena S. Phagocytosis and antigen presentation in dendritic cells. *Immunol Rev.* 2007;219:143-156.

252. Savina A, Jancic C, Hugues S, et al. NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. *Cell.* 2006;126(1):205-218.

253. Trombetta ES, Ebersold M, Garrett W, Pypaert M, Mellman I. Activation of lysosomal function during dendritic cell maturation. *Science.* 2003;299(5611):1400-1403.

254. Wolf AJ, Linas B, Trevejo-Nunez GJ, et al. Mycobacterium tuberculosis infects dendritic cells with high frequency and impairs their function in vivo. *J Immunol.* 2007;179(4):2509-2519.

255. Meraviglia S, El DS, Dieli F, Martini F, Martino A. γδ T cells cross-link innate and adaptive immunity in Mycobacterium tuberculosis infection. *Clin Dev Immunol.* 2011;2011:587315.

256. Cohen NR, Garg S, Brenner MB. Antigen presentation by CD1 lipids, T cells, and NKT cells in microbial immunity. *Adv Immunol.* 2009;102:1-94.

257. Brigl M, Brenner MB. CD1: antigen presentation and T cell function. *Annu Rev Immunol.* 2004;22:817-890.

258. Schaible UE, Winau F, Sieling PA, et al. Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. *Nat Med.* 2003;9(8):1039-1046.

259. Behar SM, Martin CJ, Booty MG, et al. Apoptosis is an innate defense function of macrophages against Mycobacterium tuberculosis. *Mucosal Immunol.* 2011;4(3):279-287.

260. Harding CV, Boom WH. Regulation of antigen presentation by Mycobacterium tuberculosis: a role for toll-like receptors. *Nat Rev Microbiol.* 2010;8(4):296-307.

261. Munz C. Antigen processing via autophagy—not only for MHC class II presentation anymore? *Curr Opin Immunol.* 2010;22(1):89-93.

262. Werninghaus K, Babiak A, Gross O, et al. Adjuvanticity of a synthetic cord factor analogue for subunit *Mycobacterium tuberculosis* vaccination requires FcRgamma-Syk-Card9-dependent innate immune activation. *J Exp Med.* 2009;206(1):89-97.

263. Torchinsky MB, Garaude J, Martin AP, Blander JM. Innate immune recognition of infected apoptotic cells directs T(H)17 cell differentiation. *Nature.* 2009;458(7234):78-82.

264. Kaufmann SH, McMichael AJ. Annulling a dangerous liaison: vaccination strategies against AIDS and tuberculosis. *Nat Med.* 2005;11(4 Suppl):S33-S44.

265. Yagi R, Junttila IS, Wei G, et al. The transcription factor GATA3 actively represses RUNX3 protein-regulated production of interferongamma. *Immunity.* 2010;32(4):507-517.

266. Ivanov II, McKenzie BS, Zhou L, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell.* 2006;126(6):1121-1133.

267. Iwakura Y, Ishigame H, Saijo S, Nakae S. Functional specialization of interleukin-17 family members. *Immunity.* 2011;34(2):149-162.

268. Khader SA, Bell GK, Pearl JE, et al. IL-23 and IL-17 in the establishment of

protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. *Nat Immunol.* 2007;8(4):369-377.

269. Lin Y, Ritchea S, Logar A, et al. Interleukin-17 is required for T helper 1 cell immunity and host resistance to the intracellular pathogen Francisella tularensis. *Immunity.* 2009;31(5):799-810.

270. Dussurget O, Pizarro-Cerda J, Cossart P. Molecular determinants of Listeria monocytogenes virulence. *Annu Rev Microbiol.* 2004;58:587-610.

271. Stenger S, Rosat JP, Bloom BR, Krensky AM, Modlin RL. Granulysin: a lethal weapon of cytolytic T cells. *Immunol Today.* 1999;20(9):390-394.

272. Voskoboinik I, Smyth MJ, Trapani JA. Perforin-mediated target-cell death and immune homeostasis. *Nat Rev Immunol.* 2006;6(12):940-952.

273. Cooper AM, D'Souza C, Frank AA, Orme IM. The course of Mycobacterium tuberculosis infection in the lungs of mice lacking expression of either perforin- or granzyme-mediated cytolytic mechanisms. *Infect Immun.* 1997;65(4):1317-1320.

274. Turner J, D'Souza CD, Pearl JE, et al. CD8- and CD95/95L-dependent mechanisms of resistance in mice with chronic pulmonary tuberculosis. *Am J Respir Cell Mol Biol.* 2001;24(2):203-209.

275. Rottenberg ME, Gigliotti Rothfuchs AC, Gigliotti D, Svanholm C, Bandholtz L, Wigzell H. Role of innate and adaptive immunity in the outcome of primary infection with Chlamydia pneumoniae, as analyzed in genetically modified mice. *J Immunol.* 1999;162(5):2829-2836.

276. Perry LL, Feilzer K, Hughes S, Caldwell HD. Clearance of Chlamydia trachomatis from the murine genital mucosa does not require perforin- mediated cytolysis or Fas-mediated apoptosis. *Infect Immun.* 1999;67(3):1379-1385.

277. Kagi D, Ledermann B, Burki K, Hengartner H, Zinkernagel RM. CD8+ T cell-mediated protection against an intracellular bacterium by perforin-dependent cytotoxicity. *Eur J Immunol.* 1994;24(12):3068- 3072.

278. Stenger S, Hanson DA, Teitelbaum R, et al. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science.* 1998;282(5386):121-125.

279. Lenz LL, Dere B, Bevan MJ. Identification of an H2-M3-restricted Listeria epitope: implications for antigen presentation by M3. *Immunity.* 1996;5(1):63-72.

280. Gold MC, Cerri S, Smyk-Pearson S, et al. Human mucosal associated invariant T cells detect bacterially infected cells. *PLoS Biol.* 2010;8(6):e1000407.

281. Bonneville M, O'Brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol.* 2010;10(7):467-478.

282. Mombaerts P, Arnoldi J, Russ F, Tonegawa S, Kaufmann SH. Different roles of alpha beta and gamma delta T cells in immunity against an intracellular bacterial pathogen. *Nature.* 1993;365(6441):53-56.

283. Lockhart E, Green AM, Flynn JL. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. *J Immunol.* 2006;177(7):4662-4669.

284. Tupin E, Kinjo Y, Kronenberg M. The unique role of natural killer T cells in the response to microorganisms. *Nat Rev Microbiol.* 2007;5(6):405-417.

285. Mattner J, Debord KL, Ismail N, et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature.* 2005;434(7032):525-529.

286. Darmoise A, Teneberg S, Bouzonville L, et al. Lysosomal alpha-galactosidase controls the generation of self lipid antigens for natural killer T cells. *Immunity.* 2010;33(2):216-228.

287. Brennan PJ, Tatituri RV, Brigl M, et al. Invariant natural killer T cells recognize lipid self antigen induced by microbial danger signals. *Nat Immunol.* 2011;12(12):1202-1211.

288. King IL, Fortier A, Tighe M, et al. Invariant natural killer T cells direct B cell responses to cognate lipid antigen in an IL-21-dependent manner. *Nat Immunol.* 2011;13(1):44-50.

289. Joller N, Weber SS, Oxenius A. Antibody-Fc receptor interactions in protection against intracellular pathogens. *Eur J Immunol.* 2011;41(4):889-897.

290. Neves P, Lampropoulou V, Calderon-Gomez E, et al. Signaling via the MyD88 adaptor protein in B cells suppresses protective immunity during Salmonella typhimurium infection. *Immunity.* 2010;33(5):777-790.

291. Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. *Nat Immunol.* 2005;6(4):353-360.

292. Belkaid Y, Tarbell K. Regulatory T cells in the control of host-microorganism interactions (*). *Annu Rev Immunol.* 2009;27:551-589.

293. Rappuoli R, Aderem A. A 2020 vision for vaccines against HIV, tuberculosis and malaria. *Nature.* 2011;473(7348):463-469.

294. Wherry EJ. T cell exhaustion. *Nat Immunol.* 2011;12(6):492-499.

295. Lanzavecchia A, Sallusto F. Understanding the generation and function of memory T cell subsets. *Curr Opin Immunol.* 2005;17(3):326-332.

296. Scott P. Immunologic memory in cutaneous leishmaniasis. *Cell Microbiol.* 2005;7(12):1707-1713.

297. Williams MA, Bevan MJ. Shortening the infectious period does not alter expansion of CD8 T cells but diminishes their capacity to differentiate into memory cells. *J Immunol.* 2004;173(11):6694-6702.

298. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol.* 2007;81(1):1-5.

299. Nathan C, Ding A. Nonresolving inflammation. *Cell.* 2010;140(6):871-882.

300. Tobin DM, Vary JC Jr, Ray JP, et al. The lta4h locus modulates susceptibility to mycobacterial infection in zebrafish and humans 3025. *Cell.* 2010;140(5):717-730.

301. Randolph GJ, Ochando J, Partida-Sanchez S. Migration of dendritic cell subsets and their precursors. *Annu Rev Immunol.* 2008;26:293-316.

302. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. *Annu Rev Immunol.* 2009;27:485-517.

303. Szabo SJ, Sullivan BM, Peng SL, Glimcher LH. Molecular mechanisms regulating Th1 immune responses. *Annu Rev Immunol.* 2003;21:713-758.

304. Sivori S, Falco M, Della CM, et al. CpG and double-stranded RNA trigger human NK cells by toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. *Proc Natl Acad Sci U S A.* 2004;101(27):10116-10121.

305. Hart OM, Athie-Morales V, O'Connor GM, Gardiner CM. TLR7/8-mediated activation of human NK cells results in accessory cell-dependent IFN-gamma production. *J Immunol.* 2005;175(3):1636-1642.

306. Kabelitz D. Expression and function of toll-like receptors in T lymphocytes. *Curr Opin Immunol.* 2007;19(1):39-45.

307. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol.* 2009;27:451-483.

308. Potian JA, Rafi W, Bhatt K, McBride A, Gause WC, Salgame P. Preexisting helminth infection induces inhibition of innate pulmonary anti-tuberculosis defense by engaging the IL-4 receptor pathway. *J Exp Med.* 2011;208(9):1863-1874.

309. Perry S, Hussain R, Parsonnet J. The impact of mucosal infections on acquisition and progression of tuberculosis. *Mucosal Immunol.* 2011;4(3):246-251.

310. Wammes LJ, Hamid F, Wiria AE, et al. Regulatory T cells in human geohelminth infection suppress immune responses to BCG and Plasmodium falciparum. *Eur J Immunol.* 2010;40(2):437-442.

311. Bunting M, Harris ES, McIntyre TM, Prescott SM, Zimmerman GA. Leukocyte adhesion deficiency syndromes: adhesion and tethering defects involving beta 2 integrins and selectin ligands. *Curr Opin Hematol.* 2002;9(1):30-35.

312. Medzhitov R. Origin and physiological roles of inflammation. *Nature.* 2008;454(7203):428-435.

313. Nathan C. Points of control in inflammation. *Nature.* 2002;420(6917): 846-852.

314. Allen SJ, Crown SE, Handel TM. Chemokine: receptor structure, interactions, and antagonism. *Annu Rev Immunol.* 2007;25:787-820.

315. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. *Immunity.* 2000;12(2):121-127.

316. Kurihara T, Warr G, Loy J, Bravo R. Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. *J Exp Med.* 1997;186(10):1757-1762.

317. Peters W, Scott HM, Chambers HF, Flynn JL, Charo IF, Ernst JD. Chemokine receptor 2 serves an early and essential role in resistance to Mycobacterium tuberculosis. *Proc Natl Acad Sci U S A.* 2001;98(14):7958-7963.

318. Lu B, Rutledge BJ, Gu L, et al. Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J Exp Med.* 1998;187(4):601-608.

319. Serbina NV, Pamer EG. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol.* 2006;7(3):311-317.

320. Di LD, Locati M, Caccamo N, et al. Role of the chemokine decoy receptor D6 in balancing inflammation, immune activation, and antimicrobial resistance in Mycobacterium tuberculosis infection. *J Exp Med.* 2008;205(9):2075-2084.

321. Jacobs M, Togbe D, Fremond C, et al. Tumor necrosis factor is critical to control tuberculosis infection. *Microbes Infect.* 2007;9(5):623-628.

322. Koebernick H, Grode L, David JR, et al. Macrophage migration inhibitory factor (MIF) plays a pivotal role in immunity against Salmonella typhimurium. *Proc Natl Acad Sci U S A.* 2002;99(21):13681-13686.

323. Tramontana JM, Utaipat U, Molloy A, et al. Thalidomide treatment reduces tumor necrosis factor alpha production and enhances weight gain in patients with pulmonary tuberculosis. *Mol Med.* 1995;1(4):384-397.

324. Sampaio EP, Kaplan G, Miranda A, et al. The influence of thalidomide on the clinical and immunologic manifestation of erythema nodosum leprosum. J Infect Dis. 1993;168(2):408-414.

325. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol.* 2007;7(9):678-689.

326. Abram CL, Lowell CA. The ins and outs of leukocyte integrin signaling. *Annu Rev Immunol.* 2009;27:339-362.

327. Sallusto F, Mackay CR, Lanzavecchia A. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu Rev Immunol.* 2000;18:593-620.

328. Zielinski CE, Corti D, Mele F, Pinto D, Lanzavecchia A, Sallusto F. Dissecting the human immunologic memory for pathogens. *Immunol Rev.* 2011;240(1):40-51.

329. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol.* 2004;22:745-763.

330. Dinarello CA. Immunological and inflammatory functions of the interleukin- 1 family. *Annu Rev Immunol.* 2009;27:519-550.

331. Schroder K, Tschopp J. The inflammasomes. *Cell.* 2010;140(6):821-832.

332. Lamkanfi M, Dixit VM. Modulation of inflammasome pathways by bacterial and viral pathogens. *J Immunol.* 2011;187(2):597-602.

333. Broz P, Monack DM. Molecular mechanisms of inflammasome activation during microbial infections. *Immunol Rev.* 2011;243(1):174-190.

334. Davis BK, Wen H, Ting JP. The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu Rev Immunol.* 2011;29: 707-735.

335. Broz P, Newton K, Lamkanfi M, Mariathasan S, Dixit VM, Monack DM. Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella. *J Exp Med.* 2010;207(8):1745-1755.

336. Flynn JL, Chan J. Immunology of tuberculosis. *Annu Rev Immunol.* 2001;19:93-129.

337. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM. Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J Exp Med.* 1993;178(6):2243-2247.

338. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. *J Exp Med.* 1993;178(6):2249-2254.

339. Jouanguy E, Altare F, Lamhamedi S, et al. Interferon-gamma-receptor deficiency in an infant with fatal bacille Calmette-Guérin infection. *N Engl J Med.* 1996;335(26):1956-1961.

340. Newport MJ, Huxley CM, Huston S, et al. A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. *N Engl J Med.* 1996;335(26):1941-1949.

341. Holland SM. Therapy of mycobacterial infections. *Res Immunol.* 1996;147(8-9):572-581.

342. O'Connell RM, Saha SK, Vaidya SA, et al. Type I interferon production enhances susceptibility to Listeria monocytogenes infection. *J Exp Med.* 2004;200(4):437-445.

343. Auerbuch V, Brockstedt DG, Meyer-Morse N, O'Riordan M, Portnoy DA. Mice lacking the type I interferon receptor are resistant to Listeria monocytogenes. *J Exp Med.* 2004;200(4):527-533.

344. Carrero JA, Calderon B, Unanue ER. Lymphocytes are detrimental during the early innate immune response against Listeria monocytogenes. *J Exp Med.* 2006;203(4):933-940.

345. Manca C, Tsenova L, Bergtold A, et al. Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha/beta. *Proc Natl Acad Sci U S A.* 2001;98(10):5752-5757.

346. Stanley SA, Johndrow JE, Manzanillo P, Cox JS. The Type I IFN response to infection with Mycobacterium tuberculosis requires ESX- 1-mediated secretion and contributes to pathogenesis. *J Immunol.* 2007;178(5):3143-3152.

347. Antonelli LR, Gigliotti RA, Goncalves R, et al. Intranasal Poly-IC treatment exacerbates tuberculosis in mice through the pulmonary recruitment of a pathogen-permissive monocyte/macrophage population. *J Clin Invest.* 2010;120(5):1674-1682.

348. Nagarajan UM, Prantner D, Sikes JD, et al. Type I interferon signaling exacerbates Chlamydia muridarum genital infection in a murine model. *Infect Immun.* 2008;76(10):4642-4648.

349. Henry T, Kirimanjeswara GS, Ruby T, et al. Type I IFN signaling constrains IL-17A/F secretion by gammadelta T cells during bacterial infections. *J Immunol.* 2010;184(7):3755-3767.

350. Rayamajhi M, Humann J, Penheiter K, Andreasen K, Lenz LL. Induction of IFN-alphabeta enables Listeria monocytogenes to suppress macrophage activation by IFN-gamma. *J Exp Med.* 2010;207(2):327-337.

351. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol.* 2010;11(10):889-896.

352. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol.* 2008;8(12):958-969.

353. Ishii M, Wen H, Corsa CA, et al. Epigenetic regulation of the alternatively activated macrophage phenotype. *Blood.* 2009;114(15):3244- 3254.

354. Satoh T, Takeuchi O, Vandenbon A, et al. The Jmjd3-Irf4 axis regulates M2

macrophage polarization and host responses against helminth infection. *Nat Immunol.* 2010;11(10):936-944.

355. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol.* 2011;11(11):723-737.

356. Pesce J, Kaviratne M, Ramalingam TR, et al. The IL-21 receptor augments Th2 effector function and alternative macrophage activation. *J Clin Invest.* 2006;116(7):2044-2055.

357. Kurowska-Stolarska M, Stolarski B, Kewin P, et al. IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. *J Immunol*. 2009;183(10):6469-6477.

358. Saenz SA, Siracusa MC, Perrigoue JG, et al. IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature.* 2010;464(7293):1362-1366.

359. Neill DR, Wong SH, Bellosi A, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature.* 2010;464(7293):1367-1370.

360. Price AE, Liang HE, Sullivan BM, et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci U S A.* 2010;107(25):11489-11494.

361. Jenkins SJ, Ruckerl D, Cook PC, et al. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science.* 2011;332(6035):1284-1288.

362. Van den Bossche J, Bogaert P, van HJ, et al. Alternatively activated macrophages engage in homotypic and heterotypic interactions through IL-4 and polyamine-induced E-cadherin/catenin complexes. *Blood.* 2009;114(21):4664-4674.

363. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. *Immunity.* 2010;32(5):593-604.

364. Stout RD, Watkins SK, Suttles J. Functional plasticity of macrophages: in situ reprogramming of tumor-associated macrophages. *J Leukoc Biol.* 2009;86(5):1105-1109.

365. Auffray C, Fogg D, Garfa M, et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science.* 2007;317(5838):666-670.

366. Shirey KA, Cole LE, Keegan AD, Vogel SN. Francisella tularensis live vaccine strain induces macrophage alternative activation as a survival mechanism. *J Immunol.* 2008;181(6):4159-4167.

367. Kahnert A, Seiler P, Stein M, et al. Alternative activation deprives macrophages of a coordinated defense program to Mycobacterium tuberculosis. *Eur J Immunol.* 2006;36(3):631-647.

368. Rajaram MV, Brooks MN, Morris JD, Torrelles JB, Azad AK, Schlesinger LS. Mycobacterium tuberculosis activates human macrophage peroxisome proliferator-activated receptor gamma linking mannose receptor recognition to regulation of immune responses. *J Immunol.* 2010;185(2):929-942.

369. Berry MP, Graham CM, McNab FW, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature.* 2010;466(7309):973-977.

370. Maertzdorf J, Repsilber D, Parida SK, et al. Human gene expression profiles of susceptibility and resistance in tuberculosis. *Genes Immun.* 2011;12:15-22.

371. Thompson LJ, Dunstan SJ, Dolecek C, et al. Transcriptional response in the peripheral blood of patients infected with Salmonella enterica serovar Typhi. *Proc Natl Acad Sci U S A.* 2009;106(52):22433-22438.

372. Montoya D, Cruz D, Teles RM, et al. Divergence of macrophage phagocytic and antimicrobial programs in leprosy. *Cell Host Microbe.* 2009;6(4):343-353.

373. Reece ST, Kaufmann SHE. Floating between the poles of pathology and protection: can we pin down the granuloma in tuberculosis? *Curr Opin Microbiol.* 2012;15(1):63-70.

374. Ulrichs T, Kaufmann SHE. New insights into the function of granulomas in human tuberculosis. *J Pathol.* 2006;208(2):261-269.

375. Flynn JL, Goldstein MM, Chan J, et al. Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice. *Immunity.* 1995;2(6):561-572.

376. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. *J Exp Med.* 1993;178(6):2249-2254.

377. Keane J, Gershon S, Wise RP, et al. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med.* 2001;345(15):1098-1104.

378. Samuel G, Maier LA. Immunology of chronic beryllium disease. *Curr Opin Allergy Clin Immunol.* 2008;8(2):126-134.

379. Shigemitsu H, Azuma A. Sarcoidosis and interstitial pulmonary fibrosis; two distinct disorders or two ends of the same spectrum. *Curr Opin Pulm Med.* 2011;17(5):303-307.

380. Okamoto YY, Umemura M, Yahagi A, et al. Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung. *J Immunol.* 2010;184(8):4414-4422.

381. Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity.* 2008;28(4):454-467.

382. Redford PS, Boonstra A, Read S, et al. Enhanced protection to Mycobacterium

tuberculosis infection in IL-10-deficient mice is accompanied by early and enhanced Th1 responses in the lung. *Eur J Immunol.* 2010;40(8):2200-2210.

383. Cruz A, Fraga AG, Fountain JJ, et al. Pathological role of interleukin 17 in mice subjected to repeated BCG vaccination after infection with Mycobacterium tuberculosis. *J Exp Med.* 2010;207(8):1609-1616.

384. Khader SA, Partida-Sanchez S, Bell G, et al. Interleukin 12p40 is required for dendritic cell migration and T cell priming after Mycobacterium tuberculosis infection. *J Exp Med.* 2006;203(7):1805-1815.

385. Robinson RT, Khader SA, Martino CA, et al. Mycobacterium tuberculosis infection induces il12rb1 splicing to generate a novel IL-12Rbeta1 isoform that enhances DC migration. *J Exp Med.* 2010;207(3):591-605.

386. Kahnert A, Hopken UE, Stein M, Bandermann S, Lipp M, Kaufmann SH. Mycobacterium tuberculosis triggers formation of lymphoid structure in murine lungs. *J Infect Dis.* 2007;195(1):46-54.

387. Khader SA, Rangel-Moreno J, Fountain JJ, et al. In a murine tuberculosis model, the absence of homeostatic chemokines delays granuloma formation and protective immunity. *J Immunol.* 2009;183(12): 8004-8014.

388. Day TA, Koch M, Nouailles G, et al. Secondary lymphoid organs are dispensable for the development of T-cell-mediated immunity during tuberculosis. *Eur J Immunol.* 2010;40(6):1663-1673.

389. Ulrichs T, Kosmiadi GA, Trusov V, et al. Human tuberculous granulomas induce peripheral lymphoid follicle-like structures to orchestrate local host defence in the lung. *J Pathol.* 2004;204(2):217-228.

390. Rangel-Moreno J, Carragher DM, de la Luz Garcia-Hernandez M, et al. The development of inducible bronchus-associated lymphoid tissue depends on IL-17. *Nat Immunol.* 2011;12(7):639-646.

391. Enright FM, Araya LN, Elzer PH, Rowe GE, Winter AJ. Comparative histopathology in BALB/c mice infected with virulent and attenuated strains of Brucella abortus. *Vet Immunol Immunopathol.* 1990;26(2):171-182.

392. Martirosyan A, Moreno E, Gorvel JP. An evolutionary strategy for a stealthy intracellular Brucella pathogen. *Immunol Rev.* 2011;240(1): 211-234.

393. Zumla A, James DG. Granulomatous infections: etiology and classification. *Clin Infect Dis.* 1996;23(1):146-158.

394. Dougan G, John V, Palmer S, Mastroeni P. Immunity to salmonellosis. *Immunol Rev.* 2011;240(1):196-210.

395. Ghon A. *Der primäre Lungenherd bei der Tuberkulose der Kinder (The primary*

lung lesion of childhood tuberculosis). Berlin & Wien: Urban & Schwarzenberg; 1912.

396. Chen M, Divangahi M, Gan H, et al. Lipid mediators in innate immunity against tuberculosis: opposing roles of PGE2 and LXA4 in the induction of macrophage death. *J Exp Med.* 2008;205(12):2791-2801.

397. Divangahi M, Chen M, Gan H, et al. Mycobacterium tuberculosis evades macrophage defenses by inhibiting plasma membrane repair. *Nat Immunol.* 2009;10(8):899-906.

398. Rivera-Marrero CA, Stewart J, Shafer WM, Roman J. The downregulation of cathepsin G in THP-1 monocytes after infection with Mycobacterium tuberculosis is associated with increased intracellular survival of bacilli. *Infect Immun.* 2004;72(10):5712-5721.

399. Liu PT, Stenger S, Li H, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science.* 2006;311(5768): 1770-1773.

400. Kaplan G, Post FA, Moreira AL, et al. Mycobacterium tuberculosis growth at the cavity surface: a microenvironment with failed immunity. *Infect Immun.* 2003;71(12):7099-7108.

401. Ulrichs T, Kosmiadi GA, Jörg S, et al. Differential organization of the local immune response in patients with active cavitary tuberculosis or with nonprogressive tuberculoma. *J Infect Dis.* 2005;192(1):89-97.

402. Egen JG, Rothfuchs AG, Feng CG, Winter N, Sher A, Germain RN. Macrophage and T cell dynamics during the development and disintegration of mycobacterial granulomas. *Immunity.* 2008;28(2):271-284.

403. Egen JG, Rothfuchs AG, Feng CG, Horwitz MA, Sher A, Germain RN. Intravital imaging reveals limited antigen presentation and T cell effector function in mycobacterial granulomas. *Immunity.* 2011;34(5):807-819.

404. Davis JM, Ramakrishnan L. The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell.* 2009;136(1):37-49.

405. Ly LH, Barhoumi R, Cho SH, Franzblau SG, McMurray DN. Vaccination with Bacille-Calmette Guérin promotes mycobacterial control in guinea pig macrophages infected in vivo. *J Infect Dis*. 2008; 198(5):768-771.

406. Kjellsson MC, Via LE, Goh A, et al. Penetration of anti-tuberculosis agents in rabbit pulmonary lesions: a pharmacokinetic evaluation. *Antimicrob Agents Chemother.* 2012;56(1):446-457.

407. Lin PL, Rodgers M, Smith L, et al. Quantitative comparison of active and latent tuberculosis in the cynomolgus macaque model. *Infect Immun.* 2009;77(10):4631-4642.

408. Mehra S, Pahar B, Dutta NK, et al. Transcriptional reprogramming in nonhuman

primate (rhesus macaque) tuberculosis granulomas. *PLoS One.* 2010;5(8):e12266.

409. Grosset J. Mycobacterium tuberculosis in the extracellular compartment: an underestimated adversary. *Antimicrob Agents Chemother.* 2003;47(3):833-836.

410. Reece ST, Loddenkemper C, Askew DJ, et al. Serine protease activity contributes to control of Mycobacterium tuberculosis in hypoxic lung granulomas in mice. *J Clin Invest.* 2010;120(9):3365-3376.

411. Pan H, Yan BS, Rojas M, et al. Ipr1 gene mediates innate immunity to tuberculosis. *Nature.* 2005;434(7034):767-772.

412. Elkington P, Shiomi T, Breen R, et al. MMP-1 drives immunopathology in human tuberculosis and transgenic mice. *J Clin Invest.* 2011;121(5):1827-1833.

413. Kim MJ, Wainwright HC, Locketz M, et al. Caseation of human tuberculosis granulomas correlates with elevated host lipid metabolism. *EMBO Mol Med.* 2010;2(7):258-274.

414. Peyron P, Vaubourgeix J, Poquet Y, et al. Foamy macrophages from tuberculous patients' granulomas constitute a nutrient-rich reservoir for M. tuberculosis persistence 2987. *PLoS Pathog.* 2008;4(11):e1000204.

415. Garton NJ, Waddell SJ, Sherratt AL, et al. Cytological and transcript analyses reveal fat and lazy persister-like bacilli in tuberculous sputum. *PLoS Med.* 2008;5(4):e75.

416. Alcais A, Abel L, Casanova JL. Human genetics of infectious diseases: between proof of principle and paradigm. *J Clin Invest.* 2009;119(9): 2506-2514.

417. Casanova JL, Abel L. Human genetics of infectious diseases: a unified theory. *EMBO J.* 2007;26(4):915-922.

418. Vannberg FO, Chapman SJ, Hill AV. Human genetic susceptibility to intracellular pathogens. *Immunol Rev.* 2011;240(1):105-116.

419. Hill AV. Aspects of genetic susceptibility to human infectious diseases. *Annu Rev Genet.* 2006;40:469-486.

420. Bellamy R. Genome-wide approaches to identifying genetic factors in host susceptibility to tuberculosis. *Microbes Infect.* 2006;8(4):1119-1123.

421. Reiter HE. *Die Säuglingstuberkulose in Lübeck.* Berlin, Germany: Julius Springer; 1935.

422. Stead WW, Senner JW, Reddick WT, Lofgren JP. Racial differences in susceptibility to infection by Mycobacterium tuberculosis. *N Engl J Med.* 1990;322(7):422-427.

423. Fortin A, Abel L, Casanova JL, Gros P. Host genetics of mycobacterial diseases in mice and men: forward genetic studies of BCG-osis and tuberculosis. *Annu Rev*

Genomics Hum Genet. 2007;8:163-192.

424. Burgner D, Jamieson SE, Blackwell JM. Genetic susceptibility to infectious diseases: big is beautiful, but will bigger be even better? *Lancet Infect Dis.* 2006;6(10):653-663.

425. Jouanguy E, Lamhamedi-Cherradi S, Lammas D, et al. A human IFNGR1 small deletion hotspot associated with dominant susceptibility to mycobacterial infection. *Nat Genet.* 1999;21(4):370-378.

426. Jouanguy E, Lamhamedi-Cherradi S, Altare F, et al. Partial interferon- gamma receptor 1 deficiency in a child with tuberculoid bacillus Calmette-Guérin infection and a sibling with clinical tuberculosis. *J Clin Invest.* 1997;100(11):2658-2664.

427. Dorman SE, Picard C, Lammas D, et al. Clinical features of dominant and recessive interferon gamma receptor 1 deficiencies. *Lancet.* 2004;364(9451):2113-2121.

428. Doffinger R, Jouanguy E, Dupuis S, et al. Partial interferon-gamma receptor signaling chain deficiency in a patient with bacille Calmette- Guérin and Mycobacterium abscessus infection. *J Infect Dis*. 2000; 181(1):379-384.

429. Dorman SE, Holland SM. Mutation in the signal-transducing chain of the interferon-gamma receptor and susceptibility to mycobacterial infection. *J Clin Invest.* 1998;101(11):2364-2369.

430. Dupuis S, Dargemont C, Fieschi C, et al. Impairment of mycobacterial but not viral immunity by a germline human STAT1 mutation. *Science.* 2001;293(5528):300-303.

431. Chapgier A, Boisson-Dupuis S, Jouanguy E, et al. Novel STAT1 alleles in otherwise healthy patients with mycobacterial disease. *PLoS Genet.* 2006;2(8):e131.

432. Altare F, Durandy A, Lammas D, et al. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science.* 1998;280(5368):1432-1435.

433. Altare F, Ensser A, Breiman A, et al. Interleukin-12 receptor beta1 deficiency in a patient with abdominal tuberculosis. *J Infect Dis.* 2001;184(2):231-236.

434. Hambleton S, Salem S, Bustamante J, et al. IRF8 mutations and human dendritic-cell immunodeficiency. *N Engl J Med.* 2011;365(2): 127-138.

435. MacLennan C, Fieschi C, Lammas DA, et al. Interleukin (IL)-12 and IL-23 are key cytokines for immunity against Salmonella in humans. *J Infect Dis.* 2004;190(10):1755-1757.

436. Gros P, Schurr E. Immunogenetics of the host immune response to bacteria in mice. In: Kaufmann SHE, Sher A, Ahmed R, eds. *Immunology of Infectious Diseases.* Washington, DC: ASM Press; 2002:407-420.

437. Vidal SM, Malo D, Vogan K, Skamene E, Gros P. Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. *Cell.* 1993;73(3):469-485.

438. Abel L, Casanova JL. Immunogenetics of the host response to bacteria and parasites in humans. In: Kaufmann SHE, Sher A, Ahmed R, eds. *Immunology of Infectious Diseases.* Washington, DC: ASM Press; 2002:395-406.

439. Abel L, Sanchez FO, Oberti J, et al. Susceptibility to leprosy is linked to the human NRAMP1 gene. *J Infect Dis.* 1998;177(1):133-145.

440. Turcotte K, Gauthier S, Tuite A, Mullick A, Malo D, Gros P. A mutation in the Icsbp1 gene causes susceptibility to infection and a chronic myeloid leukemia-like syndrome in BXH-2 mice. *J Exp Med.* 2005;201(6):881-890.

441. Mitsos LM, Cardon LR, Fortin A, et al. Genetic control of susceptibility to infection with Mycobacterium tuberculosis in mice. *Genes Immun.* 2000;1(8):467-477.

442. Turcotte K, Gauthier S, Mitsos LM, et al. Genetic control of myeloproliferation in BXH-2 mice. *Blood.* 2004;103(6):2343-2350.

443. Sanchez F, Radaeva TV, Nikonenko BV, et al. Multigenic control of disease severity after virulent Mycobacterium tuberculosis infection in mice. *Infect Immun.* 2003;71(1):126-131.

444. Lavebratt C, Apt AS, Nikonenko BV, Schalling M, Schurr E. Severity of tuberculosis in mice is linked to distal chromosome 3 and proximal chromosome 9. *J Infect Dis.* 1999;180(1):150-155.

445. Kramnik I, Dietrich WF, Demant P, Bloom BR. Genetic control of resistance to experimental infection with virulent Mycobacterium tuberculosis. *Proc Natl Acad Sci U S A.* 2000;97(15):8560-8565.

446. Turcotte K, Gauthier S, Malo D, Tam M, Stevenson MM, Gros P. Icsbp1/IRF-8 is required for innate and adaptive immune responses against intracellular pathogens. *J Immunol.* 2007;179(4):2467-2476.

447. Thye T, Vannberg FO, Wong SH, et al. Genome-wide association analyses identifies a susceptibility locus for tuberculosis on chromosome 18q11.2. *Nat Genet.* 2010;42(9):739-741.

448. Baghdadi JE, Orlova M, Alter A, et al. An autosomal dominant major gene confers predisposition to pulmonary tuberculosis in adults. *J Exp Med.* 2006;203(7):1679-1684.

449. Zhang FR, Huang W, Chen SM, et al. Genomewide association study of leprosy. *N Engl J Med.* 2009;361(27):2609-2618.

450. Wong SH, Gochhait S, Malhotra D, et al. Leprosy and the adaptation of human toll-like receptor 1. *PLoS Pathog.* 2010;6:e1000979.

451. Hawn TR, Verbon A, Lettinga KD, et al. A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to

legionnaires' disease. *J Exp Med.* 2003;198(10):1563-1572.

452. Dunstan SJ, Hawn TR, Hue NT, et al. Host susceptibility and clinical outcomes in toll-like receptor 5-deficient patients with typhoid fever in Vietnam. *J Infect Dis.* 2005;191(7):1068-1071.

453. Hawn TR, Verbon A, Janer M, Zhao LP, Beutler B, Aderem A. Tolllike receptor 4 polymorphisms are associated with resistance to Legionnaires' disease. *Proc Natl Acad Sci U S A.* 2005;102(7):2487-2489.

454. Kleinnijenhuis J, Oosting M, Joosten LA, Netea MG, Van CR. Innate immune recognition of Mycobacterium tuberculosis. *Clin Dev Immunol*. 2011;2011:405310.

455. Netea MG, van der Meer JW. Immunodeficiency and genetic defects of pattern-recognition receptors. *N Engl J Med.* 2011;364(1):60-70.

456. Hill AV. The genomics and genetics of human infectious disease susceptibility. *Annu Rev Genomics Hum Genet.* 2001;2:373-400.

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Chapter 41

Immunity to Extracellular Bacteria

Moon H. Nahm

Jannet Katz

INTRODUCTION

Human interactions with bacteria are complex, with a consortial relationship having been developed between humans and microbes. Each human being is composed of 10^{12} human cells and is inhabited by 10^{14} bacteria composed of innumerable species.[1] More than 500 distinct bacterial species are estimated to reside in the human oropharynx alone.[2] Recent studies of gut microbiota have begun to reveal both the extensive diversity of microbes in the gut as well as their complex relationship with the host.[3] Relatively few of these bacteria are harmful to us in any way. We know little about the innate or acquired immune mechanisms that maintain this equilibrium. In large part, many diseases caused by bacteria are mistakes in which this consortial relationship breaks down and the lines that define the relationship are crossed. The innate and adaptive responses to these transgressions can in themselves lead to dire consequences. In *Lives of the Cell* , Lewis Thomas points out:

The microorganisms that seem to have it in for us in the worst way—the ones that really appear to wish us ill—turn out on close examination to be rather more like bystanders, strays, strangers in from the cold. They will invade and replicate if they get the chance, and some of them will get into our deepest tissues and set forth in the blood, but it is our response to their presence that makes the disease. Our arsenals for fighting off bacteria are so powerful, and involve so many different defense mechanisms, that we are in more danger from them than from the invaders. We live in the midst of explosive devices; we are mined.[4]

While certain bacterial species are classified as pathogens, they can live in harmony on our surfaces for long periods and never cause disease. The bacteria that can cause human disease are quite diverse. Based on the pathogenesis of infection and the resulting immune response, these bacteria can be categorized into two general types: those causing intracellular infections and those causing extracellular infections. Most bacteria causing intracellular infections avoid being killed after phagocytosis by either interfering with phagosome-lysosome fusion or by escaping from the phagosome and into the cytoplasm. Cellular immunity is critical against bacteria that reside mainly within an intracellular milieu. In contrast, the bacteria causing extracellular infections survive in the host by avoiding engulfment by professional phagocytic cells such as neutrophils and macrophages. They do this by presenting a surface that minimizes the opsonic and lytic effects of antibody, complement, and other opsonins. Although extracellular bacteria have the ability to enter and

pass through cells as a means of moving from one in vivo environment to another, they are readily killed once captured by phagocytes. Accordingly, the host defense against extracellular bacteria is critically dependent on humoral immunity: complement and the production of specific antibody. Table 41.1 lists many of the important bacteria that can cause extracellular infections in humans, together with the diseases they cause and some of their major virulence factors. In this chapter, we describe the surface structures of many of these bacteria and provide examples of how they are able to infect their hosts and cause disease. We also describe the salient aspects of the innate immunity and antigen-induced immunity important in the host's defense against these bacteria.

SURFACE STRUCTURE OF GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

Differences among bacteria contribute to their specific adaptation to either a particular host species or to microenvironments within their host. In general, we currently have only a limited understanding of the limitations in nutrients and the specificity of enzymes and bacterial adhesins, or host receptors, that account for these highly specific microbial tropisms. The diversity of bacterial structures encountered by the host offers a major challenge to immune detection of potential pathogens. There are no structural features that reliably differentiate pathogens from nonpathogens. Moreover, many important extracellular pathogens exist mainly as commensals, only causing damage when the balance between host and microbe is perturbed (opportunistic pathogens). Thus, their diversity requires that initial recognition through innate immunity be focused on the more conserved structural features or “molecular patterns” of bacteria. These molecular patterns are, in general, structures such as peptidoglycan, lipoteichoic acid (LTA), and lipopolysaccharide (LPS) that are also essential for bacterial viability and are, thus, unlikely to be modified so as to evade innate immunity. A further consideration is that these structural differences between types of bacteria may dictate differences in host responses and contribute to distinct patterns of disease. That said, different types of bacteria may also give rise to remarkably similar host responses. The syndrome of sepsis, for instance, looks very similar regardless whether it is caused by a gram-positive or gram-negative organism, although there may be little overlap in the specific bacterial mediators involved.

TABLE 41.1 Extracellular Bacteria Commonly Associated With Diseases

Species	Disease	Important Virulence Structures/Molecules	Special Adaptations Critical to Host Infection
Neisseria gonorrhoeae	Urethritis, cervicitis, salpingitis, endometritis, prostatitis, arthritis, proctitis, pharyngitis	Lipopolysaccharide, fimbria, peptidoglycan, Opa protein adhesin, IgA1 protease	Phase and antigenic variation, molecular mimicry of human antigens

Neisseria meningitidis	Bacteremia, meningitis, septic arthritis	Capsule, lipopolysaccharide, fimbria, IgA1 protease	Phase and antigenic variation, molecular mimicry of human antigens, asymptomatic carriage common
Haemophilus influenzae	Otitis media, bronchitis, pneumonia, sepsis, meningitis (encapsulated strains)	Lipopolysaccharide with phosphorylcholine, fimbria, high-molecular-weight adhesions, IgA1 protease	Phase and antigenic variation, molecular mimicry of human antigens
Bordetella pertussis	Whooping cough in children, chronic cough syndrome in adults	Pertussis toxin, pertactin, filamentous hemagglutinin, fimbria	Coordinate regulation of multiple virulence factors upon exposure to the host environment
Pseudomonas aeruginosa	Infections in compromised hosts, pneumonia, sepsis	Lipopolysaccharide, proteases, lipases, lecithinases, exotoxin A, elastase, flagella	Relatively large genomic size (approximately six megabases) allows considerable adaptability to changes in environmental conditions, biofilms
Escherichia coli	Urinary tract infection, sepsis, traveler's diarrhea, dysentery, neonatal meningitis, hemolytic-uremic syndrome	Capsular polysaccharide, lipopolysaccharide, fimbria, toxins, siderophores	Antigenic heterogeneity of capsule and lipopolysaccharide
Vibrio cholerae	Diarrhea	Cholera toxin, fimbria	Bacterial dispersal via cholera toxin, which induces

			copious watery diarrhea
Helicobacter pylori	Peptic ulcer disease	Urease, flagella, CagA	Ability to survive at low pH provides a niche lacking bacterial competition or efficient immune surveillance
Streptococcus pneumoniae	Pneumonia, otitis media, meningitis, sinusitis	Capsule, PspA and C, pneumolysin, neuraminidase, hyaluronidase, teichoic acids with phosphorylcholine, IgA1 protease	Asymptomatic colonization, genetic transformation permitting continual generation of new genotypes
Streptococcus pyogenes (group A *Streptococcus*)	Acute pharyngitis, scarlet fever, necrotizing fasciitis, streptococcal toxic shock syndrome, rheumatic fever, and glomerulonephritis	Hyaluronic acid capsule, M-protein, streptococcal pyrogenic exotoxins, streptolysin O, streptolysin S, NAD-glycohydrolase, C5a peptidase	Molecular mimicry of human antigens, high diversity of M-proteins
Streptococcus agalactiae (Group B *Streptococcus*)	Bacteremia, neonatal pneumonia and meningitis	Capsule, beta hemolysin, hyaluronidase, C5a peptidase	Asymptomatic colonization, acquisition by infants during parturition
Staphylococcus aureus	Impetigo, folliculitis, boils, cellulitis, wound infections, toxic shock, osteomyelitis, endocarditis, bacteremia, pneumonia, food poisoning	Tissue-degrading enzymes, alpha toxin and other membranedamaging toxins, epidermolytic toxins, enterotoxins, capsule, protein A, TSST1, pigment	Resistant to dehydration, asymptomatic colonization
Bacillus	Cutaneous infection,	Capsule, lethal and	Opportunistic

anthracis	inhalation anthrax	edema factors	infection by a spore-forming soil organism
Corynebacterium diphtheria	Diphtheria (pharyngitis/tonsillitis)	Diphtheria toxin	Toxin gene contained intemperate phage and expression regulated by iron concentration
Clostridium tetani	Tetanus (spastic paralysis)	Tetanus toxin (blocks inhibitory neurotransmitters)	Opportunistic infection by a spore-forming soil anaerobe
Clostridium perfringens	Gas gangrene, anaerobic cellulitis, endometritis, food poisoning	More than 10 exotoxins	Opportunistic infection of wounds
Clostridium botulinum	Flaccid paralysis: cutaneous, infant, and ingestion forms	Botulism toxin blocks acetylcholine release at synapses	Opportunistic infection by a spore-forming anaerobe

CagA, cytotoxin-associated gene A, Ig, immunoglobulin; Psp, pneumococcal surface protein; TSST1, toxic shock syndrome toxin 1.

Most extracellular, as well as intracellular, pathogenic bacteria can be divided into two major groups (gram-negative and gram-positive) based on their response to staining with Gram stain. To illustrate the surface of the bacteria in the two groups, the surface structures of *Streptococcus pneumoniae* (Panel A) and *Neisseria meningitidis* (Panel B) are shown in Figure 41.1. Three layers are commonly recognized: cytoplasmic membrane, cell wall, and outer layer. Although these layers are described in detail in the following, it is important to note that these definitions are operational and that, in reality, the layers are not entirely distinct. For instance, molecules anchored in the cytoplasmic membrane or cell wall may extend into or through other layers. It is also important to note that the capsule, O antigens, and cell wall are not contiguous shields; rather they are permeable enough to allow through secreted products and nutrients as well as some immunologic factors (eg, antibodies and complement).

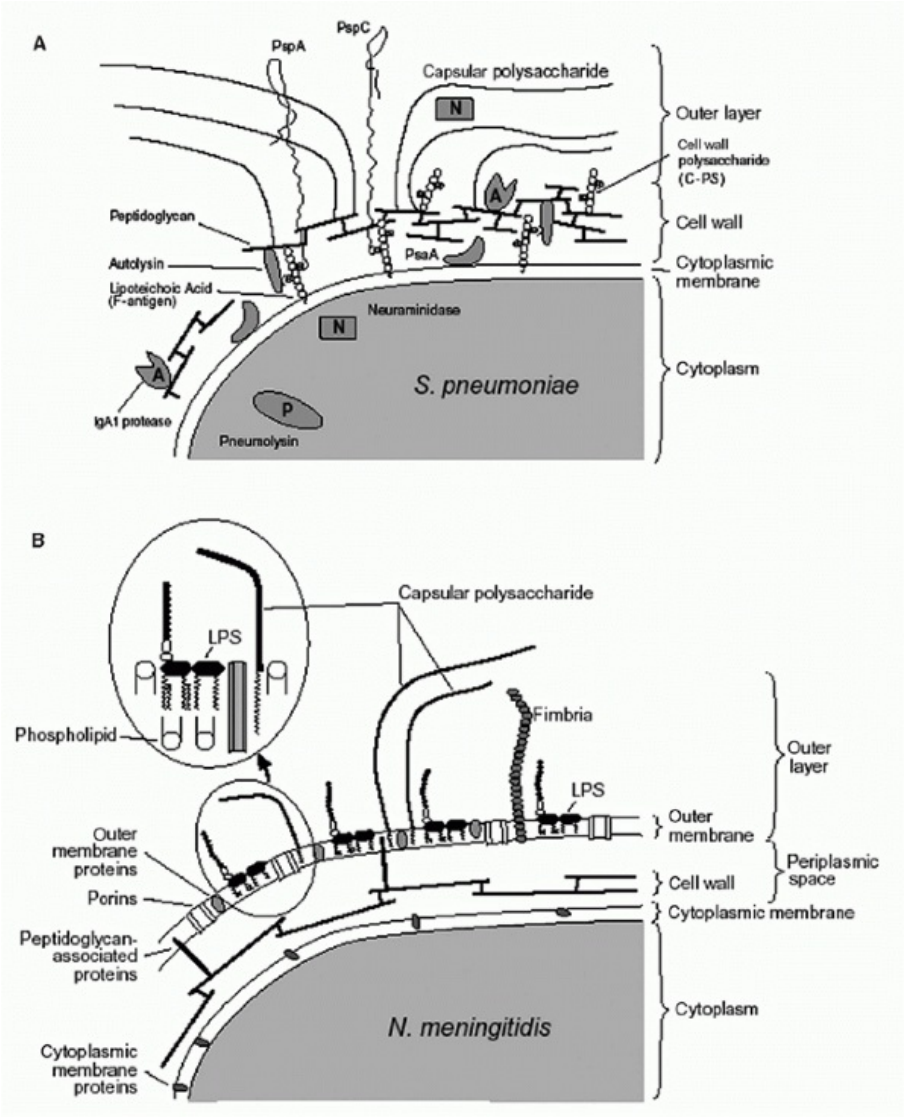

FIG. 41.1. Schematic representation of the surfaces of *Streptococcus pneumoniae* **(A)** and *Neisseria meningitidis* **(B)** as examples of gram-positive and gram-negative bacteria, respectively. The cell wall polysaccharide of *S. pneumoniae* is often called C-polysaccharide. The inset in *B* shows lipopolysaccharide anchored to the outer leaflet of the outer membrane.

All bacteria have a cytoplasmic membrane, a non-sterol-containing phospholipid bilayer. This membrane is an osmotic barrier and also forms a barrier for most molecules. The cytoplasmic membrane has various proteins, many of which function in transport. Some of these proteins, referred to as lipoproteins (eg, pneumococcal surface adhesion A, which is a manganese permease in *S. pneumoniae*), are noncovalently anchored to the membrane through

lipid modifications and are especially common among some bacteria (eg, *Borrelia burgdorferi* and *Mycobacterium tuberculosis*). Proteins not exposed on the surface generally display a greater degree of structural and functional conservation compared with those exposed to the selective pressure of host immunity.

A cell wall is found in all of the pathogenic bacteria of both groups, with the exception of

mollicutes (which include the genus *Mycoplasma*). The cell wall surrounds the cytoplasmic membrane and is made of peptidoglycan, which is a polymer of alternating sugars N-acetylglucosamine and N-acetylmuramic acid, the latter being connected to a stem peptide. The stem peptides include four to five D- and L-amino acids that are extensively cross-linked by bridges that provide rigidity to the cell wall and protect it from environmental extremes (especially differences in osmolarity). These cell wall peptides include atypical amino acids such as diamino pimelic acids, which are the anchoring site for Braun lipoprotein of gram-negative bacteria and are found in most gram-negative bacteria but in few gram-positive bacterial species. Peptidoglycan polymerization is carried out by enzymes, many of which are the target of β-lactam antibiotics and are referred to as penicillinbinding proteins. Compared with gram-negative bacteria, gram-positive organisms may have different stem peptides and cross-linking as well as a thicker (20 to 30 nm compared with 2 to 4 nm) cell wall layer that can retain the Gram stain better. The thick cell wall of the gram-positive bacteria may be responsible for their greater resistance to complementmediated lysis. Other features of the cell wall such as the O-acetylation of N-acetylmuramic acid or the deacetylation of N-acetylglucosamine[5,6] found in some species mediate resistance to lysozyme, an enzyme that lyses bacteria by cleaving the peptidoglycan backbone.[6]

In addition to peptidoglycan, many gram-positive bacteria have polysaccharide (PS) associated with their cell walls, with this cell wall PS often extending into the outer layer. The structure of the cell wall PS of gram-positive bacteria varies between species but is relatively invariant within a species. Differences in the antigenicity of the cell wall PS have been used to distinguish species (eg, separate streptococci into groups A, B, C, etc.).[7,8] Cell wall PS often has phosphate group(s) in repeating units of glycerol or ribitol in a structure known as teichoic acid. Teichoic acid may also be linked to lipid molecules and is then called LTA, which is anchored to the cytoplasmic membrane and extends out through the cell wall.[9] In pneumococci, the overall PS structures of LTA and cell wall teichoic acid (also referred to as C-polysaccharide) are very similar, with the difference being their mode of attachment to the bacterial surface.[10,11]

Another major difference between the surface structures of gram-negative and gram-positive bacteria is the presence of an outer membrane on gram-negative bacteria. The outer membrane contains many proteins, including channel-forming porins. The outer membrane is an asymmetrical lipid bilayer. The inner leaflet is comprised primarily of phospholipids while the outer leaflet contains lipid A, the hydrophobic component of LPS. LPS, also called endotoxin, is an amphipathic glycolipid with four distinct regions: lipid A, the inner core, the outer core, and, in some species, the O antigen. Lipid A is composed of a dihexosamine backbone to which between five and seven saturated (12- to 16-carbon) fatty acids are attached through amide and ester linkages. Lipid A is the principal “toxin” associated with most gramnegative bacteria, although it is now clear that lipid A is not a true toxin. Rather, its ability to induce cytokines accounts for its potentially detrimental effects. The carbohydrate portion of the LPS, which makes a minimal contribution to its endotoxin activity, is attached to lipid A through a molecule unique to gram-negative bacteria called ketodeoxyoctanoate. Together with heptose moieties, this molecule forms the inner core of the LPS.

The outer core is composed of 7 to 10 monosaccharide units whose arrangement is relatively conserved among gram-negative species.[12] In many gram-negative bacteria, the outer core of LPS is connected to a repeating series of carbohydrates called the O antigen. The O

antigen forms a hydrophilic shield around the bacterium that provides a barrier to complement deposition on the bacterial cell surface. The O antigen is variable in length, is antigenically diverse, and confers serotypic specificity. The O antigens of *Escherichia coli*, *Klebsiella*, and *Salmonella* have as many as 30 repeating units composed of four to six sugars each.[12] Members of the genera *Neisseria* and *Haemophilus*, on the other hand, lack LPS with O antigens but instead have lipooligosaccharides, which have short oligosaccharides that do not exceed 7000 daltons.

For many pathogenic extracellular bacteria, PS components dominate the outer layer. In addition to the PS on LPS (gram-negative) and teichoic acid (gram-positive), there is often another thick layer of carbohydrate referred to as "capsule" that may account for more than half of the bacterial mass. An exception to this general rule is *Bacillus anthracis*, whose capsule is made of poly-D-glutamic acid rather than a polysaccharide.[13] *S. pneumoniae* has capsular PS that is covalently attached to the cell wall in most (but not all) serotypes.[14] In contrast, the capsule PS is anchored to the outer membrane by acyl chains in *N. meningitidis*[15] and *Haemophilus influenzae* type b.[16] Capsular PSs may be highly diverse both within and between species. In the case of *S. pneumoniae*, each strain expresses a single type of capsular PS, with members of this species being capable of synthesizing more than 90 structurally distinct types.[17,18] This diversity limits immune recognition until antibody is generated to the capsular PS of the infecting strain (antigenic variation).

The outer layer is well developed in bacteria that cause extracellular infections and has many features that help the bacteria circumvent the host immune system. First, the outer layer has properties that reduce the attachment of extracellular bacteria to eukaryotic surfaces, including those of phagocytes. Generally, the PS capsules render the bacteria hydrophilic and negatively charged like eukaryotic cell surfaces, which are rich in sialic acid. By enhancing the degradation of C3b, the negatively charged surface makes the bacteria partly resistant to the deposition of complement by the alternative pathway.[19] Second, in some cases, elicitation of antibody is minimized because the capsular PS or

LPS mimics host antigens, as is more fully described in the section on Antigen-Specific Host Defense Response of this chapter. Third, by physically masking most of the other bacterial surface components, the outer layer minimizes the number of exposed epitopes that can be recognized by the antibody and complement. Although the capsule is porous to antibodies and complement, the binding of antibodies and fixing of complement beneath the capsule surface are relatively ineffective in promoting opsonophagocytosis and clearance.[20]

BACTERIAL VIRULENCE FACTORS

Extracellular bacteria often elaborate molecules called "virulence factors" that are useful to their survival and proliferation in the host. For example, the shielding function of the outer layer is further augmented by the presence of surface proteins that can interfere with host clearance mechanisms. Proteins inhibiting effective complement deposition include M-protein in *Streptococcus pyogenes*,[21] pneumococcal surface protein A (PspA), and pneumococcal surface protein C, which is alternatively called choline binding protein A or the C3-binding protein in *S. pneumonia.*[22,23] An example of a protein that interferes with antibody is protein A, which is expressed on the surface of *Staphylococcus aureus* and binds immunoglobulin

(Ig) in a manner that precludes recognition of its target antigen.[24] In addition, many successful mucosal pathogens, including members of the *Neisseria*, *Haemophilus*, and *Streptococcus* genera, express proteases with specificity for the hinge region of human IgA1.[25] These IgA1 proteases remove the Fc_{α} component required to promote the inflammatory process, leaving the organisms' antigens obscured by the binding of inert Fab_{α} fragments. By inhibiting the deposition of complement or antibody, many of these proteins act to diminish phagocytosis.

The best-known virulence factors are toxins, which interrupt specific host functions. These proteinaceous molecules (also referred to as exotoxins to differentiate them from endotoxin) can be grouped on the basis of their molecular structure and their mechanism of action.[26] The largest group are called A-B toxins, which are comprised of two subunits, each with a different function. The A subunit has enzymatic activity, and the B subunit targets the A subunit to the host cells. This group includes diphtheria toxin, cholera toxin, pertussis toxin, and two anthrax toxins (lethal factor and edema factor). For instance, the lethal factor of *B. anthracis* behaves as the A subunit and requires a B subunit protein named “protective antigen” to enter into target cells. In some cases, the toxin alone is sufficient to account for the detrimental symptoms of its respective infection. Cholera toxin causes ADP ribosylation of G proteins, which stimulates adenylate cyclase and increases cyclic adenosine monophosphate in cells lining the gut. This results in the secretion of electrolytes and is responsible for a severe diarrhea, which promotes transmission of *Vibrio cholerae* but often also causes dehydration that, if not treated, may be fatal. Uptake of botulism toxin by nerve endings leads to retrograde transport that interrupts synaptic transmission and causes a flaccid paralysis.[27] Staphylococcal enterotoxin A (a toxin that acts from the gut lumen), which is one of five membrane-damaging toxins produced by staphylococci, is the primary cause of staphylococcal food poisoning and plays a major role in invasive infections.[28] Some strains of *E. coli* produce a protein synthesis-inhibiting verotoxin, which may damage the microvasculature of the kidney and cause hemolytic uremic syndrome.[29] Another group of proteins secreted by *S. aureus* and *S. pyogenes* have toxin-like effects but lack any enzymatic activity. These “superantigens” cause a nonclonal stimulation of T cells by bridging major histocompatibility complex (MHC) class II molecules (outside the antigen-binding site) on antigen-presenting cells with the Vβ region of the T-cell receptor on T cells. The ensuing massive release of cytokines by localized release of a superantigen, such as the toxic shock syndrome toxin 1 expressed by some strains of *S. aureus*, results in systemic symptoms that are collectively known as “toxic shock syndrome.”[30]

Another class of virulence factors neutralizes host defenses. As stated previously, many virulence factors interfere with complement deposition on bacteria. *S. pyogenes* and group B streptococci produce a C5a peptidase that inhibits the chemotaxic effects (recruitment of host phagocytes to the sites of infections) of C5a, a product of complement activation.[31] Pneumolysin produced by *S. pneumoniae* is a member of a large class of cholesterol-dependent cytotoxins that oligomerize to form large pores, which interfere with a number of host cell functions or induce cell death when present in higher concentrations.[32] Pneumolysin also depletes complement at a distance from the pneumococci and interferes with both the function of phagocytes and the development of protective immunity.[33] *Helicobacter pylori* produces urease, which can generate ammonia that can neutralize acid

in the stomach and thereby promotes the survival or the organism. *S. aureus* produces a pigment that makes the bacterium more resistant to oxidative stress and killing by neutrophils.[34]

While evasion of professional phagocytes is critical for extracellular pathogens, the ability to attach to other cell types, including both mucosal and nonmucosal surfaces, is important for their persistence. Many bacterial surface proteins have an adhesive function that confers a high affinity for binding to specific host cell receptors. Nasopharyngeal carriage of pneumococci is mediated largely by adherence to the host molecules N-acetylglucosamine β1→3 galactose or N-acetylglucosamine β1→4 galactose.[35] Bacteria often mimic host ligands in order to coopt host receptors for their own purposes. Many pathogens of the airway express phosphorylcholine (phosphocholine [PC]) on their surfaces.[36] This molecule, which is otherwise unusual in bacteria, is found on platelet-activating factor (PAF) and allows bacterial binding to its receptor (rPAF).[37] To facilitate their attachment to host cells, many bacteria use pili (fimbriae), long filamentous structures extending from the organism. The Pap pilus of *E. coli* binds the galactose α1→4 galactose unit of cell surface globoside in urethral epithelial cells.[38] The *V. cholerae* pilus allows that bacterium to attach to the enterocyte for more efficient toxin delivery.[39,40] *Bordetella pertussis* has three adherence factors—a filamentous hemagglutinin,

pertactin, and a pilus—that allow it to attach to ciliated respiratory epithelial cells in the trachea and bronchi and thus resist the cleansing action of mucus flow.[41,42]

Another group of virulence factors helps bacteria acquire essential nutrients. Motile bacteria (eg, *Pseudomonas aeruginosa*) express flagellin, a complex motor apparatus that allows the bacterial cell to transit along a concentration gradient of nutrients.[43] While in some cases the host and microbe provide nutrients for one another, for other nutrients there is fierce competition. Mucosal fluid and blood are low in free ferric iron due to the presence of iron-binding proteins such as lactoferrin and transferrin. To successfully compete with the host for this vital metabolite, *N. meningitidis*, *Neisseria gonorrhoeae*, and *H. influenzae* have complex surface transport systems that can obtain iron from human transferrin, lactoferrin, and hemoglobin.[44] Other bacteria such as *E. coli* and salmonella use a different mechanism to acquire iron; they secrete small, high-affinity iron chelators, called siderophores, that remove iron from human proteins in the environment surrounding the bacteria. The iron-siderophore complex is then taken up by the bacterium, which then degrades the siderophore so that the iron can be freed for its use.[45]

Production of virulence factors is often highly regulated by bacteria in order to adapt to different environments, such as the natural environment outside of a host, the mucosa of a host, or more invasive sites within a host. For staphylococci, it has been shown that the amount of capsule is regulated in response to environmental stimuli.[46] One of the best studied of such regulatory systems is the BvgAS, a two-component regulatory system in *B. pertussis*.[47] This system, which regulates the expression of adhesins, toxins, and other virulence factors, is controlled by external signals including Mg^{2+}, temperature, and nicotinic acid. Two proteins are involved in this regulatory system: BvgS and BvgA. BvgS, the sensor, is a kinase and is able to autophosphorylate itself in response to the environmental signal.

BvgA, the response regulator, is in turn phosphorylated by BvgS. Phosphorylated BvgA is able to activate transcription of virulence genes through a change in its interaction with a 70-bp consensus sequence repeated in bvg-regulated promoters.[47] Analogous two-component regulatory systems in other pathogens are frequently used to regulate the expression of genes associated with virulence.[48]

Another strategy used by extracellular pathogens depends on selection among a heterogeneous population for those members with permissive characteristics. This heterogeneity in a population is commonly generated through genomic rearrangements, such as recombinational events or slipstranded mispairing in highly repetitive deoxyribonucleic acid (DNA) sequences.[49] This latter mechanism allows for reversible on-off switching (phase variation) and is especially prevalent in genes encoding cell surface structures subject to immune pressure. For instance, the capsular PS on *N. meningitidis* is needed to protect the organism during invasive infection but inhibits adherence on the mucosal surface where complement is less abundant. Phase variation of a gene required for capsule synthesis allows for selection of organisms without capsule (phase-off).[50] This change facilitates the bacterial adhesion to the epithelial cells, perhaps by exposing the bacterial adhesins. Alternatively, by decreasing capsule production, the bacteria become less hydrophilic and less negatively charged. This change facilitates their entry into the epithelial cells and their subsequent invasion into deeper tissues. Upon the emergence of the bacteria from the epithelial cells into the submucosa, capsule synthesis is restored (phase-on) because of the selective pressure of complementmediated clearance and the requirement for the capsule to survive where the concentration of complement is higher. The flexibility to express different surface properties helps bacteria successfully evade the host immune system and survive in many niches within the host.

Bacteria-to-bacteria signaling is another important mechanism for the control of virulence factors. This phenomenon, called “quorum sensing,”[51] has been shown to be operative in a large number of gram-negative and grampositive species. The signal transmitted between the bacteria can be an acylated lipid (eg, homoserine lactone) in gramnegative bacteria (eg, vibrios) or a peptide in gram-positive bacteria (eg, *S. aureus*). Quorum sensing has been shown to be important in biofilm formation in a number of bacterial species, in the expression of “competence” for the uptake and incorporation of exogenous DNA (transformation), and in the regulation of a number of virulence factors.[52] Biofilms are communities of one or multiple bacterial species that adhere both to each other and to a target surface. Bacteria in biofims are particularly resistant to many host clearance mechanisms and to antibiotics that are effective against free-living (planktonic) bacteria. Biofilms, therefore, are often a contributing factor in more chronic bacterial infections such as those involving foreign bodies or chronic otitis media.[53]

An important characteristic of virulence factors is their structural polymorphism. For instance, there are at least 100 different serologic types of M proteins of *S. pyogenes*.[54] Similarly, pneumococci have more than 90 serologically distinct capsular PSs.[17,18] The polymorphism in the structure of many virulence factors allows the bacteria making them to avoid antigen-specific host immunity. For example, antibodies to the immunodominant region on one serotype of M protein do not cross-react with M proteins of other serotypes and thus do not provide protection against strains expressing other serotypes. Similarly, newly acquired

pneumococci can escape recognition by anticapsular antibodies produced in response to previous pneumococcal infections with other serotypes.

The polymorphism in virulence factors is achieved by various genetic mechanisms. Variation in M proteins is the result of sequence differences in the N-terminal (but not C-terminal) half of M proteins.[55] *S. pneumoniae* has the genes for synthesizing capsular PS as a "genetic cassette" that can be exchanged among different strains[56] and may result in the shift in the serotype distribution following the use of vaccines designed to elicit serotype-specific protection.[57,58] *Neisseria* has genetic machinery for rapid gene rearrangement[59] through gene conversion using the multiple "silent" pili genes with different sequences. This process, similar to gene rearrangements that generate specific Ig, permits an individual bacterium to quickly produce progeny expressing pili with different characteristics. The number of potential

pilus-antigen variants within the progeny of a single organism is estimated to be greater than 100,000.[60] In addition, *Neisseria* contain large numbers of genes with tandem repeats that undergo phase variation through slip-stranded mispairing of these sequences. Based on predictions from whole genome sequencing, through this mechanism alone the organism may be able to generate more than 2^{65} different phenotypic variants.[61]

Sequencing of the entire genomes of bacteria has shown that the genes for virulence factors have generally originated from other organisms and exist as a part of large blocks of DNA containing multiple genes. These DNA blocks are called "pathogenicity islands" (PAIs). For instance, strains of "enteropathogenic" *E. coli* contain a PAI encompassing about 41 genes encoding a surface ligand required for intimate association of the bacterial and host cells and for a bacterial secretion apparatus.[62] This system (type IV secretion system) allows for delivery of the receptor for its own adhesin, encoded on the same PAI, into host cells. Elaborate secretion mechanisms (types III and IV) and pore-forming toxins are now known to be mechanisms whereby extracellular organisms gain access to the host cell cytoplasm to modify its activity to suit their needs. Another example is *H. pylori*, which injects cytotoxin-associated gene A (CagA) molecules directly into host cells using a syringe-like type IV secretory apparatus. CagA is then phosphorylated by the host cells, and the phosphorylated CagA alters host cell function, with the *H. pylori* strains producing the CagA molecules being more likely to cause ulcers.[63] In the case of *S. pyogenes*, its pore-forming toxin, streptolysin O, allows for translocation of an enzyme (NAD-glycohydrolase) that is capable of producing the potent cytoplasmic second messenger, cyclic ADP-ribose.[64]

BACTERIAL INVASION OF THE HOST

Both keratinized skin and mucosal surfaces have inherent nonimmune defense mechanisms that modulate bacterial growth and minimize the risk of invasion. Healthy human skin is an effective physical barrier to infection by most human extracellular and intracellular pathogens. The keratinization of fully differentiated skin epithelium results in a relatively impermeable surface. In addition, lysozymes, toxic lipids, and hydrogen ions secreted by cutaneous glands offer bacteriostatic protection for cutaneous pores and hair follicles. Occasionally, this defense can be breached by extracellular bacteria such as *S. pyogenes* or *S. aureus*, causing cellulitis and abscess. More commonly, bacterial invasion through intact skin requires physical damage, such as abrasions, burns, or other trauma. For instance, cutaneous anthrax

develops when *B. anthracis* enters the body through a break in the skin. *Staphylococcus epidermidis*, a member of the commensal skin flora, can infect indwelling catheters by invading through the puncture site in the skin and may lead to bacteremia or colonization of prosthetic devices, including artificial heart valves and shunts. A major factor allowing these bacteria to cause disease is their ability to form a biofilm, which facilitates their adhesion, is antiphagocytic, and acts as a barrier to antibiotic penetration.[65]

Unlike the skin, the mucosal epithelium is not keratinized. Instead, mucosal areas, such as the gastrointestinal tract, nasopharynx, upper airway, and vagina, are moist and nutritionally rich. Thus, it is not surprising that mucosal areas contain a large number of bacteria. In oral secretions and gastrointestinal products, 10^8 and 10^{11} bacteria/mL may be found, respectively. To ensure their survival in the mucosal environment, extracellular bacteria elaborate many virulence factors required for the acquisition of essential nutrients or for their adherence to the host cells. In some cases, bacteria may subvert the host inflammatory response. Salmonella species can block the activation of NF-κB and the subsequent activation of the inflammatory response. They achieve this by preventing the degradation of IκB, which is essential for the translocation of NF-κB from the cytoplasm to the nucleus.[66] In some cases, pathogens locate in a less well-protected microenvironment within the mucosal areas. *H. pylori* survives in the very acidic stomach by burying itself in the mucus, which protects it from direct exposure to the acid and from phagocytes. There are also few other species for *H. pylori* to compete with in this more hostile environment.

Mucosal sites play host to an especially diverse array of bacterial species. Most of the bacteria species found at mucosal sites are harmless. In addition, polymerase chain reaction analysis of 16S ribosomal ribonucleic acid sequences suggests the presence of many additional unidentified (and so far unculturable) bacterial species on the mucosal surface.[67] Many potentially pathogenic bacterial strains are also often found in the mucosal areas of healthy individuals without causing symptoms. *S. pneumoniae*, *N. meningitidis*, *H. influenzae*, and *S. aureus* are examples of pathogenic extracellular bacteria that are frequently carried in the nasopharynx of healthy individuals. The carriage rate of pathogenic bacteria can be relatively high; for example, 50% to 60% of healthy young children may carry *S. pneumoniae* in their throats.[68]

Maintenance of species diversity in mucosa is dynamic. In some situations, collaboration among bacteria is essential for their successful colonization, as seen among the complex hierarchical communities adhering to tooth surfaces. In other situations, bacterial species compete and regulate diversity among themselves.[36] Many bacterial species produce molecules that suppress the growth of other bacterial species. These molecules may include bacteriocins, small molecules that target members of the same or different species that do not express the same bacteriocins.[69] Some species can take advantage of host responses to which they are resistant to outcompete another member of the same niche that is less resistant.[36] The host may also control the diversity of colonizing bacteria by modifying the pH or other environmental conditions in the mucosal area. Interference with these homeostatic mechanisms, as occurs with antibiotic therapy, may alter the flora and predispose the host to disease. As noted previously, stomach acid is an effective barrier to reaching the nutrient-rich environment of the gut. When stomach acid is pharmacologically reduced, the inoculum of organisms like *V. cholerae* required to infect the intestines is greatly diminished.

Several explanations have been advanced to explain why many pathogens that colonize mucosal sites do not cause disease. One explanation is that the maintenance of diverse bacterial population is responsible for the prevention of the disease. For instance, the destruction of the normal gastrointestinal bacterial flora with some antibiotics can be associated with the selective expansion of *Clostridium difficile* and the development of pseudomembranous colitis.[70] Another explanation may be that the pathogenic bacteria carried in healthy persons are different from those isolated in disease settings. For instance, during nonepidemic periods, approximately 5% to 10% of the population carries *N. meningitidis*, which are mostly nonencapsulated.[71] During epidemics, 30% to 60% of the population may carry meningococci, which are mostly encapsulated and the majority of which are of the same capsular type as the case strain causing the epidemic.[72] A third explanation is that the pathogenic bacteria are effectively confined to the mucosal surface where they do not cause damage or induce inflammation. Group B streptococci are carried asymptomatically in the lower intestine and the female genital tract. In the same host, in the setting of parturition, group B streptococci may access the bloodstream and cause septic infection. A fourth explanation is centered on the differences among hosts. Group B streptococci that colonize the mother may cause life-threatening infection when the same strain is passed to the neonate at or before birth.[73]

Although pathogenic extracellular bacteria can exist asymptomatically in the mucosa, they can passively enter into less-well-defended sites and cause focal infections. For instance, *E. coli*, normally present in the gut, may enter the normally sterile urogenital tract and cause urinary tract infections. *S. pneumoniae* and *H. influenzae* are often carried in the nasopharyngeal space, but they can invade nearby normally sterile cavities (eg, lungs, sinuses, and the middle ear) and cause focal infections. Aspiration of bacteria from the nasopharynx into the lungs most likely occurs frequently with no ill effects; however, aspiration may lead to an infection when there is damage to the epithelial surface, particularly when the protective effects of mucociliary activity are lost, as often occurs in a smoker or during recent viral infection (respiratory syncytial virus or influenza).[7] Some bacteria produce enzymes such as hyaluronidase,[74] which may aid in their passage through tissue barriers.

Bacteria can actively invade deeper tissues by multiple pathways. They can enter through specialized cells. Shigella, for example, can breach the gut mucosa by transcytosing through the M cells in the gut.[75] Alternatively, extracellular bacteria can breach a cellular barrier (epithelium or endothelium) by going through (transcytosis) or between (paracellular pathway) the cells.[75] *Porphyromonas gingivalis*, an organism associated with adult periodontitis, may breach the epithelial layer by the paracellular pathway through the production of enzymes useful in digesting the tight junction.[76] Two different mechanisms of transcytosis have been described for pneumococci. In one, pneumococci may cross the bronchial epithelial cells by binding the polymeric Ig receptor of the epithelial cells and traveling in a retrograde manner by the IgA secretory pathway.[77] In the other, pneumococci may use PC to bind to rPAF, which is abundant on activated endothelial cells, epithelial cells, or pneumocytes.[35,78] In many cases, bacterial adhesion triggers changes in the host cell function, and these changes can assist transcytosis. For instance, nontypeable *H. influenzae* with LPS glycoform-containing PC can bind to rPAF on endothelial cells and initiate signaling

through this receptor.[79]

ANTIGEN-NONSPECIFIC HOST DEFENSE RESPONSE

To protect from infections caused by highly adaptable bacteria, the host employs a multilayered defense. This includes the mechanical barriers and iron sequestration described previously as well as phagocytes, complement fixation, lysozyme, and (cytokine-induced) local inflammation. In addition, the host is protected with antigen-specific antibody (see section on Antigen-Specific Host Defense Response) and T-cell-mediated cellular immunity. Antigen-specific immunity, although exquisitely protective, takes several days to weeks to develop following exposure to a pathogen. As many extracellular pathogens are capable of causing overwhelming infection in periods of hours to days, other more rapidly acting forms of protection are needed. Consequently, the primary defense against bacteria during the early phase of infection remains antigen-nonspecific host immunity. The importance and significance of nonspecific immunity is readily demonstrated by the relative ease with which colonies of SCID mice, which lack antigen-specific immunity, can be maintained.[80] This section describes several antigennonspecific host defense mechanisms, but see the chapter on innate immunity for additional information.

Mucosal Defense

Although mucosal areas are rich with nutrients for bacteria, uncontrolled local proliferation of bacteria is held in check by mechanical cleansing actions and the lack of available iron. In the gastrointestinal tract, normal peristaltic motility, the secretion of mucus, and the detergent action of bile limit the number of bacteria. The normally sterile lower respiratory tract is protected by the movement of mucus by cilia lining the airway, which continually remove aspirated bacteria. Normal epithelial and tissue architecture are essential for drainage and expulsion of bacteria, and disruption of this mechanism by smoking, viral infections (eg, influenza), or bacterial infection (eg, pertussis) makes the host markedly susceptible to infection by bacteria that otherwise exist only as commensals of the upper airway. The increased frequency of lower respiratory tract infections in the elderly is due, in large part, to the loss of function of the mucociliary elevator and the increased aspiration from the upper respiratory tract of secretions containing bacteria.[81,82]

In addition to the removal of bacteria by mucus flow, mucosal fluid contains many antibacterial products such as lactoferrin, lactoperoxidase, mucin, lysozyme, and defensins.[83] Lactoferrin—found in various body fluids such as milk, saliva, and tears—binds iron and lowers the level

of available iron (especially in areas with a low pH).[84] Mucin traps microbes and facilitates their removal.[85] IgA antibodies in mucosal fluid may inhibit colonization by interfering with microbial adherence or by inactivating toxins.[86] In addition, the polymeric structure of secretory IgA promotes agglutination, which in turn facilitates removal by mucus. Lysozyme reduces the bacterial load by cleaving the 1→4 linkage between N-acetylmuramic acid and N-acetylglucosamine of peptidoglycan. A number of antimicrobial peptides, including defensins, disrupt bacterial membranes.[87] In the intestine, Paneth cells at the base of intestinal crypts produce defensins that are important in defense against intestinal

infections.[88] In the lungs, collectin-like surfactant proteins, such as SP-A and SP-D, may be important in host defense by opsonizing bacteria for alveolar macrophages.[89] SP-A-deficient mice, for example, are more susceptible to group B streptococcal infection of the lungs.[90] Epithelial cells that interface with the microbial world must exist in a quiescent state in response to colonizing organisms. If not, chronic inflammation may result in a disease (eg, chronic inflammatory bowel disease). When the epithelial barrier is breached, these cells are able to elaborate cytokines and chemokine as an early trigger to the inflammatory response.

Local Response to Bacterial Invasion (Acute Inflammation)

Upon entry into the host, many bacteria initiate local inflammatory processes by providing various inflammatory products such as peptidoglycan, LPS, LTA, exotoxins, lipoproteins, and glycolipids.[91] Antibiotics used for treatment may destroy bacteria and consequently release additional inflammatory products. These molecules are called pathogen-associated molecular patterns and trigger responses through their interaction with pathogen pattern recognition receptors (PRR), which often reside on the host cell membrane or in the cytoplasm.

The best-known PRRs residing on the cell membrane are the toll-like receptors (TLRs), which have a transmembrane region that separates the cytoplasmic signaling domain from the extracellular ligand binding domain. TLRs are generally most abundant on inflammatory cells but are present in lower levels in the epithelial barrier, which is continuously exposed to microbial products.[92] TLR2 detects lipoproteins and lipoteichoic acid and requires a binding partner (TLR1 or 6) to transmit signals leading to cytokine production.[93] Mice lacking the TLR2 gene are more susceptible to mucosal and systemic infection with staphylococci and streptococci.[94,95] LPS binds to TLR4 with the help of MD2, cluster of differentiation (CD)14, and lipid-binding proteins. Mice without functional TLR4 are unresponsive to LPS. Bacterial flagellin signals through TLR5.[96] Bacterial DNA, rich in unmethylated CpG motif, is a potent inducer of inflammation through its binding to TLR9. TLR9 receptors are found inside the cells as they occur in endocytic vesicle membranes and react to phagocytosed bacteria. TLR activation increases expression of inflammatory cytokines (eg, tumor necrosis factor [TNF]-α) through increased transcription of their genes. The protective effect of TLR can be seen early in an infection. For instance, TLR9 can protect against pneumonia caused by *S. pneumoniae* even before circulating inflammatory cells enter into the lungs.[97]

Bacterial invasion is also detected by various intracellular PRRs. The best-known PRR family may be the nucleotide oligomerization domain (NOD)-like receptors (NLRs). At least 23 NLR family members have been identified in humans, with each member having a central nucleotide-binding domain (NACHT domain), an N-terminal effector domain, and a C-terminal receptor domain with leucine-rich repeats. Ligands may bind the leucine-rich repeat domain and initiate oligomerization of the NATCH domain to form a signaling platform that allows the binding of adaptor and effector molecules, ultimately leading to activation of caspase 1 and then interleukin (IL)-1β.[98,99,100] This molecular assembly is named an “inflammasome,”[101] with the best-known infammasome being Nlrp3 (also known as NALP3 or cyropyrin), which can sense various bacterial products as well as alum, silica, and urate crystals.[101] Another inflammasome is Nlrc4 (also known as CARD12 or IPAF), which can detect flagellin. Flagellin, however, is also detected by TLR5.[102]

Other well-known NLRs are NOD1 and NOD2. NOD1 is expressed in various cell types, but NOD2 is expressed primarily among the epithelial cells of the lungs and intestine, macrophages, and dendritic cells (DCs).[103,104,105,106] NOD1 recognizes peptidoglycan fragments containing *meso*-diaminopimelic acid primarily from gram-negative bacteria,[107] but NOD2 recognizes muramyl dipeptide, the minimal motif of peptidoglycan that is shared by both gram-positive and gram-negative bacteria.[108] Interestingly, the lack of signaling by muramyl dipeptide results from the NOD2 gene mutation involved in Crohn disease.[98,108,109]

During the initial phase of inflammation following a bacterial invasion, many cell types residing in the mucosa or skin (eg, keratinocytes) may produce molecules important in controlling infections. Several studies revealed that mast cells are one of the important resident host cells involved in the innate immune response. Mast cells, classically known for their stores of histamine and serotonin,[110] are abundant along the bronchial tree and the epidermis of the skin. They are now known to both contain preformed TNF-α as well as be a major source of various cytokines. Mast cells account for 90% of IL-4- and IL-6-producing cells in the nasal cavity.[111] Upon exposure to various bacterial products (eg, LPS), mast cells release these cytokines, which are essential for the recruitment of neutrophils to the site of inflammation. The absence of mast cells can increase the susceptibility of animals to bacterial infections in the peritoneum or the lungs; their absence can be partially compensated for by administration of TNF-α.[112]

As the inflammatory process persists, additional cell types come to the site of inflammation. In the case of experimental pneumococcal pneumonia, neutrophils come to the lungs in 12 to 24 hours, followed by the appearance of monocytes and macrophages in 48 hours.[113] Similar phagocyte entry sequence was observed for nasopharyngeal

colonization by *S. pneumoniae.*[114] Few lymphocytes are observed in the lungs during this time period. Upon their arrival at the site of infection, neutrophils and macrophages, which can rapidly phagocytize and kill the bacteria, become activated by the bacterial products (eg, LPS) and chemokines (eg, IL-8). Phagocytosis can occur when phagocytic cells recognize certain native molecular structures of the bacteria such as lectins, PS, and peptides (RGD sequence),[115] or aided by their CR3 and Fc receptors, recognize the host opsonins on the bacterial surface.

Inflammatory processes trigger the cascade of chemokine and cytokine release at the site of inflammation. Sequential appearance of chemokines has been noted in the pneumonia model.[113] The peak levels of chemokines macrophage inflammatory protein (MIP)-2 and KC are achieved in the lungs less than 6 hours after infection. The peak levels of MIP-1a and MCP-1 are observed in 12 to 24 hours. Neutralizing MIP-1a and MCP-1 along with RANTES reduces macrophage recruitment. The cytokines produced during acute inflammation can be divided into two groups: proinflammatory cytokines (eg, IL-1 and TNF-α) and anti-inflammatory cytokines (eg, IL-4). The molecules produced during inflammation can induce the expression of ELAM, ICAM, and VCAM on endothelial cells and of selectins and integrins on leukocytes, thereby modifying the properties of the cells (cell adhesion, vascular permeability, etc.) at the site of inflammation.

Inflammation also draws phagocytes to the site of pathogen invasion, where the phagocytes generally efficiently recognize, ingest, and kill the extracellular pathogens. Among many surface receptors, CR3 may be the most relevant to recognizing extracellular bacteria. Indeed, persons with CR3 deficiency (leukocyte adhesion deficiency type 1) have leukocytes ineffective in phagocytic killing.[116] The phagocytic killing occurs rapidly (generally within 15 minutes) when phagosomes fuse with lysosomes and the ingested bacteria are exposed to lysosomal enzymes, although some extracellular bacteria can survive in phagocytes for a significant period.[117]

In addition to this classic killing mechanism, a new mechanism called “autophagy” has been described. This process is characterized by the engulfment of portions of the cytosol into a characteristic double-membrane vacuole called an “autophagosome.”[118,119] After maturation, autophagosomes fuse with lysosomes followed by degradation of the sequestered structures and recycling of the degraded products.[120] While the autophage is important in controlling infections by intracellular pathogens (eg, virus or intracellular bacteria), it may also be involved in defense against extracellular pathogens. *S. pyogenes* may be killed by autophagy.[121,122] However, *S. aureus* may also exploit autophages to its advantage as *S. aureus* activates an autophagic response to enter the double-membraned autophagosomes but prevents autophagosome maturation.[123,124]

Another phagocyte response involves neutrophil extracellular traps (NETs).[125] In this situation, when phagocytes encounter pathogens, the phagocytes decondense the phagocytes' DNA and release their DNA as well as their cellular contents. This decondensed DNA forms a net with cellular debris with the NET being able to capture and destroy many bacteria. However, some pathogens such as *S. pneumoniae* can escape the NET by destroying it with endonuclease.[126]

Systemic Response to Bacterial Invasion

In response to inflammatory bacterial products, cytokines such as IL-1, TNF-a, and IL-6 are released into the systemic circulation and trigger many systemic changes, such as fever and accumulation of leukocytes at the sites of infection. The cytokines also trigger an acute-phase response by the liver.[127,128] The acute-phase response occurs when hepatocytes, in response to the cytokines, activate transcription factors such as NF-κB and STAT3,[127,128] and increase production and secretion of a variety of molecules that are termed acute-phase reactants, such as coagulation factors, serum amyloid A protein, C-reactive protein (CRP), TREM-1,[129] and collectins.[130]

CRP was named for its ability to bind to pneumococcal C-polysaccharide. Its serum level begins to rise 2 to 3 hours after infection and increases more than 1000-fold within 2 to 3 days after infection.[131] CRP recognizes pathogens by binding to PC, which is expressed on many respiratory tract pathogens, and activates complement on the bacteria or functions like anti-PC antibody by engaging FcR. Indeed, CRP can kill PC-expressing *H. influenzae* in vitro in the presence of complement. In addition, while many pathogens use PC to bind to the host cells via rPAF, CRP also blocks bacterial adhesion that involves this receptor.[132] Transgenic mice expressing human CRP are more resistant to systemic pneumococcal infection.[133]

The liver produces mannan-binding lectin (MBL), which is a member of the collectin family. The structural hallmark of these family members is a cystine-rich N terminus, a collagen-like domain, and a C-type lectin domain. These molecular features allow MBL to assemble into a C1q-like structure. Although it is an acute-phase protein, MBL levels change only two- and threefold during the course of an infection.[134] However, the population distribution of MBL levels ranges between 100- and 1000-fold,[135,136] with about 5% of Europeans being MBL deficient (< 100 ng/mL).[137] MBL uses the lectin domain to recognize the target carbohydrate on a microbe, activates MBL-associated protease-2, and initiates the lectin pathway of the complement system. MBL is the innate opsonin for baker's yeasts, and MBL-deficient individuals may have an increased incidence of infection.[138] MBL significantly reduces bacteremia from *N. meningitides.*[139] Although this relationship is controversial,[140,141] MBL-deficient persons may be more susceptible to infections by *S. pneumoniae* with a low invasion index.[142] Interestingly, transplant patients who received the liver from MBL-deficient donors are more susceptible to infections.[143] The collectin family includes SP-A and SP-D, which may play important roles in lung immunity.[144,145]

The liver produces another group of innate opsonins, called ficolins. Humans produce three ficolins: L- and H-ficolins are from the liver,[146] but M-ficolin is in the secretory granules of neutrophils, monocytes, and type II alveolar

epithelial cells.[147] Ficolins have a fibrinogen-like domain at the C terminus instead of a C-type lectin domain, but they assume an overall structure similar to those of MBL and C1q. Ficolins also trigger the lectin pathway of the complement system by activating MBL-associated protease-2.[148,149] L-ficolin can bind many structures with acetyl groups[150] but many bacterial surface molecules that appear to be the natural ficolin ligands contain carbohydrate moieties. Thus, ficolins display lectin-like functional properties.

Once extracellular bacteria enter the systemic circulation, these opsonins together with antibodies opsonize bacteria for rapid removal by the spleen or the liver.[20,151] Persons lacking splenic function (due to sickle cell disease or splenectomy) are at an increased risk of overwhelming sepsis from encapsulated pathogens (eg, *S. pneumoniae*).[133] Clearance of bacteria from the blood by these organs is facilitated because phagocytes are abundant and blood circulates slowly in these organs. *S. pneumoniae* serotype 27 produce capsular PS-containing PCs. CRP can bind and opsonize serotype 27 pneumococci for phagocytes,[152] which may be why serotype 27 pneumococci are nonpathogenic.[153]

The liver is also the major source of transferrin, which increases iron storage by tissues and lowers the serum concentration of iron. Iron at the site of inflammation may be reduced by neutrophil-secreted lactoferrin. The reduction in the amount of iron available to bacteria can be a significant defensive measure.[154] Moreover, even a moderate reduction in iron intake[155] and the use of an iron chelator have both been shown to be beneficial against infections by extracellular bacteria. In contrast, an excess of iron may predispose an individual to infections.[156]

ANTIGEN-SPECIFIC HOST DEFENSE RESPONSE

Accompanying antigen-nonspecific responses, the host also mounts an adaptive, antigen-specific immune response. For protective responses to extracellular bacteria, B-cell-mediated (but not T-cell-mediated) immune responses are critical, as shown by clinical observations of patients with Bruton agammaglobulinemia. These patients, who have relatively normal T-cell function but lack B cells, suffer primarily from infections caused by extracellular bacteria, infections that can be successfully treated with the passive administration of pooled gammaglobulin.[157] Consequently, protective B-cell responses are described in detail subsequently.

Responses of the Host (B Cell) Immune System to Bacteria

Following an asymptomatic exposure to extracellular bacteria or to an infection, the host develops antibodies to many different bacterial antigens. For instance, the level of antibodies to various pneumococcal antigens increases in young children as they age, even if they never have clinical infections.[158] However, the antibody levels remain low in those young children without evidence of asymptomatic nasopharyngeal carriage of pneumococci. This finding suggests that asymptomatic carriage of pneumococci is sufficient to raise antibody levels.[158]

When an infection occurs, it presents the host with a large load of free antigens released from bacteria such as capsular PSs and proteins (eg, toxins). Bacterial proteins induce strong immune responses in a conventional T-cell-dependent manner; indeed, the antibody response induced by bacterial proteins has been used to diagnose infections.[159] In addition, the released PSs that are readily detectable in the urine of many patients[160] are used to diagnose pneumococcal pneumonia.[161] The released PSs may neutralize the anti-PS antibodies in the host. For example, vaccination with hemophilus vaccines may neutralize preexisting anti-PS antibody and briefly increase disease susceptibility immediately after the vaccination.[162]

In contrast to proteins, bacterial PSs generally elicit antibody responses with minimal help from T cells.[163] As bacterial PSs usually have many repeating units and multiple epitopes, they can efficiently cross-link B-cell receptors and stimulate B cells. The PSs primarily stimulate two subsets of B cells: B1 B cells[164] and marginal zone (MZ) B cells.[165,166] These two types of B cells together with follicular B cells are the three recognized subsets of mature B cells with preferential anatomic locations. B1 B cells are associated with the peritoneum, MZ B cells are found in the splenic marginal zone, and follicular B cells are in splenic follicles. In mice, the subsets can be distinguished by their surface phenotypes. Follicular B cells are IgM^{lo}, IgD^{hi}, CD23+, $CD21^{int}$, and $CD1d^{low}$, whereas MZ B cells are IgM^{hi}, IgD^{lo}, $CD23^{low}$, $CD21^{hi}$, and $CD1d^{hi}$.[167] B1 B cells express CD11b and B220, with CD5 expression being used to divide them into B1a (CD5+) and B1b (CD5-) subsets.[164] Furthermore, these subsets have distinct developmental requirements. MZ B cells require a proline-rich tyrosine kinase (Pyk-2),[165] Aiolos, and Notch2.[167] B1 B-cell deficiency was noted in mice without the regulatory B1 subunit of calcineurin.[168] B1a B cells are absent in CD19-deficient mice,[169] and the development of B1 and follicular B cells requires BTK.[170]

Several observations support the contention that the antibody response to PS antigens is largely independent of T cells. Athymic mice can produce antibodies to PS antigens. PS antigens do not bind class II molecules as protein antigens do[171] and may actually interfere with the presentation of protein antigens.[172] In addition, they do not usually induce the formation of germinal centers,[173] they elicit poor immune memory,[174] and they easily tolerize B cells.[175] Nonetheless, there have been past reports of T-cell involvement in the antibody response to PS antigens,[176] and recent studies suggest that CD40 is involved in this response.[177] Because the PS antigens used for the studies may have had contaminants that affected their immune properties[178] and as zwitter ionic PSs may behave differently from other PSs,[179] additional studies with pure PSs would be needed to define the role of non-B cells in the antibody response to PS antigens.

PS antigens commonly elicit oligoclonal antibodies, which utilize a restricted number of V region genes[180,181] even among genetically unrelated humans.[182] In addition, the antibodies to PS exhibit few somatic mutations[182,183] and generally have a low affinity to the antigen.[184] However, because the capsular PS and LPS O antigens present repeating epitopes, even low-affinity antibodies can bind with enough

avidity to fix complement and cause opsonization and/or bacteriolysis.

Capsular PSs have been used as vaccines because antibodies specific for them are protective. Young children, however, do not produce antibodies to most PS antigens until they are several years old,[185] and they are particularly susceptible to infections by encapsulated bacteria during their first few years of life.[186] However, young children readily produce antibodies to PS when it is conjugated to a protein carrier. The clinical use of “conjugate” vaccines to induce antibodies to *H. influenzae* type b (Hib)-PS in young children has virtually eliminated Hib meningitis as well as oropharyngeal colonization by Hib.[187] Similar “conjugate” vaccine approaches have been used to produce 4-valent meningococcal and 7-, 10-, and 13-valent pneumococcal conjugate vaccines.[188,189,190] The pneumococcal conjugate vaccine has been used for young children since 2000 and has markedly reduced the incidence of invasive pneumococcal infections in both young children as well as old adults.[191]

The immunobiology of conjugate vaccines can differ based on the protein carriers used. Among hemophilus conjugate vaccines, Hib-PS conjugated to the meningococcal outer membrane protein complex can elicit antibody responses after only one immunization,[192] presumably because, unlike other protein molecules used as carriers, outer membrane protein complex stimulates TLR2.[193]

Not all bacterial antigens are presented to the host as free molecules, and some antigens remain associated with the bacteria. When bacteria enter the blood circulation, they preferentially localize at the marginal zone of the spleen. The marginal zone has features useful in capturing particles in the blood: the zone is where the terminal arteriole ends and empties into sinuses and has several characteristic macrophages and DCs.[167] Following the localization of bacteria at the marginal zone of the spleen, even without T cells, within 2 to 3

days MZ B cells can be activated and become plasma cells secreting antibodies to bacterial PSs.[166] MZ B cells have additional unique characteristics. They are rapidly stimulated by LPS,[194] and their maturation may be facilitated by other bacterial molecules as well. MZ B cells may facilitate the activation of follicular B cells as they can capture IgM immune complex and transport it to follicular DCs.[195,196]

Although MZ B cells can mature and differentiate independently of T cells,[166] other cells, including T cells, may help their maturation and antibody responses to various bacterial antigens. MZ B cells can present bacterial (protein) antigens to naïve T cells.[195] In addition to protein antigens, antibody responses to bacterial PSs require cofactors such as B7-2[197] and CD40,[197,198] and can be reduced with the simultaneous injection of anti-CD4 and anti-CD8 antibodies.[199,200] Indeed, studies have shown antibody responses to PSs attached to bacteria to be T-cell-dependent.[201] MZ B-cell response may depend on DCs as well. Upon taking up dead bacteria, $CD11c^{low}$ $CD11b^{high}$ DCs in the blood locate to the spleen and may provide transmembrane activator and CAML interactor ligand(s) helpful in MZ B-cell survival.[202] Adoptive transfer of live DCs after an in vitro exposure to dead pneumococci can transfer antibody responses to pneumococcal proteins and PS antigens.[203] Natural killer T cells may be involved in MZ B-cell maturation as MZ B cells express CD1 and may activate natural killer T cells.[195]

Preimmune animals have antibodies that cross-react with many structurally unrelated antigens. These antibodies are often labeled as “natural antibodies.” The majority of these antibodies is of the IgM isotype and frequently bind autologous antigens. Anti-PC antibodies may be an example of natural antibodies. Recent studies suggest that these natural antibodies are important in the early phase of bacterial and viral infections.[204] For instance, anti-PC antibodies react with a PC epitope found on *S. pneumoniae*, *H. influenzae*, and *Wuchereria bancrofti* (a tissue nematode).[205,206,207] Anti-PC antibodies can reduce the susceptibility of mice to pneumococcal infections.[206] A recent study suggests that the natural antibodies are from B1a B cells. CD19-deficient mice lack B1a B cells and have a reduced number of MZ B cells. These mice also lack anti-PC antibodies and are susceptible to pneumococcal infections.[169] In contrast, another mouse strain that exhibits both a reduced number of MZ B cells and a deficiency of B1b cells can produce anti-PC antibodies and is as resistant to pneumococcal infections as are normal mice.[169]

In addition to natural antibodies, animals often have preexisting antibodies to a PS that cross-react with structurally similar PSs,[208,209,210] probably because many PS molecules have very similar structures. Sometimes it is difficult to distinguish usual “anti-PS antibodies” from “natural antibodies.” Cross-reactions may play an important role in protecting the host against its first exposure to a bacterial species. For instance, human adults carry detectable amounts of antibodies to the Hib-PS—even in the absence of vaccination—and are thus relatively resistant to *H. influenzae* infections.[186] While some of the antibodies may be the result of immunization by subclinical infections, the majority of human preimmune (but not postimmune) anti-Hib-PS antibodies cross-react with *E. coli* K100, the PS capsule of which is an isopolymer of Hib-PS.[211] Experimental colonization of rats with *E. coli* K100 can protect

them against Hib.[212] About 1% of human IgG binds a carbohydrate epitope (galactose [1→3] galactose),[213] and this antibody can kill *Trypanosoma* and *Leishmania* in vitro.[214] Cross-reactive antibodies binding the LPS core components are thought to be responsible for the protection from bacteremic dissemination of gonococci in nonimmune patients,[215] although they cannot prevent infection of the genital tract.[215]

Normal gut flora may be the antigenic stimulus for many of the cross-reactive anti-PS antibodies. About 1% of the human population carries *E. coli* K100 in their gut at any moment.[216] Antibodies to (galactose [1→3] galactose) bind many species of bacteria isolated from normal stool specimens.[213] The gut flora may have additional interesting impacts on the immune system. For example, in some transgenic mice, inflammatory bowel diseases develop in the presence of normal intestinal flora but not in the absence of gut flora. In addition, in some animals, such as chickens and rabbits, microbial colonization of the gut

appears to be necessary for the normal development of antibody V region repertoires.[217] Bacteria should therefore be considered as active participants in shaping the host immune system.

Protective Mechanisms of Antibodies

Antibodies to virulence factors may act by neutralizing the function of those factors. Anti-toxin antibodies can protect a host by blocking the action of the toxins (eg, blocking the binding of the toxins to the host cell receptors) or by increasing the removal rate of the toxins. Antibodies to superantigens or tetanus toxin can inactivate them and thereby provide protection to the host. Antibodies to an *E. coli* adhesin can prevent experimental infections by *E. coli*.[218] Antibodies to M protein neutralize its ability to interfere with complement and provide protection against *S. pyogenes* infections. Antibodies to LPS,[219,220] and perhaps to LTA,[221] can be protective. Antibodies to PspA, pneumolysin, autolysin, or pneumococcal surface protein C can protect animals from fatal pneumococcal sepsis. Although these antigens are being investigated as potential replacements for the expensive pneumococcal conjugate vaccines, the protective mechanisms that they employ are still unclear. The most recent hypothesis suggests that antibodies to PspA may inhibit its decomplementation properties and the antibodies may increase the complement fixation on pneumococci.[33,222] Antibodies to IgA1 protease or iron-transport systems[223,224] can also protect against bacterial infections, most likely by neutralizing the normal functions of the target antigens. Finally, in the presence of antibodies and complement, the ability of the liver to remove bacteria increases significantly.[20] Thus, another protection mechanism provided by antibodies may be to facilitate the in vivo removal of bacteria from circulation by enhancing the ability of the reticuloendothelial system to clear bacteria.

Antibodies to capsule PS can provide protection by fixing complement on the surface of bacteria and by inducing bacteriolysis or opsonization. The bacteriolysis pathway can provide significant in vivo protection against gramnegative bacteria, as illustrated by the susceptibility to *N. meningitidis* infections of persons with deficiencies in C5-9 components.[225] In contrast, antibodies and complement do not lyse gram-positive bacteria but opsonize them for

phagocytic killing, as explained in the following.[226] Host phagocytes cannot readily recognize and kill the intact encapsulated gram-positive bacteria. However, once bacteria are coated with antibodies and complement, the host phagocytes can readily recognize the bacteria via various recognition receptors and engulf them for intracellular killing. The Fc receptor (CD16b) and the complement receptor (CR3) are some of the important recognition receptors. CR3, an integrin molecule, is a heterodimer of CD11b and CD18. Protection mediated by this antibody/complementmediated opsonization is probably important in vivo, as both complement deficiency and agammaglobulinemia predispose individuals to infections by many different extracellular bacteria.[157,225] To be effective for opsonization, the epitope of the surface antigens must be exposed on the surface of the bacteria. Effective antibodies to the porins of *N. meningitidis* recognize the surface loop of the molecule.[227] In most pneumococci, C-polysaccharide (C-PS) is mostly buried underneath the PS capsule. Although antibodies to the C-PS can fix complement,[20] anti-C-PS antibodies were ineffective in protecting mice against most *S. pneumoniae* unlike antibodies to capsular PS, which are protective.[228] However, a recent study found that purified human anti-C-PS antibodies can opsonize pneumococci[229]; thus, additional studies are needed.

Because antibody-mediated opsonization and bacteriolysis are dependent upon the complement-fixing properties of the Fc region, the relative efficacies of antibodies of different Ig isotypes have been compared. IgM antibodies are produced early in the course of infections and should be important in the early phase of infections because they fix complement very efficiently and can opsonize bacteria. Selective deficiency of IgM antibodies was found to increase susceptibility to bacterial infections.[230] Studies found that specific IgM antibodies agglutinate erythrocytes, fix complement, and lyse erythrocytes more readily than IgG antibodies,[231] and IgM antibodies are more effective in complement-mediated bacteriolysis[232]; however, IgG antibodies are more effective than IgM antibodies in preventing pneumococci infections of mice[233] or in opsonizing Hib in vitro.[234] Moreover, antibodies of some IgG subclasses have been reported to be more protective against specific viral[235] and fungal[236] infections than antibodies of other subclasses. These results suggest that optimal opsonization requires not only complement receptors but also Fc receptors for IgG. In the absence of inflammation, IgM antibodies are confined to the intravascular space, whereas IgG antibodies can enter the extracellular space. However, inflammation can make the vessels at the infection site permeable, at which point, antibodies of all isotypes may enter the infection site. Compared with IgM antibodies, IgG antibodies may be especially efficient at neutralizing toxins because they have a longer half-life, generally have a higher affinity, and are already present in extravascular spaces prior to infection.[237]

IgG subclasses differ in their ability to fix complement and to bind Fc receptors.[238,239] It has also been reported that IgG1 mouse monoclonal antibody is protective against *Cryptococcus neoformans* but that IgG3 mouse antibody is not.[236] Consequently, the fact that antibodies to bacterial PS are found to be largely restricted to a single IgG subclass (IgG2 in humans and IgG3 in mice) has led to many studies of the differences in the protective properties of anti-PS antibodies of different isotypes. Mouse IgG3 antibodies (but not antibodies of other IgG subclasses) can associate with each other through their Fc regions.[240] This feature may make the IgG3 antibodies with a low affinity to PS more effective in binding the antigen than

antibodies of other isotypes of the same affinity. Although these observations provide a theoretical advantage for mouse IgG3 antibodies, this same aggregation phenomenon has not been observed for human IgG2 antibodies even though some human IgG2 can form covalently joined dimers.[241] The full significance of IgG3 aggregation is not clear, however, as anti-PS antibodies of the IgG3 isotype have not been observed to be any more efficacious

against pneumococcal infections than antibodies of other isotypes.[242] IgG2 antibody levels can be significant, however. People expressing the Gm23+ IgG2 allele have higher IgG2 antibody levels than people with the Gm23- allele. Among C2-deficient persons, Gm23- persons are more susceptible to bacterial infections than Gm23+ individuals.[243]

Moreover, in contrast to expectations, many studies found that human IgG1 antibodies are slightly more effective at opsonization and bacteriolysis than are human IgG2 antibodies.[239,244] Neither of these isotypes appears to be essential, however, as individuals lacking IgG1 and IgG2 subclass genes are healthy.[245] Furthermore, human IgG2 antibodies bind less strongly to CD16, CD32, and CD64 than do IgG1 or IgG3 antibodies[246] and may not be effective for neutrophil opsonization in individuals homozygous for a specific CD32 allele.[244] These observations, taken together, strongly suggest that the human IgG2 (or mouse IgG3) subclass may not provide any unique advantage in defense against bacteria.

IgA is highly heterogeneous in structure: it can exist as a monomer, a polymer, or in secretory forms. In addition, its function is still unclear. Although it has been reported that IgA can opsonize,[247] fix complement,[248] and facilitate the lysis of *N. meningitides*,[249] other studies have found that IgA does not fix complement in vitro[250] and may even inhibit IgG-mediated complement-dependent killing.[251] The ability of IgA to fix complement may also depend upon its denaturation or its glycosylation status.[252,253] Nevertheless, other studies indicate that IgA antibodies may fix complement by the MBL pathway[254] and that human IgA antibodies against pneumococcal capsular PS can opsonize pneumococci for killing by neutrophils.[255] Bacteria that commonly colonize or infect mucosal areas often produce IgA1 protease, and IgA antibody has been found to provide protection in at least some of these cases.[256] These findings suggest that IgA may play an important role as a part of the complex mucosal immune defense. For example, IgA antibodies may be important in reducing nasopharyngeal colonization by bacteria inasmuch as the mice deficient in IgA or polymeric Ig receptor can carry pneumococci in the nasopharynx even after an immunization against pneumococci.[257] IgA may function by aggregating the bacteria and facilitating their expulsion from mucosal areas. IgA may also block the invasion of bacteria through mucosal epithelial cells, as endocytosed IgA has been found to block the transport of virus through epithelial cells.[258] However, IgA-deficient persons or mice are relatively healthy, and IgA-deficient mice can elicit normal protective immunity to experimental infections with influenza virus. IgM antibodies may function as secretory antibodies in IgA-deficient individuals.[259]

T-Cell Immune Responses to Extracellular Bacteria

Although immune responses to toxins from extracellular bacteria are T-cell-dependent,

antitoxin antibodies mediate protection; therefore, the protective immunity against extracellular bacteria is clearly centered on the B-cell responses. However, recent studies suggest additional roles for T cells in responses to extracellular bacteria and their products. PS associated with lipid can stimulate T cells in association with CD1 molecules.[260] Also, studies of abscess formation in response to *Bacteroides fragilis* infections led to a discovery that zwitterionic PS can be taken up by antigen-presenting cells, which can process the PS via a nitric oxide-dependent mechanism and present it in association with MHC class II molecules[179] to stimulate CD4+ T cells to produce IL-17.[261] IL-17-producing CD4+ T cells are now named "Th17 cells" and are found to be distinct from Th1 and Th2 cells. Th17 cells produce unique set of cytokines, such as IL-17 (IL-17A), IL-17F, IL-21, and IL-22, and require a distinct cytokine milieu (IL-1, IL-6, and TGF-β) for their development.[262,263,264,265]

Th17 cells may be involved in abscess formation by *B. fragilis* infections because Th17 cells are found in these abscess.[266] Several studies found that IL-17 and Th17 cells are important in the nasopharyngeal carriage of *S. pneumoniae* in mice.[114,267] A recent study showed that Th17 cells may recruit monocytes and macrophages to the nasopharynx where the monocytes/macrophages may actually remove pneumococci. Clinical examples clearly demonstrate the importance of IL-17 on some extracellular bacterial infections in humans. Patients with autosomal dominant hyper-IgE syndrome are deficient in Th17 cells and are very susceptible to infections by fungus and *S. aureus.*[268] Patients with mutations in IL-17F or IL-17 receptor A (IL-17RA) are susceptible to fungal and staphylococcal infections.[269] In view of these new findings, some researchers are investigating the possibility of using antigens stimulating Th17 cells as vaccines against *S. pneumoniae.*

DELETERIOUS HOST RESPONSES

Inflammatory responses by the host inevitably cause some tissue damage. In some bacterial infections such as pneumonia and meningitis, this damage plays a significant role in disease pathology and symptoms. For instance, animal models of meningitis have shown that inflammation associated with bacterial products (primarily bacterial cell walls) is the primary cause of neurologic damage. Treatment of animals with antibiotics alone can eradicate the bacteria, but it does not prevent neurologic damage. In contrast, when inflammation was controlled by steroids administered along with the antibiotics, neurologic damage was considerably reduced.[270]

Antigen-Nonspecific Deleterious Response

Uncontrolled inflammation at the systemic level can produce septic shock, which can be triggered by several factors, including exotoxins (eg, staphylococcal enterotoxin B) of gram-positive bacteria, the combination of LTA and peptidoglycan from gram-positive bacteria,[271] or LPS from gram-negative bacteria. The staphylococcal enterotoxin B superantigen binds the host's class II molecules of the MHC region and can stimulate large numbers of helper T cells to release cytokines. Septic shock can also be initiated when LPS from gram-negative bacteria binds CD14 and a TLR and stimulates macrophages or monocytes to secrete inflammatory cytokines. In addition to resulting in the release of cytokines, the stimulation of host cells by bacterial

products leads to the release of other mediators of inflammation, such as arachidonic acid metabolites, activation of the complement cascade, and activation of the coagulation cascade. Excess release of the mediators leads to the failure of the vascular system and, finally, the failure of multiple organ systems. Studies using transgenic mice with defective genes have identified several molecules critical in developing septic shock, such as TNF-α, one of its receptors TNFRI, caspase 1, and ICAM-1.[272] This approach also showed that CD14 and TLR4 are critical for LPS-induced septic shock and that CD28, a T-cell costimulation molecule, is necessary for superantigen-induced septic shock.[272]

Anthrax infections provide another example of uncontrolled host responses. “Lethal factor” binds the “ protective antigen” immobilized on the macrophages and then stimulates the cells to secrete cytokines and reactive oxygen intermediates. These macrophage products are thought to kill the host, as the host dies even when the proliferation of the bacteria is controlled. When macrophage cells are removed from animals, the animals are resistant to anthrax toxins.[273] This suggests that the macrophage response to the toxins is actually responsible for the death of the host.

Although inflammation is a significant cause of morbidity and mortality, it must also be regarded as the host's primary protection against bacterial infections. Evidence for this hypothesis comes from studies with TLR4-deficient mice, which, although nonreactive to LPS and completely resistant to LPS shock, are more susceptible to infection with gram-negative bacteria than are normal mice.[274,275] Perhaps LPS is “toxic” because the host has evolved to use this common bacterial component as a trigger for host responses.

Autoimmune Disorders

Autoimmune diseases are characterized by an overactive host immune response toward self, the host's own cells and tissues. Various factors are involved in autoimmune diseases, including genetic predisposition and environmental triggers. The pathogenesis of autoimmune diseases has at its core the development of autoreactive effector lymphocytes, and these can involve, among others, T-cell bypass and molecular mimicry. T-cell bypass is based on the notion that activated T helper cells provide the necessary factors to activated B cells for the production of antibodies. Some microorganisms can provide the bypass with superantigens, which can bind to many T cells expressing certain types of Vβ regions and stimulate them to create a nonspecific polyclonal T-cell activation.[276,277] The best-studied examples of superantigens are the exotoxins secreted by *S. aureus* and *S. pyogenes.*[278,279,280]

Molecular mimicry occurs when a bacterial antigen shares structural similarities with a host antigen, and hence, antibodies produced against the bacterial antigen could also bind to the host antigen, thereby casuing an autoimmune disease. For instance, the LPS of many strains of *N. meningitidis*, *N. gonorrhoeae*, *H. influenzae*, and *Haemophilus ducreyi* expresses the epitope of blood group antigen pK.[281] The PS capsule of *N. meningitidis* group B mimics epitopes expressed in the central nervous system,[282] such as the N-acetylneuramic acid epitope in the embryonic N-CAM.[283] While infection by these pathogens may cause autoimmune diseases, epidemiologic studies have clearly associated *Campylobacter jejuni* infections, a leading cause of acute gastroenteritis, with development of antiganglioside

antibodies and autoimmune diseases: Guillain-Barre syndrome[284] or its variant Miller-Fisher syndrome. Patients with Miller-Fisher syndrome have ophthalmoplegia and generally have autoantibodies to GQ1b, but Guillain-Barre syndrome is associated with autoantibodies to GM1 or GD1a.[285] Production of the two different types of autoantibodies has been associated with an allelism of sialyl transferase II of *C. jejuni*. One allele produces lipooligosaccharide-mimicking GM1 and GD1a, and infection by *C. jejuni* with this allele has been associated with Guillain-Barre syndrome. On the other hand, the other allele produces lipooligosaccharide-mimicking GQ1b, with its infection being associated with Miller-Fisher syndrome.

Perhaps the most classical example of infection-associated autoimmunity may be rheumatic fever and acute glomerulonephritis associated with *S. pyogenes* infections. Studies found that *S. pyogenes* can be divided into two classes with a monoclonal antibody to M protein[286] and that rheumatic fever develops only after infections with class I strains of *S. pyogenes*.[286] Class I and class II strains of *S. pyogenes* can also be readily distinguished by the linkage relationship of the M protein genes with the genes encoding related surface proteins.[286,287] M proteins from some class I *S. pyogenes* express epitopes highly cross-reactive with epitopes of cardiac myosin, tropomyosin, vimentin, laminin, and keratin.[288,289,290] An antibody molecule may bind to all of these protein molecules because a major portion of these proteins is coiled-coil α-helix.[290] The polyreactive antibodies to M protein may directly damage myocardial and endothelial cells.[291] In addition to antibodies, CD4+ and CD8+ T cells are found at rheumatic heart valves,[292] and the T cells proliferate to M protein peptides and heart proteins.[293] These observations suggest that the T cells with crossreactivity between M protein and myosin may be involved in the pathogenesis of rheumatic fever as well.

CONCLUSION

Because extracellular bacteria can grow rapidly and produce toxins, some are potent pathogens. To combat these bacteria, higher organisms primarily depend on two arms of the immune system: innate immunity and adaptive immunity centered on antibody molecules. The two arms of the immune system are comprised of multiple layers of protection. In the early stage of an infection, innate immunity involving pattern recognition receptors, complement, phagocytes, and natural antibodies cross-reacting with many antigens are important in host defense. During the late stage of an infection, pathogen-specific antibodies appear. These antibodies generally mediate the ultimate protection against extracellular bacteria by triggering the protective effects of complement and phagocytes. Nevertheless, innate and adaptive immune responses may cause damage instead of protection. A better understanding of how our immune system protects against each pathogen will aid in the development of more effective preventive and therapeutic measures against these pathogens.

REFERENCES

1. Hooper LV, Gordon JI. Commensal host-bacterial relationships in the gut. *Science.* 2001;292(5519):1115-1118.

2. Aas J, Paster B, Stokes L, Olsen I, Dewhirst F. Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol.* 2005;43:5721-5732.

3. Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI. Human nutrition, the gut microbiome and the immune system. *Nature.* 2011;474(7351):327-336.

4. Thomas L. *The Lives of a Cell: Notes of a Biology Watcher.* New York, NY: Bantam Books; 1974.

5. Crisostomo MI, Vollmer W, Kharat AS, et al. Attenuation of penicillin resistance in a peptidoglycan O-acetyl transferase mutant of Streptococcus pneumoniae. *Mol Microbiol.* 2006;61(6):1497-1509.

6. Bera A, Biswas R, Herbert S, Gotz F. The presence of peptidoglycan O-acetyltransferase in various staphylococcal species correlates with lysozyme resistance and pathogenicity. *Infect Immun.* 2006;74:4598-4604.

7. Gray BM. Streptococcal infections. In: Evans AS, Brachman PE, eds. *Bacterial Infections of Humans.* 3rd ed. New York, NY: Plenum Medical Book Co; 1998:673-711.

8. Lancefield RC. A serological differentiation of human and other groups of hemolytic streptococci. *J Exp Med.* 1933;57(4):571-595.

9. Fischer W. Lipoteichoic acid and lipoglycans. *New Compr Biochem.* 1994;27:199-215.

10. Fischer W, Behr T, Hartmann R, Peter-Katalinic J, Egge H. Teichoic acid and lipoteichoic acid of Streptococcus pneumoniae possess identical chain structures. A reinvestigation of teichoid acid (C polysaccharide). *Eur J Biochem.* 1993;215(3):851-857.

11. Seo HS, Cartee RT, Pritchard DG, Nahm MH. A new model of pneumococcal lipoteichoic acid structure resolves biochemical, biosynthetic, and serologic inconsistencies of the current model. *J Bacteriol.* 2008;190(7):2379-2387.

12. Nikaido H. Outer membrane. In: Neidhardt FC, ed. *Escherichia coli and Salmonella: Cellular and Molecular Biology.* Washington, DC: ASM Press; 1996:29-47.

13. Ezzell JW, Welkos SL. The capsule of bacillus anthracis, a review. *J Appl Microbiol.* 1999;87(2):250.

14. Yother J. Capsules of Streptococcus pneumoniae and other bacteria: paradigms for polysaccharide biosynthesis and regulation. *Annu Rev Microbiol.* 2011;65:563-581.

15. Gotschlich EC, Fraser BA, Nishimura O, Robbins JB, Liu TY. Lipid on capsular polysaccharides of gram-negative bacteria. *J Biol Chem.* 1981;256:8915-8921.

16. Kuo JS-C, Doelling VW, Graveline JF, McCoy DW. Evidence for covalent attachment of phospholipid to the capsular polysaccharide of Haemophilus influenzae type b. *J Bacteriol.* 1985;163:769-773.

17. Bentley SD, Aanensen DM, Mavroidi A, et al. Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. *PLoS Genet.* 2006;2(3):e31.

18. Park IH, Pritchard DG, Cartee R, Brandao A, Brandileone MC, Nahm MH. Discovery of a new capsular serotype (6C) within serogroup 6 of Streptococcus pneumoniae. *J Clin Microbiol.* 2007;45:1225-1233.

19. Kazatchkine MD, Fearon DT, Austen KF. Human alternative complement pathway: membrane-associated sialic acid regulates the competition between B and beta 1H for cell-bound C3b. *J Immunol.* 1979;122:75-81.

20. Brown EJ, Hosea SW, Hammer CH, Burch CG, Frank MM. A quantitative analysis of the interactions of antipneumococcal antibody and complement in experimental pneumococcal bacteremia. *J Clin Invest.* 1982;69:85-98.

21. Horstmann RD, Sievertsen HJ, Knobloch J, Fischetti VA. Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H. *Proc Natl Acad Sci U S A.* 1988;85(5):1657-1661.

22. Tu AH, Fulgham RL, McCrory MA, Briles DE, Szalai AJ. Pneumococcal surface protein A inhibits complement activation by Streptococcus pneumoniae. *Infec Immun.* 1999;67:4720-4724.

23. Cheng Q, Finkel D, Hostetter MK. Novel purification scheme and functions for a C3-binding protein from Streptococcus pneumoniae. *Biochemistry.* 2000;39(18):5450-5457.

24. Roben P, Salem A, Silverman G. VH3 family antibodies bind domain D of staphylococcal protein A. *J Immunol.* 1995;154:6437-6445.

25. Kilian M, Reinholdt J. Immunoglobulin A1 proteases of pathogenic and commensal bacteria of the respiratory tract. In: Nataro JP, ed. *Colonization of Mucosal Surfaces.* Washington, DC: American Society for Microbiology Press; 2005:119-130.

26. Finlay BB, Falkow S. Common themes in microbial pathogenicity revisited. *Microbiol Mol Biol Rev.* 1997;61(2):136-169.

27. Salyers AA, Whitt DD. *Bacterial Pathogenesis: A Molecular Approach.* Washington, DC: ASM Press; 1994:47-60.

28. Barg NL, Harris T. Toxin-mediated syndromes. In: Crossely KB, ed. *The Staphylococci.* New York, NY: Churchill Livingstone; 1997: 527-543.

29. Noel JM, Boedeker EC. Enterohemorrhagic Escherichia coli: a family of emerging pathogens. *Dig Dis.* 1997;15:67-91.

30. Krakauer T. Immune response to staphylococcal superantigens. *Immunol Res.* 1999;2063-73.

31. Ji Y, Carlson B, Kondugunta A, Cleary PP. Intranasal immunization with C5a peptidase prevents nasopharyngeal colonization of mice by the group A streptococcus. *Infect Immun.* 1997;65:2080-2087.

32. Tweten R. Cholesterol-dependent cytolysins, a family of versatile poreforming toxins. *Infect Immun.* 2005;73:6199-6209.

33. Briles DE, Paton JC, Nahm MH, Swiatlo E. Immunity to Streptococcus pneumoniae. In: Cunningham MW, Fujinami RS, eds. *Effects of Microbes on the Immune System.* Philadelphia, PA: Lippincott-Raven; 1999:263-280.

34. Liu G, Essex A, Buchanan J, et al. Staphylococcus aureus golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *J Exp Med.* 2005;202:209-215.

35. McCullers JA, Tuomanen EI. Molecular pathogenesis of pneumococcal pneumonia. *Front Biosci.* 2001;6:D877-D889.

36. Lysenko ES, Ratner AJ, Nelson AL, Weiser JN. The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces. *PLoS Pathog.* 2005;1(1):e1.

37. Cundell DR, Gerard NP, Gerard C, Idanpaan-Heikkila I, Tuomanen EI. Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor. *Nature.* 1995;377:435-438.

38. Striker R, Nilsson U, Stonecipher A, Magnusson G, Hultgren SJ. Structural requirements for the glycolipid receptor of human uropathogenic Escherichia coli. *Mol Microbiol.* 1995;16:1021-1029.

39. Sengupta TK, Sengupta DK, Ghose AC. A 20-kDa pilus protein with haemagglutination and intestinal adherence properties expressed by a clinical isolate of a non-01 Vibrio cholerae. *FEMS Microbiol Lett.* 1993;112:237-242.

40. Chiang SL, Taylor RK, Koomey M, Mekalanos JJ. Single amino acid substitutions in the N-terminus of Vibrio cholerae TcpA affect colonization, autoagglutination, and serum resistance. *Mol Microbiol.* 1995;17:1133-1142.

41. Brennan MJ, Shahin RD. Pertussis antigens that abrogate bacterial adherence and

elicit immunity. *Am J Respir Crit Care Med.* 1996;154:- S145-S149.

42. Geuijen CA, Willems RJ, Bongaerts M, Top J, Gielen H, Mooi FR. Role of the Bordetella pertussis; minor fimbrial subunit, FimD, in colonization of the mouse respiratory tract. *Infect Immun.* 1997;65: 4222-4228.

43. Prince A. Flagellar activation of epithelial signaling. *Am J Respir Cell Mol Biol.* 2006;34:548-551.

44. Otto B, Verweij-van Vught A, MacLaren D. Transferrins and hemecompounds as iron sources for pathogenic bacteria. *Crit Rev Microbiol.* 1992;18:217-233.

45. Earhart CF. Uptake and metabolism of iron and molybdenum. In: Neidhardt FC, ed. *Escherichia Coli and Salmonella: Cellular and Molecular Biology.* Washington, DC: ASM Press; 1996:1075-1090.

46. Dassy B, Hogan T, Foster TJ, Fournier JM. Involvement of the accessory gene regulator (agr) in expression of type-5 capsular polysaccharide by Staphylococcus aureus. *J Gen Microbiol.* 1993;139: 1301-1306.

47. Uhl MA, Miller JF. Autophosphorylation and phosphotransfer in the Bordetella pertussis BvgAS siganl transduction cascade. *Proc Nat Acad Sci U S A.* 1994;91:1163-1167.

48. Hoch JA. Two-component and phosphorelay signal transduction. *Curr Opin Microbiol*. 2000;3(2):165-170.

49. Moxon E, Lenski R, Rainey P. Adaptive evolution of highly mutable loci in pathogenic bacteria. *Perspect Biol Med.* 1998;42:154-155.

50. Hammerschmidt S, Müller A, Sillmann H, et al. Capsule phase variation in Neisseria meningitidis serogroup B by slipped-strand mispairing in the polysialyltransferase gene (siaD): correlation with bacterial invasion and the outbreak of meningococcal disease. *Mol Microbiol.* 1996;20:1211-1220.

51. de Kievit TR, Iglewski BH. Bacterial quorum sensing in pathogenic relationships. *Infect Immun.* 2000;68(9):4839-4849.

52. Withers H, Swift S, Williams P. Quorum sensing as an integral component of gene regulatory networks in Gram-negative bacteria. *Curr Opin Microbiol.* 2001;4(2):186-193.

53. Hall-Stoodley L, Hu FZ, Gieseke A, et al. Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *JAMA.* 2006;296(2):202-211.

54. Fischetti VA. Streptococcal M protein: molecular design and biological behavior. *Clin*

Microbiol Rev. 1989;2:286-314.

55. Fischetti VA, Bessen DE, Schneewind O, Hruby DE. Protection against streptococcal pharyngeal colonization with vaccines composed of M protein conserved regions. *Adv Exp Med Biol.* 1991;303:159-167.

56. Dillard JP, Vandersea MW, Yother J. Characterization of the cassette containing genes for type 3 capsular polysaccharide biosynthesis in Streptococcus pneumoniae. *J Exp Med.* 1995;181:973-983.

57. Singleton RJ, Hennessy TW, Bulkow LR, et al. Invasive pneumococcal disease caused by nonvaccine serotypes among Alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. *JAMA.* 2007;297(16):1784-1792.

58. Pilishvili T, Lexau C, Farley MM, et al. Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. *J Infect Dis.* 2010;201(1):32-41.

59. Zhang QY, DeRyckere D, Lauer P, Koomey M. Gene conversion in Neisseria gonorrhoeae: evidence for its role in pilus antigenic variation. *Proc Nat Acad Sci.* 1992;89:5366-5370.

60. Seifert HS. Questions about gonococcal pilus phase- and antigenic variation. *Mol Microbiol.* 1997;21(3):433-440.

61. Saunders N, Jeffries A, Peden J, et al. Repeat-associated phase variable genes in the complete genome sequence of Neisseria meningitidis strain MC58. *Mol Microbiol.* 2000;37:207-215.

62. Nougayrede J, Fernandes P, Donnenberg M. Adhesion of enteropathogenic Escherichia coli to host cells. *Cell Microbiol.* 2003;5:359-372.

63. Covacci A, Rappuoli R. Tyrosine-phosphorylated bacterial proteins: Trojan horses for the host cell. *J Exp Med.* 2000;191(4):587-592.

64. Madden J, Ruiz N, Caparon M. Cytolysin-mediated translocation (CMT): a functional equivalent of type III secretion in gram-positive bacteria. *Cell.* 2001;104:143-152.

65. Rupp ME, Archer GL. Coagulase-negative staphylococci: pathogens associated with medical progress. *Clin Infect Dis.* 1994;19:231-245.

66. Neish AS, Gewirtz AT, Zeng H, et al. Prokaryotic regulation of epithelial responses by inhibition of IkappaB- alpha ubiquitination. *Science.* 2000;289(5484):1560-1563.

67. Eckburg P, Bik E, Bernstein C, et al. Diversity of the human intestinal microbial flora. *Science.* 2005;308:1635-1638.

68. Mbelle N, Huebner RE, Wasas AD, Kimura A, Chang I, Klugman KP. Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. *J Infect Dis.* 1999;180:1171-1176.

69. Riley M, Wertz J. Bacteriocins: evolution, ecology and application. *Annu Rev Microbiol.* 2002;56:117-137.

70. Bartlett JG, Chang TW, Taylor NS, Onderdonk AB. Colitis induced by Clostridium difficile. *Rev Infect Dis.* 1979;1:370-378.

71. Caugant DA, Hoiby EA, Magnus P, et al. Asymptomatic carriage of Neisseria meningitidis in a randomly sampled population. *J Clin Microbiol.* 1994;32:323-330.

72. Kuhns DM, Nelson CT, Feldman HA, et al. The prophylactic value of sulfadiazine in the control of meningococcic meningitis. *JAMA.* 1943;123(6):335-339.

73. Zangwill KM, Schuchat A, Wenger JD. Group B streptococcal disease in the United States, 1990: report from a multistate active surveillance system. *MMWR.* 1992;41:25-32.

74. Berry AM, Lock RA, Paton JC. Cloning and characterization of nanB, a second Streptococcus pnuemoniae neuraminidase gene, and purification of the NanB enzyme from recombinant Escherichia coli. *J Bacteriol.* 1996;178:4854-4860.

75. Kazmierczak BI, Mostov K, Engel JN. Interaction of bacterial pathogens with polarized epithelium. *Annu Rev Microbiol.* 2001;55:407-435.

76. Katz J, Sambandam V, Wu JH, Michalek SM, Balkovetz DF. Characterization of Porphyromonas gingivalis-induced degradation of epithelial cell junctional complexes. *Infect Immun.* 2000;68(3): 1441-1449.

77. Zhang JR, Mostov KE, Lamm ME, et al. The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells. *Cell.* 2000;102:827-837.

78. Cundell DR, Weiser JN, Shen J, Young A, Tuomanen EI. Relationship between colonial morphology and adherence of Streptococcus pneumoniae. *Infect Immun.* 1995;63:757-761.

79. Swords WE, Ketterer MR, Shao J, Campbell CA, Weiser JN, Apicella MA. Binding of the non-typeable Haemophilus influenzae lipooligosaccharide to the PAF receptor initiates host cell signalling. *Cell Microbiol.* 2001;3(8):525-536.

80. Bancroft GJ, Kelly JP. Macrophage activation and innate resistance to infection in SCID mice. *Immunobiology.* 1994;191:424-431.

81. Musher DM. Streptococcus pneumoniae. In: Mandell GL, Bennett JE, Dolin R, eds. *Principles and Practice of Infectious Diseases.* 4th ed. New York, NY: Churchill Livingstone; 1995:1811-1826.

82. Donowitz GR, Mandell GL. Acute pneumonia. In: Mandell GL, Bennett JE, Dolin R, eds. *Principles and Practice of Infectious Diseases.* 4th ed. New York, NY: Churchill Livingstone; 1995:619-637.

83. Pruitt KM, Rahemtulla F, Mansson-Rahemtulla B. Innate humoral factors. In: Ogra PL, ed. *Handbook of Mucosal Immunology.* New York, NY: Academic Press; 1994:53-70.

84. Vorland LH. Lactoferrin: a multifunctional glycoprotein. *APMIS.* 1999;107(11):971-981.

85. Nelson AL, Roche AM, Gould JM, Chim K, Ratner AJ, Weiser JN. Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance. *Infect Immun.* 2007;75(1):83-90.

86. Mestecky J, McGhee J. Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune response. *Adv Immunol.* 1987;40:153-245.

87. Fellermann K, Stange EF. Defensins—innate immunity at the epithelial frontier. *Eur J Gastroenterol Hepatol.* 2001;13(7):771-776.

88. Bevins CL. Events at the host-microbial interface of the gastrointestinal tract. V. Paneth cell alpha-defensins in intestinal host defense. *Am J Physiol Gastrointest Liver Physiol.* 2005;289(2):G173-G176.

89. Crouch E, Wright JR. Surfactant proteins a and d and pulmonary host defense. *Annu Rev Physiol.* 2001;63:521-554.

90. LeVine AM, Bruno MD, Huelsman KM, Ross GF, Whitsett JA, Korfhagen TR. Surfactant protein A-deficient mice are susceptible to group B streptococcal infection. *J Immunol.* 1997;158(9):4336-4340.

91. Henderson B, Poole S, Wilson M. Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. *Microbiol Rev.* 1996;60:316-341.

92. Melmed G, Thomas L, Lee N, et al. Human intestinal epithelial cells are broadly unresponsive to toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut. *J Immunol.* 2003;170:1406-1415.

93. Kirschning C, Schumann R. TLR2: cellular sensor for microbial and endogenous molecular patterns. *Curr Top Microbiol Immunol.* 2002;270:121-144.

94. Echchannaoui H, Frei K, Schnell C, Leib SL, Zimmerli W, Landmann R. Toll-like receptor 2-deficient mice are highly susceptible to Streptococcus pneumoniae meningitis because of reduced bacterial clearing and enhanced inflammation. *J Infect Di* s. 2002;186(6):798-806.

95. Takeuchi O, Hoshino K, Akira S. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. *J Immunol.* 2000;165(10):5392-5396.

96. Hayashi F, Smith KD, Ozinsky A, et al. The innate immune response to bacterial flagellin is mediated by toll-like receptor 5. *Nature.* 2001;410(6832):1099-1103.

97. Albiger B, Dahlberg S, Sandgren A, et al. Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection. *Cell Microbiol.* 2007;9:633-644.

98. Fritz JH, Ferrero RL, Philpott DJ, Girardin SE. Nod-like proteins in immunity, inflammation and disease. *Nat Immunol.* 2006;7(12):1250-1257.

99. Proell M, Riedl SJ, Fritz JH, Rojas AM, Schwarzenbacher R. The Nodlike receptor (NLR) family: a tale of similarities and differences. *PLoS One.* 2008;3:e2119.

100. Meylan E, Tschopp J, Karin M. Intracellular pattern recognition receptors in the host response. *Nature.* 2006;442:39-44.

101. Lamkanfi M, Dixit VM. The inflammasomes. *PLoS Pathog.* 2009;5(12): e1000510.

102. Miao EA, Andersen-Niessen E, Warren SE, Aderem A. TLR5 and Ipaf: dual sensors of bacterial flagellin in the innate immune system. *Semin Immunopathol.* 2007;29:275-288.

103. Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S, Nunez G. Nod2, A Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J Biol Chem.* 2001;276:4812-4818.

104. Tada H, Aiba S, Shibata K, Ohteki T, Takada H. Synergistic effect of Nod1 and Nod2 agonists with toll-like receptor agonists on human dendritic cells to generate interleukin-12 and T helper type 1 cells. *Infect Immun.* 2005;73(12):7967-7976.

105. Hisamatsu T, Suzuki M, Reinecker HC, Nadeau WJ, McCormick BA, Podolsky DK. CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. *Gastroenterology.* 2003;124: 993-1000.

106. Uehara A, Fujimoto Y, Fukase K, Takada H. Various human epithelial cells express functional toll-like receptors, NOD1 and NOD2 to produce anti-microbial peptides, but not proinflammatory cytokines. *Mol Immunol.* 2007;44:3100-3111.

107. Chamaillard M, Hashimoto M, Horie Y, et al. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol.* 2003;4:702-707.

108. Chamaillard M, Girardin SE, Viala J, Philpott DJ. Nods, Nalps and Naip: intracellular regulators of bacterial-induced inflammation. *Cell Microbiol.* 2003;5:581-592.

109. Inohara N, Chamaillard M, McDonald C, Nunez G. NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. *Annu Rev Biochem.* 2005;74:355-383.

110. Galli SJ. New concepts about the mast cell. *N Engl J Med.* 1993;328: 257-265.

111. Bradding P, Feather IH, Wilson S, et al. Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects. The mast cell as a source of IL-4, IL-5, and IL-6 in human allergic mucosal inflammation. *J Immunol.* 1993;151(7):3853-3865.

112. Malaviya R, Ikeda T, Ross E, Abraham SN. Mast cell modulation of neutorphil influx and bacterial clearance at sites of infection through TNFa. *Nature.* 1996;381:77-80.

113. Fillion I, Ouellet N, Simard M, Bergeron Y, Sato S, Bergeron MG. Role of chemokines and formyl peptides in pneumococcal pneumonia-induced monocyte/macrophage recruitment. *J Immunol.* 2001;166(12): 7353-7361.

114. Zhang Z, Clarke TB, Weiser JN. Cellular effectors mediating Th17- dependent clearance of pneumococcal colonization in mice. *J Clin Invest.* 2009;119(7):1899-1909.

115. Ofek I, Goldhar J, Keisari Y, Sharon N. Nonopsonic phagocytosis of microorganisms. *Annu Rev Microbiol.* 1995;49:239-276.

116. Hixson P, Smith CW, Shurin SB, Tosi MF. Unique CD18 mutations involving a deletion in the extracellular stalk region and a major truncation of the cytoplasmic domain in a patient with leukocyte adhesion deficiency type 1. *Blood.* 2004;103(3):1105-1113.

117. Gresham HD, Lowrance JH, Caver TE, Wilson BS, Cheung AL, Lindberg FP. Survival of Staphylococcus aureus inside neutrophils contributes to infection. *J Immunol.* 2000;164(7):3713-3722.

118. Espert L, Codogno P, Biard-Piechacyk M. Involvement of autophagy in viral infections: antiviral function and subversion by viruses. *J Mol Med.* 2007;85:811-823.

119. Levine B, Klionsky DJ. Development of self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell.* 2004;6:463-477.

120. Klionsky DJ. Autophagy. *Curr Biol.* 2005;15:R282-R283.

121. Nakagawa I, Amano A, Mizushima N, et al. Autophagy defends cells against invading group A Streptococcus. *Science.* 2004;306:1037-1040.

122. Huang J, Klionsky DJ. Autophagy and human disease. *Cell Cycle.* 2007;6:1837-1849.

123. Belen-Mestre M, Fader CM, Sola C, Colombo MI. a-hemolysin is required fort he activation of the autophagic pathway in Staphylococcus aereus-infected cells. *Autophagy.* 2010;6:110-125.

124. Schnaith A, Kashkar H, Leggio SA, Addicks K, Kronke M, Krut O. Staphylococcus aereus subvert autophagy for induction of caspaseindependent host cell death. *J Biol Chem.* 2007;282:2695-2706.

125. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. *Science.* 2004;303(5663):1532-1535.

126. Beiter K, Wartha F, Albiger B, Normark S, Zychlinsky A, Henriques- Normark B. An endonuclease allows Streptococcus pneumoniae to escape from neutrophil extracellular traps. *Curr Biol.* 2006;16(4): 401-407.

127. Quinton LJ, Jones MR, Robson BE, Mizgerd JP. Mechanisms of the hepatic acute-phase response during bacterial pneumonia. *Infect Immun.* 2009;77(6):2417-2426.

128. Quinton LJ, Mizgerd JP. NF-kappaB and STAT3 signaling hubs for lung innate immunity. *Cell Tissue Res.* 2011;343(1):153-165.

129. Gibot S, Cravoisy A, Levy B, Bene MC, Faure G, Bollaert PE. Soluble triggering receptor expressed on myeloid cells and the diagnosis of pneumonia. *N Engl J Med.* 2004;350(5):451-458.

130. Steel DM, Whitehead AS. The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. *Immunol Today.* 1994;15:81-88.

131. Pue CA, Mortensen RF, Marsh CB, Pope HA, Wewers MD. Acute phase levels of C-reactive protein enhance IL-1 beta and IL-1ra production by human blood monocytes but inhibit IL-1 beta and IL- 1ra production by alveolar macrophages. *J Immunol.* 1996;156(4): 1594-1600.

132. Gould JM, Weiser JN. The inhibitory effect of C-reactive protein on bacterial phosphorylcholine platelet-activating factor receptor-mediated adherence is blocked by surfactant. *J Infect Dis.* 2002;186(3): 361-371.

133. Szalai AJ, Briles DE, Volanakis JE. Human C-reactive protein is protective against

fatal Streptococcus pneumoniae infection in transgenic mice. *J Immunol.* 1995;155:2557-2563.

134. Thiel S, Holmskov U, Hviid L, Laursen SB, Jensenius JC. The concentration of the C-type lectin, mannan-binding protein, in human plasma increases during an acute phase response. *Clin Exp Immunol.* 1992;90(1):31-35.

135. Ip WK, Takahashi K, Ezekowitz RA, Stuart LM. Mannose-binding lectin and innate immunity. *Immunol Rev.* 2009;230(1):9-21.

136. Ram S, Lewis LA, Rice PA. Infections of people with complement deficiencies and patients who have undergone splenectomy. *Clin Microbiol Rev.* 2010;23(4):740-780.

137. Degn SE, Jensenius JC, Thiel S. Disease-causing mutations in genes of the complement system. *Am J Hum Genet.* 2011;88(6):689-705.

138. Turner MW. Mannose-binding lectin: the pluripotent molecule of the innate immune system. *Immunol Today.* 1996;17(11):532-540.

139. Jack DL, Lee ME, Turner MW, Klein NJ, Read RC. Mannose-binding lectin enhances phagocytosis and killing of Neisseria meningitidis by human macrophages. *J Leuk Biol.* 2005;77:328-336.

140. Roy S, Knox K, Segal S, et al. MBL genotype and risk of invasive pneumococcal disease: a case-control study. *Lancet.* 2002;359(9317): 1569-1573.

141. Kronborg G, Weis N, Madsen HO, et al. Variant mannose-binding lectin alleles are not associated with susceptibility to or outcome of invasive pneumococcal infection in randomly included patients. *J Infect Dis.* 2002;185(10):1517-1520.

142. Valles X, Roca A, Lozano F, et al. Serotype-specific pneumococcal disease may be influenced by mannose-binding lectin deficiency. *Eur Respir J.* 2010;36(4):856-863.

143. Bouwman LH, Roos A, Terpstra OT, et al. Mannose binding lectin gene polymorphisms confer a major risk for severe infections after liver transplantation. *Gastroenterology.* 2005;129(2):408-414.

144. van de Wetering JK, van Eijk M, Lambert M, et al. Characteristics of surface protein A and D binding to lipoteichoic acid and peptidoglycan, 2 major cell wall components of Gram-positive bacteria. *J Infect Dis.* 2001;184:1143-1151.

145. van de Wetering JK, van Golde LM, Batenburg JJ. Collectins: players of the innate immune system. *Eur J Biochem.* 2004;271:1229-1249.

146. Kilpatrick DC, Fujita T, Matsushita M. P35, an opsonic lectin of the ficolin family, in

human blood from neonates, normal adults, and recurrent miscarriage patients. *Immunol Letters.* 1999;67:109-112.

147. Liu Y, Endo Y, Iwaki D, et al. Human M-ficolin is a secretory protein that activates the lectin complement pathway. *J Immunol.* 2005;175:3150-3156.

148. Endo Y, Matsushita M, Fujita T. The role of ficolins in the lectin pathway of innate immunity. *Int J Biochem Cell Biol.* 2011;43:705-712.

149. Matsushita M. Ficolins: complement-activating lectins involved in innate immunity. *J Innate Immun.* 2009;2(1):24-32.

150. Krarup A, Thiel S, Hansen A, Fujita T, Jensenius JC. L-ficolin is a pattern recognition molecule specific for acetyl groups. *J Biol Chem.* 2004;279(46):47513-47519.

151. Schiffmann E, Corcoran B, Wahl S. N-formylmethionyl peptides as chemoattractants for leucocytes. *Proc Natl Acad Sci U S A.* 1975;72: 1059-1062.

152. Edwards KM, Gewurz H, Lint TF, Mold C. A role for C-reactive protein in the complement-mediated stimulation of human neutrophils by type 27 Streptococcus pneumoniae. *J Immunol.* 1982;128(6):2493-2496.

153. Chudwin DS, Artrip SG, Korenblit A, Schiffman G, Rao S. Correlation of serum opsonins with in vitro phagocytosis of Streptococcus pneumoniae. *Infect Immun.* 1985;50(1):213-217.

154. Bullen JJ, Griffiths E, eds. *Iron and Infection.* 2nd ed. New York, NY: John Wiley & Sons; 1999.

155. Weinberg ED, Weinberg GA. The role of iron in infection. *Curr Opin Infect Dis.* 1997;8:164-169.

156. Patruta SI, Horl WH. Iron and infection. *Kidney Int Suppl.* 1999; 69:S125-S130.

157. Lederman HM, Winkelstein JA. X-linked agammaglobulinemia: an analysis of 96 patients. *Medicine.* 1985;64(3):145-156.

158. Rapola S, Jäntti V, Haikala R, et al. Natural development of antibodies to pneumococcal surface protein A, pneumococcal surface adhesion A, and pneumolysin in relation to pneumococcal carriage and acute otitis media. *J Infect Dis.* 2000;182:1146-1152.

159. Scott JA, Obiero J, Hall AJ, Marsh K. Validation of immunoglobulin G enzyme-linked immunosorbent assay for antibodies to pneumococcal surface adhesin A in the diagnosis of pneumococcal pneumonia among adults in Kenya. *J Infect Dis.* 2002;186(2):220-226.

160. Scott JA, Hannington A, Marsh K, Hall AJ. Diagnosis of pneumococcal pneumonia in epidemiological studies: evaluation in Kenyan adults of a serotype-specific urine latex agglutination assay. *Clin Infect Dis.* 1999;28(4):764-769.

161. Smith MD, Derrington P, Evans R, et al. Rapid diagnosis of bacteremic pneumococcal infections in adults by using the Binax NOW Streptococcus pneumoniae urinary antigen test: a prospective, controlled clinical evaluation. *J Clin Microbiol.* 2003;41(7):2810-2813.

162. Marchant CD, Band E, Froeschle JE, McVerry PH. Depression of anticapsular antibody after immunization with Haemophilus influenzae type b polysaccharide-diphtheria conjugate vaccine. *Pediatr Infect Dis J.* 1989;8:508-511.

163. Humphrey JH, Parrott DMV, East J. Studies of globulin and antibody production in mice thymectomized at birth. *Immunology.* 1964;7: 419-439.

164. Herzenberg LA, Stall AM, Lalor PA, Sidman C, Moore WA, Parks DR. The LY-1 B cell lineage. *Immunolol Rev.* 1986;93:81-102.

165. Guinamard R, Okigaki M, Schlessinger J, Ravetch JV. Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. *Nat Immunol.* 2000;1(1):31-36.

166. Martin F, Oliver AM, Kearney JF. Marginal zone and B1 B cells unite in the early response against T- independent blood-borne particulate antigens. *Immunity.* 2001;14(5):617-629.

167. Pillai S, Cariappa A, Moran ST. Marginal zone B cells. *Annu Rev Immunol.* 2005;23:161-196.

168. Winslow MM, Gallo EM, Neilson JR, Crabtree GR. The calcineurin phosphatase complex modulates immunogenic B cell responses. *Immunity.* 2006;24(2):141-152.

169. Haas KM, Poe JC, Steeber DA, Tedder TF. B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S. pneumoniae. *Immunity.* 2005;23(1):7-18.

170. Khan WN, Alt FW, Gerstein RM, et al. Defective B cell development and function in Btk-deficient mice. *Immunity.* 1995;3(3):283-28/9.

171. Harding CV, Roof RW, Allen PM, Unanue ER. Effects of pH and polysaccharides on peptide binding to class II major histocompatibility complex molecules. *Proc Natl Acad Sci U S A.* 1991;88(7):2740-2744.

172. Leyva-Cobian F, Unanue ER. Intracellular interference with antigen presentation. *J*

Immunol. 1988;141:1445-1450.

173. Weissman IL, Gutman GA, Friedberg SH, Jerabek L. Lymphoid tissue architecture. III. Germinal centers, T cells, and thymus-dependent vs thymus-independent antigens. *Adv Exp Med Biol.* 1976;66:229-237.

174. Baker PJ, Stashak PW, Amsbaugh DF, Prescott B. Characterization of the antibody response to type III pneumococcal polysaccharide at the cellular level. I. Dose-response studies and the effect of prior immunization on the magnitude of the antibody response. *Immunol.* 1971;20:469-480.

175. Klaus GG, Humphrey JH. The immunological properties of haptens coupled to thymus-independent carrier molecules. I. The characteristics of the immune response to dinitrophenol-lysine-substituted pneumococcal polysaccharide (SIII) and levan. *Eur J Immunol.* 1974;4: 370-377.

176. Baker PJ. Regulation of magnitude of antibody response to bacterial polysaccharide antigens by thymus-derived lymphocytes. *Infect Immun.* 1990;58:3465-3468.

177. Jeurissen A, Billiau AD, Moens L, et al. CD4+ T lymphocytes expressing CD40 ligand help the IgM antibody response to soluble pneumococcal polysaccharides via an intermediate cell type. *J Immunol.* 2006;176(1):529-536.

178. Sen G, Khan AQ, Chen Q, Snapper CM. In vivo humoral immune responses to isolated pneumococcal polysaccharides are dependent on the presence of associated TLR ligands. *J Immunol.* 2005;175(5): 3084-3091.

179. Cobb BA, Kasper DL. Zwitterionic capsular polysaccharides: the new MHCII-dependent antigens. *Cell Microbiol.* 2005;7(10):1398-1403.

180. Carroll WL, Adderson EE, Lucas AH, et al. Molecular basis of antibody diversity. In: Ellis RW, Granoff DM, eds. *Development and Clinical Uses of Haemophilus b Conjugate Vaccines.* 1st ed. New York, NY: Marcel Dekker, Inc; 1994:207-229.

181. Claflin JL, Hudak S, Maddalena A. Anti-phosphocholine hybridoma antibodies. I. Direct evidence for three distinct families of antibodies in the murine response. *J Exp Med.* 1981;153:352-364.

182. Scott MG, Crimmins DL, McCourt DW, et al. Clonal characterization of the human IgG antibody repertoire to Haemophilus influenzae type b polysaccharide. III. A single VKII gene and one of several JK genes are joined by an invariant arginine to form the most common L chain V region. *J Immunol.* 1989;143:4110-4116.

183. Gearhart PJ, Johnson ND, Douglas R, Hood L. IgG antibodies to phosphorylcholine exhibit more diversity than their IgM counterparts. *Nature.* 1981;291:29-34.

184. Hetherington SV. The intrinsic affinity constant (K) of anticapsular antibody to oligosaccharides of Haemophilus influenzae type b. *J Immunol.* 1988;140:3966-3970.

185. Robbins JB, Austrian R, Lee CJ, et al. Considerations for formulating the second-generation pneumococcal capsular polysaccharide vaccine with emphasis on the cross-reactive types within groups. *J Infect Dis.* 1983;148:1136-1159.

186. Fothergill LD, Wright J. Influenzal meningitis: the regulation of age incidence to the bactericidal power of blood against the causal organism. *J Immunol.* 1933;24:273-284.

187. Takala AK, Eskola J, Leinonen M, et al. Reduction of oropharyngeal carriage of Haemophilus influenzae type b (Hib) in children immunized with an Hib conjugate vaccine. *J Infect Dis.* 1991;164:982-986.

188. Girard MP, Preziosi MP, Aguado MT, Kieny MP. A review of vaccine research and development: meningococcal disease. *Vaccine.* 2006;24(22):4692-4700.

189. Shinefield HR, Black S. Efficacy of pneumococcal conjugate vaccines in large scale field trials. *Pediatr Infect Dis J.* 2000;19:394-397.

190. Anonymous. Licensure of a 13-valent pneumococcal conjugate vaccine (PCV13) and recommendations for use among children-Advisory Committee on Immunization Practices (ACIP), 2010.*MMWR.* 2010;59(9):258-261.

191. Whitney CG, Farley MM, Hadler J, et al. Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. *N Engl J Med.* 2003;348(18):1737-1746.

192. Donnelly JJ, Deck RR, Liu MA. Immunogenicity of A Haemophilus influenzae polysaccharide-Neisseria meningitidis outer membrane protein complex conjugate vaccine. *J Immunol.* 1990;145:3071-3079.

193. Latz E, Franko J, Golenbock DT, Schreiber JR. Haemophilus influenzae type b-outer membrane protein complex glycoconjugate vaccine induces cytokine production by engaging human toll-like receptor 2 (TLR2) and requires the presence of TLR2 for optimal immunogenicity. *J Immunol.* 2004;172(4):2431-2438.

194. Oliver AM, Martin F, Gartland GL, Carter RH, Kearney JF. Marginal zone B cells exhibit unique activation, proliferative and immunoglobulin secretory responses. *Eur J Immunol.* 1997;27(9):2366-2374.

195. Lopes-Carvalho T, Foote J, Kearney JF. Marginal zone B cells in lymphocyte activation and regulation. *Curr Opin Immunol.* 2005;17(3): 244-250.

196. Ferguson AR, Youd ME, Corley RB. Marginal zone B cells transport and deposit

IgM-containing immune complexes onto follicular dendritic cells. *Int Immunol.* 2004;16(10):1411-1422.

197. Wu ZQ, Vos Q, Shen Y, et al. In vivo polysaccharide-specific IgG isotype responses to intact Streptococcus pneumoniae are T cell dependent and require CD40- and B7-ligand interactions. *J Immunol.* 1999;163: 659-667.

198. Hwang Y, Nahm M, Briles D, Thomas D, Purkerson J. Acquired, but not innate, immune responses to Streptococcus pneumoniae are compromised by neutralization of CD40L. *Infect Immun.* 2000;68(2): 511-517.

199. Wu ZQ, Khan AQ, Shen Y, et al. B7 requirements for primary and secondary protein- and polysaccharide-specific Ig isotype responses to Streptococcus pneumoniae. *J Immunol.* 2001;165:6840-6848.

200. Snapper CM, Shen Y, Khan AQ, et al. Distinct types of T-cell help for the induction of a humoral immune response to Streptococcus pneumoniae. *Trends Immunol.* 2001;22(6):308-311.

201. Briles DE, Nahm M, Marion TN, Perlmutter RM, Davie JM. Streptococcal group A carbohydrate has properties of both a thymusindependent (TI-2) and a thymus-dependent antigen. *J Immunol.* 1982;128:2032-2035.

202. Balazs M, Martin F, Zhou T, Kearney J. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. *Immunity.* 2002;17(3):341-352.

203. Colino J, Shen Y, Snapper CM. Dendritic cells pulsed with intact Streptococcus pneumoniae elicit both protein- and polysaccharidespecific immunoglobulin isotype responses in vivo through distinct mechanisms. *J Exp Med.* 2002;195(1):1-13.

204. Bouvet J, Dighiero G. From natural polyreactive autoantibodies to à la carte monoreactive antibodies to infectious agents: is it a small world after all? *Infect Immun.* 1998;66:1-4.

205. Lal RB, Paranjape RS, Briles DE, Nutman TB, Ottesen EA. Circulating parasite antigen(s) in lymphatic filariasis: use of monoclonal antibodies to phosphocholine for immunodiagnosis. *J Immunol.* 1987;138: 3454-3460.

206. Briles DE, Nahm M, Schroer K, et al. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 Streptococcus pneumoniae. *J Exp Med.* 1981;153:694- 705.

207. Weiser JN, Shchepetov M, Chong ST. Decoration of lipopolysaccharide with phosphorylcholine: a phase-variable characteristic of Haemophilus influenzae. *Infect Immun.* 1997;65:943-950.

208. Heidelberger M, Rebers PA. Immunochemistry of the pneumococcal types II, V, and VI. The relation of type VI to type II and other correlations between chemical constitution and precipitation in antisera to type VI. *J Bacteriol.* 1960;80:145-153.

209. MacPherson CF, Heidelberger M, Alexander HE, Leidy G. The specific polysaccharides of types A, B, C, D, and F Hemophilus influenzae. *J Immunol.* 1946;52:207-219.

210. Heidelberger M, Bernheimer AW. Cross-reactions of polysaccharides of fungi, molds, and yeasts in anti-pneumococcal and other antisera. *Proc Nat Acad Sci U S A.* 1984;81:5247-5249.

211. Lucas AH, Langley RJ, Granoff DM, Nahm MH, Kitamura MY, Scott MG. An idiotypic marker associated with a germ-line encoded kappa light chain variable region that predominates the vaccine-induced human antibody response to the Haemophilus influenzae b polysaccharide. *J Clin Invest.* 1991;88:1811-1818.

212. Moxon ER, Anderson P. Meningitis caused by Haemophilus influenzae in infant rats: protective immunity and antibody priming by gastrointestinal colonization with Escherichia coli. *J Infect Dis*. 1979; 140:471-478.

213. Galili U, Mandrell RE, Hamadeh RM, Shohet SB, Griffiss JM. Interaction between human natural anti-alpha-galactosyl immunoglobulin G and bacteria of the human flora. *Infect Immun.* 1988;56:1730-1737.

214. Avila JL, Rojas M, Galili U. Immunogenic Gal-alpha-1—3Gal carbohydrate epitopes are present on pathogenic American Trypanosoma and Leishmania. *J Immunol.* 1989;142:2828-2834.

215. Apicella MA, Westerink MA, Morse SA, Schneider H, Rice PA, Griffiss JM. Bactericidal antibody response of normal human serum to the lipooligosaccharide of Neisseria gonorrhoeae. *J Infect Dis.* 1986;153: 520-526.

216. Ginsburg CM, McCracken GH Jr, Schneerson R, Robbins JB, Parke JC Jr. Association between cross-reacting Escherichia coli K100 and disease caused by Haemophilus influenzae type b. *Infect Immun.* 1978;22: 339-342.

217. Knight KL, Crane MA. Generating the antibody repertoire in rabbit. *Advan Immunol*. 1994;56:179-218.

218. Langermann S, Palaszynski S, Barnhart M, et al. Prevention of mucosal Escherichia coli infection by FimH-adhesin-based systemic vaccination. *Science.* 1997;276:607-611.

219. Cross AS, Opal S, Cook P, Drabick J, Bhattacharjee A. Development of an anti-core

lipopolysaccharide vaccine for the prevention and treatment of sepsis. *Vaccine.* 2004;22(7):812-817.

220. Ziegler EJ, McCutchan JA, Fierer J, et al. Treatment of gram-negative bacteremia and shock with human antiserum to a mutant Escherichia coli. *N Engl J Med.* 1982;307(20):1225-1230.

221. Theilacker C, Kaczynski Z, Kropec A, et al. Opsonic antibodies to Enterococcus faecalis strain 12030 are directed against lipoteichoic acid. *Infect Immun.* 2006;74(10):5703-5712.

222. Ren B, McCrory MA, Pass C, et al. The virulence function of Streptococcus pneumoniae surface protein A involves inhibition of complement activation and impairment of complement receptor-mediated protection. *J Immunol.* 2004;173(12):7506-7512.

223. Lewis LA, Gray E, Wang YP, Roe BA, Dyer DW. Molecular characterization of hpuAB, the haemoglobin-haptoglobin-utilization operon of Neisseria meningitidis. *Mol Microbiol.* 1997;23:737-749.

224. Pettersson A, Poolman JT, van der Ley P, Tommassen J. Response of Neisseria meningitidis to iron limitation. *Antonie Van Leeuwenhoek.* 1997;71:129-136.

225. Winkelstein JA. The complement system. In: Gorbach SL, Bartlett JG, Blacklow NR, eds. *Infectious Diseases.* 1st ed. Philadelphia, PA: WB Saunders Co; 1992:37-43.

226. Frank MM, Fries LF. The role of complement in defense against bacterial disease. *Baillieres Clin Immunol Allergy.* 1988;2:335-361.

227. Van der Lay P, Heckels JE, Virji M, Hoogerhout P, Poolman JT. Topology of outer membrane porins in pathogenic Neisseria spp. *Infect Immun.* 1991;59:2963-2971.

228. Nielsen SV, Sorensen UBS, Henrichsen J. Antibodies against pneumococcal C-polysaccharide are not protective. *Microb Pathog.* 1993;14:299-305.

229. Goldenberg HB, McCool TL, Weiser JN. Cross-reactivity of human immunoglobulin G2 recognizing phosphorylcholine and evidence for protection against major bacterial pathogens of the human respiratory tract. *J Infect Dis.* 2004;190(7):1254-1263.

230. Boes M, Prodeus AP, Schmidt T, Carroll MC, Chen J. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. *J Exp Med.* 1998;188(12):2381-2386.

231. Cooper NR. The classical complement pathway: activation and regulation of the first complement component. *Adv Immunol.* 1985;37: 151-216.

232. Mostov KE. Transepithelial transport of immunoglobulins. *Annu Rev Immunol.* 1994;12:63-84.

233. McDaniel LS, Benjamin WH, Forman C, Briles DE. Blood clearance by anti-phosphocholine antibodies as a mechanism of protection in experimental pneumococcal bacteremia. *J Immunol.* 1984;133: 3308-3312.

234. Schreiber JR, Barrus V, Cates KL, Siber GR. Functional characterization of human IgG, IgM, and IgA antibody directed to the capsule of Haemophilus influenzae type b. *J Infect Dis.* 1986;153:8-16.

235. Schlesinger JJ, Foltzer M, Chapman S. The Fc portion of antibody to yellow fever virus NS1 is a determinant of protection against YF encephalitis in mice. *Virology.* 1993;192:132-141.

236. Yuan R, Casadevall A, Spira G, Scharff MD. Isotype switching from IgG3 to IgG1 converts a nonprotective murine antibody to Cryptococcus neoformans into a protective antibody. *J Immunol.* 1995;154(4): 1810-1816.

237. Possee RD, Schild GC, Dimmock NJ. Studies on the mechanism of neutralization of influenza virus by antibody: evidence that neutralizing antibody inactivates influenza virus by inhibiting virion transcriptase activity. *J Gen Virol.* 1997;58:373-386.

238. Jefferis R, Pound J, Lund J, Goodall M. Effector mechanisms activated by human IgG subclass antibodies: clinical and molecular aspects. *Ann Biol Clin.* 1994;52:57-65.

239. Burton DR, Woof JM. Human antibody effector function. *Adv Immunol.* 1992;51:1-84.

240. Cooper LJ, Shikhman AR, Glass DD, Kangisser D, Cunningham MW, Greenspan NS. Role of heavy chain constant domains in antibody-antigen interaction. Apparent specificity differences among streptococcal IgG antibodies expressing identical variable domains. *J Immunol.* 1993;150:2231-2242.

241. Yoo EM, Wims LA, Chan LA, Morrison SL. Human IgG2 can form covalent dimers. *J Immunol.* 2003;170(6):3134-3138.

242. Briles DE, Forman C, Hudak S, Claflin JL. The effects of subclass on the ability of anti-phosphocholine antibodies to protect mice from fatal infection with Streptococcus pneumoniae. *J Mol Cell Immunol.* 1984;1:305-309.

243. Jonsson G, Oxelius VA, Truedsson L, Braconier JH, Sturfelt G, Sjoholm AG. Homozygosity for the IgG2 subclass allotype G2M(n) protects against severe infection in hereditary C2 deficiency. *J Immunol.* 2006;177(1):722-728.

244. Bredius RGM, de Vries CEE, Troelstra A, et al. Phagocytosis of Staphylococcus aureus and Haemophilus influenzae type b opsonizied with polyclonal human IgG1 and IgG2 antibodies. *J Immunol.* 1993;151:1463-1472.

245. Lefranc M, Lefranc G, Rabbitts TH. Inherited deletion of immunoglobulin heavy chain constant region genes in normal human individuals. *Nature.* 1982;300:760-762.

246. Ravetch JV, Kinet J. Fc receptors. *Annu Rev Immunol.* 1991;9:457-492.

247. Gorter A, Hiemstra PS, Leijh PCJ, et al. IgA- and secretory IgA-opsonized S. aureus induce a respiratory burst and phagocytosis by polymorphonuclear leucocytes. *Immunol.* 1987;61:303-309.

248. Hiemstra PS, Gorter A, Stuurman ME, van Es LA, Daha MR. Activation of alternative pathway of complement by human serum IgA. *Euro J Immunol.* 1987;17:321-326.

249. Jarvis GA, Griffiss JM. Human IgA1 initiates complement-mediated killing of Neisseria meningitidis. *J Immunol.* 1989;143:1703-1709.

250. Imai H, Chen RJ, Wyatt RJ, Rifai A. Lack of complement activation by human IgA immune complexes. *Clin Exper Immunol.* 1988;73: 479-483.

251. Jarvis GA, Griffiss JM. Human IgA1 blockade of IgG-initiated lysis of Neisseria meningitidis is a function of antigen-binding fragment binding to the polysaccharide capsule. *J Immunol.* 1991;147:1962-1967.

252. Nikolova EB, Tomana M, Russell MW. All forms of human IgA antibodies bound to antigen interfere with complement (C3) fixation induced by IgG or by antigen alone. *Scand J Immunol.* 1994;39(3):275-280.

253. Russell MW, Mansa B. Complement-fixing properties of human IgA antibodies. Alternative pathway complement activation by plastic-bound, but not specific antigen-bound, IgA. *Scand J Immunol.* 1989;30(2):175-183.

254. Roos A, Bouwman LH, van Gijlswijk-Janssen DJ, Faber-Krol MC, Stahl GL, Daha MR. Human IgA activates the complement system via the mannan-binding lectin pathway. *J Immunol.* 2001;167(5):2861-2868.

255. Janoff EN, Fasching C, Orenstein JM, Rubins JB, Opstad NL, Dalmasso AP. Killing of Streptococcus pneumoniae by capsular polysaccharidespecific polymeric IgA, complement, and phagocytes. *J Clin Invest.* 1999;104(8):1139-1147.

256. Michetti P, Mahan MJ, Slauch JM, Mekalanos JJ, Neutra MR. Monoclonal secretory immunoglobulin A protects mice against oral challenge with the invasive pathogen Salmonella typhimurium. *Infect Immun.* 1992;60:1786-1792.

257. Sun K, Johansen FE, Eckmann L, Metzger DW. An important role for polymeric Ig receptor-mediated transport of IgA in protection against Streptococcus pneumoniae nasopharyngeal carriage. *J Immunol.* 2004;173(7):4576-4581.

258. Lamm ME. Interaction of antigens and antibodies at mucosal surfaces. *Ann Rev Microbiol.* 1997;51:311-340.

259. Brandtzaeg P, Fjellanger I, Gjeruldsen ST. Immunoglobulin M: local synthesis and selective secretion in patients with immunoglobulin A deficiency. *Science.* 1968;160:789-791.

260. Sieling PA, Chatterjee D, Porcelli SA, et al. CD1-restricted T cell recognition of microbial lipoglycan antigens. *Science.* 1995;269:227-230.

261. Tzianabos AO, Finberg RW, Wang Y, et al. T cells activated by zwitterionic molecules prevent abscesses induced by pathogenic bacteria. *J Biol Chem.* 2000;275(10):6733-6740.

262. Harrington LE, Hatton RD, Mangan PR, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol.* 2005;6:1123-1132.

263. Langrish CL, Chen Y, Blumenschein WM, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med.* 2005;201:233-240.

264. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17 producing T cells. *Immunity.* 2006;24:179-189.

265. Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol.* 2007;25:821-852.

266. Chung DR, Kasper DL, Panzo RJ, et al. CD4+ T cells mediate abscess formation in intra-abdominal sepsis by an IL-17-dependent mechanism. *J Immunol.* 2003;170(4):1958-1963.

267. Lu YJ, Gross J, Bogaert D, et al. Interleukin-17A mediates acquired immunity to pneumococcal colonization. *PLoS Pathog.* 2008;4(9):e1000159.

268. Minegishi Y, Saito M, Nagasawa M, et al. Molecular explanation for the contradiction between systemic Th17 defect and localized bacterial infection in hyper-IgE syndrome. *J Exp Med.* 2009;206:1291-1301.

269. Puel A, Cypowyj S, Bustamante J, et al. Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science.* 2011;332(6025):65-68.

270. Bhatt SM, Cabellos C, Nadol JB Jr, et al. The impact of dexamethasone on hearing loss in experimental pneumococcal meningitis. *Pediatr Infect Dis J.* 1995;14(2):93-96.

271. Kengatharan KM, De Kimpe S, Robson C, Foster SJ, Thiemermann C. Mechanism of gram-positive shock: identification of peptidoglycan and lipoteichoic acid moieties essential in the induction of nitric oxide synthase, shock, and multiple organ failure. *J Exp Med.* 1998; 188(2):305-315.

272. Gutierrez-Ramos JC, Bluethmann H. Molecules and mechanisms operating in septic shock: lessons from knockout mice. *Immunol Today.* 1997;18:329-334.

273. Hanna PC, Acosta D, Collier RJ. On the role of macrophages in anthrax. *Proc Natl Acad Sci U S A.* 1993;90(21):10198-10201.

274. O'Brien AD, Rosenstreich DL, Scher I, Campbell GH, MacDermott RP, Formal SB. Genetic control of susceptibility to Salmonella typhimurium in mice: role of the Lps gene. *J Immunol.* 1980;124:20-24.

275. Hagberg L, Briles DE, Eden CS. Evidence for separate genetic defects in C3H/HeJ and C3HeB/FeJ mice, that affect susceptibility to gramnegative infections. *J Immunol.* 1985;134:4118-4122.

276. Llewelyn M, Cohen J. Superantigens: microbial agents that corrupt immunity. *Lancet Infect Dis.* 2002;2:156-162.

277. Petersson K, Forsberg G, Walse B. Interplay between superantigens and receptors. *Scand J Immunol.* 2004;59:345-355.

278. Alouf JE, Muller-Alouf H. Staphylococcal and streptococcal superantigens: molecular, biological and clinical aspects. *Int J Med Microbiol.* 2003;292:429-440.

279. Bachert C, Gevaert P, van Cauwenberge P. Staphylococcus aureus enterotoxins: a key in airway disease? *Allergy.* 2002;57:480-487.

280. Sriskandan S, Faulkner L, Hopkins P. Streptococcus pyogenes: Insight into the function ofthe streptococcal superantigen. *Intl J Biochem Cell Biol.* 2007;39:12-19.

281. Moran AP, Prendergast MM, Appelmelk BJ. Molecular mimicry of host structures by bacterial lipopolysaccharides and its contribution to disease. *FEMS Immunol Med Microbiol.* 1996;16:105-115.

282. Finne J, Leinonen M, Makela PH. Antigenic similarities between brain components and bacteria causing meningitis. Implications for vaccine development and pathogenesis. *Lancet.* 1983;2(8346):355-357.

283. Rougon G, Dubois C, Buckley N, Magnani JL, Zollinger W. A monoclonal antibody against meningoccus group B polysaccharides distinguishes embryonic from adult N-CAM. *J Cell Biol.* 1986;103: 2429-2437.

284. Rees JH, Soudain SE, Gregson NA, Hughes RA. Campylobacter jejuni infection and Guillain-Barré syndrome. *N Eng J Med.* 1995;333: 1374-1379.

285. Yuki N. Campylobacter sialyltransferase gene polymorphism directs clinical features of Guillain-Barré syndrome. *J Neurochem.* 2007;103(suppl 1):150-158.

286. Bessen D, Jones KF, Fischetti VA. Evidence for two distinct classes of streptococcal M protein and their relationship to rheumatic fever. *J Exp Med.* 1989;169:269-283.

287. Hollingshead SK, Bessen DE. Evolution of the emm gene family: virulence gene clusters in group A streptococci. *Dev Biol Standard.* 1995;85:163-168.

288. Cunningham MW, McCormack JM, Fenderson PG, Ho MK, Beachey EH, Dale JB. Human and murine antibodies cross-reactive with streptococcal M protein and myosin recognize the sequence gln-lys-serlys- gln in M protein. *J Immunol.* 1989;143:2677-2683.

289. Cunningham MW, Antone SM, Gulizia JM, McManus BA, Gauntt CJ. Alpha-helical coiled-coil molecules: a role in autoimmunity against the heart. *Clin Immunol Immunopathol.* 1993;68:118-123.

290. Cunningham MW. Streptococci and rheumatic fever. In: Friedman H, Rose NR, Bendinelli M, eds. *Microorganisms and Autoimmune Disease.* New York, NY: Plenum Press; 1996:13-66.

291. Cunningham MW, Antone SM, Gulizia JM, McManus BM, Fischetti VA, Gauntt CJ. Cytotoxic and viral neutralizing antibodies crossreact with streptococcal M protein, enteroviruses and human cardiac myosin. *Proc Nat Acad Sci U S A.* 1992;89:1320-1324.

292. Chow LH, Yuling Y, Linder J, McManus BM. Phenotypic analysis of infiltrating cells in human myocarditis. An immunohistochemical study in paraffin-embedded tissue. *Arch Pathol Lab Med.* 1989;113:1357-1362.

293. Guilherme L, Chuna-Neto E, Coelho V, et al. Human heart-infiltrating T cell clones from rheumatic heart disease patients recognize both streptococcal and cardiac proteins. *Circulation.* 1995;92:415-446.

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Chapter 42

Immunology of Human Immunodeficiency Virus Infection

Douglas S. Kwon

Bruce D. Walker

INTRODUCTION

In June 1981, the U.S. Centers for Disease Control and Prevention (CDC) reported five cases of *Pneumocystis* pneumonia (PCP) in otherwise healthy young gay men in Los Angeles, California,[1] representing the first published evidence of what was to become the acquired immunodeficiency syndrome (AIDS) epidemic. At that time PCP was rare, known as an “opportunistic infection” that occurred in the setting of severe immune compromise resulting from cancer treatment. At the same time, there were also reports of an aggressive form of Kaposi sarcoma, a generally benign cancer, in the same demographic, namely young gay men.[2] Like PCP, Kaposi sarcoma was infrequent and was typically seen in older men or those who were immunosuppressed following organ transplantation. It made sense in the setting of severe immune compromise to see PCP or Kaposi sarcoma, but what was alarming in those initial reports was that it was being seen in otherwise seemingly healthy people.

At that time pentamidine, the main drug used to treat PCP, was only available through the CDC. Coincident with the first PCP cases reported in Los Angeles, there was a dramatic increase in requests for pentamidine from many other cities across the United States. A consistent and disturbing pattern was quickly noted—the drug was largely being requested for young men who had no known reason to be immune compromised and at risk for PCP. An emerging epidemic was thus recognized, and already from those first patients there were clues pointing to the central problem induced by what was ultimately recognized to be infection by a new human retrovirus: that human immunodeficiency virus (HIV) is an infection of the immune system.

When an antibody test for HIV became available in 1985, the kinetics of the expanding epidemic first began to be realized through retrospective examination of stored serum samples. Results from analysis of samples from a hepatitis B vaccine trial that had coincidentally been initiated in 1980 in gay and bisexual men in San Francisco, California, were startling.[3] By 1982, the cumulative incidence of infection in those at-risk men had risen to 42.6%. HIV has since gone on to infect over 60 million people worldwide and has caused over 30 million deaths.[4] In some of the most affected areas of the world, HIV prevalence exceeds 50% in certain communities and age groups (Fig. 42.1).[5]

Thankfully, the development of effective HIV drug therapy has turned HIV infection into a

treatable condition for those fortunate to have access to these medications,[6] but viral integration into the host chromosome makes the need for treatment lifelong.[7] Regrettably, for each person who begins antiretroviral therapy (ART) in resource-limited settings, there are two who become newly infected.[4] The ultimate resolution of the epidemic will almost certainly require an effective vaccine, but despite nearly a billion dollars invested annually in AIDS vaccine development, this has remained an elusive goal. Unlike other infections for which effective vaccines exist, for HIV there is a lack of natural clearance and subsequent durable protection.[8] As natural protective immunity does not exist, an effective HIV vaccine will have to do better than nature does.

HUMAN IMMUNODEFICIENCY VIRUS ORIGIN, TRANSMISSION, AND DISEASE PROGRESSION

Origin of Human Immunodeficiency Virus and Relationship to Other Retroviruses

The isolation of a retrovirus in 1983 from a patient with AIDS in France led to wider sampling of at-risk persons and was paralleled by the discovery of other primate retroviruses. Sequencing of HIV revealed enormous variability among isolates, which were ultimately phylogenetically characterized as belonging to four distinct groups. Group M is the largest and accounts for over 98% of infections worldwide.[9] Based on geographically targeted sequencing, it appears that the epicenter of the global group M epidemic was in Kinshasa in the Democratic Republic of the Congo,[10] and that a cross-species transmission of simian immunodeficiency virus (SIV) from chimpanzees occurred in the early 1900s.[11,12] In turn, chimpanzees had acquired SIV from cross-species transmission of two viruses in monkeys that recombined. The actual origin of the group M epidemic in humans appears to have been in a remote region of southeastern Cameroon, likely by exposure through butchering of a chimpanzee.[13] Much less frequent are groups N, O, and P, the result of separate cross-species transmissions from chimpanzees (groups N and O) and gorillas (group P), which have remained localized to Cameroon and neighboring countries in west Africa.[9,14,15]

HIV is a typical retrovirus, morphologically characterized by a core containing the viral ribonucleic acid (RNA) and two copies of reverse transcriptase (RT), surrounded by an outer envelope that is heavily glycosylated. The errorprone RT leads to the rapid generation of new mutations, resulting in enormous genetic variation within group M viruses globally. This is largely due to lack of 3′ exonuclease proofreading activity of RT, which gives rise to the mutation of approximately 1 in every 10,000 nucleotides during viral genome transcription; in other words, once per replication

cycle[16]. Additional factors are the frequent viral recombination and the lifelong nature of infection. Within an individual, this produces an enormously diverse quasispecies of viruses whose intraindividual diversity exceeds the global diversity of influenza in a single season[17] (Fig. 42.2). This represents a significant challenge to the immunologic control of infection and the development of broadly prophylactic vaccines. Currently, there are at least nine distinct clades, or subtypes, of group M HIV-1 worldwide, and many additional subtypes that are the result of recombination events between these major clades.

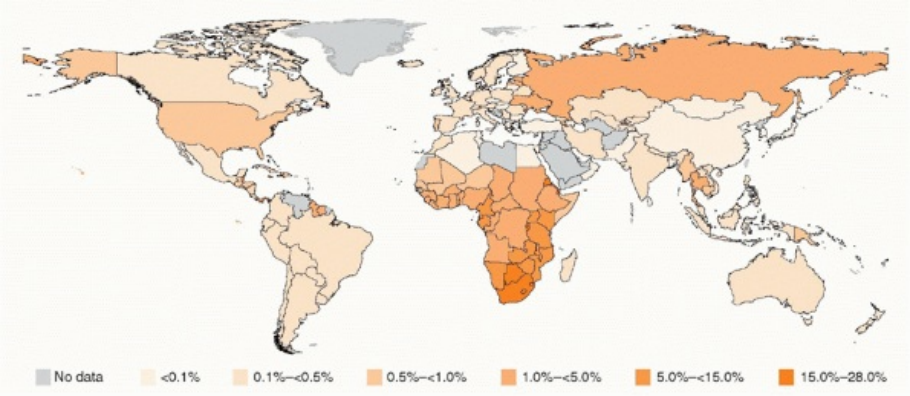

FIG. 42.1. Global Human Immunodeficiency Virus Prevalence by Country. From World Health Organization.[4]

HIV-1 is closely linked to HIV-2, a less pathogenic human retrovirus that is endemic in west Africa. HIV is also closely related to SIVs, a number of nonhuman primate retroviruses.[18] Although SIV was initially considered nonpathogenic in primates, infection of certain wild chimpanzee species results in CD4 T-cell decline and increased mortality. It is important to note that some wild nonhuman primate species tolerate their species-specific SIV infections quite well, including African green monkeys and sooty mangabeys, in whom the infection is characterized by persistent high viral load but a lack of CD4 cell decline or development of immune deficiency.[19]

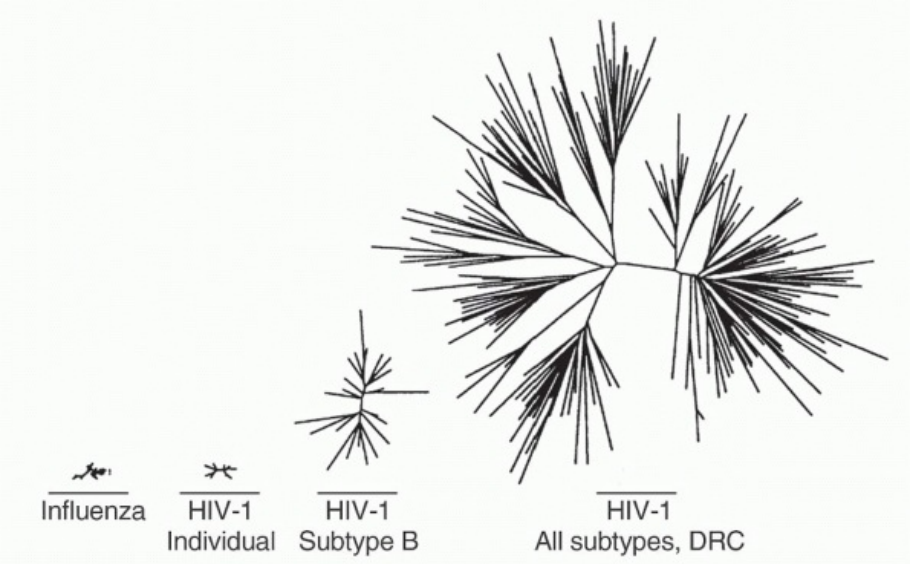

FIG. 42.2. Relative Viral Diversity of Influenza and Human Immunodeficiency Virus (HIV)-1. Comparison of influenza HA1 domain and HIV-1 envelope V2-C5 regions, starting from left with diversity of all H3N2 influenza sequences available from the 1996 influenza season, then HIV-1 subtype B sequence diversity from a single individual, HIV-1 sequences from a subtype B epidemic occurring in Amsterdam in 1990 to 1991, and

finally HIV-1 sequences from the Democratic Republic of Congo obtained in 1997. Adapted from Korber et al.[17]

Exploiting Knowledge of the Viral Life Cycle to Develop Antiviral Agents

Early in the HIV epidemic, it was realized that expression of CD4 was necessary for cells to become infected—a finding that was assisted by the recognition that CD4+ cells were specifically lost in progressive disease. However, it was also clear that CD4 alone was not sufficient. Expression of recombinant CD4 on murine cells failed to allow viral infection. The discovery of chemokine receptors as necessary coreceptors for HIV entry took over a decade to be realized and was facilitated by a remarkable convergence of clinical observation and basic science. Blood samples from persons identified to be very heavily exposed to HIV, who had never become infected, showed that a group of β chemokines: MIP-1α, MIP-1β, and RANTES, were secreted from CD8 T cells and could inhibit HIV replication.[20] These chemokines were known to be linked through a common receptor, CCR5, a member of a family of G-protein coupled seven transmembrane domain chemokine receptors. In the next year, CCR5 and another chemokine receptor, CXCR4, were identified as the long-sought HIV entry coreceptors whose expression along with CD4 were both necessary and sufficient to allow viral entry.[21,22,23] Shortly thereafter, it was discovered that homozygosity of a naturally occurring 32 base pair deletion of

CCR5 (CCR5 δ32) that abrogated surface expression of the protein and occurred in 1% of Caucasians imparted resistance to HIV acquisition, thus demonstrating the biologic significance of chemokine receptors for HIV infection.[24,25,26] As individuals lacking functional CCR5 were found to be largely immunologically intact, CCR5 became an attractive target for ART; in 2007, maraviroc, a negative allosteric modulator of the CCR5 receptor, became the first chemokine receptor antagonist approved for treatment of HIV infection.

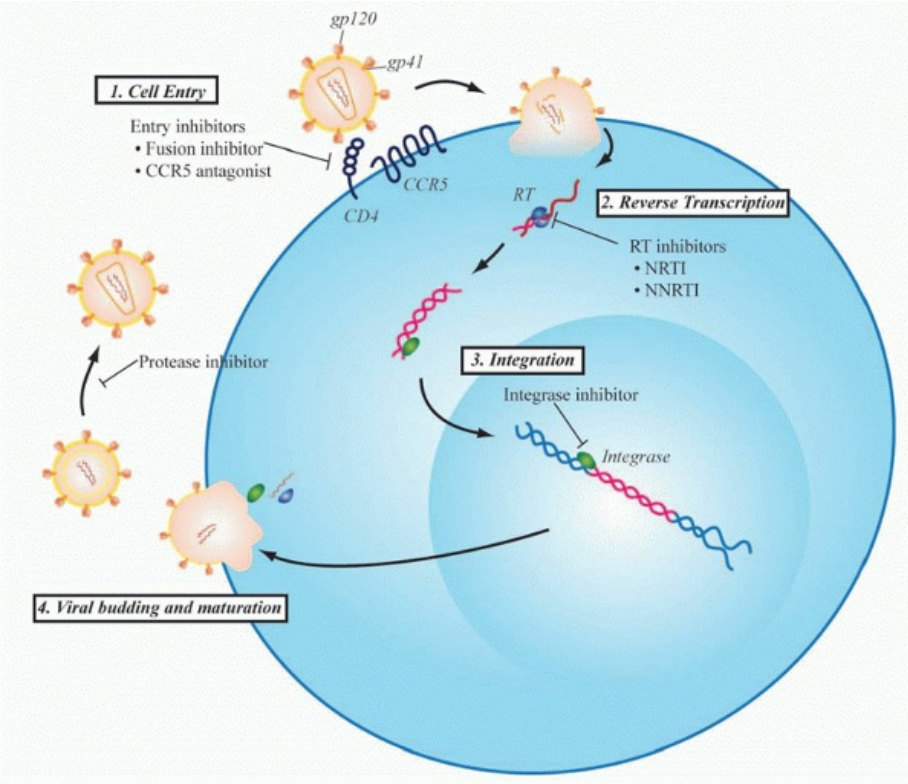

FIG. 42.3. Human Immunodeficiency Virus (HIV) Viral Life Cycle and Targets for Antiretroviral Therapy. The HIV envelope protein gp120 first contacts CD4 on the target cell, which then induces conformational changes in gp120 that exposes the chemokine receptor binding site. Following binding of chemokine receptor, HIV gp41 mediates fusion of target and viral membranes. The viral single-stranded ribonucleic acid genome is then reverse transcribed, and the viral genome is transported to the nucleus where it is integrated in the host genome. Viral genes are then transcribed and translated, and virions then bud from the host membrane. Following budding, virions mature in a process that requires cleavage of HIV polyproteins by the viral protease. Currently approved antiviral agents target entry, reverse transcription, viral genome integration, and viral protease function.

Even before the development of maraviroc, however, multiple viral proteins had been successfully targeted to treat HIV infection based upon a rapidly developing understanding of the viral life cycle (Fig. 42.3). HIV RT was the first viral protein to be successfully targeted by drug therapy, and the class of RT inhibitors remains the backbone of firstline regimens of combination ART strategies used today. Zidovudine was the first clinically approved HIV drug. It is a thymidine analog initially developed in 1964 as a potential anticancer agent. Over 20 years later, however, it was found to inhibit replication of HIV as part of a unique collaboration between the National Cancer Institute and the pharmaceutical company Burroughs Wellcome. When zidovudine was first tested for activity against HIV in 1985, there were over 10,000 people suffering from AIDS, a disease for which there was no available therapy at that time.[27] After proving to be safe in a small phase I trial, zidovudine quickly went on to efficacy studies and remarkably went on to be approved for clinical use by the U.S. Food and Drug Administration just 2 years after its first testing in the laboratory. This would be the first of

several drugs that target RT that now constitute one of the mainstays of HIV treatment.

The next major breakthrough in ART was the development of drugs that target the HIV protease. Protease is required for cleavage of viral polyproteins in immature virions released from the infected cell, a step necessary for virion assembly and maturation. The first protease inhibitor, saquinavir, was approved in 1995, and its development marked the age of modern multidrug ART. Up to that point, only four drugs had been developed, all nucleoside RT inhibitors (NRTIs). However, therapy with these NRTIs showed toxicity, and none of them alone or in combination could durably control HIV. Combination therapy with agents that inhibited both RT and protease showed strong in vitro synergy,[28] could achieve virologic suppression in vivo, and resulted in a dramatic decrease in AIDS deaths (Fig. 42.4).[29] This was quickly followed by the development of non-NRTIs, which also target RT and can also be used effectively with NRTIs to suppress viral replication. This marked the

modern era of combination ART and has changed HIV from a nearly uniformly terminal illness to a chronic treatable disease for those with access to medications.

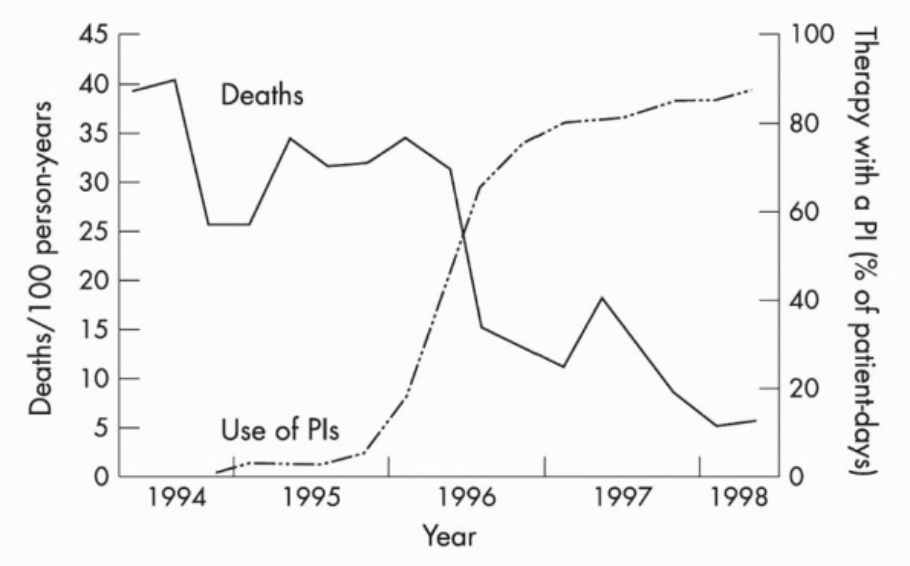

FIG. 42.4. Protease Inhibitors and Decline in Acquired Immunodeficiency Syndrome (AIDS) Mortality. Mortality from AIDS showed a significant decrease following the introduction of protease inhibitors, which were used in combination with agents that target human immunodeficiency virus (HIV) reverse transcriptase to treat HIV-infected individuals with combination therapy to suppress viral replication. From Palella et al.[29]

Since the development of RT and protease inhibitors, two other steps in the viral life cycle, entry and viral genome integration, have been successfully targeted by approved drugs. HIV entry is initiated when the viral envelope protein, gp120, contacts CD4 and a chemokine receptor on the target cell membrane. This triggers conformational changes in the transmembrane envelope glycoprotein, gp41, which leads to fusion of viral and host membranes. There are currently two antiretroviral agents that act at this early stage of entry:

maraviroc, a CCR5 antagonist that blocks the interaction of gp120 with CCR5, and enfuvirtide (Fuzeon, T-20), approved in 2003, a 36 amino acid peptide that binds to gp41 and prevents formation of a hairpin intermediate necessary for fusion of the viral and target cell membranes. Raltegravir targets HIV integrase and represents the newest class of antiretroviral agents to be developed. Integrase is the viral enzyme that catalyzes the deoxyribonucleic acid strand transfer reactions necessary for incorporation of the viral genome into the host genome. It represents one of the most potent of currently available HIV drugs and is generally one of the best tolerated therapies.

Although there are currently over 30 approved antiretroviral drugs in various formulations, demand continues for novel agents, especially as drug resistance becomes increasingly common. Studies in Europe have demonstrated that approximately 10% of all newly diagnosed patients harbor virus with at least one major resistance-associated mutation.[30] New drugs remain in development including maturation inhibitors, CD4 receptor inhibitors, and additional agents in existing classes.[31] The development of suppressive multidrug ART has saved millions of lives and has only been possibly with a detailed understanding of the molecular mechanisms of HIV pathogenesis. These drugs are now being used not only to treat those who are infected, but also are being tested for use in preventing infection in individuals at high risk of acquisition.[32]

Mucosal Transmission and Early Local Viral Replication

Transmission of HIV is relatively inefficient with a risk of approximately 1 in 1,000 sexual acts for male-to-female sexual transmission and 1 in 100 for receptive anal intercourse.[33] Over 90% of HIV transmissions worldwide occur across a mucosal barrier, either through sexual exposure or maternalto-child transmission, with the remaining cases due to direct blood exposure such as injection drug use. These mucosal surfaces are exposed to an enormously diverse quasispecies of genetically distinct virions, and yet the majority of transmissions are mediated by a single founder virus,[34] although as many as 16 viruses have been documented to be transmitted simultaneously.[35] Multiple transmitting founder viruses appear to be more prevalent in transmission occurring via intravenous drug use, suggesting that mucosal-specific factors are an important determinant for this restriction.[35] Following mucosal exposure, a small founder population establishes host infection by initially replicating locally within the exposed mucosal tissue for 7 to 10 days before disseminating to the draining lymph node and then moving quickly into the blood.[36] Once infection has spread to the periphery, a widespread reservoir of latently infected cells is rapidly established at multiple sites and subsequent viral eradication becomes enormously challenging due to this extensive reservoir.

The fact that HIV transmission is inherently inefficient and typically initiated by a single virion that requires days of local expansion before systemic infection can take place suggests that these early events represent a vulnerable stage in the process of transmission. Unfortunately, few HIVspecific B- or T-cell responses are detected locally in the mucosal site of exposure until well after viral dissemination, despite the fact that these sites contain abundant antigenpresenting cells and numerous lymphocytes.[37,38] This lack of early responses likely plays a critical role in the failure of the immune system to contain viral replication within the genital mucosa. Although some studies suggest that a subset of persons with multiple

exposures will develop adaptive immune responses to HIV,[39] and have suggested that these can protect against acquisition, this remains controversial.[40]

Transmission depends on specific properties of the envelope glycoprotein of HIV. Acute infection is typically established with R5 viruses that utilize CCR5 as a coreceptor. Over time, some viruses within the quasispecies of an infected person can evolve to an X4 phenotype, which is characterized by the use of CXCR4 as its coreceptor, but these viruses somehow are either prevented from establishing the initial infection or rapidly revert to R5 viruses upon transmission.

The mechanism of the transmission "bottleneck" remains incompletely understood. The efficiency of HIV transmission is actually quite low and highly linked to the viral load in the donor. A population study of 45 discordant couples, in which one partner is HIV positive and the other is not, showed a complete lack of transmission in the 15 couples in whom the infected partner had a viral load of < 1,500 RNA copies/uL plasma.[41] More recently, treatment of couples discordant for HIV infection has confirmed the impact of viral load on transmission: when the HIV-infected partner is on therapy, the risk of HIV transmission to his or her partner is reduced by 96%.[42] Periods of highest transmissibility tend to be the acute phase of infection, before viral load has declined to a steady state, and end stage disease, when viral load again rises.[43] Importantly, the highest viral loads are seen in acute infection, before antibodies become detectable, such that standard enzyme-linked immunosorbent assay antibody testing is typically negative at the time of highest risk of transmission. This is being addressed at least in part through next generation tests that detect both antibody as well as p24 antigen, decreasing the "window period" when persons are infected but test negative.

Acute Infection Syndrome and Early Infection

Most persons have had the symptoms that could be consistent with acute HIV infection at some point in their lives as these are not specific to HIV but rather are typical of many viral infections. Indeed, the diagnosis of acute HIV infection is frequently missed by health care providers because at the time of acute symptoms, anti-HIV antibodies have not yet

developed and thus persons test negative by the standard antibody tests that are used to diagnose infection. Most but not all patients are symptomatic with their initial acute infection, with fever, sore throat, swollen lymph nodes, fatigue, malaise, and often transient oral or genital ulcers.[44] The syndrome looks very much like acute infectious mononucleosis.

The acute symptoms, which are nonspecific, eventually resolve, and subsequent disease course is characterized by a gradual CD4 cell decline and slowly rising viral load, with the ultimate development of clinical AIDS when the CD4 count is < 200 cells/uL or certain AIDS-defining illnesses arise.

Initial peak viremia in newly infected adults is often 10 million viral particles per mL or more,[45] followed by a decline over the first few months to a quasisteady state at a mean of around 30,000 RNA copies/mL (Fig. 42.5).[46]

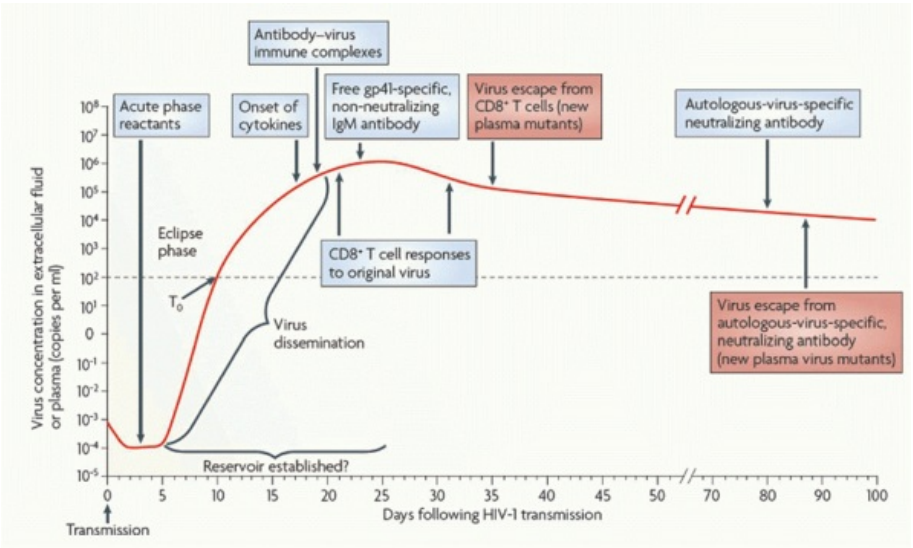

FIG. 42.5. Early Immune Responses in Acute Human Immuno deficiency Virus (HIV)-1 Infection. Acute infection is typically characterized by high viral loads and flu-like symptoms. There is an initial "eclipse phase" in which virus is present but antibody-based HIV tests remain negative. Early adaptive responses are comprised of HIVspecific CD8 T-cell responses and nonneutralizing antibody responses. The emergence of CD8 T-cell responses coincides with the decline of peak viral loads and the establishment of a viral set point. From McMichael et al.[190]

Current views of the very earliest stages in acute infection stem largely from studies in the nonhuman primate model of AIDS virus infection, in which rhesus macaques are infected with SIV. In high-dose challenge models of macaques, virus crosses the mucosal site of exposure within hours and initially establishes a small founder population of infected cells locally.[47] It is unclear how the virus crosses the mucosal epithelial barrier, but studies have suggested a variety of mechanisms, including transcytosis across epithelial cells, facilitation by mucosal dendritic cells (DCs), and/or entry through mechanical disruption or physiological intercellular spaces in the epithelium. Once across, the first cells to be infected are CD4 T cells. Surprisingly, though HIV preferentially infects activated CD4 cells, the first targets appear to be resting CD4 T cells, or cells that have been previously activated and still express CCR5 coreceptor but have entered into a more quiescent state. Once infection within the tissue is established, there is a period of local expansion that lasts for 7 to 10 days before the virus is disseminated to regional lymph nodes. This is then followed by rapid progression to the peripheral circulation and seeding of gut-associated lymphoid tissue (GALT). This gut reservoir contains 60% to 80% of the body's CD4 T cells, a large majority relative to the 2% to 3% of lymphocytes present in the peripheral circulation.

Based upon studies initially conducted in macaques and subsequently corroborated in humans, a hallmark of HIV infection is the rapid and profound depletion of GALT CD4 T cells in the lamina propria within the first weeks of HIV infection (Fig. 42.6). It appears that these cells never fully recover, even with prolonged treatment with antiviral medications that suppress peripheral viral loads and result in

rebound of CD4 T-cell counts in the peripheral blood.[48,49] This CD4 T-cell depletion is accompanied by extensive apoptosis of bystander cells, associated immune activation, and disruption of normal epithelial barrier function in the gut. Recent data suggest that bystander death actually involves abortive infection, with accumulation of cytoplasmic nucleic acids that elicit proapoptotic and proinflammatory responses.[50]

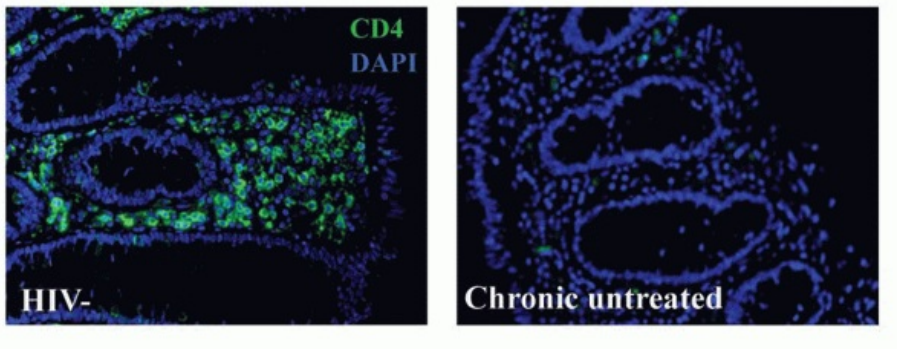

FIG. 42.6. Depletion of CD4 T Cells in the Intestinal Mucosa. Gut-associated lymphoid tissue contains the majority of the body's lymphocytes, which reside within both the lamina propria and lymphoid follicles. Lamina propria CD4 T cells are rapidly and massively depleted early in human immunodeficiency virus (HIV) infection. Shown are representative stains from colonic mucosa from HIV-infected and -uninfected individuals stained by immunofluorescence for CD4 (*green*) and nuclei (4′,6-diamidino-2-phenylindole [DAPI], *blue*).

Natural History of Untreated Infection

The typical course of untreated HIV infection until the development of AIDS, defined as a CD4 count of < 200 cells/μL or the presence of certain AIDS defining illnesses is approximately 10 years[51] and involves progressive immunologic decline. As the epidemic has matured, however, it has become clear that not all persons progress. There are now persons with documented untreated infection for more than 30 years who have maintained normal CD4 cell numbers and an undetectable viral load, whereas others progress from acute infection to AIDS in as little as 6 months without therapy.[52]

The best HIV natural history data derive from the Multicenter AIDS Cohort Study and other population studies established in the early days of the epidemic. The Multicenter AIDS Cohort Study, which involved recruitment of both infected and at risk men in Baltimore, Maryland, Chicago, Illinois, Los Angeles, California, and Pittsburgh, Pennsylvania, was established in 1984 and continues today. The study currently has almost 7,000 participants with an accumulated 87,000 person years of follow-up,[53] with a large repository of archived specimens. These multicenter seroprevalent and seroincident cohorts were pivotal in identifying laboratory predictors of prognosis, including CD4 T-cell count and viral load, which are both independent predictors of disease progression. Following acute infection, viral load reaches a quasi-set point within approximately 6 months of infection, with a median viral load around 30,000 RNA copies/mL and interquartile ranges from 12,000 to 80,000 copies/mL.

There is a marked increase in the development of opportunistic infections when CD4 count drops below 200 cells/μL, which led to the current recommendations that prophylactic treatment of opportunistic infections begin when this level is reached. The sequelae of chronic untreated HIV infection are the development of these opportunistic diseases, which are what led to the original identification of the new epidemic of HIV infection. What was being seen clinically was the end stage of a chronic infection in which immune dysfunction allowed the establishment of a number of otherwise rare illnesses.

Among the CDC AIDS-defining illnesses are candidiasis of the respiratory tract, *Pneumocystis jiroveci* pneumonia, cytomegalovirus disease, extrapulmonary cryptococcal disease, toxoplasmosis of the brain, and mycobacterial infection,[54] all indicative of an underlying defect in cellular immunity. *Mycobacterium tuberculosis* is a particularly devastating coinfection, in part because its widespread prevalence, with almost one-third of HIV-infected individuals globally also infected with tuberculosis. Both infections are far more prevalent in resource-limited settings, and coinfection is associated with worse outcomes, with tuberculosis being the leading cause of death in coinfected individuals.[55] Although opportunistic infections are a hallmark of progressive HIV, AIDS-defining illnesses are not confined to infectious diseases. There are notably several cancers that are also associated with HIV, including Kaposi sarcoma, lymphoma, and cervical cancer, all of which are likely due to loss of protective immune surveillance and the presence of other viral coinfections. Additionally, although not listed by the CDC as AIDS defining, there is also evidence that HIV-infected individuals are at increased risk for anal cancer, hepatocellular carcinoma, and lung cancer.[56] The risks for associated infectious diseases decrease with therapy and subsequent immune reconstitution; however, the risk for some cancers, such as cervical and anal cancer, remains elevated.

With the advent of combination ART, HIV has in many ways become a chronic treatable disease. However, despite the immunologic and virologic benefits of ART, life expectancy in HIV-infected individuals remains approximately 14% shorter than the average for uninfected persons.[57] The ART Cohort Collaboration study, a group composed of 13 independent studies, found that deaths were due largely due to age-related clinical diseases, such as cardiovascular disease, cancer, fractures, and renal dysfunction, despite apparent immune reconstitution with effective ART. Additionally, treated HIV-infected adults older than 50 years old had three times greater risk of death compared to those younger than 30 years. The precise mechanism of this age-related morbidity and mortality is unclear, but it is increasingly relevant as the population of HIV-infected individuals ages, a result of the success of ART. It is projected that by 2015 more than half of all HIV-infected individuals in the United States will be over 50 years of age,[58] and given their increased risk of morbidity and mortality despite what is considered successful treatment, the mechanism of these age-related diseases is an area of critical research. One key to understanding this is the observation that HIV-infected individuals have increased systemic immune activation and that with therapy the activation level decreases but does not reach the same state of those who are uninfected. Persistent immune activation, even at low levels, may therefore be one mechanism by which HIV creates progressive impairment.

Chronic Immune Activation and Immune Dysregulation

Peripheral markers of T-cell activation predict the rate of disease progression and CD4 T-cell

decline in untreated infection more accurately than plasma viral load.[59,60,61] This persistent immune activation is a hallmark of chronic HIV infection; although it is clear that HIV viremia itself plays a direct role, it does not fully account for the entirety of observed T-cell activation seen in infected patients. For example, in sooty mangabeys, a natural host of SIV, high levels of viremia are supported but without a significant increase in immune activation and with the remarkable absence of clinically advanced disease.[62,63,64] Additionally,

patients who are successfully treated with ART and have suppressed viral loads continue to have elevated markers of T-cell activation relative to HIV-uninfected subjects.[65]

Recent studies have suggested that the gut may be critically involved in chronic immune activation. Individuals with untreated HIV infection have been found to exhibit elevated serum levels of bacterial lipopolysaccharide (LPS), which is thought to be a consequence of increased microbial translocation from the intestinal lumen in the setting of a damaged gut mucosa.[66] Serum LPS levels are closely correlated with markers of systemic T-cell activation; in patients who receive allogeneic bone marrow transplants complicated by graft-versus-host disease, it has been shown that plasma LPS levels closely correlate with biochemical and histologic measures of mucosal damage.[67] Thus, elevated serum LPS in HIV-infected subjects is believed to reflect the loss of mucosal epithelial integrity concomitant with HIV infection, and it defines a mechanism by which the gut may play a central role in chronic immune activation through increased microbial translocation. The ultimate anatomic cause of this increased translocation remains to be fully defined The ultimate consequence of this chronic immune activation appears to be higher turnover of CD4 and CD8 T cells, clonal exhaustion, and the progressive impairment of T-cell function.

The development of immune dysfunction or “exhaustion” in the presence of ongoing antigen exposure is a cardinal feature of chronic viral infections with persistent viremia.[68,69] Exhaustion results in the hierarchical impairment of T-cell effector functions, with an initial loss of proliferative capacity and interleukin (IL)-2 production and the late loss of the ability to secrete proinflammatory cytokines such as interferon (IFN)γ.[70] This exhaustion is mediated by several immunoregulatory pathways,[69,71] including programmed death 1 (PD-1), cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), T-cell immunoglobulin and mucin-3 (TIM-3), and IL-10, which are all upregulated in vivo in chronic untreated HIV infection and have been shown to mediate a reversible virus-specific immune dysfunction in vitro. These immunoregulatory pathways are therefore being explored as potential targets for therapeutic intervention in chronic HIV infection.[69,72,73,74]

IMMUNE RESPONSE TO HUMAN IMMUNODEFICIENCY VIRUS

Unlike many other viral infections, there does not appear to be natural clearance of HIV infection. The only known case in which virus has been apparently successfully eliminated occurred in a patient who underwent a bone marrow transplant from a donor who was homozygous for the 32 base pair deletion in CCR5, rendering the newly transplanted cells not susceptible to infection by the R5 virus strain with which he was infected.[75] The fact that people do not spontaneously clear virus indicates by definition that natural immunity is insufficient to eliminate HIV. Despite this, there is clear evidence of initial partial control in the

early stages of infection, when viral load is decreased from peak levels often in excess of 10 million viral particles per mL of plasma to a set point mean of around 30,000 copies/mL.[45] Moreover, some rare individuals termed elite controllers maintain undetectable viral loads and normal CD4 cell counts, in some cases for over 30 or more years, suggesting that partial host control is possible. Evidence exists to implicate both innate and adaptive immune responses in situations of partial control in acute and chronic infection, but the actual correlates of protective immunity remain unclear.

Natural Killer Cell Response

The potential protective role of natural killer (NK) cells in human viral infections is clear from examples of genetic defects in NK cells. For example, severe herpes simplex virus infection was observed in an adolescent with no NK cells.[76] Aside from the fact that NK cells would be the first possible line of defense against HIV, HIV downregulates human leukocyte antigen (HLA)-A and -B molecules on infected cells, which would render them targetable by certain NK-cell subsets. NK-cell interactions with HIV-infected cells do not depend on expression of an antigen-specific receptor, but instead are dependent on alterations in activating and inhibitory signaling between HIV-infected cells and NK cells. This occurs via interactions with a subset of highly polymorphic killer immunoglobulin-like receptors (KIRs) with HLA class I molecules, as well as through indirect mechanisms involving antibody-dependent cellular cytotoxicity and an arsenal of nonspecific inhibitory and activating receptors.[77,78,79]

The evidence for a beneficial effect of NK cells in HIV infection is mounting, both in protection from acquisition and in subsequent control of viremia. A potential functional role for NK cells in blocking viral acquisition is supported by studies in exposed, uninfected persons who appear to be protected from infection. Persons who have been heavily exposed to HIV and yet remain uninfected have increased NK cell activity[80] and a higher KIR3DS1 to KIR3DL1 transcript ratio,[81] consistent with the genetic studies indicating a protective effect of the compound genotype consisting of the activating KIR KIR3DS1 and Bw4-80I.[78] Studies in persons who are acutely infected also support an antiviral role for NK cells. There is an expansion of KIR3DS1+ NK cells in acute infection in persons expressing the putative ligand Bw4-80I, and these cells are able to lyse HIV infected cells in vitro, in a Bw4-80I-dependent manner.[82]

The mechanism of putative protection by NK cells in HIV infection likely relates to the selective downregulation of HLA A and B alleles by HIV. This certainly would be consistent with the role of activating ligands, but the inhibitory KIR allelic association is more challenging to explain. It may relate to the strength of the KIR-HLA interaction during NK-cell licensing, when stronger engagement of inhibitory KIRs by HLA molecules enhances their potential functional capacity. Stronger inhibitory signals during NK-cell development could lead to greater activation when infected cells downregulate HLA-A and -B, thus leading to enhanced function.[78]

There are a number of defects in NK cells that have been identified in HIV-infected persons. There is an inverse correlation between viral load and NK cell-mediated suppression of viral replication, suggesting that NK-cell dysfunction

may contribute to disease progression.[83] NK cells in HIVinfected persons have impaired cytotoxic capacity associated with decreased levels of granzyme A and perforin,[84] whereas other studies suggest that NK cells may be incompletely activated in chronic HIV infection and that functionally anergic subsets may be expanded.[85] It has also been suggested that NK cells can become HIV-infected and might contribute to the persistent HIV reservoir in vivo.[86]

More recently, in vivo evidence for antiviral selection pressure mediated by NK cells has been reported. Functional roles for NK cells are suggested by studies in infected persons, showing expansion of a subset of NK cells in acute infection.[82] But the most compelling data implicating NK cells in the host defense against HIV come from the identification of NK-cell "footprints" in viral sequences obtained from infected persons. Analysis of KIR genotypes and fulllength virus sequencing in a large cohort of persons with chronic HIV infection demonstrated 22 positions throughout the HIV genome in which amino acid polymorphisms were associated with the presence of specific KIR genes.[87] In vitro studies showed that these HIV sequence polymorphisms increased the binding of inhibitory KIRs to HIVinfected cells, suggesting a functional consequence favoring the virus. Moreover, these polymorphisms reduced the antiviral activity of KIR-positive NK cells, indicating not only that NK cells exert immune selection pressure on HIV, but also that HIV evolves to escape from detection by this response.

CD8 T-Cell Response

Systemic spread of HIV infection from an initial localized mucosal infection is associated with a dramatic increase in plasma viremia followed by decline to a quasi-set point level. The appearance of a cytotoxic T-lymphocyte (CTL) response coincides with the drop in viremia a month or more before neutralizing antibodies are detectable, suggesting that these cells may be the main effector mechanism of this decline.[88,89]

The data in support of a primary role of CTL in initial containment of systemic viremia also come from CD8 T-cell depletion studies in SIV-infected macaques. Use of a monoclonal antibody to achieve complete CD8 depletion in peripheral blood and near complete depletion in tissues resulted in persistence of peak viremia in acute infection.[90,91] When CD8 T cells are depleted in chronic infection, well after set point has been established, viral load increases and then declines again as CD8 cells reappear. CD8 depletion also resulted in more rapid disease progression.[90] These studies also may have depleted CD8-bearing NK cells, so one cannot entirely rule out a contribution of the innate immune response. Support for a central role for CTL is also implied from large cohort studies, in which the most striking association with viral load and CD4-cell decline is the expression of certain HLA class I alleles[92] (Fig. 42.7). Among these are so called "protective" alleles associated with natural control, in particular HLA B*57 and B*27,[92,93] and "risk" alleles, such as certain HLA B*35 subtypes associated with higher viral loads and more rapid disease progression.[94]

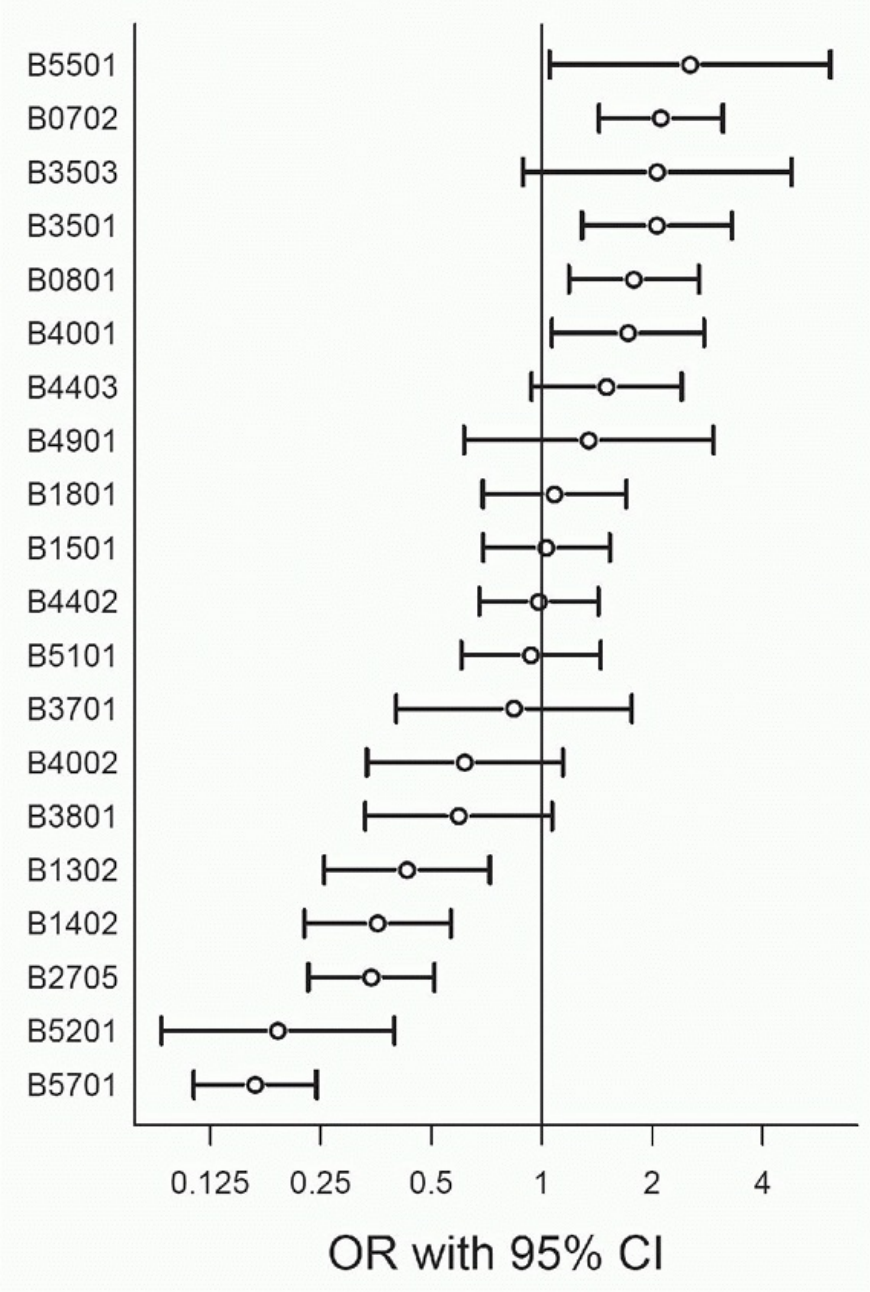

FIG. 42.7. Human Leukocyte Antigen (HLA)-B alleles and Human Immunodeficiency Virus (HIV) Control. Association of HIV immune control and imputed classical HLA-B alleles in a European cohort were calculated using a logistic regression model where all alleles with frequency of 2% or greater were used as covariables in the model. Odds ratio (OR) < 1 indicates association with virologic control of HIV, and OR > 1 indicates association with progression. Raw data was taken from Pereyra et al.[173] Courtesy of M. Carrington.

Because acute HIV infection first becomes symptomatic at the time of peak viremia, very few studies have been able to identify patients early enough to characterize CTL responses during the rapid initial increase in viral load. In the studies that have been done, however, it is clear that the initial CD8 T-cell response is narrowly directed, and it remains narrow when initial set point is achieved.[95,96] Comprehensive screening of cellular immune responses to

sets of 410 overlapping peptides representing the entire HIV proteome at an estimated 28 days after virus exposure revealed an average of only three peptides targeted per person.[96] Because most studies characterizing CD8 T-cell responses have relied on IFNγ ELIspot assays, and have used a reference strain of virus, it is possible that some responses may be missed due to sequence differences in the infecting strain. Indeed, when autologous peptides have been used, the breadth of responses detectable can be up to 30% higher, but even with this caveat most studies

suggest that the initial breadth of responses consists of one to three epitope-specific responses.[95,97] Thus the breadth of responses associated with the dramatic initial decline in viremia is actually quite narrow. The narrowness of these responses is further supported by sequencing of autologous virus in acute infection, where epitopes that subsequently become targeted in the chronic phase of infection are present in wild-type form that should be normally processed and presented, so the lack of detection of responses cannot be ascribed to sequence divergence of the infecting strain from the reference strain used in the assay.[96]

The initial CD8 T-cell response is predominantly targeted to variable regions in the Nef protein, particularly the central region of Nef. Following the transition to chronic infection, targeting of Nef declines, and instead responses to Gag, Pol, and Env become prominent.[98] The fact that these other proteins are subsequently targeted is clear evidence of the preferential immunogenicity of Nef in early infection, although the reason for this remains unclear.

At the time of acute infection, certain HLA alleles predominate in terms of presenting HIV epitopes for CD8 T-cell recognition, and certain epitopes are preferentially targeted, for reasons that remain unclear. For example, of more than a dozen epitopes that have been identified to be presented and targeted through HLA A3 in chronic HIV infection, the same two are consistently dominant in the earliest stages of acute infection, and the other responses arise over time.[99] In genetically identical twins acutely infected with the same virus through injection drug use, the initial dominant and subdominant responses were almost identical, again pointing to a high degree of predictability in terms of the major targets for initial responses.[100] Studies in larger cohorts have confirmed the finding that many epitopes are not targeted in the acute stage of infection, and for each restricting HLA allele there are marked differences in the evolution of responses to different epitopes. Moreover, certain HLA alleles consistently contribute more to the initial CTL response.[101] Interestingly, HLA B*57, the allele most strongly associated with favorable outcome following HIV infection, is associated with less symptomatic acute infection,[97] and the protective alleles were also found to exhibit immunodomination in the early stages of acute infection, in that the presence of protective alleles reduces the absolute magnitude of responses restricted by other alleles.[101,102] The importance of early responses is also reflected in data showing that the immunodominance patterns in acute infection are associated with subsequent viral set point, whereas this relationship is lost during chronic infection.[102]

In addition to differences in terms of epitope targeting in acute compared to chronic infection, the characteristics of the CTL are also different. Functional avidity of responses in acute and chronic infection differ—initial high avidity responses are subsequently lost in persons with

progressive infection, whereas the original clonotypes persist in those who control spontaneously.[103] Studies in persons who control HIV spontaneously, so called "elite controllers,"[52] have shown that the ability of CD8 T cells to proliferate to cognate antigen is associated with control of viremia.[104] Strong proliferative responses by HIV-specific CD8 T cells are detected in acute infection, but decline with the transition to chronic uncontrolled infection.[105] At least part of the proliferative defect in chronic infection is due to a lack of sufficient CD4 T helper cell responses and can be restored in vitro by addition of autologous CD4 T cells obtained at the time of acute infection. The defect in CD8 T-cell proliferation in chronic infection can also be corrected by vaccine-induced HIV-specific CD4 T helper cell responses, suggesting the presence of a reversible defect in CD8 T-cell function.[105,106]

Although Nef is predominantly targeted in acute infection, data from numerous studies indicate that the targeting of Gag during chronic infection is associated with better outcomes. Studies of persons who control virus spontaneously without medication have shown preferential targeting of Gag, whereas responses are more broadly directed in persons with progressive infection.[107] The magnitude and breadth of CD8 T-cell responses to the entire Gag protein or just the p24 protein was found to correlate inversely with viral load and directly with CD4 cell counts; in contrast, there was no association with Env or Nef peptides.[108] In a large study of persons with chronic clade C virus infection in South Africa, the broader the Gag-specific CD8 T-cell response, the lower was the viral load, whereas the broader the targeting of Env, the higher the viral load (Fig. 42.8).[109] Moreover, median viral load in those persons in whom Gag was the dominant target of the CD8 T-cell response was lower than in those in whom the response was predominantly targeted to non-Gag epitopes.[110]

Although the precise correlates of a protective CD8 T-cell response remain to be defined, there is growing consensus that certain functional properties of these cells are associated with better outcome. The ability of CD8 T cells to proliferate in response to cognate antigen is perhaps the strongest association with lower viremia.[104] When CD8 T cells from "low viremic" individuals were exposed to autologous CD4 T cells infected with HIV, not only was the ability to proliferate better maintained, but it was also associated with upregulation of perforin production[104,111] and the ability to load lytic granules upon in vitro stimulation.[112] The ability of cells to produce multiple cytokines, termed polyfunctionality, has also been associated with persistent control of viremia.[113] Analysis of TCR usage comparing persons who control and do not control suggests that TCR clonotypes modulate the antiviral function of CD8 T cells.[113a]

Most studies of HIV-specific CD8 T cells have relied on the use of peptide-pulsed target cells rather than infected cells. As such, multiple steps in antigen processing are not assessed, nor are potential differences in the ability of infected cells to process and present epitopes.[114] When compared to monocytes, CD4 T-lymphocytes have significantly lower proteolytic activity, which impacts epitope generation, but whether this impacts the ability of CTL to clear virus-infected cells is not clear.[114] CTLs clearly have the ability to lyse infected cells and thereby limit virus replication, and in vitro CD8 T cells can actually clear virus infection from infected autologous CD4 T cells.[115] Specificity may make a difference, as preformed Gag protein appears to be processed and presented for recognition,

whereas epitopes in non-Gag proteins must first be synthesized and then processed, making these responses delayed in comparison.[116,117] Similar effects have also been seen with Pol, which is also part of the incoming virus.[117]

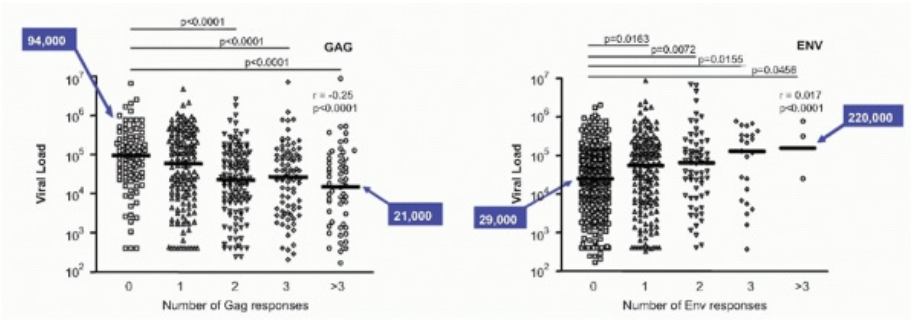

FIG. 42.8. Breadth of Gag and Env CD8 T-Cell Responses and Relation to Viral Load. Broader Gag targeting is associated with lower viral loads whereas dominant Env responses are associated with higher viral loads. Adapted from Kiepiela et al.[109]

In vitro studies show marked differences in the ability of CD8 T cells from different patient groups to inhibit virus replication. CD8 T cells from persons who control HIV spontaneously have the capacity to inhibit virus replication in vitro, without any need for in vitro stimulation, and these cells are phenotypically different from those from progressors, in that they express the activation marker HLA-DR but are negative for another activation marker, CD38. Other studies involving in vitro expansion of CD8 T cells show that lytic granule loading is enhanced in HIV-specific CD8 T cells from controllers compared to progressors.[112] The observed activity appears to be due to a cytotoxic mechanism rather than secretion of soluble factors such as the β chemokines, which are clearly antiviral. Similar studies in persons with chronic infection have revealed that Gag-specific CD8 T cells have enhanced antiviral function compared to those with other specificities.[118] But the relative contribution of lytic activity versus secretion of soluble factors remains controversial, particularly given recent results in animal models suggesting that CD8 T cells can control viremia without reducing the lifespan of infected cells.[119]

CD4 T-Cell Response

Immunologic studies performed at the early stages of the HIV epidemic revealed a striking lack of HIV-specific CD4 T-cell responses in most infected persons. This has long been seen as a central defect in HIV infection, as virus-specific CD4 cells are central to immune control in every setting in which this has been evaluated. They play a major role in immune induction through the production of IL-2 and the activation of antigen-presenting cells via CD40-CD40L interactions and are essential for long-term control of viremia due to help provided to CD8 T cells.[120] HIV preferentially infects CD4 T cells, and during acute infection there is a dramatic loss of CD4 T cells in the GALT as well as the peripheral blood. Unlike the recovery of

peripheral CD4 cell counts when a quasi-set point viremia is achieved, the CD4 cell loss in GALT persists and is not restored even with prolonged antiviral therapy. However, with very early treatment of acute HIV infection, gradual emergence of virus-specific CD4 T-cell responses is observed, and similar responses are characteristic of persons who are able to control virus spontaneously without the need for antiviral therapy.[121,122] It has been hypothesized that these antigen-specific CD4 T cells are preferentially deleted at the time of high-level viremia, as these cells are being maximally activated at this time. However, the majority of HIV-specific CD4 cells remain uninfected, suggesting that these cells may contribute to the level of relative control that is observed.[123]

Renewed interest in HIV-specific CD4 T cells has been sparked in part by results from the RV144 phase III trial of a canarypox prime-recombinant gp120 boost HIV vaccine in which a modest protective effect was correlated with the presence of HIV-specific CD4 T-cell responses but not CD8 T-cell responses.[124] The role of HIV-specific CD4 T cells in immune control and the need to induce these for successful immunization remains controversial. HIV-specific CD4 T cells predominantly target Gag and Env proteins[125] with some interesting associations. A recent study demonstrated that the breadth of total HIV-specific CD4 T-cell responses, and the breadth and magnitude of Gag-specific responses are both inversely correlated with HIV viral load.[125] Additionally, this study suggested that dominant targeting of Gag was more frequently seen in HIV controllers, whereas individuals with progressive disease more frequently targeted Env epitopes. Additional recent data suggest that the presence of cytolytic CD4 T cells at the time of peak and declining viremia in acute infection is associated with a lower viral set point, suggesting an effector role for CD4 T cells.[126]

Immune regulatory networks also appear to play a role and indicate that there is not a total loss of HIV-specific CD4 T cells but rather that these are impaired. In chronic progressive infection, cytotoxic T-lymphocyte-associated antigen 4 is upregulated on CD4 T cells but not CD8 T cells and correlates positively with viral load and negatively

with the ability of HIV-specific CD4 T cells to produce IFNγ.[127,128] Other studies show impaired IL-2 production by HIV-specific CD4 T cells in persons with persistent viremia, although these cells are present in persons who control viremia in the absence of therapy.[128] In settings of relative control, HIV-specific CD4 T cells consist of both central memory cells capable of producing exclusively IL-2 and effector memory cells producing IL-2 and INFγ. In contrast, in viremic patients there are elevated levels of effector memory cells that exclusively produce IFNγ.[128] Functional studies are consistent with a defect in these cells in progressive infection, in that HIV-specific CD4 proliferation is negatively associated with viral load,[122] whereas this association is not seen when one measures the ability to produce IFNγ.[129]

B-Cell Responses

In 1984, Margaret Heckler, the secretary of the Department of Health and Human Services, stated that a vaccine for HIV could be expected within 2 years. Unfortunately, the development of an effective vaccine has proved to be much more challenging. For the majority of currently licensed vaccines, neutralizing viral-specific antibodies have been a

reliable correlate of protection. In HIV, however, several challenges exist to the induction of protective antibody responses. First and foremost is the enormous diversity of the surface Env proteins. This is compounded by the dense glycosylation of the viral Env proteins, which creates a barrier to antibody recognition. This has made it difficult to induce the production of broadly neutralizing antibodies (nAbs) using conventional vaccination strategies. Nonetheless, it appears that 10% to 25% of chronically infected individuals are able to induce antibodies with narrow neutralization properties, with 2% to 4% being able to produce broadly nAbs.[130,131] These antibodies, however, appear to arise only after years of infection. The initial antibody responses shortly after infection are nonneutralizing but are followed by the appearance of responses 12 weeks or more later that are capable of neutralizing autologous viruses, but to which escape mutations quickly develop.[132] These antibody responses do not appear to play a significant role in the control of viremia, as they are directed not against contemporaneous viral populations but against viruses from which escape has quickly developed.

Although antibody responses may not be critical for immunologic control of viremia, they are considered essential to achieve protection from acquisition. There is significant evidence from nonhuman primate models of infection that nAbs are able to prevent HIV acquisition. Passive immunization of rhesus macaques with nAbs protects against infection with vaginal challenge with SIV-HIV chimeric virus.[133,134] Such antibodies have also been shown to be protective when applied directly at the female genital mucosa prior to challenge.[135] Until relatively recently, only a small number of nAbs had been identified. New methods of identification and recovery of single antibody-producing cells, however, have allowed for the identification of many others. This has revealed several sites of vulnerability to nAbs on the Env proteins, including the membrane proximal region of gp41, the CD4 binding site on gp120, Env glycans, and gp120 variable loops, which recognize a quaternary epitope on trimeric gp120 (Fig. 42.9).[136,137,138,139]

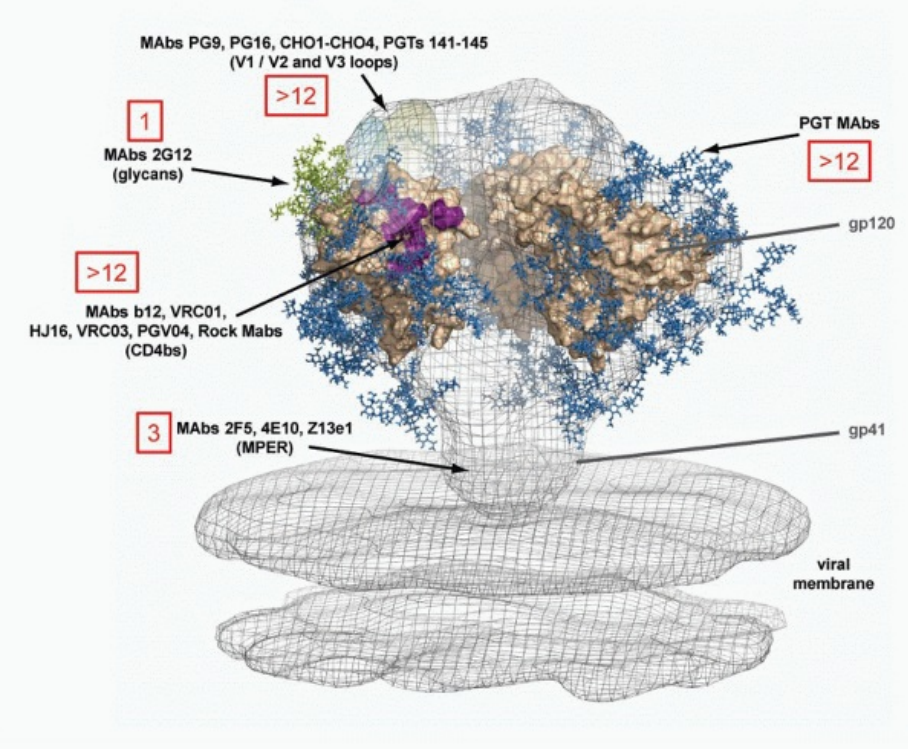

FIG. 42.9. Human Immunodeficiency Virus Envelope Recognition Sites of Broadly Neutralizing Monoclonal Antibodies. Identified neutralizing antibodies target the membrane proximal external region of gp41, and multiple sites on gp120 including the CD4 binding site, a conserved region involving the V2/V3 loops and a patch including a number of high mannose glycans (2G12 and PGT antibodies). In *red* are the approximate number of monoclonal neutralizing antibodies that have been identified that recognize the indicated region of Env. Courtesy of D. Burton.

Although much of the early antibody-directed work was focused on the induction on nAb responses, more recent interest has developed regarding Fc-mediated antibody effector functions such as antibody-dependent cell-mediated cytotoxicity, in part due to studies that show that alterations to the Fcγ receptor binding region of the nAb b12 show reduced protection from SIV-HIV chimeric virus challenge in rhesus macaques.[79,140] Additionally, recent data from the RV144 HIV vaccine trial suggest the possibility of nonneutralizing antibody responses being important in mediating a modest protection from infection. The exact mechanism of this antibody associated protection, however, remains uncertain.

Dendritic Cells in Human Immunodeficiency Virus Infection

A major hallmark of chronic untreated HIV infection is immune suppression. CD4 cell loss certainly contributes to this, but additional dysregulation occurs within DCs, which play a key role in providing cross-talk between innate and adaptive immune responses to viral infections.[141] Both plasmacytoid and conventional DCs can harbor HIV, and both are reduced in numbers in HIV infection; this correlates with increasing viral load and with increased disease progression.[142] This depletion occurs early in acute infection and appears to persist in the chronic phase.

Although DCs express CD4 and conventional coreceptors CCR5 and CXCR4, it remains controversial to what extent these cells are productively infected and

subsequently deleted versus rendered dysfunctional. DC infection ex vivo is inefficient, suggesting that the observed decrease in numbers in HIV may be due to indirect mechanisms or redistribution of DCs to secondary lymphoid tissues. In addition to coreceptors, DC also express the C-type lectin DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN), which viruses can use to enter DCs through the capacity of gp120 to bind to DC-SIGN. This results in the uptake of virions into early endosomal compartments where HIV is not degraded but maintained and then released after migration to draining lymph nodes to efficiently infect T cells.[143,144] Clusterin, a glycoprotein found in a number of tissues and blood, specifically blocks HIV-DC-SIGN interactions and prevents transmission of HIV to CD4 T cells when present in semen. It may help explain why sexual transmission is less effective than bloodborne exposure, as clusterin in blood is differently glycosylated and does not have this effect.[145]

The HIV single-stranded RNA genome encodes tolllike receptor 7/8 agonists that are recognized by DCs and lead to the induction of inflammatory cytokines through a mechanism that involves internalization of virions.[146,147] There is also evidence that infected DCs may be able to recognize HIV through interaction of newly transcribed HIV capsid with the cellular protein cyclophilin A, which results in the induction of type I IFNs by the transcription factor IFN regulatory factor 3.[148] Although the interaction of DCs and HIV is complex and the overall numbers of DCs appear to be reduced in the blood during HIV infection, the degree to which DCs are functionally impaired during infection remains unclear. Plasmacytoid DCs from persons with progressive disease only partially mature upon stimulation and may contribute to chronic immune activation and further CD4 T-cell infection by production of copious amounts of IFNα that induces a chronic inflammatory response.[149] There is evidence that myeloid DCs from HIVinfected individuals appear to have reduced responsiveness to toll-like receptor agonists and are less efficient at T-cell activation.[150,151,152] However, other studies indicate that both myeloid and plasmacytoid DCs from HIV-infected individuals continue to be capable of activating T cells and producing cytokines in response to toll-like receptor agonists.[153,154] Evidence also exists that DCs can become tolerized in vivo in infected persons, reducing the ability to induce immune responses.[155]

Immune Selection Pressure and Escape from Cellular Immune Responses

HIV has an enormous ability to escape from immune responses, including NK and antibody responses, as discussed previously. However, our most detailed understanding of immune escape comes from the study of viral adaptation to CD8 T-cell responses. In most infected persons, virus continues to replicate in the presence of a CD8 T-cell response that is insufficient to completely contain viremia. In the same way that incomplete control of viral replication leads to emergence of drug-resistant viruses, incomplete immune control is associated with escape mutations that diminish recognition by the established T-cell response. Analysis of large datasets of patient-derived sequences reveals a profound impact of CTL responses in driving HIV evolution.[156,157,158] The clinical significance of this has been shown in infants born to HLA B*27-positive HIV-infected mothers.[159] HLA B*27-positive

infants who received the HLA allele from their fathers and the virus from their mothers were able to control viremia through induction of a strong HLA B*27-restricted T-cell response to a dominant epitope in Gag. In contrast, in B*27-positive infants whose mothers were also B*27-positive, transmitted viruses were found to have already escaped from the dominant B*27-restricted response in the mother, and thus there was no targeting of this otherwise dominant B*27-restricted Gag epitope in their children.

The kinetics of immune escape are just beginning to be understood, in part because of the paucity of studies in the earliest stages of infection, before the decline in viremia from peak levels. What is clear is that escape can occur within days to weeks of infection. In studies in which autologous virus was sequenced and responses screened with peptides representing the autologous strain, clear evidence of immune escape was evident within 25 to 32 days of infection, coinciding with peak viremia.[95] Longitudinal studies in persons with acute infection have shown selection for CTL escape mutations arise at quite variable rates depending on the epitope and the restricting HLA allele.[160] Deep sequencing has revealed that the virus explores multiple pathways to immune escape, and that there can be numerous variants that arise at low levels within targeted epitopes—so low that they are missed by traditional sequencing methods.[161,162] What remain unclear are the factors that contribute to immunodominance and immunodomination. For a given HLA allele, it has become increasingly clear that the earliest immune responses are predictable, as are the pathways to immune escape.[163] Likewise, it is also clear that certain responses do not arise in the presence of other HLA alleles, a phenomenon called immunodomination.[101]

One of the challenges in defining the role of escape mutations in disease progression is defining mutations that lead to an immunologic impact. Mutations within targeted epitopes are most frequent and can lead to full or partial loss of recognition by the T-cell receptor or loss of peptide-binding to the restricting HLA class I allele. Mutations within residues flanking epitopes also account for immune escape through alterations in antigen processing.[164,165] Detection of immune escape presents a potential problem using simple IFNγ ELIspot assays, as these may still indicate recognition because they do not incorporate potential confounding effect of antigen processing. More recent focus on assays that incorporate infected target cells, in which antigen-processing events are required for recognition, may provide a better sense of the true measure of immune escape, and may be needed in order to finally define the contribution of immune escape to disease progression.

It is important to note that immune escape can have either positive or negative impact on viral control, due to the possibility that escape mutations can confer a fitness cost

to the virus. In other words, there are competing factors: on the one hand, escape leads to loss of recognition by an established immune response, and on the other hand, it can result in a virus that is markedly impaired in its pathogenic potential. This effect may be most important in the Gag protein, where recent data suggest multidimensional constraints of HIV evolution due to structural constraints on capsid formation.[166] Indeed, viruses obtained from elite controllers have been shown to be less fit overall, and selective targeting of regions of Gag under multidimensional constraints on evolution by persons who control HIV spontaneously may contribute to durable control.[166] Transmission of escape variants with

reduced fitness has been linked to less severe disease in recipients[167,168] and has also been associated with subsequent reversion to wild type, a clear indication of a fitness cost to the virus that can revert in the absence of ongoing immune selection pressure in a recipient lacking the restricting HLA allele for that epitope.

HUMAN IMMUNODEFICIENCY VIRUS IMMUNOGENETICS

The associations between class I alleles and viral control suggest that class I-restricted CTLs play a role in limiting HIV replication, and large population studies coupling HLA typing with sequence analysis of the viruses circulating in vivo add to this evidence. HLA class I-associated mutational "footprints" have been identified in HIV-1, suggesting that this rapidly mutating virus is adapting to immune selection pressure.[156,169] Given the role of HLA molecules in determining the repertoire and specificity of the immune response, these data point most strongly toward the CD8 T-cell responses being involved in immune control.

Additional insight into the immunogenetics of HIV infection has come from genome-wide association studies. In a study of persons with acute infection, two single nucleotide polymorphisms (SNPs) in the major histocompatibility complex locus were independently associated with viral control, one associated with HLA-C expression and another that is a proxy for HLA B57,[170] already known to be associated with better outcome. These two plus an additional two independently associated SNPs were subsequently identified in genome-wide association study analyses of persons termed HIV controllers, who maintain viral loads of < 2,000 RNA copies/mL in the absence of therapy.[171,172] Genome-wide association study analysis of these HIV controllers also demonstrated that aside from the signal in the region of HLA-B and -C in chromosome 6, most of the previously reported host genetic influences on HIV control could not be replicated (Fig. 42.10A). Indeed, only the chemokine cluster in chromosome 3, which harbors the gene encoding CCR5, the coreceptor associated with HIV entry, was found to be statistically significantly associated with viral control when controlled for population structure and corrected for multiple comparisons.[173,174]

Further insight into the mechanism of the effects of these SNPs has been revealed by the observations that they "tag" specific features within HLA sequences themselves: specific amino acids within the HLA-B peptide binding groove, as well as an independent SNP associated with HLA-C expression.[173] Specific amino acids at the HLA-B positions, particularly positions 67, 70, and 97, are associated with differential ability to control HIV viremia, providing a mechanism of action for the SNP associations and suggesting that the nature of the peptide-HLA-B interaction is key to HIV control (Fig. 42.10B). In addition to these HLA-B associations, a SNP 35 kb upstream of the HLA-C gene was also strongly associated with control of viral load and with levels of HLA-C expression. The mechanism of this effect has been linked to variation with the 3′ untranslated region of HLA-C that is posttranscriptionally regulated by a microRNA, resulting in differences in HLA-C expression. The variant that does not bind this microRNA results in high expression of HLA-C and better control of HIV.[175] Unlike HLA-A and -B, which are downregulated during HIV infection, HLA-C is not.[176] Thus higher levels of HLA-C may prolong the ability of infected cells to be targeted by CTLs, after HLA-A and -B have been downregulated, thereby conferring a benefit. However, the antiviral efficacy of HLA-C-restricted responses does not approach the level of immune selection pressure applied through -A and -B, as evidenced by the paucity of

HLA-C-associated footprints on the viral genome. Another possibility is that high expression of HLA-C may lead to more effective education and maturation of NK

cells due to better engagement of inhibitory NK-cell receptors with their HLA-C ligands, resulting in stronger NK-cell responses to infected cells.

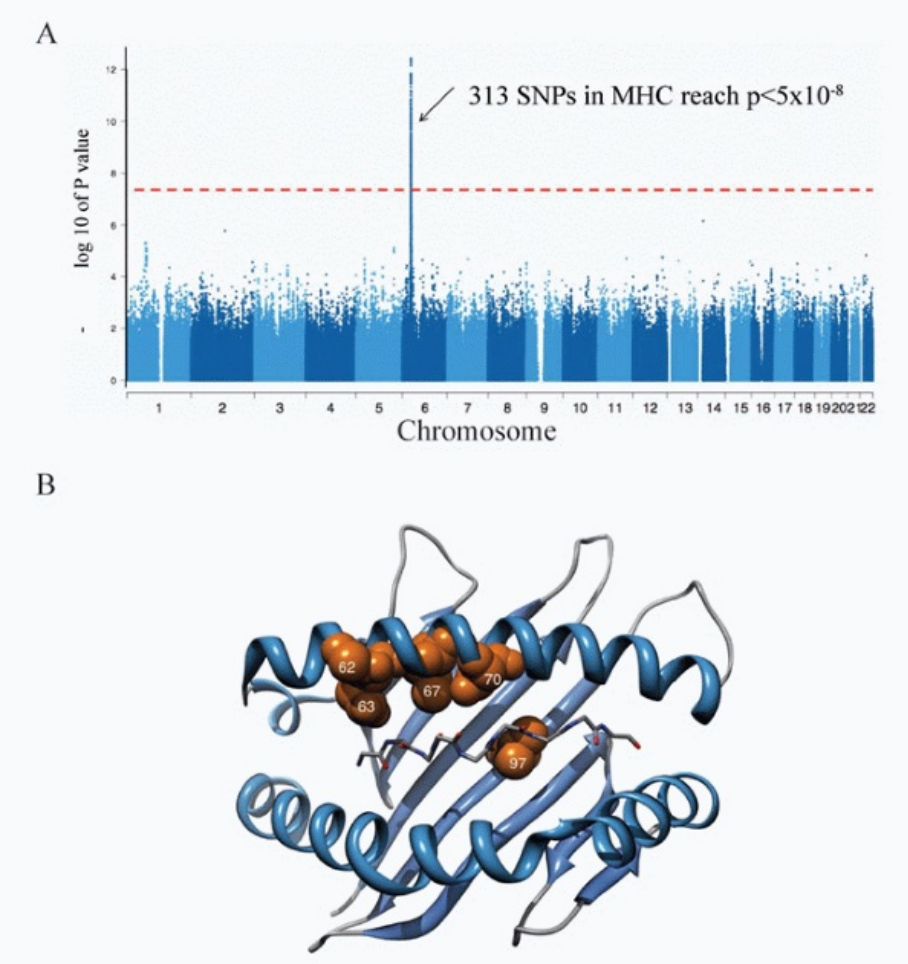

FIG. 42.10. Human Leukocyte Antigen (HLA) and Immune Control of Human Immunodeficiency Virus (HIV). A: Genome-wide association study of 1.3 million single nucleotide polymorphisms show a significant association with a region in chromosome 6 encoding HLA-B and C. **B:** Discrete amino acids within the HLA-B peptide biding groove at positions 67, 70, and 97 are associated with immunologic control of HIV. Adapted from Pereyra et al.[173]

HLA class II associations with HIV disease outcome have also been reported, but the data are less convincing than for class I. Class II "footprints" have not been convincingly identified, suggesting that responses restricted by HLA class II either exert less selection pressure or that epitopes are presented so promiscuously on different class II alleles that clear signatures cannot be discerned.

In addition to HLA class I alleles, the KIR locus has also been implicated in modulating control of HIV viremia, suggesting the potential involvement of NK cells in human control of HIV. KIR,

like HLA, is one of the most polymorphic loci in the human genome. The *KIR* genes, located on chromosome 19, encode molecules that regulate NK-cell activity, with stimulatory and inhibitory effects. Analysis of AIDS progression rates in untreated persons with HIV infection have shown a protective effect of the activating KIR allele *KIR3DS1* and its putative ligand, HLA-B Bw4 alleles that contain an isoleucine at position 80 (Bw4-80I).[77] In contrast, in persons who express KIR3DS1 in the absence of HLA Bw4-80I progression is more rapid, whereas expression of HLA B Bw4-80I in the absence of KIR3DS1 has no effect. In addition to these effects of an activating KIR on disease progression, other studies have shown that certain alleles of the inhibitory receptor KIR3DL1 also provide protection against disease progression, and that protection relates to the relative expression levels of KIR3DL1.[78] Interestingly, this allotype acts synergistically with HLA B*57, which contains the KIR3DS1 ligand Bw4-80I.[78] Thus, data exist suggesting a protective role of both activating and inhibitory KIRs in control of HIV.

HUMAN IMMUNODEFICIENCY VIRUS PREVENTION

Developing a Human Immunodeficiency Virus Vaccine

Despite the success of potent ART, it has been a challenge to deliver medicines to all those in need. Even with billions of dollars spent on rollout of drug delivery in developing regions, only 5 million of the 23 million HIV-infected individuals in sub-Saharan Africa are currently receiving HIV medicines.[177] The prospect of delivering lifelong HIV treatment to individuals living in regions of the world where there is little health care infrastructure and unreliable access to food and safe drinking water is truly daunting. Although treatment is a critical component of the global fight against HIV, the development of an efficacious preventative vaccine remains paramount to ending the AIDS pandemic. It has been estimated that a prophylactic vaccine with only 50% efficacy, delivered to just 30% of the population, could reduce annual infections by up to one-third, averting 17 million infections over the next 15 years.[178] And yet, the HIV vaccine field has not been able to make significant headway toward this goal. To date, only three HIV vaccine candidates have been tested in large-scale trials, and all of them have failed to provide compelling evidence that an effective vaccine will be produced in the near future.[179]

The first candidate vaccine to be tested in a large efficacy trial was the VaxGen recombinant gp120 protein, which was used in over 5,000 participants in the AIDSVAX trial. The vaccine utilized a recombinant subunit protein to induce antibody responses, an approach that is the basis for the currently licensed hepatitis B vaccine. Initial results in chimpanzees were promising, showing that gp120 vaccination could protect from subsequent HIV challenge with a laboratory isolate.[180] Yet after 3 years of follow-up in the AIDSVAX study, acquisition rates in vaccine recipients and those who received a placebo were statistically indistinguishable.[181,182]

In part because of the difficulty in inducing broadly neutralizing antibody responses against HIV and an appreciation of the important role of CD8 T-cell responses in virologic control of infection, interest increased in vaccines that induce T-cell responses. Although such vaccines may not protect from HIV acquisition, they may be able to reduce viral set point and delay development of AIDS. Cohort studies have shown that viral set point inversely correlates with

disease progression and thus such vaccines may be able to delay the time before ART is needed.[183] The STEP trial, the second large HIV vaccine trial to be undertaken, tested the T-cell vaccine concept in a phase IIb trial of an adenovirus serotype 5 vaccine. The vaccine consisted of an equal mixture of three replication defective adenoviral vectors, each containing a near consensus clade B gag, pol, or nef gene. The trial was opened in 2005 and cosponsored by Merck and the National Institute of Allergy and Infectious Disease, but in September 2007 it was halted early when interim analysis showed that the vaccine failed to prevent HIV infection and had no effect on viral load at 3 months after infection.[184,185] Although this test-of-concept vaccine failed to show a result in either of the two primary endpoints (HIV acquisition and viral load), it generally has been seen as a first step and not as a condemnation of the T-cell vaccine approach per se. In the wake of STEP, the most recent efficacy trial was the RV144 "Thai vaccine" trial.[124] This approach utilized a recombinant canarypox vector prime with a recombinant gp120 boost strategy and was designed to induce both antibody and T-cell responses. Although there were no significant differences in acquisition or viral load using intention-to-treat analysis, a modified intention-to-treat analysis showed a 31% decrease in acquisition in vaccine recipients with no effect on viremia or CD4 T-cell count. Interestingly, this effect did not correlate with HIV-specific CD8 T-cell responses as measured by ELIspot or intracellular cytokine staining but did correlate with the presence of nonneutralizing antibody responses and Env directed CD4 T-cell responses.

The path to an effective vaccine is likely to be long. There are few new concepts currently in early-stage clinical trials, and some have argued that more focus needs to be placed on understanding basic immunologic mechanisms of vaccine-induced protection. Nevertheless, the apparent partial efficacy of the Thai trial has boosted morale and has led to a resurgence in vaccine development and testing that hopefully will pay out dividends in the future. Nonetheless, although a vaccine will likely be necessary, it may ultimately

be only one component of efforts to successfully control the epidemic.

Other Human Immunodeficiency Virus Prevention Strategies

Globally, over 90% of HIV is transmitted following heterosexual intercourse. Besides abstinence and barrier contraception, the most effective methods of transmission prevention utilize ART (Fig. 42.11). The best established method of antiretroviral prevention of transmission is in the setting of prevention of mother-to-child transmission. Peripartum treatment of the mother can reduce the risk of transmission to 5% in breastfeeding children and less than 2% in nonbreastfeeding children, even with short-course therapy.[186] The use of ART for prevention of mother-to-child transmission has been widely applied and is the major driver of the global reduction in mother-to-child transmission in the developing world. A newer application of "treatment as prevention" was studied in the HIV Prevention Trails Network 052 study of serodiscordant couples, where ART treatment of the infected partner was shown to reduce transmission by 96%, thus providing compelling evidence that treatment for prevention is effective.[42] A 73% reduction was also seen in the Partners PrEP Study of serodiscordant couples in which the uninfected partner was given ART. Clearly, these strategies makes sense for individuals at high risk of transmitting within serodiscordant couples who do not use barrier contraception, but how widely these strategies should be applied to other populations remains controversial.

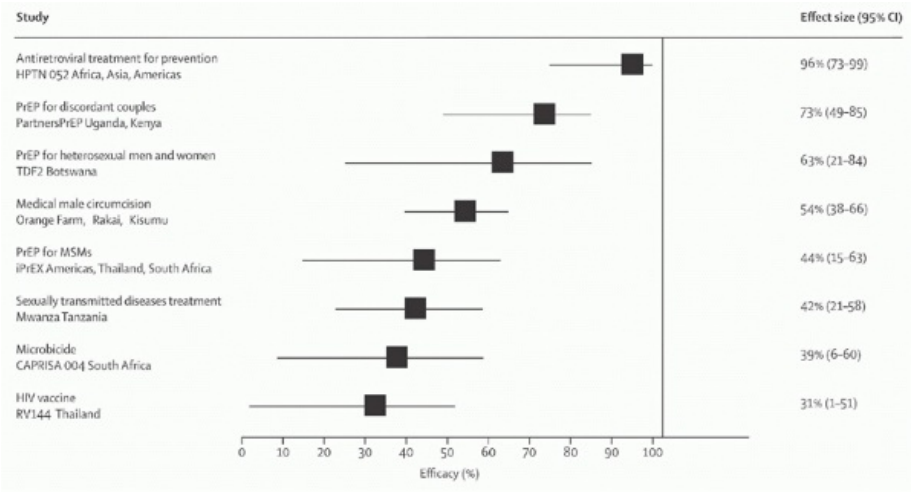

FIG 42.11. Human Immunodeficiency Virus Prevention Methods Testing in Randomized Controlled Trials. From Karim and Karim.[191]

The potential utility of the use of ART in at-risk uninfected individuals as “pre-exposure prophylaxis” to prevent transmission remains uncertain. Initial promising results in the use of an oral daily fixed-dose combination of tenofovir and emtricitabine as pre-exposure prophylaxis in HIV-negative men who have sex with men taking part in the Iniciative Profilaxis Pre Exposicion or Preexposure Prophylaxis (iPrEX) study showed a 44% decrease in risk of HIV acquisition.[187] However, a similar strategy when used in at-risk women exposed via heterosexual intercourse failed to show any protective effects.[188] Researchers have suggested that this difference may have to do with the pharmacologic properties of tenofovir and emtricitabine, or may be related to adherence in the female participants, as the protective effects observed in the men who have sex with men study were only seen in those who took ART regularly. Additional research is underway to better elucidate these differences.

Besides oral therapy, antiretroviral treatment has been incorporated into microbicides to topically delivery drugs at the area of exposure. This approach has the advantage of low toxicity and up to 100× higher levels of drugs within the mucosal site of exposure. The landmark Center for the AIDS Programme of Research in South Africa (CAPRISA) 004 trial of a vaginal microbicide containing tenofovir showed a 39% reduction in HIV acquisition among at-risk heterosexual South African women.[189] However, it was recently announced that a tenofovir microbicide arm of the Vaginal and Oral Interventions to Control the Epidemic (VOICE) trial was halted due to a lack of efficacy. This has generated questions regarding why differences were seen be

tween the two studies, and closer examination of the results are currently underway.

CONCLUSION

Overall, it is clear that although tremendous advances have been made in the treatment and prevention of HIV, much more remains to be done. Halting the AIDS pandemic will require a multipronged approach, relying on expanding treatment rollout, defining the most effective

antiretroviralbased methods for prevention, and implementing structural and behavior interventions such as male circumcision and prevention education and programs to reduce risk behaviors. Importantly, a safe and effective HIV vaccine will also be needed as a primary component of these efforts. However, to produce such a vaccine will require a more detailed understanding of HIV immunity and host interactions. In the first 30 years since the discovery of HIV as the causative agent of AIDS, an enormous amount has been learned, but significant challenges clearly remain. To overcome these challenges will require the continued dedication and support of clinicians, researchers, patients, funders, and the public at large. The pace of discovery continues to be rapid and hopes high that this is a disease that can be eventually prevented in those not yet infected and potentially eradicated in those who are.

ACKNOWLEDGMENTS

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REFERENCES

1. Centers for Disease Control and Prevention. Pneumocystis pneumonia —Los Angeles. *MMWR*. 1981;30:250-252.

2. Hymes KB, Cheung T, Greene JB, et al. Kaposi's sarcoma in homosexual men-a report of eight cases. *Lancet*. 1981;2:598-600.

3. van Griensven GJ, Hessol NA, Koblin BA, et al. Epidemiology of human immunodeficiency virus type 1 infection among homosexual men participating in hepatitis B vaccine trials in Amsterdam, New York City, and San Francisco, 1978-1990. *Am J Epidemiol*. 1993;137:909-915.

4. World Health Organization. *UNAIDS Report on the Global AIDS Epidemic 2010*. Geneva, Switzerland: World Health Organization; 2010.

5. Virgin HW, Walker BD. Immunology and the elusive AIDS vaccine. *Nature*. 2010;464:224-231.

6. Walensky RP, Paltiel AD, Losina E, et al. The survival benefits of AIDS treatment in the United States. *J Infect Dis*. 2006;194:11-19.

7. Finzi D, Blankson J, Siliciano JD, et al. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. *Nat Med*. 1999;5:512-517.

8. Johnston MI, Fauci AS. HIV vaccine development—improving on natural immunity. *N*

Engl J Med. 2011;365:873-875.

9. Sharp PM, Hahn BH. The evolution of HIV-1 and the origin of AIDS. *Philos Trans R Soc Lond B Biol Sci*. 2010;365:2487-2494.

10. Vidal N, Peeters M, Mulanga-Kabeya C, et al. Unprecedented degree of human immunodeficiency virus type 1 (HIV-1) group M genetic diversity in the Democratic Republic of Congo suggests that the HIV-1 pandemic originated in Central Africa. *J Virol*. 2000;74:10498-10507.

11. Korber B, Muldoon M, Theiler J, et al. Timing the ancestor of the HIV-1 pandemic strains. *Science*. 2000;288:1789-1796.

12. Worobey M, Gemmel M, Teuwen DE, et al. Direct evidence of extensive diversity of HIV-1 in Kinshasa by 1960. *Nature*. 2008;455:661-664.

13. Hahn BH, Shaw GM, De Cock KM, et al. AIDS as a zoonosis: scientific and public health implications. *Science*. 2000;287:607-614.

14. Plantier JC, Leoz M, Dickerson JE, et al. A new human immunodeficiency virus derived from gorillas. *Nat Med*. 2009;15:871-872.

15. Vallari A, Holzmayer V, Harris B, et al. Confirmation of putative HIV-1 group P in Cameroon. *J Virol*. 2011;85:1403-1407.

16. Roberts JD, Bebenek K, Kunkel TA. The accuracy of reverse transcriptase from HIV-1. *Science*. 1988;242:1171-1173.

17. Korber B, Gaschen B, Yusim K, et al. Evolutionary and immunological implications of contemporary HIV-1 variation. *Br Med Bull* . 2001;58:19-42.

18. Gao F, Bailes E, Robertson DL, et al. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature*. 1999;397:436-441.

19. Sodora DL, Allan JS, Apetrei C, et al. Toward an AIDS vaccine: lessons from natural simian immunodeficiency virus infections of African nonhuman primate hosts. *Nat Med*. 2009;15:861-865.

20. Cocchi F, DeVico AL, Garzino-Demo A, et al. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science*. 1995;270:1811-1815.

21. Alkhatib G, Combadiere C, Broder CC, et al. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophagetropic HIV-1. *Science*. 1996;272:1955-1958.

22. Paxton WA, Martin SR, Tse D, et al. Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposure. *Nat Med*. 1996;2:412-417.

23. Deng H, Liu R, Ellmeier W, et al. Identification of a major co-receptor for primary isolates of HIV-1. *Nature*. 1996;381:661-666.

24. Samson M, Libert F, Doranz BJ, et al. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature*. 1996;382:722-725.

25. Liu R, Paxton WA, Choe S, et al. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell*. 1996;86:367-377.

26. Huang Y, Paxton WA, Wolinsky SM, et al. The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. *Nat Med*. 1996;2:1240-1243.

27. Yarchoan R, Mitsuya H, Broder S. AIDS therapies. *Sci Am*. 1988;259: 110-119.

28. Chow YK, Hirsch MS, Merrill DP, et al. Use of evolutionary limitations of HIV-1 multidrug resistance to optimize therapy. *Nature*. 1993;361:650-654.

29. Palella FJ Jr, Delaney KM, Moorman AC, et al. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV outpatient study investigators. *N Engl J Med* . 1998; 338:853-860.

30. Vercauteren J, Wensing AM, van de Vijver DA, et al. Transmission of drug-resistant HIV-1 is stabilizing in Europe. *J Infect Dis*. 2009;200: 1503-1508.

31. Ghosh RK, Ghosh SM, Chawla S. Recent advances in antiretroviral drugs. *Expert Opin Pharmacother*. 2011;12:31-46.

32. Okwundu CI, Okoromah CA. Antiretroviral pre-exposure prophylaxis (PrEP) for preventing HIV in high-risk individuals. *Cochrane Database Syst Rev*. 2009:CD007189.

33. Boily MC, Baggaley RF, Wang L, et al. Heterosexual risk of HIV-1 infection per sexual act: systematic review and meta-analysis of observational studies. *Lancet Infect Dis*. 2009;9:118-129.

34. Salazar-Gonzalez JF, Salazar MG, Keele BF, et al. Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. *J Exp Med*. 2009;206:1273-1289.

35. Bar KJ, Li H, Chamberland A, et al. Wide variation in the multiplicity of HIV-1 infection among injection drug users. *J Virol*. 2010;84:6241-6247.

36. Haase AT. Early events in sexual transmission of HIV and SIV and opportunities for interventions. *Annu Rev Med*. 2011;62:127-139.

37. Haase AT. Perils at mucosal front lines for HIV and SIV and their hosts. *Nat Rev Immunol*. 2005;5:783-792.

38. Reynolds MR, Rakasz E, Skinner PJ, et al. CD8+ T-lymphocyte response to major immunodominant epitopes after vaginal exposure to simian immunodeficiency virus: too late and too little. *J Virol*. 2005;79: 9228-9235.

39. Rowland-Jones SL, Dong T, Fowke KR, et al. Cytotoxic T cell responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi. *J Clin Invest*. 1998;102:1758-1765.

40. Addo MM, Altfeld M, Brainard DM, et al. Lack of detectable HIV-1-specific CD8(+) T cell responses in Zambian HIV-1-exposed seronegative partners of HIV-1-positive individuals. *J Infect Dis*. 2011;203:258-262.

41. Quinn TC, Wawer MJ, Sewankambo N, et al. Viral load and heterosexual transmission of human immunodeficiency virus type 1. Rakai Project Study Group. *N Engl J Med*. 2000;342:921-929.

42. Cohen MS, Chen YQ, McCauley M, et al. Prevention of HIV-1 infection with early antiretroviral therapy. *N Engl J Med*. 2011;365:493-505.

43. Cohen MS, Shaw GM, McMichael AJ, et al. Acute HIV-1 infection. *N Engl J Med*. 2011;364:1943-1954.

44. Kahn JO, Walker BD. Acute human immunodeficiency virus type 1 infection. *N Engl J Med*. 1998;339:33-39.

45. Kassutto S, Maghsoudi K, Johnston MN, et al. Longitudinal analysis of clinical markers following antiretroviral therapy initiated during acute or early HIV type 1 infection. *Clin Infect Dis*. 2006;42:1024-1031.

46. Lyles RH, Munoz A, Yamashita TE, et al. Natural history of human immunodeficiency virus type 1 viremia after seroconversion and proximal to AIDS in a large cohort of homosexual men. Multicenter AIDS Cohort Study. *J Infect Dis*. 2000;181:872-880.

47. Haase AT. Targeting early infection to prevent HIV-1 mucosal transmission. *Nature*. 2010;464:217-223.

48. Brenchley JM, Schacker TW, Ruff LE, et al. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med*. 2004;200:749-759.

49. Mattapallil JJ, Douek DC, Hill B, et al. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature*. 2005;434:1093-1097.

50. Doitsh G, Cavrois M, Lassen KG, et al. Abortive HIV infection mediates CD4 T cell depletion and inflammation in human lymphoid tissue. *Cell*. 2010;143:789-801.

51. Moss AR, Bacchetti P. Natural history of HIV infection. *AIDS*. 1989;3:55-61.

52. Deeks SG, Walker BD. Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy. *Immunity*. 2007;27:406-416.

53. Detels R, Jacobson L, Margolick J, et al. The multicenter AIDS Cohort Study, 1983 to …. *Public Health*. 2012;126(3):196-198.

54. Centers for Disease Control and Prevention. Revised surveillance case definitions for HIV infection among adults, adolescents, and children aged <18 months and for HIV infection and AIDS among children aged 18 months to <13 years — United States, 2008. *MMWR*. 2008;57: No. RR-10.

55. Corbett EL, Watt CJ, Walker N, et al. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Arch Intern Med*. 2003;163:1009-1021.

56. Sigel K, Dubrow R, Silverberg M, et al. Cancer screening in patients infected with HIV. *Curr HIV/AIDS Rep*. 2011;8:142-152.

57. Life expectancy of individuals on combination antiretroviral therapy in high-income countries: a collaborative analysis of 14 cohort studies. *Lancet*. 2008;372:293-299.

58. Effros RB, Fletcher CV, Gebo K, et al. Aging and infectious diseases: workshop on HIV infection and aging: what is known and future research directions. *Clin Infect Dis*. 2008;47:542-553.

59. Fahey JL, Taylor JM, Manna B, et al. Prognostic significance of plasma markers of immune activation, HIV viral load and CD4 T-cell measurements. *AIDS*. 1998;12:1581-1590.

60. Fahey JL, Taylor JM, Detels R, et al. The prognostic value of cellular and serologic markers in infection with human immunodeficiency virus type 1. *N Engl J Med*. 1990;322:166-172.

61. Giorgi JV, Hultin LE, McKeating JA, et al. Shorter survival in advanced human

immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. *J Infect Dis*. 1999;179:859-870.

62. Silvestri G, Sodora DL, Koup RA, et al. Nonpathogenic SIV infection of sooty mangabeys is characterized by limited bystander immunopathology despite chronic high-level viremia. *Immunity*. 2003;18:441-452.

63. Silvestri G, Fedanov A, Germon S, et al. Divergent host responses during primary simian immunodeficiency virus SIVsm infection of natural sooty mangabey and nonnatural rhesus macaque hosts. *J Virol*. 2005;79:4043-4054.

64. Broussard SR, Staprans SI, White R, et al. Simian immunodeficiency virus replicates to high levels in naturally infected African green monkeys without inducing immunologic or neurologic disease. *J Virol*. 2001;75:2262-2275.

65. Hunt PW, Brenchley J, Sinclair E, et al. Relationship between T cell activation and CD4+ T cell count in HIV-seropositive individuals with undetectable plasma HIV RNA levels in the absence of therapy. *J Infect Dis*. 2008;197:126-133.

66. Brenchley JM, Price DA, Schacker TW, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med*. 2006;12:1365-1371.

67. Cooke KR, Hill GR, Crawford JM, et al. Tumor necrosis factor-alpha production to lipopolysaccharide stimulation by donor cells predicts the severity of experimental acute graft-versus-host disease. *J Clin Invest*. 1998;102:1882-1891.

68. Blackburn SD, Wherry EJ. IL-10, T cell exhaustion and viral persistence. *Trends Microbiol*. 2007;15:143-146.

69. Shin H, Wherry EJ. CD8 T cell dysfunction during chronic viral infection. *Curr Opin Immunol*. 2007;19:408-415.

70. Wherry EJ, Blattman JN, Murali-Krishna K, et al. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol*. 2003;77:4911-4927.

71. Virgin HW, Wherry EJ, Ahmed R. Redefining chronic viral infection. *Cell*. 2009;138:30-50.

72. Kaufmann DE, Walker BD. PD-1 and CTLA-4 inhibitory cosignaling pathways in HIV infection and the potential for therapeutic intervention. *J Immunol*. 2009;182:5891-5897.

73. El-Far M, Halwani R, Said E, et al. T-cell exhaustion in HIV infection. *Curr HIV/AIDS Rep*. 2008;5:13-19.

74. Kwon DS, Kaufmann DE. Protective and detrimental roles of IL-10 in HIV pathogenesis. *Eur Cytokine Netw* . 2010;21:208-214.

75. Hutter G, Nowak D, Mossner M, et al. Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *N Engl J Med* . 2009;360:692-698.

76. Biron CA, Byron KS, Sullivan JL. Severe herpesvirus infections in an adolescent without natural killer cells. *N Engl J Med* . 1989;320:1731-1735.

77. Martin MP, Gao X, Lee JH, et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet*. 2002;31:429-434.

78. Martin MP, Qi Y, Gao X, et al. Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. *Nat Genet*. 2007;39:733-740.

79. Hessell AJ, Hangartner L, Hunter M, et al. Fc receptor but not complement binding is important in antibody protection against HIV. *Nature*. 2007;449:101-104.

80. Scott-Algara D, Truong LX, Versmisse P, et al. Cutting edge: increased NK cell activity in HIV-1-exposed but uninfected Vietnamese intravascular drug users. *J Immunol* . 2003;171:5663-5667.

81. Ravet S, Scott-Algara D, Bonnet E, et al. Distinctive NK-cell receptor repertoires sustain high-level constitutive NK-cell activation in HIVexposed uninfected individuals. *Blood*. 2007;109:4296-4305.

82. Alter G, Martin MP, Teigen N, et al. Differential natural killer cellmediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. *J Exp Med*. 2007;204:3027-3036.

83. Kottilil S, Chun TW, Moir S, et al. Innate immunity in human immunodeficiency virus infection: effect of viremia on natural killer cell function. *J Infect Dis*. 2003;187:1038-1045.

84. Portales P, Reynes J, Pinet V, et al. Interferon-alpha restores HIVinduced alteration of natural killer cell perforin expression in vivo. *AIDS*. 2003;17:495-504.

85. Fogli M, Costa P, Murdaca G, et al. Significant NK cell activation associated with decreased cytolytic function in peripheral blood of HIV-1-infected patients. *Eur J Immunol*. 2004;34:2313-2321.

86. Bernstein HB, Wang G, Plasterer MC, et al. CD4+ NK cells can be productively infected with HIV, leading to downregulation of CD4 expression and changes in function. *Virology*. 2009;387:59-66.

87. Alter G, Heckerman D, Schneidewind A, et al. HIV-1 adaptation to NK-cell-mediated immune pressure. *Nature*. 2011;476:96-100.

88. Borrow P, Lewicki H, Hahn BH, et al. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol*. 1994;68: 6103-6110.

89. Koup RA, Safrit JT, Cao Y, et al. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol*. 1994;68:4650-4655.

90. Schmitz JE, Kuroda MJ, Santra S, et al. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science*. 1999;283:857-860.

91. Jin X, Bauer DE, Tuttleton SE, et al. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med*. 1999;189:991-998.

92. Kaslow RA, Carrington M, Apple R, et al. Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat Med*. 1996;2:405-411.

93. Migueles SA, Sabbaghian MS, Shupert WL, et al. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc Natl Acad Sci U S A* . 2000;97:2709-2714.

94. Carrington M, Nelson GW, Martin MP, et al. HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. *Science*. 1999;283: 1748-1752.

95. Goonetilleke N, Liu MKP, Salazar-Gonzalez JF, et al. The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. *J Exp Med*. 2009;206:1253-1272.

96. Radebe M, Nair K, Chonco F, et al. Limited immunogenicity of HIV CD8+ T-cell epitopes in acute clade C virus infection. *J Infect Dis*. 2011;204:768-776.

97. Altfeld M, Addo MM, Shankarappa R, et al. Enhanced detection of human immunodeficiency virus type 1-specific T-cell responses to highly variable regions by using peptides based on autologous virus sequences. *J Virol*. 2003;77:7330-7340.

98. Lichterfeld M, Yu XG, Cohen D, et al. HIV-1 Nef is preferentially recognized by CD8 T cells in primary HIV-1 infection despite a relatively high degree of genetic diversity. *AIDS*. 2004;18:1383-1392.

99. Yu XG, Addo MM, Rosenberg ES, et al. Consistent patterns in the development and

immunodominance of human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T-cell responses following acute HIV-1 infection. *J Virol*. 2002;76:8690-8701.

100. Draenert R, Allen TM, Liu Y, et al. Constraints on HIV-1 evolution and immunodominance revealed in monozygotic adult twins infected with the same virus. *J Exp Med*. 2006;203:529-539.

101. Altfeld M, Kalife ET, Qi Y, et al. HLA alleles associated with delayed progression to AIDS contribute strongly to the initial CD8(+) T cell response against HIV-1. *PLoS Med*. 2006;3:e403.

102. Streeck H, Jolin JS, Qi Y, et al. Human immunodeficiency virus type 1-specific CD8+ T-cell responses during primary infection are major determinants of the viral set point and loss of CD4+ T cells. *J Virol*. 2009;83:7641-7648.

103. Lichterfeld M, Yu XG, Mui SK, et al. Selective depletion of high-avidity human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T cells after early HIV-1 infection. *J Virol*. 2007;81:4199-4214.

104. Migueles SA, Laborico AC, Shupert WL, et al. HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol*. 2002;3:1061-1068.

105. Lichterfeld M, Kaufmann DE, Yu XG, et al. Loss of HIV-1-specific CD8+ T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific CD4+ T cells. *J Exp Med*. 2004;200: 701-712.

106. Robbins GK, Addo MM, Troung H, et al. Augmentation of HIV-1-specific T helper cell responses in chronic HIV-1 infection by therapeutic immunization. *AIDS*. 2003;17:1121-1126.

107. Pereyra F, Addo MM, Kaufmann DE, et al. Genetic and immunologic heterogeneity among persons who control HIV infection in the absence of therapy. *J Infect Dis*. 2008;197:563-571.

108. Edwards BH, Bansal A, Sabbaj S, et al. Magnitude of functional CD8+ T-cell responses to the gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma. *J Virol*. 2002;76:2298-2305.

109. Kiepiela P, Ngumbela K, Thobakgale C, et al. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nat Med*. 2007;13:46-53.

110. Ramduth D, Chetty P, Mngquandaniso NC, et al. Differential immunogenicity of HIV-1 clade C proteins in eliciting CD8+ and CD4+ cell responses. *J Infect Dis*. 2005;192:1588-1596.

111. Hersperger AR, Pereyra F, Nason M, et al. Perforin expression directly ex vivo by HIV-specific CD8+ T-cells is a correlate of HIV elite control. *PLoS Pathog*. 2010;6:e1000917.

112. Migueles SA, Osborne CM, Royce C, et al. Lytic granule loading of CD8+ T cells is required for HIV-infected cell elimination associated with immune control. *Immunity*. 2008;29:1009-1021.

113. Betts MR, Nason MC, West SM, et al. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood*. 2006;107:4781-4789.

114. Lazaro E, Godfrey SB, Stamegna P, et al. Differential HIV epitope processing in monocytes and CD4 T cells affects cytotoxic T lymphocyte recognition. *J Infect Dis*. 2009;200:236-243.

115. Yang OO, Kalams SA, Trocha A, et al. Suppression of human immunodeficiency virus type 1 replication by CD8+ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms. *J Virol*. 1997;71:3120-3128.

116. Sacha JB, Chung C, Rakasz EG, et al. Gag-specific CD8+ T lymphocytes recognize infected cells before AIDS-virus integration and viral protein expression. *J Immunol*. 2007;178:2746-2754.

117. Sacha JB, Chung C, Reed J, et al. Pol-specific CD8+ T cells recognize simian immunodeficiency virus-infected cells prior to Nef-mediated major histocompatibility complex class I downregulation. *J Virol*. 2007;81:11703-11712.

118. Julg B, Williams KL, Reddy S, et al. Enhanced anti-HIV functional activity associated with Gag-specific CD8 T-cell responses. *J Virol*. 2010;84:5540-5549.

119. Klatt NR, Shudo E, Ortiz AM, et al. CD8+ lymphocytes control viral replication in SIVmac239-infected rhesus macaques without decreasing the lifespan of productively infected cells. *PLoS Pathog*. 2010;6:e1000747.

120. Soghoian DZ, Streeck H. Cytolytic CD4(+) T cells in viral immunity. *Expert Rev Vaccines*. 2010;9:1453-1463.

121. Rosenberg ES, Altfeld M, Poon SH, et al. Immune control of HIV-1 after early treatment of acute infection. *Nature*. 2000;407:523-526.

122. Rosenberg ES, Billingsley JM, Caliendo AM, et al. Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science*. 1997;278:1447-1450.

123. Douek DC, Brenchley JM, Betts MR, et al. HIV preferentially infects HIV-specific

CD4+ T cells. *Nature*. 2002;417:95-98.

124. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, et al. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med* . 2009;361:2209-2220.

125. Ranasinghe S, Flanders M, Cutler S, et al. HIV-specific CD4 T cell responses to different viral proteins have discordant associations with viral load and clinical outcome. *J Virol*. 2012;86(1):277-283.

126. Soghoian DZ. HIV-specific cytolytic CD4 T cell responses during acute HIV infection predict disease outcome. *Sci Transl Med*. 2012;4(123):123ra25.

127. Kaufmann DE, Kavanagh DG, Pereyra F, et al. Upregulation of CTLA-4 by HIV-specific CD4+ T cells correlates with disease progression and defines a reversible immune dysfunction. *Nat Immunol*. 2007;8:1246-1254.

128. Younes SA, Yassine-Diab B, Dumont AR, et al. HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity. *J Exp Med*. 2003;198:1909-1922.

129. Pitcher CJ, Quittner C, Peterson DM, et al. HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. *Nat Med*. 1999;5:518-525.

130. Stamatatos L, Morris L, Burton DR, et al. Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine? *Nat Med*. 2009;15:866-870.

131. Gray ES, Madiga MC, Moore PL, et al. Broad HIV-1 neutralization mediated by plasma antibodies against the gp41 membrane proximal external region. *J Virol*. 2009;83(21):11265-11274.

132. Wei X, Decker JM, Wang S, et al. Antibody neutralization and escape by HIV-1. *Nature*. 2003;422:307-312.

133. Mascola JR, Lewis MG, Stiegler G, et al. Protection of macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. *J Virol*. 1999;73:4009-4018.

134. Shibata R, Igarashi T, Haigwood N, et al. Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. *Nat Med*. 1999;5:204-210.

135. Baba TW, Liska V, Hofmann-Lehmann R, et al. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. *Nat Med*. 2000;6: 200-206.

136. Walker LM, Huber M, Doores KJ, et al. Broad neutralization coverage of HIV by multiple highly potent antibodies. *Nature*. 2011;477(7365): 466-470.

137. Walker LM, Phogat SK, Chan-Hui PY, et al. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science*. 2009;326:285-289.

138. Scheid JF, Mouquet H, Feldhahn N, et al. Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals. *Nature*. 2009;458:636-640.

139. Scheid JF, Mouquet H, Ueberheide B, et al. Sequence and structural convergence of broad and potent HIV antibodies that mimic CD4 binding. *Science*. 2011;333:1633-1637.

140. Hessell AJ, Poignard P, Hunter M, et al. Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. *Nat Med*. 2009;15:951-954.

141. Altfeld M, Fadda L, Frleta D, et al. DCs and NK cells: critical effectors in the immune response to HIV-1. *Nat Rev Immunol* . 2011;11:176-186.

142. Donaghy H, Pozniak A, Gazzard B, et al. Loss of blood CD11c(+) myeloid and CD11c(-) plasmacytoid dendritic cells in patients with HIV-1 infection correlates with HIV-1 RNA virus load. *Blood*. 2001;98: 2574-2576.

143. Kwon DS, Gregorio G, Bitton N, et al. DC-SIGN-mediated internalization of HIV is required for trans-enhancement of T cell infection. *Immunity*. 2002;16:135-144.

144. Geijtenbeek TB, Kwon DS, Torensma R, et al. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell*. 2000;100:587-597.

145. Sabatte J, Ceballos A, Raiden S, et al. Human seminal plasma abrogates the capture and transmission of human immunodeficiency virus type 1 to CD4+ T cells mediated by DC-SIGN. *J Virol*. 2007;81: 13723-13734.

146. Meier A, Alter G, Frahm N, et al. MyD88-dependent immune activation mediated by human immunodeficiency virus type 1-encoded Tolllike receptor ligands. *J Virol*. 2007;81:8180-8191.

147. Beignon AS, McKenna K, Skoberne M, et al. Endocytosis of HIV-1 activates plasmacytoid dendritic cells via toll-like receptor-viral RNA interactions. *J Clin Invest*. 2005;115:3265-3275.

148. Manel N, Hogstad B, Wang Y, et al. A cryptic sensor for HIV-1 activates antiviral innate immunity in dendritic cells. *Nature*. 2010;467: 214-217.

149. O'Brien M, Manches O, Sabado RL, et al. Spatiotemporal trafficking of HIV in human plasmacytoid dendritic cells defines a persistently IFN-alpha-producing and partially matured phenotype. *J Clin Invest*. 2011;121:1088-1101.

150. Yonkers NL, Rodriguez B, Asaad R, et al. Systemic immune activation in HIV infection is associated with decreased MDC responsiveness to TLR ligand and inability to activate naive CD4 T-cells. *PLoS One*. 2011;6:e23884.

151. Donaghy H, Gazzard B, Gotch F, et al. Dysfunction and infection of freshly isolated blood myeloid and plasmacytoid dendritic cells in patients infected with HIV-1. *Blood*. 2003;101:4505-4511.

152. Martinson JA, Roman-Gonzalez A, Tenorio AR, et al. Dendritic cells from HIV-1 infected individuals are less responsive to toll-like receptor (TLR) ligands. *Cell Immunol*. 2007;250:75-84.

153. Sabado RL, O'Brien M, Subedi A, et al. Evidence of dysregulation of dendritic cells in primary HIV infection. *Blood*. 2010;116:3839-3852.

154. Smed-Sorensen A, Lore K, Vasudevan J, et al. Differential susceptibility to human immunodeficiency virus type 1 infection of myeloid and plasmacytoid dendritic cells. *J Virol*. 2005;79:8861-8869.

155. Lichterfeld M, Kavanagh DG, Williams KL, et al. A viral CTL escape mutation leading to immunoglobulin-like transcript 4-mediated functional inhibition of myelomonocytic cells. *J Exp Med*. 2007;204:2813-2824.

156. Moore CB, John M, James IR, et al. Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science*. 2002;296:1439-1443.

157. Allen TM, Altfeld M, Geer SC, et al. Selective escape from CD8+ T-cell responses represents a major driving force of human immunodeficiency virus type 1 (HIV-1) sequence diversity and reveals constraints on HIV-1 evolution. *J Virol*. 2005;79:13239-13249.

158. Brumme ZL, Brumme CJ, Heckerman D, et al. Evidence of differential HLA class I-mediated viral evolution in functional and accessory/regulatory genes of HIV-1. *PLoS Pathog*. 2007;3:e94.

159. Goulder PJ, Brander C, Tang Y, et al. Evolution and transmission of stable CTL escape mutations in HIV infection. *Nature*. 2001;412:334-338.

160. Brumme ZL, Brumme CJ, Carlson J, et al. Marked epitope- and allelespecific differences in rates of mutation in human immunodeficiency type 1 (HIV-1) Gag, Pol, and Nef cytotoxic T-lymphocyte epitopes in acute/early HIV-1 infection. *J Virol*. 2008;82:9216-

9227.

161. Fischer W, Ganusov VV, Giorgi EE, et al. Transmission of single HIV-1 genomes and dynamics of early immune escape revealed by ultra-deep sequencing. *PLoS One*. 2010;5:e12303.

162. Henn MR, Boutwell CL, Charlebois P, et al. Whole genome deep sequencing of HIV-1 reveals the impact of early minor variants upon immune recognition during acute infection. *PLoS pathogens*. 2012;8:e1002529.

163. Brumme ZL, John M, Carlson JM, et al. HLA-associated immune escape pathways in HIV-1 subtype B Gag, Pol and Nef proteins. *PLoS One*. 2009;4:e6687.

164. Draenert R, Le Gall S, Pfafferott KJ, et al. Immune selection for altered antigen processing leads to cytotoxic T lymphocyte escape in chronic HIV-1 infection. *J Exp Med*. 2004;199:905-915.

165. Allen TM, Altfeld M, Yu XG, et al. Selection, transmission, and reversion of an antigen-processing cytotoxic T-lymphocyte escape mutation in human immunodeficiency virus type 1 infection. *J Virol*. 2004;78:7069-7078.

166. Dahirel V, Shekhar K, Pereyra F, et al. Coordinate linkage of HIV evolution reveals regions of immunological vulnerability. *Proc Natl Acad Sci U S A* . 2011;108:11530-11535.

167. Crawford H, Lumm W, Leslie A, et al. Evolution of HLA-B*5703 HIV-1 escape mutations in HLA-B*5703-positive individuals and their transmission recipients. *J Exp Med*. 2009;206:909-921.

168. Miura T, Brumme ZL, Brockman MA, et al. Impaired replication capacity of acute/early viruses in persons who become HIV controllers. *J Virol*. 2010;84:7581-7591.

169. Kawashima Y, Pfafferott K, Frater J, et al. Adaptation of HIV-1 to human leukocyte antigen class I. *Nature*. 2009;458:641-645.

170. Fellay J, Shianna KV, Ge D, et al. A whole-genome association study of major determinants for host control of HIV-1. *Science*. 2007;317:944-947.

171. Pereyra F, Addo MM, Kaufmann DE, et al. Genetic and immunologic heterogeneity among persons who control HIV infection in the absence of therapy. *J Infect Dis*. 2008;197:563-571.

172. Dalmasso C, Carpentier W, Meyer L, et al. Distinct genetic loci control plasma HIV-RNA and cellular HIV-DNA levels in HIV-1 infection: the ANRS Genome Wide Association 01 study. *PLoS One*. 2008;3:e3907.

173. Pereyra F, Jia X, McLaren PJ, et al. The major genetic determinants of HIV-1 control affect HLA class I peptide presentation. *Science*. 2010;330:1551-1557.

174. Fellay J, Ge D, Shianna KV, et al. Common genetic variation and the control of HIV-1 in humans. *PLoS Genet*. 2009;5:e1000791.

175. Kulkarni S, Savan R, Qi Y, et al. Differential microRNA regulation of HLA-C expression and its association with HIV control. *Nature*. 2011;472:495-498.

176. Cohen GB, Gandhi RT, Davis DM, et al. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity*. 1999;10: 661-671.

177. World Health Organization. *Global HIV/AIDS Response*. Geneva, Switzerland: World Health Organization; 2011.

178. Stover J, Bollinger L, Hecht R, et al. The impact of an AIDS vaccine in developing countries: a new model and initial results. *Health Aff (Millwood)* . 2007;26:1147-1158.

179. Barouch DH, Korber B. HIV-1 vaccine development after STEP. *Annu Rev Med*. 2010;61:153-167.

180. Berman PW, Gregory TJ, Riddle L, et al. Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. *Nature*. 1990;345:622-625.

181. Check E. AIDS vaccines: back to “plan A.” *Nature*. 2003;423:912-914.

182. Flynn NM, Forthal DN, Harro CD, et al. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. *J Infect Dis*. 2005;191:654-665.

183. Gupta SB, Jacobson LP, Margolick JB, et al. Estimating the benefit of an HIV-1 vaccine that reduces viral load set point. *J Infect Dis*. 2007;195:546-550.

184. McElrath MJ, De Rosa SC, Moodie Z, et al. HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. *Lancet*. 2008;372:1894-1905.

185. Buchbinder SP, Mehrotra DV, Duerr A, et al. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a doubleblind, randomised, placebo-controlled, test-of-concept trial. *Lancet*. 2008;372:1881-1893.

186. Siegfried N, van der Merwe L, Brocklehurst P, et al. Antiretrovirals for reducing the risk of mother-to-child transmission of HIV infection. *Cochrane Database Syst Rev*. 2011:CD003510.

187. Grant RM, Lama JR, Anderson PL, et al. Preexposure chemoprophylaxis for HIV prevention in men who have sex with men. *N Engl J Med* . 2010;363:2587-2599.

188. Early end for FEM-PrEP HIV prevention trial. *AIDS Patient Care STDS*. 2011;25:383.

189. Abdool Karim Q, Abdool Karim SS, Frohlich JA, et al. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science.* 2010;329:1168-1174.

190. McMichael AJ, Borrow P, Tomaras GD, et al. The immune response during acute HIV-1 infection: clues for vaccine development. *Nat Rev Immunol* . 2010;10:11-23.

191. Karim SS, Karim QA. Antiretroviral prophylaxis: a defining moment in HIV control. *Lancet*. 2011;378:e23-e25.

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Chapter 43

Vaccines

Ennio De Gregorio
Ugo D'Oro
Sylvie Bertholet
Rino Rappuoli

INTRODUCTION

Over the last century, vaccination has been the most effective medical practice to control infectious diseases. Smallpox has been eradicated worldwide, and polio has been almost eliminated. Most viral and bacterial diseases traditionally affecting children worldwide are now preventable with vaccines (see www.who.int/vaccines-documents). Vaccination is estimated to save two to three million lives per year. Traditional vaccines designed following the basic Pasteur principle (isolate, inactivate, and inject) have been extremely successful in preventing infections by pathogens expressing relatively conserved antigens mainly through antibody-mediated mechanisms. However, there is still a long list of diseases that are not preventable by vaccination, and infectious diseases are still a major cause of death and disability worldwide. Some of these diseases are caused by pathogens that have a high degree of antigen variability and cannot be controlled only by antibodies but require a mix of humoral and cellular immune responses. As an example, the world is still waiting for effective vaccines against the three big killers: acquired immunodeficiency syndrome (AIDS), tuberculosis (TB), and malaria, which collectively kill almost five million people per year. Therefore, the development of new vaccines can still have a tremendous impact in reducing the mortality caused by infectious diseases throughout the world. Novel technologies for antigen identification, structural design, and formulation now allow for the development of safer vaccines that can better cope with pathogen diversity. In addition, progress in the field of innate immunity is translating into the development of novel classes of vaccine adjuvants that can promote, better than in the past, protective humoral and cellular immune responses. Significant research efforts are also being applied toward the development of therapeutic vaccines against cancer, allergy, autoimmunity, and other inflammatory disorders that could have a major impact on human health across the world. However, in this context, we will focus mainly on preventive vaccines against infectious diseases, although therapeutic vaccines against cancer will not be covered here. This chapter will give a short overview of the history of vaccine development and will describe most of the existing licensed vaccines for the prevention of infectious diseases. In addition, we will give details on novel approaches to develop effective vaccines against human immunodeficiency virus (HIV), malaria, and TB. One paragraph is dedicated to licensed and experimental vaccine adjuvants, with special focus on their immunologic mechanism of action. Other sections of this chapter will analyze new technologies that can either improve existing vaccines or allow the development of novel effective vaccines where conventional technologies have failed. Another section will concentrate on novel needle-free vaccine delivery systems. Finally, the last paragraph will review the major challenges pertaining to vaccines in the 21st century society.

HISTORICAL PERSPECTIVES

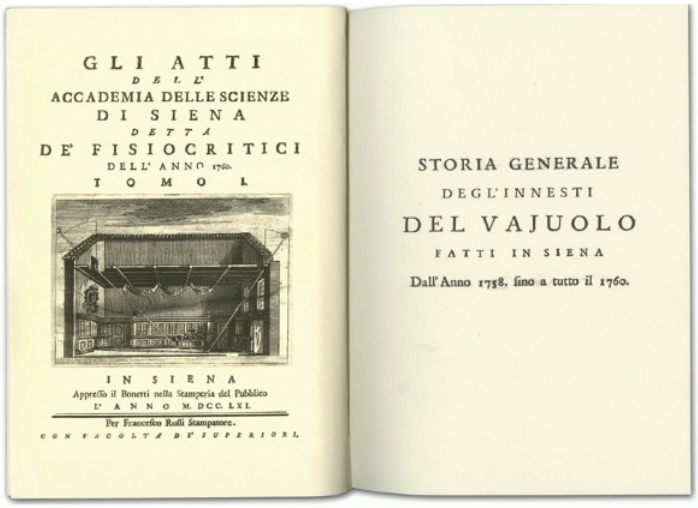
Smallpox: From Variolation to Vaccination

Smallpox, one of the most severe human viral diseases, is transmitted by inhalation of orthopoxvirus *Variola*. Before vaccination, smallpox outbreaks where quite frequent, killing more than 30% of infected people. It is estimated that in the late 18th century in Europe, 400,000 people died annually of smallpox. A method for preventing naturally acquired smallpox was discovered in India before AD 1000 and spread to China and western Asia. This method, called “variolation,” was introduced in Europe in 1721 by Lady Mary Wortley

Montagu after her return to England from Constantinople were she observed the use of the technique. Variolation consisted in the inoculation of pustule material from patients infected with smallpox in the skin or in the nose of noninfected people to protect them from the outbreaks. Of course, the practice was not safe, with a mortality rate ranging from 1% to 2%; however, it generally produced an illness that was less severe than natural *Variola* infection by inhalation. A variolation campaign was introduced in Siena, Italy in 1758 as reported in a dedicated publication by the local Academy of Science in 1760 (Fig. 43.1). The symptoms experienced by a child in the days following variolation are described in the following translated excerpt.

> In the year 1756, on the 8th of June, day on which the weather was very hot, Gherardo, son of Mr. Giovanni Pavolotti, was inoculated with smallpox…. A small cut was performed on each arm, and a wad of cotton wool soaked in the pus of a smallpox pustule of his brother, who had naturally acquired the disease, was applied. His arms were wrapped and the bandages were removed for the first time on the second day to treat the wounds. The cuts were almost dry and showed no change in color or other signs on the surrounding skin. The child showed not even the slightest discomfort; he went about the house enjoying his childish babble as usual; his pulse was steady, normal, the same as before the operation….
>
> Fourth day. The cuts started to hurt occasionally and to a small degree, they were red and started to fester…
>
> Sixth day. All became more serious. He complained a lot about the cuts; they were more open, festering,

> swollen, and quite uneven around the edges. At midday he felt cold, then he became hot, feverish. In the evening, he vomited bitter, biliary fluids. His eyes were teary, light did not bother him very much. The cuts had small blisters in the middle and around the uneven edges. They were fetid. Moderate fever, no thirst. He was kept under watch overnight. Moderate urination with dark yellow sediment.
>
> Seventh day. The cuts were more swollen, more festered, more fetid, the blisters were white, some were open. He complained about shooting pain in the armpits. At midday he became very hot, the fever rose, he was thirsty, somewhat shivering, frightened, spoke senselessly…
>
> Eighth day. At sunrise he slept at length. All was very pleasant. The cuts were very festered. The blisters that had appeared on the sixth day were open. There were some smallpox pustules around the cuts…
>
> Ninth day. No fever at all…
>
> Tenth day. The rash stopped. The blisters started to fester. The child got up from bed and walked about the house.
>
> Sixteenth day. The blisters were dry and the scabs started falling. The cuts were red, there was less pus, which was not so fetid…
>
> Twenty-fourth day. The cuts had cicatrized. On that day, the child went outdoors for a walk and the blister marks were hardly visible.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IN SIENA
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L' ANNO M. DCC. LXI.
Per Francesco Rossi Stampatore.
CON FACOLTÀ DE' SUPERIORI.
STORIA GENERALE
DEGL'INNESTI
DEL VAJUOLO
FATTI IN SIENA
Dall'Anno 1758. fino a tutto il 1760.

FIG. 43.1. Front page of the publication by the Academy of Science of Siena reporting the history of the variolation campaign made in Siena between 1758 and 1760. Reproduction by courtesy of the Accademia de' Fisiocritici, Siena, Italy.

A real vaccination practice was introduced when Edward Jenner replaced smallpox-infected material with pustule material from humans infected by cowpox. Cows can be infected by a virus similar to *Variola*, called *Vaccinia* virus, and develop a smallpox-like diseases called cowpox that can be transmitted to humans. It was quite frequent that people in close contact with cows, such as milkmaids, would contract cowpox and develop sores on fingers and hands. In the 18th century, several physicians noticed that people exposed to cowpox were protected from smallpox and did not react to variolation. In 1796, Edward Jenner demonstrated for the first time that pustule material taken from a milkmaid infected by cowpox when inoculated into the skin of another person produced a localized self-limiting infection, which, a few months afterward, was able to protect from smallpox challenge (by variolation). He also demonstrated that the same individual was still protected from smallpox variolation 5 years later, confirming the persistence of immunity. Jenner called the material used for the inoculum *Vaccinia* (from "vacca," the Latin name of cow) and the process "vaccination." For many years, vaccination became a standard practice in several countries and replaced variolation. At the beginning, vaccination was still relying on the arm-to-arm inoculation of human infected material. This process led to a progressive attenuation of the inoculum and was often associated with the transmission of other human diseases from the donor. Therefore, by 1890, human infected material was replaced by the use of

vesicle fluid from infected cows. In 1940, Collier developed a modern commercial process to produce a stable freeze-dried vaccine for large-scale smallpox prevention. Because *Variola* virus is transmitted from human to human and does not have any animal reservoir, it was hypothesized that eradication of smallpox through vaccination was possible. In 1959, the World Health Organization (WHO) set global smallpox eradication as a goal, but it was only in 1966 that a Smallpox Eradication Unit was formed. The global eradication program started in 1967. It is estimated that at that time, there were still 10 to 15 million cases of smallpox worldwide. The vaccination campaign lasted about 10 years and was extremely successful. The last case of naturally contracted smallpox was recorded in 1977 in Somalia, and in 1979, the Global Commission for Smallpox Eradication certified that global smallpox eradication had been achieved.

Nineteenth Century Vaccines

The first successful vaccine was discovered by Jenner without any understanding of its mechanism of action. The microbial origin of infectious diseases was discovered many years later by Louis Pasteur and Robert Koch. Both scientists gave a dramatic impulse to vaccine research. Pasteur discovered that bacteria grown in vitro for a sufficient amount of time lose their virulence and become attenuated. He proposed that these live-attenuated microorganisms could be used to inoculate individuals similarly to what Jenner had done in

the case of cowpox. In honor of Jenner, Pasteur extended the word “vaccine” to all preparations used for immunization. Initially, Pasteur worked on animal models demonstrating that live-attenuated chicken cholera provides immunity against an experimental challenge with virulent organisms. Later, he showed that attenuated anthrax bacilli protected sheep from infection. After demonstrating the vaccination principle in animals, Pasteur worked on an attenuated rabies preparation that was first administered in humans in 1885. This vaccine was prepared by infecting rabbits with rabiesinfected material for several passages and then recovering rabbit spinal cord. The virulence of spinal cord material was further attenuated by exposing it to dry air. Pasteur's rabies vaccine was administered for the first time subcutaneously in 13 doses over a 10-day period to Joseph Meister, a boy who had been bitten by a rabid dog 60 hours before and had no chance to survive. Thanks to vaccination, the patient resisted the development of the disease. At that time, Pasteur did not know that rabies was caused by a virus belonging to the family of *Rhabdoviridae* that takes several days from the time of infection to reach the central nervous system, making postexposure prophylaxis possible. Today, several antirabies vaccines exist and are all made of chemically inactivated virus grown in duck embryos or in cell culture.

Pasteur thought that only live-inactivated pathogens could be used for effective vaccination. The concept of killed vaccines was introduced in 1886. After Robert Koch discovered *Vibrio cholerae*, Daniel Elmer Salomon and Theobald Smith showed that a killed suspension of *V. cholerae* from hogs protected pigeons from the disease. Similar data were presented by a group working at the Pasteur Institute. These findings paved the way for the development of human killed whole-organism vaccines against typhoid fever (1896), cholera (1896), and plague (1897) (Table 43.1).

TABLE 43.1 Development of Human Vaccines

Live Attenuated	Killed Whole Organism	Toxoid/Protein	Polysaccharide	Glycoconjugate	Recombinant	Century
Smallpox						18th
Rabies	Typhoid Cholera Plague					19th
Tuberculosis (BCG) Yellow fever Polio (OPV) Measles Mumps Rubella Typhoid Varicella Rotavirus Cholera	Pertussis Influenza Typhus Polio (IPV) Rabies JE TBE HAV	Diphtheria Tetanus Acellular Pertussis Anthrax Influenza subunit	Pneumococcus Meningococcus Hib Typhoid (Vi)	Hib	HBV Lyme disease Cholera toxin B	20th
Cold-adapted influenza Rotavirus reassortants Zoster				Pneumococcus MenACWY	HPV	21st

BCG, Bacille Calmette-Guérin; HAV, hepatitis A virus; HBV, hepatitis B virus; Hib, *Haemophilus influenzae* type b; IPV, inactivated polio vaccine; JE, Japanese encephalitis; Men, meningococcus; OPV, oral polio vaccine; TBE, tick-borne

encephalitis.

Adapted from Plotkin S, Orenstein WA, Offit P. Vaccines. 5th ed. New York, NY: Saunders-Elsevier; 2008, with permission.

Twentieth Century Vaccines

At the end of the 19th century, the basic principle acknowledged in vaccinology was to isolate the causative agent of disease, attenuate or kill the agent, and immunize. This principle allowed for the development of many live-attenuated and killed vaccines. The first important innovation in vaccinology in the first half of the 20th century was the discovery of vaccines based on inactivated toxins, also called toxoids. Bacterial toxins had already been identified in the 19th century. In 1888, Roux and Yersin identified diphtheria toxin. Two years later, Emil von Behring and Shibasaburo Kitasato discovered the presence of antitoxins in serum. In 1891, passive immunization using sera of animals immunized with low doses of diphtheria and tetanus toxins became available. However, toxins were not used as vaccines because they were too dangerous. At the beginning of the 20th century, Theobald Smith demonstrated that vaccines made from chemically inactivated toxins protected guinea pigs from infection. In 1923, Alexander Glenny showed that diphtheria toxin can be chemically inactivated by formalin, leading to the first human toxoid vaccine against diphtheria. In 1926, another formalin-inactivated toxoid vaccine against tetanus was introduced by Gaston Ramon and Christian Zoeller.

During the same period, the application of Pasteur's principle led to the development of two new live-attenuated vaccines against TB and yellow fever. The TB vaccine was developed by Albert Calmette and Camille Guérin at the Pasteur Institute. They started from a *Mycobacterium bovis* strain that was attenuated for 13 years by 230 passages in culture media. The resulting Bacille Calmette-Guérin (BCG) strain became available for human vaccination in 1927. The first yellow fever vaccine was developed by attenuating the virus in a culture medium made of mouse brain tissue and was introduced in 1932. A few years later, the introduction of embryonated eggs as a medium for growing viruses allowed for the development of a new, safer, more attenuated vaccine to prevent yellow fever infection from a strain called 17D. Embryonated eggs were also used to develop the first two human vaccines against type A influenza. In the same year (1936), a live-attenuated influenza vaccine was introduced by Wilson Smith while a killed whole virus vaccine was developed by Thomas Francis and Thomas Magill. In the first half of the 20th century, two additional whole-killed bacterial vaccines against typhus and pertussis were developed, and in 1948, the first combination vaccine against diphtheria, tetanus, and pertussis became available.

The second half of the 20th century is considered the golden age of vaccine development. Probably, the most important technologies introduced at that time are related to the production of human viruses in controlled cell culture conditions, which allowed for the development of several new virus vaccines. Among them, the two vaccines against poliovirus are perhaps the ones that gained the greatest public interest. Before vaccination, more than 20,000 cases of polio were reported annually in the United States. A formalin-inactivated polio vaccine (IPV), developed by Jonas Salk, was licensed in 1955. A few years later (1960), a liveattenuated polio vaccine for oral administration (oral polio vaccine [OPV]) was developed by Albert Sabin. Owing to global vaccination programs, polio has been eradicated in most countries and remains endemic only in some areas of Afghanistan, India, Pakistan, and Nigeria. Other liveattenuated virus vaccines licensed between 1950 and 1970 are against measles, mumps, rubella, and varicella. More recently (1998), an oral live-attenuated vaccine against rotavirus infection was licensed in the United States, Europe, and other countries. In addition to live-attenuated vaccines, new whole-killed virus vaccines have also been licensed, including a hepatitis A virus (HAV) vaccine in 1979 (Table 43.1).

In the second part of the 20th century, several bacterial vaccines were developed using purified polysaccharides. Monovalent vaccines to prevent meningococcus (Men) group A was first licensed in 1974 followed by a MenC and a bivalent MenAC vaccine in 1975, and a quadrivalent MenACWY later in 1982. A 14-valent pneumococcus vaccine was licensed in 1977 followed by a 23-valent vaccine in 1983, whereas a vaccine against *Haemophilus*

influenzae type b (Hib) was licensed in 1985. However, polysaccharide vaccines are not protective in children younger than 18 months, who are at most risk of contracting the disease. This problem was solved when it was shown that the immunogenicity of polysaccharides could be enhanced by linking them to a carrier protein such as diphtheria toxoid (DT) or tetanus toxoid (TT). This new class of vaccines, called glycoconjugates, is immunogenic in all age groups, including infants. The first glycoconjugate to be licensed was a Hib vaccine developed by Robbins and Schneerson (1987).[1]

Another important innovation in vaccinology introduced in the 20th century has been the use of a genetically modified toxin as a vaccine first applied to pertussis.[2] Finally, the use of recombinant proteins instead of the antigens purified directly from the pathogens has been a major breakthrough. A vaccine made of hepatitis B surface antigen (HBsAg) expressed in yeast and adsorbed to alum was the first recombinant protein vaccine for human use licensed in 1987. The vaccine takes advantage of the property of HBsAg to self-assemble in a viral-like particle (VLP), resulting in an increased immunogenicity. A second recombinant protein vaccine based on the OspA antigen from *Borrelia burgdorferi* expressed in *Escherichia coli* was licensed in 1999 to prevent Lyme disease. However, the vaccine was withdrawn from the market in 2002 due to a lack of demand.

Twenty-first Century Vaccines

Several novel vaccines were licensed in the first decade of the 21st century. Three of them (cold-adapted influenza, novel rotavirus, and zoster) are based on live-attenuated pathogens. Others, such as 7-valent, 10-valent, and 13-valent pneumococcal conjugate vaccines or quadrivalent meningococcal vaccines, are based on the glycoconjugate technology. Finally, two vaccines to prevent human papillomavirus (HPV) infection are based on recombinant antigens (Table 43.1). Most of the vaccines listed in this paragraph will be described in more detail in the section dedicated to licensed vaccines. Another paragraph will describe new experimental vaccines that have not been licensed yet, such as vaccines to prevent AIDS, TB, and malaria.

CLASSIFICATION OF HUMAN VACCINES

To be efficacious, a vaccine needs to generate an immunologic status that is sufficient to block infection by the pathogen or at least to inhibit the establishment of disease. Depending on the type of infection to be prevented, an effective vaccine may require the induction of different humoral and cellular immune effector mechanisms. Protective humoral responses include neutralizing antibodies, opsonizing antibodies, or antibodies able to induce complement-mediated killing. Protective cellular mechanisms include different classes of cluster of differentiation (CD)4+ T cells and CD8+ cytotoxic T cells (CTLs). Based on the type of material from which they are made, licensed vaccines can be broadly classified into three general categories: live-attenuated vaccines, whole-killed vaccines, and subunit vaccines. Subunit vaccines can be further divided into polysaccharide, glycoconjugate, toxoid, or protein subunit and recombinant subunit vaccines. Different types of vaccines target diverse adaptive immune responses (Fig. 43.2). For example, glycoconjugate vaccines elicit high-avidity bactericidal antibodies but do not trigger any antigen-specific effector T-cell responses. Proteinbased vaccines can induce bactericidal and neutralizing antibodies and can also trigger robust CD4+ T-cell responses; however, they are not efficient in inducing CTLs. Finally, live-attenuated vaccines are able to generate all humoral and cellular responses, including CTLs. This section will describe all classes of licensed vaccines with a special focus on their immunologic correlates of protection. In addition, some promising technologies for vaccine design such as viral vectored and nucleic acid-based vaccines will be presented, even if they have not been applied yet to vaccine licensed for use in humans.

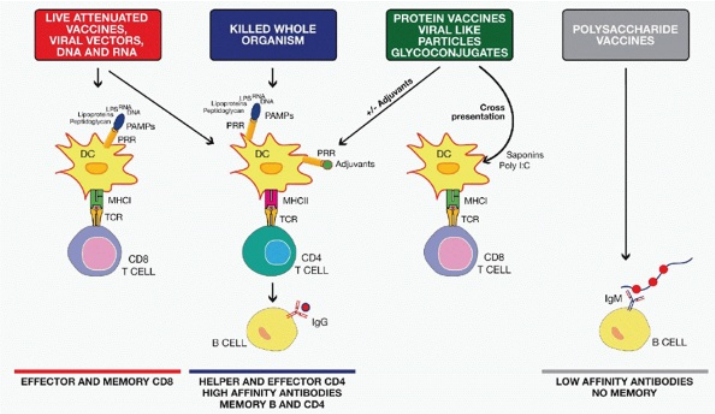

FIG. 43.2. Mode of Action of Different Classes of Vaccines. Live attenuated, viral vectors and nucleic acid vaccines induce both major histocompatibility complex (MHC) class I and MHC class II antigen presentation and activate pattern recognition receptors (PRRs) expressed by dendritic cells (DCs) through several pathogen-associated molecular patterns (PAMPs) including peptidoglycan, lipopolysaccharide, and bacterial or viral ribonucleic acid and deoxyribonucleic acid. They generally induce robust cluster of differentiation (CD)8 and CD4 T-cell responses, high antibody titers, and good memory. Killed whole-organism vaccines activate DCs through the same PAMPs and induce good CD4 and B-cell responses. Protein vaccines, viral-like particles, and glycoconjugate vaccines may need an adjuvant for optimal DC activation and CD4 T-cell priming. Some adjuvants such as polyinosinic:polycytidylic acid or saponins can also induce CD8 T-cell responses to subunit vaccines through cross-presentation mechanisms. Finally, polysaccharide vaccines induce a T-cell-independent antibody response.

Live-Attenuated Vaccines

As described in the previous paragraph on the history of vaccination, the very first vaccine of the modern era, Jenner's vaccine against smallpox, was a live-attenuated viral vaccine, prepared from a natural strain of the cowpox or *Vaccinia* virus, which is not pathogenic for humans, to protect humans against *Variola* virus infection. Indeed, one type of live-attenuated vaccine is prepared from a microorganism that normally infects other animal species and does not cause disease in humans but is similar enough to the human pathogen to trigger a specific immune response that protects against the human infectious organism.

For most pathogens, however, a related animal pathogen that is inoffensive to humans does not exist, but luckily, a live-attenuated vaccine can also be obtained using a mutated strain of the infectious microorganism that has a reduced pathogenicity and therefore is able to infect the human being without inducing disease. It is not uncommon for attenuated strains of a pathogen to spontaneously develop during natural infection of the human population. These attenuated strains can therefore be isolated in order to develop a vaccine.[3] However, attenuated viral strains can also be obtained by culturing the virus in vitro in nonhuman cells and selecting for mutant viruses that are adapted to this environment but are less fit to grow in the human cells. Most of the vaccines against viral infections in use today belong to this group of vaccines (eg, mumps, rubella, measles, varicella, and rotavirus vaccines). Although more difficult to obtain, and thus more rare, live-attenuated vaccines may also be developed against bacterial infections. Examples of live-attenuated bacterial vaccines among the currently licensed products are the vaccine against TB and an oral vaccine against typhoid fever. As the attenuated microorganism is capable of replication in the host mimicking a natural infection, this type of vaccine is generally able to induce strong and persistent antigen-specific immune responses in the vaccinees, resulting in a significant clinical efficacy. On the other hand, these vaccines may cause clinical manifestations associated with the infection, especially in immunocompromised subjects. Another potential risk associated with the use of attenuated strains is that they could revert to virulent form, therefore causing pathology in the vaccinated subject.

Whole-Killed or Inactivated Vaccines

Vaccines in this class contain the entire infectious agent that has been made harmless and incapable of replication by either chemical treatment (eg, formaldehyde) or a physical treatment like heating or radiations. Similar to live-attenuated vaccines, whole-killed or inactivated vaccines target bacterial and viral infections. Although this approach has been widely exploited in the past, only a few of the vaccines in use today are based on whole-killed microorganisms. In particular, only one whole-inactivated bacterial vaccine is still extensively used around the world, the whole-cell pertussis vaccine, whereas an oral vaccine against cholera, containing both heat-killed and formalin-inactivated strains of *V. cholerae*, is currently administered only to specific populations, such as travelers to an area where the infection is endemic. Examples of efficient vaccines prepared with whole-killed viruses are Salk's IPV, rabies vaccine, and HAV vaccine. Inactivated vaccines are not able to cause infection or reproduce in the host, and, therefore they generally induce a lower antigenic stimulation compared to live-attenuated vaccines, requiring multiple immunizations to achieve efficacy. In addition, whole-killed vaccines can be reactogenic because they generally include microbe-derived proinflammatory molecules, also known as pathogen-associated molecular patterns (PAMPs). The high reactogenicity associated with some vaccines containing killed microorganisms has led to a progressive reduction of their use in favor of more purified vaccine preparations represented by the subunit vaccines.

Subunit Vaccines

A vaccine can be made of single or multiple antigenic components of a microorganism that are capable of stimulating a specific immune response sufficient to protect from the relevant pathogen infection or from the clinical manifestation of the disease. Depending on the molecular composition of the purified antigen used to prepare the vaccine, and on the techniques applied to obtain the final material used as a vaccine, different types of subunit vaccines can be defined.

Toxoids

Toxoids are toxins that are inactivated by chemical or physical treatment, but that nonetheless preserve their antigenic structure. This approach allows the formation of a protein that has completely lost toxic activity but is still capable of inducing the production of antibodies that will recognize and block the native toxin. This approach can be applied in case of infections in which the pathology exclusively depends on the damage induced by a single toxin produced by a pathogen, and, therefore, production of antibodies neutralizing the activity of the toxin or rapidly causing its elimination will be enough to avoid any disease. Vaccines against tetanus and diphtheria are based on toxoids; as in both cases, the pathology is caused entirely by a single toxic protein secreted by *Clostridium tetani* or *Corynebacterium diphtheriae*, respectively. Another commonly used toxoid is the inactivated pertussis toxin from *Bordetella pertussis*, which is one of the components of the acellular pertussis vaccine.

Protein Vaccines

In case immunization with a single protein or a combination of proteins from a pathogen is sufficient to stimulate a protective immune response against that particular microorganism, the approach of a protein-based vaccine is appropriate. Proteins can be purified from in vitro cultures of a pathogenic microorganism. The resulting vaccine preparations contain different amounts of contaminants depending on the efficiency of the purification process. Licensed acellular pertussis vaccines currently available contain from two to four different proteins purified from *B. pertussis* and are able to confer protection against whooping cough comparable to that obtained with the whole cell vaccine. One of the most widely used subunit protein vaccines is the influenza vaccine composed of hemagglutinin (HA) and neuraminidase (NA) purified from the inactivated influenza virus.

Recombinant Protein Vaccines

Development of the recombinant deoxyribonucleic acid (DNA) technology has made possible the expression of protective protein antigens in heterologous expression systems such as *E. coli*, yeast, mammalian cells, or baculovirus. This technology avoids the problems related to growing and manipulating large amounts of a pathogen from which the antigen is purified. Moreover, recombinant proteins are generally better purified from cultured microorganisms

resulting in cleaner vaccine preparations with a better safety

profile. A drawback of a clean vaccine preparation containing pure recombinant protein(s) is their reduced immunogenicity that may require the addition of an adjuvant to achieve enhanced efficacy.

Viral-Like Particles

VLPs are supramolecular protein structures formed by viral proteins in vitro in the absence of a viral genome, which are structurally similar to the natural viral infectious particles while they are not infectious. One example of a VLP is the vaccine used to prevent hepatitis B virus (HBV). HBsAg spontaneously assembles into spherical particles of 22 nm diameter when expressed in yeast or mammalian cells.[4,5,6] Many theoretical and practical aspects of VLP technology make this approach attractive to vaccine design. The viral antigens expressed in VLPs usually exhibit a conformational structure that is similar to what they present in the real virus, and this is usually associated with conservation of critical conformational epitopes that are targets of a neutralizing immune response. Another important characteristic of VLPs is that they express the antigenic epitopes in multiple repetitive structures in one single particle, which potentially can induce an efficient engagement of the antigen receptor on the surface of B cells, resulting in an improved antibody response compared to what is obtained with soluble protein antigens. As VLPs are not infectious, there are no issues related to manipulation of high amounts of infectious material. Moreover, no inactivation procedures, which may result in damage of important immune relevant epitopes, are required. VLPs are at the base of the two HPV licensed vaccines. The VLP approach has been applied to many vaccine candidates, some of which are currently being tested in clinical trials.[7,8] This approach has also been used for antigens that do not naturally form VLPs by developing chimeric VLPs using two different methods. In one case using DNA recombinant techniques, a candidate vaccine antigen is genetically fused with a protein that is able to form VLPs, resulting in its expression in the context of the VLP structure. Alternatively, an antigen is chemically linked to heterologous VLPs that will provide the multimeric structure able to induce an appropriate immune stimulation. When these chimeric particles are administered, they are able to elicit a strong immune response to all components in the VLPs, including the epitopes from the fused candidate vaccine antigen. The chimeric VLP approach has been evaluated for development of vaccines against viral infections such as hepatitis C virus (HCV)[9] and HIV,[10] or against malaria,[11] as well as of therapeutic vaccines for cancer or for addictive substances such as nicotine.[12] However, thus far, none of these experimental chimeric VLP vaccines have progressed beyond phase I/II clinical trials.

Polysaccharide-Based Vaccines

The surface of many pathogenic bacteria is covered by a capsular shell that is mainly assembled from ordered polymeric glycans. This extensive polysaccharide coat entirely shields the bacteria outer membrane, preventing other surface bacterial components from becoming a target of the host immune response. Nevertheless, antibodies to bacterial surface polysaccharides can clear the bacteria from the host by different mechanisms, such as complement-mediated killing and opsonophagocytosis. Hence, stimulation of an antibody response against the surface polysaccharide of pathogenic bacteria is a valid strategy for the development of vaccines against capsulated bacteria. Experiments using the capsular polysaccharide isolated from *Streptococcus pneumoniae* demonstrated for the first time the immunogenicity of a bacterial carbohydrate and a protective effect of a polysaccharide-based vaccination.[13] The chemical structure of capsular polysaccharides varies not only between bacteria of different species but also between different strains within a single species, which are usually differentiated and typed based on their capsular polysaccharides. As a consequence, a limitation of polysaccharide-based vaccine is that the immune responses they elicit are often serotype specific. Thus, if disease burden is caused by multiple polysaccharide types, a vaccine would optimally contain multiple polysaccharides in order to confer broad protection. In addition to *S. pneumoniae*, for which a vaccine against 23 serotypes is available, polysaccharide-based vaccines have been developed for MenACWY, Hib, and *Salmonella typhimurium*.

Glycoconjugate-Based Vaccines

Purified polysaccharides are able to stimulate only B-cell responses because they cannot enter the major histocompatibility complex (MHC) cavity (which evolved to bind peptides) and cannot be presented to T cells. For this reason, they are classically known as “T-independent” antigens. Thus, the antibody response generated by a polysaccharide vaccine is a typical T-cell-independent response that presents two major disadvantages from a vaccine point of view. First, the response does not take place in germinal centers, and it is therefore not associated with affinity maturation of the antibodies or the generation of memory B cells. As a consequence, a polysaccharide vaccine will induce low- to intermediate-affinity antibody responses that is not followed by immunologic memory, resulting in the need for booster administration to maintain immunity. Moreover, a T-cell-independent antibody response is deficient or absent in young children (younger than 18 months of age) and in the elderly, two populations at risk of infection with capsulated bacteria such as pneumococcus or meningococcus, and for which a polysaccharide-based vaccine is not efficacious. A way to circumvent the limitations of these vaccines is to conjugate a polysaccharide to a carrier protein, such as TT or DT, in order to induce a carrier-specific T-cell response. Following vaccination with glycoconjugate vaccines, T follicular helper cells promote differentiation of the polysaccharide-specific B cells in the germinal center, where somatic mutation and affinity maturation take place, resulting in higher affinity antibody response and generation of long-lived memory B cells conferring long-term protection. The first demonstration that conjugation to a protein increases immunogenicity of a capsular polysaccharide was obtained in 1929; however, it took 50 years for this strategy to be applied to vaccine design, when a glycoconjugated vaccine for Hib was first tested in animals.[1] Compared to polysaccharide vaccines, glycoconjugated vaccines are indeed

more efficacious, being able to induce a long-lasting antibody response not only in adults but also in infants, young children, and the elderly. Similarly to the polysaccharidebased vaccines, the glycoconjugate vaccines are also serotype specific, and, therefore, multivalent formulations are required to achieve protection against multiple serotypes.

Combination Vaccine

In clinical practice, vaccines for different pathogens or for different strains of a particular pathogen are combined in one single formulation, allowing a reduction in the number of injections administered, with a concomitant reduction in costs and distress for the individuals. This last advantage is particularly important for childhood vaccinations that are frequently associated with psychological distress for parents, which may affect compliance with the vaccination schedule and be accompanied by possible negative effects on vaccination coverage. Indeed, it has been reported that the use of combination vaccines has improved both coverage rates and timeliness of vaccination.[14,15] A combination of diphtheria and tetanus toxoids with the whole-killed pertussis vaccine (DTwP) was the first multipathogen vaccine to be licensed. Years later, a similar combination containing the acellular pertussis component (DTaP) has been used as the basis to build new expanded combinations that may include one or more components such as IPV, HBsAg, and Hib glycoconjugate. Another commonly used pediatric combination is a mix of measles, mumps, and rubella vaccine, although a quadrivalent vaccine containing also the varicella vaccine became available only recently after solving problems related to compatibility and stability of the different components. Together with the continuous development of new vaccines, demands for additional combinations will be increasing with the aim of reducing costs and simplifying the vaccination schedule, in particular in the infant population. However, the increase in complexity of combination vaccines will raise the risk of a possible interference on antigen stability and immunogenicity.

Novel Technologies for Vaccine Design

Novel technologies and new strategies are continuously tested in preclinical research and applied to the development of new candidate vaccines. Novel types of vaccines can therefore be identified among the experimental vaccines currently evaluated in both preclinical and clinical research, some of which may become established products in the future.

Viral Vector-Based Vaccines

Priming of CTL responses requires presentation of the antigen by the MHC class I molecules, a pathway usually followed by endogenously synthesized proteins (eg, viral antigens) but not by exogenously administered antigens. For this reason, inactivated or subunit vaccines are not per se able to stimulate a CTL response, which is instead induced by live-attenuated viral vaccines. The observation that a gene encoding an external antigen could be inserted in the genome of the *Vaccinia* virus and expressed in the virus-infected cells has prompted the idea of genetically manipulating an attenuated or nonpathogenic virus to express an exogenous antigen from a pathogenic virus against which vaccines need to be developed. Because these antigens are synthesized by the host cells, they undergo all posttranslational modifications and acquire a correct conformation and should be able to stimulate a strong neutralizing antibody response. Moreover, antigens are presented in the context of the MHC class I pathway generating therefore a CTL response. Examples of attenuated strains of *Vaccinia* virus are the modified *Vaccinia* Ankara (MVA) strain obtained by repetitive passages through in vitro cell cultures and the New York *Vaccinia* strain obtained by genetic manipulation of the viral genome. These strains have been evaluated in both nonhuman primates and humans as vector vaccines for HIV, malaria, influenza, TB, and other infectious diseases.[16,17,18] Other poxviruses tested as vector vaccines include fowlpox and canarypox.[19] Despite extensive testing of these vectors as vaccine candidates, so far, no successful vaccine has been developed using this approach.

Because of their immunogenicity and efficacy in the induction of a robust cellular response, replication-incompetent recombinant adenoviral vectors (rAds) have been successfully tested in many animal models as vaccine vectors for many pathogens such as Ebola, HIV, anthrax, influenza, and malaria.[20,21,22,23] Adenoviral vaccine vectors have also been evaluated in many human clinical trials, particularly for HIV and cancer. On the other hand, natural adenovirus circulates in the human population, and, therefore, preexisting immunity, in particular neutralizing antibodies, may be present in the subject who will receive the vaccine, compromising efficacy of these vaccines.[24] It is important to note that tens of adenovirus serotypes exist but most of the clinical studies have used a vector derived from a single serotype (Ad5). Serotype 5 is one of the most common serotypes spread in the human population, and a high frequency of subjects possesses anti-Ad5 antibodies. In order to overcome potential problems related to preexisting anti-Ad5 antibodies, new vectors based on different human serotypes[25] or on adenovirus from other species[26,27,28] were developed. At the present, the most promising are the chimpanzee-derived adenoviruses.[27]

Alphaviruses are positive single-stranded ribonucleic acid (RNA) viruses in which the genome can be manipulated to obtain replication-defective infectious particles that express an exogenous antigen. Besides this favorable safety feature, upon entering the target cell, alphavirus RNA self-replicates, producing many copies of the coding RNA, which can then direct the synthesis of a very high amount of the antigen, making it a promising platform for a vector-based vaccine.[29] Alphavirus-based vaccines have been tested in animal models for several pathogens and have demonstrated excellent immunogenicity.[30,31,32,33] Some clinical trials have been conducted, but the results have not been published.

The prime-boost vaccination strategy using viral vectors has received wide evaluation in the vaccine field. Heterologous prime-boost regimens mainly use a viral vector or a DNA vaccine (see subsequent discussion) at priming

followed by a boost with a protein-based vaccine, although other options have also been used. This unconventional immunization schedule usually results in the induction of a strong cellular immune response, with generation of both CD4+ and CD8+ antigen-specific T cells. This is in turn associated with a higher and more specific antibody response against the vaccine target compared to homologous immunization with either DNA or viral vector. The immunologic mechanisms responsible for these improved responses are not entirely known. It is hypothesized that while DNA or viral vector priming is required to induce a strong T-cell response, boosting with only the target protein focuses the B-cell response on a single protein antigen for which high-affinity antibodies are desirable and at the same time avoiding interference by antivector immunity.[34]

Nucleic Acid-Based Vaccines

At the beginning of the 1990s, experiments designed to evaluate new approaches for gene therapy were performed to test if intramuscular injection of a liposome formulation containing a plasmid DNA or a messenger RNA coding for a reporter protein could result in in vivo expression of the protein. Surprisingly, animals in control groups that were injected with pure DNA or RNA showed a significant and prolonged expression of the reporter protein in muscle cells.[35] This observation inspired a series of studies that demonstrated that mouse immunization with an antigen encoded by DNA could induce a strong antigen-specific immune response characterized by antibody production and generation of both T-helper and CTL responses.[36,37,38] One of these studies also established the protective effect of a DNA-mediated immune response, showing that mice injected with a plasmid encoding the nucleoprotein from a H1N1 strain of influenza A virus were completely protected from challenge with a virulent H3N2 strain.[38] Based on these promising results, a huge amount of experimental work has been performed in the last two decades to apply DNA vaccination to many disease targets with both prophylactic and therapeutic approaches. As with vector-based vaccines, the advantage of a DNA-encoded vaccine is that the antigen is synthesized within host cells, facilitating correct folding of conformational neutralizing epitopes and MHC class I antigen presentation, allowing generation of CTL responses. An additional advantage of DNA vaccines compared to viral vectors is that the former contains only DNA, therefore nullifying any effects of preexisting vector immunity. DNAbased vaccines are composed of plasmid DNA containing an antigen gene sequence. To be efficiently expressed in the host mammalian cells, the antigen sequence has to be under the control of a strong eukaryotic promoter, such as those present in many viral genomes (eg, the early promoter of the human cytomegalovirus [HCMV]). Classical plasmids used for DNA vaccination also contain antibiotic resistance genes and prokaryotic origin of replication sequences, both required for selection and replication in bacteria. These prokaryotic sequences may contain unmethylated CpG motifs that are recognized by toll-like receptor (TLR)9 and were originally shown to play an immunostimulatory role during DNA vaccination.[39] However, subsequent investigations have excluded a role for TLR9 and CpG motifs in the immunogenicity of DNA vaccines and have suggested that the immunostimulatory activity might be due to the double-stranded structure of DNA and its capacity to activate other innate immune receptors.[40,41,42,43] After intramuscular injection, DNA is primarily localized within myocytes where the encoded antigens are expressed, although expression can also be observed in antigen-presenting cells (APCs).[44,45] Indeed, several studies have demonstrated that expression of antigen by myocytes is critical for induction of a CTL response, although they do not directly present the antigen to T cells. Experiments using parental bone marrow transplantation into F1 mice established that generation of antigen-specific CTL responses by DNA immunization depends on a cross-priming mechanism. Transfected myocytes transfer the antigen to professional APC, driving its processing and presentation by MHC class I molecules, which then leads to triggering of a CD8+ T-cell response.[46,47] Manufacturing of DNA vaccines has several benefits over all other type of vaccines: 1) design of DNA construct is straightforward; 2) production of DNA plasmids is uncomplicated and cheap and does not require manipulation of infectious materials; and 3) plasmids are stable even at room temperature, reducing the complexity and the cost of distribution. Because of the potential advantages of a DNAbased vaccine, this approach has been extensively tested for many pathogen such as rabies, HBV, and HIV in different animal models with promising immunogenicity results.[48,49,50] Furthermore, the approach has also been evaluated in humans in several clinical trials.[51,52] The human studies have demonstrated that DNA vaccination is generally safe and able to stimulate an immune response; however, immunogenicity is usually lower than expected based on the results of animal studies, resulting in a diminished confidence in this approach. The initial failure of DNA vaccine in the clinical setting led to the exploration of a series of techniques to improve their immunogenicity. To improve expression in vivo, the antigen-coding sequences were modified in order to contain only codons that match transfer RNAs with high frequency in mammalian cells, whereas stronger promoters were included in the plasmids. In an attempt to increase stability of the administered plasmid, different formulations and delivery systems, such as liposomes or microparticles, have also been

used.[53] In addition, several new injection techniques have been tested to improve in vivo transfection efficiency. A forced delivery of DNA into the skin can be obtained by the use of gold microparticles in gene gun techniques[54] or by a high-pressure liquid stream created by the Biojector device (Bioject Medical Technologies, Inc., Tigard, OR, U.S.A.),[55] whereas electroporation can greatly increase efficiency of intramuscular injection of DNA.[56]

Peptide-Based Vaccines

Although B cells can recognize linear epitopes within a protein, most of the epitopes recognized by a protective antibody response during a natural infection are conformational. Linear epitopes are usually not efficient at inducing a good neutralizing response. The goal of peptide vaccines is therefore to create T-cell epitopes capable of stimulating

T-cell responses and in particular CTLs, in cases of viral infections[57] or cancer,[58] where these type of immune responses are required to obtain a complete protection. Although the peptide approach is able to induce protection from viral infections and cancer in the mouse model, polymorphism of the MHC system has limited the application of peptides to human preventive vaccines. The use of peptides has been mostly restricted to therapeutic vaccines against cancer.[59]

VACCINE ADJUVANTS

Live-attenuated and inactivated vaccines have been successful in preventing and, in some cases, eradicating infectious diseases in the last century. However, there is an increasing demand for subunit vaccines composed of highly purified antigens because they are generally better tolerated compared to vaccines made of inactivated or live-attenuated pathogens. The limitation of subunit vaccines is that they are in general poorly immunogenic and often require the addition of an adjuvant to achieve protective immune responses. The term vaccine adjuvant has been used to define several compounds that enhance the immunogenicity of a coadministered antigen in vivo. As a consequence of this functional and empirical definition, the vaccine adjuvants group is composed of diverse classes of molecules such as microbial products, emulsions, mineral salts, small molecules, microparticles, and liposomes that have different mechanisms of action.[60] In general, adjuvants are believed to boost vaccine response by increasing the persistency of the antigen in vivo or by targeting innate immune pathways normally associated with response to infection[60,61] (Box 43.1).

Several studies have linked the mechanism of action of known adjuvants to the upregulation of major histocompatibility complex proteins, costimulatory molecules, and cytokines in dendritic cells (DCs). All these events increase the potential of DCs to prime naive T cells, a central step in the activation of both humoral and cellular adaptive responses. Interestingly, DC activation following adjuvant injection can occur either directly, through the stimulation of innate immune receptors, or indirectly, through the stimulation of blood cells or stromal cells at the injection site.[62] It has been shown that various cell types can react to adjuvant injection by producing cytokines or danger-associated molecular patterns, such as endogenous DNA and uric acid crystals that in turn can activate DCs.

BOX 43.1. ACTIVITIES ASSOCIATED WITH VACCINE ADJUVANT MECHANISM OF ACTION

- Increase persistency of antigen at injection site
- Enhanced antigen uptake by APCs
- Production of cytokines and danger signals at injection site
- Activation of APCs and other cells of the innate immune system
- Increased cross-presentation by DCs
- Recruitment of DCs and other immune cells to the injection site
- Migration of antigen-positive APCs to draining lymph nodes
- More efficient B-cell receptor engagement by making antigen multivalent
- Activation of B-cell proliferation and differentiation into plasma cells

APC, antigen-presenting cell; DC, dendritic cell.

Many independent studies have suggested that although activation of DCs is probably required for all vaccine formulations, it is not sufficient for optimal adjuvanticity, which is achieved when an adjuvant able to activate DC (generally called immunopotentiator) is formulated with a second adjuvant that enhance antigen uptake (also known as antigen delivery systems).[63]

Examples of immunopotentiators are microbial compounds, small molecules, and nucleic acids that are able to activate pattern recognition receptors (PRRs) such as TLRs, nucleotide oligomerization domain-like receptors (NLRs), and C-type lectins receptors (CLRs); cytokines such as granulocyte macrophage-colony stimulating factor, interleukin (IL)-12, IL-2, and interferon (IFN)-α saponins and microbial compounds targeting invariant natural killer T cells (αGal-Cer and derivatives).

Examples of antigen delivery systems are mineral salts (alum), emulsions, microparticles (polylactide coglycolide), liposomes, and monoclonal antibodies targeting DC internalization receptors such as DEC-205. All these compounds are able to enhance antigen uptake by APCs in vitro. However, alum and oil-in-water emulsions can also induce the expression of cytokines and other innate immune mediators at the site of injection that may contribute to DC activation (described in detail in following paragraphs).

It has been shown that codelivery of antigen and an immunopotentiator in the same APC can greatly enhance adaptive responses to vaccination. In most studies, codelivery has been achieved by adsorbing the antigens (generally recombinant proteins or purified subunits) and immunopotentiators to the same antigen delivery system. However, codelivery can also be achieved by linking directly immunopotentiators such as flagellin, oligonucleotides, lipopeptides, and small molecules to the vaccine antigen.

As a consequence of the innate immune stimulation, adjuvants can have a strong impact on adaptive responses. In preclinical and clinical studies, it has been demonstrated that adjuvants can increase the amount, avidity, and the cross-reactivity of antigen-specific antibodies, potentiate T-cell responses, and modulate the immune response toward a targeted CD4+ T-cell phenotype adapted against defined pathogens. Finally, adjuvants can improve adaptive responses to vaccination in immunologically hyporesponsive populations including the elderly and infants and can allow for antigen dose sparing. Despite the importance of adjuvants, only very few compounds selected in preclinical studies have been licensed for human use (Table 43.2). Among them, alum has been widely used for more than 70 years; oil-in-water emulsions have been licensed for

adjuvanted influenza vaccines; and AS04, a combination adjuvant composed of the TLR4 agonist monophosphoryl lipid A (MPL) adsorbed to alum, has been approved for HBV and HPV vaccines. The identification and development of new adjuvants is necessary because the limited number of currently approved vaccine adjuvants do not always elicit the desired immune responses capable of preventing the targeted infection. In particular, there is a need for new adjuvants eliciting potent CD8+ T-cell and Th1 responses. A large number of compounds have demonstrated vaccine adjuvant activities in preclinical models, and it would be difficult to review all of them in this chapter. Therefore, we will focus only on the adjuvants that have been either licensed (Table 43.2) or already tested in human (see Table 43.3), with a special focus on their use with subunit vaccines targeting infectious diseases.

TABLE 43.2 Licensed Human Vaccine Adjuvants

Name	Class	Components	Vaccine	Reference
Alum[a]	Mineral salts	Aluminum phosphate, aluminum hydroxide	Diphtheria, tetanus, pneumococcus, HAV, HBV, anthrax, tick-borne encephalitis, MenC, HPV	64
MF59	Oil-in-	Squalene,	Seasonal and	84,85,88

	water emulsion	Tween 80, Span 85	pandemic influenza	
AS03	Oil-in-water emulsion	Squalene, Tween 80, α-tocopherol	Pandemic influenza	89
AF03[b]	Oil-in-water emulsion	Squalene, Montane 80, Eumulgin B1PH	Pandemic influenza	86
Virosomes	Liposomes	Phospholipids, cholesterol, HA	Seasonal influenza, HAV	115,360
AS04[a]	Alum-adsorbed TLR4 agonist	Aluminum hydroxide, MPL	HBV, HPV	111,361
RC-529[c]	Alum-adsorbed TLR4 agonist	Aluminum hydroxide, synthetic MPL	HBV	114

HAV, hepatitis A virus; HBV, hepatitis B virus; HPV, human papillomavirus; Men, meningococcus, MPL, monophosphoryl lipid A; TLR, toll-like receptor.

[a] Adjuvants approved by the U.S. Food and Drug Administration.

[b] Approved in Europe but not marketed.

[c] Licensed only in Argentina.

Licensed Vaccine Adjuvants

Alum

Numerous subunit vaccines in use today, including those against HAV, HBV, HPV, anthrax, diphtheria, tetanus, MenC, tick-borne encephalitis, and pneumococcal conjugate vaccines, are adsorbed to aluminum salts.[64] The most used aluminum salts for licensed vaccines are aluminum hydroxide and aluminum phosphate, and they are generically called “alum.” Alum adsorption enhances the antibody responses to the coadministered antigens and, in some cases, can also increase antigen stability allowing for more extended vaccine shelf life. Only recently has the mechanism of action of alum been deeply investigated.[65,66,67] Early work suggested that alum is able to form a depot with the antigen at injection site increasing antigen persistency and availability. However, in the last 20 years, the depot effect of alum has been challenged by several studies that reported a similar half-life of antigen formulated in the presence or absence of alum.[68,69,70] Alum absorption increases antigen uptake by DCs in vitro, suggesting that it acts as an antigen delivery system.[71] More recently, it has been shown that the interaction between alum and DCs requires membrane sphingomyelin and cholesterol.[72] Moreover, alum injection results in cell recruitment to the injection site,[73,74] especially local recruitment of monocytes, which migrate to the draining lymph nodes and differentiate into inflammatory DC capable of priming T cells.[75] Altogether, these

data suggest that alum has a dual function in promoting antigen uptake and a local proinflammatory environment at injection site. In vitro experiments conducted on mouse and human blood cells led to the identification of at least one of the molecular targets of alum inflammatory activity. Alum activates a cytoplasmic NLR protein called NLRP3, which associates with the adaptor protein ASC and the caspase-1 protease to form a protein complex called inflammasome.[76,77,78] Activation of NLRP3 ultimately results in the processing of pro-caspase-1 into its active form, which cleaves several proinflammatory cytokines including IL-1β and IL-18. It is still not clear how alum activates NLRP3; however, it has been proposed that phagosomal destabilization induced by aluminum and by other crystal structures (silica crystals) following phagocytosis plays an important role.[79]

In vivo studies conducted in NLRP3-deficient mice have shown that direct NLRP3 activation contributes to alum adjuvanticity.[75,77,80] However, the requirement of NALP3 for the adjuvant activity of alum has been challenged by other studies using alternative antigens or different immunization routes.[76,81,82] Probably, other proinflammatory pathways triggered by alum exist and are independent from inflammasome activation. Recently, it has been proposed that host DNA released from dying cells acts as a danger signal that mediates alum adjuvanticity through the activation of several innate immune pathways including interferon regulatory factor 3 (IRF3). In addition, it has been demonstrated that (IRF3) is required for immunoglobulin (Ig)E antibody response to alum adjuvanted vaccines.[83]

Oil-in-Water Emulsions

Oil-in-water emulsions are liquid dispersions of oil droplets stabilized by one or more surfactants. The first emulsion to be licensed for human use was MF59 composed of squalene

oil, emulsified with two surfactants (Tween 80 and Span 85). MF59 has been licensed in Europe since 1997 in an adjuvanted seasonal influenza vaccine for its capacity to increase flu immunogenicity in the elderly.[84] More recently, MF59 and a second squalene-based oil-in-water emulsion called AS03, composed of squalene, Tween 80 and α-tocopherol, have been licensed in Europe for pandemic influenza vaccines and have been widely used for the 2009 H1N1 pandemic flu campaign.[85] A third squalene-based oil-in-water emulsion called AF03 has been approved for pandemic influenza in Europe but has not been marketed yet.[86] Clinical trials demonstrated that avian H5 pandemic influenza vaccines containing oil-in-water emulsions are superior to alum-adjuvanted or nonadjuvanted H5 vaccines in the induction of protective antibody titers.[87] Both AS03 and MF59 allowed for antigen dose sparing and increased seroconversion and cross-protection.[88,89] It is important to notice that in the case of H5 avian flu, for which most of the population has not been previously exposed, nonadjuvanted vaccines did not reach the antibody titers considered to be the serologic correlate of protection, whereas emulsion-adjuvanted vaccines were able to elicit protective antibody titers after two doses. A phage display library approach demonstrated that MF59 can enhance the breadth and the affinity of the antibody responses to H1 and H5 pandemic influenza vaccines.[90,91,92] In particular, the addition of MF59 enhanced the recognition of the HA globular head region that contains most of the neutralizing epitopes. Two additional clinical trials have been conducted on individuals vaccinated several years before with H5 vaccines either nonadjuvanted or mixed with MF59 or AS03 by administering a new dose of oil-in-water emulsion-adjuvanted H5 vaccine (boost). These studies demonstrated that priming with avian H5 vaccines mixed with MF59 or AS03 allows for better and more rapid responses to a booster dose compared to individuals primed with nonadjuvanted vaccines.[93,94] Priming with adjuvanted vaccines was effective even when H5 antigens from different influenza clades were used for priming and boosting, inducing a rapid increase of cross-neutralizing antibody titers. These studies suggest that adjuvanted influenza vaccines may be used for a prepandemic vaccination strategy. Another recent study highlighting the strong impact that adjuvants can have on vaccine efficacy was recently performed on children between 6 and 72 months of age. The addition of MF59 to seasonal flu subunit vaccine increased the efficacy of the vaccine from 43% to 86% against polymerase chain reaction confirmed influenza-like illness.[95] Interestingly, in the age subgroup 6 to 24 months,

nonadjuvanted influenza vaccine was unable to protect, whereas the efficacy of MF59-adjuvanted vaccine was 77%.

Several studies using the mouse model and human blood cells have addressed the mechanism of action of oil-in-water emulsion. In contrast to alum, which physically binds antigens through an adsorption process, oil-in-water emulsions are simply mixed with antigens. MF59 neither increases antigen persistence at the injection site nor induce any depot effect.[96] However, MF59 can enhance antigen uptake by activated DCs in mouse muscle[97] and phagocytosis in vitro in human peripheral blood mononuclear cells.[98] These data suggest that MF59 can act as an antigen delivery system; however, other studies have shown that it has also immunostimulating properties. In vitro analysis of human blood cells showed that MF59 induces the differentiation of monocytes toward DCs and can stimulate chemokine secretion (CCL2, CCL3, CCL4, and CXCL8) in human macrophages, monocytes, and granulocytes.[98] Intramuscular administration of MF59 in mice induced the recruitment of mononuclear cells partially dependent on chemokine receptor 2 (CR2).[99] The local effect of MF59 at an injection site has been evaluated in detail by combining DNA microarray and immunofluorescence techniques. Transcriptome analysis showed that the injection of MF59 in mouse muscle induced the activation of several innate immune genes, including Ccr2 and its ligands (CCL2, CCL7, and CCL8), further supporting the data described previously on CR2-dependent cell recruitment.[100] In addition, MF59 promoted the upregulation of many proinflammatory cytokines and other innate immunity genes and genes involved in leukocyte migration.[100] Two of the early genes modulated by MF59, JunB and pentraxin 3, were upregulated in the muscle, suggesting that the activation of muscle cells by MF59 might play some role in its adjuvant activity.

Intramuscular injection of fluorescently labeled MF59 and antigen revealed that MF59 colocalizes with the antigen in APCs in the muscle and enhances the number of antigenpositive DCs and B cells in the draining lymph node.[101]

Similarly to MF59, AS03 induces transient cytokine activation at the injection site and triggers the secretion of cytokines in human monocytes and macrophages.[102] In addition, AS03 induces cytokine expression in the draining lymph node in a mechanism that is at least partially dependent on the presence of α-tocopherol in the formulation.

In summary, the data described previously suggest that squalene-based oil-in-water emulsion function by enhancing antigen uptake and migration of antigen-positive APCs in the draining lymph nodes and by inducing an immunostimulatory environment at the injection site. However, the innate immune pathways required for the mechanism of action of emulsions are not well characterized. Unlike alum, MF59 does not activate NLRP3 in vitro and is not dependent on NLRP3 or caspase-1 in mouse immunization studies.[82,103] Despite the fact that MF59 adjuvanticity is inflammasome independent, adaptive responses induced by an H5 influenza subunit vaccine adjuvanted with MF59 largely depend on the inflammasome component ASC. Therefore, it has been proposed that ASC plays an inflammasome-independent function required for MF59 adjuvanticity.[103] Surprisingly, MF59 adjuvanticity to a recombinant MenB subunit vaccine requires another adapter protein called MyD88, which plays an essential role in TLR and IL-1R signaling. However, MF59 does not have any TLR agonist activity and the MyD88-dependent pathway required for its adjuvanticity is still unknown.[82]

Monophosphoryl Lipid A and Derivatives

TLRs are key pathogen sensors that modulate the host immune system and play a fundamental role in response to microbial infection.[104] TLRs are PRRs expressed on innate immune cells including DCs that sense PAMPs. Upon pathogen recognition, DCs are activated and provide essential

signals for naive T-cell priming and development of an adaptive immune response. In addition to pathogen infection, microbial products from live-attenuated or heat-killed vaccines can also stimulate TLR pathways providing adjuvant activities.[105,106] For example, the yellow fever

vaccine activates DCs by signaling through TLR2, 7, 8, and 9.[107] Many preclinical and clinical studies using purified TLR agonists have been conducted and demonstrated that all TLRs examined can be exploited to enhance adaptive responses to vaccination. However, despite the large amount of work performed, so far, only the TLR4 agonist 3-O-desacyl-4′-MPL, a component of lipopolysaccharide (LPS) purified from *Salmonella minnesota* has been licensed for human use. MPL is approved for two vaccines against HPV and HBV. In both vaccines, MPL has been adsorbed to alum to form a combination adjuvant called AS04. The HBV vaccine formulated with AS04 was developed for patients with renal insufficiency and was tested in several immunocompromised individuals including the elderly and patients on hemodialysis. The incorporation of MPL allowed for a more rapid increase of antibody titers and enhanced seroprotection rates using fewer vaccine doses.[108] HPV vaccine formulated in AS04 induced higher neutralizing antibody titers and memory B-cell responses compared to the same vaccine formulated only with alum.[109] A head-to-head trial was conducted to compare the alum-MPL adjuvanted HPV vaccine versus another HPV licensed vaccine that is adjuvanted only by alum.[110,111] Both vaccines exploit VLP technologies for the production of antigens and are highly efficacious in preventing HPV16/18-associated cervical intraepithelial neoplasia in women. However, the AS04-adjuvanted vaccine produced significantly higher neutralizing antibody titers to HPV16 and HPV18, and higher frequencies of memory B cells compared to the other vaccine.

The molecular mechanism of action of AS04 is well understood. The MPL component activates directly the LPS sensor TLR4 that is expressed by human innate immune cells including DCs and macrophages. Adsorption of MPL to alum does not interfere with its ability to activate human immune cells including DC.[112] AS04 injection in the mouse muscle lead to a transient production of interferon and proinflammatory cytokines at injection site and to an increased number of antigen-loaded DCs in the draining lymph node.[112] TLR4 stimulation on the surface of conventional DCs induces the production of IL-12 and activates the expression of MHC class II and costimulatory molecules leading to efficient CD4+ T-cell priming and to strong Th1 adaptive responses.

Synthetic TLR4 agonists have the advantage over MPL to be more pure and less expensive. Several synthetic TLR4 agonists such as RC-529 and GLA have been developed and tested as vaccine adjuvants.[113] RC-529, a fully synthetic monosaccharide mimetic of MPL, is the most advanced and has been approved in Argentina for a vaccine against HBV.[114]

Virosomes and Liposomes

Virosomes were licensed for an influenza vaccine in 1994 and are composed of a mixture of phospholipids and cholesterol that are assembled in vitro with purified influenza HA and NA to form synthetic influenza viral-like structure. Influenza virosomes can be also used as adjuvants to incorporate additional antigens from other viruses or other pathogens in general. One formulation composed of H1N1 virosomes and inactivated HAV was licensed in 1997 to target HAV. The mechanism of action of virosomes is mainly linked to antigen delivery functions. Virosomes increase antigen uptake by APCs and display the antigen in draining lymph nodes in a multimeric form, facilitating B-cell-receptor engagement. The presence of preexisting immunity against influenza further enhance the adjuvant effect of virosomes probably through the formation of immune complexes that are rapidly internalized by APCs.[115]

Other liposome adjuvants made of phospholipid bilayers have been tested in preclinical models and in clinical trials in combination with various antigens and in some cases with other adjuvants, as described in the following paragraphs.

Vaccine Adjuvants in Clinical Development Phase

New Toll-Like Receptor Agonist Adjuvants

There is a great interest worldwide in exploiting TLRs as targets for vaccine adjuvants. Besides MPL, several additional TLR agonists are known to be effective adjuvants in preclinical studies.[116] Among them, agonists of TLR2 and TLR9 and novel TLR4 agonists have been extensively tested in human as vaccine adjuvants (Table 43.3). Clinical data are

also available for vaccine formulations including TLR5 and TLR7 agonists. Human data on TLR3 agonists are not yet published; however, clinical trials have already started.

***Toll-Like Receptor 2*.** Bacterial lipoproteins targeting TLR2/1 or TLR2/6 dimers are validated vaccine adjuvants in humans and multiple TLR2 ligands have undergone clinical evaluation. The most advanced TLR2 agonist is the lipopeptide moiety of OspA, the surface protein of *B. burgdorferi*, the major component of a vaccine preventing Lyme disease.[117] The vaccine was efficacious and licensed by the U.S. Food and Drug Administration (FDA) in 1998. However, despite the fact that the vaccine was safe and well tolerated, it was withdrawn by the manufacturer 3 years later following media coverage of possible autoimmune side effects.

***Toll-Like Receptor 3*.** Agonists of the double-stranded RNA sensor TLR3 have not been extensively tested in human yet. However, a synthetic analogue of double-stranded RNA called polyinosinic:polycytidylic acid (Poly I:C) has been tested in multiple animal models with various antigens. Poly I:C can activate TLR3 and a second double-stranded RNA sensor called MDA-5, both leading to the production of type I IFN. In mice, Poly I:C was as effective as other TLR agonists in boosting antibody responses to HIV gag antigen and more efficient than any other TLR agonist tested in inducing specific IFN-γ+ CD4+ T cells.[118] The same study showed that the adjuvanticity of Poly I:C depends on type I IFN. A study conducted with a *Plasmodium falciparum* antigen has shown that Poly I:C is effective in inducing specific antibodies and promoting multifunctional CD4+ T-cell responses in nonhuman primates.[119]

TABLE 43.3 Vaccine Adjuvants in Clinical Development

Name	Class	Components	Vaccine	Reference
CpG 7909	TLR9 agonist	CpG ODN	HBV, malaria, influenza, anthrax, cancer	116
CpG 1018	TLR9 agonist	CpG ODN	HBV, cancer	126
Imiquimod	TLR7 agonist	Small molecule formulated in a topical cream	Cancer	124
Pam3Cys[a]	TLR2 agonist	Lipopeptide	Lyme disease	117
Flagellin	TLR5 agonist	Bacterial flagellin fused to antigen	Influenza	121
Iscomatrix	Combination	Saponin, cholesterol, dipalmitoylphosphatidylcholine	HCV, influenza, HPV, cancer	133
AS01	Combination	Liposome, MPL, saponin (QS21)	Malaria	140,141
AS02	Combination	Oil-in-water emulsion, MPL, saponin (QS21)	Malaria, TB, cancer	131,139
IC31	TLR9	ODN, cationic peptides	TB	129

	agonists			
Montanide ISA 51	Water-in-oil emulsion	Mineral oil, surfactants	Malaria, HIV, cancer	23
Montanide ISA 720	Water-in-oil emulsion	Squalene, surfactants	Malaria, HIV, cancer	23
LT	Bacterial toxins	A subunit + B pentamer of *Escherichia coli* heat-labile enterotoxin	Influenza (intranasal), ETEC, pandemic influenza (Patch)	149,154,155
LTK63	Bacterial toxins	Genetically modified A subunit +B pentamer of *E. coli* heat-labile enterotoxin	Influenza, TB, HIV (intranasal)	152,153

ETEC, enterotoxigenic *Escherichia coli*; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HPV, human papillomavirus; MPL, monophosphoryl lipid A; ODN, oligodeoxynucleotide; TB, tuberculosis; TLR, toll-like receptor.

[a] Licensed and then withdrawn from market.

***Toll-Like Receptor 5*.** TLR5 recognizes bacterial flagellin and is a validated adjuvant target in animal models. A vaccine composed of a recombinant fusion protein consisting of flagellin and four tandem copies of the ectodomain of the conserved influenza matrix protein M2e protects mice from a lethal challenge with influenza A virus.[120] Furthermore, a recombinant fusion protein made of *S. typhimurium* flagellin type 2 and the globular head of influenza HA could increase HA inhibition (HAI) titers by 12-fold over baseline in subjects over 65 years old.[121] However, seasonal influenza vaccines are known to be immunogenic even in the absence of a coadministered adjuvant, and the absence of a control group in this vaccine trial makes it impossible to assess the real contribution of flagellin in HA responses.

***Toll-Like Receptor 7/8*.** Preclinical data in mice and in nonhuman primates showed that small molecules agonist of the single-stranded RNA sensors TLR7 and TLR8 can improve the immunogenicity of various vaccine antigens if adequately formulated or directly conjugated to protein antigens.[122,123] Agonists of TLR7/8 have not been used as adjuvant in human vaccine formulations. However, a cream formulation of the TLR7 agonist imiquimod, which is licensed for dermatologic diseases, has been applied at the site of injection of several tumor antigens.[116] The application of imiquimod-containing cream at the site of injection of recombinant human NYESO-1 protein antigen in melanoma patients induced measurable CD4+ T-cell and antibody responses in a subset of subjects. Unfortunately, the trial was designed without a control group that did not receive the adjuvant, and it is not possible to estimate the impact of imiquimod on the adaptive immune responses directed against NY-ESO-1.[124]

***Toll-Like Receptor 9*.** DNA oligodeoxynucleotides (ODNs) targeting TLR9 are in advanced development stage.[125] Two synthetic CpG-containing ODNs called 1018 and 7909 have been incorporated in various vaccine formulations and tested in humans. Both ODNs belongs to the B type CpG class; therefore, they are strong activators of B cells and induce large amounts of type I IFN by plasmacytoid DCs. ODN 1018 has been evaluated as an adjuvant to HBsAg vaccine and induced more rapid and sustained seroprotection in healthy adults and vaccine hyporesponsive populations.[126] The ODN 7909 has been tested with HBV, malaria, influenza, anthrax, and various cancer antigens.[116] Clinical data demonstrated that 7909 enhances antibody responses to vaccines directed against infectious diseases. In addition, in

some vaccine trials targeting cancer, 7909 had also a significant impact on CD8+ T-cell responses.[116] Another class of TLR9 agonist adjuvant called IC31 consists of a cationic peptide KLKL(5)KLK complexed with the immunostimulatory ODN1a sequence.[127] IC31 was found to promote highly efficient Th1 responses through a MyD88- and TLR9-dependent manner.[128] The complex between the cationic peptide and the immunostimulatory ODN results in a depot effect at the injection site. Furthermore, the presence of the peptide reduces the amount of ODN needed to elicit an immune response. IC31 has been combined to the

Mycobacterium tuberculosis (Mtb) antigens Ag85B-ESAT-6 and tested in a phase I clinical trial.[129] IC31 significantly boosted antigen-specific IFN-γ-producing T cells.

Saponins

Saponins are natural products extracted from plants, which have been known to exert adjuvant activities for more than 80 years. Most of the saponins used as vaccine adjuvants today are extracted from the bark of *Quillaja saponaria*. The QS21 is a fraction of *Quillaia saponaria* extract purified by reverse-phase chromatography.[130] QS21 is known to promote cross-presentation and stimulate specific CD8+ T-cell responses to subunit vaccines in preclinical models and has been formulated with liposomes, emulsions, MPL, and CpG to obtain various combination adjuvants that have been tested in human.[131] Two of these combinations, AS01 and AS02, have been successfully adopted for a vaccine preventing malaria transmission.

Saponins have also been formulated with cholesterol and phospholipids to obtain a particulate adjuvant called Iscomatrix® (CSL, Victoria, Australia).[132] Iscomatrix® has been formulated with several vaccine antigens and tested in various animal species including mice, guinea pigs, cattle, and nonhuman primates.[132] In addition, experimental vaccines containing Iscomatrix® targeting HCV, influenza, and HPV16 have been tested in humans.[133] More recently, a subunit vaccine composed of a recombinant NY-ESO-1 testis cancer antigen formulated with Iscomatrix was tested in a phase I trial.[134] In animal models, Iscomatrix® is an efficient adjuvant in boosting antibody and both CD4+ and CD8+ T-cell responses even to recombinant antigens.[133] Mechanism of action studies showed that Iscomatrix® increases antigen translocation from the endosome to the cytosol of DCs in vitro and can promote cross-presentation in both proteasome-dependent and tripeptidyl peptidase II-dependent pathways.[135] Accordingly, when Iscomatrix® was combined to ovalbumin and injected in mice, it potently enhanced the number of DCs in draining LN able to activate OT-I CD8+ T cells ex vivo.[136] More recently, it was shown that Iscomatrix® injection in mice induced cytokine expression and antigen-independent cell recruitment in draining lymph nodes and enhanced antigen uptake by CD8+ and CD8- DCs; however, CD8+ DCs were more efficient in cross-presenting ovalbumin peptides to CD8+ T cells.[137] Unfortunately, CD8+ T-cell responses induced in human by subunit vaccines are in general less strong compared to those induced in animal models, suggesting that cross-presentation in humans is more difficult to achieve. For example, the experimental vaccine using HCV core protein antigen formulated in Iscomatrix® induced high antibody titers in all patients and specific CD4+ T-cell responses in seven out of eight patients; however, in only two of eight patients with HCV could core-specific CD8+ T cells be detected.[138]

Water-in-Oil Emulsions

Water-in-oil emulsions were developed by Freund in 1937 and are still largely used in preclinical models. Incomplete Freund adjuvant is composed of mineral oil emulsified with mannide monooleate and is a potent adjuvant with a large number of antigens. However, when incomplete Freund adjuvant was tested in humans, it produced several local adverse reactions including sterile abscesses. Later, novel water-in-oil formulations were developed by using less reactogenic oils and surfactants. The most advanced are Montanide ISA 51 and ISA 720 (Seppic, Paris, France) that have been used in malaria, HIV, and cancer vaccine trials.[139] Montanide ISA 51 is composed of mineral oil and surfactants, whereas ISA 720 is

made of squalene oil emulsified in mannide monooleate to obtain droplets of 1 μm of diameter. The different oil composition affects the reactogenicity profiles of this class of adjuvants: Mineral oils persist at injection site for a long time, whereas squalene is more rapidly metabolized. The mechanism of action of water-inoil surfactants has been linked to their ability to 1) increase the persistence of the antigens that are slowly released at the injection site (depot effect), 2) increase antigen uptake by APCs like other antigen delivery systems, and 3) induce local inflammation that promotes blood cell recruitment at injection site. In a clinical trial, Montanide enhanced antibody and cellular adaptive responses to coadministered antigen. Cellular responses could be further enhanced by the inclusion of CpG ODNs.

Novel Adjuvant Combinations

In some cases, a single adjuvant may not be sufficient to achieve a protective immune response. For this reason, several combinations composed of various classes of adjuvants have been tested in preclinical and clinical settings. The most successful example of adjuvant combination is AS02 composed of MPL, QS21 saponin and an oil-in-water emulsion. When *P. falciparum* recombinant antigen RTS,S was formulated with AS02, a 37% reduction of malaria infection was observed in a clinical trial.[140] Recently, the same vaccine has been optimized with the adjuvant combination AS01, in which the oil-in-water emulsion is substituted with liposomes, further increasing the protection rate.[141] The AS02 combination has been also used for the non-small cell lung cancer antigen MAGE-A3. The vaccine gave a significant antibody and CD4+ T-cell responses. However, only a small subset of patients developed antigen-specific CD8+ T-cell responses.[142] In the future, adjuvant combinations may be further improved by using different TLR agonists. In vitro data on human cells showed that TLR3 and TLR4 agonists can synergize with TLR7, TLR8, and TLR9 inducers for cytokine production and DC activation.[143] Accordingly, preclinical data in mice showed that combining TLR3 with TLR9 or TLR2 agonists resulted in a synergistic effect on the development of antigen-specific CD8+ T cells.[144] Furthermore, the small molecule TLR7 agonist imiquimod/R837 synergized with the TLR4 agonist MPL in mice inducing more persistent germinal centers, increased antibody titers, and increased frequencies of antigen-specific CD4+ and CD8+ T cells and plasma cells.[145]

Mucosal Adjuvants LT and LTK63

Mucosal vaccines elicit protective immune responses at the site of pathogen infection and have the additional advantage to be needle free, thereby improving accessibility and

cost-effectiveness. Out of five licensed mucosal vaccines, four are live attenuated (intranasal flu, oral rotavirus, OPV, and typhoid) and one is made of killed *V. cholerae* given with cholera toxin (CT) B antigen. Notably, no subunit vaccine has been licensed for mucosal administration due mainly to the absence of safe and effective human mucosal adjuvants. In fact, the administration of antigens in the absence of adjuvants in respiratory or gastrointestinal tracts can induce tolerance. Mucosal adjuvants are required to 1) protect antigens from degradation by mucosal proteases, 2) enhance internalization of vaccine antigen by intraepithelial DCs or M cells, and 3) overcome regulatory mechanisms that prevent the development of effector B- and T-cell responses in the mucosal-associated lymphoid tissue. Mucosal immunity can protect from pathogen infection by various mechanisms including local production of IgA, IgG, and IgD antibodies by mucosal B cells; activation of mucosal CD4+ T cells producing IL-17 (Th17); and activation of mucosal CD8+ T cells.[146] A large number of animal studies showed that some TLR agonists such as CpG ODNs, MPL, and flagellin can act as mucosal adjuvants; however, they have not been tested in humans yet.[147] The most advanced mucosal adjuvants are CT and heat-labile toxin (LT) from *E. coli*. Both are adenosine diphosphate (ADP)-ribosyl tranferase enterotoxins composed of a pentameric B subunit and a catalytic A subunit. The B pentameric subunit binds the ganglioside GM1 expressed on the surface of several host cells including mucosal M cells. The binding of the B pentamer promotes internalization of LT and CT into target cells followed by processing of the complex and release of the A subunit into the cytosol, where it catalyzes the ADP-ribosylation of trimeric G proteins.[148] Both CT and LT induce potent

mucosal responses to coadministered antigens when administered intranasally in mice. However, the use of wild-type toxins as adjuvants in human has been prevented by a nonfavorable safety profile. LT was included in an inactivated virosome influenza intranasal vaccine licensed in Switzerland. After licensure, the vaccine was associated with a 19-fold greater risk of facial nerve paralysis (Bell palsy) compared to unvaccinated controls and therefore was withdrawn by the manufacturer.[149] In order to improve the safety profile of CT and LT, several nontoxic mutants have been designed. LTK63 and CTK63 have a single amino acid substitution in the nicotinamide adenine dinucleotide-binding site of the subunit A of LT and CT, respectively, which results in a complete loss of enzymatic activity. Both protein complexes assemble into a holotoxin and induce mucosal immune responses.[150,151] LTK63 was superior to CTK63 in inducing mucosal responses in mice and was selected for further investigation in human clinical trials. In a phase I clinical trial, LTK63 was tested as mucosal adjuvant with an intranasal trivalent inactivated influenza vaccine. LTK63 was simply coadministered with influenza HA antigens or formulated in a nanoparticle called biovector made of polysaccharide, phospholipids, and cholesterol. Both intranasal formulations were safe and enhanced IgA mucosal responses to HA in nasal secretions. The presence of the biovector enhanced both IgA responses and HAI titers.[152] Later, LTK63 was also administered intranasally with Mtb Ag85B-ESAT6 fusion protein or with HIV glycoprotein 140 in two phase I clinical trials. In both trials, one subject developed a transient form of Bell palsy, questioning further development of LTK63 in intranasal administration.[153] It is important to report that LT has been used also for transdermal immunization using a skin patch system. In a first approach, the LT patch has been used to induce anti-LT immune responses and protect from enterotoxigenic *E. coli* infection. In a phase II placebocontrolled field trial, the LT patch protected 75% from moderate-to-severe diarrhea and 84% from severe diarrhea.[154] The same patch has been used as an adjuvant to enhance immune responses to a coadministered intramuscular H5 influenza vaccine. The application of the LT patch to the vaccine injection site increased HAI titers and seroconversion.[155] The mechanism of action of CT and LT as mucosal adjuvants is not well characterized. However, the data on genetically modified toxins showed that the enzymatic activity is not required. Experiments conducted in mouse lungs demonstrated that LTK63 activated the expression of several innate immune genes that may be involved in adjuvanticity by activating mucosal APCs.[156] The same study demonstrated that LTK63 induced recruitment of DCs and activation of alveolar macrophages in the airways. These events may increase antigen capture and presentation in the lung. Recently, one study demonstrated that LT and LTK63 can have direct effects on DC in vitro. Mouse bone marrow -derived DC stimulated ex vivo with LT or LTK63, and antigen have an increased capacity to stimulate specific IL-17, IL-10, or IFN-γ-positive CD4+ T cells in recipient mice. The same study showed that LT and LTK63 can stimulate the production of MIP-2 by mouse DCs and synergize with TLR agonists for the production of other cytokines including IL-1α and IL-1β.[157] Similarly to what has been previously observed for alum (see previous paragraph), LT and LTK63 increased IL-1β production in vitro by activating the NLRP3 inflammasome complex. In agreement with in vitro data, IL-1R has been implicated in IL-17 and IFN-γ CD4+ T-cell responses elicited by parenteral immunization with LT, LTK63, and keyhole limpet hemocyanin antigens. It remains to be demonstrated if the same pathway is involved in mucosal adjuvanticity.

ROUTES OF ADMINISTRATION

Most vaccines are administered parenterally and stimulate systemic immune responses such as serum antibodies and in some cases effector T cells. Currently, the preferred immunization route is intramuscular, whereas the subcutaneous and intradermal routes are used less frequently. However, most pathogens enter the host through mucosal epithelia; thus, an ideal vaccine should induce protective immune responses in these tissues. Dimeric secretory IgA (sIgA) is the predominant Ig found in respiratory and gastrointestinal tract mucosal secretions, saliva, and breast milk. SIgA is concentrated within and on the apical side of the mucosal epithelium and functions by neutralizing toxins and preventing pathogen attachment to and translocation across epithelial cells. Parenteral vaccines are inefficient at stimulating primary mucosal immune responses such

as sIgA; therefore, the development of mucosal vaccines is actively being pursued.

Mucosal routes of administration include nasal, oral, rectal, and vaginal. Although all these routes are amenable to the application of vaccines, in practical terms, only nasal and oral administrations are suitable options for all ages, genders, and cultures. Intranasal administration of vaccines by spraying, nebulization, or aerosolization induces sIgA in the airway mucosa and in the genital tract but is rather inefficient at generating intestinal antibody responses. However, intranasal vaccine delivery has raised safety concerns after reports of Bell palsy adverse effects associated with retrograde passage of inhaled antigens or adjuvants through the olfactory epithelium. To date, the only licensed vaccine administered intranasally is a live-attenuated influenza vaccine. Conversely, the ingestion of antigens (orogastric route) can induce sIgA responses in the small intestine, proximal colon, and mammary and salivary glands but is poorly efficient for the distal segments of the gut and the respiratory and reproductive tracts. All licensed vaccines against enteric pathogens are given by the orogastric route (Sabin OPV, cholera, typhoid, and rotavirus). A promising alternative to intranasal and orogastric routes might be the sublingual route of vaccine delivery, which is gaining increased attention from recent studies indicating that sublingual immunization may induce broadly disseminated mucosal and systemic immune responses including sIgA and CTL.[158]

Finally, transcutaneous immunization, a technique that introduces vaccines onto the skin in the form of a patch or topical ointment, targets the numerous resident professional APCs known as Langerhans cells in the superficial layer of the skin epidermis. Vaccine delivery in the skin may offer advantages over the intramuscular route of immunization including dose sparing, broad mucosal and systemic antibody responses (gut, lung, saliva, oral and nasal cavity, and female reproductive tract), and CD4+ T helper and CTL. The major obstacle to transcutaneous immunization, however, resides in the stratum corneum, the outermost layer of the skin that provides a natural barrier against foreign materials. Gentle disruption of this outer layer is necessary to improve vaccine delivery in the skin epidermis and requires the usage of special devices.[154]

Needle-free delivery of vaccines remains a high priority for both the industrialized and developing world due to increasing aversion to injections; improper practices involving nonsterile needles and syringes causing abscesses and transmitting blood-borne pathogens such as HBV, HCV, and HIV; and safety risks of needlestick for health care providers. Sublingual and transcutaneous routes of delivery of vaccines are alternatives that bypass the need for needles and represent promising new venues.

LICENSED VACCINES

In this section, we will discuss some of the licensed vaccines that are more commonly used. Other vaccines are either licensed for emergency use (eg, anthrax vaccine) or are used in selected populations (eg, Japanese encephalitis or cholera vaccines, which are indicated for travelers in endemic area) and will not be discussed.

Viral Vaccines

Poliomyelitis

Development of a vaccine against poliomyelitis represents an important achievement in the history of vaccines. It demonstrated that two different approaches, based on inactivated or live-attenuated formulations, could be successfully applied to prevention of this disabling disease. Although generally associated with the names of Salk and Sabin, discovery of the polio vaccine benefited from several key findings that preceded their work. These were the isolation of the poliovirus in monkeys, the identification of three immunologic different serotypes of the virus, the demonstration that neutralizing antibodies against one serotype could not protect against the other two, and finally the propagation of the virus in vitro in human embryonic cells of nonneural origin. Indeed, early efforts in the 1930s to obtain a vaccine using inactivated poliovirus isolated from monkey spinal cord were unsuccessful, with induction of the disease at an unacceptable rate of 1 in 1000 vaccinated individuals. Application of all the discoveries on the biology of poliovirus allowed Salk to develop the first IPV, obtained using three serotypes grown on monkey primary kidney cells, inactivated by formalin and then formulated in a final trivalent vaccine. This vaccine was 80% to 90% efficacious in one of the biggest field trials in the history of vaccines,[159] and its introduction

in the United States resulted in a dramatic decline in the cases of poliomyelitis in few years. Unfortunately, the use of IPV is also associated with the most famous and dramatic case of adverse effects related to vaccine administration. In the summer of 1955, 204 cases of vaccine-associated poliomyelitis were reported a few months after IPV was licensed in the United States. The infections were caused by two lots of vaccine produced by Cutter Laboratories, in which the virus was not adequately inactivated, despite having passed safety controls. Although isolated, the Cutter case stimulated a revision of the production procedures for IPV with inclusion of more stringent safety controls, which on the other hand also resulted in a reduction of the immunogenicity of the vaccine preparations. Subsequent technical advances, in particular culturing of the virus in long-term cell lines such as Vero monkey kidney cells and concentration of the virus before inactivation, improved the potency of IPV that is currently used in all developed countries. The presence of neutralizing antibodies at levels above a 1:8 dilution in the serum of vaccinated subjects is considered a correlate of protection. Several studies showed that following four immunizations with IPV, protective antibodies titers were still present in at least 95% of the vaccinees up to 7 years after the priming dose, whereas another study demonstrated neutralizing titers 18 years later in 100% of the subjects.[160] The Cutter incident encouraged further research on development of an attenuated vaccine, which was finally obtained by Sabin in 1961. The efficacy of this vaccine depends on the capacity of the virus to infect and multiply in the gut of the vaccinated individuals.

Initially, the OPV was manufactured as three separate monovalent formulations and then as a single trivalent vaccine. The three strains, one for each serotype, contained in OPV were selected for their reduced neurotropism in monkeys, but they preserved the capacity to infect the human intestinal tract.[3] After three doses of OPV, more than 95% of the individuals showed seroconversion for all three serotypes of virus and developed a long-lasting immunity, similar to that induced by infection with a natural poliovirus. After licensing in the United States and Europe, OPV rapidly replaced IPV for polio vaccination in all countries because of its lower costs and because oral vaccination was easier to administer than an intramuscular injection. Moreover, revisions in the IPV production that followed the Cutter incident had reduced the immunogenicity of this vaccine, resulting in cases of paralytic polio in IPV-vaccinated persons that were related to vaccine failure and led to a diminished confidence in the IPV. However, one drawback of the OPV is a low risk of inducing vaccine-associated paralytic poliomyelitis because of reversion to neurovirulence for one of the vaccine strains, serotype 3 in particular. The incidence of vaccineassociated paralytic poliomyelitis can be estimated in one case per 1.4 million of doses in case of first administration of OPV, but it is higher in case of immunocompromised individuals. To reduce the incidence of vaccine-associated paralytic poliomyelitis in many developed countries, a sequential vaccination schedule was adopted in which IPV was administered for the first two doses followed by two doses of OPV. Today, in most developed countries, including the United States, Canada, and most of Europe, polio vaccination is based only on IPV, whereas a few other countries continue to use a sequential IPV/OPV schedule. The rest of the world, which includes all developing countries and therefore the majority of all newborns who need to be vaccinated, is using only OPV. Indeed, the international community relies solely on OPV-based vaccination programs to achieve polio eradication, which is the goal for the Polio Global Eradication initiative of the WHO.[161]

Influenza

Influenza is a systemic infection that is mainly manifested with respiratory symptoms. It is broadly diffuse around the world with epidemic outbreaks associated with the winter season in temperate climate regions, whereas it has no seasonal pattern in tropical regions. Although often asymptomatic or self-limited, seasonal influenza infection can be debilitating, resulting in the loss of many working days every year. Moreover, it can be associated with life-threatening complications, especially in some at-risk populations such as the very young, the elderly, and the chronically ill. Annual seasonal influenza epidemics result in about three to five million cases of severe illness and about 250,000 to 500,000 deaths worldwide. Periodically, the human population is affected by influenza pandemics, characterized by rapid global spread of this infection and an increase in morbidity and mortality compared to seasonal influenza. For years *H. influenzae* was erroneously believed to be the origin of this

disease, and the etiologic agent of influenza, the influenza virus, was identified only in 1933. Part of the *Orthomyxoviridae* family, influenza viruses are enveloped viruses with a genome made by eight separate negative strand RNA segments and can be differentiated in three major types named A, B, and C. Influenza types B and C viruses show low variability and infect mainly humans, but only virus B is responsible of epidemic episodes. On the other hand, type A virus can also infect mammals other than humans, such as pigs, dogs, and horses, and domestic or wild birds. Based on differences in the major envelope glycoproteins, HA, and NA, influenza type A virus can be further divided in many subtypes. Sixteen major variants for HA and nine types of NA exist, and type A viruses with many different combinations of these two proteins can be isolated. Whereas HA is the protein responsible for attachment to the receptor on the host cells, and is the major target of protective neutralizing antibodies, NA is required for maturation and egress of viral particles from infected cells, and anti-NA antibodies can reduce the spread of infection. Receptor specificity of the different HA subtypes influences the host specificity of the different subtypes. Indeed, HA of human influenza viruses preferentially binds to sialic acid 2,6 galactose residues, which are expressed by human upper respiratory tract, whereas that of avian virus has a preference for sialic acid 2,3 galactose residues expressed in birds. Influenza viruses are characterized by a great variability of HA and NA that complicates the production of a protective vaccine.

Point mutations are continuously introduced during influenza virus replication because the enzymes that replicate the viral genome have no proofreading activity. These mutations, which are called “antigenic drifts,” are responsible throughout the years of small changes in the antigens of the circulating viruses and can be observed in both types A and B viruses. These changes confer a selective advantage to the new virus, against which large segments of the population have insufficient immunity, requiring the preparation of a new vaccine every year to immunize the susceptible populations. The high genetic mutability of the influenza virus requires continuous monitoring of influenza cases by the WHO surveillance system in order to identify the prevalent virus antigens in the population. Following the recommendations of this surveillance system, yearly vaccines for active prophylaxis of influenza are produced ad hoc and distributed worldwide to protect against the new variants of the influenza virus that are predicted to be the most widely circulating. A second type of variations that is observed only in influenza virus A is called “antigenic shifts.” These depend on the reassortment of RNA segments from two different virus subtypes that occurs when both viruses infect the same host, ultimately resulting in the formation of a new subtype. This reassortment can take place when both avian-specific and human-specific subtypes infect pigs, which express both sialic acid 2,3 and 2,6 galactose and therefore are susceptible to both viruses. The exchange of genetic material can lead to the formation of a new subtype that is able to infect humans and will be very different from the previously circulating viruses. As no immunologic memory for this virus exists in the human population, the new virus will rapidly spread worldwide resulting in a global pandemic outbreak. The so-called Spanish flu in 1918, the

most severe pandemic flu in the modern era, was caused by a highly pathogenic virus A/H1N1 that is estimated to have resulted in 50 to 100 million deaths worldwide. The other two pandemics which occurred in the 20th century were the Asian flu in 1957 and the Hong Kong flu in 1968, which were caused by H2N2 and H3N2 viruses, respectively. In 1977, there was a reemergence of a H1N1 virus which, however, had a limited spread and did not cause a real pandemic but only several outbreaks. However, this H1N1 virus continued to circulate and therefore was added as a third strain in the yearly vaccine formulation. Indeed, H3N2 subtype was still responsible of seasonal influenza A outbreaks from 1968 to 2009 and therefore was kept in the yearly vaccine composition.

More recently, in April 2009, a new strain of H1N1 was isolated in Mexico and California and was associated with severe to fatal cases of flu. The new virus derived from the reassortment of a previous triple reassortment of bird, swine, and human flu viruses, which was circulating among pigs in North America, with a Eurasian pig flu virus; it was called “swine flu.” This new virus rapidly spread worldwide and by June 27,737 cases of influenza A/H1N1 2009 virus were confirmed across 74 countries with 141 deaths, inducing the WHO to declare a pandemic. The WHO and national health agencies agreed that a specific vaccine was needed due to the severity of human infections and due to the risk of more pathogenic new reassortment. Vaccine manufacturers initiated efforts to develop vaccines against the

pandemic strain, and different adjuvanted and nonadjuvanted monovalent pandemic vaccines were licensed and used in national vaccination campaigns.

Alternatively, a pandemic influenza can originate when an avian virus infects humans and can be easily transmitted from person to person. This is the fear of what might happen with a highly pathogenic strain of bird influenza, the A/H5N1 virus, which was isolated in humans for the first time in 1997 in Hong Kong. The infection has spread to wild birds and chickens in many countries in Asia, Africa, and Europe, directly causing the death of tens of million of birds and requiring the sacrifice of hundreds of million of domestic chickens in the attempt to contain spreading of the infection. The WHO has recorded 564 human infections by H5N1 with 330 deaths through August 2011. Although no transmission from human to human has been observed, the H5N1 virus has the potential of originating a new severe influenza pandemic.

The first vaccine against influenza was made available to the public in 1945 and was a formalin-inactivated whole virus vaccine from viruses grown in embryonated hen eggs. Most vaccines currently in use is still produced from virus cultured in eggs and then inactivated; however, additional steps in the production process have been added to obtain more purified viral preparations. A detergent treatment that eliminates the viral envelope and other purification steps are used in the production of "split" influenza vaccines, which contain only some viral components. Even cleaner vaccine preparations, called "subunits," are obtained by additional purification procedures and mainly contain the viral surface proteins. Nonetheless, two main reasons have convinced vaccine manufacturers to search for an alternative to eggs for growing influenza virus for vaccine production. First, the spreading of an avian infection caused by flu or other viruses could greatly reduce the availability of hens and thus the eggs required for vaccine manufacturing. Second, each new influenza virus has to be adapted to grow in eggs with the risk that antigenic mutations will rise in the egggrown virus compared to the virus that is circulating in the human population, ultimately negatively affecting the vaccine efficacy. Therefore, several cell lines such as Vero or MDCK have been evaluated for growing the influenza virus, and recently, the first vaccine using cell culture-derived virus has been licensed in Europe.[162] This cell-derived influenza vaccine offers a safe alternative for individuals with egg allergies. All seasonal influenza vaccines are currently trivalent containing one strain of B type and two subtypes of A virus according to WHO recommendations. Not all influenza virus strains grow well in eggs, and this can affect yield of vaccine production. During the 1970s, the influenza virus A Puerto Rico/8/34 (PR8) strain, which shows a high growth capacity in eggs, was isolated. PR8 strain can be used together with a wild-type virus to obtain in vitro a reassortant strain that contains only HA and NA from wild-type virus and all other genetic material from the PR8 strain, usually maintaining a high growth capacity in eggs. To increase yield in vaccine production, most of the influenza A vaccine strains are reassortant of PR8 with the wild-type seasonal strains. In the 1990s, the circulating B strain diverged into two separate subtypes, which in the last two decades circulated with a different prevalence during each season, but the vaccine contains only the strain that will most likely circulate. However, during some seasons, the vaccine strain did not match the most widely circulating strain, resulting in reduced efficacy for the vaccine, especially in children who are the population most affected by influenza B virus. For this reason, the proposal by the WHO and vaccine manufacturers to include in the seasonal vaccine both B subtypes is currently under discussion.

An adjuvanted vaccine containing the emulsion MF59 is licensed in Europe and many countries worldwide for use in the elderly. This adjuvanted vaccine shows increased immunogenicity and better clinical protection compared with the split conventional influenza vaccine in the elderly population.[163,164] A virosome-based formulation of the flu vaccine is also in use in Europe and is immunogenic in children.[165]

Several live attenuated strains of influenza virus have been obtained either by growing in nonhuman cells, the host range mutant strains, or by isolation of temperature-sensitive strains, which do not grow at 39°C. However, none of these strains was able to give a really attenuated strain to be used as vaccine. On the contrary, cold-adapted strains, which grow at 25°C, were used as master strains for reassortment to successfully obtain an attenuated virus containing HA and NA coding RNAs from wild-type virus and the other six RNAs from the master strain. A live-attenuated influenza vaccine can therefore be obtained each year

from two cold-adapted strains of A and B subtypes by reassortment with the seasonal wild-type viruses. The reassortant strains are attenuated and immunogenic but are not able to be transmitted by

the vaccinated person to other individuals.[166] A live-attenuated influenza vaccine has been licensed in the United States and is administered as an intranasal aerosol spray. This vaccine contains three attenuated reassortant strains that express the antigens from the seasonal recommended strains and are grown in pathogen-free eggs.

Neutralizing HA-specific serum antibodies are the main mechanism of protection against influenza, although mucosal sIgA antibodies and cell-mediated immune responses may also play a role. Therefore, titration of serum antibodies is used as a surrogate of protection to evaluate vaccine efficacy. Three serologic assays can be used for measuring the antibody response to influenza vaccination: hemagglutination inhibition (HAI), single radial hemolysis, and microneutralization. The HAI test is widely used to assess vaccine immunogenicity because of its reproducibility. Several studies of influenza infection suggested that HAI antibody titers ranging from 1:18 to 1:65 were associated with protection in 50% of adult individuals, and that protection directly correlated with HAI titers.[167,168] This finding led to the decision that a HAI titer ≥1:40 is accepted as a surrogate marker for protection in adult individuals, although a similar marker for children has not been established. However, the HAI test is not accurate to measure neutralizing antibodies against avian viruses, and a modified test is used for assessment of vaccination against avian strains. It is indeed accepted that the microneutralization assay is a more reliable method to specifically evaluate the antibody response against H5N1 virus in patients or vaccinated subjects.

As also observed during the 2009 pandemic, in case of the unexpected emergence of a new influenza pandemic strain, it may be difficult to produce within a short time frame sufficient quantities of the specific vaccine to block spreading of the emerging strain and reduce casualties. For this reason, one option of the pandemic preparedness strategy is the development and licensing of prepandemic vaccines against a strain that has the potential of becoming a pandemic strain. The H5N1 avian strain is still considered as a likely candidate for future pandemic influenza, and prepandemic vaccines for this virus have been produced and licensed. During the interpandemic period, these vaccines can be used to immunize individuals at high risk of both avian and human virus infection (eg, veterinarians, poultry workers, operators involved in the manufacturing of vaccines with pandemic-like strains, laboratory workers) to reduce the chance of an emergence of a reassortant pandemic strain. At the beginning of a pandemic caused by a strain that matches the vaccine strain, a rapid use of these vaccines can allow early protection of the human population. A pandemic by a not completely matched strain could still benefit from the use of such prepandemic vaccines if the prepandemic vaccine is able to elicit a crossreactive immune responses in the vaccinees. Indeed, it is calculated that even a vaccine with low efficacy can mitigate the effects of a pandemic.[169]

Hepatitis A and B

Described by Hippocrates as transmittable jaundice, infectious hepatitis has been responsible for epidemics throughout the centuries and has influenced military strategies in the past because of frequent outbreaks among soldiers. Only at the beginning of the 1950s was infectious hepatitis definitively differentiated from serum hepatitis, and these two diseases were classified as hepatitis A and B, respectively. Hepatitis A is caused by HAV, an enterovirus of the *Picornaviridae* family. HAV is transmitted by the fecal-oral route and has an incubation period of 2 to 6 weeks. As with many other infectious disease, key steps on the road to a vaccine were the identification of its etiologic agent[170] and the establishment of an infection animal model in the marmoset, which permitted isolation of the virus,[171] and testing of a prototype vaccine using formalin-killed virus.[172] Subsequent advancements in virus propagation in cell culture were then developed to obtain the first licensed vaccine for humans.[173] Currently, two vaccines are licensed in the United States, whereas four vaccines are registered in Europe, Canada, and other regions. HAV is grown in the human diploid lung fibroblast cell line MRC-5, purified from lysed cells and inactivated with formalin. However, the manufacturing process is characterized by low yields. The inactivated HAV vaccine

induces anti-HAV neutralizing antibody titers that are considered protective. Two immunizations are indicated for both children and adults and provide anti-HAV antibody levels that are predicted to persist for many years. Efficacy of the vaccine has been demonstrated by several studies showing a decline in disease occurrence in vaccinated communities.[174] The virus strains used for preparation of the inactivated vaccine are attenuated by cell culture passages, and a live-attenuated vaccine has been successfully tested in humans while a similar vaccine is registered and used in China. An attenuated vaccine would present no advantages over the inactivated formulation, as the high production costs due to the low yields are not addressed. On the contrary, a promising VLP-based vaccine could be a feasible cheaper alternative.[175]

Hepatitis B, which was known in the past as serum hepatitis, is transmitted by parenteral inoculation with infectious materials, particularly blood or blood-derived products, and has a long incubation period from 40 days to 6 months. Infection with HBV is a worldwide health problem, with more than two billion people presenting evidence of previous infection and more than 350 million who are carriers of the virus according to the WHO estimates. The age of the first encounter with HBV greatly influences the outcome of the disease; 90% of children infected when younger than 1 year of age develop a chronic disease. As 25% to 40% of these chronic infections progress to liver cirrhosis or cancer, HBV represents one of the major causes of mortality globally. HBV is a double-strand DNA virus of the *Hepadnaviridae* family that does not propagate in vitro. Vaccine development was facilitated by the following findings: 1) discovery of the so-called Australia antigen in the serum of people who had received blood transfusions, 2) recognition of HBsAg, and 3) identification of VLPs in the serum of patients with hepatitis that contained the Australia antigen. The first vaccine against HBV was prepared with formaldehyde-inactivated particles isolated from the serum of chronically infected people and was proven safe and efficacious.[176] However, as mentioned previously, cloning of the viral DNA allowed development of a new vaccine made

by recombinant HBsAg produced in yeast or mammalian cells, which self-assembles into VLPs and is as protective as the previous vaccine.[4] The recombinant vaccine is currently used in most countries worldwide and is able to induce anti-HBsAg antibodies above the protective concentration of 10 IU per mL after the standard three-dose schedule in more than 95% of normal children or adolescents. Although 90% of vaccinated adults younger than 40 years old achieved this protective level, this percentage declines with age with only 75% of 60-year-old vaccinees generating adequate levels of anti-HBsAg antibodies. Vaccine-induced antibodies tend to decline with time and, depending on the study, return to undetectable levels in up to 60% of vaccinees 10 years after the last immunization. The effectiveness of the vaccine has been demonstrated by reduction in the prevalence of HBV chronic infection following the implementation of routine vaccination programs. Because one of the long-term consequences of HBV infection is development of hepatocellular carcinoma, the HBV vaccine is considered the first licensed preventive human cancer vaccine. Indeed, infant HBV vaccination has been associated with a reduction of more than 50% in the incidence of hepatocellular carcinoma.[177]

Measles, Mumps, Rubella, and Varicella

Measles, mumps, rubella, and varicella are common childhood diseases that can be associated with complications and in rare cases may result in death. All these diseases can be prevented by live-attenuated vaccines.

Measles is still one of the leading causes of death among young children worldwide. It was responsible for 164,000 deaths globally in 2008, with 95% of them occurring in developing countries. One of the most severe complications of measles is subacute sclerosing panencephalitis, which is particularly frequent at those younger than 1 year of age and results in brain damage and death. Measles was mistaken with smallpox until the 17th century, and by the 18th century, it was considered an infectious disease, although the measles virus was only isolated in 1954 by Enders and Peebles in tissue culture from the blood of 13-year-old boy named Edmonston.[178] The Edmonston strain of measles virus was then attenuated by many passages in cell culture and adapted to grow in chicken embryos and finally used to develop the first measles vaccine in 1963 containing the Edmonston B

strain. However, coadministration with Igs was required to reduce the high frequency of side effects associated with this attenuated vaccine. Further passages in cell culture resulted in the isolation of more attenuated Edmonston strains, which were as efficacious as the original strain in inducing protective antibodies but were not associated with side effects and did not require coadministration of Igs.[179,180] Immunization with measles vaccine is able to induce specific IgG, IgM, and IgA in both serum and mucosal secretions of vaccinees, and seroconversion can be observed in more than 95% of individuals. The efficacy of this vaccine is demonstrated by the dramatic reduction in the incidence of measles cases in all populations after the implementation of vaccination and by a 78% drop in measles deaths between 2000 and 2008 worldwide.

Mumps is a highly infectious disease that was first described by Hippocrates. Parotitis is the classical symptom of mumps, which can be complicated by meningitis, pancreatitis, and orchitis in 20% to 30% of postpubertal males. Mumps virus, a negative-sense RNA virus of the genus *Rubulavirus* in the *Paramyxoviridae* family, was isolated in 1945 by growing on chick embryos and propagated in in vitro tissue culture, but attempts to attenuate this isolate for vaccine purposes were not successful. Hilleman's group then isolated the Jeryl Lynn strain which was attenuated by culturing on embryonated hens's eggs and chick embryos.[181] This vaccine induced seroconversion in 98% of the vaccinated subjects and demonstrated a 95% efficacy in protection.[161,182] Most of the mumps vaccines currently in use are still based on the Jeryl Lynn strain or its derivatives, whereas few other products are made with different attenuated strains.

Rubella is usually a benign and self-limiting disease in children characterized by maculopapular exanthema and is caused by a positive-strand RNA virus of the genus *Rubivirus* in the *Togavirus* family. However, infection of a pregnant woman can result in spreading of the virus to the fetus leading to fetal death or congenital rubella, which is associated with severe birth defects in many organs. In particular, infection during the first trimester of pregnancy results in developmental defects of the eyes and the heart, whereas deafness is the major consequence of infection in the second trimester. Between 1962 and 1965, a large outbreak of rubella spread throughout Europe and then to North America, infecting a billion people and tens of thousands of pregnant women, resulting in thousands of newborns with congenital defects. The rubella virus was isolated in 1962 by two different groups and different live-attenuated strains were then used to produce different vaccines, which were licensed in the United States, Europe, and other countries in 1969 and 1970.[183,184,185] Seroconversion rates ranging from 90% to 98% resulted in a remarkable reduction in the rate of both acquired and congenital rubella cases after introduction of the vaccine in many countries. However, as vaccines based on the RA27/3 attenuated strain have shown a better immunogenicity and a reduced incidence of side effects, these strains are currently used for the production of most rubella vaccines in use worldwide. Rubella virus can be detected in mucosal secretion of vaccinees starting 1 week after immunization and can persist for 2 weeks, but no spread of the virus to contacts could be observed, excluding the risk that the vaccine strain can pass to pregnant women causing congenital rubella.[186]

Varicella or chickenpox is caused by a double-strand DNA virus of the *Alphaherpesviridae* family, which is also the etiologic agent of herpes zoster and therefore has been named varicella-zoster virus. Similar to measles, varicella was clinically mistaken with smallpox until the 18th century and is characterized by a maculopapular rash rapidly transforming into vesicles, pustules, and crusts. Varicella-zoster virus was isolated in 1952, but no sufficiently attenuated strain suitable for vaccine purpose was derived for many years. Such a strain was indeed obtained in 1974 by Michiaki Takahashi in Japan with a virus isolated from a boy suffering from a mild form of varicella, which was passed several times in human embryo lung fibroblasts and then in guinea

pig fibroblasts, and was named the Oka strain.[187,188] This strain was used to prepare a vaccine that was shown to be efficacious in clinical trials and was first licensed for use in immunocompromised children and their healthy contacts in several European countries in 1984, followed by Japan and Korea. Ten years later, Oka-based varicella vaccines were approved for use in all children in Europe and North America. The immunogenicity of this vaccine has been evaluated in many clinical studies that showed that the Oka strain induced

seroconversion from 76% to 96% of vaccinated individuals,[189,190] although no clear correlation could be established between the levels of serum antibodies and protection from varicella infection. Varicella-zoster virus-specific antibodies tend to decrease with time but can still be detected many years after vaccination.[191]

Measles, mumps, and rubella vaccines have been combined in a trivalent vaccine, and more recently, a tetravalent formulation with addition of varicella Oka strain has been developed. The immunogenicity and the side effect rate of this combined vaccine are comparable to that obtained with administration of each single vaccine separately.

Rotavirus

Rotavirus infection is the leading cause of severe diarrhea in children younger than 5 years of age in all countries in the world, resulting in a large number of hospitalizations in developed nations and more than 500,000 deaths a year worldwide, most of them by dehydration in developing countries. Rotaviruses represent a genus of the *Reoviridae* family, containing many members that can infect humans or other animal species such as cows or nonhuman primates. Rotaviruses have a double-stranded DNA genome segmented in 11 fragments that can reassort during infection of a cell by more than one strain of virus, resulting in a novel virus type. Many strains of rotavirus can be identified based on differences in two surface proteins VP4 and VP7, which define, respectively, the P- and the G-types. Many combinations of VP4 and VP7 were isolated from humans, although most cases of gastroenteritis in children are associated with five strains, with the P1A[8]-type associated to the G1, G3, G4, or G9 serotypes, and the P1B[4]-type associated with the G9 serotype. Both VP4 and VP7 can induce typespecific neutralizing antibodies that can prevent infection by a specific serotype of rotavirus, although some level of crossreactivity can be observed.[192] Moreover, natural sequential infections are associated with a progressively reduced risk for subsequent rotavirus infection even if caused by another serotype,[193] suggesting that broad protective immunity may be stimulated by an attenuated oral vaccine.

The first attempts to develop an oral rotavirus vaccine followed Jenner's approach, using more easily attenuated nonhuman strains and relying on a potential protective effect from a live-attenuated heterologous strain. Two candidate vaccines used two different bovine rotavirus strains, whereas a third was based on a simian virus strain. All these vaccines were well tolerated and immunogenic, and induced serum-neutralizing antibodies against the vaccine strain. Although some clinical studies showed that each of these vaccines could induce protection against rotavirus-mediated gastroenteritis, efficacy data were not consistent. This suggested that animal strains do not induce efficient protection against human rotaviruses, and this approach was eventually abandoned.

An alternative approach has been the selection of reassortant strains expressing VP7 or VP4 protein from human rotavirus in a genetic background of a simian- or bovineattenuated strain. A first vaccine of this type contained three rhesus-human reassortants each expressing the human VP7 protein from one of the serotypes 1, 2, or 4, plus the parental simian strain that expressed a simian VP7 protein of serotype 3. Many clinical trials demonstrated that this tetravalent vaccine was immunogenic, inducing neutralizing antibodies in more than 90% of vaccinated children and had 69% to 100% efficacy against severe rotavirus-mediated gastroenteritis.[194] This vaccine was licensed in 1998, but after 1 year, in which more than 1.5 million of doses were administered, a small increase in the risk of intussusception (a rare bowel obstruction in which one segment of bowel prolapses into a more distal segment) in the vaccinated population was observed, leading to the withdrawal of the product. The incidence of intussusception attributed to this vaccine was 1 in 10,000 and was considered unacceptable for children in developed countries such as the United States where only 1 in 150,000 cases of rotavirus infection results in death. However, this tetravalent vaccine was still beneficial for young populations in developing countries where 1 in 250 children die because of rotavirus-induced gastroenteritis and where the use of this vaccine could have saved many more lives compared to the number of lives saved avoiding the adverse event associated with the vaccine.

Another rotavirus vaccine based on bovine-human reassortants was developed and contains five in vitro derived strains expressing either the human VP7 protein from one of the G1, G2, G3, or G4 serotypes, or the human VP4 protein of P1A[8] genotype in the genetic

background of a bovineattenuated strain. Because administration of the first licensed rotavirus vaccine had been associated with increased risk of intussusception, licensing of this pentavalent vaccine required an extensive phase III trial on nearly 70,000 young children worldwide. This, as well as subsequent studies, demonstrated that administration of three doses of this oral vaccine was not associated with an increase of any severe adverse event and was efficacious against severe gastroenteritis or any grade of disease due to rotavirus, reducing hospitalizations associated with rotavirus infection.[195] In 2006, this vaccine was approved for use in the United States and is now licensed in more than 95 countries.

Analysis of the immune response to natural infections has shown that although the first infection primarily induces serospecific neutralizing antibodies, subsequent infections are able to generate more broadly cross-reactive responses.[196] This observation has stimulated an alternative approach to rotavirus vaccine design, which consists of the use of a single human attenuated strain with a G1PA1[8] serotype. Similar to the pentavalent recombinant vaccine, this monovalent oral formulation has been evaluated in a large phase III trial on more than 70,000 subjects that showed that the vaccine was safe and efficacious in preventing

rotavirus-mediated gastroenteritis after two doses.[197] This vaccine is now licensed in Europe, the United States, and many other countries.

After introduction of these vaccines in the United States, a great reduction in rotavirus-mediated gastroenteritis was observed. Recently, the WHO recommended the inclusion of rotavirus vaccine for infants in all national immunization programs, particularly in countries where diarrhea is responsible for more than 10% of deaths among children younger than 5 years old. Implementation of the rotavirus vaccination in developing countries is expected to have a great impact on mortality rates among infants worldwide.

Human Papillomavirus

HPV is a nonenveloped virus with an icosahedral capsid made up of only two structural proteins, L1 and L2, which surround a double-stranded DNA genome. Each viral particle contains 72 pentamers of L1, the major capsid protein, and up to 72 monomers of the L2 protein. HPV can infect different types of human epithelial cells. Until the beginning of 1980s, HPV infection was not considered clinically relevant because most were asymptomatic or associated with benign cutaneous papillomatosis and genital warts. However, in the last 30 years, it has been clearly demonstrated that infection of the female genital tract by some types of HPV is associated with development of cervical cancer, and that 100% of these malignancies are caused by infection with one of the “high-risk” HPV types, so called for their capacity to induce a malignant transformation of the cervical cells. More than 100 genotypes of HPV have been identified, and at least 15 of them have been associated with cervical cancer. Two genotypes, HPV16 and HPV18, are responsible for 70% of these malignancies. After breast cancer, cervical cancer is the most frequent type of cancer in women around the world, affecting mostly young women in their 20s to 40s. Cancer of the cervix was responsible of 275,000 deaths worldwide in 2008, with most of them occurring in developing countries. The disease, therefore, represents a significant social and economic burden due to the costs for screening and treatment as well as the young age of the affected patients.

HPV specifically infects human cells, and no animal model of infection is available for testing potential vaccine efficacy. However, experiments with homologous bovine or canine papillomavirus showed that an inactivated virus preparation was able to induce neutralizing antibodies that protected cattle and dogs from infection.[198,199] This suggested that a preventive vaccine able to induce neutralizing antibodies to HPV was feasible. HPV replicates only in differentiated epithelial cells, and it is difficult to grow in culture, making propagation of this virus in order to prepare a live-attenuated or killed vaccine complicated. Early attempts to use a recombinant L1 protein of bovine papillomavirus as a vaccine against this virus were not successful. A breakthrough in the development of an HPV vaccine came from the demonstration that when expressed in insect cells using a baculovirus system, recombinant L1 capsid proteins were able to self-assemble in VLPs that induced neutralizing antibodies upon immunization.[200] However, these antibodies are genotype specific, suggesting that a broadly protective vaccine would need to include VLPs from multiple genotypes. Fifteen years of further research and clinical trials were required to develop the first licensed vaccine for

prevention of HPV infection in 2006. Two types of vaccines are currently available in the United States, Europe, and many other countries. One product contains VLPs produced in yeast cells adjuvanted with alum and protects from infection by the two oncogenic HPV16 and HPV18 and two other genotypes, HPV6 and HPV11, which are responsible of 90% of genital warts. VLPs from HPV16 and HPV18 produced in insect cells are present in the second vaccine, which also contains the AS04 adjuvant system. Both vaccines were efficacious in preventing infection by the HPV genotypes contained in the vaccine preparation and development of cervical intraepithelial neoplasia, an endpoint that was required by FDA in the efficacy trials.[201] Although no correlate of protection for HPV has been established, the observation that transfer of antibodies from VLP-immunized animals prevents infection by papillomaviruses in animal models indicates that the presence of serum-neutralizing antibodies could be considered as an indicator of vaccine efficacy. Although the current licensed HPV vaccines are administered intramuscularly, they are nevertheless able to protect from infection by HPV at the genital mucosa, probably by secretion of neutralizing IgG in the mucosa fluids. The high production costs of the licensed HPV vaccines are reflected in the relatively high cost of these products, limiting their use in developing countries where 80% of cases of cervical cancers occur. Alternative and less costly vaccines are therefore required to reduce the economic burden of broad HPV vaccine implementation in developing countries.

Bacterial Vaccines

Diphtheria, Tetanus, and Pertussis

The diphtheria-tetanus-pertussis (DTP) combination vaccine is the oldest but still the most used combination vaccine. Two types of this vaccine combination are available today: the classical DTwP, which contains the whole cell pertussis vaccine, and the DTaP in which the diphtheria and tetanus toxoids are combined with an acellular pertussis vaccine.

Tetanus is a nontransmissible disease caused by a toxin produced by *C. tetani*, a gram-positive bacterium that can grow in anaerobic conditions in infected tissues. Tetanus toxin is one of the most powerful neurotoxins that affects particularly inhibitory neurons, resulting in localized and generalized spasm of the muscles and leading to death in almost 100% of cases if left untreated. The identification of tetanus toxin and the demonstration that the toxin induced the production of protective neutralizing antibodies in animals when injected in small amounts led to the discovery that an inactivated toxin, anatoxin or toxoid, could induce protective immunity against tetanus, as described previously. The toxoid obtained by formaldehyde treatment became available at the end of the 1930s when it started to be widely used for tetanus prophylaxis. Although no formal study has been performed to directly evaluate the vaccine efficacy, the great reduction of tetanus cases in all vaccinated populations demonstrates the effectiveness of

toxoid immunization. Several studies have evaluated persistence of immunity following three immunizations with tetanus toxoid. Vaccine-induced antitoxin antibodies decline over the years. Therefore, after a three- to four-dose primary immunization series, a booster dose every 10 years is recommended to maintain protective levels of antibodies.

Diphtheria is a lethal infectious disease that was a major cause of childhood death in the prevaccination era. It is caused by *C. diphtheriae*, a gram-positive bacterium that infects the upper respiratory tract and secretes a potent toxin that induces cell death, producing local and systemic damages, and is responsible for diphtheria lethality. Identification of diphtheria toxin in 1888 was followed by the discovery that antisera produced in animals immunized with inactivated bacteria could protect other animals or humans from disease, which led to the use of antisera or antitoxin for the treatment of diphtheria. In 1907, a mixture of toxin-antitoxin was successfully used to immunize animals and humans, and its use became a common practice to prevent children disease. When in 1923 Ramon showed that a formaldehyde-inactivated toxin could induce protective antitoxins, a toxoid-based vaccine became available and widely used. Although no clinical trials to measure efficacy of this vaccine have been performed, the vaccine effectiveness has been demonstrated by the disappearance of diphtheria disease in the developed countries where routine vaccination is in place. After a three-dose schedule during the first year of life, serum antibodies reach a protective concentration but then diminish with time. Indeed, decreased immunity has probably been responsible for many

diphtheria outbreaks among adults observed in several states of the former Soviet Union in the 1990s. As with tetanus, a booster dose every 10 years is therefore recommended to maintain protective levels of antibodies. Finally, although a mutated inactive form of diphtheria toxin, called CRM197,[202] induces antitoxin protective antibodies, this has not been used alone as a diphtheria vaccine; rather, it has found application as a protein carrier in many glycoconjugate vaccines.

A whole-killed **pertussis** vaccine (wP) was the first of the three components of DTP to be licensed in 1914, a few years after the identification of *B. pertussis* as the etiologic agent of whooping cough or pertussis. It is obtained by a suspension of the *B. pertussis* killed by formaldehyde. Efficacy of the wP vaccine was demonstrated by early clinical trials during whooping cough epidemics on the Faroe Islands and by a rapid decline of pertussis in the United States after the introduction of the universal vaccination for children in the 1940s. Although efficacious, the wP vaccine is reactogenic, inducing sometimes serious although transient adverse events. However, several controlled studies have definitively excluded any causative relation between the wP vaccine and all particularly worrisome reactions such as encephalopathy, sudden infant death syndrome, and infantile spasms, which in the past were associated with this vaccine. Nevertheless, in the 1970s, the adverse events associated with DTwP vaccines containing the wP started to create alarm among parents and doctors in developed countries where pertussis had almost disappeared and where the benefits of the vaccination were becoming less appreciated. Public concerns for wP-mediated adverse reactions were so strong that vaccination rates declined leading in some countries to recurrence of pertussis outbreaks, which resulted in the death of hundreds of children. An example of the effects of the decline in pertussis vaccine coverage was observed in Japan, where the universal pertussis vaccination was initiated in 1947, leading to a remarkable reduction of whooping cough cases to such a low rate of 0.1/100,000 by 1971. In 1975, the vaccine-unrelated death of two vaccinated children increased fears for the vaccine-related side effects, leading to a dramatic decline of the vaccination rate in the following years, resulting in an increase of pertussis cases to 8.4/100,000 in 1979 with many associated deaths.[203] Reactogenicity of the wP vaccine encouraged the search for a better tolerated vaccine, leading to the development of the first acellular pertusis vaccine (aP), which was licensed in Japan in 1981 as a DTaP combination. Many DTaP vaccines have been developed during the years, leading to replacement of DTwP in all developed countries. All DTaP vaccines contain the inactivated pertussis toxin, a protein complex with ADP-ribosyl transferase enzymatic activity, which is the major pathogenetic component of *B. pertussis*, with the addition of one or more bacterial components such as filamentous HA, pertactin, and fimbrial proteins. One vaccine, which has been an important innovation in this field, was based on a genetically inactivated form of pertussis toxin, in which two amino acid substitutions in the catalytic site abolish toxin activity.[2] Many clinical trials have evaluated the efficacy of the different DTaP vaccines, some also comparing one or more DTaP vaccines to one DTwP vaccine. Differences in the execution of these trials render a comparative analysis complicated. However, a general conclusion that can be drawn is that DTaP vaccines are as efficacious as the DTwP vaccine in protecting from pertussis disease, with an efficacy from 71% to 96%, depending on the study. Evidence of efficacy for the DTaP vaccines is also the persistent reduction of pertussis cases in Japan after the introduction of this vaccine. More than 5000 cases were reported in this country in 1980, the year before licensing of the DTaP, whereas only 131 were reported in 1993. All clinical studies also showed fewer local and systemic adverse events for the different DTaP vaccines compared to the DTwP. Although a significant increase of serum antibodies against the different components of the aP vaccine can be detected in subjects immunized with both DTaP and DTwP vaccines, no clear correlate of protection could be established. Data from various trials conducted with DTaP and DTwP vaccines have also suggested that after a three-dose schedule, the immunity persists for 5 to 6 years, after which protection starts to decline, leading to the recommendation for a booster immunization at the age of 5 to 6 years old. An adolescent boost with diphtheria-tetanus or DTaP is also recommended, after which a boost every 10 years can help to maintain a protective immunity. Unfortunately, whooping cough is still a major cause of childhood mortality worldwide, in particular in countries with low vaccination coverage. The WHO estimates that in 2008, about 16 million cases of pertussis occurred worldwide, resulting in 195,000 deaths, 95% of which were in developing countries. The WHO currently recommends a primary three-dose course

of DTP within the first year of life, followed by a booster immunization in the second year of life. This first series should be able to confer protection for at least 6 years.

Haemophilus Influenzae Type b

Bacterial meningitis and septicemia are caused mainly by three capsulated pathogens, *H. influenzae*, *S. pneumoniae*, and *Neisseria meningitidis*. *H. influenzae* infection can also result in other clinical outcomes such as pneumonia, otitis media, sinusitis, epiglottitis, septic arthritis, and osteomyelitis. *H. influenzae* was originally described by Koch in 1883. Two major groups of *H. influenzae* can be identified: capsulated and uncapsulated. Bacteria in the first group are the most virulent types and contain a polysaccharide capsule that allows the identification of six antigenically different serotypes named from A to F. In the prevaccination era, capsulated Hib strains were responsible for more than 95% of severe infections caused by this species of pathogens. Uncapsulated or nontypeable strains (nontypeable *H. influenzae* [NTHi]) generally cause pneumonia, otitis media, and sinusitis. Hib capsular polysaccharide is made of polyribosylribitol phosphate (PRP) and is a major virulence factor, as it renders the bacteria particularly resistant to complement-mediated killing. A polysaccharide-based vaccine containing PRP was the first vaccine against Hib to be evaluated in the early 1980s. Efficacy trials showed that this vaccine was 90% effective in protecting children 18 months and older from Hib infection, although no efficacy could be observed in subjects below this age, who are most at risk for Hib invasive disease. These results stimulated the development of a DT glycoconjugated vaccine that was licensed in 1988. Efficacy of this vaccine, however, was inconsistent, in particular in very young children. Three other vaccines were developed, in which Hib PRP has been conjugated to CRM197, the outer membrane protein complex of *N. meningitidis* serogroup B, or TT, respectively. All these vaccines are highly immunogenic in all age groups after three doses, although the outer membrane protein complex conjugated vaccine induces higher antibody titers after the first or second immunization.[204] Many clinical trials have shown an overall efficacy ranging from 87% to 100% in prevention of Hib infection after a three-dose immunization schedule, although no study has compared all three vaccines in parallel. Efficacy of the conjugated Hib vaccines is also demonstrated by the reduction of more than 90% in the incidence of Hib disease in all countries where routine vaccination has been implemented. Several studies have shown that after a threedose primary immunization series, serum antibody levels tend to decrease to a concentration thought to be insufficient to confer protection, although a minimum protective antibody level has not been clearly established. However, a booster immunization is able to drastically increase serum antibodies that then decline to a concentration that is higher than the preboost concentration and remains constant with time.[205] Therefore, the WHO recommends a three-dose immunization schedule for Hib starting from 6 weeks of age and given at a 4-week intervals. In most developed countries, a booster dose at 12 to 18 months is also included in the routine schedule. The WHO has actively supported Hib routine vaccination in developing countries, but in 2009, vaccine coverage was still below 50% for the world population. Glycoconjugated Hib vaccines are currently combined with DTwP or DTaP vaccines, which may also include IPV and HBV vaccine.

Pneumococcus

S. pneumoniae is a major cause of diseases such as meningitis, pneumonia, sepsis, and otitis media. Children younger than 2 years of age and the elderly are the most susceptible populations. In 2005, the WHO estimated that pneumococcal infection is responsible for 1.6 million deaths every year and most of these occur in developing countries *(*http://www.who.int/nuvi/pneumococcus/en/*)*. Soon after its isolation in 1881, pneumococcus was identified as the major cause of pneumonia. *S. pneumoniae* are gram-positive bacteria coated by a polysaccharide capsule that protects them from the host immune system. Indeed, an antibody response against capsular polysaccharide is able to stimulate opsonophagocytosis and bacterial killing, resulting in protection from pneumococcus infection. Structural and antigenic differences in the capsular polysaccharide allow the identification of more than 90 serotypes, although the number of pathogenic serotypes is lower. As antibody to the capsular polysaccharide is protective but serotype specific, in order to induce broad protection against pathogenic serotypes, a pneumococcal vaccine needs to

contain different polysaccharides.

A first pneumococcal vaccine containing polysaccharides from six serotypes was successfully developed in the mid-1940s.[13] At that time, however, it was believed that pneumococcal infection could be easily cured by penicillin, and there was no interest in the use of this vaccine, which was therefore withdrawn soon after its licensure. In the 1970s, 6-valent, 12-valent, or 13-valent polysaccharide vaccines were tested in clinical trials performed in South Africa in adult gold miners, a population at high risk for pneumococcal pneumonia. These studies reported a vaccine efficacy between 76% and 92% against pneumonia caused by the pneumococcus serotypes contained in the vaccine[206,207] and supported licensure of a 14 serotype vaccine in 1977. This vaccine was then replaced in 1983 by a 23 polysaccharide vaccine that covered 85% of the serotypes causing invasive pneumococcal disease (IPD) at the time of vaccine introduction. These polysaccharide-based vaccines stimulate a T-cell-independent antibody response and therefore are able to induce an increase in serotype-specific antibodies in adults but not in children younger than 2 years of age. Indeed, no protection against otitis media was observed in clinical trials among children younger than 2 years of age. Results of several controlled clinical trials evaluating these vaccines in different adult populations were inconsistent and did not confirm efficacy for these polysaccharide-based vaccines against pneumococcal pneumonia as endpoint. However, the conclusions of these studies might have been negatively affected by the fact that pneumococcal infection with bacteremia is relatively rare in an adult population, that the clinical diagnosis of nonbacteremic pneumonia is based on not very specific and sensitive criteria, and that the sample size of

some trials was probably not adequate to correctly measure vaccine efficacy. On the contrary, many postlicensure observational studies have reported effectiveness for these vaccines against IPD in adult subjects, supporting the use of the currently licensed 23-valent vaccine in elderly and other adult subjects at risk of pneumococcal infection.

Induction of polysaccharide-specific antibodies in children younger than 2 years of age requires a T-cell-dependent response, which can be stimulated by a glycoconjugate vaccine. A 7-valent pneumococcal conjugate vaccine (PCV7) with CRM197 as carrier protein and polysaccharide from serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F was licensed in 2000 in the United States, then in Europe, and in many other countries for use in children younger than 2 years of age. When PCV7 was licensed, 65% to 80% of IPD in children from industrialized countries were associated with serotypes contained in this vaccine. In a large PCV7 clinical trial performed in California before licensure, 40 cases of IPD associated with the vaccine serotypes were identified with only one in the vaccinated population, resulting in an efficacy of 97%.[208] After the introduction of PCV7 in routine immunization schedule, a 95% reduction of IPD by vaccine serotype and a 75% decrease in all serotype IPD has been observed as well as an indirect effect on unvaccinated population as a reduced incidence was observed also in the adult population.[209] Similar observations have been made in other countries where PCV7 has been introduced. Several studies have documented that a wide use of PCV7 has resulted in carriage reduction for the vaccine serotypes, which is associated with a parallel increase in carriage of nonvaccine serotypes. The nonvaccine serotypes are more likely to produce a disease compared to the prevaccine era, resulting in a serotype replacement among disease-associated strains. Immunogenicity of pneumococcal glycoconjugate vaccines can be evaluated by measuring serum-specific antibodies against capsular polysaccharides using enzyme-linked immunosorbent assay or opsonophagocytic assay. This latter method detects functional antibodies that are able to kill bacteria in the presence of complement and phagocytic cells. Despite the lack of a clear correlate of protection for pneumococcal vaccines, WHO guidelines suggest the use of a concentration of 0.35 µg per mL for polysaccharide-specific IgG as a threshold when comparing immune responses to each serotype for a new PCV.

A high proportion of IPD in developing countries, particularly in Africa, are associated with serotypes 1 and 5, which are not included in this 7-valent vaccine formulation. A 9-valent CRM197 conjugated vaccine, which also includes serotypes 1 and 5, has been evaluated in two trials in South Africa and in The Gambia, where efficacy against IPD by vaccine serotypes was 85% and 77%, respectively, supporting the use of such type of vaccine for children in developing countries where the burden for pneumococcal infection is highest.

Two new glycoconjugate vaccines against pneumococcus have recently been licensed in the United States, Europe, and other countries. One is an evolution of the PCV7 and contains 13 serotype (PCV13), including serotypes 1, 3, 5, 6A, 7F, and 19A and has shown to meet the WHO criteria of immunogenicity and noninferiority compared to PCV7.[210] The other licensed product is a 10-valent vaccine that contains polysaccharides of the PCV7 serotypes plus serotypes 1, 5, and 7F but uses a different carrier protein than that of PCV7. One serotype is conjugated to TT, one to DT, and all other serotypes to protein D from NTHi.[211] The advantage of this last vaccine should be the capacity of protecting from otitis media induced by both pneumococcus and NTHi. With the inclusion of more serotypes, both these new vaccines should broaden coverage of protection, in particular including serotypes 1 and 5 which are most spread in many developing countries. Moreover, PCV13 has included invasive serotypes that have been increasingly causing severe disease both in the United States and Europe because of strain replacement following PCV7 introduction. Future epidemiologic observations will inform whether new pathogenic strains will continue to replace the vaccine strains after the introduction of these new vaccines. The need for a universal pneumococcal vaccine has stimulated research for a protein-based vaccine. Such a vaccine should not induce strain replacement; it should theoretically have a broader coverage compared to the glycoconjugate vaccines, and it should be cheaper to produce, helping the extension of vaccine use to less developed countries. Several protein-based vaccines are currently being evaluated in preclinical models.[212,213,214]

Meningococcus

N. meningitidis is the major cause of epidemics of bacterial meningitis and still represents a major worldwide threat to public health. Children younger than 2 years of age are at a higher risk of contracting the infection, although some protection seems to be provided by persisting maternal antibodies for up to 6 months of age. Incidence then declines with increasing age but increases again in the teenage years. In 10% to 20% of cases, meningococcal infection is associated with severe sepsis that rapidly progress to death 40% of the time. Today, the overall fatality rate for all meningococcal infections is 10% to 15%, whereas 10% to 20% of subjects who survive have lifelong disabling sequelae. Humans are the only natural reservoir of the bacterium that colonizes the nasopharynx and is transmitted by aerosol or secretions. This makes eradication feasible. It is estimated that 5% to 10% of the adult population in nonendemic areas are asymptomatic carriers. In only a small fraction of carriers the bacterium penetrates the mucosa entering the bloodstream, causing the systemic disease, although for most individuals bacterial carriage is associated with the acquisition of a protective antibody response.[215] The precise mechanism of progression from carriage of bacterium to invasive disease is still poorly understood.

N. meningitidis is a gram-negative aerobic encapsulated diplococcus. There are 13 known serogroups based on the immunologic reactivity of the capsular polysaccharides, of which the A, B, C, W-135, Y, and occasionally X account for virtually all meningococcal disease cases. Protection is usually group specific, and for serogroups A, C, Y, and W-135, protection appears largely to be due to antipolysaccharide antibodies.

Most cases in Europe and the Americas are due to MenB and MenC, whereas MenA and MenC are the most common in Asia and Africa. The major worldwide burden of the disease is, however, caused by MenA epidemics in Africa, in particular in “the meningitis belt,” which includes the sub-Saharan region extending from Senegal to Ethiopia. These epidemics occur at irregular cycles every 5 to 12 years, lasting for 2 to 3 years, and peaking for still unknown reasons at the end of the dry season. The burden of the epidemics is heavy, with case fatality rates of 10% to 20% and 75% of cases occurring in individuals younger than 15 years of age. MenB is the main cause of endemic meningococcal disease in the industrialized world and accounts for 30% to 40% of cases in North America and up to 80% of cases in some European countries. Incidence is highest in winter and in infants younger than 1 year of age, for which the fatality rate is also higher. MenB strains have also been the cause of severe persistent epidemics such as those in South American countries (Cuba, Colombia, Brazil, and Chile), in Norway, and in New Zealand. MenC is the cause of most of the remaining cases of disease in North America and Europe. MenW-135 accounts for only a small number of cases

in the United States but recently has caused outbreaks in Africa. MenY cases have recently increased in the United States, becoming a predominant cause of invasive infection in the elderly.[216]

The licensure of meningococcal vaccines has been based on a serological surrogate marker for protection, the serum bactericidal assay (SBA). This assay measures the level of antibodies that are capable of directing complement-mediated bacterial lysis. Based on the work by Goldschnider et al.,[217] the accepted correlate of protection from meningococcal disease is currently considered an SBA titer ≥4.

Initial vaccines against bacterial meningitis were based on heat-killed whole bacterial preparations; however, they had variable efficacy and caused severe adverse reactions probably due to the high endotoxin content. The first polysaccharide vaccines to be developed were those against MenA and MenC. They were developed in response to disease outbreaks in military recruits and proved to be a safe and effective preventive measure. Trials in army recruits during MenC outbreaks demonstrated an estimated 90% efficacy at 8 weeks following vaccination.[218,219] Meningococcal polysaccharide vaccines presently in clinical use are either bivalent, covering serogroups A and C, or tetravalent, covering serogroups A, C, W-135, and Y. These vaccines are currently used for the control of meningococcal disease epidemics in Africa.

The main disadvantage of polysaccharide vaccines is the poor immunogenicity in the young (younger than 2 years of age), who are at the highest risk for of infection, in addition to the short-lived protection induced in all age groups. Following the successful introduction of Hib conjugated vaccine, conjugation of the polysaccharide to a carrier protein has been exploited also for meningococcal vaccines. Monovalent glycoconjugated meningococcal vaccines against MenC were developed using CRM197 or TT as protein carrier. Three MenC vaccines were introduced in 1999 in the United Kingdom as additional vaccines in the routine infant immunization schedule at 2, 3, and 4 months of age, combined with a catch-up campaign for a single dose at 1 to 18 years of age in response to high rates of serogroup C infection. The immunization campaign has been a great success, resulting in a decrease >90% in the number of clinical cases and deaths, and a decrease of 66% in asymptomatic carriage The incidence of the disease also decreased by 70% among the nonvaccinated population, an effect probably due to reduced circulation of the bacterium and to the effect on nasopharyngeal colonization.[220]

The Meningitis Vaccine Project, a joint project between the WHO and PATH, was initiated in 2001 aiming at the development of affordable conjugated vaccines against MenA (MenAfriVac) for developing countries. The goal was to license a conjugate vaccine that is expected to be more effective than the existing polysaccharide vaccine currently in use and to cost only 40 cents per dose. A single dose in mass immunization campaigns in those 1 to 29 years old in the meningitis belt in Africa has been initially considered. At the end of 2010, MenAfriVac was introduced in Burkina Faso, Mali, and Niger, and after 6 months, these countries reported the lowest number of confirmed MenA cases ever recorded during an epidemic season.

Two slightly different tetravalent conjugate vaccines against serogroups ACW-135Y were developed in recent years. One vaccine, which uses DT as protein carrier, has been licensed in the United States in 2005 for use from 9 months to 55 years old. Licensure was exclusively based on the noninferiority to the PS vaccine, and no efficacy trial was conducted. In a trial in individuals aged 11 to 18 years old, both the polysaccharide and conjugated vaccines were able to elicit a fourfold or greater increase in SBA titers against the four serogroups (range 80% to 97%). However, in a 3-year follow-up, the persistence of bactericidal antibodies and booster response to a second dose was only measured in subjects who had received the glycoconjugate vaccine.[221] Studies in the 2-year-olds showed that a higher immune response against the four meningococcal serogroups was obtained with one dose of the tetravalent glycoconjugate vaccine than with the tetravalent polysaccharide vaccine.[222] Although antibodies persisted for 2 years for a large proportion of children, SBA titers were <1:4. This suggests that a booster dose might be needed in order to maintain long-lasting protection.[223]

CRM197 is the carrier for the second conjugate vaccine against serogroups ACW-135Y,

which has been licensed in both the United States and Europe, and is indicated for individuals 2 to 55 years of age, although an extension of the license for use in those younger than 2 years old has been filed with the FDA and the European Medicines Agency. Immunogenicity of this vaccine in children aged 2 to 10 years old is comparable to the other licensed tetravalent conjugated vaccine,[224] whereas in a higher proportion of vaccinated subjects, a persistence of bactericidal response could be observed.[225] Moreover, after three doses in infants, SBA titers ≥1:4 could be detected in 93% to 97% of subjects depending on the serogroup,[226] suggesting a good efficacy in this age group.

Currently, there is no vaccine available for broad protection against MenB disease. Classical approaches, such as those using polysaccharide-based vaccines, are not feasible because

MenB polysaccharide is structurally and antigenically identical to polysialosyl glycopeptides present in fetal and adult human neural tissue and, therefore, is poorly immunogenic and has the potential to trigger autoimmunity. Most vaccines developed against MenB are based on outer membrane vesicles (OMV) prepared by detergent extraction, which contain outer membrane proteins and lipooligosaccharides. These vaccines elicit functional bactericidal antibodies mainly directed against PorA, the most abundant protein component of these vaccines, and confer serosubtype-specific protection. Although PorA is almost ubiquitously expressed by meningococci, it has different antigenic specificities in the different strains, making it unlikely that a universal vaccine based exclusively on PorA could be generated. It has been estimated that 20 PorA proteins should be included in a MenB OMV vaccine in order to cover 80% of the prevalent circulating strains causing sporadic cases in the United States.[227] MenB vaccines based on OMV have been developed in response to disease outbreaks caused by a single strain in Cuba, Brazil, Chile, Norway, and New Zealand. The use of these OMV vaccines to combat MenB disease has been limited, however, due to the strain-specific nature of the protection.

A breakthrough in development of a MenB vaccine was the sequencing of the *N. meningitidis* genome, which opened the way to the identification of new potential antigens that could not have been characterized by conventional immunologic and microbiologic approaches. This approach, called “reverse vaccinology,” has led to the identification of 570 open reading frames encoding novel surface-exposed or exported proteins.[228] A total of 350 open reading frames were expressed as fusion proteins and tested in mice. Of these 350, 28 recombinant proteins were able to elicit serum bactericidal antibodies in the mouse. Five of the more conserved antigens (GNA2132, GNA1870, NadA, GNA1030, and GNA2091) were selected based on their function and used to prepare a multicomponent MenB vaccine, which was able to induce high SBA titers in mice. Bactericidal antibodies were obtained against 78% of a panel of 65 meningococcal strains representative of the global population, and protection was broadened by using adjuvants such as CpG and MF59.[229] A final vaccine formulation, containing three recombinant proteins and OMV from the New Zealand strain (rMenB+OMVnz), was shown to be immunogenic against strains expressing homologous proteins or homologous PorA.[230] This vaccine has progressed to phase III clinical trials, and a license authorization has been submitted to the European Medicines Agency. It could be the first vaccine to allow broad protection against the great majority of MenB strains responsible for invasive disease.

VACCINES IN DEVELOPMENT

In this section, we will review vaccines that have completed human studies and for which clinical data are available. Particular attention will be devoted to the big three: HIV, TB, and malaria. Interested readers will find more information on vaccines still in preclinical stages of development by consulting corresponding chapters in *New Generation Vaccines*, Fourth Edition.[231]

Human Immunodeficiency Virus

HIV-1 is the causative agent of the global AIDS epidemic, with about 7000 persons newly infected each day. The burden of the disease affects resource-poor countries where education, diagnosis, and treatment are most difficult to obtain. Therefore, the development of prophylaxis and/or therapeutic vaccines would have a profound impact on the spread of

this disease.

HIV-1 is naturally transmitted through sexual intercourse and from mother to infant during pregnancy. The virus infects predominantly CD4+ T cells, seeds in mucosaassociated lymphoid tissue, and reaches maximal viremia with up to 10^7 particles per mL of blood by week 3 after infection. By 2 to 6 months after infection, the viral load usually falls to reach a relatively stable level (the set point) due to the host immune response. A low set point has been shown to correlate positively with a slower progression to AIDS and with a greatly reduced risk of sexual and maternal-to-infant HIV transmission.[232,233,234,235] Eventually, the level of CD4+ T cells gets so low that resistance essentially disappears and the disease progresses to AIDS. The two pillars of the host immune response against HIV infection are envelope-specific neutralizing antibodies[236] and CTL and multifunctional CD8+ T cells.[237] Hence, it is natural that efforts at developing HIV vaccines have predominantly targeted these two arms of the immune response.

HIV-1 represents a formidable challenge to the host's immune system and to vaccine developers alike. The virus has evolved potent immunoevasion strategies that include the following: 1) high genetic diversity, 2) structural features of the envelope involved in infectivity that interfere with the induction of broadly neutralizing antibodies, 3) high rate of mutation of the envelope allowing the virus to escape neutralizing antibodies, 4) infection of and persistence in memory CD4+ T cells, 5) persistence of the virus out-of-reach of immune responses in extracellular spaces in lymph nodes and immunoprivileged sites such as the brain, and 6) destruction of CD4+ T cells. The efficacy of HIV-1 at escaping immune defenses is demonstrated by the failure in developing a vaccine for preventing HIV-1 infection to date, despite more than two decades of intense scientific research, more than 120 phase I, 7 phase II, and 3 phase III clinical trials (IAVI Database of AIDS Vaccines in Human Trials: www.iavireport.org).

Subunit Vaccines against Human Immunodeficiency Virus-1

Vaccine-induced envelope-specific neutralizing antibodies as a key to HIV-1 prophylaxis is supported by two observations: First, passive antibody administration to macaques can prevent infection with a simian immunodefeciency virus-HIV hybrid,[238] and second, at least a dozen broadly neutralizing antibodies have been isolated from HIV-1-infected individuals.[239] Early attempts to develop an HIV-1 vaccine followed the alum/subunit protein path. These subunit vaccines consisted in the most prominent antigens on the viral surface, namely, the envelope (*env*) glycoprotein gp160 and its products surface gp120 and transmembrane

gp41 because of their functional importance for virus attachment and entry into CD4+ T cells and as primary targets for neutralizing antibodies. The most advanced adjuvantedsubunit vaccine is **AIDSVAX** that consists in recombinant monomeric gp120 adjuvanted with alum (Table 43.4). AIDSVAX progressed to phase III efficacy evaluation in two large trials. The vaccines were safe and induced gp120-specific antibodies and CD4+ T-cell responses. However, the antibodies failed to neutralize commonly transmitted primary HIV-1 isolates, and there was no efficacy detected in either trial.[240,241]

TABLE 43.4 Most Advanced Human Immunodeficiency Virus Vaccine Candidates

Category	Candidate	Trial Phase	Key Investigator
Adjuvanted subunit vaccine	**AIDSVAX**, recombinant monomeric gp120/alum (B/B and B/E)	III	VaxGen
Vectored subunit vaccine	**ALVAC-vCP1521** prime, canarypox virus expressing gp120, transmembrane gp41, gag, and protease, followed by boosting	III	VaxGen

with **AIDSVAX (B/E)**		
MRKAd5 vector expressing clade B gag, pol, and nef	III	Merck
VRC-HIVDNA016-00-VP/VRC-HIVADV014-00-VP DNA prime; encoding clade B gag; and pol genes, and clade A, B, C modified env genes; followed by boosting with rAd5 vector expressing the same genes	IIb	NIH VRC

DNA, deoxyribonucleic acid; NIH VRC, National Institutes of Health Vaccine Research Center.

Vectored Vaccines against Human Immunodeficiency Virus-1

Several gene delivery approaches of viral antigens have shown promises for inducing strong CD8+ T-cell responses, including CTL. The most advanced vector-based strategies involve replication-defective recombinant canarypox and rAd in prime-boost regimen. **ALVAC-vCP1521** is a canarypox-derived vector expressing clade E gp120, transmembrane gp41, gag, and protease. ALVAC-vCP1521 was tested in a prime-boost vaccine strategy followed by AIDSVAX (B/E) in an efficacy trial in persons at risk from heterosexual HIV-1 transmission and, for the first time for an HIV vaccine, demonstrated a 31% reduction in HIV-1 acquisition.[242] It remains to be determined which vaccineinduced immune responses correlate with this protection. Previous phase I and II trials showed that all vaccinees developed neutralizing antibodies, more than 80% of them developed antibody-dependent cell-mediated cytotoxicity, and 64% had measurable CD8+ CTL after the prime-boost strategy.[243,244] Two other HIV candidate vaccines based on multivalent rAd5 vectors have reached efficacy trials in humans. **MRKAd5** is a vaccine containing rAd5 vectors expressing clade B gag, pol, and nef (see Table 43.4) in two efficacy trials (STEP and Phambili) enrolling a total of 3801 persons. Despite the induction of HIV-1 gag- and pol-specific CD8+ T-cell responses in a majority of subjects, early viral loads were not decreased, and a trend to increased risk of acquisition in vaccine recipients was observed, leading to a premature termination of both trials in September 2007.[245] The National Institutes of Health is testing a DNA prime-rAd5 boost vaccine strategy (**VRC-HIVDNA016-00-VP/VRC-HIVADV014-00-VP**). Both gene delivery systems include HIV-1 modified env genes from clades A, B, and C, and *gag* and *pol* genes from clade B. Preliminary endpoint analysis is expected mid-2012. In previous phase I and II clinical trials, this heterologous prime-boost consisting of DNA followed by rAd5 showed increased antibody and T-cell responses and more polyfunctional T cells than for either vaccine modality given alone.[246] Preexisting immunity to Ad5 is high throughout the world and can reach 90% of adults in developing countries where an HIV-1 vaccine is the most needed. High titers against Ad5 have been associated with diminished T-cell responses induced by the rAd5-based vaccine[246,247]; therefore, Ad26 and Ad35, less common serotypes, and an Ad5/Ad48 chimera (resistant to anti-Ad5 neutralizing antibody) are being further developed.[247] In addition to canarypox and rAd5 vectors, new replication-defective viral vectors such as MVA and New York *Vaccinia* (a derivative from the Copenhagen vaccinia strain) evaluated with DNA priming have shown promising immunogenicity characterized by high levels of polyfunctional T-cell responses and are moving toward phase II testing.[248,249]

The results of two decades of intensive scientific research aimed at developing an HIV vaccine is quite sobering with most candidate vaccines failing in phase II and III evaluation. There is a glimmer of hope with the 31% efficacy observed with GenVax's canarypox prime-recombinant protein boost strategy, which might provide critical information on vaccine-induced correlates of protection.

Human Cytomegalovirus

HCMV is an enveloped double-stranded DNA virus member of the *Herpesviridae* family. It causes a spectrum of diseases in adults and children ranging from asymptomatic to a mononucleosis-like disease and can be fatal in immunocompromised individuals. HCMV is the most prevalent pathogen transmitted to the fetus by infected mothers resulting in severe neurologic sequelae in about 10% of

congenitally infected infants. Prevention of congenital disease is the main incentive for developing HCMV vaccines. A secondary objective would be to provide HCMV immunity to individuals prior to becoming immunosuppressed due to transplantation, HIV, or other reasons.

In vivo, epithelial cells, endothelial cells, fibroblasts, smooth muscle cells, and macrophages can support replication of the virus while latent virus can be found in macrophages and other myeloid cells. Protection against HCMV is multifactorial and involves innate immunity (natural killer cells); neutralizing antibodies primarily to glycoproteins gB, gH, and gM; and pp65-, IE1-, and gB-specific CD8+ T cells. Approaches to HCMV vaccines have included live-attenuated and whole-killed viruses, vectored, DNA, protein, and peptide-based subunit vaccines.[250] The live-attenuated Towne vaccine is the most widely evaluated HCMV vaccine. It was shown to be safe and well tolerated, induced seroconversion in healthy adults, and protected against challenge with the Toledo strain.[251,252] Furthermore, the Towne vaccine, albeit not preventing HCMV infection, reduced severe disease by 85% in renal transplant recipients,[253,254] although it failed to provide protection to women naturally exposed to HCMV from their infant.[255] Attempts were made at enhancing the immunogenicity of the Towne vaccine by adjuvanting it with IL-12[256] or by adding back gene regions from the more virulent Toledo strains that had been deleted in the Towne strain during the attenuation process. The recombinant Towne/Toledo appeared safe in seropositive adults.[257] Subunit HCMV vaccines tested in the clinic predominantly target gB, pp65, and IE1. The most advanced subunit vaccine in human studies is gB/MF59 (developed by Sanofi Pasteur MSD, Lyon, France), a recombinant glycoprotein gB formulated with MF59 adjuvant. In two large phase I studies in healthy adults, gB/MF59 was found safe and immunogenic and induced higher anti-gB titers compared with alum gB recipients.[258,259] A third study in toddlers revealed that immunization with gB/MF59 resulted in titers higher than found in vaccinated adults.[260] A phase II efficacy study in seronegative women within 1 year of giving birth revealed a significant increase in the number of vaccine recipients that remained uninfected during the follow-up period.[261]

Another vaccine strategy used alphavirus-like replicon particles from Venezuelan equine encephalitis virus to express HCMV antigens. Alphavirus-like replicon particles have been shown to specifically target DCs in vivo and induce broad immune responses including neutralizing antibodies and CTLs. AVX601, a bivalent alphavirus replicon vaccine expressing three HCMV proteins (gB, pp65, and IE1) (developed by AlphaVax Inc., Research Triangle Park, NC, U.S.A.), was found safe and induced neutralizing antibody and multifunctional CD4+ and CD8+ T-cell responses.[262] The bivalent DNA vaccine, VCL-CB01 (developed by Vical Inc., San Diego, CA, U.S.A.), consisting of plasmids encoding for gB and pp65 formulated with poloxamer CRL1005 and benzalkonium chloride to enhance immune responses, was evaluated in HCMV-seropositive and -seronegative adults.[263] VCL-CB01 was immunogenic as measured by IFN-γ ELISpot in 45.5% of seronegative and 25% of seropositive vaccine recipients. VCL-CB01 boosted pp65-specific T-cell responses but not anti-gB antibodies in seropositive individuals, suggesting that this vaccine is more effective at inducing T-cell than gB-specific antibodies.

Finally, prime-boost strategies have been also evaluated. Trivalent HCMV DNA vaccine VCL-CT02 (developed by Vical Inc.), encoding gB, pp65, and IE1, was evaluated in three phase I studies using a DNA prime/Towne liveattenuated boost approach.[264] HCMV-specific memory T-cells were detected by IFN-γ ELISpot in 20% and 60% of unprimed and primed subjects, respectively. The median time to first pp65 T-cell and gB antibody response after Towne was 14 days for DNA-primed subjects versus 28 days for controls administered Towne only. Similar observations were made with a canarypox gB prime/Towne live-attenuated boost.[265]

Tuberculosis

TB caused by Mtb complex bacilli is one of the leading causes of death worldwide. The WHO has declared TB a global public health emergency and predicts that almost one billion people will be infected, with 35 million dying from the disease, by 2020. TB is prevalent in developing regions of the world such as sub-Saharan Africa and Southeast Asia, where it is often associated with the HIV epidemic. Upon exposure to Mtb, 30% to 40% of close contacts will develop TB infection, of whom 5% would be expected to develop active disease within a 24-month period, whereas the other 95% enter a state of controlled latent TB infection (LTBI), which can reactivate later in life following decreased immunocompetence of the host.

Despite the availability of drugs against Mtb and the directly observed therapy short-course campaign initiated by the WHO, TB persists as a global health concern in part because infected individuals do not have access to point of care and/or are often noncompliant with the 6-month or longer drug treatment. This is particularly true in the developing world where more than 95% of infections occur. Treatment noncompliance has contributed to the current TB pandemic by increasing the probability of transmission and sustaining the development of multidrug-resistant strains of Mtb. Multidrug-resistant strains are resistant to the two most powerful first-line drugs: rifampicin and isoniazid. Since the discovery of multidrug-resistant TB in the 1990s, the resistance pattern of TB has continued to evolve, and isolates resistant to both first- and second-line drugs have been identified. Therefore, development of safe, effective, and affordable prophylactic vaccines that also provides long-lasting protection in BCG-immunized people is a critical step toward controlling TB.

Promptly after inhalation, Mtb is engulfed by lung alveolar macrophages in an attempt by the host to destroy the invader. Mtb, however, has adapted to survive in macrophages by preventing phagosome acidification and fusion with lysosomes. T cells, both CD4+ Th1 and CD8+, and IFN-γ and tumor necrosis factor (TNF) cytokines play important roles in the prevention of active disease and the control of LTBI, as demonstrated by gene-knockout animal models and

human subjects with mutations affecting the expression of these two cytokines.[266] As a result of macrophage activation by these inflammatory cytokines, Mtb bacilli stop multiplying and enter a state of dormancy.[267] Once the activation status of macrophages is lowered, however, mycobacteria can resuscitate, leading to disease reactivation. Current vaccine design strategies focuses on stimulation of a highly potent T-cell response in an attempt to contain or even eradicate Mtb after it has established itself in the phagosome of macrophages.

BCG, the only licensed vaccine currently available against TB, is likely the most widely used vaccine in the world with more than four billion doses administered. BCG is a live-attenuated strain of *Mycobacterium bovis* that was developed by Albert Calmette and Camille Guérin by serial passage on media.[268] During the attenuation process, BCG lost a large number of genes clustered in numerous regions of difference.[269] The BCG vaccine strain was introduced in 1921 and was further distributed to numerous institutions all over the world. Today, due to differences in culture conditions over decades, the BCG vaccine exists in more than one genetic background, possibly resulting in differences in efficacy. BCG is administered to newborns and is effective in preventing severe childhood forms of TB such as miliary TB and TB meningitis, whereas its efficacy in adults against pulmonary TB has been highly variable and ranged from 0% to 80% protection.[270] In adults, the lowest BCG efficacy was found in countries with the highest incidence of tuberculin skin test positivity due to prior exposure to Mtb or other environmental bacteria, suggesting that preexisting immune responses to mycobacterial antigens shared in BCG prevent necessary bacterial replication and vaccine take. Therefore, although safe in all immunocompetent individuals, BCG was efficacious only in the skin test negative population, primarily children.[271] As a consequence, vaccine protection against adult pulmonary TB, the most common form of TB, in high-endemic countries is very limited[272] and demonstrates the urgency of developing novel TB vaccines. Other drawbacks of BCG include waning efficacy over time and interference with the tuberculin skin test diagnosis of TB.

Novel TB vaccines currently in development target different needs and individuals. Some efforts are made at improving the current BCG vaccine for newborns and nonimmunized tuberculin skin test-negative adults. In addition, a heterologous booster vaccine, consisting of an adjuvanted or vectored subunit vaccine, is a high priority for individuals who have already received BCG, as BCG efficacy wanes after 10 to 15 years and boosting with BCG has proven ineffective in both preclinical and clinical studies.[273,274,275,276,277,278] Ideally, this booster vaccine would be given during adolescence and possibly repeated during adulthood as necessary. In theory, such a booster vaccine could also boost the immunity of individuals already exposed to Mtb. Finally, a therapeutic vaccine as an adjunct to antibiotics in the therapy of TB has been considered with the hope of reducing the overall duration of drug treatment. All novel TB vaccines that are being tested in humans or are about to enter clinical trials demonstrated immunogenicity and protective efficacy in mouse, guinea pig, and/or nonhuman primate models of TB.[279]

Vaccines Replacing Bacille Calmette-Guérin

Due to the long history of BCG's safety and efficacy in children, it is likely to remain on the WHO Expanded Program on Immunization vaccination schedule. Therefore, a line of improved BCG vaccines was developed and showed superior efficacy compared to BCG in different animal models of TB, including the stringent guinea pig infection model.

The first two such vaccines to complete phase I clinical trials were rBCG30, a recombinant BCG overexpressing Mtb extracellular Ag85B antigen (developed by Dr. Horwitz's group at the University of California Los Angeles, CA, U.S.A.), and VPM1002, a urease-deficient BCG expressing listeriolysin (rBCGδureC::Hly+::Hyg+) that delivers mycobacterial antigens to the cytosol for enhanced presentation to T cells (developed by Dr. Kaufmann's group at the Max Plank Institute, Berlin, Germany) (Table 43.5). rBCG30 was as safe as nonrecombinant BCG and significantly enhanced the population of Ag85B-specific CD4+ and CD8+ T cells capable of proliferation and IFN-γ secretion.[280] Similarly, no safety concerns were reported for VPM1002 in studies including individuals with or without preexposure to BCG (Germany) and with preexposure to BCG in endemic areas (South Africa). Furthermore, VPM1002 induced antigenspecific multifunctional CD4+ and CD8+ T cells and showed a trend of superiority compared to BCG at equivalent dosage (for more information, see www.vakzine-manager.de/en/resources/Produkte/VPM1002_en.pdf).

Another approach still in preclinical evaluation is worth mentioning and consists of an attenuated Mtb strain resulting from the inactivation of the phoP virulence factor and has extensively been tested for safety and efficacy, including in the guinea pig model of TB.[281,282]

Subunit Tuberculosis Vaccines

Hundreds of Mtb proteins have been screened in experimental animal models of TB, including systematic functional genomic antigen discovery and prioritization.[283,284,285] Combinations of one or more among six protective Mtb antigens (Ag85A, Ag85B, ESAT-6, TB10.4, Rv0125, and Rv1196) comprise the subunit vaccines currently in human studies. Ag85A and 85B are major secreted Mtb proteins; ESAT-6 and TB10.4 are virulence factors belonging to the Esx family of proteins; Rv0125 is a serine protease; and Rv1196 belongs to the PE/PPE family of proteins. All but ESAT-6 antigens are present in BCG vaccine strains, allowing for heterologous prime-boost vaccination strategy using BCG as the prime and subunit as the boost.

Three adjuvanted recombinant subunit TB vaccines have been tested in human studies. Mtb72F, a fusion protein made of Rv0125 and Rv1196 formulated with the AS02A adjuvant system has completed phase I. The Mtb72F/AS02A subunit vaccine was found safe and well tolerated in purified protein derivative-negative adults, with some mild reactogenicity. It induced good production of IL-2 and IFN-γ in the ELISpot assay, and polyfunctional CD4+ T cells expressing CD40L, IL-2, TNF, and/or IFN-γ were observed with intra-cellular staining while no CD8+ T-cell responses were detected.[286,287] Mtb72F is currently being evaluated in phase II studies. H1, a fusion protein consisting of Ag85B and ESAT-6, has been

tested with IC31 adjuvant in different cohorts including negative tuberculin skin test, BCG

vaccinated, and subjects with LTBI in phase I clinical trials. Vaccination with H1/IC31 did not cause local or systemic adverse effects besides transient soreness at the injection site, but it elicited strong antigenspecific T-cell responses against H1 and both the Ag85B and the ESAT-6 components.[129]

TABLE 43.5 Most Advanced Tuberculosis Vaccine Candidates

Category	Candidate	Trial Phase	Key Investigator
Genetically engineered BCG	**rBCG30**: BCG overexpressing *Mycobacterium tuberculosis* antigen Ag85B	I	University of California Los Angeles, Aeras
	VPM1002: Urease-deficient BCG expressing listeriolysin	I	Vakzine Projekt Management GmbH
	AERAS-422: Urease-deficient recombinant BCG, overexpressing *Mycobacterium tuberculosis* antigens Ag85A, Ag85B, and TB10.4 as well as listeriolysin	I	Aeras
Adjuvanted subunit vaccine	**Hybrid-1 (H1):** Fusion protein of Ag85B and ESAT-6 administered with IC31 adjuvant	I	Statens Serum Institute and TBVAC
	Hybrid-1 (H1): Fusion protein of Ag85B and ESAT-6 administered with LTK63 adjuvant	I	Statens Serum Institute, Novartis Vaccines, MUVAPRED
	Mtb72F: Fusion protein of Rv1196 and Rv0125 administered with AS02 adjuvant	II	GSK and Aeras
	HyVac4 (**AERAS-404**): Fusion protein of Ag85B and TB10.4 administered with IC31 adjuvant	I	Statens Serum Institute, Intercell, Aeras
Vectored vaccine	**MVA85A**, a modified vaccinia virus Ankara expressing *Mycobacterium tuberculosis* antigen Ag85A, as booster of BCG-priming or naturally acquired TB immunity	IIb	Oxford University
	FP85A, a recombinant fowlpox virus 9 expressing *Mycobacterium tuberculosis* antigen Ag85A, as booster of BCG-	I	Oxford University

	priming or naturally acquired TB immunity		
	AERAS-402: Replication-incompetent adenovirus 35 vector expressing *Mycobacterium tuberculosis* antigens Ag85A, Ag85B and TB10.4	IIb	Crucell Holland B.V. and Aeras

BCG, Bacille Calmette-Guérin; TB, tuberculosis.

Two additional vaccines ready to enter clinical evaluation are worth mentioning:

1. *ID93*, a fusion protein comprising Rv3619 (Esx Mtb protein family), Rv1813 (involved in Mtb latency), Rv3620 (Esx Mtb protein family), and Rv2608 (PE/PPE Mtb protein family). Rv2608 formulated with the adjuvant system GLA-SE was found immunogenic in mice, guinea pigs, and nonhuman primates. ID93/GLA-SE boosted BCG-primed immune responses and increased protective efficacy in the stringent guinea pig TB challenge model.[288]
2. *H56*, a fusion protein based on H1 and further including Rv2660c (Mtb latency antigen) (developed by P. Andersen and colleagues at the Statens Serum Institute, Copenhagen, Denmark) adjuvanted with CAF01 induced polyfunctional CD4+ T-cell responses in vaccinated mice, was associated with more efficient containment of late-stage TB infection than the H1 and BCG, controlled reactivation, and significantly lowered the bacterial load in two postexposure mouse models.[289]

Vectored Tuberculosis Vaccines

MVA85A, a MVA expressing the extracellular Mtb antigen Ag85A, and AERAS-402, a modified adenovirus 35 expressing Mtb antigens Ag85A, Ag85B, and TB10.4, are in phase IIb efficacy trials and the most clinically advanced novel TB vaccines (see Table 43.5). MVA85A is the first vector-based TB vaccine tested in humans, and it was found safe and well tolerated in multiple phase I studies with BCG-naive and BCG-vaccinated healthy adults[290,291,292,293]; in adults with LTBI[294]; and in BCG-vaccinated adolescents, children, and infants in an endemic area.[295,296] In all studies, immunization with MVA85A induced high levels of Ag85A-specific polyfunctional CD4+ T cells producing IFN-γ, TNF, and IL-2 with additional subsets secreting IL-17 or granulocyte macrophage-colony stimulating factor. Surface staining showed the responding CD4+ T cells to be relatively immature ($CD45RO+CD27^{int}CD57-$); this observation was supported by the robust proliferative responses observed following antigenic stimulation.[297] AERAS-402 was found safe and well tolerated in healthy Mtb-uninfected BCG-vaccinated adults from a TB-endemic region of South Africa.[298] The vaccine induced a robust polyfunctional CD4+ T-cell response dominated by cells coexpressing IFN-γ, TNF, and IL-2. AERAS-402 also induced a potent CD8+ T-cell response, characterized by cells expressing IFN-γ and/or TNF, which persisted for the duration of the study.

Therapeutic Tuberculosis Vaccines

Therapeutic vaccines have a potential role as adjunctive therapy during or after completion of antibiotic treatment against active TB as well as LTBI. Heat-killed *Mycobacterium vaccae* has been extensively studied in humans as an immunotherapeutic vaccine for active TB with variable success.[299,300,301] In a recent clinical trial, patient treated with *M. vaccae* regimen showed faster and more complete clinical improvement than patients administered with placebo.[300] In contrast, RUTI, a therapeutic vaccine made from virulent Mtb grown in stressful conditions, fragmented, detoxified, heat inactivated, and formulated in liposomes has been designed to be used against LTBI after one month of chemotherapeutic treatment. In a phase I study, RUTI induced T-cell responses to purified protein derivative and a number of Mtb antigens in healthy adults.[302] In conclusion, TB vaccine research, development, and testing has become an active area in the recent years; thanks to the increasing investment

from public funds such as the European Union, National Institutes of Health, and the Bill & Melinda Gates Foundation. Several vaccines have entered human clinical trials and there is hope for new and better TB vaccines soon.

Staphylococcus aureus

Staphylococcus aureus (SA) are mostly capsulated grampositive extracellular bacteria that exist as human commensals in the nares and on the skin of about one-third of individuals. A breach in natural barriers (skin epithelium and mucosal membranes) predisposes the host to SA invasion of the tissues and associated disease manifestations. In addition to skin and soft-tissue infection, SA can also cause pneumonia, osteomyelitis, endocarditis, and septicemia. Historically, significant SA infections were most often nosocomial, as hospitalized patients have weakened natural immunity and are more vulnerable.

There are no vaccines currently licensed against SA, and antibiotics are the only treatment to fight off SA infection. Penicillin, introduced in the 1940s, and semisynthetic methicillin in the 1960s were potent antistaphylococcal drugs; however, SA quickly developed resistance, and today, more than 60% of hospital-acquired strains are methicillin resistant.[303] Over the past decade, more virulent methicillin-resistant SA strains developed outside the hospital environment, frequently transmitted among otherwise healthy individuals, and designated as community-acquired methicillin-resistant SA. Antimicrobial resistance and the emergence of more virulent strains call for the development of vaccines that are capable of inducing protection against a wide array of diseases caused by SA infection in the population at large.

Vaccine targets against SA are multiple and include capsular polysaccharides type 5 and type 8 (expressed by 80% of all strains), surface poly-*N*-acetylglucosamine polysaccharide, microbial surface components recognizing adhesive matrix molecules such as fibrinogen- and fibronectin-binding proteins (ClfA, ClfB and FnbA, FnbB, respectively), collagen adhesin (Cna) and matrix-binding proteins (Sdr), iron-regulated surface determinants (IsdA, IsdB), Ess secretion system (EsxA, EsxB), exotoxins, and a collection of proteases. In addition, targeting molecules from SA involved in immunoevasion, including protein A (SpA) that binds the Fc portion of host IgG; CHIPS, a modulator of phagocyte chemotaxis; and SCIN, a modulator of the complement, are alternative vaccine strategies. SA has developed a myriad of molecules to escape host immunity; therefore, it is likely that vaccines targeting more than one factor will have improved chances of success. Based on experimental evidences such as passive immunization, it is believed that protection against SA is largely mediated by bioactive antibodies.

Human studies of SA vaccines have been mostly disappointing to date. In the 1940s, intramuscular vaccination with 3 to 4 hour cultures of **live SA** in individuals suffering from chronic ocular staphylococcal infection resulted in improvement in two-thirds of the patients.[304] However, further controlled human studies have not been carried out since. Vaccine-induced protection with live-attenuated strain in multiple animal models highlights the potential of this approach against SA. In the 1960s, protein-based vaccination strategies with phage- or enzyme-induced **lysates of SA** strains were evaluated for efficacy in the treatment of human skin infection with some success[305,306,307] but were not developed further. An attempt with **SA whole-killed** bacteria plus **toxoids** in patient receiving kidney replacement therapy induced an increase in antistaphylococcal antibodies but failed to demonstrate efficacy against peritonitis.[308] IsdB protein-based subunit vaccine **V710** was well tolerated and immunogenic in healthy adults in a phase I studies with and without alum.[309] V710 was further tested in a phase II and in a phase II/III study, but data are not available yet. Finally, **StaphVAX** (developed by Nabi Biopharmaceuticals, Rockville, MD, U.S.A.) consists of types 5 and 8 capsular polysaccharides coupled to a nontoxic recombinant form of *Pseudomonas aeruginosa* exotoxin A as protein carrier. In phase III clinical trials, StaphVAX was found to be safe, induced protective levels of SA-specific IgG in more than 70% of the individuals, and conferred approximately 60% protection for 10 months against bacteremia caused by SA in patients on hemodialysis.[310,311] From 2004 to 2006, StaphVAX has completed three phase III human studies in. Despite its immunogenicity, StaphVAX development was dropped when a large multicenter phase III study failed to demonstrate reduction in SA infections among kidney transplant patients in the 1-year follow-up period.

In summary, no SA vaccine has resisted the efficacy test in human studies yet, and

researchers are back to the drawing board with a handful of promising candidates still in preclinical development.

Malaria

Malaria is the most prevalent vector-borne disease in the world with 2 billion people at risk of contracting this disease, 500 to 600 million cases each year, and 1 to 2 million deaths per year, mostly in children younger than 5 years old. Malaria is caused by protozoa of four species of *Plasmodium* among which *P. falciparum*, responsible for deadly cerebral malaria, and *Plasmodium vivax* are the most prominent and dangerous.

The feasibility of a vaccine against malaria is supported by two observations: People living in endemic regions

eventually become relatively immune to the disease, although this immunity is not sterilizing and disappears when individuals move to a malaria-free region, and antibodies can be therapeutic. The challenges facing those trying to develop a malaria vaccine are daunting and reflected by the considerable amount of research that has been done and the absence of a licensed vaccine. *Plasmodium* protozoa are exceedingly complex pathogens that 1) consist of 5300 genes among which to select vaccine candidates,[312] 2) have a multistage life cycle characterized by stage-specific protein expression, and 3) display extensive allelic polymorphism of candidate antigens. To further complicate the task of vaccine developers, both *P. falciparum* and *P. vivax* are difficult to grow in vitro, and no animal model truly reflects the human disease. On the positive side and unlike HIV and TB, controlled *P. falciparum* challenges are possible in humans to test vaccines for efficacy.

Malaria candidate vaccines targeting each of the four stages of the parasite life cycle are being developed. These include vaccines against the sporozoites, the infected hepatocytes (liver stage), the merozoites (blood stage), and the gametocytes (transmission-blocking vaccines). Preerythrocytic malaria vaccines target sporozoites and hepatic stages to completely prevent blood-stage infection and thus manifestations of the disease, whereas bloodstage vaccines aim at reducing parasite burden, therefore decreasing the severity and resulting complications of the disease. Finally, transmission-blocking malaria vaccines are designed to arrest the development of sexual stages inside mosquitoes through passive ingestion of specific antibodies during the insect's blood meal.

Four preerythrocytic stage antigens have reached clinical testing: the circumsporozoite protein (CSP), thrombospondin-related adhesive protein (TRAP/SSP2), and liver-associated antigen 1 and 3 (LSA1, LSA3); seven blood-stage antigens: merozoite surface proteins 1 to 3 (MSP1, MSP2, MSP3), apical membrane antigen 1 (AMA-1), serine-repeat antigen 5 (SERA/P126), glutamate-rich protein (GLURP), and erythrocyte-binding antigen^{175} (EBA175); and one sexual-stage antigen: Pfs^{25}. From animal models and human naturally acquired immunity to malaria, it is assumed that immune responses required for protection against sporozoites, merozoites, and gametocytes are mostly antibody dependent, whereas protection against liver-stage likely requires CD8+ T-cell-mediated immune responses for clearance.[313] There is evidence that all of these immune mechanisms are involved in protecting against malaria.[314] Therefore, choosing the proper antigen delivery system is a key element in determining protective-efficacy of candidate malaria vaccines. Wholekilled or live attenuated parasites, gene-based approaches, and adjuvanted recombinant proteins and peptides (Table 43.6) will be discussed.

Whole-Organism Malaria Vaccines

Immunization of volunteers with radiation-attenuated *P. falciparum* sporozoites (PfSPZ), a candidate malaria vaccine targeting preerythrocytic stage, resulted in sterile immunity and completely prevented infection upon challenge with fully virulent sporozoites.[315,316,317,318] High levels of antibodies to sporozoites and CSP-specific antibody and T-cell responses were observed in individuals receiving the PfSPZ vaccine.[316,319] Since 2003, scientists at Sanaria Inc. (Rockville, MD, U.S.A.) have made significant progress in developing manufacturing processes that are scalable and meet regulatory requirements for producing irradiated sporozoites. Clinical lots of PfSPZ have been made and are currently tested in

phase I/II clinical trials (NCT01001650, NCT01441167).

Adjuvanted Malaria Subunit Vaccines

A variety of preerythrocytic and blood-stage recombinant proteins and long synthetic peptides have been evaluated with alum, emulsions, and liposomal adjuvant formulations in numerous phase I/II studies. The only candidate malaria vaccine with a proven track record of protecting people against sporozoite challenge in clinical settings and in the field is **RTS/S** developed by GSK and WRAIR (see Table 43.6). RTS/S includes hepatitis B surface antigen, which promotes assembly into VLPs, and a portion of the CSP molecule consisting of 1) the immunodominant repeat region that is a target for neutralizing antibodies against sporozoites and 2) a protein domain that contains CD4+ and CD8+ T-cell epitopes. RTS/S formulated in AS02A and AS01B is safe and immunogenic, and has consistently shown 30% to 50% protection on immunized individuals against experimental sporozoite challenge.[320,321,322] Meta-analyses of 11 human clinical trials concluded that in semiimmune children, RTS/S reduced clinical malaria episodes by 26% and severe malaria by 58% for up to 18 months with no severe adverse events.[323] Furthermore, RTS/S in AS02D and in AS01E reduced the new onset of parasitemia in infants by 66% over a 6-month observation period[324] and 61.6% over a 19-month follow-up period, respectively.[325,326] Strong multifunctional CD4+ T-cell responses were detected in RTS/S immunized infants.[327] Two malaria vaccine candidates against LSA1 and LSA3 have been tested in phase I/II trials. LSA-NRC formulated in AS02A and AS01B induced high levels of LSA1-specific antibodies and IFN-γ producing T-cells but failed to protect against sporozoite challenge.[328] Malaria blood-stage AMA1-C1 candidate vaccine formulated with alhydrogel and alhydrogel plus CpG 7909 reached phase IIb clinical trials. AMA1-C1 consists of two forms of AMA-1 and was developed to reduce the effect of antigen polymorphism. AMA1-C1 in alhydrogel was safe and immunogenic; however, the antibodies only moderately inhibited merozoite growth in vitro and the vaccine later failed to show efficacy in a phase IIb field trial.[329] Addition of CpG 7909 to the vaccine greatly increased levels of functional antibodies but failed to protect immunized volunteers against a controlled challenge with *P. falciparum*-infected erythrocytes.[330] In a phase II study in Mali, FMP2.1, another AMA-1-based vaccine, in AS02A, did not provide significant protection (20%) against clinical malaria on the basis of the primary end point, but on the basis of secondary results, it may have strain-specific efficacy (64%).[331] Several vaccine candidates targeting the 42-kDa C-terminal region of MSP-1 (MSP-1_{42}) and MSP-3 formulated with AS02A, ISA720, and alhydrogel

± CpG 7909 were safe and immunogenic but failed to induce high levels of functional antibodies and were not efficacious against clinical outcomes. Finally, transmission-blocking vaccines, Pfs25 and Pvs25, formulated with Montanide ISA 51 induced functional antibodies, but the vaccines were too reactogenic,[332] requiring new vaccine formulations to be developed.

TABLE 43.6 Most Advanced Malaria Vaccine Candidates

Category	Candidate	Stage	Trial Phase	Key Investigator
Live-attenuated vaccine	**PfSPZ**, live radiation-attenuated sporozoites	PE	I/II	Sanaria Inc./NIAID
Adjuvanted subunit vaccine	**RTS,S:** fusion protein of CSP repeat region (R), T-cell epitopes region (T), and hepatitis B surface antigen (S), formulated with **AS02A**	PE	IIb/III	GSK

	and **AS02D** oil-in-water emulsion or **AS01B** and **AS01E** liposomal adjuvant formulations			
	LSA-NRC, N- and C-terminal regions of LSA1 flanking two 17-aa repeats formulated with **AS01B** or **AS02A** adjuvants	PE	I/II	U.S. Army Medical Research
	PfLSA-3rec formulated with alum or Montanide ISA 720	PE	I/II	Radboud University
	AMA1-C1, FVO and 3D7 forms of AMA-1 formulated with **alhydrogel ± CpG7909**	Blood	IIb	NIAID/University of Oxford
	FMP2.1, 3D7 form of AMA-1 formulated with **AS02A**	Blood	II	NIAID
	FMP1, 3D7 form of MSP-1_{42} formulated with AS02A	Blood	IIb	U.S. Army Medical Research
	MSP1_{42}-C1, FVO and 3D7 forms of MSP-1_{42} formulated with **alhydrogel ± CpG7909**	Blood	I/II	NIAID
	MSP-3 LSP formulated with **alum**	Multiple	IIb	Vac4All
	Pfs25, Pvs25, formulated with Montanide **ISA 51**	Transmission	I	NIAID
Vectored vaccine	**DNA**, ME-TRAP	PE	I/II	Oxford University
	DNA, CSP, TRAP/SSP2, LSA1, LSA3, Emp1	PE	I/II	U.S. Army Medical Research
	DNA/MVA, ME-TRAP	PE	IIb	Oxford University
	DNA/Ad5, expressing CSP and AMA-1 antigens	Multiple	I/II	U.S. Army Medical Research
	FP9/MVA, ME-TRAP	PE	IIb	Oxford University
	NMRC-M3V-Ad-PfCA, Ad5 vectors expressing CSP and AMA-1 antigens	Multiple	I/II	U.S. Army Medical Research

Ad35.CS.01/Ad26.CS.0.1, CSP	PE	I/II	Crucell
Ad35.CS.01/RTS,S/AS02A, CSP	PE	II	GSK
AdCh63/MVA, AMA-1, MSP-1, ME-TRAP	Multiple	I/II	Oxford University
AdCh63/MVA, MSP-1	Blood	I/II	Oxford University

CSP, circumsporozoite protein; DNA, deoxyribonucleic acid; GSK, GlaxoSmithKline; MVA, modified *Vaccinia* Ankara; NIAID, National Institute of Allergy and Infectious Diseases; PE, preerythrocytic-stage antigens.

Vectored Malaria Vaccines

Gene-based malaria vaccines can be divided in three groups: DNA, poxvirus, and adenovirus vectors. In human studies, DNA malaria vaccines consisting of single antigens or mixtures of plasmids showed minimal antibody, modest T-cell responses, and no evidence of protective efficacy.[333,334] A DNA prime-MVA boost vaccination strategy targeting the ME-TRAP preerythrocytic antigen showed good immunogenicity and some level of efficacy in sporozoite-challenged volunteers with an approximately 80% reduction in parasite load but later failed in a phase IIb field efficacy trial (11% efficacy).[335,336] Despite higher METRAP-specific T-cell responses obtained with fowlpox FP9 prime-MVA boost than previously observed with the DNA/MVA vaccination strategy,[337] no efficacy was observed in field trials.[338,339] In humans, DNA, MVA, and FP9 coding for the CSP antigen were poorly immunogenic. Finally, adenovirus (Ad5, Ad26, Ad35, and AdCh63) vectors expressing preerythrocytic stage CSP and ME-TRAP, and blood-stage AMA-1 and MSP-1 antigens are being evaluated in phase I/II for efficacy against sporozoites challenge by different groups (see Table 43.6).

In summary, with the exception of RTS/S, most malaria vaccine candidates failed to show efficacy despite good safety and immunogenicity in humans. Novel combinations of antigens targeting multiple life stages of the parasite and heterologous prime-boost vaccination strategies are being pursued.

Leishmania

Leishmania species are protozoan parasites causing a wide range of disease manifestations from simple self-healing cutaneous leishmaniasis (CL) lesions to highly disfiguring mucosal leishmaniasis (ML) and lethal visceral leishmaniasis (VL). All species of *Leishmania* are naturally transmitted by the bite of female sandflies that inoculate low numbers (100 to 1000) of flagellated metacyclic promastigotes during a blood meal. Infected humans (anthroponotic leishmaniasis) and animals (zoonotic leishmaniasis) represent reservoirs for the parasites. Once in its mammalian host, *Leishmania* becomes an obligate intracellular pathogen that resides and replicates as nonflagellated amastigotes in the host's macrophages. Acquired resistance to leishmaniasis is mediated by CD4+ Th1 cells and CD8+ T cells, but like for TB, surrogate markers of protection are not fully identified.

There is currently no effective prophylactic immunization available against *Leishmania*, outside the cutaneous inoculation of live virulent *Leishmania major*, a practice known as “Leishmanization,” to produce a self-healing lesion that induces lifelong immunity due to the chronic persistence of low levels of parasites in the host. Leishmanization was mostly practiced in the military from the 1960s to the 1980s, but stopped after the Iran-Iraq war mostly due to unwanted side effects, prolonged duration of lesions, and a few nonhealing

cases that were difficult to treat.[340,341] In school-aged children and migrants in a high endemic region in Uzbekistan, a mixture of dead and live *L. major* continues to be given.

First-generation *Leishmania* vaccines consisted of wholekilled parasites (from one to five different *Leishmania*) with or without BCG as an adjuvant. In absence of adjuvant, New World (Americas) vaccines were found safe albeit weakly immunogenic[342] but failed to afford protection against CL.[343] Inclusion of BCG as an adjuvant increased both immunogenicity and protective efficacy of the vaccine[344]; however, in another trial, volunteers refused to take the last vaccine dose because of side effects (active lesion and scarring due to live BCG).[345] Interestingly, three doses of whole-killed parasites + BCG vaccine given a month apart as immunotherapeutic might be a safer (less serious side effects) and more costeffective than drug treatment against CL, ML, and early diffuse CL.[346,347,348,349] A similar approach, using autoclaved *L. major* + BCG in single or multiple injections was initially applied with Old World vaccines against homologous or heterologous leishmaniasis. The vaccine was found safe and induced *Leishmania* skin test conversion in up to 36% of the individuals; however, it failed to demonstrate protective efficacy superior to BCG-alone control groups.[350,351,352,353] A meta-analysis of all clinical trial data available confirmed the lack of protective efficacy of whole-killed vaccine.[354] More recently, a new formulation of alum-precipitated autoclaved *L. major* + BCG demonstrated enhanced immunogenicity with 25% to 56% *Leishmania* skin test conversion.[355,356] More than 20 second-generation *Leishmania* vaccines, consisting of adjuvanted-protein or DNA subunit vaccines that demonstrated protective efficacy against CL and/or VL in animal models of leishmaniasis, are currently in preclinical development.[357] The most advanced one to enter human clinical trials is a fusion protein made of *L. major* stress-inducible protein 1, thiol-specific antioxidant, and *Leishmania* elongation/initiation factor, called LEISH-F1 and LEISH-F2 (modified-F1 version lacking histidine (His) tag and including a single amino acid mutation to reduce protein breakdown), formulated with MPL-SE adjuvant. Phase I studies completed in healthy LTS-negative and LTS-positive individuals living in *Leishmania* nonendemic and endemic regions demonstrated that the vaccine was well tolerated and immunogenic.[358] In addition, the vaccine was safe and well tolerated when administered as an adjunct to drug treatment in CL- and MLinfected individuals,[359,360] although data in post Kala-azar dermal leishmaniasis (a complication of VL) patients are not yet available.

To date, the best efficacy data with first- or second-generation *Leishmania* vaccines were obtained in therapeutic rather than prophylactic immunization strategies.

CHALLENGES FOR THE FUTURE

During the recorded history, infectious diseases had profound influence on the human population. From 1347 to 1351, the Black Death or bubonic plague caused by *Yersinia pestis* killed approximately 30% of the European population. During the 18th century, it is calculated that smallpox used to kill approximately 0.7% of the total European population (1 person per 140) every year. The 1918 pandemic influenza killed approximately 1% of the global population. In the United States, at the beginning of the 20th century, there were at least 1.7 million cases of infectious diseases with an average mortality rate of 1%. Today, most of these infectious diseases have been eliminated at least from highincome countries, mostly thanks to the availability of clean water and vaccination. In low-income countries, infectious disease are still a major problem, and it is estimated that at least 8 million people die annually from infectious diseases for which vaccines already exist, and >10 million die from infectious diseases for which vaccines do not exist such as HIV, TB, malaria, *Salmonella*, and *E. coli*.

Our children are routinely vaccinated against approximately 10 infectious diseases, and we have forgotten that it was not a long time ago that infectious diseases used to be one of the major problems of mankind. Given the great success that vaccines already had in eliminating infectious diseases, a question that is often asked is whether there is a role for vaccines in the 21st century.

During the last 30 years, we have experienced several waves of innovation that made possible vaccines that were previously impossible. Examples are recombinant DNA that made possible the hepatitis B vaccine; conjugate vaccines that made possible vaccines against

Hib, pneumococcus, and meningococcus; and reverse vaccinology that allowed the discovery of new antigens for MenB, adjuvants to improve the efficacy of influenza vaccines. Novel technologies are emerging every day, and it is likely that in a few years we will be able to do things that are still impossible today. These technologies will allow the development

of novel vaccines that will be used for the following purposes: 1) eliminate the remaining infant infectious diseases such as meningococcus, respiratory syncytial virus, group B streptococcus, and influenza; 2) develop vaccines against cytomegalovirus, Epstein-Barr virus, and herpes simplex virus; 3) develop vaccines against antibiotic-resistant bacteria such as *S. aureus, Clostridium difficile*, *E. coli*, and *P. aeruginosa*; 4) develop vaccines able to prevent or delay cancer; 5) develop vaccines against HIV, TB, malaria, and other infections of developing countries such as typhoid and nontyphoidal *Salmonella* and *E. coli*; 6) prepare better vaccines against emerging infectious diseases such as pandemic influenza; 7) improve therapy of cancer, chronic infectious diseases, and allergies; and 8) treat metabolic diseases such as diabetes, obesity, etc.

In conclusion, we expect that in the 21st century, vaccines will be able to 1) take care of the remaining infant infectious diseases; 2) provide vaccines for all ages, including adolescents, adults, the elderly, and special groups such as travelers and patients with chronic diseases; 3) mitigate the effect of emerging infectious diseases; and 4) help to eliminate poverty from low-income countries by providing the vaccines that will eliminate the burden of disease and help to develop an healthy economy.

REFERENCES

1. Schneerson R, Barrera O, Sutton A, et al. Preparation, characterization, and immunogenicity of Haemophilus influenzae type b polysaccharideprotein conjugates. *J Exp Med*. 1980;152:361-376.

2. Rappuoli R. Rational design of vaccines. *Nat Med*. 1997;3:374-376.

3. Sabin AB, Hennessen WA, Winsser J. Studies on variants of poliomyelitis virus. I. Experimental segregation and properties of avirulent variants of three immunologic types. *J Exp Med*. 1954;99:551-576.

4. McAleer WJ, Buynak EB, Maigetter RZ, et al. Human hepatitis B vaccine from recombinant yeast. *Nature*. 1984;307:178-180.

5. Scolnick EM, McLean AA, West DJ, et al. Clinical evaluation in healthy adults of a hepatitis B vaccine made by recombinant DNA. *JAMA*. 1984;251:2812-2815.

6. Tron F, Degos F, Brechot C, et al. Randomized dose range study of a recombinant hepatitis B vaccine produced in mammalian cells and containing the S and PreS2 sequences. *J Infect Dis*. 1989;160:199-204.

7. Bernstein DI, Sahly HM, Keitel WA, et al. Safety and immunogenicity of a candidate parvovirus B19 vaccine. *Vaccine*. 2011;29(43):7357-7363.

8. Landry N, Ward BJ, Trepanier S, et al. Preclinical and clinical development of plant-made virus-like particle vaccine against avian H5N1 influenza. *PLoS One*. 2010;5:e15559.

9. Mihailova M, Boos M, Petrovskis I, et al. Recombinant virus-like particles as a carrier of B- and T-cell epitopes of hepatitis C virus (HCV). *Vaccine*. 2006;24:4369-4377.

10. Peters BS, Cheingsong-Popov R, Callow D, et al. A pilot phase II study of the safety and immunogenicity of HIV p17/p24:VLP (p24-VLP) in asymptomatic HIV seropositive subjects. *J Infect*. 1997;35:231-235.

11. Nardin EH, Oliveira GA, Calvo-Calle JM, et al. Phase I testing of a malaria vaccine composed of hepatitis B virus core particles expressing Plasmodium falciparum circumsporozoite epitopes. *Infect Immun*. 2004;72:6519-6527.

12. Cornuz J, Zwahlen S, Jungi WF, et al. A vaccine against nicotine for smoking cessation: a randomized controlled trial. *PLoS One*. 2008;3:e2547.

13. Macleod CM, Hodges RG, Heidelberger M, et al. Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides. *J Exp Med*. 1945;82:445-465.

14. Kalies H, Grote V, Verstraeten T, et al. The use of combination vaccines has improved timeliness of vaccination in children. *Pediatr Infect Dis J* . 2006;25:507-512.

15. Marshall GS, Happe LE, Lunacsek OE, et al. Use of combination vaccines is associated with improved coverage rates. *Pediatr Infect Dis J* . 2007;26:496-500.

16. Ockenhouse CF, Sun PF, Lanar DE, et al. Phase I/IIa safety, immunogenicity, and efficacy trial of NYVAC-Pf7, a pox-vectored, multiantigen, multistage vaccine candidate for Plasmodium falciparum malaria. *J Infect Dis*. 1998;177:1664-1673.

17. Currier JR, Ngauy V, de Souza MS, et al. Phase I safety and immunogenicity evaluation of MVA-CMDR, a multigenic, recombinant modified vaccinia Ankara-HIV-1 vaccine candidate. *PLoS One*. 2010;5:e13983.

18. Berthoud TK, Hamill M, Lillie PJ, et al. Potent CD8+ T-cell immunogenicity in humans of a novel heterosubtypic influenza A vaccine, MVA-NP+M1. *Clin Infect Dis*. 2011;52:1-7.

19. Paoletti E. Applications of pox virus vectors to vaccination: an update. *Proc Natl Acad Sci U S A*. 1996;93:11349-11353.

20. Sullivan NJ, Geisbert TW, Geisbert JB, et al. Immune protection of nonhuman primates against Ebola virus with single low-dose adenovirus vectors encoding modified GPs. *PLoS Med*. 2006;3:e177.

21. Rodrigues EG, Zavala F, Eichinger D, et al. Single immunizing dose of recombinant adenovirus efficiently induces CD8+ T cell-mediated protective immunity against malaria. *J Immunol* . 1997;158:1268-1274.

22. Shiver JW, Fu TM, Chen L, et al. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature*. 2002;415:331-335.

23. Aucouturier J, Dupuis L, Deville S, et al. Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines. *Expert Rev Vaccines*. 2002;1:111-118.

24. Zaiss AK, Machado HB, Herschman HR. The influence of innate and pre-existing immunity on adenovirus therapy. *J Cell Biochem*. 2009;108:778-790.

25. Lemckert AA, Sumida SM, Holterman L, et al. Immunogenicity of heterologous prime-boost regimens involving recombinant adenovirus serotype 11 (Ad11) and Ad35 vaccine vectors in the presence of anti-ad5 immunity. *J Virol* . 2005;79:9694-9701.

26. McCoy K, Tatsis N, Korioth-Schmitz B, et al. Effect of preexisting immunity to adenovirus human serotype 5 antigens on the immune responses of nonhuman primates to vaccine regimens based on human-or chimpanzee-derived adenovirus vectors. *J Virol* . 2007;81:6594-6604.

27. Folgori A, Capone S, Ruggeri L, et al. A T-cell HCV vaccine eliciting effective immunity against heterologous virus challenge in chimpanzees. *Nat Med*. 2006;12:190-197.

28. Patel A, Tikoo S, Kobinger G. A porcine adenovirus with low human seroprevalence is a promising alternative vaccine vector to human adenovirus 5 in an H5N1 virus disease model. *PLoS One*. 2010;5:e15301.

29. Perri S, Greer CE, Thudium K, et al. An alphavirus replicon particle chimera derived from Venezuelan equine encephalitis and Sindbis viruses is a potent gene-based vaccine delivery vector. *J Virol*. 2003;77:10394-10403.

30. Lin Y, Kwon T, Polo J, et al. Induction of broad CD4+ and CD8+ T-cell responses and cross-neutralizing antibodies against hepatitis C virus by vaccination with Th1-adjuvanted polypeptides followed by defective alphaviral particles expressing envelope glycoproteins gpE1 and gpE2 and nonstructural proteins 3, 4, and 5. *J Virol*. 2008;82:7492-7503.

31. Pan CH, Greer CE, Hauer D, et al. A chimeric alphavirus replicon particle vaccine expressing the hemagglutinin and fusion proteins protects juvenile and infant rhesus macaques from measles. *J Virol*. 2010;84:3798-3807.

32. Reap EA, Dryga SA, Morris J, et al. Cellular and humoral immune responses to alphavirus replicon vaccines expressing cytomegalovirus pp65, IE1, and gB proteins. *Clin Vacc Immunol*. 2007;14:748-755.

33. Barnett SW, Burke B, Sun Y, et al. Antibody-mediated protection against mucosal simian-human immunodeficiency virus challenge of macaques immunized with alphavirus replicon particles and boosted with trimeric envelope glycoprotein in MF59 adjuvant. *J Virol*. 2010; 84:5975-5985.

34. Ranasinghe C, Ramshaw IA. Genetic heterologous prime boost vaccination strategies for improved systemic and mucosal immunity. *Exp Rev Vaccines*. 2009;8:1171-1181.

35. Wolff JA, Malone RW, Williams P, et al. Direct gene transfer into mouse muscle in vivo. *Science*. 1990;247:1465-1468.

36. Cox GJ, Zamb TJ, Babiuk LA. Bovine herpesvirus 1: immune responses in mice and cattle injected with plasmid DNA. *J Virol*. 1993;67:5664-5667.

37. Tang DC, DeVit M, Johnston SA. Genetic immunization is a simple method for eliciting an immune response. *Nature*. 1992;356:152-154.

38. Ulmer JB, Donnelly JJ, Parker SE, et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science*. 1993;259:1745-1749.

39. Sato Y, Roman M, Tighe H, et al. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science*. 1996;273:352-354.

40. Babiuk S, Mookherjee N, Pontarollo R, et al. TLR9-/- and TLR9+/+ mice display similar immune responses to a DNA vaccine. *Immunol*. 2004;113:114-120.

41. Ishii KJ, Kawagoe T, Koyama S, et al. TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. *Nature*. 2008;451:725-729.

42. Spies B, Hochrein H, Vabulas M, et al. Vaccination with plasmid DNA activates dendritic cells via toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice. *J Immunol*. 2003;171:5908-5912.

43. Stetson DB, Medzhitov R. Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. *Immunity*. 2006;24:93-103.

44. Dupuis M, Denis-Mize K, Woo C, et al. Distribution of DNA vaccines determines their immunogenicity after intramuscular injection in mice. *J Immunol*. 2000;165:2850-2858.

45. Ulmer JB, Otten GR. Priming of CTL responses by DNA vaccines: direct transfection of antigen presenting cells versus cross-priming. *Dev Biol (Basel)*. 2000;104:9-14.

46. Ulmer JB, Deck RR, DeWitt CM, et al. Expression of a viral protein by muscle cells in vivo induces protective cell-mediated immunity. *Vaccine*. 1997;15:839-841.

47. Fu TM, Ulmer JB, Caulfield MJ, et al. Priming of cytotoxic T lymphocytes by DNA vaccines: requirement for professional antigen presenting cells and evidence for antigen transfer from myocytes. *Mol Med*. 1997;3:362-371.

48. Letvin NL, Montefiori DC, Yasutomi Y, et al. Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination. *Proc Natl Acad Sci U S A*. 1997;94:9378-9383.

49. Davis HL, McCluskie MJ, Gerin JL, et al. DNA vaccine for hepatitis B: evidence for immunogenicity in chimpanzees and comparison with other vaccines. *Proc Natl Acad Sci U S A*. 1996;93:7213-7218.

50. Osorio JE, Tomlinson CC, Frank RS, et al. Immunization of dogs and cats with a DNA vaccine against rabies virus. *Vaccine*. 1999;17:1109-1116.

51. MacGregor RR, Ginsberg R, Ugen KE, et al. T-cell responses induced in normal volunteers immunized with a DNA-based vaccine containing HIV-1 env and rev. *AIDS*. 2002;16:2137-2143.

52. Le TP, Coonan KM, Hedstrom RC, et al. Safety, tolerability and humoral immune responses after intramuscular administration of a malaria DNA vaccine to healthy adult volunteers. *Vaccine*. 2000;18:1893-1901.

53. O'Hagan D, Singh M, Ugozzoli M, et al. Induction of potent immune responses by cationic microparticles with adsorbed human immunodeficiency virus DNA vaccines. *J Virol*. 2001;75:9037-9043.

54. Roy MJ, Wu MS, Barr LJ, et al. Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. *Vaccine*. 2000;19:764-778.

55. Mumper RJ, Cui Z. Genetic immunization by jet injection of targeted pDNA-coated nanoparticles. *Methods*. 2003;31:255-262.

56. Otten G, Schaefer M, Doe B, et al. Enhancement of DNA vaccine potency in rhesus macaques by electroporation. *Vaccine*. 2004;22:2489-2493.

57. Schulz M, Zinkernagel RM, Hengartner H. Peptide-induced antiviral protection by cytotoxic T cells. *Proc Natl Acad Sci U S A* . 1991;88:991-993.

58. Feltkamp MC, Smits HL, Vierboom MP, et al. Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. *Eur J Immunol* . 1993;23:2242-2249.

59. Rosenberg SA, Yang JC, Schwartzentruber DJ, et al. Immunologic and therapeutic

evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat Med*. 1998;4:321-327.

60. Fraser CK, Diener KR, Brown MP, et al. Improving vaccines by incorporating immunological coadjuvants. *Expert Rev Vaccines*. 2007;6: 559-578.

61. Hoebe K, Janssen E, Beutler B. The interface between innate and adaptive immunity. *Nat Immunol*. 2004;5:971-974.

62. De Gregorio E, D'Oro U, Wack A. Immunology of TLR-independent vaccine adjuvants. *Curr Opin Immunol* . 2009;21:339-345.

63. O'Hagan DT, Valiante NM, Recent advances in the discovery and delivery of vaccine adjuvants. *Nat Rev Drug Discov* . 2003;2:727-735.

64. Plotkin S, Orenstein WA, Offit P. *Vaccines*. 5th ed. New York, NY: Saunders-Elsevier; 2008.

65. De Gregorio E, Tritto E, Rappuoli R. Alum adjuvanticity: unraveling a century old mystery. *Eur J Immunol* . 2008;38:2068-2071.

66. Lambrecht BN, Kool M, Willart MA, et al. Mechanism of action of clinically approved adjuvants. *Curr Opin Immunol* . 2009;21(1):23-29.

67. Marrack P, McKee AS, Munks MW. Towards an understanding of the adjuvant action of aluminium. *Nat Rev Immunol* . 2009;9(4):287-293.

68. Brewer JM. (How) do aluminium adjuvants work? *Immunol Lett*. 2006;102:10-15.

69. Hem SL, Hogenesch H. Relationship between physical and chemical properties of aluminum-containing adjuvants and immunopotentiation. *Expert Rev Vaccines*. 2007;6:685-698.

70. HogenEsch H, Mechanisms of stimulation of the immune response by aluminum adjuvants. *Vaccine*. 2002;20(suppl 3):S34-S39.

71. Morefield GL, Sokolovska A, Jiang D, et al. Role of aluminumcontaining adjuvants in antigen internalization by dendritic cells in vitro. *Vaccine*. 2005;23:1588-1595.

72. Shi Y, Flach TL, Ng G, et al. Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity. *Nat Med*. 2011;17:479-487.

73. Goto N, Kato H, Maeyama J, et al. Local tissue irritating effects and adjuvant activities of calcium phosphate and aluminium hydroxide with different physical properties. *Vaccine*. 1997;15:1364-1371.

74. Goto N, Akama K. Histopathological studies of reactions in mice injected with aluminum-adsorbed tetanus toxoid. *Microbiol Immunol*. 1982;26:1121-1132.

75. Kool M, Petrilli V, De Smedt T, et al. Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. *J Immunol*. 2008;181:3755-3759.

76. Franchi L, Nunez G. The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1beta secretion but dispensable for adjuvant activity. *Eur J Immunol* . 2008;38:2085-2089.

77. Eisenbarth SC, Colegio OR, O'Connor W, et al. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature*. 2008;453:1122-1126.

78. Petrilli V, Dostert C, Muruve DA, et al. The inflammasome: a danger sensing complex triggering innate immunity. *Curr Opin Immunol* . 2007;19:615-622.

79. Hornung V, Bauernfeind F, Halle A, et al. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* . 2008;9:847-856.

80. Li H, Willingham SB, Ting JP, et al. Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3. *J Immunol* . 2008;181:17-21.

81. McKee AS, Munks MW, MacLeod MK, et al. Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity. *J Immunol* . 2009;183:4403-4414.

82. Seubert A, Calabro S, Santini L, et al. Adjuvanticity of the oil-in-water emulsion MF59 is independent of Nlrp3 inflammasome but requires the adaptor protein MyD88. *Proc Natl Acad Sci U S A*. 2011;108:11169-11174.

83. Marichal T, Ohata K, Bedoret D, et al. DNA released from dying host cells mediates aluminum adjuvant activity. *Nat Med*. 2011;17:996-1002.

84. Podda A. The adjuvanted influenza vaccines with novel adjuvants: experience with the MF59-adjuvanted vaccine. *Vaccine*. 2001;19:2673-2680.

85. Clark TW, Pareek M, Hoschler K, et al. Trial of 2009 influenza A (H1N1) monovalent MF59-adjuvanted vaccine. *N Engl J Med* . 2009; 361:2424-2435.

86. Vogel FR, Caillet C, Kusters IC, et al. Emulsion-based adjuvants for influenza vaccines. *Expert Rev Vaccines*. 2009;8:483-492.

87. Leroux-Roels I, Leroux-Roels G. Current status and progress of prepandemic and pandemic influenza vaccine development. *Expert Rev Vaccines*. 2009;8:401-423.

88. Banzhoff A, Gasparini R, Laghi-Pasini F, et al. MF59-adjuvanted H5N1 vaccine induces immunologic memory and heterotypic antibody responses in non-elderly and elderly adults. *PLoS One*. 2009;4:e4384.

89. Schwarz TF, Horacek T, Knuf M, et al. Single dose vaccination with AS03-adjuvanted H5N1 vaccines in a randomized trial induces strong and broad immune responsiveness to booster vaccination in adults. *Vaccine*. 2009;27:6284-6290.

90. Dormitzer PR, Galli G, Castellino F, et al. Influenza vaccine immunology. *Immunol Rev*. 2011;239:167-177.

91. Khurana S, Chearwae W, Castellino F, et al. Vaccines with MF59 adjuvant expand the antibody repertoire to target protective sites of pandemic avian H5N1 influenza virus. *Sci Transl Med*. 2010;2: 15ra5.

92. Khurana S, Verma N, Yewdell JW, et al. MF59 adjuvant enhances diversity and affinity of antibody-mediated immune response to pandemic influenza vaccines. *Sci Transl Med*. 2011;3:85ra48.

93. Galli G, Hancock K, Hoschler K, et al. Fast rise of broadly cross-reactive antibodies

after boosting long-lived human memory B cells primed by an MF59 adjuvanted prepandemic vaccine. *Proc Natl Acad Sci U S A* . 2009;106:7962-7967.

94. Leroux-Roels I, Roman F, Forgus S, et al. Priming with AS03 A-adjuvanted H5N1 influenza vaccine improves the kinetics, magnitude and durability of the immune response after a heterologous booster vaccination: an open non-randomised extension of a doubleblind randomised primary study. *Vaccine*. 2010;28:849-857.

95. Vesikari T, Knuf M, Wutzler P, et al. Oil-in-water emulsion adjuvant with influenza vaccine in young children. *N Engl J Med* . 2011;365:1406-1416.

96. Dupuis M, McDonald DM, Ott G. Distribution of adjuvant MF59 and antigen gD2 after intramuscular injection in mice. *Vaccine*. 1999;18:434-439.

97. Dupuis M, Murphy TJ, Higgins D, et al. Dendritic cells internalize vaccine adjuvant after intramuscular injection. *Cell Immunol* . 1998;186:18-27.

98. Seubert A, Monaci E, Pizza M, et al. The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells. *J Immunol* . 2008;180:5402-5412.

99. Dupuis M, Denis-Mize K, LaBarbara A, et al. Immunization with the adjuvant MF59 induces macrophage trafficking and apoptosis. *Eur J Immunol* . 2001;31:2910-2918.

100. Mosca F, Tritto E, Muzzi A, et al. Molecular and cellular signatures of human vaccine adjuvants. *Proc Natl Acad Sci U S A* . 2008;105:10501-10506.

101. Calabro S, Tortoli M, Baudner BC, et al. Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes. *Vaccine*. 2011;29(9):1812-1823.

102. Morel S, Didierlaurent A, Bourguignon P, et al. Adjuvant system AS03 containing alpha-tocopherol modulates innate immune response and leads to improved adaptive immunity. *Vaccine*. 2011;29:2461-2473.

103. Ellebedy AH, Lupfer C, Ghoneim HE, et al. Inflammasomeindependent role of the apoptosis-associated speck-like protein containing CARD (ASC) in the adjuvant effect of MF59. *Proc Natl Acad Sci U S A* . 2011;108:2927-2932.

104. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* . 2004;5:987-995.

105. Koyama S, Coban C, Aoshi T, et al. Innate immune control of nucleic acid-based vaccine immunogenicity. *Expert Rev Vaccines*. 2009;8:1099-1107.

106. Steinman RM, Banchereau J. Taking dendritic cells into medicine. *Nature*. 2007;449:419-426.

107. Querec T, Bennouna S, Alkan S, et al. Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. *J Exp Med*. 2006;203:413-424.

108. Tong NK, Beran J, Kee SA, et al. Immunogenicity and safety of an adjuvanted hepatitis B vaccine in pre-hemodialysis and hemodialysis patients. *Kidney Int*. 2005;68:2298-2303.

109. Giannini SL, Hanon E, Moris P, et al. Enhanced humoral and memory B cellular

immunity using HPV16/18 L1 VLP vaccine formulated with the MPL/aluminium salt combination (AS04) compared to aluminium salt only. *Vaccine*. 2006;24:5937-5949.

110. Einstein MH, Baron M, Levin MJ, et al. Comparison of the immunogenicity and safety of Cervarix and Gardasil human papillomavirus (HPV) cervical cancer vaccines in healthy women aged 18-45 years. *Hum Vaccin*. 2009;5:705-719.

111. Harper DM. Currently approved prophylactic HPV vaccines. *Expert Rev Vaccines*. 2009;8:1663-1679.

112. Didierlaurent AM, Morel S, Lockman L, et al. AS04, an aluminum salt-and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. *J Immunol*. 2009;183:6186-6197.

113. Fox CB, Friede M, Reed SG, et al. Synthetic and natural TLR4 agonists as safe and effective vaccine adjuvants. *Subcell Biochem*. 2010;53:303-321.

114. Dupont J, Altclas J, Lepetic A, et al. A controlled clinical trial comparing the safety and immunogenicity of a new adjuvanted hepatitis B vaccine with a standard hepatitis B vaccine. *Vaccine*. 2006;24:7167-7174.

115. Moser C, Amacker M, Zurbriggen R. Influenza virosomes as a vaccine adjuvant and carrier system. *Expert Rev Vaccines*. 2011;10:437-446.

116. Steinhagen F, Kinjo T, Bode C, et al. TLR-based immune adjuvants. *Vaccine*. 2010;29(17):3341-3355.

117. Steere AC, Sikand VK, Meurice F, et al. Vaccination against Lyme disease with recombinant Borrelia burgdorferi outer-surface lipoprotein A with adjuvant. Lyme Disease Vaccine Study Group. *N Engl J Med*. 1998;339:209-215.

118. Longhi MP, Trumpfheller C, Idoyaga J, et al. Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. *J Exp Med*. 2009;206:1589-1602.

119. Tewari K, Flynn BJ, Boscardin SB, et al. Poly(I:C) is an effective adjuvant for antibody and multi-functional CD4+ T cell responses to Plasmodium falciparum circumsporozoite protein (CSP) and alphaDEC-CSP in non human primates. *Vaccine*. 2010;28:7256-7266.

120. Huleatt JW, Nakaar V, Desai P, et al. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. *Vaccine*. 2008;26:201-214.

121. Taylor DN, Treanor JJ, Strout C, et al. Induction of a potent immune response in the elderly using the TLR-5 agonist, flagellin, with a recombinant hemagglutinin influenza-flagellin fusion vaccine (VAX125, STF2.HA1 SI). *Vaccine*. 2011;29:4897-4902.

122. Wille-Reece U, Flynn BJ, Lore K, et al. HIV Gag protein conjugated to a toll-like receptor 7/8 agonist improves the magnitude and quality of Th1 and CD8+ T cell responses in nonhuman primates. *Proc Natl Acad Sci U S A*. 2005;102:15190-15194.

123. Wille-Reece U, Wu CY, Flynn BJ, et al. Immunization with HIV-1 Gag protein conjugated to a TLR7/8 agonist results in the generation of HIV-1 Gag-specific Th1 and CD8+ T cell responses. *J Immunol*. 2005;174:7676-7683.

124. Adams S, O'Neill DW, Nonaka D, et al. Immunization of malignant melanoma patients

with full-length NY-ESO-1 protein using TLR7 agonist imiquimod as vaccine adjuvant. *J Immunol*. 2008;181:776-784.

125. Krieg AM. Therapeutic potential of toll-like receptor 9 activation. *Nat Rev Drug Discov*. 2006;5:471-484.

126. Barry M, Cooper C. Review of hepatitis B surface antigen-1018 ISS adjuvant-containing vaccine safety and efficacy. *Expert Opin Biol Ther* . 2007;7:1731-1737.

127. Schellack C, Prinz K, Egyed A, et al. IC31, a novel adjuvant signaling via TLR9, induces potent cellular and humoral immune responses. *Vaccine*. 2006;24:5461-5472.

128. Schijns VE. Mechanisms of vaccine adjuvant activity: initiation and regulation of immune responses by vaccine adjuvants. *Vaccine*. 2003;21:829-831.

129. van Dissel JT, Arend SM, Prins C, et al. Ag85B-ESAT-6 adjuvanted with IC31 promotes strong and long-lived Mycobacterium tuberculosis specific T cell responses in naive human volunteers. *Vaccine*. 2010;28:3571-3581.

130. Kensil CR, Patel U, Lennick M, et al. Separation and characterization of saponins with adjuvant activity from Quillaja saponaria Molina cortex. *J Immunol* . 1991;146:431-437.

131. Garcon N, Chomez P, Van Mechelen M. GlaxoSmithKline adjuvant systems in vaccines: concepts, achievements and perspectives. *Expert Rev Vaccines*. 2007;6:723-739.

132. Sun HX, Xie Y, Ye YP. ISCOMs and ISCOMATRIX. *Vaccine*. 2009;27:4388-4401.

133. Drane D, Gittleson C, Boyle J, et al. ISCOMATRIX adjuvant for prophylactic and therapeutic vaccines. *Expert Rev Vaccines*. 2007;6:761-772.

134. Mischo A, Bubel N, Cebon JS, et al. Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad antibody responses in humans, a RAYS-based analysis. *Int J Oncol* . 2011;39:287-294.

135. Schnurr M, Orban M, Robson NC, et al. ISCOMATRIX adjuvant induces efficient cross-presentation of tumor antigen by dendritic cells via rapid cytosolic antigen delivery and processing via tripeptidyl peptidase II. *J Immunol* . 2009;182:1253-1259.

136. Maraskovsky E, Schnurr M, Wilson NS, et al. Development of prophylactic and therapeutic vaccines using the ISCOMATRIX adjuvant. *Immunol Cell Biol* . 2009;87:371-376.

137. Duewell P, Kisser U, Heckelsmiller K, et al. ISCOMATRIX adjuvant combines immune activation with antigen delivery to dendritic cells in vivo leading to effective cross-priming of CD8+ T cells. *J Immunol* . 2011;187:55-63.

138. Drane D, Maraskovsky E, Gibson R, et al. Priming of CD4+ and CD8+ T cell responses using a HCV core ISCOMATRIX vaccine: a phase I study in healthy volunteers. *Hum Vaccin*. 2009;5:151-157.

139. Aucouturier J, Dupuis L, Deville S, et al. Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines. *Expert Rev Vaccines*. 2002;1:111-118.

140. Alonso PL, Sacarlal J, Aponte JJ, et al. Efficacy of the RTS,S/AS02A vaccine

against Plasmodium falciparum infection and disease in young African children: randomised controlled trial. *Lancet*. 2004;364:1411-1420.

141. Lell B, Agnandji S, von Glasenapp I, et al. A randomized trial assessing the safety and immunogenicity of AS01 and AS02 adjuvanted RTS,S malaria vaccine candidates in children in Gabon. *PLoS One*. 2009;4:e7611.

142. Atanackovic D, Altorki NK, Cao Y, et al. Booster vaccination of cancer patients with MAGE-A3 protein reveals long-term immunological memory or tolerance depending on priming. *Proc Natl Acad Sci U S A* . 2008;105:1650-1655.

143. Napolitani G, Rinaldi A, Bertoni F, et al. Selected toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol* . 2005;6:769-776.

144. Zhu Q, Egelston C, Vivekanandhan A, et al. Toll-like receptor ligands synergize through distinct dendritic cell pathways to induce T cell responses: implications for vaccines. *Proc Natl Acad Sci U S A* . 2008;105:16260-16265.

145. Kasturi SP, Skountzou I, Albrecht RA, et al. Programming the magnitude and persistence of antibody responses with innate immunity. *Nature*. 2011;470:543-547.

146. Chen K, Cerutti A. Vaccination strategies to promote mucosal antibody responses. *Immunity*. 2010;33:479-491.

147. Lawson LB, Norton EB, Clements JD. Defending the mucosa: adjuvant and carrier formulations for mucosal immunity. *Curr Opin Immunol* . 2011;23:414-420.

148. Rappuoli R, Pizza M, Douce G, et al. Structure and mucosal adjuvanticity of cholera and Escherichia coli heat-labile enterotoxins. *Immunol Today*. 1999;20:493-500.

149. Mutsch M, Zhou W, Rhodes P, et al. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *N Engl J Med* . 2004;350:896-903.

150. Douce G, Fontana M, Pizza M, et al. Intranasal immunogenicity and adjuvanticity of site-directed mutant derivatives of cholera toxin. *Infect Immun*. 1997;65:2821-2828.

151. Pizza M, Fontana MR, Giuliani MM, et al. A genetically detoxified derivative of heat-labile Escherichia coli enterotoxin induces neutralizing antibodies against the A subunit. *J Exp Med*. 1994;180:2147-2153.

152. Stephenson I, Zambon MC, Rudin A, et al. Phase I evaluation of intranasal trivalent inactivated influenza vaccine with nontoxigenic Escherichia coli enterotoxin and novel biovector as mucosal adjuvants, using adult volunteers. *J Virol* . 2006;80:4962-4970.

153. Lewis DJ, Huo Z, Barnett S, et al. Transient facial nerve paralysis (Bell's palsy) following intranasal delivery of a genetically detoxified mutant of Escherichia coli heat labile toxin. *PLoS One*. 2009;4:e6999.

154. Frech SA, Dupont HL, Bourgeois AL, et al. Use of a patch containing heat-labile toxin from Escherichia coli against travellers' diarrhoea: a phase II, randomised, double-blind, placebo-controlled field trial. *Lancet*. 2008;371:2019-2025.

155. Glenn GM, Thomas DN, Poffenberger KL, et al. Safety and immunogenicity of an influenza vaccine A/H5N1 (A/Vietnam/1194/2004) when coadministered with a heat-labile enterotoxin (LT) adjuvant patch. *Vaccine*. 2009;27(suppl 6):G60-G66.

156. Tritto E, Muzzi A, Pesce I, et al. The acquired immune response to the mucosal adjuvant LTK63 imprints the mouse lung with a protective signature. *J Immunol*. 2007;179:5346-5357.

157. Brereton CF, Sutton CE, Ross PJ, et al. Escherichia coli heat-labile enterotoxin promotes protective Th17 responses against infection by driving innate IL-1 and IL-23 production. *J Immunol*. 2011;186:5896-5906.

158. Czerkinsky C, Cuburu N, Kweon MN, et al. Sublingual vaccination. *Hum Vaccin*. 2011;7:110-114.

159. Francis T Jr, Korns RF, Voight RB, et al. An evaluation of the 1954 poliomyelitis vaccine trials. *Am J Public Health Nations Health*. 1955;45:1-63.

160. Bottiger M. Polio immunity to killed vaccine: an 18-year follow-up. *Vaccine*. 1990;8:443-445.

161. World Health Organization. *Global Polio Eradication Initiative*. Available from: http://www.polioeradication.org/.

162. Szymczakiewicz-Multanowska A, Groth N, Bugarini R, et al. Safety and immunogenicity of a novel influenza subunit vaccine produced in mammalian cell culture. *J Infect Dis*. 2009;200:841-848.

163. Gasparini R, Pozzi T, Montomoli E, et al. Increased immunogenicity of the MF59-adjuvanted influenza vaccine compared to a conventional subunit vaccine in elderly subjects. *Eur J Epidemiol* . 2001;17:135-140.

164. Iob A, Brianti G, Zamparo E, et al. Evidence of increased clinical protection of an MF59-adjuvant influenza vaccine compared to a nonadjuvant vaccine among elderly residents of long-term care facilities in Italy. *Epidemiol Infect*. 2005;133:687-693.

165. Herzog C, Metcalfe IC, Schaad UB. Virosome influenza vaccine in children. *Vaccine*. 2002;20(suppl 5):B24-B28.

166. Maassab HF, DeBorde DC. Development and characterization of cold-adapted viruses for use as live virus vaccines. *Vaccine*. 1985;3:355-369.

167. Hobson D, Curry RL, Beare AS, et al. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *J Hyg (Lond)*. 1972;70:767-777.

168. de Jong JC, Palache AM, Beyer WE, et al. Haemagglutination-inhibiting antibody to influenza virus. *Dev Biol (Basel)*. 2003;115:63-73.

169. Ferguson NM, Cummings DA, Fraser C, et al. Strategies for mitigating an influenza pandemic. *Nature*. 2006;442:448-452.

170. Feinstone SM, Kapikian AZ, Purceli RH. Hepatitis A: detection by immune electron microscopy of a viruslike antigen associated with acute illness. *Science*. 1973;182:1026-1028.

171. Mascoli CC, Ittensohn OL, Villarejos VM, et al. Recovery of hepatitis agents in the marmoset from human cases occurring in Costa Rica. *Proc Soc Exp Biol Med*. 1973;142:276-282.

172. Provost PJ, Hilleman MR. An inactivated hepatitis A virus vaccine prepared from

infected marmoset liver. *Proc Soc Exp Biol Med*. 1978;159:201-203.

173. Werzberger A, Mensch B, Kuter B, et al. A controlled trial of a formalin-inactivated hepatitis A vaccine in healthy children. *N Engl J Med*. 1992;327:453-457.

174. Zamir C, Rishpon S, Zamir D, et al. Control of a community-wide outbreak of hepatitis A by mass vaccination with inactivated hepatitis A vaccine. *Eur J Clin Microbiol Infect Dis*. 2001;20:185-187.

175. Stapleton JT, Raina V, Winokur PL, et al. Antigenic and immunogenic properties of recombinant hepatitis A virus 14S and 70S subviral particles. *J Virol*. 1993;67:1080-1085.

176. Buynak EB, Roehm RR, Tytell AA, et al. Vaccine against human hepatitis B. *JAMA*. 1976;235:2832-2834.

177. Chang MH, You SL, Chen CJ, et al. Decreased incidence of hepatocellular carcinoma in hepatitis B vaccinees: a 20-year follow-up study. *J Natl Cancer Inst* . 2009;101:1348-1355.

178. Enders JF, Peebles TC. Propagation in tissue cultures of cytopathogenic agents from patients with measles. *Proc Soc Exp Biol Med*. 1954;86: 277-286.

179. Schwarz AJ. Immunization against measles: development and evaluation of a highly attenuated live measles vaccine. *Ann Paediatr*. 1964;202:241-252.

180. Hilleman MR, Buynak EB, Weibel RE, et al. Development and evaluation of the Moraten measles virus vaccine. *JAMA*. 1968;206:587-590.

181. Buynak EB, Hilleman MR. Live attenuated mumps virus vaccine. 1. Vaccine development. *Proc Soc Exp Biol Med*. 1966;123:768-775.

182. Hilleman MR, Weibel RE, Buynak EB, et al. Live attenuated mumpsvirus vaccine. IV. Protective efficacy as measured in a field evaluation. *N Engl J Med*. 1967;276:252-258.

183. Meyer HM Jr, Parkman PD, Hobbins TE, et al. Clinical studies with experimental live rubella virus vaccine (strain HPV-77). Evaluation of vaccine-induced immunity. *Am J Dis Child*. 1968;115:648-654.

184. Plotkin SA, Farquhar JD, Katz M, et al. Attenuation of RA 27-3 rubella virus in WI-38 human diploid cells. *Am J Dis Child*. 1969;118:178-185.

185. Prinzie A, Huygelen C, Gold J, et al. Experimental live attenuated rubella virus vaccine. Clinical evaluation of Cendehill strain. *Am J Dis Child*. 1969;118:172-177.

186. Halstead SB, Diwan, AR. Failure to transmit rubella virus vaccine. A close-contact study in adults. *JAMA*. 1971;215:634-636.

187. Takahashi M, Otsuka T, Okuno Y, et al. Live vaccine used to prevent the spread of varicella in children in hospital. *Lancet*. 1974;2: 1288-1290.

188. Takahashi M, Okuno Y, Otsuka T, et al. Development of a live attenuated varicella vaccine. *Biken J*. 1975;18:25-33.

189. White CJ, Kuter BJ, Hildebrand CS, et al. Varicella vaccine (VARIVAX) in healthy children and adolescents: results from clinical trials, 1987 to 1989. *Pediatrics*. 1991;87:604-610.

190. Krause PR, Klinman DM. Efficacy, immunogenicity, safety, and use of live attenuated chickenpox vaccine. *J Pediatr* . 1995;127:518-525.

191. Kuter B, Matthews H, Shinefield H, et al. Ten year follow-up of healthy children who received one or two injections of varicella vaccine. *Pediatr Infect Dis J* . 2004;23:132-137.

192. Chiba S, Yokoyama T, Nakata S, et al. Protective effect of naturally acquired homotypic and heterotypic rotavirus antibodies. *Lancet*. 1986;2:417-421.

193. Velazquez FR, Matson DO, Calva JJ, et al. Rotavirus infections in infants as protection against subsequent infections. *N Engl J Med* . 1996;335:1022-1028.

194. Foster RH, Wagstaff AJ. Tetravalent human-rhesus reassortant rotavirus vaccine: a review of its immunogenicity, tolerability and protective efficacy against paediatric rotavirus gastroenteritis. *BioDrugs*. 1998;9:155-178.

195. Ciarlet M, Schödel F. Development of a rotavirus vaccine: clinical safety, immunogenicity, and efficacy of the pentavalent rotavirus vaccine, RotaTeq®. *Vaccine*. 2009;27(suppl 6):G72-G81.

196. Green KY, Taniguchi K, Mackow ER, et al. Homotypic and heterotypic epitope-specific antibody responses in adult and infant rotavirus vaccinees: implications for vaccine development. *J Infect Dis*. 1990;161:667-679.

197. Ruiz-Palacios GM, Perez-Schael I, Velazquez FR, et al. Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis. *N Engl J Med* . 2006;354:11-22.

198. Bell JA, Sundberg JP, Ghim SJ, et al. A formalin-inactivated vaccine protects against mucosal papillomavirus infection: a canine model. *Pathobiology*. 1994;62:194-198.

199. Jarrett WF, O'Neil BW, Gaukroger JM, et al. Studies on vaccination against papillomaviruses: the immunity after infection and vaccination with bovine papillomaviruses of different types. *Vet Rec*. 1990;126: 473-475.

200. Kirnbauer R, Booy F, Cheng N, et al. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc Natl Acad Sci U S A*. 1992;89:12180-12184.

201. Schiller JT, Castellsague X, Villa LL, et al. An update of prophylactic human papillomavirus L1 virus-like particle vaccine clinical trial results. *Vaccine*. 2008;26(suppl 10):K53-K61.

202. Giannini G, Rappuoli R, Ratti G. The amino-acid sequence of two nontoxic mutants of diphtheria toxin: CRM45 and CRM197. *Nucleic Acids Res*. 1984;12:4063-4069.

203. Kanai K. Japan's experience in pertussis epidemiology and vaccination in the past thirty years. *Jpn J Med Sci Biol* . 1980;33:107-143.

204. Granoff DM, Anderson EL, Osterholm MT, et al. Differences in the immunogenicity of three Haemophilus influenzae type b conjugate vaccines in infants. *J Pediatr* . 1992;121:187-194.

205. Scheifele DW, Halperin SA, Guasparini R, et al. Extended follow-up of antibody levels and antigen responsiveness after 2 Haemophilus influenzae type b conjugate vaccines. *J Pediatr* . 1999;135:240-245.

206. Austrian R, Douglas RM, Schiffman S, et al. Prevention of pneumococcal pneumonia by vaccination. *Trans Assoc Am Physicians*. 1976;89:184-194.

207. Smit P, Oberholzer D, Hayden-Smith S, et al. Protective efficacy of pneumococcal polysaccharide vaccines. *JAMA*. 1977;238:2613-2616.

208. Black S, Shinefield H, Fireman B, et al. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. *Pediatr Infect Dis J* . 2000;19:187-195.

209. Centers for Disease Control and Prevention. Direct and indirect effects of routine vaccination of children with 7-valent pneumococcal conjugate vaccine on incidence of invasive pneumococcal disease—United States, 1998-2003. *MMWR Morb Mortal Wkly Rep*. 2005;54:893-897.

210. Frenck R Jr, Thompson A, Yeh SH, et al. Immunogenicity and safety of 13-valent pneumococcal conjugate vaccine in children previously immunized with 7-valent pneumococcal conjugate vaccine. *Pediatr Infect Dis J* . 2011;30(12):1086-1091.

211. Vesikari T, Wysocki J, Chevallier B, et al. Immunogenicity of the 10-valent pneumococcal non-typeable Haemophilus influenzae protein D conjugate vaccine (PHiD-CV) compared to the licensed 7vCRM vaccine. *Pediatr Infect Dis J* . 2009;28:S66-S76.

212. Gianfaldoni C, Censini S, Hilleringmann M, et al. Streptococcus pneumoniae pilus subunits protect mice against lethal challenge. *Infect Immun*. 2007;75:1059-1062.

213. Giefing C, Meinke AL, Hanner M, et al. Discovery of a novel class of highly conserved vaccine antigens using genomic scale antigenic fingerprinting of pneumococcus with human antibodies. *J Exp Med*. 2008;205:117-131.

214. Godfroid F, Hermand P, Verlant V, et al. Preclinical evaluation of the Pht proteins as potential cross-protective pneumococcal vaccine antigens. *Infect Immun*. 2011;79:238-245.

215. Stephens DS. Uncloaking the meningococcus: dynamics of carriage and disease. *Lancet*. 1999;353:941-942.

216. Cohn AC, MacNeil JR, Harrison LH, et al. Changes in Neisseria meningitidis disease epidemiology in the United States, 1998-2007: implications for prevention of meningococcal disease. *Clin Infect Dis*. 2010;50:184-191.

217. Goldschneider I, Gotschlich EC, Artenstein MS. Human immunity to the meningococcus. I. The role of humoral antibodies. *J Exp Med*. 1969;129:1307-1326.

218. Gotschlich EC, Rey M, Triau R, et al. Quantitative determination of the human immune response to immunization with meningococcal vaccines. *J Clin Invest*. 1972;51:89-96.

219. Gotschlich EC, Goldschneider I, Artenstein MS. Human immunity to the meningococcus. IV. Immunogenicity of group A and group C meningococcal polysaccharides in human volunteers. *J Exp Med*. 1969;129:1367-1384.

220. Campbell H, Borrow R, Salisbury D, et al. Meningococcal C conjugate vaccine: the experience in England and Wales. *Vaccine*. 2009;27(suppl 2):B20-B29.

221. Keyserling H, Papa T, Koranyi K, et al. Safety, immunogenicity, and immune memory of a novel meningococcal (groups A, C, Y, and W-135) polysaccharide diphtheria toxoid

conjugate vaccine (MCV-4) in healthy adolescents. *Arch Pediatr Adolesc Med* . 2005;159: 907-913.

222. Granoff DM, Harris SL. Protective activity of group C anticapsular antibodies elicited in two-year-olds by an investigational quadrivalent Neisseria meningitidis-diphtheria toxoid conjugate vaccine. *Pediatr Infect Dis J* . 2004;23:490-497.

223. Granoff DM, Morgan A, Welsch JA. Persistence of group C anticapsular antibodies two to three years after immunization with an investigational quadrivalent Neisseria meningitidis-diphtheria toxoid conjugate vaccine. *Pediatr Infect Dis J* . 2005;24:132-136.

224. Halperin SA, Gupta A, Jeanfreau R, et al. Comparison of the safety and immunogenicity of an investigational and a licensed quadrivalent meningococcal conjugate vaccine in children 2-10 years of age. *Vaccine*. 2010;28:7865-7872.

225. Gill CJ, Baxter R, Anemona A, et al. Persistence of immune responses after a single dose of Novartis meningococcal serogroup A, C, W-135 and Y CRM-197 conjugate vaccine (Menveo(R)) or Menactra(R) among healthy adolescents. *Hum Vaccin*. 2010;6:881-887.

226. Snape MD, Perrett KP, Ford KJ, et al. Immunogenicity of a tetravalent meningococcal glycoconjugate vaccine in infants: a randomized controlled trial. *JAMA*. 2008;299:173-184.

227. Tondella ML, Popovic T, Rosenstein NE, et al. Distribution of Neisseria meningitidis serogroup B serosubtypes and serotypes circulating in the United States. The Active Bacterial Core Surveillance Team. *J Clin Microbiol* . 2000;38:3323-3328.

228. Pizza M, Scarlato V, Masignani V, et al. Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. *Science*. 2000;287:1816-1820.

229. Giuliani MM, Adu-Bobie J, Comanducci M, et al. A universal vaccine for serogroup B meningococcus. *Proc Natl Acad Sci U S A* . 2006;103:10834-10839.

230. Findlow J, Borrow R, Snape MD, et al. Multicenter, open-label, randomized phase II controlled trial of an investigational recombinant Meningococcal serogroup B vaccine with and without outer membrane vesicles, administered in infancy. *Clin Infect Dis*. 2010;51: 1127-1137.

231. Levine MM, Dougan G, Good MF, et al. *New Generation Vaccines*. 4th ed. New York, NY: Informa Healthcare USA, Inc; 2010.

232. Garcia PM, Kalish LA, Pitt J, et al. Maternal levels of plasma human immunodeficiency virus type 1 RNA and the risk of perinatal transmission. Women and Infants Transmission Study Group. *N Engl J Med* . 1999;341:394-402.

233. Mayaux MJ, Dussaix E, Isopet J, et al. Maternal virus load during pregnancy and mother-to-child transmission of human immunodeficiency virus type 1: the French perinatal cohort studies. SEROGEST Cohort Group. *J Infect Dis*. 1997;175:172-175.

234. Mellors JW, Rinaldo CR Jr, Gupta P, et al. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science*. 1996;272: 1167-1170.

235. Quinn TC, Wawer MJ, Sewankambo N, et al. Viral load and heterosexual transmission of human immunodeficiency virus type 1. Rakai Project Study Group. *N Engl J Med*. 2000;342:921-929.

236. McMichael AJ. HIV vaccines. *Annu Rev Immunol*. 2006;24:227-255.

237. Betts MR, Nason MC, West SM, et al. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood*. 2006;107:4781-4789.

238. Parren PW, Marx PA, Hessell AJ, et al. Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. *J Virol*. 2001;75:8340-8347.

239. Kwong PD, Mascola JR, Nabel GJ. Rational design of vaccines to elicit broadly neutralizing antibodies to HIV-1. *Cold Spring Harb Perspect Biol* . 2011;3:a007278.

240. Flynn NM, Forthal DN, Harro CD, et al. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. *J Infect Dis*. 2005;191:654-665.

241. Pitisuttithum P, Gilbert P, Gurwith M, et al. Randomized, doubleblind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. *J Infect Dis*. 2006;194:1661-1671.

242. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, et al. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med*. 2009;361:2209-2220.

243. Karnasuta C, Paris RM, Cox JH, et al. Antibody-dependent cellmediated cytotoxic responses in participants enrolled in a phase I/II ALVAC-HIV/AIDSVAX B/E prime-boost HIV-1 vaccine trial in Thailand. *Vaccine*. 2005;23:2522-2529.

244. Belshe RB, Gorse GJ, Mulligan MJ, et al. Induction of immune responses to HIV-1 by canarypox virus (ALVAC) HIV-1 and gp120 SF-2 recombinant vaccines in uninfected volunteers. NIAID AIDS Vaccine Evaluation Group. *AIDS*. 1998;12:2407-2415.

245. Buchbinder SP, Mehrotra DV, Duerr A, et al. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a doubleblind, randomised, placebo-controlled, test-of-concept trial. *Lancet*. 2008;372:1881-1893.

246. Catanzaro AT, Koup RA, Roederer M, et al. Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 candidate vaccine delivered by a replication-defective recombinant adenovirus vector. *J Infect Dis*. 2006;194:1638-1649.

247. Roberts DM, Nanda A, Havenga MJ, et al. Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. *Nature*. 2006;441:239-243.

248. Precopio ML, Betts MR, Parrino J, et al. Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. *J Exp Med*. 2007;204:1405-1416.

249. Sandstrom E, Nilsson C, Hejdeman B, et al. Broad immunogenicity of a multigene, multiclade HIV-1 DNA vaccine boosted with heterologous HIV-1 recombinant modified vaccinia virus Ankara. *J Infect Dis*. 2008;198:1482-1490.

250. Bernstein DI, Plotkin SA. Cytomegalovirus vaccines. In: Levine MM, ed. *New Generation Vaccines*. New York, NY: Informa Healthcare USA, Inc; 2009:631-645.

251. Plotkin SA, Farquhar J, Horberger E. Clinical trials of immunization with the Towne 125 strain of human cytomegalovirus. *J Infect Dis*. 1976;134:470-475.

252. Plotkin SA, Starr SE, Friedman HM, et al. Protective effects of Towne

cytomegalovirus vaccine against low-passage cytomegalovirus administered as a challenge. *J Infect Dis*. 1989;159:860-865.

253. Plotkin SA, Higgins R, Kurtz JB, et al. Multicenter trial of Towne strain attenuated virus vaccine in seronegative renal transplant recipients. *Transplantation*. 1994;58:1176-1178.

254. Plotkin SA, Smiley ML, Friedman HM, et al. Towne-vaccine-induced prevention of cytomegalovirus disease after renal transplants. *Lancet*. 1984;1:528-530.

255. Adler SP, Starr SE, Plotkin SA, et al. Immunity induced by primary human cytomegalovirus infection protects against secondary infection among women of childbearing age. *J Infect Dis*. 1995;171:26-32.

256. Jacobson MA, Sinclair E, Bredt B, et al. Safety and immunogenicity of Towne cytomegalovirus vaccine with or without adjuvant recombinant interleukin-12. *Vaccine*. 2006;24:5311-5319.

257. Heineman TC, Schleiss M, Bernstein DI, et al. A phase 1 study of 4 live, recombinant human cytomegalovirus Towne/Toledo chimeric vaccines. *J Infect Dis*. 2006;193:1350-1360.

258. Frey SE, Harrison C, Pass RF, et al. Effects of antigen dose and immunization regimens on antibody responses to a cytomegalovirus glycoprotein B subunit vaccine. *J Infect Dis*. 1999;180:1700-1703.

259. Pass RF, Duliege AM, Boppana S, et al. A subunit cytomegalovirus vaccine based on recombinant envelope glycoprotein B and a new adjuvant. *J Infect Dis*. 1999;180:970-975.

260. Mitchell DK, Holmes SJ, Burke RL, et al. Immunogenicity of a recombinant human cytomegalovirus gB vaccine in seronegative toddlers. *Pediatr Infect Dis J* . 2002;21:133-138.

261. Pass RF. Development and evidence for efficacy of CMV glycoprotein B vaccine with MF59 adjuvant. *J Clin Virol*. 2009;46(Suppl 4):S73-S76.

262. Bernstein DI, Reap EA, Katen K, et al. Randomized, double-blind, phase 1 trial of an alphavirus replicon vaccine for cytomegalovirus in CMV seronegative adult volunteers. *Vaccine*. 2009;28:484-493.

263. Wloch MK, Smith LR, Boutsaboualoy S, et al. Safety and immunogenicity of a bivalent cytomegalovirus DNA vaccine in healthy adult subjects. *J Infect Dis*. 2008;197:1634-1642.

264. Jacobson MA, Adler SP, Sinclair E, et al. A CMV DNA vaccine primes for memory immune responses to live-attenuated CMV (Towne strain). *Vaccine*. 2009;27:1540-1548.

265. Adler SP, Plotkin SA, Gonczol E, et al. A canarypox vector expressing cytomegalovirus (CMV) glycoprotein B primes for antibody responses to a live attenuated CMV vaccine (Towne). *J Infect Dis*. 1999;180:843-846.

266. Flynn JL, Chan J. Immunology of tuberculosis. *Annu Rev Immunol* . 2001;19:93-129.

267. Boshoff HI, Barry CE III. Tuberculosis—metabolism and respiration in the absence of growth. *Nat Rev Microbiol* . 2005;3:70-80.

268. Calmette A, Guérin C, Boquet A, et al. *La vaccination préventive contre la tuberculose par le "BCG."* Paris, France: Masson et Cie; 1927.

269. Behr MA, Wilson MA, Gill WP, et al. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science*. 1999;284: 1520-1523.

270. Andersen P, Doherty TM. The success and failure of BCG—implications for a novel tuberculosis vaccine. *Nat Rev Microbiol* . 2005;3: 656-662.

271. Colditz GA, Berkey CS, Mosteller F, et al. The efficacy of bacillus Calmette-Guérin vaccination of newborns and infants in the prevention of tuberculosis: meta-analyses of the published literature. *Pediatrics*. 1995;96:29-35.

272. Fifteen year follow up of trial of BCG vaccines in south India for tuberculosis prevention. Tuberculosis Research Centre (ICMR), Chennai. *Indian J Med Res*. 1999;110:56-69.

273. Karonga Prevention Trial Group. Randomised controlled trial of single BCG, repeated BCG, or combined BCG and killed Mycobacterium leprae vaccine for prevention of leprosy and tuberculosis in Malawi. Karonga Prevention Trial Group. *Lancet*. 1996;348:17-24.

274. Basaraba RJ, Izzo AA, Brandt L, et al. Decreased survival of guinea pigs infected with Mycobacterium tuberculosis after multiple BCG vaccinations. *Vaccine*. 2006;24:280-286.

275. Kubit S, Czajka S, Olakowski T, et al. Effectiveness of BCG vaccination [in Polish]. *Pediatr Pol* . 1983;58:775-781.

276. Leung CC, Tam CM, Chan SL, et al. Efficacy of the BCG revaccination programme in a cohort given BCG vaccination at birth in Hong Kong. *Int J Tuberc Lung Dis* . 2001;5:717-723.

277. Sepulveda RL, Parcha C, Sorensen RU. Case-control study of the efficacy of BCG immunization against pulmonary tuberculosis in young adults in Santiago, Chile. *Tuber Lung Dis*. 1992;73:372-377.

278. Tala-Heikkila MM, Tuominen JE, Tala EO. Bacillus Calmette-Guérin revaccination questionable with low tuberculosis incidence. *Am J Respir Crit Care Med* . 1998;157:1324-1327.

279. Horwitz MA, Andersen P, Kaufmann SH. Novel vaccines against tuberculosis. In: Levine MM, ed. *New Generation Vaccines*. New York, NY: Informa Healthcare USA, Inc; 2009:516-531.

280. Hoft DF, Blazevic A, Abate G, et al. A new recombinant bacille Calmette-Guerin vaccine safely induces significantly enhanced tuberculosis-specific immunity in human volunteers. *J Infect Dis*. 2008;198:1491-1501.

281. Cardona PJ, Asensio JG, Arbues A, et al. Extended safety studies of the attenuated live tuberculosis vaccine SO2 based on phoP mutant. *Vaccine*. 2009;27:2499-2505.

282. Martin C, Williams A, Hernandez-Pando R, et al. The live Mycobacterium tuberculosis phoP mutant strain is more attenuated than BCG and confers protective immunity against tuberculosis in mice and guinea pigs. *Vaccine*. 2006;24:3408-3419.

283. Orme IM. Preclinical testing of new vaccines for tuberculosis: a comprehensive

review. *Vaccine*. 2006;24:2-19.

284. Reed S, Lobet Y. Tuberculosis vaccine development; from mouse to man. *Microbes Infect*. 2005;7:922-931.

285. Bertholet S, Ireton GC, Kahn M, et al. Identification of human T cell antigens for the development of vaccines against Mycobacterium tuberculosis. *J Immunol*. 2008;181:7948-7957.

286. Von Eschen K, Morrison R, Braun M, et al. The candidate tuberculosis vaccine Mtb72F/AS02A: tolerability and immunogenicity in humans. *Hum Vaccin*. 2009;5:475-482.

287. Leroux-Roels I, Leroux-Roels G, Ofori-Anyinam O, et al. Evaluation of the safety and immunogenicity of two antigen concentrations of the Mtb72F/AS02(A) candidate tuberculosis vaccine in purified protein derivative-negative adults. *Clin Vaccine Immunol*. 2010;17:1763-1771.

288. Bertholet S, Ireton GC, Ordway DJ, et al. A defined tuberculosis vaccine candidate boosts BCG and protects against multidrug-resistant Mycobacterium tuberculosis. *Sci Transl Med*. 2010;2:53ra74.

289. Aagaard C, Hoang T, Dietrich J, et al. A multistage tuberculosis vaccine that confers efficient protection before and after exposure. *Nat Med*. 2011;17:189-194.

290. McShane H, Pathan AA, Sander CR, et al. Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. *Nat Med*. 2004;10:1240-1244.

291. Pathan AA, Sander CR, Fletcher HA, et al. Boosting BCG with recombinant modified vaccinia ankara expressing antigen 85A: different boosting intervals and implications for efficacy trials. *PLoS One*. 2007;2:e1052.

292. Hawkridge T, Scriba TJ, Gelderbloem S, et al. Safety and immunogenicity of a new tuberculosis vaccine, MVA85A, in healthy adults in South Africa. *J Infect Dis*. 2008;198:544-552.

293. Brookes RH, Hill PC, Owiafe PK, et al. Safety and immunogenicity of the candidate tuberculosis vaccine MVA85A in West Africa. *PLoS One*. 2008;3:e2921.

294. Sander CR, Pathan AA, Beveridge NE, et al. Safety and immunogenicity of a new tuberculosis vaccine, MVA85A, in Mycobacterium tuberculosis-infected individuals. *Am J Respir Crit Care Med*. 2009;179:724-733.

295. Scriba TJ, Tameris M, Mansoor N, et al. Modified vaccinia Ankaraexpressing Ag85A, a novel tuberculosis vaccine, is safe in adolescents and children, and induces polyfunctional CD4+ T cells. *Eur J Immunol* . 2010;40:279-290.

296. Scriba TJ, Tameris M, Mansoor N, et al. Dose-finding study of the novel tuberculosis vaccine, MVA85A, in healthy BCG-vaccinated infants. *J Infect Dis*. 2011;203:1832-1843.

297. Beveridge NE, Price DA, Casazza JP, et al. Immunisation with BCG and recombinant MVA85A induces long-lasting, polyfunctional Mycobacterium tuberculosis-specific CD4+ memory T lymphocyte populations. *Eur J Immunol* . 2007;37:3089-3100.

298. Abel B, Tameris M, Mansoor N, et al. The novel tuberculosis vaccine, AERAS-402, induces robust and polyfunctional CD4+ and CD8+ T cells in adults. *Am J Respir Crit Care Med*. 2010;181:1407-1417.

299. Dlugovitzky D, Stanford C, Stanford J. Immunological basis for the introduction of immunotherapy with Mycobacterium vaccae into the routine treatment of TB. *Immunotherapy*. 2011;3:557-568.

300. Dlugovitzky D, Fiorenza G, Farroni M, et al. Immunological consequences of three doses of heat-killed Mycobacterium vaccae in the immunotherapy of tuberculosis. *Respir Med*. 2006;100:1079-1087.

301. Stanford J, Stanford C, Grange J. Immunotherapy with Mycobacterium vaccae in the treatment of tuberculosis. *Front Biosci* . 2004;9:1701-1719.

302. Vilaplana C, Montane E, Pinto S, et al. Double-blind, randomized, placebo-controlled phase I clinical trial of the therapeutical antituberculous vaccine RUTI. *Vaccine*. 2010;28:1106-1116.

303. Klevens RM, Edwards JR, Tenover FC, et al. Changes in the epidemiology of methicillin-resistant Staphylococcus aureus in intensive care units in US hospitals, 1992-2003. *Clin Infect Dis*. 2006;42:389-391.

304. Mangiaracine AB, Goodale RL. Clinical observations on the use of autogenous vaccine made from cultures of coagulase positive hemolytic Staphylococcus aureus. *Laryngoscope*. 1952;62:299-310.

305. Salmon GG Jr, Symonds M. Staphage lysate therapy in chronic staphylococcal infections. *J Med Soc N J* . 1963;60:188-193.

306. Greenberg L, Le Riche WH. Staphylococcal enzyme lysed soluble vaccine. *Can J Public Health*. 1961;52:479-485.

307. Bryant RE, Sanford JP, Alcoze T Treatment of recurrent furunculosis with staphylococcal bacteriophage-lysed vaccine. *JAMA*. 1965;194: 11-14.

308. Poole-Warren LA, Hallett MD, Hone PW, et al. Vaccination for prevention of CAPD associated staphylococcal infection: results of a prospective multicentre clinical trial. *Clin Nephrol* . 1991;35:198-206.

309. Harro C, Betts R, Orenstein W, et al. Safety and immunogenicity of a novel Staphylococcus aureus vaccine: results from the first study of the vaccine dose range in humans. *Clin Vaccine Immunol* . 2010;17: 1868-1874.

310. Fattom A, Fuller S, Propst M, et al. Safety and immunogenicity of a booster dose of Staphylococcus aureus types 5 and 8 capsular polysaccharide conjugate vaccine (StaphVAX) in hemodialysis patients. *Vaccine*. 2004;23:656-663.

311. Shinefield H, Black S, Fattom A, et al. Use of a Staphylococcus aureus conjugate vaccine in patients receiving hemodialysis. *N Engl J Med* . 2002;346:491-496.

312. Gardner MJ, Hall N, Fung E, et al. Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature*. 2002;419:498-511.

313. Doolan DL, Martinez-Alier N. Immune response to pre-erythrocytic stages of malaria parasites. *Curr Mol Med* . 2006;6:169-185.

314. Marsh K, Kinyanjui S. Immune effector mechanisms in malaria. *Parasite Immunol* . 2006;28:51-60.

315. Clyde DF. Immunity to falciparum and vivax malaria induced by irradiated

sporozoites: a review of the University of Maryland studies, 1971-75. *Bull World Health Organ*. 1990;68(suppl):9-12.

316. Herrington D, Davis J, Nardin E, et al. Successful immunization of humans with irradiated malaria sporozoites: humoral and cellular responses of the protected individuals. *Am J Trop Med Hyg* . 1991;45:539-547.

317. Hoffman SL, Goh LM, Luke TC, et al. Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites. *J Infect Dis*. 2002;185:1155-1164.

318. Rieckmann KH. Human immunization with attenuated sporozoites. *Bull World Health Organ*. 1990;68(suppl):13-6.

319. Egan JE, Hoffman SL, Haynes JD, et al. Humoral immune responses in volunteers immunized with irradiated Plasmodium falciparum sporozoites. *Am J Trop Med Hyg* . 1993;49:166-173.

320. Kester KE, Cummings JF, Ofori-Anyinam O, et al. Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. *J Infect Dis*. 2009;200:337-346.

321. Kester KE, McKinney DA, Tornieporth N, et al. Efficacy of recombinant circumsporozoite protein vaccine regimens against experimental Plasmodium falciparum malaria. *J Infect Dis*. 2001;183:640-647.

322. Agnandji ST, Lell B, Soulanoudjinhgar SS, et al. First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. *N Engl J Med* . 2011;365:1863-1875.

323. Graves P, Gelband H. Vaccines for preventing malaria (pre-erythrocytic). *Cochrane Database Syst Rev*. 2006:CD006198.

324. Aponte JJ, Aide P, Renom M, et al. Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial. *Lancet*. 2007;370:1543-1551.

325. Asante KP, Abdulla S, Agnandji S, et al. Safety and efficacy of the RTS,S/AS01(E) candidate malaria vaccine given with expanded-programmeon-immunisation vaccines: 19 month follow-up of a randomised, openlabel, phase 2 trial. *Lancet Infect Dis*. 2011;11:741-749.

326. Olotu A, Lusingu J, Leach A, et al. Efficacy of RTS,S/AS01E malaria vaccine and exploratory analysis on anti-circumsporozoite antibody titres and protection in children aged 5-17 months in Kenya and Tanzania: a randomised controlled trial. *Lancet Infect Dis*. 2011;11:102-109.

327. Ansong D, Asante KP, Vekemans J, et al. T cell responses to the RTS,S/AS01(E) and RTS,S/AS02(D) malaria candidate vaccines administered according to different schedules to Ghanaian children. *PLoS One*. 2011;6:e18891.

328. Cummings JF, Spring MD, Schwenk RJ, et al. Recombinant Liver Stage Antigen-1 (LSA-1) formulated with AS01 or AS02 is safe, elicits high titer antibody and induces IFN-gamma/IL-2 CD4+ T cells but does not protect against experimental Plasmodium falciparum infection. *Vaccine*. 2010;28:5135-5144.

329. Sagara I, Dicko A, Ellis RD, et al. A randomized controlled phase 2 trial of the blood stage AMA1-C1/Alhydrogel malaria vaccine in children in Mali. *Vaccine*. 2009;27:3090-

3098.

330. Duncan CJ, Sheehy SH, Ewer KJ, et al. Impact on malaria parasite multiplication rates in infected volunteers of the protein-in-adjuvant vaccine AMA1-C1/Alhydrogel+CPG 7909. *PLoS One*. 2011;6: e22271.

331. Thera MA, Doumbo OK, Coulibaly D, et al. A field trial to assess a blood-stage malaria vaccine. *N Engl J Med* . 2011;365:1004-1013.

332. Wu Y, Ellis RD, Shaffer D, et al. Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51. *PLoS One*. 2008;3:e2636.

333. McConkey SJ, Reece WH, Moorthy VS, et al. Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. *Nat Med*. 2003;9:729-735.

334. Wang R, Richie TL, Baraceros MF, et al. Boosting of DNA vaccineelicited gamma interferon responses in humans by exposure to malaria parasites. *Infect Immun*. 2005;73:2863-2872.

335. Moorthy VS, Imoukhuede EB, Milligan P, et al. A randomised, double-blind, controlled vaccine efficacy trial of DNA/MVA METRAP against malaria infection in Gambian adults. *PLoS Med*. 2004; 1:e33.

336. Dunachie SJ, Walther M, Epstein JE, et al. A DNA prime-modified vaccinia virus Ankara boost vaccine encoding thrombospondin-related adhesion protein but not circumsporozoite protein partially protects healthy malaria-naive adults against Plasmodium falciparum sporozoite challenge. *Infect Immun*. 2006;74:5933-5942.

337. Webster DP, Dunachie S, Vuola JM, et al. Enhanced T cell-mediated protection against malaria in human challenges by using the recombinant poxviruses FP9 and modified vaccinia virus Ankara. *Proc Natl Acad Sci U S A* . 2005;102:4836-4841.

338. Bejon P, Mwacharo J, Kai O, et al. A phase 2b randomised trial of the candidate malaria vaccines FP9 ME-TRAP and MVA ME-TRAP among children in Kenya. *PLoS Clin Trials*. 2006;1:e29.

339. Bejon P, Ogada E, Mwangi T, et al. Extended follow-up following a phase 2b randomized trial of the candidate malaria vaccines FP9 METRAP and MVA ME-TRAP among children in Kenya. *PLoS One*. 2007; 2:e707.

340. Koufman Z, Egoz N, Greenblatt CL, et al. Observations on immunization against cutaneous leishmaniasis in Israel. *Isr J Med Sci* . 1978;14:218-222.

341. Nadim A, Javadian E, Tahvildar-Bidruni G, et al. Effectiveness of leishmanization in the control of cutaneous leishmaniasis. *Bull Soc Pathol Exot Filiales*. 1983;76:377-383.

342. Genaro O, de Toledo VP, da Costa CA, et al. Vaccine for prophylaxis and immunotherapy, Brazil. *Clin Dermatol*. 1996;14:503-512.

343. Velez ID, Gilchrist K, Arbelaez MP, et al. Failure of a killed Leishmania amazonensis vaccine against American cutaneous leishmaniasis in Colombia. *Trans R Soc Trop Med Hyg*. 2005;99:593-598.

344. Armijos RX, Weigel MM, Aviles H, et al. Field trial of a vaccine against New World cutaneous leishmaniasis in an at-risk child population: safety, immunogenicity, and

efficacy during the first 12 months of follow-up. *J Infect Dis*. 1998;177:1352-1357.

345. Velez ID, del Pilar Agudelo S, Arbelaez MP, et al., Safety and immunogenicity of a killed Leishmania (L.) amazonensis vaccine against cutaneous leishmaniasis in Colombia: a randomized controlled trial. *Trans R Soc Trop Med Hyg*. 2000;94:698-703.

346. Convit J, Castellanos PL, Rondon A, et al. Immunotherapy versus chemotherapy in localised cutaneous leishmaniasis. *Lancet*. 1987;1: 401-405.

347. Convit J, Castellanos PL, Ulrich M, et al. Immunotherapy of localized, intermediate, and diffuse forms of American cutaneous leishmaniasis. *J Infect Dis*. 1989;160:104-115.

348. Convit J, Ulrich M, Polegre MA, et al. Therapy of Venezuelan patients with severe mucocutaneous or early lesions of diffuse cutaneous leishmaniasis with a vaccine containing pasteurized Leishmania promastigotes and bacillus Calmette-Guérin: preliminary report. *Mem Inst Oswaldo Cruz*. 2004;99:57-62.

349. Convit J, Ulrich M, Zerpa O, et al. Immunotherapy of American cutaneous leishmaniasis in Venezuela during the period 1990-99. *Trans R Soc Trop Med Hyg*. 2003;97:469-472.

350. Bahar K, Dowlati Y, Shidani B, et al. Comparative safety and immunogenicity trial of two killed Leishmania major vaccines with or without BCG in human volunteers. *Clin Dermatol*. 1996;14:489-495.

351. Khalil EA, El Hassan AM, Zijlstra EE, et al. Autoclaved Leishmania major vaccine for prevention of visceral leishmaniasis: a randomised, doubleblind, BCG-controlled trial in Sudan. *Lancet*. 2000;356:1565-1569.

352. Momeni AZ, Jalayer T, Emamjomeh M, et al. A randomised, doubleblind, controlled trial of a killed L. major vaccine plus BCG against zoonotic cutaneous leishmaniasis in Iran. *Vaccine*. 1999;17:466-472.

353. SharifiI, FeKri AR, Aflatonian MR, et al. Randomised vaccine trial of single dose of killed Leishmania major plus BCG against anthroponotic cutaneous leishmaniasis in Bam, Iran. *Lancet*. 1998;351: 1540-1543.

354. Noazin S, Khamesipour A, Moulton LH, et al. Efficacy of killed wholeparasite vaccines in the prevention of leishmaniasis: a meta-analysis. *Vaccine*. 2009;27:4747-4753.

355. Kamil AA, Khalil EA, Musa AM, et al. Alum-precipitated autoclaved Leishmania major plus bacille Calmette-Guérin, a candidate vaccine for visceral leishmaniasis: safety, skin-delayed type hypersensitivity response and dose finding in healthy volunteers. *Trans R Soc Trop Med Hyg*. 2003;97:365-368.

356. Khalil EA, Musa AM, Modabber F, et al. Safety and immunogenicity of a candidate vaccine for visceral leishmaniasis (alum-precipitated autoclaved Leishmania major + BCG) in children: an extended phase II study. *Ann Trop Paediatr*. 2006;26:357-361.

357. Modabber F, Coler RN, Reed SG. Vaccines against Leishmania. In: Levine MM, ed. *New Generation Vaccines*. New York, NY: Informa Healthcare USA, Inc; 2009:790-807.

358. Chakravarty J, Kumar S, Trivedi S, et al. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1+MPL-SE vaccine for use in the prevention of visceral leishmaniasis. *Vaccine*. 2011;29: 3531-3537.

359. Llanos-Cuentas A, Calderon W, Cruz M, et al. A clinical trial to evaluate the safety

and immunogenicity of the LEISH-F1+MPL-SE vaccine when used in combination with sodium stibogluconate for the treatment of mucosal leishmaniasis. *Vaccine*. 2010;28:7427-7435.

360. Nascimento E, Fernandes DF, Vieira EP, et al. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1+MPL-SE vaccine when used in combination with meglumine antimoniate for the treatment of cutaneous leishmaniasis. *Vaccine*. 2010;28:6581-6587.

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Chapter 44

Autoimmunity and Autoimmune Diseases

Ken T. Coppieters

Matthias G. von Herrath

Dirk Homann

INTRODUCTION

The concept of autoimmunity (ie, the phenomenon of immune reactivity directed against an organism's constituent organs, tissues, cells, and/or extracellular factors) comprises both physiologic and pathophysiologic components that attain practical relevance in the context of autoimmune diseases, a collection of more than 80 disorders with circumscribed pathologic features that are thought to result primarily from the induction and perpetuation of aberrant immune reponses. The precise etiopathology for most human autoimmune diseases remains incompletely defined but appears to be promoted by the complex interplay of genetic predispositions, environmental insults such as viral infections and, in an acknowledgment of the many questions yet to be answered, bad luck. However, a role for immune-mediated processes and pathologies has by now been documented in at times compelling detail and provides important direction for novel and improved diagnostic, prophylactic, and therapeutic modalities. Herein, we discuss the evolution of major conceptual and practical advances and challenges in autoimmunity and autoimmune diseases in general, and consider individual autoimmune disorders regarding the contribution of genetic and environmental components, specific pathology and autoimmune features, experimental models and ongoing research efforts, as well as current and potential future therapeutics.

GENERAL CONCEPTS IN AUTOIMMUNITY

Immunity and Autoimmunity

> *"… weil, so schliesst er messerscharf, nicht sein kann, was nicht sein darf"*
>
> *("… for, he reasons pointedly, that which must not, can not be")*

—Christian Morgenstern, *The Impossible Fact* (1910)

Autoimmunity is "the Other"[1] of immunity. And in true dialectical fashion, immunity's inception as a scientific discipline encapsulates the conceptual problem that was to shape the immunologic debates in the first 15 and last 50 years of the past century. As much as the prefix *auto* assigns a specific place apart from immunity proper, the idea of immunity is not

conceivable, for better or worse, without the notion of the self.[2] Immunity's distinct association with the individual self stretches from its etymologic roots in the Roman legal concept of an *individual's* exemption from duty, service, or tax; to its official induction into the canon of medical terminology (defined as “idiosyncratic condition” in the 1878 edition of Littré's Dictionnaire de Médecine[3]); and beyond. However, with the dawn of a new century, in the wake of the seminal discoveries of immune protection by active immunization with attenuated pathogens[4] or passive transfer of convalescent serum[5] and the seemingly unstoppable success of the “New Immunology,” the notion of the immunologic self underwent a dramatic reconfiguration. The price for immunity, it appeared, was autoimmunity, a concept so problematic that its existence had to be relegated to the realm of the almost unspeakable, that is, the Greco-Latin neologism of a “horror autotoxicus.”[6] Paradoxically, the time that witnessed the concept of horror autotoxicus acquire quasi-dogmatic status as the “law of immunity research”[7] also became what A. Silverstein[8] has termed the “classical period” of autoimmunity research. Though declared anathema, autoimmune phenomena were reported in a quick succession of widely publicized observations. In 1902, Portier and Richet[9] reported the phenomenon of anaphylaxis (as opposed to *pro*-phylaxis); Maurice Arthus[10] characterized the local inflammatory response that was to bear his name in 1903. Donath and Landsteiner[11] described the first human autoimmune disease (paroxysmal cold hemoglobinuria) in 1904, and the term *allergy* (“altered reactivity”) was coined by von Pirquet and Schick[12] in their analysis of serum sickness in 1905. The

idea that tissue destruction may lead to expanded immunopathology (nowadays referred to as “determinant spreading”) was proposed by Weil and Braun in 1907: In the course of an infectious disease, “tissue destruction results in the generation of antibodies that […] will be directed not only against the degraded products of the destroyed cells but also against human cell products as such. […] These autoantibodies could attack cells, [and] liberate antigen which in turn could induce the generation of autoantibodies.”[13] Even the concepts of organ specificity,[14] immunoprivilege, and a breakdown of regulatory mechanisms as a cause for autoimmunity[7] were developed in the early days of autoimmunity research.

This period of extraordinary productivity was followed by an almost 40-year hiatus, “the dark ages of autoimmunity research.”[8] The reasons for a generalized disinterest in autoimmune phenomena are manifold and include political reconfigurations after World War I, the death of both Ehrlich (1915) and Metchnikoff (1916), a misconception of horror autotoxicus as the immune system's *inability* to generate responses against “self,” as well as a paradigm shift in the field of immunology in favor of immunochemical approaches.[8] The renaissance of autoimmunity research had to await observations about immunologic tolerance of mice congenitally infected with lymphocytic choriomeningitis virus (LCMV),[15] description of tolerance in chimeric cattle twins,[16] and Peter Medawar's work on skin transplant rejection,[17,18,19,20] as well as the integration of these findings into a conceptual framework of self and nonself as determinants for immunologic reactivity.[21] Burnet subsequently developed and extended these ideas into the “clonal selection theory of antibody formation, ”[22] thereby establishing the conceptual centrality of self, nonself, and immunologic tolerance.

Although the dogmatic reading of horror autotoxicus was still prevalent (Ernest Witebsky delayed his publication about thyroid antibodies[23] for several years assuming an experimental error[8]), Ehrlich's original conception as “regulatory contrivances” that prevent autoimmunity was now validated within the context of the clonal selection theory.

Again, however, the usefulness of self and nonself as distinguishing parameters was challenged at the very time they began their rise to prominence. Ludwik Fleck, in his singular study *Genesis and Development of a Scientific Fact* , questioned the capacity of an immune system that only interacts with structures that are strictly nonself: “… it is very doubtful whether an invasion in the old sense is possible, involving as it does an inference by completely foreign organisms in natural conditions. A completely foreign organism could find no receptors capable of reaction and thus could not generate a biological process.”[24] This view is echoed and elaborated upon in the work of Jerne[25] and Coutinho et al.,[26] and even the possibility of a beneficial role for autoimmune processes was postulated by concepts such as “physiologic autoimmunity,”[27] “positive autoimmunity,”[28] and “protective autoimmunity.”[29] The importance of autoreactivity as an integral aspect of immunity is furthermore demonstrated by the process of “positive selection” of developing T cells in the thymus and the T cell-mediated destruction of transformed or infected tissues that is based on the recognition of “foreign” (eg, viral peptides or even self as in the case of some tumorderived antigens) in the context of self (major histocompatibility complexes [MHCs]). In fact, events associated with “danger” or the preservation of “tissue integrity” rather than the discrimination between “self/nonself” have been postulated as a primary driving force that engages the immune system.[30,31] More recently, the notion that immune reactivity is generated by the introduction of strong antigenic discontinuities (“criterion of continuity”) has been proposed and may offer an elegant solution to some of the conceptual problems outlined previously.[32,33] Still, the idea of “recognition”[2,34,35] remains a central element common to all these models as the immune system's principal task is cast as a differentiation between organismal states (self vs nonself, safety vs danger, integrity vs damage, continuity vs discontinuity, etc.). Such operational distinction is indeed a powerful tool to conceptualize, with both impressive success and some obvious shortcomings, the functions of the immune system. Yet, it may also insinuate a proximity of logical and functional categories that can culminate in the postulate of a principal objective, a veritable raison d'être for the immune system, and thus reintroduces a tinge of teleology. Rather, we favor an evolutionary perspective that conceives of the immune system, devoid of a particular purpose,[36] as “the cause of its own necessity.”[37]

The notion of autoimmunity as an aberrant phenomenon has informed much of our current understanding about the immune system and its functions, and there appears to be a growing awareness that immunity and autoimmunity are both historically and conceptually inextricably intertwined. An emerging consensus indicates that the anthropomorphisms of “self” and “non-self” should be overcome (eg, as suggested in the respective forewords to two major autoimmunity textbooks[38,39]), and that autoimmunity is likely a universal phenomenon in the evolution of the vertebrate immune system. As part of the evolving organism, the immune system processes antigen stimuli in a deterministic fashion restricted by genetics, previous antigenic experience of the host, nature of the antigen, and the conditions of its

presentation.[38] However, imbuing the immune system's function with an overriding purpose, no matter how important for our conceptualization and experimentation, has to consider that evolution is ignorant to teleology. In this respect, the remark by the Darwinist Paul Ehrlich that production of autoantibodies is “dysteleological in the extreme”[6] may be extended to the functionality of the immune system as a whole: There is no teleology in autoimmunity nor immunity, just the workings of a complex system under evolutionary constraints. The rules that inform immunity are the same ones that govern autoimmunity.

The Burden of Autoimmune Diseases

The existence of autoimmune diseases in humans has been known for 100 years. By now, autoimmune pathogenesis has been attributed to more than 80 human diseases,[40] yet it is still far from clear which features can conclusively prove an underlying autoimmune pathogenesis. It has been suggested, somewhat provocatively, that with knowledge about

an infectious origin, diseases are called immunopathologically mediated, whereas lack of such knowledge results in reference to such diseases as autoimmune.[41] While this argument is akin to the medical taxonomy where diseases of unknown origin are assigned to the domain of the “endogenous,” “idiotypic,” “essential,” or “primary,” a “positive” definition for autoimmune diseases is needed to provide a specific diagnostic framework that allows for unequivocal identification of distinct autoimmune disorders, yet remains flexible enough to accommodate new insights in etiologic and symptomatologic processes. A first attempt to provide such a basis for the establishment of the autoimmune origin of human diseases was formulated by Witebsky et al.[42] who modeled their postulates on those of Koch: recognition of an autoimmune response (autoantibody or cell mediated), identification of a corresponding autoantigen, as well as induction of an analogous autoimmune response and disease in experimental animals. A timely update for these criteria has been proposed by Rose and Bona,[43] who suggested a combination of direct evidence (transfer of pathogenic antibodies or T cells), indirect evidence (reproduction of disease in experimental animals), and circumstantial evidence (clinical clues) to determine an underlying autoimmune etiology for human diseases. However, it is important to note that any specific guidelines have to be tailored to individual autoimmune disorders. An example for a catalog of diagnostic criteria to be evaluated in a scoring system for identification of patients with a specific autoimmune disease is the report of the International Autoimmune Hepatitis Group.[44] This report also illustrates the importance of distinguishing between an autoimmune and infectious origin for hepatitis[44]: Immunosuppressive therapy has a beneficial effect on the course of autoimmune hepatitis (AIH); responsiveness to such therapy is in fact one of the diagnostic criteria for AIH but may be detrimental when employed for treatment of virus-induced hepatitis.

As a first of its kind, a meta-study by Jacobson et al.[45] provided the comprehensive evaluation of prevalence and incidence studies conducted for 24 autoimmune diseases between 1965 and 1995. Overall, 1 in 31 Americans (˜8.5 million people, 3.2% prevalence) were estimated to be afflicted by autoimmune diseases, and the most common disorders, divided into organ specific and systemic conditions, and ranked in order of prevalence, are listed in Table 44.1. More recent epidemiologic studies have provided even higher estimates for the comtemporary burden of autoimmune diseases. Eaton et al.,[46] using national

hospitalization registry data in Denmark from 1977 to 2001, arrived at a prevalence of 5.3%, and Cooper et al.[47] calculated a global prevalence rate of 7.6% to 9.4% for the period from 1989 to 2008. Although clearly only approximations, it therefore appears that autoimmune diseases are much more frequent than previously thought. Walsh and Rau[48] approached this subject in a different manner by

determining the relative ranking of autoimmune diseases in terms of mortality risk among women under the ages of 65. Remarkably, the collection of 24 autoimmune diseases specified by Jacobson et al. ranked within the top 10 causes of death.[48] Thus, autoimmune conditions not only decrease the quality of life among afflicted individuals but also due to their high prevalence constitute a major public health burden. In addition, a trend toward rising incidence rates among most autoimmune disease has been noticed over the past few decades. For example, the incidence of multiple sclerosis (MS) in Italy has doubled between 1981 and 2002,[49] and in the United States, the incidence of celiac disease increased fivefold in 15 years.[50] These trends are not likely to abate, and predictions for the number of new type 1 diabetes (T1D) cases in Europe are as high as 24,400 by 2020, whereas while the prevalence under the age of 15 years is estimated to rise from 94,000 in 2005 to 160,000 in 2020[51]; in Finland, the country with the highest incidence of T1D, the number of new cases diagnosed at or before the age of 14 years will likely double in the next 15 years.[52] The economic challenges associated with these developments are indeed staggering as shown by the health care-related expenses in the United States and provided by the American Autoimmune Related Diseases Association for Crohn disease ($10.9 to $15.5 billion in 2008), rheumatoid arthritis (RA; $19.3 billion in 2005), or psoriasis ($11.2 in 2010). Treatment costs are expected to increase even further due to the nature of some of the more succesful therapeutic modalities developed for chronic autoimmune conditions, for example, the introduction of antitumor necrosis factor (TNF) biologics. While clearly an important advance and of great benefit to patients, these drugs do not promote a cure for the underlying disease, and the need for continuous treatment will exacerbate associated health care costs.

TABLE 44.1 Prevalence of Autoimmune Diseases

Autoimmune Disease	Organ	Weighted Mean Prevalence Rate/100,000	Weighted Mean Incidence Rate/100,000
Thyroiditis/hypothyroidism	**Thyroid**	**1323.8**	**21.8**
Grave disease/hyperthyroidism	**Thyroid**	**1151.5**	**13.9**
Rheumatoid arthritis	Joints, lung, heart, other	**860.0**	**23.7**

Vitiligo	**Skin**	**400.2**	
Type 1 diabetes	**Pancreatic B cells**	**192.0**	**12.2**
Pernicious anemia	**Stomach**	**150.9**	__a
Multiple sclerosis	**Brain/spinal cord**	**58.3**	**3.2**
Glomerulonephritis (primary)	**Kidney**	**40.0**	**3.6**
Systemic lupus erythematosus	Skin, joints, kidney, brain, lung, heart, other	**23.8**	**7.3**
Glomerulonephritis (immunoglobulin A)	**Kidney**	**23.2**	**2.4**
Sjögren syndrome		14.4	__a
Addison disease	**Adrenal**	**5.0**	__a
Myasthenia gravis	**Muscle**	**5.1**	**0.4**
Polymyositis/dermatomyositis	Muscle, lung, heart, joints, other	**5.1**	**1.8**
Scleroderma	Skin	**4.4**	**0.8**
Primary biliary cirrhosis	**Liver bile ducts**	**3.5**	**0.9**
Uveitis	**Eye**	**1.7**	**18.9**
Chronic active hepatitis	**Liver**	**0.4**	**0.7**

Data taken from Jacobson et al.45

Organ-specific autoimmune diseases in **bold**, systemic autoimmune diseases are in regular type face.

The prevalence/incidence rate from each study within a disease category contributed proportionately to the mean prevalence/incidence rate based on the population size of that study. The proportion or weight was calculated by dividing the study population denominator by the total of all the study population denominators for each disease.

For details see Jacobson et al.45

[a] No studies on disease incidence available.

These epidemiologic studies also permit several additional, if not entirely unexpected, conclusions. Many autoimmune conditions are clearly understudied, and some of the most frequently studied diseases exhibit comparatively low prevalence rates. The cause for the seeming imbalance between the public health burden posed by some autoimmune disorders and their attraction as objects for scientific study remains to be elucidated but will likely include the presence or absence of effective therapy. Pernicious anemia, the sixth most common autoimmune disease in the United States, can be effectively managed, and therefore elicits only limited epidemiologic interest. In contrast, some rare conditions may pose a pronounced burden to afflicted individuals and thus warrant continued efforts to develop more effective prophylactic and therapeutic interventions. Further, the availability of certain models for autoimmune diseases, again not necessarily a reflection of the epidemiologic importance of the corresponding human autoimmune disease, will have an impact on choices made by researchers charting their field of study. Additionally, as in other areas of research or clinical medicine, the funds and resources available are the result of multiple factors that may or may not include the public health burden exerted by a particular autoimmune disease. Balancing these aspects to appropriately appreciate and address the burden of autoimmune diseases, based on both the afflicted individual and society at large, is a challenge that will require our continued efforts to identify, investigate, inform, and, hopefully, improve the therapies for many autoimmune diseases.

Spectra and Continua: Organ-Specific and Systemic Autoimmune Disorders, Autoinflammatory Diseases, and the Challenges of Taxonomy

A perennial approach in our quest to make sense of the complex phenomena we encounter is the establishment of dichotomies, however, fraught with shortcomings, inconsistencies, and exceptions to the rule. Steeped in clinical traditions and immediately intelligible, the distinction between systemic and organ-specific autoimmune diseases is as useful as it is inadequate. Given that our evolving understanding of autoimmune diseases requires a constant reevaluation of our concepts pertaining to etiopathogenesis and effective treatment modalities, it would be premature to abandon such a simple and still useful classification. Rather, that porous juncture between systemic and organ-specific disorders may reveal hitherto unappreciated aspects of pathogenesis. On the surface, the patterns of pathology result from the distribution of anatomic niches that provide a suitable environment to

“interface” antigens and immune effectors. Leaving for the moment aside the difficulties pertaining to the identification of initiating autoantigens in many human autoimmune diseases and the challenging task to correlate markers of immunologic activity (eg, autoantibodies) with cause or consequence of tissue destruction, a particularly puzzling phenomenon is the seeming organ specificity of some disorders in the face of autoimmune responses that target ubiquitous antigens. For instance, the ribonucleoprotein antigens implicated in Sjögren syndrome or the transfer ribonucleic acid (RNA) synthetases targeted in polymyositis are widely expressed intracellular antigens, yet the pathology of these diseases is relatively circumscribed. Another intriguing example is the K/BxN arthritis model in which pathogenic antibodies recognize the ubiquitous cytoplasmic enzyme glucose-6-phosphate isomerase. Here, the preferential involvement of the joints apparently results from unique properties of the regional vasculature that allow for an antibody-mediated increase of vasopermeability and amplification of pathology by extracellular glucose-6-phosphate isomerase deposition in the articular cavities.[53,54] The observation that autoimmune damage is critically dependent on aspects of the local microanatomy emphasizes the importance to consider autoimmune processes in the larger context of interdependent organ systems.

In addition, an examination of some animal models used for the study of particular organ-specific autoimmune disorders further challenges the simple notion of restricted pathology and may provide clues about etiologic commonalities of ostensibly disparate clinical autoimmune syndromes. The nonobese diabetic (NOD) mouse is the most widely used animal model for the study of T1D, a severe condition caused by autoimmune destruction of

the insulin-producing β cells in the pancreas.[55,56] However, NOD mice also exhibit aspects of type 2 diabetes[57] and are prone to autoimmune sialitis, thyroiditis, peripheral neuropathy, prostatitis, a lupus erythematosus-like syndrome that develops after exposure to killed mycobacteria, as well as, under certain circumstances, exocrine pancreatitis.[56] Similar to the etiology of T1D, specific T cells are involved in the pathogenesis of all these disorders, although antigenic targets and requirements for costimulatory interactions are distinct. Thus, as in human T1D, the NOD mouse combines a generalized genetic susceptibility to multiorgan autoimmunity that is focused on pancreatic β cells but not limited to endocrine organs.

While the preceding considerations argue against a rigorous opposition of systemic versus organ-specific autoimmune diseases and rather support the notion of a spectrum of clinicopathologic features that variously emphasize prominent organ-specific and systemic features, even the conceptual distinction of autoimmune disorders at large is suffused with certain limitations (eg, absence of MHCs or autoantibody associations with some diseases tentatively labeled “autoimmune”) that have prompted new taxonomic proposals for immunologic diseases in general. Perhaps most prominently, McGonagle and McDermott introduced the concept of autoinflammation defined as “self-directed tissue inflammation, where local factors at disease-prone sites determine the activation of the innate immune system.”[58] Accordingly, a continuum of immunologic diseases is demarcated by rare monogenic disorders that are purely autoimmune and demonstrate preferential involvement of adaptive immune responses (eg, autoimmune polyendocrine syndrome-1 resulting from mutations of the AIRE gene), and purely autoinflammatory conditions defined by mutations in cells or molecules of the innate immune system and localized pathologies that escape the

traditional purview of “classical” autoimmune mechanisms (eg, TNF receptor-associated periodic syndrome). Between these boundaries, the vast majority of other immunologic disorders can be organized along a continuum of pathologies that range from classic polygenic autoimmune diseases to mixed pattern diseases and to polygenic autoinflammatory diseases.[58] The major appeal of this expanded notion of autoimmunity lies in the recognition of innate immune mechanisms as an important component of autoimmune disorders (with inevitable echoes of the Ehrlich-Metchnikoff rivalry informing the current discussions) and in the provision of an inclusive classification of immunologic diseases.

Central and Peripheral Tolerance: Implementing an Operational Concept

A detailed historical discussion of the concept of tolerance is beyond the scope of this chapter, but some aspects of the usage of the term tolerance require clarification at the outset. Tolerance in adaptive immunity, sensu stricto, is the absence of specific lymphocyte activity, the consequence of physical deletion, or functional silencing of specific T and B cells. Some researchers refer to these tolerance mechanisms as “passive” or “recessive” tolerance to explicitly distinguish them from “active” or “dominant” tolerance. While the latter mechanisms constitute bona fide immune responses (therefore, other researchers do not categorize them as a mode of tolerance), their particular nature results in a phenotype that is comparable to that achieved by means of passive/recessive tolerance. Distinct effector mechanisms (eg, immunosuppressive cytokines) and possibly dedicated classes of immune cells (eg, “regulatory T” [T_{reg}] cells) assure that local or systemic autoimmunity is avoided.

The concept of T-cell suppressors, first proposed in the early 1970s by Gershon and Kondo,[59] was resurrected in the form of “professional” cluster of differentiation (CD)25+FoxP3+CD4+ and “adaptive” T_{reg}s cells (TR1 and other T_{reg}s) and has since attracted considerable attention. However, while there is indeed a CD25+ lineage of T cells committed to regulatory activity in naive, nonimmunized mice, we wish to underscore that regulatory functions, including those that limit autoimmunity, are a feature of the immune system as a whole and can be exercised by other classes of immune cells as well (eg, CD8+ T cells, γδT-cell receptor (TCR) T cells, natural killer T cells, etc.). Thus, while CD25+CD4+ T_{reg} cells occupy a distinct and important niche in the complex dynamic network of immune functions, not all T-cell regulators are CD25+CD4+ nor do all CD25+CD4+ T cells function as suppressors. Indeed, novel markers might characterize regulatory function better; among these are expression of the transcription factor FoxP3[60,61,62,63,64,65,66] and secretion of cytokines with regulatory function such as interleukin (IL)-10[67,68] or transforming growth factor (TGF)-β.[69] The multiplicity of current efforts to understand the nature of CD25+CD4+ T_{reg} cells has been expertly reviewed elsewhere,[70,71] and we note in particular more recent observations on the transcriptional, phenotypic, and functional instability of T_{reg} population.[72,73] This has important repercussions in the context of autoimmunity, as T_{reg}s can lose their regulatory capacities when exposed to local conditions of autoimmune inflammation. On the one hand, these results suggest that aberrant conversion from regulatory to effector phenotype may partly underlie progression to overt autoimmune disease in susceptible individuals. On the other hand, such propensity may jeopardize the long-term efficacy of therapeutic strategies based on the induction or adoptive transfer of autoantigen-specific T_{reg}, as these cells could potentially convert to effectors once they

arrive in the inflamed tissue. Therefore, these recent insights clearly warrant caution regarding the popular endeavor of assigning T cells to defined subpopulations based on their "signature" transcription factors and functionalities and emphasize the importance of plasticity and mutability the mechanistic foundation and relevance of which remains to be explored in further detail. Lastly, we emphasize that changes in T_{reg} functionality rather than a mere numerical decrease appear to constitute a correlate for some human autoimmune diseases.[74] Thus, the simple quantification of peripheral T_{reg} numbers as a measure of risk in autoimmunity-prone indviduals or as a biomarker in clinical studies will have to be complemented with functional assays to generate relevant data.

T-Cell Tolerance

Autoreactivity, by definition, designates a specific immune response to self-antigens. Antigen nonspecific responses such as inflammatory and innate immune processes should not be considered autoimmune in the strict sense, although they may accompany, enhance, or even trigger autoimmune processes proper. Thus, antigen-specific T- or B-cell immunity will have to underlie a genuine autoimmune disorder. Furthermore, for organ-specific autoimmune diseases, antigen specificity of primary effector lymphocytes must be largely restricted to autoantigens derived from defined organs or tissues. Once initiated, specific responses that precipitate or "drive" the localized autoimmune reaction may diversify to comprise additional specificities (determinant spreading) and pathogenic mechanisms.

How does the adaptive immune system restrict generation and activation of autoreactive lymphocytes? The central process by which the generation of TCR diversity is limited is called thymic selection. Thymic selection is a developmental process that selects T cells with a biased repertoire for export into the periphery.[75,76,77,78,79] T cells that interact at least weakly with self-peptides presented in the context of MHC molecules are chosen in the course of positive selection,[80,81] while those that do not effectively interact with MHC/peptide complexes die "by neglect." However, interactions above a certain avidity threshold result in elimination by negative selection and constitute the basis for "central tolerance."[75,79] Thus, central tolerance prevents widespread autoimmunity as a function of lymphocyte/antigen-complex avidity and preferentially selects T cells with specificity for antigens not expressed in thymic epithelium for export into the periphery. However, central tolerance is not a complete mechanism, and a sizable pool of T cells with intermediate avidity can escape negative selection and constitutes most autoreactive T cells found in the peripheral immune system. The presence of these autoreactive T cells can be considered physiologic, and they are not noxious for two reasons: either they are usually not activated and exhibit a "naive" phenotype or they exhibit regulatory effector functions and act as adaptive T_{reg}s following activation. Thus, not all self-reactive lymphocytes need to necessarily exhibit an aggressive phenotype. Depending on their specific effector functions, autoreactive T cells may exhibit regulatory functions and may critically modulate or even abort local autoimmune processes. Such autoreactive regulators might occur physiologically and constitute most autoimmune responses present in healthy individuals. Only an encounter under appropriate stimulatory conditions (ie, presentation of autoantigen-derived peptides presented in the context of MHC class I or II molecules accompanied by antigen-nonspecific costimulatory interactions and strong inflammatory signals) can lead to their full activation and

detrimental effector functions in the periphery. As such “armed effectors,” autoreactive T cells are now potentially very dangerous and may initiate specific autoimmunity, if they recognize the autoantigens or closely related proteins in a defined tissue. It is thought that a few autoaggressive “driver clones” with highly detrimental effector function can sustain a localized autoimmune process. High receptor/MHC/self-peptide avidity likely, but not necessarily, predisposes to this phenotype.[82,83,84]

The presence of autoreactive T cells in the periphery might suggest that detrimental autoimmunity should occur quite frequently if organ-specific autoantigens are not expressed in the thymus, or alternatively or in addition, such physiologically occurring autoimmunity is not of a regulatory nature. Yet, there are several additional mechanisms that maintain tolerance in the periphery. “Peripheral tolerance” involves a set of mechanisms that ensures that autoreactive T lymphocytes are not activated in the periphery. It should be noted that these mechanisms pertain to both autoreactive and “heteroreactive” T cells and involve the following pathways. First, it has been observed that naive T cells triggered by a strong signal through the TCR alone may lose the ability to proliferate, and some but not all effector functions become “anergic.”[85,86,87] Presence of certain cytokines or costimulatory interactions can avoid the induction of anergy or may reverse an anergic state. Second, highly activated T cells will eventually undergo activationinduced cell death (AICD).[88] AICD is thought to be essential for the downmodulation of immune responses and the reestablishment of immune homeostasis. Impairment of AICD may lead to continued immune activation and generalized autoreactivity. For CD4 lymphocytes, AICD is FAS/FAS-L dependent[89,90]; it is not clear which interactions precisely control AICD in CD8 cells. Third, molecules that can deliver specific negative signals, such as the B7-binding cytotoxic T-lymphocyte antigen (CTLA)-4 are involved in “turning off” of antigen-specific T cells.[91,92] Finally, other factors such as regulatory lymphocytes and regulatory antigen-presenting cells (APCs) might play important roles in maintaining peripheral tolerance.[59,70,93]

B-Cell Tolerance

Although they are not selected in the thymus, similar paradigms as those for T cells apply to autoreactive B lymphocytes. Clonal selection occurs after recognizing antigens, is avidity dependent, and allows the B cells to undergo further receptor editing. Both central (ie, bone marrow) as well as peripheral tolerance mechanisms are in place to avoid generating mature B cells with specificity for self-antigens. After B cells mature in the bone marrow, they clonally expand after recognizing antigens in the periphery. T-helper (Th) lymphocytes are needed for this process in response to most protein antigens, and these B-cell responses are therefore termed “thymus dependent” or, in other words, require T cells with specificity linked to an epitope on the antigen they are reacting with. Th cell-independent B-cell responses occur mostly to bacterial and lipid antigens, for example, to lipopolysaccharide, and are therefore rarely autoaggressive. Thus, T-cell tolerance directly controls B-cell reactivity to autoantigens. In general, systemic autoimmune disorders such as systemic lupus erythematosus (SLE) are B-cell dependent, and organ-specific diseases such as MS and T1D are less dependent on autoantibodies, although

B cells can play important roles as APCs, and antibodies can possibly enhance disease pathogenesis. In both diabetes and MS, autoantibodies correlate with disease progression. In

thyroiditis, autoantibodies are instrumental for causing disease and in AIH, their role is thought to be crucial.

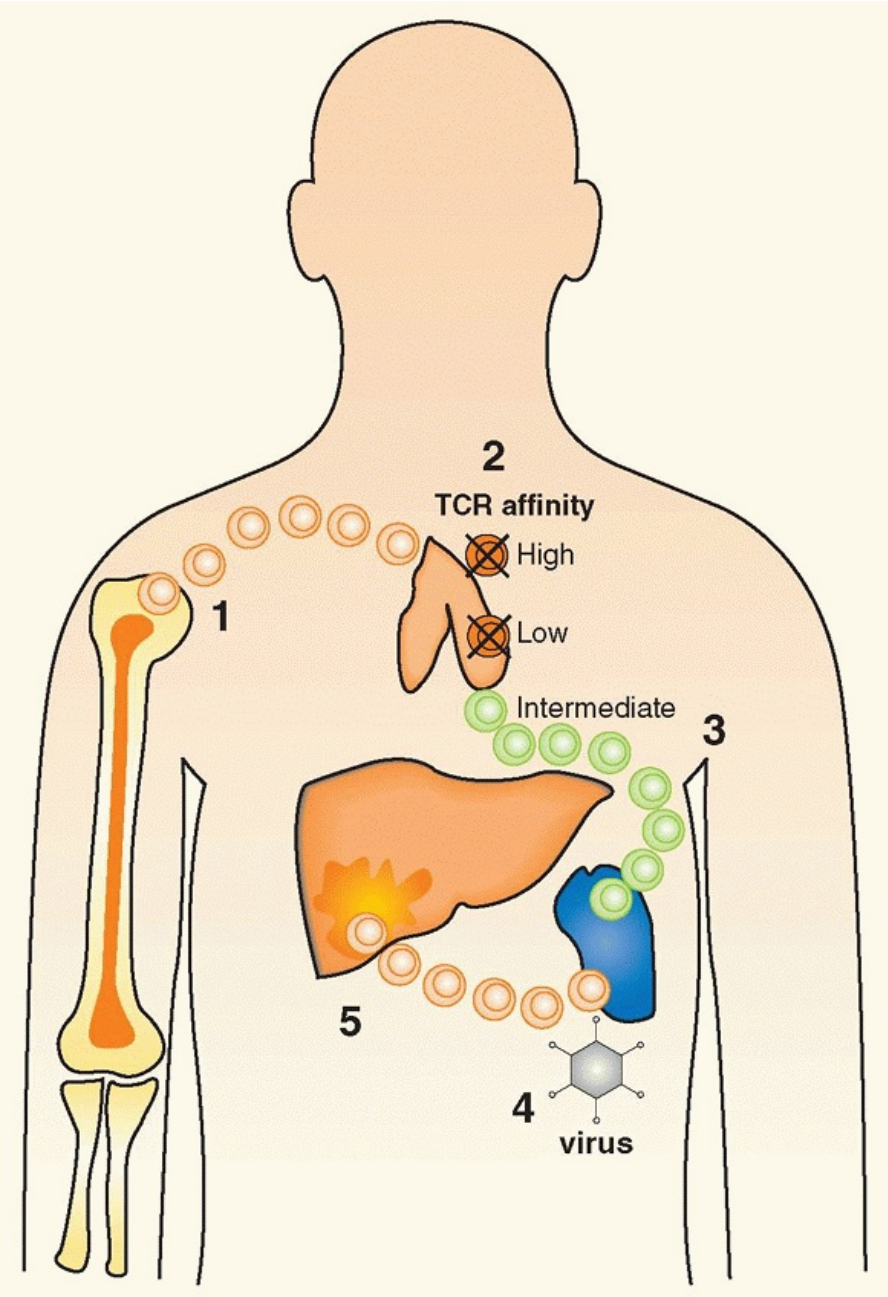

FIG. 44.1 Events Important for the Pathogenesis of Autoimmunity. 1: Lymphocyte precursors migrate from the bone marrow to the thymus to undergo maturation. **2:** In the thymus of susceptible individuals, autoreactive lymphocytes can escape thymic selection. Whereas cells with high or low T-cell receptor affinity are generally deleted; "intermediate" lymphocytes fail to undergo negative selection. **3:** Such initially naive cells migrate to peripheral organs such as the spleen and may remain unresponsive for many years. **4:** Certain environmental triggers have the ability to activate these naive cells by bystander activation, molecular mimicry, or antigenpresenting cell cross-presentation. **5:** As a consequence, activated autoreactive lymphocytes migrate to target organs such as the liver and mediate inflammation and tissue damage, leading to clinical autoimmune disease symptoms.

In conclusion, central (thymic) and peripheral tolerance mechanisms will effectively control the vast majority of autoreactive lymphocytes, which assures that autoaggressive immune responses are relatively rare (< 5% overall population). Some of the considerations described in this paragraph are illustrated in Figure 44.1.

Organ-Specific Tuning—Regulatory and Destructive Autoimmunity

As indicated previously, while autoimmune disorders must conform with a set of general criteria, organ-specific autoimmunity must be considered in the context of the target organ affected. Certain effector functions exerted by autoreactive lymphocytes will be detrimental only to particular cells or tissues. For example, the pancreatic β cells are more sensitive to the damaging effect of inflammatory cytokines than neighboring α cells (both cell types are part of the islets of Langerhans) and other cells in their vicinity (eg, fibroblasts or acinar tissue of the pancreas).[94] On the other hand, some organs provide a microenvironment that suppresses inflammatory responses. For example, both the gut and the central nervous system (CNS) contain relatively large amounts of TGF-β, which can have direct anti-inflammatory effector functions on T cells and APCs, and activate T_{reg}s, unless IL-6 is present.[69,95,96,97,98] Also, certain large organs such as the liver may better tolerize lymphocyte responses directed toward them because they contain a high number of cells that are incapable of costimulation (eg, hepatocytes) and will, therefore, more likely shut down naive autoreactive lymphocytes that stimulate them.[30] Finally, the precise activation state, phenotype, and effector function of an autoreactive cell is critical in determining their impact on tissues expressing their cognate antigen. Some molecules will exert beneficial functions, for example, IL-4 and probably IL-10 and TGF-β in T1D.[99,100,101] Antigen-specific adaptive and professional regulatory cells are likely participants of every local autoimmune process and can therefore be essential for delay or prevention of clinical disease altogether. While their induction is a clearly therapeutic goal, phenotype and mechanistic aspects essential for regulatory function are not yet understood in complete detail. Likely, professional and adaptive T_{reg}s may exert their function by targeted alteration of APC function. Recent evidence even suggests that T_{reg}s may safeguard against autoimmunity by specifically killing APCs that present autoantigens to effector T cells.[102] They may also act as true bystander processors if their suppressive action via APC modulation is extended to aggressive immune responses regardless of specificity found in the microenvironment of the affected organ (Fig. 44.2). Indeed, such cells have been found in several animal models,[100,103,104,105] but their existence and function in humans is not well defined. Alternatively, regulatory lymphocytes may directly affect activated or naive aggressive lymphocytes and induce anergy or apoptosis. Lastly, by changing the overall cytokine milieu of a given inflammatory process, the number and function of aggressive cells and cytokines/chemokines may be dampened in a localized area.[106] Probably, the best understood balancing circuit employed by autoreactive adaptive T_{reg}s are the T_H1/T_H2 paradigm[107,108,109] and signaling by IL-10[68] and/or TGF-β.[69] In light of these observations, autoreactivity is not necessarily detrimental and maybe even essential in the sense of T_{reg}s.

Last, it might be difficult or even impossible to define a general phenotype of autoaggressive lymphocytes that will cause damage at any site or organ. Rather, autoaggression has to be defined in relation to the target organ or cell that is under

attack, and lymphocytes detrimental in one organ/disease will not necessarily be detrimental to other organs, and ubiquitous autoimmunity will therefore occur very rarely.

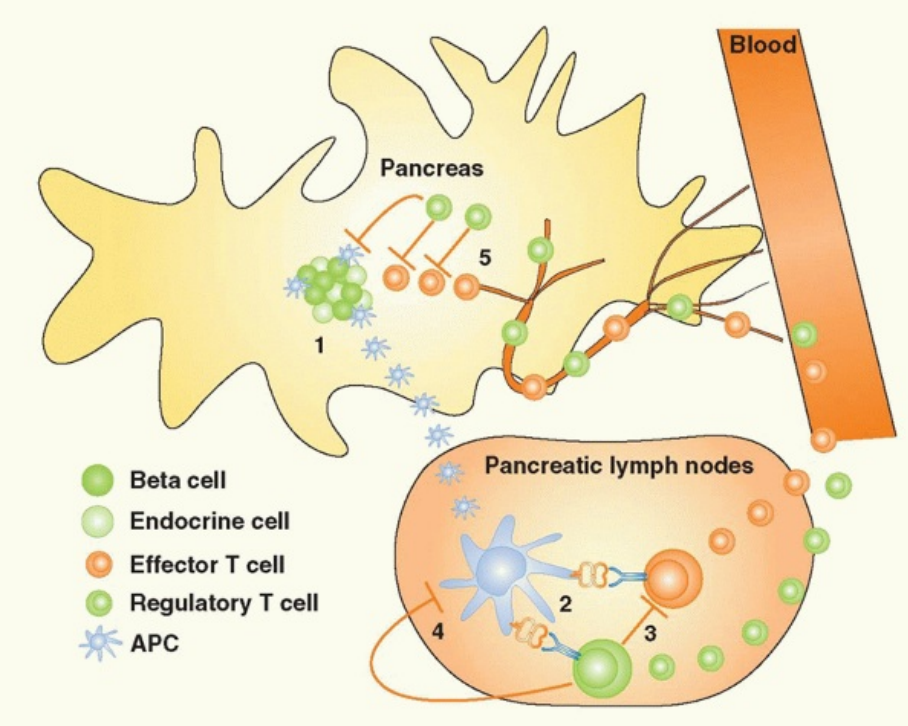

FIG. 44.2. The Concepts of Bystander Suppression and Infectious Tolerance. 1: Autoantigens are taken up by professional antigen-presenting cells (APCs) in the target organ, here depicted as the pancreas in type 1 diabetes. Upon maturation, APCs migrate to the draining lymph nodes. **2:** In the lymph nodes, lymphocytes specific for distinct β cell antigens are activated by the APCs. **3:** As a consequence, aggressive T cells directed toward one antigen could be regulated by regulatory T (T_{reg}) cells specific for another autoantigen, a process termed “bystander suppression” by Howard Weiner.[561] **4:** In theory, T_{reg}s could also modulate APC function and foster induction of more T_{reg}s to the same or other antigens, a process termed “infectious tolerance” by Hermann Waldman.[562] **5:** Identical mechanisms may then regulate autoimmune responses in the target organ, after both effector cells and T_{reg}s gain access via the circulation.

Initiating Autoimmunity: Antigens, Genes, and Environment

Antigens

A central challenge in most human autoimmune disorders is the identification of an initiating autoantigen or autoantigens. For example, several candidate autoantigens have been identified in T1D. However, to date, it remains unclear and controversial whether any are

actually involved in initiation of the disease. Similar situations exist for multiple sclerosis and arthritis. Thus, distinguishing features that constitute an ideal candidate autoantigen and parameters by which potential candidates can be searched for and identified as the initiating autoantigen antigen for a given organ-specific disease must be determined with much greater precision to yield insights applicable to preventative or therapeutic interventions.

The concept that "processing of antigens determines the self" has been introduced by Sercarz.[110,111] Autoantigens that are not expressed in the thymus or are of cryptic nature[110,112,113] may encounter a more extensive T-cell repertoire that is likely of higher avidity. Such antigens appear to be better targets. Low levels of thymic antigen expression or limited numbers of thymic medullary cells may lead to partial tolerance.[114,115] In addition, the precise timing of antigen expression during embryonic development may play a major role for the establishment of tolerance. Antigens that are cryptic or not expressed during early embryonic development might become better targets later because more autoreactive T cells will be present in the periphery. It should be considered that certain "embryonic antigens" are involved in initiating autoimmune diseases.[116]

Of further importance are the constraints with which particular antigens can be presented to the immune system in a given target organ. Exogenous uptake of soluble antigens usually results in processing via the MHC class II presentation pathway. Such antigens may also be processed through the MHC class I pathway by dendritic cells, a mechanism termed *cross-presentation*, if the antigen itself was originally expressed by a cell different from the APC.[117,118] Cross-presentation appears to be less efficient than direct endogenous presentation of antigens through the MHC class I pathway. At this point, it is not clear which properties will make an antigen a more efficient candidate for cross-presentation. The class I pathway may be important in the effector phase for many autoimmune disorders because CD8+ T cells with cytotoxic function (cytotoxic T lymphocyte [CTL]) can be induced in this fashion, and CTLs have profound detrimental effector functions (interferon [IFN]γ and TNF-α secretion, lysis of target cells).[119] Clearly, cross-presentation can lead to immunity, as well as tolerance, and in this way propagates or, alternatively, halts autoimmune disease.[117,120] It is less likely that autoantigens that are not presented by professional APCs or within lymphoid organs after their release from target cells/organs can initiate autoimmunity. Only under special circumstances (ie, extremely high-density presentation of an antigen on a target cell or highly inflammatory conditions including chemotaxis) would this appear possible. This might occur during localized or systemic viral infections and could also be an important event in lymphocyte entry into target organs, especially if chemotaxis is involved and endothelial cells present autoantigens.[121] In addition, experienced or primed autoaggressive lymphocytes will be capable of becoming activated after seeing antigen in the absence of costimulation; thus, accumulating a memory pool of autoaggressive T cells might be inherently dangerous to an organism.

Thus, the type of autoantigen will define its own potential "candidacy" in an autoimmune process. Autoantigens

that are not expressed during development, or were cryptic for an extended period of time, as well as antigens secreted and cross-presented in lymphoid organs by APCs, appear to be better suited for assuming the role as primary culprits.

Genes

An abundance of empiric evidence indicates the association of many organ specific autoimmune diseases with certain human leukocyte antigen (HLA) haplotypes as well as other susceptibility or protective genes. The principal specific genetic linkages will be discussed separately for each condition in part II. In general, MHC class I (such as HLA-B27)[122,123] or class II (such as DR4) genes might predispose to a certain disease by enhanced presentation of pathogenic peptides in the periphery or inefficacious presentation of autoantigenderived peptides in the thymus. For example, the human HLA-DQ8 molecule has a striking structural similarity to mouse I-A^{g7} class II that predisposes NOD mice to spontaneous autoimmune diabetes.[124,125] However, the link to disease appears not to be as simple as reasoned previously, and more complex mechanisms might be in place. Presentation of the pathogenic peptide in gluten-induced celiac disease involves acidic modification of the protein to generate the peptide ultimately presented by MHC class II,[126] and other chemical modifications can be expected to alter peptide binding to MHC and the resulting conformation of the peptide/MHC complex.

Other genes that encode immunoregulatory or inflammatory proteins may be involved in the disease process, and, finally, genes that support tissue or wound repair (eg, islet cell regeneration in T1D) may be of help in preventing disease development.[127] For most autoimmune disorders, the genetic links are complex, not absolute, and many susceptibility and resistance genes act in concert to modulate the clinical phenotype. Noteworthy, newly emerging themes in the genetics of autoimmunity are the fact that small nucleotide polymorphisms might result in alternate forms of the same gene and the fact that the character of the full array of MHC region genes is becoming understood.[128] A striking example for the former is the existence of an alternate spliced form of the inhibitor of cellular function, CTLA-4. Kuchroo and colleagues discovered that the presence of a secondary CTLA-4 lacking the B7.1/B7.2 binding motif can be instrumental in the genetic penetrance of diabetes pathogenesis.[129] An important area still largely unexplored is the nature of genes responsible for transcriptional control of other proteins. Experimental models indicate that transcription factors can have profound effects on the development of autoimmune disease, and variations in expression and activity levels may be found between susceptible and protected individuals. In summary, a complex interplay of many genes will predispose for a certain autoimmune disease, but the concordance of clinical manifestation is frequently not higher than 30% to 40% in monozygotic twins.[130,131,132,133,134,135,136] For this reason, other factors are to be considered in triggering or propagating the pathogenic autoimmune process.

Our understanding of the genetic component to autoimmunity has greatly benefited from the arrival of a first wave of so-called genome-wide association studies (GWAS) in the past 5 years. Prior to the use of this technology, genetic contribution was analyzed by two different approaches: linkage analysis and association studies. Linkage analysis tests for correlation between a genomic region and phenotype in families, whereas association analysis searches for correlations in the entire population.[137] In a genetic association study of autoimmune disease, sequence variants are genotyped in patients, and controls and the frequencies of these genotypes are compared. It is then evaluated whether certain alleles are significantly

more associated with patients than with controls. Most autoimmune conditions are polygenic diseases, meaning that they are caused by (a range of) combinations of polymorphisms within different genes. As mentioned previously, HLA-associated risk was discovered early in many autoimmune disorders and turned out to be reproducable because of its highly significant association. The vast majority of common genetic variants in autoimmunity, however, display modest effects. Even extremely large linkage studies have usually been unsuccessful in discovering susceptibility genes for polygenic diseases that met stringent statistical thresholds. Likewise, most common variants require large association studies for reliable detection. Advances in the development of high-throughput genotyping, statistical correlation methods, and better insight into the organization of the genome offered the opportunity to design approaches for the unbiased interrogation of the entire genome.[138] Sufficiently large case-control GWAS thus have the potential of identifying disease-associated mutations, even if the contribution of that polymorphism is minor. It should be noted here that GWAS often cannot unequivocally identify gene associations (at best "candidate genes"), as risk loci contain multiple genes, and, therefore, follow-up fine mapping or functional studies are generally required. Although GWAS have rapidly increased the list of known risk loci for many autoimmune disorders, some have questioned whether this knowledge substantially aids our understanding of their etiology and offers clues for rational intervention. It could be argued that all associations are often eclipsed by the major contributing factor, HLA polymorphisms, and essentially pinpoint genes with key roles in the immune system that could be expected to be involved to some degree. In T1D, for instance, minor associations with important genes encoding molecules involved in immunity such as CTLA-4 and IL-2 were discovered. From a therapeutic perspective, such findings may be hard to translate as, based on the relatively small association rates, these genes and their products may not necessarily represent pivotal targets in a diverse human patient population. In summary, it can be concluded that most likely all major susceptibility genes associated with the most common autoimmune disorders as discussed in the following text are currently known. Future years will tell whether this comprehensive genetic dataset translates to a better understanding of the origins of autoimmunity and if this will open new avenues toward better treatements.

Environment

***Viral Infections*.** For many years, viral infections have been discussed as potential candidates to trigger autoimmunity in susceptible individuals because of their capacity to directly

infect target tissues and induce strong inflammatory responses and immune activation. While the association between viral infections and organ-specific autoimmune disorders is an intriguing possibility, it has been exceedingly difficult to demonstrate a causative role for specific viruses in human autoimmune diseases. Among the many obstacles are the fact that 1) all individuals undergo a multitude of viral infections during their lifetime; 2) one has to assume that viruses are frequently cleared at the time of diagnosis and viral footprints can be difficult to find in individuals affected by an autoimmune disease ("hit and run" event); 3) the precise viral strain, infection kinetics, and number of T cells and type of effector functions induced may play an instrumental role in determining its effect in an individual genetically at risk, necessitating a very detailed immunologic profiling[139]; 4) due to MHC polymorphism,

there is a significant variation in specificity of the antiviral response; 5) viral infections might not, per se, trigger autoimmunity but affect an ongoing autoimmune process in a detrimental way; and 6) there now is increasing evidence that viral and other[140] infections may rather prevent than enhance ongoing autoimmune responses, either by apoptosing aggressive T cells or by augmenting the T_{reg} pool.[141,142] Thus, a successful approach should be to first explore the underlying mechanisms in virally induced autoimmunity and then apply the precise insight and paradigms developed specifically to the human situation.

The most important mechanisms to be considered are listed here and are summarized in Figure 44.3.

1. Molecular mimicry, which implies the cross-reactivity between viral and self-determinants as a principle cause or mechanism to enhance autoimmunity[143,144]
2. Bystander activation, which postulates that APCs and autoreactive lymphocytes will become activated indirectly as a consequence of the cytokine/chemokine by virus infection of a particular organ[145]
3. Virally induced determinant spreading, which involves the presentation of autoantigens (possibly previously cryptic) by virus-activated APCs[146,147,148]

The experimental evidence for all of these three scenarios obtained in different mouse models in vivo is well documented. However, none of these have been proven for any human autoimmune disorder due to the large size of human trials and the invasive nature of in vivo diagnostics required at the present stage. Thus, in the near future, we will continue to depend on animal models until noninvasive human in vivo diagnostic strategies have advanced and allow for imaging of trafficking of antigen-specific lymphocytes and high-resolution definition of immune process present in a specific organ. A final remark should be made for the existence of a negative association between viral infections and autoimmune disease found in several experimental models.[149] These observations are in support of epidemiologic findings that the incidence of many autoimmune disorders is decreased in equatorial countries, where the presence of certain infectious diseases is significantly increased. However, no firm associations have been established to date.

Other Environmental Causes. Similar to viral infections, other inflammatory stimuli may trigger or enhance autoimmunity. The gut deserves particular attention in this respect. At this site, each individual harbors thousands of different bacterial strains, and viral infections are common. Furthermore, the mucosal lining is permeable for nutrients and constitutes a very large interactive surface with the environment. Again, the complete absence of all bacteria results in severe immune dysfunction and possibly autoimmunity. However, it would be incorrect to conclude that infections are therefore always protective. Indeed, the commensal flora appears crucial in maintaining proper immune activation and function, but certain pathogens could definitely elicit strong gut immune responses that lead to autoimmune disease.[150,151]

Regulatory Circuits in Autoimmune Processes

Cytokines

Cytokines and chemokines are essential regulators of cellular and humoral immune

responses and lymphocyte trafficking.[152,153,154] They play a central role in orchestrating autoimmune processes and constitute a multitude of positive as well as negative feedback loops.[155] It is well established that certain cytokines can negatively or positively influence the production of other cytokines (ie, the Th1/Th2/Th17 paradigm) and thus determine the balance between proinflammatory and anti-inflammatory factors in the local environment. Furthermore, autocrine production feedback can augment or shut down production of a given cytokine by one cell. Cytokine networks operate with a fair amount of redundancy, and many cytokines and chemokines share common receptors. They are the most likely mediators of "bystander activation" and "suppression" processes and also offer an effective and versatile therapeutic target via the temporally restricted use of cytokine- or chemokine-blocking antibodies. Their precise function can vary quite dramatically in respect to the autoimmune disease under investigation and will be discussed in the section on Individual Autoimmune Disorders. Further, their level and timing of expression during an ongoing disease process will determine whether they have a positive or negative effect (or any at all; see the following discussion).

Many autoimmune disorders have traditionally been classified as "Th1-" or "Th2"-driven disorders based on, among other factors, the pathogenic role of cytokines such as IFNγ and IL-4.[107] Recent years have challenged this perspective with of the description the Th17 helper subset, characterized by the production of IL-17.[156,157,158] This class of T cells is considered highly proinflammatory, and its discovery was found to be of importance in several autoimmune disorders in both animal models and humans.[159] One example of an autoimmune disease where Th17 cells have taken center stage is RA. The disease's principal animal model was long referred to as a Th1 driven, but the field struggled with the observation that mice treated with neutralizing antibodies to IFNγ- and IFNγ receptor-deficient mice developed more severe arthritis.[160,161] Dominance of Th17-directed immunity was put forward when IL-17 knockout mice were found

to develop less arthritis,[162] and treatment with neutralizing antibodies to IL-17 or soluble IL-17 receptor alleviated joint inflammation.[163] In humans, it was found that IL-17 is increased in RA sera and synovial fluid and is present in the T-cell rich areas of the synovium.[164] Furthermore, IL-17 has profound inflammatory effects on a range of cell types within the joint. Several clinical trials are underway to either block IL-17 or the cytokines that govern the generation of Th17 cells such as IL-6.

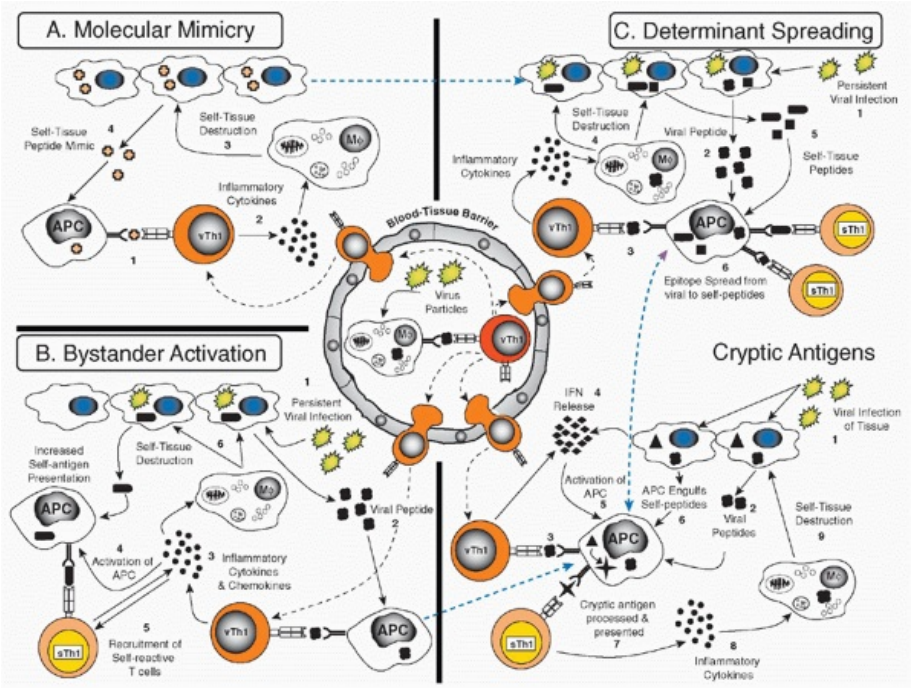

FIG. 44.3. Potential Mechanisms of Autoimmune Disease Induction. After a viral infection, activated virus specific T-helper cells (vTH1) and cluster of differentiation 8 cytotoxic T lymphocytes will migrate more readily through blood-tissue barriers to infected and noninfected organs. **A:** Molecular mimicry describes the activation of cross-reactive lymphocytes that recognize the original viral epitope and a self-epitope *(1)*, which leads to the release of cytokines and chemokines *(2)*, which enhance local inflammation, activate antigen-presenting cells (APCs), and indirectly or directly cause tissue destruction *(3)* and spreading of the autoimmune process *(4)*. **B:** In the epitope spread model, persistent viral infections *(1)* could result in the activation of virus-specific T cells *(2,3)*, which cause tissue damage by killing virally infected cells *(4)*, leading to the release *(5)* and (cross-)presentation of more autoantigens *(6)*. **C:** The bystander activation model describes the nonantigenspecific activation of autoreactive T cells. Infiltration by virally specific T cells *(1,2)* leads to inflammation and upregulation of immunity throughout the tissue *(3,4)*, involving the activation of APCs, which now differentially process autoantigens (also de novo or previously cryptoic antigens). This can lead to activation of lymphocytes by T-cell receptor (TCR)-specific or TCR-independent mechanisms *(5)*, which can then cause tissue damage *(6)*. The cryptic antigen model describes the initiation of autoimmunity by differential processing of self-antigen/peptides, which can occur under inflammatory conditions. After viral infections, interferons *(1)* are secreted by antiviral T cells and infected cells *(2-4)*. APCs are activated in this way (5), which enables them to engulf self-peptides *(6, triangle)* or to differentially process endogenous autoantigens. Cytokines can activate proteases more strongly, which might result in the presentation and processing of previously cryptic autoantigens *(7-9)*. These displayed pathways are not mutually exclusive and are probably operational at different levels in many autoimmune responses. Currently of high interest is the presentation of neoantigens and strategies to define them. Courtesy of Steve Miller and Ludovich Croxford, Northwestern University, Chicago, IL.

Apoptosis

It appears to be a general paradigm of great functional consequence that activation of the immune system is followed by a process that reverses the activation and reestablishes

homeostatic baseline levels of immunity.[88,90] In the absence of such regulatory mechanisms, immune responses will overshoot their goals and excessive immunopathology will occur. Thus, AICD is believed to play an important role in regulating autoimmunity. Apoptotic lymphocytes, for example, are easily detected in islet infiltrates in T1D,[165] and targeted induction of limited apoptosis may even prevent onset of autoimmune disease.[166] While increased apoptosis of aggressive lymphocytes that exceeds the "supply" of newly activated cells may directly limit an ongoing autoimmune process, limited apoptosis of target tissues may indirectly facilitate induction of protective regulatory responses. On the other hand, while apoptosis of target cells should at best be limited, decreased apoptosis of autoreactive aggressive lymphocytes will propagate autoimmunity. In RA, for instance, insufficient apoptosis of synovial macrophages, fibroblasts, and lymphocytes is one mechanism that might contribute to persistence of the disease and lead to synovial lining hyperplasia.[167] It is therefore important to consider precisely which cells undergo apoptosis in order to predict the possible outcome.

- If too many target cells die by apoptosis, organ destruction occurs more rapidly; however, at the same time, antigens releases from apoptotic cells appear to propagate tolerance rather than immunity.[166,168]
- If T_{reg} cells die by apoptosis, autoimmunity will be enhanced.[169]
- If aggressive lymphocytes die by apoptosis, disease should be ameliorated. However, because they have to first be activated, they might induce organ damage during their activation phase.

Thus, an ongoing autoimmune process can be viewed as a rather fine-tuned and fragile equilibrium of aggressive and regulatory components, and the precise activation kinetics and survival times of all lymphocyte types implicated in the process will determine the outcome. We are, at present, unable to delineate the precise in vivo cellular kinetics, and a more thorough understanding will require improved noninvasive diagnostic techniques.

Kinetics

One of the most important emerging areas for an improved understanding of the pathogenesis of autoimmunity is concerned with the kinetics of immune responses. The pathophysiologic or therapeutic effect of a given lymphocyte population depends not only on specificity, activation state, and effector functions but is also a function of the timing during which phase of an ongoing disease process it is present. Indeed, inflammatory cytokines such as IFNγ or TNF-α exhibit opposing effects in T1D depending on the precise time point of generation.[170] Early expression enhances islet destruction and disease development, whereas late expression ameliorates disease by inducing apoptosis of autoaggressive cells. These kinetic issues constitute a major obstacle for successful immune intervention because they preclude the use of specific blocking agents or administration of cytokines without

precise knowledge of their kinetically differential role in the disease process. Figure 44.4 illustrates these kinetic considerations in relation to target cell destruction. A better understanding of the underlying “autoimmune kinetics” is essential and treatments will likely have to be individualized, in particular for antigen-specific immune-based interventions.

Therapeutic Considerations

Efficacy, Specificity, and Undesired Effects

Treatment of autoimmune disorders is not that different conceptually from cancer therapy. A fine balance must be found between efficacy of the intervention and acceptable undesired effects. The main goal of autoimmune disorder therapy is suppression of the pathologic autoimmune response. Therapeutic options range in principle from continuous immunosuppression of the entire immune system to specific, targeted, temporally limited, and local immunosuppression. Systemic immunomodulation or anti-inflammatory therapy will affect the entire immune system and may compromise the immune status of the individual. One of the more succesful examples for effective systemic treatments is the blockade of TNF-α to ameliorate RA.[171,172] This new class of drugs is especially valuable because TNF blockade effects inflammatory pathways distinct from the targets of conventional anti-inflammatory therapy with corticosteroids or nonsteroidal anti-inflammatory drugs. TNF inhibitors can thus be used in patients that are refractory to the latter treatments with the added advantage of considerably improved outcome in terms of structural bone erosions. The remarkable therapeutic success of these drugs has since been replicated in other autoimmune conditions such as Crohn disease, ulcerative colitis, ankylosing spondylitis, and psoriasis. Whereas these diseases target different organs, common therapeutic improvements upon TNF blockade thus indicate shared mechanisms in terms of cytokine signaling. While undesired effects are relatively low, SLE-like symptoms have been observed in a few patients, as has the enhanced susceptibility to tuberculosis. Another important disadvantage, in addition to the high cost of these biologics, is that not all patients respond to TNF blockade. This has spurred a search for more potent blockers of inflammation, with the most promising developments stemming from the design of Janus kinase inhibitors for treatment of RA.[173] This class of small molecular drugs that interrupt signaling downstream of multiple rather than individual cytokines has recently cleared phase III trials in RA and is expected to revolutionarize the therapeutic landscape. While indications exist that this approach is at least safe in the short-term and that it outperforms anti-TNF biologics, long-term safety profiles obviously remain to be determined.

Autoantigen-specific immune interventions, in contrast, bear the promise of lower systemic side effects, as they can be targeted to antigens that are exclusively expressed in the diseased organ.[174,175] However, the efficacy might be lower and suitable target antigens have to be chosen carefully, because enhancement of autoimmunity is an important concern. The goal is either deletion of aggressive autoreactive T cells or induction of regulatory cells.[176] To achieve the latter, response modifiers are probably required at the time

of immunization in order to skew the resulting immune response to exert regulatory effector functions. Deletion of autoaggressive lymphocytes or anergy induction is even more risky, as only suboptimal immunization (ie, in the absence of costimulators) will result in this outcome. To control this in vivo is rather difficult. Ultimately, antigen-specific therapy will likely have to

be individualized due to MHC polymorphism and distinct T-cell repertoires between individual patients and should be combined with other systemically acting agents, for example, antibodies against CD3 or CD4 or costimulation blockade. Indeed, there is intriguing evidence along these lines in animal models: Combination of a non-Fc-binding anti-CD3 antibody with mucosal immunization of insulin or insulin-derived peptides exhibited clear synergy and enhanced efficacy in reversing recent-onset diabetes in the NOD.[177] In this case, induction of insulin specific adaptive T_{reg}s and their effector function were enhanced and protection by these T_{reg}s was transferable and highly effective in that it reversed recent-onset T1D.

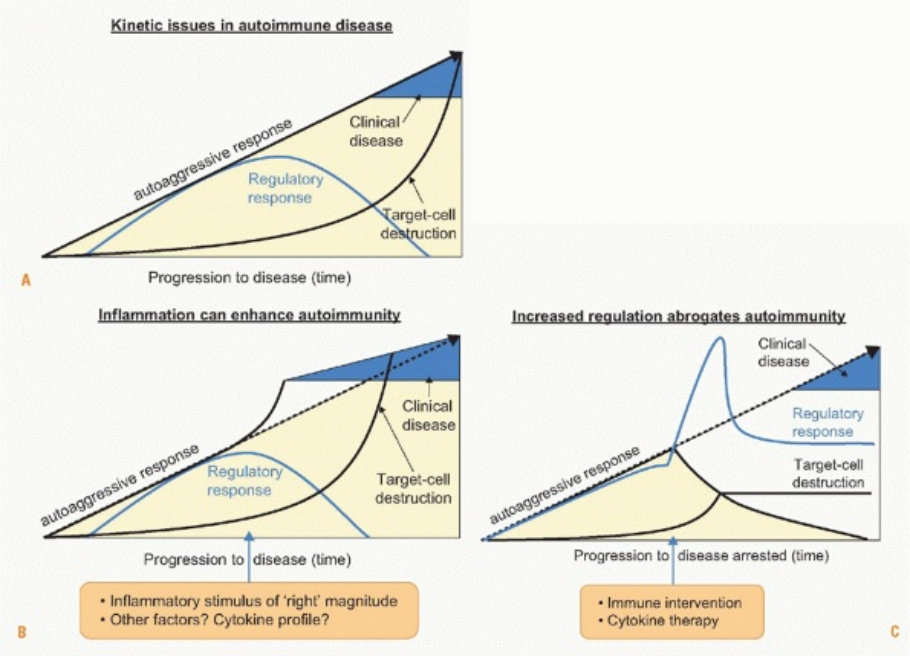

FIG. 44.4. Kinetic Issues in Autoimmune Diseases. A: Clinical manifestation of autoimmunity is the consequence of a dysequilibrium between protective (regulatory) and aggressive (effector) responses. An important consideration is that the destruction of the target cell or organ will usually lag somewhat behind the peak of the aggressive responses, because organ regeneration is common. **B:** Inflammatory stimuli will augment the effector arm of the autoreactoive response resulting in more rapid disease development. **C:** In contrast, induction of regulatory T cells can delay or dampen organ destruction.

Promising Targets

For anti-inflammatory interventions, the factor to be targeted should be as disease specific as possible. Therefore, blockade of TNF works well for RA but not for diabetes or MS.[178] Experimental evidence supports this observation because TNF is a crucial mediator found to be elevated in affected joints,[172] but has clearly positive, as well as negative effector functions in murine models of MS and diabetes.[179,180] Targeting ubiquitously present chemokines or cytokines will likely not bear much success because of the resultant

generalized immune modulation or suppression.

For antigen-specific interventions, antigens that are already targeted by regulatory autoreactivity are likely to constitute good targets to augment such a preexisting response.[181] In contrast, these antigens should not be selected to delete autoreactive cells, which results in a loss of regulation. For anergy or apoptosis induction, antigens targeted by a primarily aggressive response will be better suited. The fact that autoantigenic and epitope spreading occurs during each ongoing autoreactive process makes such interventions difficult to design, and individualization will likely be necessary.

Reestablishment of Tissue-Specific Immune Regulation

One of the factors that pose a challenge to understanding the pathogenesis of distinct autoimmune diseases may also hold a clue to developing effective and specific treatment strategies. Each target cell, tissue, or organ exhibits specific features that distinguish it from other sites of the body. These site-specific features of autoimmunity will

likely offer unique target sites for interventions with lower systemic side effects. However, one concern is that reestablishing proper immune homeostasis and regulation in one organ may still affect homeostasis systemically or at another site. Therefore, thorough preclinical evaluation and careful monitoring of undesired effects is urgently needed. Ideally, treatments have to be administered before complete organ destruction has occurred in patients identified by genetic or other screening to be at risk to develop full-blown clinical disease. During the preclinical state, frequently regulatory autoreactive responses are still strong and their augmentation can result in protection (Fig. 44.5). During advanced stages of autoimmunity, T_{reg} function or susceptibility of effector T cells to regulation might decrease.[182] The goal to reestablish homeostasis and proper regulation after an initial insult that caused organ-specific inflammation appears a natural countermeasure to which the specific immune system may be successfully harnessed. An intriguing example is the emerging concept of organ repair that can potentially be enhanced by drug therapy. In T1D, regeneration and replication of β cells can possibly occur after giving exenatide, a GLP-1 agonist, which is an established intervention in type 2 diabetes and under investigation in combination with other drugs such as anti-CD3 in T1D.

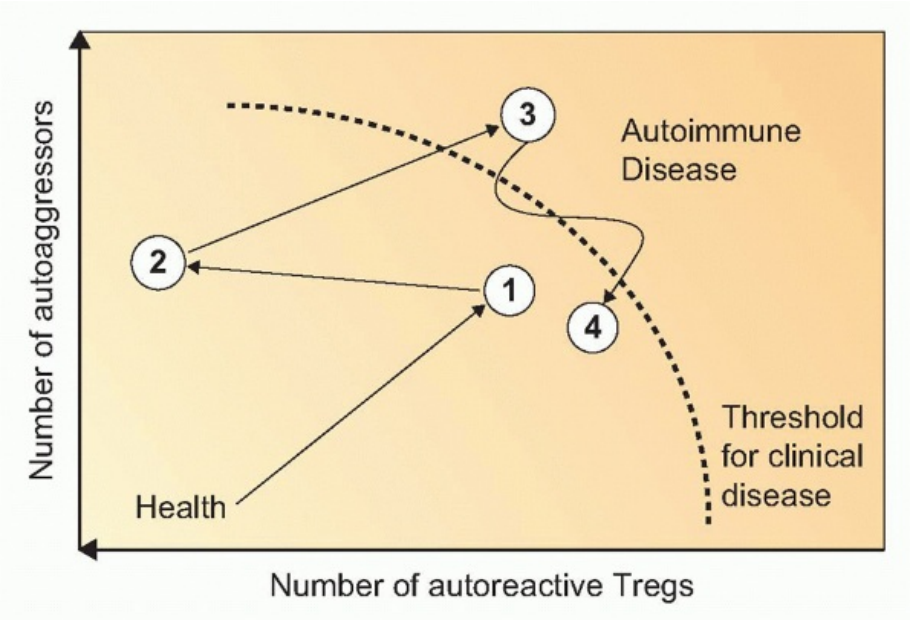

FIG. 44.5. Development of Autoimmune Disease as a Function of Aggression versus Regulation. Following induction of disease **(1)**, distinct numbers of autoaggressive and autoreactive regulatory T cells will be generated and activated. Undulations between protective states and diseases states follow **(2, 3)** (ie, the so-called honeymoon phase in T1D), which can eventually lead to an irreversible state of clinical manifestation of disease **(4)**. An additional important component to factor in is the ability of the target organ to regenerate.

INDIVIDUAL AUTOIMMUNE DISORDERS

A comprehensive and balanced discussion of autoimmune and autoinflammatory diseases at large is well beyond the scope of this chapter. Our choice of individual diseases for this section of this chapter was guided by their relevance to human health (ie, disease prevalence and severity), recent insights into pathogenetic mechanisms, as well as the development of novel treatment modalities. Given these limitations, we extend our apologies to all those scientists and clinicians whose work on autoimmune disorders is not mentioned in our discussion.

Endocrine Autoimmune Diseases

Thyroid: Graves Disease and Hashimoto Thyroiditis

***Introduction and Disease Description*.** The year 1956 was a seminal year for the field of human autoimmunity given the discoveries of Hashimoto thyroiditis as an autoimmune disease and of Graves disease as caused by an autoantibody. These discoveries have prompted some straightforward and relatively uncomplicated treatments.[183,184] In Graves disease, autoantibodies directed against the thyroid-stimulating hormone receptor on thyroid cells stimulate excessive production of thyroid hormone, which is normally controlled by feedback regulation. In contrast, in Hashimoto thyroiditis, autoantibodies to thyroid peroxidase and thyroglobulin are present over years and likely able to fix sublytic doses of complement to cells of the thyroid. The result is an inflammatory reaction, which is also

associated with T cell-mediated cytotoxicity. Thyroid damage due to painful Hashimoto thyroiditis may be associated with the development of Graves disease, indicating that there is a tendency for spreading of the autoimmune reaction in humans. Treatment of thyroiditis is relatively straightforward with antithyroid drugs (methimazole) and radioactive iodine.

Autoimmune Features. Autoantigens targeted in thyroiditis are thyroid peroxidase, a cell surface protein (Hashimoto thyroiditis), and the thyroid-stimulating hormone receptor (Graves disease). Autoimmune responses to thyroglobulin are also seen in animal models. The B-cell epitopes to these autoantigens have been mapped relatively well; however, as it is the case for other human autoimmune disorders, T-cell responses, their tracking and specificity, as well as their eliciting antigens in humans have remained largely elusive.[183] An interesting genetic link has been established to the CTLA-4 region, which is supported by the finding that NOD mice that also exhibit a link to this gene may present with thyroiditis. The usual suspects (ie, APC dysfunction, autoaggressive lymphocytes, and links to viral infections) have been examined, but no conclusive etiologic or mechanistic evidence has been obtained to date. It is noteworthy that a role for T_{reg} cells has been established in Mason's animal model for thyroiditis.[185]

Genetic Features and Environmental Factors. Interestingly, numerous viruses have been implicated in the pathogenesis of different thyroid diseases, but firm evidence for a direct involvement of viruses or virus-induced immune responses leading to clinically manifest disease is scarce. Subacute thyroiditis is a clinical and pathologic form of thyroid involvement that appears after infection with viruses such as measles, influenza, adenovirus, Epstein-Barr virus, and Coxsackie virus.[186] Again, however, a causative role in vivo has not been shown for any single infectious agent.[187] Presence of viral material in the thyroid and elevated virus-specific antibody titers were found to correlate with subacute thyroiditis.

In other instances, direct virally induced thyroiditis has been documented epidemiologically with thyroid or parathyroid disease.

Retrovirus, in particular human immunodeficiency virus (HIV) infections have generated much interest. Although HIV infection and acquired immunodeficiency syndrome (AIDS) may affect multiple endocrine organ systems,[188,189,190,191] thyroid dysfunction usually reflects weight loss, anorexia, and cachexia of advanced HIV disease, rather than a direct viral effect on the thyroid.[192,193] Thus, direct involvement of the thyroid by HIV or by opportunistic infections is uncommon and may include subclinical hypothyroidism and “euthyroid sick syndrome.” The clinical relevance is probably limited, as overt hyperthyroidism or hypothyroidism does not occur with greater frequency in patients with HIV and AIDS as compared to patients with other nonthyroidal illnesses (HIV and the thyroid gland have been reviewed in Heufelder and Hofbauer[192]). Involvement of the parathyroid in patients with AIDS could be shown by reduced basal and maximal parathyroid hormone levels, but the mechanisms underlying these findings have not yet been elucidated.[194] To account for a possible role of HIV in thyroid autoimmunity, a 66% homology between the HIV-1 nef protein and the human thyroid-stimulating hormone receptor has been noted. However, reactivity of sera of patients with Graves disease against a nef peptide showed no significant differences as compared to normal controls.[195] This does not rule out the presence conformationally shared T- or B-cell

epitopes with HIV proteins. In analyzing mechanisms of molecular mimicry, studies of potential antigenic surfaces have emerged as an important supplement to analysis of sequence similarity.[196]

In addition, antibodies from a patient with Graves disease showed reactivity to the gag proteins of another retrovirus, human foamy virus.[196] The association of human foamy virus with Graves disease or subacute thyroiditis is controversial. Whereas one study demonstrated human foamy virus-related sequences in the deoxyribonucleic acid (DNA) of peripheral blood in two-thirds of patients with Graves disease but none in normal controls,[197] another study could not confirm these findings.[198]

In other studies, an association between human T-lymphotropic virus I and II and the occurrence of autoimmune thyroiditis or Graves disease was reported.[198,199,200,201] Further, as in the case of HIV, hepatitis C virus was shown to lead to a wide variety of autoimmune disorders including involvement of the thyroid gland.[202,203,204,205,206] Moreover, treatment of chronic hepatitis B and C with IFNα leads to induction or enhancement of autoimmune disease.[207,208,209] For congenital rubella infection, which has been associated with diabetes mellitus, Addison disease, growth hormone deficiencies, as well as thyroid disorders,[210] it is not clear whether thyroid involvement is the result of a direct viral effect or a more generalized dysfunction of the immune system.[187]

***Animal Models*.** Animal models in mice and chicken have been used to study virus-induced thyroiditis.[187] Mice persistently infected with LCMV showed reductions of thyroglobulin messenger RNA and circulating thyroid hormones in the absence of thyroid cell destruction.[211] Thyroiditis characterized by focal destruction of the follicular structures, inflammatory infiltration, and generation of antibodies against thyroglobulin and thyroid peroxidase was observed in a reovirus type 1 mouse model of thyroiditis.[212] The reovirus gene responsible for autoantibody induction was identified and the encoded polypeptide shown to bind to tissue-specific surface receptors.[213] Spontaneous lymphocytic infiltration of the thyroid is observed in the obese strain of chickens. Such chickens express an endogenous retrovirus, avian leukosis virus (ev22), not found in healthy normal inbred strains.[214] Although ev22 appears to be a genetic marker rather than cause for thyroiditis, infection of normal chicken embryos with avian leukosis virus can cause hypothyroidism.[215] Moreover, aberrant MHC class II expression is demonstrated in obese strain chickens and elevated levels of 2.5-oligoadenylate synthetase as well as 2,5 oligoadenylate-polymer levels in the cytosol of thyroid epithelial cells occur, suggesting viral involvement.[216] Again, not all cross-reactivities with self-ligands need to increase autoimmunity,[217] and regulatory cells also play a major role in modulating autoimmune thyroiditis.[185]

Endocrine Pancreas: Type 1 Diabetes

***Introduction and Disease Description*.** While the distinct symptoms of diabetes mellitus have been known since antiquity, the underlying pathophysiologic processes were only identified in the late 19th and early 20th centuries. The proof of the involvement of the pancreas in diabetes etiology was conducted by von Mering and Minkowski,[218] who

demonstrated in 1890 that extirpation of the canine pancreas results in the classic symptomatology of hyperglycemia, abnormal hunger, increased thirst, polyuria, and glycosuria. Subsequently, inflammatory changes in the endocrine pancreas (ie, the islets of Langerhans) were correlated with diabetes by Schmidt[219] in 1902; two decades later, Banting and Best[220] identified insulin as a pancreatic hormone, thereby providing the basis for insulin substitution therapy, which remains to this day the cornerstone for T1D management. In a classic 1965 paper, Gepts[221] noted the histopathologic similarity between thyroiditis and insulitis and suggested an immune basis for the disease. By 1974, the concept of T1D as an autoimmune syndrome was firmly established by the discovery of islet cell antibodies and an association between T1D and certain HLA genes.[222,223,224]

Autoimmune Features. Today, a quarter century later, the possible autoimmune origin of T1D is understood in much greater detail. However, the lymphocytic infiltration of the islets of Langerhans (Fig. 44.6) and the presence of antibodies specific for β-cell antigens associated with the progressive destruction of insulin-producing β cells[225,226] still constitute the cardinal evidence for an autoimmune etiology. While there is a reasonably strong genetic linkage to certain HLA molecules, the disease has to be considered polygenic in nature,[130] and a significant discordance of disease among monozygotic twins suggests that environmental factors contribute to trigger and/or exacerbate the disease. Furthermore, it remains unclear which islet antigens are the

primary targets. The earliest islet cell-specifc antibodies in human individuals at risk are directed to insulin,[227,228] and evidence from relevant animal models and human studies points toward insulin as a primary antigenic target,[229] islet-specific glucose-6-phosphatase catalytic subunit related protein,[230,231] and possibly glutamic acid decarboxylase (GAD),[232] but definitive proof for a pathogenic role has only been ascertained in the NOD mouse model and not yet in humans.[233] The cellular infiltrates found in the islets contain both CD4+ and CD8+ T lymphocytes and their irreducible role in β-cell destruction has been documented in several animal models.[234,235,236] CD8+ T cells can exert direct cytotoxic effects toward MHC class I-expressing β cells, while CD4+ T cells secreting inflammatory cytokines and can provide help to CD8+ T cells as well as B cells. Ultimately, it is important to consider that β cells constitute about 60% of the islets, which in turn contribute only ~2% to the pancreas mass and demonstrate, unlike many other tissues targeted in autoimmune disorders, an exquisite sensitivity to cytokines such as IL-1, TNF, and IFNs that will result in their apoptotic cell death after prolonged exposure.[94]

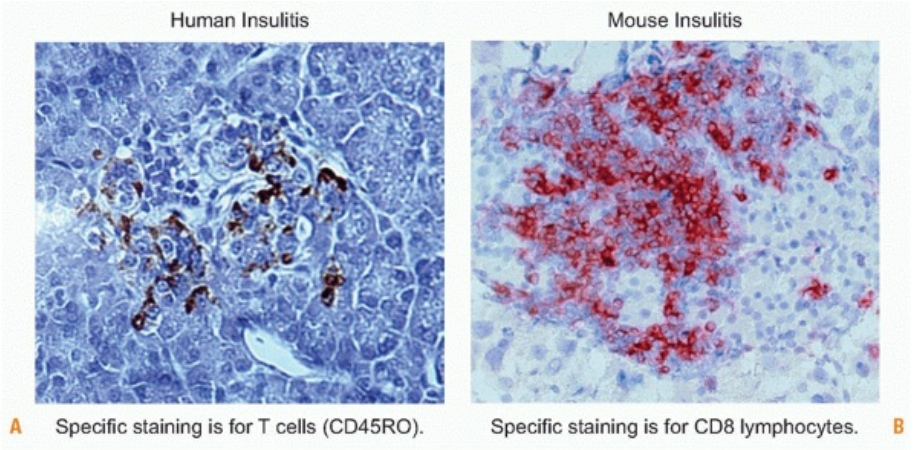

FIG. 44.6. Human and Mouse Insulitic Lesions. Comparison of human insulitis *(Left)*, courtesy of Francesco Dotta (University La Sapienzia, Rome, Italy), and mouse insulitis *(Right)*, from a rat insulin promoter-lymphocytic choriomeningitis virus mouse 14 days postinfection with lymphocytic choriomeningitis virus (von Herrath laboratory, La Jolla Institute for Allergy and Immunology, La Jolla, CA).

The detection of islet antigen-specific antibodies remains an essential tool in identifying prediabetic subjects and monitoring the progression of subclinical and clinical disease. Procedures for autoantibody determination have been substantially refined and standardized worldwide. Emerging data from clinical studies support the notion that with progression of the prediabetic phase, generation of islet antibodies also is increased.[237,238,239] Usually, antibodies to insulin become discernible first, then to GAD, thereafter to insulinoma antigen 2. Individuals with islet antibodies to three or more distinct antigens have a greater than 90% risk of developing T1D.[136] Thus, islet antibodies are an excellent marker for disease risk. However, they appear not to play a role pathogenetically, as transfer of antibodies from mothers to children does not increase the risk for T1D, and B cells are not needed for human diabetes.[240,241,242] In this crucial respect, the NOD mouse appears to provide a paradigm that might not be applicable to human diabetes, as maternal antibodies are an essential factor for diabetes development in NOD offspring.[243]

Measurements of human T-cell responses to islet antigens are not yet standardized and can vary considerably between different laboratories. One reason for this may be the source of T cells that are generally subjected to specificity analyses: blood-borne CD4+ or CD8+ T cells may not reflect the specificity distribution and frequencies of isletspecific T cells found in the target organ (ie, pancreas and its draining lymph nodes). Even the study of spleen-derived islet-specific T cells readily obtained from NOD mice and analyzed in standardized proliferation assays has shown variations between different NOD colonies.[244] Therefore, measurement of multiple effector functions (ie, cytokine production, etc.) in highly standardized assays will likely be required to assess T-cell autoreactivity on a routine basis.[245] A recent study shows that while techniques such as enzyme-linked immunosorbent spot can discriminate between patients and controls, difficulties in detection of low-frequency antigen-specific T cells probably leads to the limited reproducibility of such

measurements.[246] Another complicating fact is that T-cell responses between individuals are expected to vary and depend on the HLA haplotype and individual trigger(s) that precipitate T1D. An emerging technology that may improve specificity and sensitivity is through the use of combinatorial HLA multimers loaded with multiple disease-associated epitopes.[247] A remarkable paper[248] has shown that tracking of immune responses to naturally processed peptide epitopes can discern between healthy individuals and those afflicted with T1D: Whereas the number of overall T cells reacting with the naturally

processed peptide epitopes was similar, individuals with T1D produced more IFNγ relative to IL-10, whereas the ratio was the opposite in healthy individuals.

***Genetic Features and Environmental Factors*.** Recent data show that concordance rates among monozygotic twins are higher than previously thought.[249] Still, the remaining discordance and particularly the major temporal differences in clinical onset indicate that environmental factors have to act in concert with diabetes susceptibility genes to orchestrate the autoimmune destruction of β cells. The initial hope that only a few genes would contribute to disease pathogenesis and that genetic links would help to directly understand the mechanistic aspects of T1D pathogenesis has been progressively eroded. Instead, a complex network of susceptibility and resistance genes in both humans and animals (eg, the NOD mouse) has slowly taken shape. The recent publication of three GWAS has extended the list of gene associations in T1D. So far,[16] robust T1D-associated loci were discovered, some of which encompass important immunity genes (eg, IL-2, IL-2Rα [CD25], CTLA-4) or β cell-specific products (insulin), while other candidate genes do not have an apparent link to immune functions and/or β cells. The implications of this enhanced genetic characterization have so far not led to the identification of monogenic pathways that directly lead to disease. Rather, consensus is growing that disease susceptibility is the consequence of a global problem of immune regulation, with many genes involved in a variety of combinations in individual patients.[250]

Many of these genes exhibit direct parallels to NOD diabetes susceptibility genes.[251]

The association between particular HLA/MHC class II haplotypes and the occurrence of human diabetes has been of particular interest. The DRB1/04-DQA1/0301/B1 and DRB1/03-DQA1/0501/0201 strongly predispose to T1D, and more than 80% of patients carry either one or both alleles.[252] In contrast, other MHC class II haplotypes can protect from disease, as evidenced by the sixfold reduced risk to T1D in DRB1/15-DQA1/B1/0602-bearing individuals. Considerable evidence indicates that other genes in the MHC region likely contribute significantly to T1D risk.[128]

An intriguing mechanistic hypothesis was put forth by McDevitt's observation that predisposing HLA class II alleles appear to express small neutral amino acids at position 57 of the DQ allele of Caucasoid populations, whereas an aspartic acid is found in resistant alleles at the same position. Because position 57 is part of the peptide-anchoring pocket, amino acid substitutions in this area will affect peptide binding. Indeed, the susceptibility alleles prefer different peptides, but the contribution to T1D development is not yet clear and a mechanistic link has to be established. Both central and peripheral tolerance mechanisms have been implicated but no direct proof has been obtained. It is important to realize that the

human susceptibility MHC class II alleles share amino acids at position 57 with the I-A^{g7} alleles expressed in the NOD mouse and are required for NOD T1D predisposition. However, as previously mentioned, polymorphisms in the MHC class II coding region alone cannot explain diabetes pathogenesis. The amount of complexity involved in the immunogenetics of T1D has been well described by Serreze[253]:

> Many genes contributing to T1D may contribute to dysregulation of different biochemical steps in a common developmental or metabolic pathway. For example, sequential expression of hundreds, if not thousands, of genes would be expected in the developmental and functional maturation of a macrophage or dendritic cell from stem cell precursors. This process does not occur in a vacuum, but is contingent upon cues provided by the physical environment. In the case for APC development, the microfloral and dietary environments are crucial.

Thus, diabetes susceptibility and resistance genes contribute to disease in a polygenetic/multifactorial fashion that appears to gain in complexity as it is being unraveled. The link to environmental factors will be defined to shape gene expression and disease development. Major contributors in this respect appear to be the gut and viral infections.

With more than 400 m^2 of mucosal epithelium, the gut constitutes the largest interactive surface area of the human body connecting us with the environment and its pathogens.[150] Therefore, exposure to antigens or pathogens through the gut, mediated by the largest outpost of the immune system, the gut-associated lymphoid tissue, will strongly affect specific and general immune functions. It is intriguing that immune tolerance to the numerous foreign protein antigens found in food, as well as bacterial antigens derived from the commensal flora, is generally well maintained.[254] This may be attributable to the high levels of immunoglobulin (Ig)A and TGF-β in the gut and to the phenomenon of "oral tolerance."[255] Oral tolerance has been observed in animal models and humans and is characterized by tolerance induction to protein antigens present in the gut. It occurs via two principal mechanisms: Low amounts of antigens will induce a nonaggressive immunoregulatory response while high amounts of antigen can lead to lymphocyte anergy or deletion,[256,257,258] which is likely achieved via APC modulation. In addition, the profound immune dysregulation found in the absence of a bacterial flora in both animals and humans points to an important physiologic role that foreign antigens play in immune homeostasis in the gut.[259] Furthermore, NOD mice only exhibit high levels of autoimmunity when kept in a clean, specific pathogen-free environment; they do not develop T1D when housed under "dirty" conditions.[260] A seminal paper by Wen et al.[261] highlighted the pivotal role of the mucosal immune compartment by showing that host recognition of the digestive flora is essential in preventing T1D through engagement of a myeloid differentiation primary response gene 88-independent signaling pathway. Thus, changes in the intestinal microbiome, integrity of the mucosal barrier, infections of the gut, or certain dietary components may play a role in T1D pathogenesis.

Several reports and studies have attempted to establish a link between the introduction of

cow's milk and development of T1D in young infants. This link was not observed in the German, Australian, and American baby diabetes studies but was in a Finnish epidemiologic study.[262,263] The Finnish study differed from most of the others by an

extended observation time involving infant as well as childhood consumption of cow's milk. Therefore, a dietary link between milk feeding and T1D can be considered unlikely but not excluded after long-term exposure to cow albumin or other milk proteins. Similarly, wheat-derived gluten and milk-derived insulin have been implicated as a cause for childhood diabetes. The evidence, however, is not convincing at this point, and no firm links have been established.

Some intriguing observations have been published more recently supporting the concept of a viral etiology for T1D. The mechanistic links between viral infections and autoimmunity can be manifold and have been discussed in detail in the introductory section of this chapter. A significant association between rotavirus infection in young infants and the first occurrence of islet autoantibodies was established by Harrison's group in Australia[264] but not in Finnish populations.[265] Rotavirus is a double-stranded RNA virus, infects the intestinal mucosa, and is a common cause for seasonal childhood diarrhea. It can polyclonally activate T and B lymphocytes, and might possibly harbor antigens that could immunologically mimic islet cell-derived self-proteins. However, it is not clear whether it infects the pancreas or islets directly. The most convincing case can be made for enteroviruses, and there is now robust evidence for a significant association with T1D.[266] Coxsackie B4 virus has been isolated from islets of a child with acute-onset T1D,[267,268,269] and Coxsackie B3 and 4 strains commonly infect the gut, pancreas, and heart.[270] They lead to profound pancreatitis if they replicate at high enough titers and might harbor a mimicry antigen (P2C protein)[226] cross-reactive on the T-cell level with a human GAD epitope. However, this evidence could not be replicated by other laboratories and is still controversial. It has become apparent, however, that Coxsackie B3 viral strains can effectively trigger[271] or prevent[142] T1D in the NOD mouse, depending on the timing of infection. Thus, diabetogenicity of a viral infection may critically depend on the preexistence of insulitis and may either act as an initiating factor or by aggravating ongoing inflammatory processes. Clinical studies have recently provided indirect support for both of these scenarios.[272,273] The finding that infections under certain conditions confer protection might support the “hygiene hypothesis,” which suggests that infections protect from rather than enhance autoimmunity. It is still uncertain whether enteroviruses routinely infect or persist in pancreatic islets, although recent reports indicate that viral particles are found more frequently in islets of patients with T1D around the time of disease onset.[274] Similar to Coxsackie, other enteroviruses such as polio or echoviruses have been detected in the pancreas and might therefore at least have enhancing effects on ongoing islet destruction in prediabetic individuals at risk.[267] The establishment of a firm association between viral infections and T1D is difficult because the underlying mechanistic links established in several animal models allow for the virus to be cleared before autoimmunity develops (ie, in the rat insulin promoter [RIP]-LCMV model); viruses need not necessarily directly induce islet-reactive T-cell responses but can act as bystander activators and, in many cases, viral infections have been found capable of preventing autoimmunity.

Exciting new data suggest that the genetic constitution may (co-)determine whether viral infections will provoke islet autoimmunity in certain individuals. It was found that certain rare polymorphisms in the IFIH1 gene are associated with protection against T1D,[275] while others confer disease risk.[276] This gene encodes a helicase enzyme, IFIH1 (also known as MDA5), which triggers the secretion of IFNs in response to viral infection. Most, if not all, viral infections trigger the production of IFN by the host immune system. Being an IFN-response gene, IFIH1 allows the infected cell to sense the RNA genome of enteroviruses and increase IFN production. IFNs then primarily limit viral replication to prevent damage to the infected cell. They also, however, increase the visibility of the infected cell to the immune system by enhancing the expression of MHC molecules. A scenario could be envisioned where mutations leading to decreased IFIH1 activity are associated with milder responses against diabetogenic viruses and thus disease protection. Conversely, mutations leading to IFIH1 hyperactivity could induce inappropriate, exaggerated IFN responses and localized immunity against the infected β cells. Altogether, such a mechanism would explain why viruses that have presumed diabetogenic effects do not necessarily trigger diabetes in all infected individuals.[277]

***Animal Models*.** Because the pancreas and its draining lymphoid organs are notoriously difficult to access, many important insights about diabetes immunology have been gained from suitable animal models that continue to refine our understanding of the pathogenesis and the development of potential prophylactic and therapeutic strategies. There are a multiplicity of animal models for T1D. The most commonly employed models take advantage of natural mutations that give rise to spontaneous diabetes onset or antigen-specific induction of disease using transgenic technology. Other models make use of β-cell damage initiated by treatment with specific chemicals (eg, streptozodozin) or virus infection. Encephalomyocarditis virus is diabetogeneic in mice; the incidence of disease is dependent on both virus and mouse strains used.[144,268,269,278,279,280,281,282,283] Similarly, Coxsackie virus, associated with diabetes development in humans, causes extensive pancreatic tissue damage and release of sequestered autoantigens that lead to rapid diabetes development in some mouse strains.[284]

Models of Spontaneous Diabetes Onset. There are several animal models of spontaneous T1D.[285] The two most extensively used are the biobreeding rat, introduced in 1974 at the Bio Breeding Laboratories in Canada, and the NOD mouse strain established 1974 in Osaka, Japan.[285] Because the biobreeding rat is associated with leukopenia and other abnormalities,[285] the NOD mouse has been the model of choice due to its genetic linkages that are reminiscent of human T1D.[285] In both models, adoptive transfer of T cells can induce disease.[286] Interestingly, viral infections, first shown with LCMV, can prevent insulindependent diabetes mellitus in both biobreeding rats and NOD mice.[287,288] This occurs in the absence of a general immune suppression. While the mechanism involved is unclear, the generation of suppresser T cells has been suggested.[288,289]

Models of Antigen-Specific Diabetes Induction. Expression of influenza virus hemagglutinin (HA) under control of the RIP resulted in a low incidence (10% to 20%) of spontaneous T1D, but and no significant enhancement of disease occurred after infection of such RIP-HA mice

with influenza virus. However, in RIP-HA mice expressing a transgenic TCR specific for a determinant of the influenza virus HA, the incidence of spontaneous T1D increased to 100%.[290] These studies clearly demonstrate the importance of autoreactive T cells present at sufficient numbers for induction of autoimmune disease. Thus, the inability of influenza strain PR8 to cause T1D in RIP-HA mice likely reflects the low precursor frequency of self-reactive CD8+ T cells. Interestingly, studies from the Sherman laboratory have demonstrated that spontaneous disease in this model is associated with cross-presentation of HA. It has been postulated that crosspresentation constitutes an important mechanism for unmasking of islet-specific antigens to autoaggressive CD8+ T cells. When the model antigen ovalbumin was expressed as a self-antigen in β cells using the RIP, similar observations were made underscoring the likely importance of crosspresentation in autoimmune diabetes. These studies also demonstrated that the clinical outcome correlated with the number of autoreactive CD4+ and CD8+ cells generated and determined a "cut off level" below, which the autoimmune reaction would not take hold of the pancreas.[291,292,293]

Similar to RIP-HA and RIP-ovalbumin mice, the RIP was also used to express the nucleoprotein or glycoprotein of LCMV in the insulin producing β cells.[234,294,295] Diabetes develops 2 to 4 weeks after infection with LCMV due to a strong CD8+ or combined CD8+/CD4+ T-cell response directed to the viral/self-glycoprotein or nucleoprotein in the β cells. Insulitis is initiated only at a time when the systemic antiviral response reaches its peak and continues well after the LCMV infection has been eliminated.[130,296] Therefore, the localized, islet-specific autoimmune process, although initiated by a response to the viral/self-transgene, can be regarded as an autoimmune process that follows kinetics distinctly different from systemic antiviral immunity. Indeed, antigenic spreading to insulin and GAD is observed during the prediabetic phase.[237] Destruction of β-cells requires activation of APCs[297] in the islets and is mediated by both perforin and inflammatory cytokines, predominantly IFNγ.[298] Thus, the RIP-LCMV model reproduces many features found in human diabetes as well as other mouse models. A distinct advantage of RIP-LCMV mice is that the time point for induction of the autoaggressive LCMV nucleoprotein specific response can be chosen experimentally, and that virus-specific, destructive CD4+ and CD8+ T cells can be enumerated, functionally evaluated, and localized using limiting dilution analyses, MHC tetramers, or intracellular cytokine stains.[299,300] These aspects constituted an advantage in our studies demonstrating that feeding of insulin during the prediabetic period induces insulin β-chain specific CD4+ regulatory lymphocytes that act as bystander suppressors and can locally downregulate the autoaggressive dibetogenic response in the pancreatic draining lymph node and the islets.[300] Other studies in the RIP-LCMV model have underlined the role of thymic selection in allowing sufficient numbers of low-avidity autoaggressive T cells to emerge in the periphery,[301] the role of non-MHC-linked genes in influencing the kinetics and severity of T1D even in the presence of high numbers of autoaggressive T cells,[300] and the importance of APCs in breaking tolerance and sustaining autoaggressive T-cell responses.[302,303,304]

Under some conditions, CD4+ or CD8+ T cells can induce T1D by adoptive immunization.[286,305] In general, T1D develops more slowly or not at all in the absence of

CD8+ CTL, MHC class I, or perforin.[94,306] Similar considerations are true for MHC class II and costimulatory molecules, unless their elimination affects the generation of regulatory lymphocytes.[307,308] Cytokines, however, frequently play dual roles in T1D pathogenesis in animal models. IFNγ, in general known as a proinflammatory mediator that upregulates MHC molecules, not only can "unmask" β cells for immune recognition by induction of MHC class I expression but also exert direct antiviral effects and might be beneficial by increasing apoptosis of aggressive T lymphocytes later in the disease.[94] A similar dual role is true for TNF-α. that appears to enhance early disease (possibly by directly causing β-cell death in conjunction with other cytokines) but ameliorates advanced autoimmunity just prior to onset of clinical disease.[170,309] For these reasons, blockade of such inflammatory mediators might be problematic in T1D. Dual roles were also described for IFNα,[310,311,312] IL-2,[313] and IL-10.[314,315] In some of these studies, the level of the cytokine might play as important a role as the precise timing of expression. The only cytokine with largely beneficial effects has been IL-4, which ameliorates the disease and therefore might be a good candidate for treating or preventing T1D.[316] Figure 44.7 illustrates the pathogenetic hypotheses generated through research in multiple animal models.

***Treatment and Prevention*.** Despite intensive research, no effective prophylaxis, therapy, or cure of T1D is available to date. However, even under optimal disease management with insulin substitution therapy, T1D significantly shortens life expectancy due to eventual vascular complications in multiple organs. Currently, all strategies that look promising have either been evaluated in animal models or are in early clinical trials in humans (see following paragraphs). Part of the problem in devising such prophylactic or interventive immune-based approaches is that the only end points in human trials are disease prevention and insulin requirements as well as remaining insulin production (C-peptide levels). No precise interim staging is yet possible, which makes efficient clinical evaluation very difficult compared to MS or RA, where access to the target organ either visually by magnetic resonance imaging or directly by sampling fluids is much easier to achieve. Animal models have provided interesting ideas and evidence for a variety of antigen-specific and systemic interventions that bear promise for human diabetes.

Antigen-specific Immunoregulation. Immunization with DNA plasmids that express islet self-antigens, with or without cytokines, which act as response modifiers, can induce autoreactive regulatory CD4+ T cells. These cells are able to

suppress autoaggressive CD4 and CD8 cells locally in the pancreatic draining lymph node where they act as bystander suppressors. Phenotypically, they behave very similarly to the Th2-like regulators[181,317,318,319,320] induced after oral antigen administration. In treated mice (using several distinct diabetes models), insulitis is permanently reduced, and progression to clinical diabetes can be prevented in 50% to 80% of the animals.[175,181,317,318] To bring this approach to the clinic as a preventive therapy, a suitable marker will be needed that can predict early outcome, for example, levels and isotypes of autoantibodies. Like other antigen-specific interventions, this strategy will not likely be effective in late stages of the prediabetic phase and should therefore be thought of and tested as an early preventive therapy. The important advantage of this and other antigen-specific approaches is that the risk for systemic side effects is low, as the effector

cells will act antigen specifically only in the area, where autoimmune destruction is ongoing and their cognate antigen (eg, GAD, insulin, or heat shock protein) is presented. Discouraging results from recent clinical trials indicate that, while highly effective in animal models, translation of antigen-specific approaches to the clinic remains a challenge.[321] It may thus be possible that successful antigen-specific therapy will require a combination with systemic immune modulators. For example, we have demonstrated that a combination treatment with intranasal proinsulin peptide and anti-CD3-specific antibody can reverse recent-onset diabetes in two murine diabetes models with much higher efficacy as compared to monotherapy with anti-CD3 or antigen alone.[177] Despite the strong rationale to tackle the disease from different angles, drug companies are generally reluctant to include successful drugs in a combinatorial regimen.[322] A specific subtype of antigen-specific approaches is the design of altered peptide ligands: Modified insulin peptides that favor a Th2-like deviation of responder cells have been developed by Neurocrine (San Diego, CA) and showed great promise in the NOD mouse model. These succesfully passed preclinical safety studies,[323,324,325] but efficacy outcomes in the first clinical trial were disappointing.[326]

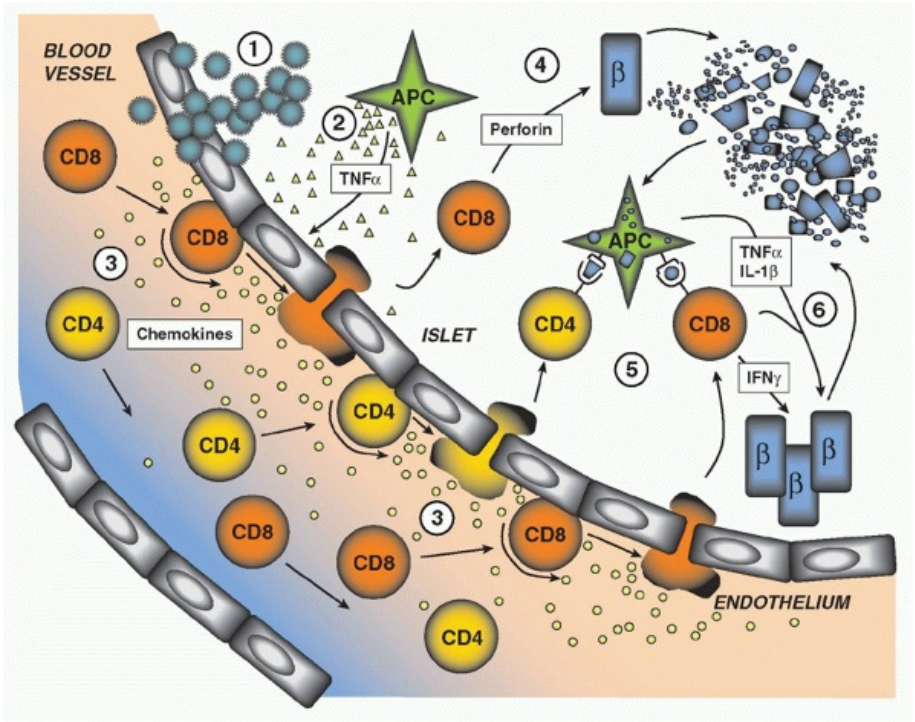

FIG. 44.7. Immunopathogenesis of Type 1 Diabetes. Viral infections and other inflammatory stimuli **(1)** can activate antigen-presenting cells (APCs) **(2)**, which secrete chemotactic mediators that entice lymphocytes in blood vessels to “rolling” and entry into the pancreatic islet tissue **(3).** Entering lymphocytes might directly destroy β cells **(4)** or activate APCs locally **(5)** and also propagate inflammation by secreting proinflammatory cytoines **(6)**. Recent intravital imaging data suggest that effectors may remain in a given islet for hours while inducing β-cell death.[559] Courtesy of Urs Christen, University of Frankfurt, Frankfurt, Germany.

Finally, induction of oral tolerance can be seen as a form of mucosally targeted antigen-

specific therapy. As for MS and RA, oral antigen trials have failed so far for T1D, except for in a distinct subgroup of patients,[327] spurring repeat investigations that are currently ongoing. The reasons for these failures after initially promising animal data have become increasingly clear from follow-up animal experimentation.

Likely, the dose of antigen was significantly too low (presumably by a factor of 10 to 100). This problem may be overcome by coupling the orally administered proteins to gut response modifiers such as the cholera toxin B subunit. Second, the choice of antigen is important, and even minor amino acid changes can modify the dose-response curves. Without a reliable preclinical marker, it will be very difficult to tune this treatment to the human situation.[259]

Therapeutic Failures and Future Perspectives

1. Anti-CD3 treatment: One of the more promising drug candidates from the past decade in terms of immune modulation in T1D was anti-CD3. The effector mechanism in mice is systemic and is likely relying on the induction of antigen nonspecific regulators[307] that are $CD62L^{hi}$ and $CD25^{hi}$ as well as anergy or deletion of activated aggressive lymphocytes. From data in animal models and humans, systemic immune suppression is not too profound, indicating reasonable safety for this strategy. Two independent randomized controlled phase III trials, however, showed minimal effectiveness of this approach for treatment of individuals recently diagnosed with T1D.[328] These notable and discouraging failures have raised questions concerning the trials' accuracy of dosing, choice of appropriate end points, and timing of administration. Finally, the inability to translate a therapy that is so potent in mice to humans may indicate that we have in the past relied too heavily on animal models to prioritize drug candidates.
2. CTLA-4Ig, anti-CD20, and anticytokine therapy: Encouraging data in mice, likely in combination with the availability of commercial compounds, have triggered a series of trials aimed at tackling islet autoimmunity from diverse angles. CTLA-4Ig (abatacept) modulates costimulation and prevents full T-cell activation. A recent trial shows that the drug initially induces a beneficial effect but also that continued administration did not offer lasting protection. The authors therefore speculated that T-cell activation in T1D may lessen with time.[329] Another study found that anti-CD20 therapy (rituximab; depletes B cells) partially preserved β-cell function over a period of 1 year in patients with T1D.[330] While the outcome was relatively modest, the finding that B lymphocytes contribute to the pathogenesis of T1D is of scientific value. Finally, clinical studies are underway to block cytokines such as TNF-α or IL-1β in order to eliminate their known deleterious effects on β-cell viability. It can be concluded from these modest successes that immunomodulatory regimens may hold potential (ie, that tackling the immune component from T1D is indeed a viable option). It is clear, however, that more potent or complementary strategies need to be evaluated in order to achieve significant and lasting protection.
3. Islet transplants: A promising therapeutic advance was made by developing a specific protocol for gentle isolation and purification of human islets (“Edmonton protocol”) that appear less prone to rejection after intraportal implantation into diabetic subjects.[331] However, continued immune suppressive treatment is still necessary in recipients of such transplants to prevent renewed autoimmune destruction of islet transplants emphasizing

the need for effective immune-based strategies of T1D prevention. A potential improvement was developed by the company Viacyte (San Diego, CA, USA) in the form of proprietary capsules that are designed to shield islet transplants from the deleterious effects of the immune system. The approach also encompasses a detailed procedure for the generation of viable β cells from human embryonic stem cells.[332,333] It is hoped that these advanced technologies will one day allow for the safe and lasting transfer of functional islets into patients with T1D.

***Perspective and Conclusion*.** No single animal model accurately reflects all aspects of human T1D. Consequently, translating the successful T1D prevention and reversion strategies developed in a variety of animal models into clinical applications faces significant obstacles, as is evident from failures in recent clinical trials. For example, to date, more than 140 therapeutic options have been developed to prevent T1D in the NOD mouse,[225] but not a single one that was tested in clinical trials is as effective in humans. To improve this situation, there is a dire need for preclinical markers that accurately predict the potential success of an intervention being evaluated by a clinical trial. Better tracking reagents for blood autoreactive T cells such as tetramers, noninvasive high-resolution in vivo imaging systems, and perhaps isotype profiles of islet antigen autoantibodies and their change after a given preclinical intervention are good candidates.[334] Similar considerations apply to the maintenance of longterm tolerance after islet transplantation, where prolonged systemic immunosuppression might not be feasible.[335,336,337,338] In addition, treatment modalities will likely have to be tailored to the specific needs of individual patients. Lastly, one could argue based on clinical studies using cyclosporine or nonmyeloablative stem cell transfer that a robust cure for T1D already exists, be it at a high cost in terms of patient safety and associated side effects. Future progress should therefore be aimed at walking the fine line between profound efficacy and optimal tolerability in order to favorably compare against lifelong insulin replacement therapy.

Central Nervous System and Occular Autoimmune Diseases

Multiple Sclerosis

***Introduction and Disease Description*.** MS is the most predominant human demyelinating disease of the CNS. An autoimmune etiology is suggested by elevated frequencies of myelin basic protein (MBP)-specific T cells documented in several independent studies of individuals diagnosed with MS.[339,340,341,342] Further, demonstrations that adoptive transfer of T cells specific for myelin or other CNS antigens can cause a CNS autoimmune syndrome in experimental animals resembling human MS support the concept of a direct T cell-mediated pathogenesis. In addition, myelin-specific and lipid-specific antibodies have been found in MS cerebrospinal fluid.[343] These antibodies are able to complement-fix

and induce antibody-dependent cellular cytotoxicity and may be involved in the demyelinating process. Despite these clues, the etiology of MS remains unclear, and a complex interplay of genetic and environmental factors (similar to T1D etiology) has to be postulated.

MS is a rather heterogeneous disease. However, we are only beginning to understand how clinical and pathologic differences may point toward distinct etiologies and, potentially, treatment strategies of differential applicability and efficacy. Different subtypes of MS are

somewhat better histologically defined (eg, the distinction between T cell-rich and macrophage-rich lesions as well as the balance between demyelination and remyelination, which can vary dramatically) than those of T1D as many tissues from patients with clinically active disease are available postmortem, whereas pancreatic islets are mostly destroyed in T1D and no immunologic correlates can be established at the end stage.[344,345] MS predominantly affects younger women, and its frequency, dependent on geographic location, may approach up to 3 in 1000.[346] Clinically, the disease can take a mild or very debilitating course, and, intriguingly, neuritis of the optical nerve is frequently the first sign for a beginning MS. Diagnosis is usually obtained by electroencephalogram and direct imaging that allows for identification of individual lesions, their development over time, and the success of therapeutic interventions. Immunebased approaches such as the use of IFNβ or Copaxone® (Teva Pharmaceutical Industries Ltd., Petach Tikva, Israel) combined with systemic immunomodulatory agents has brought some limited success for certain forms of MS. Thus, a cure for MS appears possibly a little closer than for T1D.

Autoimmune Features. Although MBP-lipoprotein and proteolipoprotein (PLP)-specific T cells have been documented in patients with MS, some follow-up studies have reported similar frequencies in normal individuals.[347] More recent studies have found increased numbers of MBP-specific T cells in patients with MS reactive to six different MBP epitopes, supporting the concept of determinant spreading as a potential pathogenetic factor. Furthermore, their activation appears to be increased selectively in patients with MS, and they are less dependent on costimulation.[348] Thus, it appears plausible that resting T cells specific for CNS antigens are present in both healthy and affected individuals, but their activation is selectively increased under conditions of disease and their epitope/antigen recognition repertoire is expanded. In addition to cellular infiltrations, MS is usually marked by edema and inflammatory signs such as MHC upregulation and cytokine and chemokine induction. A breakdown of the blood-brain barrier facilitates antibody access to the CNS. Antibodies specific for myelin structures and lipid components[349] are commonly found in patients with MS, and some studies indicate fluctuations similar to T-cell responses of antibody titers in parallel to exacerbations and remissions of disease. These findings still await confirmation in larger patient cohorts. It is important to note that such antibodies can be directed to MBP, myelin oligodendrocyte-associated protein, and other myelin constituents.[350] The fact that they can fix complement and enhance complement-mediated cytotoxicity implicates them as possible agents of demyelination.

***Genetic Features and Environmental Factors*.** There is a definite genetic link between progressive (HLA-DR3) as well as remitting relapsing MS (DR2) and human MHC class II alleles. However, similar to T1D, there is a significant discordance among monozygotic twins (>55%). A pronounced geographic gradient (higher incidence in northern as compared to southern regions) argues for additional environmental triggers or modulators.[346] Many viruses have been implicated in the pathogenesis of MS, but a causal link has not been established. For example, human herpesvirus 6 was discovered around MS lesions, but follow-up studies did not discover any differences comparing healthy, Alzheimer, or Parkinson tissues with MS lesions.[351] Furthermore, antibodies to corona virus have been detected in serum of patients with MS, which indicates that demyelination might occur similarly to the mouse hepatitis virus model. Mechanistically, the same paradigms as for diabetes might apply

(described in the introductory section), but a persistent viral infection might be more likely the culprit, based on similar observations in some mouse models (see the Theiler murine encephalitis virus [TMEV] model, for example). The difficulty in establishing conclusive proof is the multiplicity of infections during the course of a lifetime and the manifold viral traces that can usually be detected in healthy as well as diseased individuals. Even if the same levels of viral antigen, RNA, or DNA are found comparing healthy patients with patients with MS, this still does not rule out the possibility that a certain virus will only induce CNS disease in conjunction with a distinct MHC haplotype. Indeed, animal models indicate that this is in fact the case. With the advent of improved vaccination and antiviral treatment protocols for multiple infectious agents, we may be able to infer a causal relationship if the incidence of MS should recede after introduction of such an intervention.

***Animal Models*.** Experimental allergic encephalitis (EAE) is one of the oldest and most widely studied animal models for a human autoimmune disease.[111,352,353] EAE, a primarily CD4+ T cell-mediated disease, can be induced in susceptible mouse strains (SJL, PL/J) by immunization with MBP, PLP, myelin oligodendrocyte-associated protein, or respective MHC class II restricted epitopes together with complete Freund adjuvant and pertussis toxin. More recently, a role for CD8+ T cells has also been documented. The clinical picture resembles that of a relapsing/remitting disease with profound neurologic symptoms. CNS histologic findings include T-cell infiltration (mainly CD4+ T cells; Fig. 44.8), APCs and microglia activation, as well as disruption of the blood-brain barrier. Some demyelination occurs, and histologic changes can undergo periods of remission that are accompanied by remyelination. The predominant cytokine profile is Th1 like, and Th2 cytokines have protective function.[354] Epitope spreading occurs, and activated APCs take a central role as “drivers” of the autoimmune process.[355] A shortcoming of this model is the major extent of external manipulation required to break tolerance (adjuvant, pertussis). These might be necessary to affect the blood-brain barrier, skew the systemic cytokine profile to the Th1 phenotype, and support prolonged inflammation.

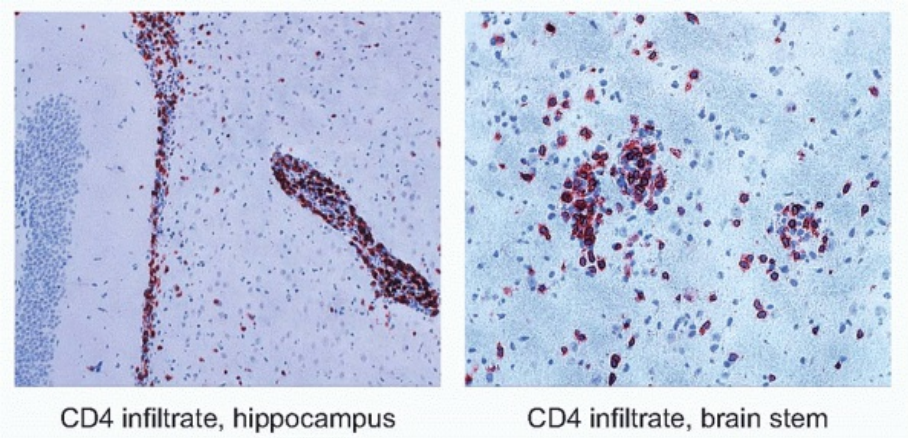

FIG. 44.8. Induction of Experimental Allergic Encephalitis in SJL Mice with PLP Peptide 139-151 and Adjuvant. Courtesy of Andreas Holz, Max Planck Institute for Neurobiology, Munich, Germany.

A lesser degree of additional nonspecific inflammatory conditions is required for some virus-induced animal models for MS. Infection with TMEV, which replicates predominantly in neurons (particularly motor neurons during initial stages of infection), results in a poliomyelitis-like syndrome including flaccid paralysis. Certain strains of mice that survive the primary infection become persistently infected and develop a chronic progressive inflammatory demyelinating disease.[355] Demyelination is the consequence of the CD4+ T-cell response to persisting TMEV and involves determinant spreading as a consequence of infection and local inflammatory processes.[355] Studies from Miller's laboratory have shown that APCs in the CNS are crucial for diversification/epitope-spreading of the initiating anti-TMEV response to other brain self-antigens such as MBP and PLP. Furthermore, when recombinant TMEV expressing PLP epitopes or modified mimic epitopes were employed, infection resulted in enhanced disease. These experiments provided in vivo evidence for a possible role of molecular mimicry in CNS autoimmune disease.[356]

Somewhat similar to TMEV, infection with neurotropic strains of mouse hepatitis virus induces neurologic disease accompanied by demyelination.[357] An interesting difference is that mouse hepatitis virus will not persist, and therefore allows for easier differentiation between the antiviral and the autoimmune response. CD4+ as well as CD8+ lymphocytes accompanied by inflammatory cytokines are needed for destruction. These virus-induced models of demyelination suggest that a transient or persisting presence of virus in the CNS may be needed in conjunction with a nontolerized antiviral response for disease induction and/or propagation.[357] Indeed, another animal model of virally induced CNS disease, the transgenic MBP-LCMV model developed in analogy to the RIP-LCMV model for T1D, only exhibits mild motor dysfunction despite good lymphocytic infiltration.[358] It is possible that activation of APCs is insufficient in this model to locally propagate systemically induced autoaggressive lymphocytes once they have entered the parenchyma of the CNS. These considerations are depicted in Figure 44.9.

Treatment and prevention. Similar to observations in the diabetes models,[319] DNA vaccines that aim to skew the autoreactive response to MBP or PLP from a Th1 to a Th2 phenotype have shown some success in the EAE model. A clinical phase II trial conducted by Bayhill Therapeutics (Palo Alto, CA) indicates that these results, at least to some extent, can be translated to humans.[359] At least for MS, there are preclinical markers that can noninvasively and continuously track the size and appearance of current lesions and in this way indicate success or failure of a given therapy in a

more immediate way as compared to T1D.[360,361] Oral tolerance induction had shown much promise in EAE models, but failed in a human trial.[362] Strong placebo effects were observed after feeding irrelevant protein versus MBP and the outcome was therefore not conclusive, leaving a smaller readout window. Blockade of TNF-α has shown promise in animal models but not in humans.[178] The antidepressive agent Rolipram® is currently under clinical evaluation after it exhibited anti-TNF effects in animal models for MS.

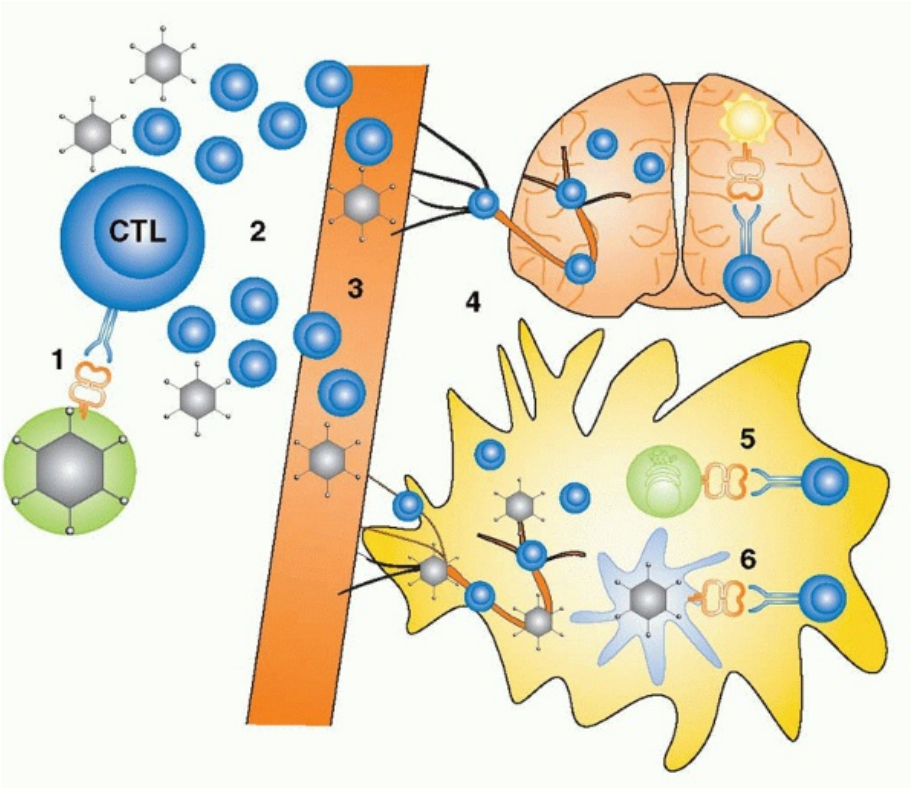

FIG. 44.9. Activated Antigen-Presenting Cells (APCs) Might Be a Crucial Local Driver Once an Autoimmune Process Has Been Initiated. Induction of profound autoimmune disease of the islets but only mild autoimmune infiltration in the central nervous system in rat insulin-promoter versus myelin basic protein-promoter lymphocytic choriomeningitis virus (LCMV) transgenic mice. **1:** In both systems, LCMV infects many peripheral organs, which leads to **(2)** activation and expansion of cytotoxic T lymphocytes (CTLs). **3:** Both virus and CTL are able to traffic via the bloodstream, but **(4)** only CTLs can cross the blood-brain barrier. In the pancreas, both transgenic β cells **(5)** and infected APCs **(6)** drive CTL activation and killing, whereas in the brain only the transgenic oligodendrocytes present antigen. The observation that clinical disease only develops in the pancreas but not the brain indicates that APCs might be crucial local drivers once an autoimmune process has been initiated.

Treatment of MS is, from the immunologic point of view, further advanced than treatment of T1D. It should be noted that life expectancy in MS is more reduced because no therapy comparable to insulin substitution is possible. Of the current regimens, IFNβ has shown strong therapeutic efficacy in phase III clinical trials; at present, more than 135,000 patients are on Avonex® (Biogen Idec, Weston, MA, USA) worldwide.[363] Interestingly, IFNγ, in contrast, has aggravated MS.[364] Furthermore, Copaxone® (Teva Pharmaceutical Industries Ltd., Petach Tikva, Israel) might act as a mixture of altered peptide ligands (as a polymer MBP analog) and in this way may skew immune responses systemically to a less aggressive phenotype, which has shown promise for certain forms of early MS.[365] Lastly, phosphodiesterase inhibitors might induce Th2 skewing, as demonstrated in a small-scale trial.[366] Finally, generalized immunosuppressive therapy with corticosteroids that are given in

conjunction with relapses, and thereafter slowly phased out, still remain an important backbone for MS treatment. Azathioprine, cyclophosphamide, and plasmapheresis are not effective. Cyclosporine A is helpful but has profound side effects. Altered peptide ligands to human MBP epitopes have shown strong allergic side effects and have worsened disease in one study indicating that APLs might activate in vivo aggressive, as well as regulatory, lymphocytes, and that the success can therefore vary from individual to individual.[326,367] Some setbacks have been encountered with monoclonal antibodies. Noteworthy is that anti-CD40L exhibited some efficacy but lead to coagulation disturbances in humans[368] and is currently on halt. In addition, integrin blockers have shown promise and are back in clinical trials, after they were on hold, because combination with IFNβ had led to reemergence of CNS viral infection in three patients.

***Perspective and Conclusion*.** Therapeutic interventions in MS are becoming more effective but will likely have to be individualized and fine-tuned in respect to the subtype of MS in a given patient. Antigen-specific therapy is still in development, but better understanding of the role of T cells in MS will be needed to translate experimental findings into clinical applications. One particularly promising strategy that is currently in clinical trials involves the intravenous administration of chemically fixed, autologous PBLs coupled with five immunodominant myelin peptides.[369] There is also interest in evaluating other therapeutic agents such as CTLA-4Ig with validated benefits in other autoimmune diseases.[370] Combination of systemically acting antibodies shows good promise and will have to be further developed and fine-tuned.

Ocular Autoimmune Diseases

Various inflammatory diseases of the eye accompany other systemic or organ-specific autoimmune disorders and are displayed in Figure 44.10. Some of these immune disorders will be discussed only briefly as they are described in more detail in other sections of this chapter or elsewhere in this book.

***Morbus Bechterew/Spondylarthoropathies*.** Iridocyclitis is frequently associated with Bechterew disease (ankylosing spondylitis) that is characterized by a chronic spondylarthrosis of the ileosacral joints resulting in profound kyphosis and is associated in 30% to 50% of cases with the HLA-B27 allele.[371,372,373] Interestingly, mucosal inflammation, ulcers, and vasculitis are frequently associated with this disease group.

The immunologic pathogenesis is not clearly known, but cross-reactive antibodies to bacterial proteins are frequently found in sera of patients with spondarthopathies such as Bechterew disease, Reiter syndrome, psoriasis, Crohn disease, or ulcerative colitis. Therefore, molecular mimicry has been hypothesized as a cause for these disorders, but proof has been difficult to obtain. Several animal models have been developed, the most intriguing of which is probably a HLA-B27 transgenic rat that develops joint diseases, genital ulcers, and eye diseases. Systemic immunosuppressive interventions are not effective and the treatment of choice is nonsteroidal anti-inflammatory drugs. Local steroids are frequently administered for symptoms affecting the eye.

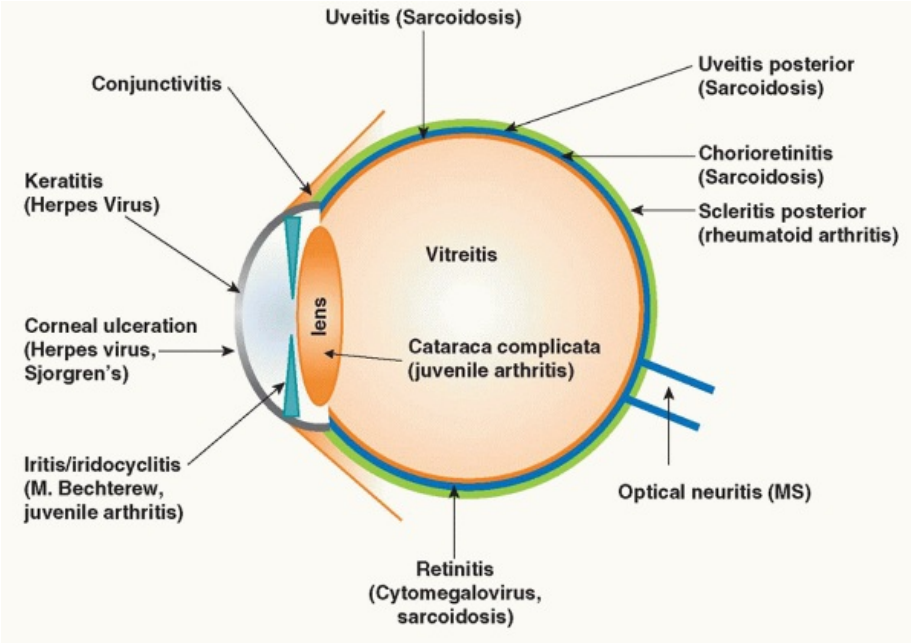

FIG. 44.10. Overview of the Structure of the Eye and Some of Its Inflammatory Diseases and Their Association with Other Systemic Disorders.

***Rheumatoid Arthritis*.** RA is an autoimmune joint disease (see following discussion) that is frequently associated with scleritis and episcleritis. Inflammatory infiltrates can be found in the peripheral cornea leading to ulcerations. Local treatments are usually without effect, and symptoms are ameliorated in conjunction with systemic immunosuppressive therapy. In contrast, juvenile arthritis is accompanied in about 20% of patients with cataracts and iridocyclitis, and ocular symptoms are often sensitive to local and systemic immunosuppression.

***Sarcoidosis*.** Sarcoidosis is a chronic, systemic granulomatous inflammatory disease that involves the eye (uveitis in 20% of cases) and constitutes the cause for uveitis in about 10% of patients. Multiple organs, in particular the lungs, are affected. The etiology is unclear, an autoimmune pathogenesis is suspected, and multiple other inflammatory changes can occur in the eye (see Fig. 44.10). Local therapy with steroids is usually helpful, and systemic immunosuppression can be considered.

***Idiopathic Uveitis*.** About half of the cases of uveitis are not associated with a known primary syndrome, and in young adults, the association (50%) with HLA B27 is striking. Only few of these patients will ultimately develop Bechterew disease. Again, the etiology is quite unclear, but some evidence suggests that molecular mimicry between ocular and viral antigens (eg, herpesvirus) could play a role. Peptide therapy is currently being evaluated; otherwise, steroids are the only effective choice.

A summary of the eye structures and some of the systemic diseases that are associated with a putative autoimmune affectation of the eye are listed in Figure 44.10.

***Animal Models*.** Small rodent models for autoimmune uveitis and herpesvirus keratitis have been very useful for understanding the underlying immunopathology of inflammatory eye disorders.

Experimental Autoimmune Uveoretinitis. Experimental autoimmune uveoretinitis (EAU) is induced, similar to EAE, by immunizing susceptible rodent strains with retinal proteins such as the receptor retinoid binding protein (IRBP).[374] Extensive studies by Tarrant et al.[375] have revealed that EAU follows similar autoimmune paradigms as other experimental autoimmune diseases including EAE and induced forms of diabetes. For example, a Th1-like response drives the aggressive EAU process,[375] whereas IL-10 in synergy with IL-4 has a pronounced protective function.[376,377,378] Similarly, diseaseresistant mice exhibit a predominantly Th2-like response to IRBP. Experimental evidence indicates that sequestration and local immune privilege of IRBP and possibly other retinal antigens leads to a lack of systemic tolerance to these proteins and, as a consequence, destructive autoimmune responses can be readily induced. Conversely, tolerance can be reestablished by application of tolerizing retinal-Ag/Ig fusion proteins or by inducing peripheral tolerance by systemic expression under an MHC class II promoter in transgenic mice. Interestingly, peptide therapy using an HLA-B27-restricted peptide appears to be effective in humans. The immunopathology of this EAU model in comparison to human uveitis, as found in sarcoidosis, is displayed in Figure 44.11. Recent evidence from the Fathman laboratory on the role of Deaf1 has revealed that multiple isoforms of this protein regulate the expression of peripheral tissue antigens, much like the gene AIRE controls antigen expression in the thymus.[379] Remarkably, the only phenotypic defect observed in Deaf1-deficient mice was the development of retina-reactive autoantibodies, which are also found in AIRE knockout mice. These data indicate that somehow the expression of an appropriate range of peripheral tissue antigens is important to maintain tolerance against retinal components.

Herpesvirus-Induced keratitis. Herpesvirus-induced keratitis has been explored in several animal models. The most intriguing studies come have come from the laboratory of Cantor. Inoculation of a genetically susceptible mouse strain with herpes simplex virus (HSV)-1 results in a viral infection of the eye and cornea that is accompanied by an immune response to HSV-1.[143] Interestingly, damage to the eye is only initiated by the virus and strongly depends on CD4 T cells that can cross-react with a self-protein. This animal model is one of the few that strongly indicate a direct immunopathologic role for molecular mimicry in an in vivo model for autoimmune disease. Studies by Gangappa et al.[380] have focused on the role of inflammatory bystander effects in the HSV keratitis model. In these studies, inflammatory cytokines were shown to play a major role in mediating local damage, and administration of DNA vaccine constructs expressing certain beneficial cytokines such as IL-10 ameliorated disease.[381] It is important to point out that these studies were performed using a different HSV strain, and that the precise immunopathogenic process (mimicry vs bystander effects) indeed depends on the strain of HSV used.[382] Additional observations have underscored the importance of regulatory cells in downmodulating a virally induced autoimmune disease. It is worthwhile mentioning that Kosiewicz et al. and Takeuchi et al.[383,384] have previously demonstrated regulatory cells in ocular autoimmune disease and found an important role for tolerizing APCs.

***Treatment and Prevention*.** Due to the associated side effects, it is desirable to avoid systemic immunosuppressive interventions as well as the local application of corticosteroids. A Munich group has identified a HLA-B27-derived peptide that can function as a mimic to the retinal S antigen

and induce EAU in Lewis rats.[385] Using this mimic peptide as an orally administered antigen has produced promising results in two human pilot trials in Germany. Thus, antigen-specific immune modulation using retinal self-antigens or their peptide mimics may be developed as an effective therapeutic choice. It remains to be seen whether combination of such a treatment with cytokine-DNA vaccines, as demonstrated by Gangappa's group for HSV keratitis or by Tarrant's group for EAU, both in mouse models, might enhance efficacy. In addition, systemic immunomodulation bears promise. Also, trials using anti-CD25 monoclonal antibody (daclizumab) for the treatment of recalcitrant ocular inflammatory disease demonstrated beneficial effects as they permitted the reduction of conventional immunosuppressive regimens.[386,387] It is noteworthy that in this case, the dampening of effector cells rather than any effects on CD25+ T_{reg}s appears to explain the efficacy.

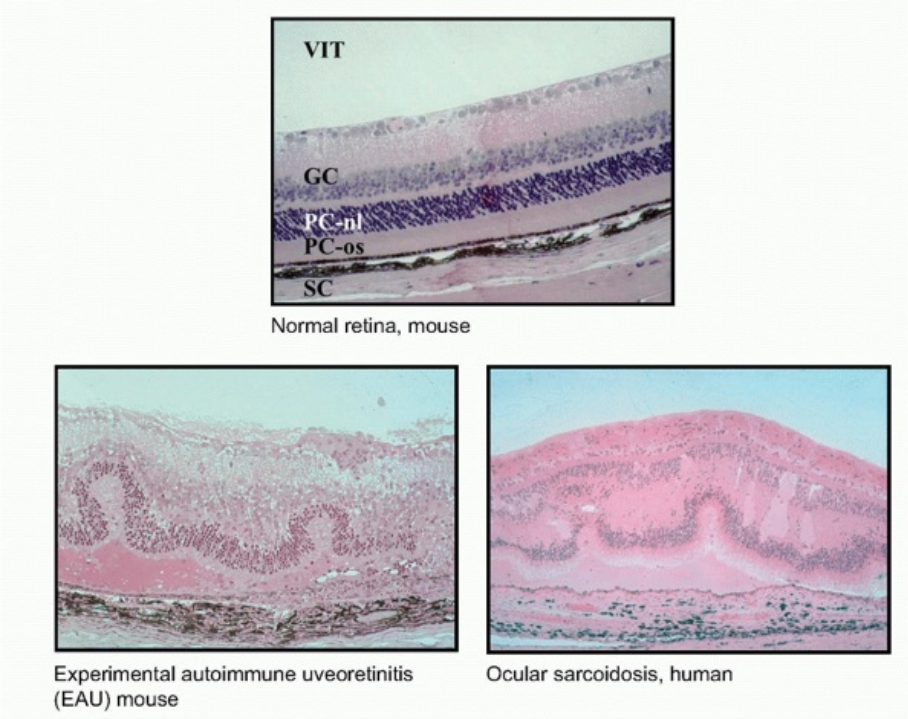

FIG. 44.11. Histopathologic Comparison of Human and Mouse Uveitis. CH, choroid; GC, ganglion cells; PO-nl, photoreceptor nuclear layer; PO-os, photoreceptor outer segments;SC, sclera; VIT: vitreous. Courtesy of Rachel Caspi, National Eye Institute, Bethesda, MD.

Gastrointestinal Autoimmune Diseases

The gut constitutes a unique immunologic environment given its large interactive mucosal surface area and the need to maintain tolerance toward food antigens and bacteria normally present within the gut flora. The gut-associated immune system has an important regulatory and barrier function. Immune responses are usually initiated within the Peyer patches that obtain a significant amount of antigens via the M cells located within the mucosa and specialized in antigen uptake and transport.[151,388,389] Lymphocytes from the bone marrow

will circulate through the Peyer patches, where B- or T-cell responses can be initiated. Interestingly, the B-cell responses are characteristically IgA high. After antigen encounter, these cells will circulate to the mesenteric lymph nodes and enter the systemic circulation from there. Specifically for the gut, there are other extralymphoid locations where immune cells are found and where immune responses (aggressive or regulatory) can be initiated. One is the intestinal lamina propria, where MHC class II is expressed and bacterial products can be presented. Predominantly CD4+ T and B cells may assume regulatory functions, and IgA is secreted with the mucus into the gut lumen. The second are intraepithelial lymphocytes. A significant proportion of intraepithelial lymphocytes expresses the γδ TCR and they have cytotoxic as well as immunoregulatory functions.[390,391] Certain studies after oral or intranasal feeding of autoantigens have attributed regulatory function to γ/δ lymphocytes. Overall, the hypothesis that the gut environment is ideally suited to induce a tolerizing or regulatory immune response to antigens present in the gut lumen is of high interest and well supported by evidence from animal models (eg, "oral tolerance").[392] The rationale is that the immune system must have evolved to put some unique mechanisms into place to deal with the multitude of "foreign" antigens present in the gut and needed as nutrients

and for digestive purposes. Indeed, mice housed within a sterile environment have a shorter life span and multiple immune defects, indicating that the gut's immune system is vital for regular immune development and functions.[261]

Pernicious Anemia and Autoimmune Gastritis

Pernicious anemia is the end stage of autoimmune gastritis observed in about 10% to 20% of patients[393] and represents the most common cause of vitamin B_{12} deficiency.[394] Autoimmune gastritis is associated with autoantibody production to parietal cells and to its secreted product, intrinsic factor. In addition, a role for CD8 and CD4 T cells has been demonstrated.[395] Animal models have been developed.[396] Standard therapy is paternal B_{12} administration. Interestingly, this disease also develops in mice after thymectomy and is characterized by a relative lack of CD25+ regulatory CD4+ T cells.[176,397,398]

Ulcerative Colitis. Ulcerative colitis is a chronic inflammatory disease of the gut that affects men somewhat more frequently than women. It usually begins in the distal colon and rectum and will spread proximally resulting in severe cases in pancolitis. The hallmark is bloody mucoid diarrhea. The etiology is unclear; evidence points toward a certain genetic predisposition that has to meet triggering environmental factors. Unlike Crohn disease, the inflammatory foci are not granulomatous and not discontinuous. Overall, inflammatory mediators are increased, but systemic symptoms are rare. Treatment is usually achieved by corticosteroids; in severe cases, ulcerative colitis may require removal of the colon.

Crohn Disease. As opposed to ulcerative colitis, Crohn disease begins as a discontinuous, granulomatous inflammation of the proximal ileum. Ulcerations are frequent, and the disease is more severe than ulcerative colitis. The permeability of the mucosal epithelium is enhanced, which leads to a local breakdown of barrier functions and upregulation of TNF, IL-1, IL-6, and other cytokines. The immune reaction involves the whole mucosa and regional

lymph nodes. Interestingly, in addition to standard immunosuppressive treatments, blockade of TNF is highly efficacious and has been approved as a therapeutic strategy in Crohn disease.[399]

Hepatic Autoimmune Diseases

As an anatomic site for the induction and perpetuation of immune responses, the liver exhibits distinctive immunologic features that appear to favor an overall "tolerogenic" microenvironment. This perspective is supported by the apparent absence of liver pathology despite the continuous exposure to nutritional antigens and components of commensal gut flora,[400] the ability of viral (hepatitis B and C) and parasitic (malaria falciparum) pathogens to establish persistence and chronic hepatitis,[401,402] as well as the ready acceptance of liver allografts without immunosuppression in experimental models.[403] Moreover, successful liver transplants in humans require comparatively less immunosuppressive therapy,[404] and in some cases, complete weaning of immunosuppressive drugs has been achieved without compromising the functionality of the transplant.[405] The prevailing notion that the liver preferentially promotes abortive immune responses and tolerance was recently challenged in a meticulous study by Klein and Crispe.[406] Having optimized the considerable surgical feat of orthotopic liver transplants in mice, the authors conclusively demonstrated that the liver effectively initiates the bona fide activation and differentiation of specific T-cell responses.[406] Thus, the mechanisms operative in "liver tolerance" are not the consequence of altered antigen presentation, maturation failure, or premature T-cell death but rather appear to rely on the activation of poorly defined regulatory circuits by persisting antigens.

Conversely, a breakdown of such tolerance mechanisms, presciently postulated almost 100 years ago as a cause for autoimmune diseases at large,[7] may contribute to pathogenesis of hepatic autoimmune disorders. Here, we will discuss the three major autoimmune diseases of the liver: AIH,[407,408,409] which primarily targets hepatocytes; primary biliary cirrhosis (PBC),[410,411,412] in which small bile ducts are destroyed; and primary sclerosing cholangitis (PSC),[413,414] in which the damage is mainly focused on medium to large bile ducts. In addition, we have included "halothane hepatitis" as a paradigm for an immune-mediated adverse drug reaction.[415]

Autoimmune Hepatitis

***Introduction and Disease Description*.** Autoimmunity was invoked as a cause for hepatitis in the early 1950s,[416,417,418] and based on the association of AIH and antinuclear antibodies (ANAs), the term lupoid hepatitis was proposed.[419] AIH preferentially affects women but is observed worldwide in children and adults of both sexes in diverse ethnic groups; it contributes an estimated 10% to 20% to all cases of chronic hepatitis (which for the most part are due to infections with hepatitis viruses B, C, and D).[407,408] Clinical symptoms range from inapparent to fulminant disease courses, and differential diagnosis is guided by an exemplary scoring system developed by the International Autoimmune Hepatitis Group.[44,420] The presence of autoantibodies targeting different subcellular components is commonly used to differentiate two types of AIH. The etiology of AIH remains undefined, but a

contribution of environmental/microbial factors and genetic predisposition, as in so many other autoimmune diseases, is invoked as a trigger for T cell-mediated, destructive autoimmunity. Finally, it is also possible that as of today, unidentified hepatitis viruses or other chronic viral infections contribute to some AIH cases.[206,421]

***Autoimmune Features*.** The presence of specific autoantibodies in AIH is a key feature in the diagnostic scoring system and the foundation for the clinical distinction of type 1 and 2 AIH (Table 44.2). Type 1 AIH is characterized by ANAs, a class of antibodies also observed in PBC, PSC, and other forms of chronic hepatitis. Smooth muscle and antiactin antibodies, atypical perinuclear antineutrophilic antibodies and antibodies directed against soluble liver antigen and liver-pancreas antigen. None of these antibodies are specific for type 1 AIH, but their combined measurements allow for a reasonable distinction from type 2 AIH and, to a certain extent, other chronic liver diseases.

Anti-liver-kidney microsomal (LKM) antibodies are a serologic marker for type 2 AIH, with the majority binding the LKM1 antigen identified as cytochrome monooxygenase P450 IID6 (CYP2D6). In addition, <20% of patients with type 2 AIH also carry autoantibodies specific for uridine diphosphate glucoronosyltransferase, designated as LKM3, while 30% to 50% of patients generate antibody responses against formiminotransferase cyclodeaminase. Of all these targets in AIH, only one, the hepatocyte-specific asialoglycoprotein receptor, is cell surface expressed and may thus function as a direct immunogen for antibody-mediated hepatocyte damage. Hepatocyte-specific asialoglycoprotein receptor-reactive antibodies, also not disease specific, are found in 50% to 75% of all patients with AIH.[407,408,422] As the generation of these autoantibodies are Th-cell dependent, it is not surprising that loss of tolerance also occurs on the T-cell level and lymphocytes isolated from livers of patients with AIH type 2 can react in vitro and expand after exposure to P4502D6 antigen. This finding supports the concept of a lymphocyte-driven autoaggression and might underlie pathogenesis. Furthermore, mononuclear infiltration of the periportal areas and liver parenchyma is usually present when the disease progresses and causes the AIH-typical “piecemeal” appearance of the liver. Although evidence from animal models and human studies gives some indication that cytotoxic killing of hepatocytes could play a role, further analysis is still hampered by the fact that intrahepatic lymphocytes are difficult to access in humans. The notion that defective suppressor T-cell activity contributes to AIH pathogenesis, proposed more than two decades ago, is supported by the finding of reduced frequencies of peripheral CD4+CD25+ T_{reg} in patients with AIH as compared to healthy controls.[423] Cytokines also appear to play an important role in human AIH. Administration of type I IFN led to strong exacerbation of AIH, showing that it likely plays a central role in liver destruction.[424] IFNs upregulate MHC class I and II molecules, enhance inflammation, and have strong antiviral effects. The outcome of these studies therefore argues against a role of an ongoing chronic (or acute) viral infection in patients with AIH who test negative for hepatitis virus antigen or antibodies. A negative regulatory role can possibly be attributed to IL-6 and TNF-α. These two cytokines are reduced in livers of patients with AIH and have a negative effect on cytochrome P450 regulation.[425] As a consequence, the lower levels of IL-6 and TNF might enhance CYP2D6 expression and in this way support autoimmunity directed to CYP2D6 in patients with type 2 AIH. Lastly, AIH pathogenesis is not necessarily controlled by the Th1/Th2 paradigm: CD4+ T cells expressing Th1 cytokines are elevated in type 1 AIH,

whereas Th2 cytokines are augmented in type 2 AIH in agreement with the high levels of CYP2D6-specific antibodies present in these patients.

TABLE 44.2 Autoantibodies and Autoantigens in Autoimmune Liver Disease

	Type I AIH	Type II AIH	PBC	PSC
Auto-Abs	ANA: centromere, ribonucleoprotein, cyclin A, histone SMA: F actin, anti-ASGPR anti-SLA/LP pANCA: myeloid-specific nuclear envelop protein	Anti-LKM-1: CYP450, 2D6 Anti-LC-1: formiminotransferase cyclodeaminase	AMA: PDC-E2, BCOADC-E2, OGDC-E2, PDC-ERBP, PDC-E1a ANA: nuclear pore complex, anti-gp210, anti-p62, nuclear dot pattern, anti-Sp100, anti-PML, anti-SUMOs	pANCA: myeloid-specific nuclear envelope protein ANA: anticardiolipin, thyroperoxidase, rheumatic factor, *Helicobacter pylori* IgG
B cell infiltrate in liver	+ with plasma cells	+ with plasma cells	+ with plasma cells	+/-

AIH, autoimmune hepatitis; ANA, antinuclear antibody; ASGPR, hepatocyte-specific asialoglycoprotein receptor; BCOADC, branched chain 2-oxo-acid dehydrogenase complex; PDCERBP, ER binding protein of the pyruvate dehydrogenase complex; Ig, immunoglobulin; LC, liver cytosol (antigen type 1); LKM, liver-kidney mircosomal; OGDC, 2-oxo-glutarate dehydrogenase complex; pANCA, perinuclear antineutrophilic antibodies; PDC, pyruvate dehydrogenase complex; PML, promyelocytic leukemia protein; SLA/LP, soluble liver antigen and liver-pancreas antigen; SMA, smooth muscle antibody; SUMO, small ubiquitin-like modifier.

From Moriotki et al.422

Is production of autoantibodies important for AIH pathogenesis, or is it just an epiphenomenon signifying the breaking of tolerance to self-antigens? Evidence suggests that autoantibodies in AIH play a role that can be situated somewhere in between T1D, where autoantibodies and B lymphocytes likely play no pathogenetically important role in humans, and SLE, where autoantibodies are complement-fixing and enhance organ destruction. LKM antibodies found in AIH, for example, can inhibit cytochrome P450 function in vitro but not in vivo, and may participate in hepatocyte dysfunction or destruction. Epitope mapping of the CYP2D6 antigen in type 2 AIH revealed two immunodominant regions spanning aa 256-269 and aa 181-245 that are recognized by most of the sera of the patient with type 2 AIH. Because there is a significant genetic polymorphism in humans for the CYP2D6 antigen, it is possible that patients with AIH target CYP2D6 protein selectively (LKM1 antibodies), whereas cytochrome P4502D9 (LKM2) is targeted in patients with drug-induced hepatitis, disulfide isomerase in halothane-induced hepatitis, or UDP-glycosyltransferases in hepatitis D virus-associated autoimmunity. Identification of the major target antigens, clearly a decisive step toward a better understanding of the underlying disease process, has so far not yielded compelling hypotheses as to the precise pathogenetic mechanisms.

***Genetic Features and Environmental Factors*.** No single genetic locus capable of explaining AIH etiology has been identified. HLA-DR3 and HLA-DR4 are associated with type 1 AIH, and the HLA-A1-B8-DR3 haplotype exhibits

a particularly strong association with early disease onset, relapse, and the eventual necessity of liver transplantation. In contrast, type 2 AIH is associated with carriage of HLA-DR7. Genes outside the HLA loci potentially conferring disease susceptibility include complement component C4, CTLA-4, vitamin D receptor, Fas receptor, and others.[407,408,422]

The association with hepatitis C, D, and E virus-induced hepatitis and autoimmunity remains very intriguing. However, at this point, the prevalence of hepatitis C virus, for example, exhibits drastic variations between studies performed in different countries, and autoantibody titers appear much higher in patients with hepatitis C virus-negative AIH. In a more recent study, it was found that sera of 38% of patients with chronic hepatitis C reacted specifically with CYP2D6, whereas none of the sera obtained from patients with chronic hepatitis B showed CYP2D6 reactivity.[424] Furthermore, it was found that hepatitis C virus has the potential to induce autoreactive CD8+ T cells that crossreactively recognize the cytochrome P450 isoforms 2A6 and 2A7 that contain sequence homology to hepatitis C virus aa 178-187. In this context, it may be important to emphasize that molecular mimicry also seems to be an important factor in other immune-mediated diseases of the liver. Hence, trifluoroacetyl (TFA)-protein adducts, as generated during the metabolism of halothane by CYP2E1, confer molecular mimicry to the lipoic acid prosthetic group of the pyruvate dehydrogenase complex (PDC) and other members of the 2-oxoacid dehydrogenase family,[82,234,295,426] which in turn are major autoantigens in PBC.[115] Consequently, halothane hepatitis and PBC may be linked on the level of cross-reactive autoantibodies that recognize similar target antigens. It remains to be seen whether novel subtypes of hepatitis viruses can be found in patients with AIH and whether further studies will corroborate such an association. It is likely, however, that

viruses play a mutifactorial role in AIH pathogenesis (similar to T1D), and that no single virus will be identified as a specific cause for liver autoimmunity.

***Animal Models*.** Over the past 30 years, many attempts have been made to develop animal models for AIH; however, to date, no reliable model is available that reproduces the spontaneously relapsing chronic disease course of type 1 AIH.[427,428]

In the hepatitis B virus surface antigen-transgenic mouse, the hepatitis B virus surface antigen is expressed in hepatocytes under control of the mouse albumin promoter. Induction of transient hepatitis is possible after adoptive transfer of activated T cells from hepatitis B virus surface antigen-primed donor mice.[429,430] Although not designed to reproduce features of AIH, this model has been extraordinarily helpful in understanding the role of IFNs in inducing liver damage as well as clearing hepatitis B virus from the liver. Results have shown that IFNs, in the absence of cytotoxicity, can purge virus from infected hepatocytes. Furthermore, induction of hepatitis B virus-specific CD4+ and CD8+ T-cell responses can be evaluated in this model. The transgenic mice exhibit profound liver damage and infiltration after transfer of hepatitis B virus-specific T lymphocytes.

In another model system, the MHC class I molecule H-2K^b is transgenically expressed in the liver of mice that also carry transgenic T cells specific for H-2K^b. Hepatitis induction was only successful when such mice were infected with a liver-specific pathogen, indicating that bystander activation within the liver microenvironment can be very potent in causing autoimmune damage.[431] A similar approach was chosen in yet another model that combines liver-specific expression of a transgene (here, the immunodominant LCMV glycoprotein epitope [GP_{33}]) and transgenic T cells specific for the transgene.[432] These mice remain tolerant to the transgene even in the presence of transferred GP_{33}-specific T cells. However, under conditions of LCMV infection and TCR-transgenic T-cell transfer, these mice develop a transient form of hepatitis as evidenced by elevated alanine aminotransferase levels.[432] Together, these findings emphasize the importance of both autoreactive T cells and generalized inflammatory alterations in the wake of microbial infection.

The potential pathogenic contribution of autoantibodies was demonstrated in a new mouse model. Injection of a monoclonal antibody, derived from a patient with type 1 AIH and specific for an undefined 190 kDa cell surface protein, promotes predominantly perivenular hepatocellular necrosis in mice.[433] However, the validity of this model remains to be critically evaluated as the pathology does not appear to closely resemble that of type 1 AIH.

Arguably, more compelling models have been developed for the study of type 2 AIH. DNA immunization of mice with human CYPD6 and formiminotransferase cyclodeaminase antigens leads to the generation of LMK1 and LC1 autoantibodies (targeting CYPD6 and formiminotransferase cyclodeaminase, respectively) and specific cytotoxic T cells, pronounced lymphocytic infiltration of the liver, and an elevation of alanine aminotransferase levels indicative of liver damage.[434] Interestingly, work with this model has reproduced some aspects of the genetic predisposition exerted on disease development as C57BL/6 mice demonstrated more pronounced disease than 129/Sv mice, and no inflammation was found in BALB/c mice.[435] Lastly, adenoviral infection with recombinant expressing the 2D6 antigen can lead to focal and confluent liver necrosis in mice resembling that of AIH[436] (Fig. 44.12).

In summary, the success of most mouse models has only been partial, as hepatitis was transient and induction of chronic disease appeared difficult to achieve. Figure 44.13 illustrates the possible pathogenetic mechanisms implicated in AIH.

Treatment and Prevention. Immunosuppression is the therapy of choice for AIH. Since their introduction in 1968,[437] prednisolone and azathioprine have become standard treatment regimens.[408,438] The impressive success rate of this therapeutic approach (more than 85% of patients with AIH) may have, to a certain extent, removed the urgency to pursue basic AIH research.[428] Immunosuppressive therapy also improves survival of patients with severe AIH,[439] but no guidelines are available for individuals with minimal symptoms. End-stage AIH is an important indication for liver transplantation.[440] Recurrence of AIH has been reported after liver transplantation[441] but interestingly is not as frequent as observed for islet

transplants in patients with T1D. Therapeutic use of IFNs, effective for treatment of viral hepatitis, can worsen autoimmune liver disease[424] and challenges the assumption that unknown chronic viral infections of the liver, while possible initiators, maintain the active disease process.

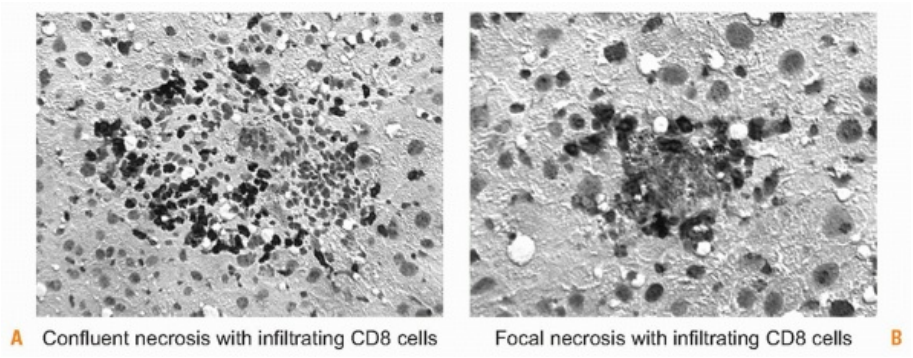

FIG. 44.12. Liver Necrosis in 2D6 Transgenic Mice Infected with an Adenoviral Recombinant Expressing 2D6. Courtesy of Urs Christen and Matthias von Herrath, University of Frankfurt, Germany, and La Jolla Institute for Allergy and Immunology, La Jolla, CA.

Primary Biliary Cirrhosis

Introduction and Disease Description. PBC is a slowly progressive hepatic autoimmune disorder that primarily affects women. The disease is typically associated with high titers of antimitochondrial antibodies (AMAs) that presage onset of clinical disease by 5 to 10 years; antinuclear antibodies (ANA) are found in about half of PBC patients. Histopathologic changes include portal inflammation and destruction of intrahepatic bile ducts that occur at various rates and degrees in different patients. The resulting decrease of bile secretion and retention of toxic substances within the liver leads to further hepatic damage, fibrosis, cirrhosis, and eventual liver failure.[410,411,412]

Autoimmune Features. Although speculation about the autoimmune pathogenesis of PBC

centers around the usual suspects of genetic and environmental/microbial factors, PBC may serve as an example how advanced knowledge about the targeted autoantigens can give rise to novel and intriguing hypotheses. The presence of AMA in PBC were first described in the 1950s, the autoantigens cloned in the 1980s and 1990s, and B cell, CD4+, and CD8+ T-cell epitopes of the major autoantigen PDC-E2 mapped in the 1990s and 2000s. More than 90% of patients with PBC have high titers of autoantibodies directed against the 2-oxoacid dehydrogenase complex E2 subunit, in particular the E2 component of the PDC. Mapping studies have identified determinants within the PDC-E2 lipoyl domains as a primary targets for antibody and T-cell reactivity, a motif that is also found in the E2 subunit of other 2-oxoacid dehydrogenase complex members (OGDC-E2, BCKD-E2; motif is also found in E3-BP).[411] Antibody reactivity to nuclear envelope proteins gp210 and p62 are found in 10% to 30% of patients and are apparently associated with more active disease. The obvious question emerging from the identification of these autoantigens pertains to the fact that they are 1) found in every cell type and 2) located on the inner surface of the inner mitochondrial membrane, thus separated

from immune effectors by three membranes. In addition, the existence of a clinical entity known as AMA-negative PBC, a disease very similar to PBC but without detectable AMAs, appears to further argue against a pathogenic role of these autoantibodies.[412] Yet, certain particularities of bile duct epithelial cells may predispose them as targets for humoral immune responses. These cells, just as salivary epithelial cells, express receptors involved in the transcytosis of IgA and high titers of PDC-E2-specific IgA are found in bile, saliva, and urine of patients with PBC. AMA IgA was shown to induce apoptosis, which in turn may lead to PDC accumulation in the cytoplasm and cell surface, as well as amplification of the immune response. Further speculation has focused on the possibility that failure of PDC glutathionylation specifically in epithelial cells may promote accumulation of immunogenic forms of PDC, a process that may be initiated by xenobiotic-modified PDC and molecular mimicry.[411] In the “determinant density model,”[442] potentially PDC-reactive T cells survive negative thymic selection due to low TCR avidity and remain “dormant” despite sporadic exposure to PDC-derived epitopes. However, strong inflammatory stimuli that allow for improved antigen presentation and activation of toll-like receptors (TLRs; monocytes from PBC patients exhibit enhanced sensitivity to TLR signals) (eg, through microbial molecular mimicry) may induce the breakdown of tolerance that precipitates the disease. It should be noted that the general validity of this hypothesis, including a key role for low-avidity T cells and TLRs, is fully supported by elegant studies in models for induction of autoimmune diabetes.[114,443] Finally, a model has been proposed in which a primary dysfunction of endothelial cells results in overproduction of endothelins, inflammatory alterations, and ischemic damage due to vasoconstriction and the generation of PDC-specific humoral and cellular responses as a secondary event.[412] These hypotheses are not mutually exclusive and may be critically evaluated in two animal models.

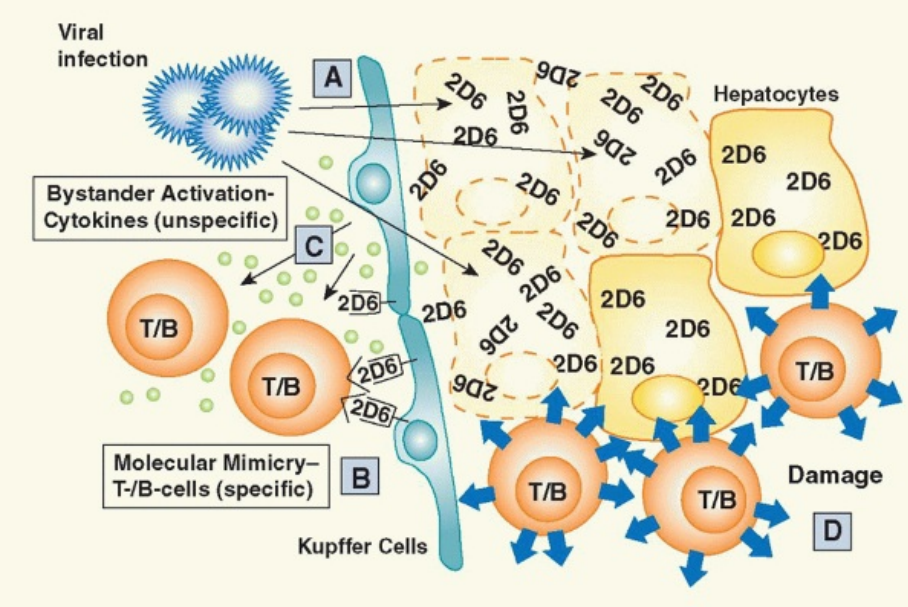

FIG. 44.13. Pathogenesis of Liver Autoimmunity after Viral Infection in the 2D6 Animal Model. A: Viral infection leads to expression of high cytokine levels in the liver. **B:** The infection will probably also activate resident macrophages (Kupffer cells). Antiviral T cells react against virally infected cells and destroy them. This leads to release of autoantigens fom hepatocytes, which can be (cross)-presented leading to pathogenic responses against autoantigens such as cytochrome P450 2D6. Chemokine and cytokine release **(C)** leads to enhanced inflammation and antigen presentation **(D).**

***Genetic Features and Environmental Factors*.** Familial clustering of PBC and a high concordance rate in monozygotic twins (63%) indicate a genetic component to PBC pathogenesis. A flurry of GWAS data has dramatically improved our insight into the genetics landscape of PBC. Before the implementation of GWAS technology, only class II HLA loci had been reproducibly shown to associate with disease. Non-HLA loci were reported on some occasions but replication often proved problematic. GWAS confirmed HLA as the strongest association and many additional non-HLA risk loci have been identified, with equivalent effect size to HLA, including IL-12A,[444] IL-12RB2,[444] STAT4,[445] IRF5-TNPO3,[446] 17q12.21,[446] MMEL1,[446] SPIB,[446] and CTLA-4.[444] As argued previously, the list of associated genes comprises many proteins with important roles in innate and adaptive immunity, and, thus, the existence of a monogenic pathway that leads to disease can be excluded. In correspondence with, for instance, T1D and RA, CTLA-4 also confers risk for this condition and many of the candidate genes signal though NF-κB, TLR, and TNF pathways, which are also important in many other autoimmune conditions. The future will tell whether this growing list of gene associations will be instumental in unraveling the immune pathology that underlies PBC.

***Animal Models*.** The absence of a suitable animal model has long been a serious obstacle to PBC research. However, two publications document important progress on this front by describing the spontaneous onset of a PBC-like disease in two mouse strains. NOD.c3c4 mice congenically derived from the NOD strain generate PDC-E2-specific autoantibodies and exhibit biliary pathology characterized by destructive cholangitis, granuloma formation, and eosinophilic infiltration. Importantly, T cells are found in affected areas of the biliary

epithelium, and transfer of splenocytes or CD4+ T cells induces disease in NOD.c3c4-scid recipients, suggesting a central role for T cells in pathogenesis.[447] In the second model, abolition of TGF-β signaling in T cells (achieved by T cell-specific expression of a dominant-negative TGF-βRII) results in production of AMAs directed at PDC-E2, BCOADC-E2, and OGDC-E2 as well as lymphocytic liver infiltration with periportal inflammation analogous to the histologic profile in human PBC. These observations implicate the TGF-β pathway in PBC pathogenesis and suggest that the activation of intrinsically self-reactive T cells is a consequence of impaired T_{reg} cell function rather than molecular mimicry.[448]

***Treatment and Prevention*.** It is noteworthy that despite its presumed autoimmune etiology, PBC is not ameliorated by immunosuppressive therapy. In the absence of optimized therapeutic protocols, treatment is tailored according to individual responsiveness of patients with PBC and includes management of disease symptoms (pruritus, osteoporosis, hyperlipidemia, portal hypertension) as well as therapy of the underlying disease by stepwise addition of ursodeoxycholic acid, colchicin, and methotrexate. Nevertheless, the use of colchicine and low-dose methotrexate, presumably acting as an immunomodulatory rather than antimetabolic agent, remains controversial. In cases of liver failure, orthotopic liver transplantation is the only effective treatment.[410]

Primary Sclerosing Cholangitis

***Introduction and Disease Description*.** PSC is a chronic cholestatic liver disease (cholestasis: suppression of biliary flow) frequently associated with inflammatory bowel disease (>75% of patients with PSC). It is characterized by fibrotic inflammation and destruction of large intrahepatic and extrahepatic bile ducts and may lead to the development of cholangiocarcinoma (in 10% to 30% of patients with PSC) and ultimately death by liver failure.

***Autoimmune Features*.** Although the etiology of PSC remains undefined, a complex multistep process has been delineated and involves cholangiocyte activation through bacterial pathogen-associated molecular patterns, production of proinflammatory cytokines in conjunction with aberrant chemokine expression and endothelial cell adhesion molecules, and the recruitment of T cells presumably specific for enterocyte antigens and primed in gut-associated lymphoid tissues.[414] The generation of autoantibodies is a common feature (see Table 44.2), but most antibodies are not specific for PSC and their contribution to disease pathogenesis remains at present unclear.[422]

***Genetic Features and Environmental Factors*.** The relative risk of PSC among first-degree relatives of patients with PSC is almost 100-fold increased as compared to the general population, indicating that there is a strong genetic component to the condition.[413] As in many other autoimmune conditions discussed here, GWAS have greatly contributed to our understanding of the array of genes that contribute to disease susceptibility. A first report revealed a similar genetic architecture as described in PBC, and it was confirmed that the association with HLA was even more pronounced.[449] The authors also found one non-HLA association at chromosome 13q31 (locus also associated with MS), and the next assayed 15 established inflammatory bowel disease (which affects 50% to 80% of patients with PSC) susceptibility loci. They were able to identify two additional non-HLA susceptibility loci

(chromosome 2q35 and chromosome 3p21) for which the G protein-coupled bile acid receptor 1 (TGR5) and macrophage-stimulating 1 (MST1) were proposed as the likely disease genes. A subsequent study from the same group added two more non-HLA loci, 2q13 and 10p15, with presumable candidate genes BCL2-like 11 (BCL2L11) and IL-2RA.[450] Interestingly, IL2ra-/- mice spontaneously develop intestinal and biliary inflammation.[451] Finally, three more inflammatory bowel disease susceptibility loci were found to be associated with PSC, encompassing the candidate genes REL, IL-2, and CARD9.[452] Collectively, these genetic data offer strong, albeit still indirect, support for an important role for innate and adaptive immune components in PSC. Moreover, the shared genetic patterns between PSC and inflammatory bowel disease indicate a certain degree of common immunopathogenesis and once again suggest the potentially shared origin of what first seemed to be distinct autoimmune disorders.

***Animal Models*.** None of the animal models developed to date reproduce all pertinent aspects of human PSC.[453] Rat models of small bowel bacterial overgrowth and administration of bacterial cell wall components have provided some support for an ethiopathogenetic role of pathogenassociated molecular patterns. Other models include injury of biliary epithelial or vascular endothelial cells (rodents, dog, monkey) and toxic, infectious, or intraluminal biliary injury (rodents) permitting the study of cytokines, chemokines, and inflammation in the development of diffuse bile duct sclerosis.[453]

***Treatment and Prevention*.** There is currently no effective medical therapy available for PSC.[454] Treatment with the hydrophilic bile acid ursodeoxycholic acid improves liver enzymes, but its effect on liver histology and prognosis remains inconclusive. Orthotopic liver transplant constitutes the only established long-term treatment for PSC with cumulative 5-year survival of about 70%.

Halothane Hepatitis

Halothane hepatitis is a severe, life-threatening form of hepatic damage that affects a small subset of individuals exposed to the anesthetic agent halothane[455] and is thought to have an immunologic basis. Sera of afflicted individuals contain autoantibodies directed against the native and the TFA form of hepatic proteins. TFA proteins are generated during the oxidative metabolism of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) and include cytochrome P450, protein disulfide isomerase, microsomal carboxylesterase, calreticulin, Erp72, GRP78 (BiP), and GRP94. Current evidence suggests that such TFA proteins arise in all individuals exposed to halothane. However, the vast majority of individuals appear to tolerate this covalent protein modification. The lack of immunologic responsiveness was suggested to occur due to tolerance induced through the presence of structures in the repertoire of self-determinants that immunochemically and structurally mimic TFA proteins very closely. In fact, lipoic acid, the prosthetic group of the constitutively expressed E2 subunits of members of the 2-oxoacid dehydrogenase complex family was demonstrated by immunochemical and molecular modeling analysis to perfectly mimic N^6-trifluoroacteyl-L-lysine, the major haptenic group of TFA proteins. Interestingly, a fraction of patients with halothane hepatitis exhibit irregularities in the hepatic expression levels of these cross-reactive proteins. Thus, molecular mimicry of TFA lysine by lipoic acid, or the impairment thereof, can be considered a susceptibility factor of individuals for the development of

halothane hepatitis.[456] A small animal model for chemically induced liver diseases has been described.[457]

***Perspective and Conclusion*.** The presence of autoantibodies in AIH, PBC, and PSC is indicative of autoimmune processes, but none of the antibodies described is liver specific and their contribution to pathogenesis remains unclear. While the complex role of T cells is subject to current investigations, the same caveats of specificity and pathogenicity discussed previously apply for autoimmune liver diseases.[458] Although the success of immunosuppressive therapy, in fact a criterion for the diagnostic scoring system for AIH,[44,420] further supports autoimmune etiology for AIH, it is conceivable that a viral infection might initiate disease as a "hit-and-run" event. Following elimination of viral antigens, disease may be perpetuated by immune-mediated processes and preclude the identification of a particular virus as causative agent at the time of liver disease diagnosis. Again, the role of "foreign antigens" and "self-antigens" becomes blurred and their interactions with the immune system in terms of immunity or autoimmunity conceptually problematic. Future investigations should seek to improve animal models and focus on human autoantigens and the application of contemporary tools for identification and isolation of specific lymphocytes. On the basis of such developments, future in vivo tracking of autoaggressive lymphocytes with noninvasive methods should substantially improve our insight into disease pathogenesis.

Renal Autoimmune Diseases

Glomerulonephritis (GN) is the major cause of chronic renal disease and kidney failure and exhibits a wide spectrum of histopathologic alterations, disease severity, and clinical outcomes. In most cases, evidence for an immunomediated pathogenesis, including humoral and cellular responses, has

been obtained.[459,460,461,462] The precise incidence of defined renal autoimmune disease is difficult to establish because definite diagnosis requires histologic evaluation of the kidneys. In this respect, the Italian Registry of Renal Biopsies provides a unique repository, and information about selected glomerular disease obtained from this resource is displayed in Table 44.3.[463,464] The identity of nephritogenic antigens targeted in most renal autoimmune diseases remain poorly defined, with the notable exception Goodpasture syndrome, a form of antiglomerular basement membrane disease in which autoantibodies bind to α3(IV) collagen in the glomerular basement membrane and are associated with rapidly progressive GN as well as pulmonary hemorrhage. Conformational epitomes have been mapped to two regions within the noncollagenous (NC1) domain of the α3(IV) chain.[465] A role for T cells, both in providing help for autoantibody production and exerting direct effector functions, has also emerged. Cast within the reigning concept of balanced Th1 and Th2 immunity, it appears that Th1 responses are associated with proliferative and crescentic forms of GN that cause severe injury while Th2 responses are associated with membranous patterns of injury.[459] These distinctions have also given rise to speculations that have harnessed the "hygiene hypothesis" as a potential explanation for the observation that proliferative GN have a relatively high incidence in developing countries, whereas minimal change disease and IgA nephropathy predominate in developed countries. Therapeutically, immunosuppression with

corticosteroids and in combination with cyclophosphamide and plasma exchange (to remove damaging antibodies) is employed to manage diseases. Based on the Th1/Th2 paradigm and its role in developing therapies for other autoimmune diseases that aim to target the Th1-Th2 balance, therapeutic approaches such as oral tolerance, cytokine-based immune modulation (anti-TNF-α and IL-12 p40 antibodies, recombinant IL-10), antiadhesion molecules, and chemokine receptor blockade are currently being evaluated for their potential use in the treatment of autoimmune renal diseases.[459]

TABLE 44.3 Incidence of Glomerular Diseases in 1993

Disease	Incidence per Million Population
Immunoglobulin A nephropathy	8.4
Membranous glomerulonephritis	4.9
Focal segmental glomerulonephritis	2.3
Minimal change disease	1.6
Mesangiocapillary glomerulonephritis	1.4
Poststreptococcal glomerulonephritis	0.7

From Schena et al.463

Cutaneous Autoimmune Diseases

Cutaneous autoimmune disorders are commonly divided into nonorgan-specific and organ-specific diseases. The former group contains skin manifestations of systemic autoimmune disorders such as SLE, scleroderma, Crohn disease, and ulcerative colitis. The organ-specific autoimmune skin disorders discussed in this section include several autoimmune bullous dermatoses as well as pigmentation disorder vitiligo. Despite a relative paucity of animal models for human autoimmune skin disorders, the skin is one of the few organs that offers direct access to target antigens and effector cells; consequently, advanced insights into mechanisms of disease induction and perpetuation have been obtained.

Autoimmune Bullous Dermatoses

Autoimmune bullous dermatoses are a group of severe, possibly fatal skin disorders characterized by erosions and blisters of skin and/or mucous membranes (Table 44.4). The term “pemphigus” (from Greek/Latin: pustule, bubble), featured in the name of several of these diseases, dates back

to “pemphigoid fever” described by Hippocrates nearly 2400 years ago and has been used to describe chronic blistering skin conditions since the mid-18th century. All forms of pemphigus have distinctive characteristics but exhibit the common feature of epidermal cell-to-cell detachment known as acantholysis. On occasion, pemphigus syndromes are associated with other autoimmune diseases such as SLE, RA, myasthenia gravis, pernicious anemia, and Hashimoto thyroiditis.

TABLE 44.4 Autoimmune Bullous Dermatoses

Disorder	Acantholysis	Autoantigen	Autoantibody Class	T-Cell Subset
Pemphigus vulgaris	Suprabasilar	Desmoglein-3 > Desmoglein-1	IgG4, IgG1, IgA (occasionally)	Th1, Th2, Tr1, CD8+CD28- T_{reg}
Pemphigus foliaceus	Subcorneal	Desmoglein-1	IgG4, IgG1	Th1, Th2
Bullous pemphigoid	Subepidermal	BP180, BP230	IgG1, IgG4, IgA (occasionally)	Th1, Th2
Cicatricial pemphigoid	Subepidermal	BP180 > BP230, Laminin 5	IgG1, IgG4, IgA (occasionally)	Th1
Pemphigus gestationis	Subepidermal	BP180, BP230	IgG1, IgG3	Th1, Th2
Dermatitis herpetiformis (associated with glutensensitive enteropathy)	Subepidermal	Epidermal transglutaminase	IgA	Th2
Linear IgA disease	Subepidermal	BP180, BP230	IgA	Th1, Th2
Epidermolysis bullosa acquisita	Subepidermal	Collagen VII	IgG, IgA and IgM (occasionally)	Th1, Th2, no T_{reg} contribution

CD, cluster of differentiation; Ig, immunoglobulin; Th, helper T; T_{reg}, regulatory T.

***Pemphigus Vulgaris*.** Pemphigus vulgaris (PV) is the most common and severe form of pemphigus in the United States, constituting more than 80% of reported cases.[466,467] With a worldwide incidence of one to five cases per million per year in the general population, PV remains a rare disease and preferentially affects individuals in the fourth to sixth decade of life. Clinical features include flaccid, fragile, and noninflammatory bullae (from Latin: bubble) that arise on normal appearing skin. These lesions tend to coalesce and rupture, leaving larger areas of denuded skin that often heal poorly.[466,467,468] About half of the patients initially present with lesions confined to the oral mucosa, although any surface with squamous epithelial tissue may be affected. Blister formation begins with edema of epidermal intracellular spaces and progresses to complete loss of cohesion between epidermal cells. In PV, blisters occur in the suprabasilar region of the epidermis while leaving attachment of basal cells to the dermis largely intact.[466,467,468,469,470]

Autoimmune Features. The hallmark of PV are autoantibodies generated against the desmosomal glycoprotein desmoglein-3 (Dsg3) (Fig. 44.14). In fact, the unequivocal identification of this antigen makes PV one of just a few human autoimmune disease for which the relevant autoantigen has been defined. Although the pathogenic contribution of Dsg3 antibodies has been demonstrated in passive transfer experiments, more recent work has emphasized the importance of specific T cells.[471] TCR transgenic mice with autoreactive CD4+ T cells that are specific for Dsg3 were recently generated. It was found that these CD4+ T cells specific for a single skin-associated antigen could induce a pemphiguslike phenotype.[472]

Peripheral CD4+ and occasional CD8+ T-cell responses targeting Dsg3 with varying functional profiles (Th1 and Th2) have been described. These autoreactive CD4+ T cells may be involved in the coordination of specific B-cell responses as evidenced by Th1-regulated IgG1 and Th2-regulated Dsg3 antibodies in patient sera. Interestingly, IL-10-producing Dsg3-reactive T cells have also been identified in the majority of healthy individuals carrying PVassociated HLA alleles (see subsequent discussion) but were found in <20% of patients with PV.[473] Given these functional attributes reminiscent of the Tr1 T_{reg} cell subset as well as the documentation of CD8+CD28- T cells with potential regulatory function in patients with remittent PV,[471] it has been proposed that clinical disease may emerge as a consequence of an imbalance between autoreactive pathogenic and T_{reg} cells.[471]

Genetic Features and Environmental Factors. Although PV can affect anyone, an enhanced prevalence is observed in people of Mediterranean or Jewish ancestry. Associations with certain HLA alleles (HLA-26, HLA-B38, SC21, HLA-DR4) in Ashkenazy Jews and non-Ashkenazy Jews (HLA-DRW6) have been detected in patients with PV, and it has been suggested that the third hypervariable region of the β-1 chain of the DRB1 allele may constitute a susceptibility locus.[466,467,468] Recent studies indicate that the H-CDR3 region within anti-Dsg antibodies is critical for disease induction and even one amino acid change

can alter their pathogenicity.[474] It was concluded that mutations in these areas can uncouple pathogenicity and binding. Finally, while PV may be associated with other autoimmune diseases,[470] its occurrence with certain malignant diseases has been defined as a separate entity.[475]

Animal Models. The pathogenic role of autoantibodies has been demonstrated by passive transfer of PV patient sera into

neonatal mice.[476] More recent studies using Dsg3-deficient mice have established that loss of tolerance and induction of autoimmunity required the presence of both autoreactive B and T cells.[477,478,479] Finally, CD4 T cells from Dsg3-immunized HLA transgenic mice target Dsg3 epitopes identical to those identified in humans and thus constitute a promising model to further analyze the regulation of Dsg3-specific T cells and autoantibody production in vivo.[471]

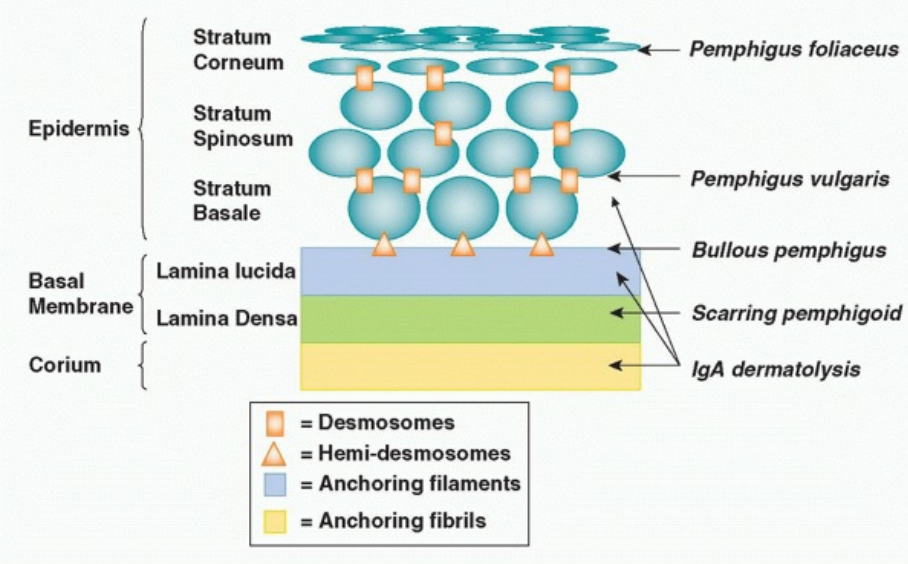

FIG. 44.14. Different Layers of the Skin and Their Involvement in Autoimmune Skin Syndromes.

Treatment and Prevention. Corticosteroids are the treatment of choice with supplementary immunosuppressive agents (cyclophosphamide, azathioprine, and methotrexate) commonly used to reduce the side effects of steroids. In severe cases, plasmapheresis may be of short-term benefit. Clinical data indicate that B-cell depletion, achieved by administrating anti-CD20 antibodies (rituximab), is an effective treatment for pemphigus, but it is generally acknowledged that, due to its potentially severe side effects, its use should be limited to the most severe types of the disease.[480]

Other Autoimmune Bullous Disorders. Other autoimmune bullous disorders include pemphigus foliaceus (PF), bullous pemphigoid (BP), cicatricial (scarring) pemphigoid, epidermolysis bullosa acquisita, dermatitis herpetiformis, and linear IgA disease (see Table 44.4). Two forms of PF are recognized: one affecting individuals irrespective of ethnic

background around the world and the other, PF fogo selvagem (Portuguese: savage fire), endemic to certain regions of Brazil. Antibodies found in the circulation of patients with PF preferentially target Dsg1, are of the IgG4 subclass, and similar to PV, can induce aspects of the disease in a neonatal mouse transfer model[466]; Th1 and Th2 T cells specific for the same antigen have also been described.[471] Information about genetic association remains incomplete due to the rarity of PF. However, PF fogo selvagem demonstrates associations with HLA-DR1 and HLA-DR4. Prior to the advent of corticosteroids, fewer than 10% of patients underwent spontaneous remission, and fatalities reached up to 40% within the first 2 years of disease. Compared to PV, patients with PF respond more rapidly and to smaller doses of corticosteroids.[466]

Together with PV, BP belongs to the best characterized bullous disorders with regard to pathogenesis. In BP, IgG autoantibodies are directed against components of the dermal-epidermal basement membrane such as BP antigens BP180 and BP230. Again, these antigens are also recognized by CD4+ T cells and comprise both Th1- and Th2-type subsets,[471] and systemic corticosteroids with facultative addition of other immunosuppressive agents are used for treatment. The principal autoimmune targets and basic pathologic features of the remaining bullous dermatoses are listed in Table 44.4.

Perspective and Conclusion. The identification of relevant autoantigens, a task that is far from complete in the majority of human autoimmune diseases, is usually considered to be a critical juncture in our understanding of pathogenetic mechanisms and an important step toward the development of targeted immunomodulatory therapies. However, the case of PV and BP is a telling reminder that even with established knowledge about such primary autoantigens, the development of specific immunotherapies faces continuing challenges.

Vitiligo

Introduction and Disease Description. Vitiligo is an acquired, noncontagious disorder of pigmentation resulting from melanocyte loss (reviewed in Passeron and Ortonne,[481] Spritz,[482] and Boissy,[482a] Theo Book). The disease is characterized by defined areas of depigmentation that range from limited numbers of macules (focal vitiligo) to bilateral, symmetric distribution of the lesions (generalized vitiligo, the most common type) to involvement of the complete body surface area (universal vitiligo). Vitiligo is arguably the most common pigmentary disorder affecting 0.5% to 2% of the population worldwide and demonstrates no predilection for gender or ethinicity. Although commonly regarded as a pathophysiologic entity with distinct clinical manifestations, the existence of a spectrum of vitiligo disorders with different etiologies was suggested. The potential autoimmune etiology of vitiligo has been discussed for some time and has gained traction through recent studies on genetic epidemiology. A combination of environmental, genetic, and immunologic factors is thought to contribute to autoimmune melanocyte destruction. This hypothesis is further strengthened by the reported association of vitiligo with other autoimmune diseases such as autoimmune thyroid disease, SLE, Addison disease, adult-onset T1D, and pernicious anemia.[483] Another interesting analogy with T1D was coined by Eisenbarth,[484] who noted that both diseases shared an unexplained focality of melanocytes or β cells. A lingering question in this regard is what differentiates one melanocyte or β cell from another in becoming a target for autoimmunity. Nevertheless, the specific causes of vitiligo remain

obscure and progress is limited due to lack of a clear definition of the disorder as well as a dearth of experimental animal models.[482]

Autoimmune Features. T-cell infiltrates have been reported in generalized vitiligo and exhibit molecular signatures indicative of CTL and Th1 responses (TNF-α and IFNγ).[485] CD8+ T cells tend to predominate in vitiligo lesions, and high frequencies of Melan-A-specific CD8+ T-cell subsets apparently correlate with severity of disease.[486,487] Additional infiltrating cells include macrophages and monocytes producing proinflammatory cytokines as well as dendritic cells. The role of dendritic cells in local immune reponses remains unclear, but the dendritic cell-mediated killing of melanocytes has been suggested as a mechanism for depigmentation.[488] Finally, circulating autoantibodies, including those specific for the melanocyte antigen TRP1, have been found in the sera of patients with vitiligo, and TRP1-specific antibodies can induce melanocyte loss in mice.[489] Nevertheless, there appears to be an emerging consensus that alterations of humoral immunity are secondary to T cell-mediated autoimmune destruction.

Genetic Features and Environmental Factors. Evidence for genetic factors contributing to onset of generalized vitiligo has been obtained in studies of the close relatives of patients with

vitiligo.[483,490] The largest vitiligo twin study to date has demonstrated concordance for generalized vitiligo in monozygotic twins of 23%, far greater than the risk for nonidentical siblings (6.3%) and the general population risk.[483] Vitiligo occurs with a frequency of 0.1-2.0% in various (general) populations.[483] At the same time, these data highlight the critical importance of environmental factors. Genetic association and linkage studies initially revealed an association between vitiligo and HLA-DRB1, HLA-DLRB4, and HLA-DQB1 alleles as well as other genes.[481,482] It is worth noting that the first vitiligo linkage data excluded a single candidate gene, the micro-ophtalmia-associated transcription factor. Microophtalmia -associated transcription factor appeared to be a relevant candidate due to numerous mutations at the mouse microphthalmia locus associated with reduced or absent pigmentation and with the mivit/vit mutation presenting postnatal depigmentation that resembles human vitiligo.[491] Many of the findings from early genetic studies were difficult to reproduce, and a recent study systematically tested the association of multiple previously reported candidate genes.[492] It was found that only HLA class I, HLA class II, and lymphoidspecific protein tyrosine phosphatase nonreceptor type 22 (PTNP22) were strongly associated, with weaker support for TSLP, XBP1, and forkhead box (FOX)D3. It can be concluded that most other previously reported associations most likely represent false-positives. One interesting finding came from association analyses that resulted in the identification of NALP1 (later renamed NLRP1) as a candidate gene.[493] This gene encodes an important regulator of the innate immune system that may monitor bacterial infection of the skin. Importantly, other reports have subsequently found genetic associations of NLRP1 with other autoimmune diseases such as T1D, Addison disease, celiac disease, and systemic sclerosis, pointing toward overlapping disease mechanisms.

The genetic basis of vitiligo was recently explored by a series of GWAS, which have drawn a more comprehensive and reliable picture of the potential disease genes in (generalized)

vitiligo. Almost all susceptibility genes that were found involve candidate genes functional in immune regulation and immune targeting of melanocytes, which has solidified the general consensus that vitiligo is a primary autoimmune disease.[494] The results from three large-scale GWAS have been reported, and major association signals in the MHC (both class I and II genes) and also numerous non-MHC associations were revealed.[495,496,497,498] The list is too long to summarize here (see Spritz[494]) but includes many important immunity genes such as *TYR* (tyrosinase), *PTPN22*, IL-2Rα (IL-2-receptor alpha chain), *GZMB* (granzyme B), and *FOXP3*. Most of these genes have been discussed in this chapter for other conditions, again highlighting potentially common immune pathways. It is estimated that the current set of known genes accounts for approximately 10% of vitiligo total risk, and it is thus expected that large GWAS in the near future will complete this picture.

Animal Models. The lack of a tractable experimental animal model has clearly hampered progress of vitiligo research. Nevertheless, the mutant Smyth line of chickens exhibits several relevant clinical features including melanocyte loss as a consequence of T-cell infiltration.[499] Furthermore, the potential contribution of herpesvirus infection to vitiligo induction in Smyth line chickens[500] emphasizes the role of environmental factors and warrants further investigations into this model.

Treatment and Prevention. Routine nonsurgical repigmentation strategies include phototherapy, topical steroids, and inhibitors of the calcineurin pathway. These treatment modalities, while interfering with a multitude of complex pathophysiologic pathways, modulate in particular the activity of local T-cell responses.

Perspective and Conclusion. In addition to genes involved in aberrant immune regulation, more than 120 genes are known to participate in the coordination of mammalian pigmentation. However, the identification of several candidate genes and genetic linkages associated with vitiligo will facilitate the elucidation of biologic pathways involved in vitiligo pathogenesis and allow for a better differentiation of the spectrum of vitiligo disorders. As a result, novel therapeutic and prophylactic approaches will likely be developed in the near future.

Cardiac Autoimmune Diseases

The etiology for primary myocarditis is unclear and an autoimmune cause has to be taken into account. The heart muscle is infiltrated mainly by CD4+ T cells and macrophages, and the infiltrates are usually diffuse and nonfocal. The infiltrating cells produce considerable amounts of INFγ and TNF-α. As a consequence of the inflammation, heart muscle cells can swell, the heart can dilate, and severe disturbances of the electrical conduction can occur. It is noteworthy that myocarditis can also occur in conjunction with certain systemic autoimmune diseases such as SLE, scleroderma, rheumatoid arthiritis, polymyositis, and polyarteritis nodosa. Two specific scenarios are noteworthy, as discussed in the following text.

The first is myocarditis occurring after Chagas disease. In this case, cross-reactivity between antigens presented by the parasite causing Chagas disease (*Trypanosoma cruzi*) and cardiac myosin has been found in patients with myocarditis (Kalil JCI). The scarcity of parasites in the chronic phase of the disease contrasts with the severe cardiac pathology observed in approximately 30% of chronic patients and suggested a role for autoimmunity as

the origin of the pathology. In these cases, various *T. cruzi* antigens, such as B13, cruzipain, and Cha, cross-react with host antigens at the B- or T-cell level, and their role in pathogenesis has been widely studied. Immunization with those antigens and/or passive transfer of autoreactive T lymphocytes in mice leads to clinical disturbances similar to those found in patients with Chagas disease. On the other hand, the parasite is becoming increasingly detected in chronically infected hosts and may also be the cause of pathology either directly or through parasite-specific mediated inflammatory responses. Thus, the issue of autoimmunity versus parasite persistence as the cause of Chagas disease pathology is hotly debated among many researchers in the field.

The second is coxsackie B virus-associated myocarditis. Also in this situation, immunopathology outlasts the direct presence of the virus in heart muscle. This disease has been extensively studied in experimental models by Rose et al.[500a] They developed two murine models of myocarditis, one elicited by cardiotropic coxsackie B3 virus infection and the other by cardiac myosin immunization, to better analyze the pathogenetic mechanisms responsible for immune-mediated heart-muscle disease. Both virus infection and myosin immunization produce myocardial inflammation and elicit heart-reactive antibodies that bind to the myocardium in vivo and which recognize the cardiac myosin heavy chain. Each model offers unique advantages. The virus-induced disease more closely resembles human myocarditis; myosin immunization isolates the autoimmune components of the disease because no virus infection is involved. They also distinguished strains of mice resistant to autoimmune myocarditis (such as B10.A) from those susceptible to the autoimmune phase of disease (such as A.CA and A/J). Mice from a resistant strain to virus- or myosin-induced autoimmune heart disease develop myocardial inflammation and myosin antibodies if cotreated with TNF-α or IL-1 when infected or immunized. Thus, cytokines can modulate the outcome of cardiotropic virus infection and enhance its autoimmune sequela. They also found that blocking IL-1R inhibits autoimmune myocarditis in genetically susceptible mice. Interestingly, there is no regulatory function of Th2 cytokines such as IL-4, illustrating that immune regulation is an organ specific concept rather than a global paradigm applicable to all autoimmune disease equally.

Rheumatoid Arthritis

Although RA is commonly classified as a systemic autoimmune disease (although it electively affects joints), we believe that RA offers insights and paradigms that are important for understanding organ-specific diseases such as T1D and MS. A particularly interesting aspect is that diagnostic or therapeutic access to the target organ is comparatively easily achieved facilitating comparatively, and that this has facilitated the development of rational and highly effective immunotherapies. In this case, high levels of TNF-α were detected in affected joints and TNF blockade has now proven to be a highly effective intervention in RA (see more detailed description in the following text).

Introduction and Disease Description

RA is a severe debilitating disease with unknown etiology. Epidemiologically, the prevalence is about 1% in the overall population, women are affected three times more frequently than men, and the peak of incidence is within the fourth and sixth decade of life.[172,501] Diagnostic criteria are the typical morning stiffness, joint swelling with fluid accumulation,

defined radiologic changes, subcutaneous rheumatic nodes, and positive rheumatoid factor (autoantibodies) (Fig. 44.15). The prognosis is worse in HLA-DR4-positive patients and, similar to diabetes and MS, there is genetic

linkage with certain MHC haplotypes. Recent advances by GWAS have added numerous non-HLA-associated disease genes, a list too long to include here (recently reviewed in Suzuki et al.[502]). Many important immunity genes, such as CTLA-4, PTPN22, and IL-2Rβ, were identified that have been found in other autoimmune conditions, in addition to genes involved in nonimmune-related pathways. One notable example in the latter category is PADI4, coding for a protein that converts the arginine residue (peptidyl arginine) to the citrulline residue (peptidyl citrulline) in a process called citrullination. Peptidyl citrulline is targeted by a class of autoantibodies in RA that have high diagnostic value, and this protein conversion is influenced by smoking. Interaction between PADI4 polymorphisms and smoking was repeatedly reported and thus offers a link between this important environmental determinant and genetic constitution of the host. Without systemic immunosuppressive or anti-inflammatory therapy, the disease will eventually result in destruction of many major joints and immobilize the patient. The major cause of death is infections that easily take a more severe course in immobile individuals.[172]

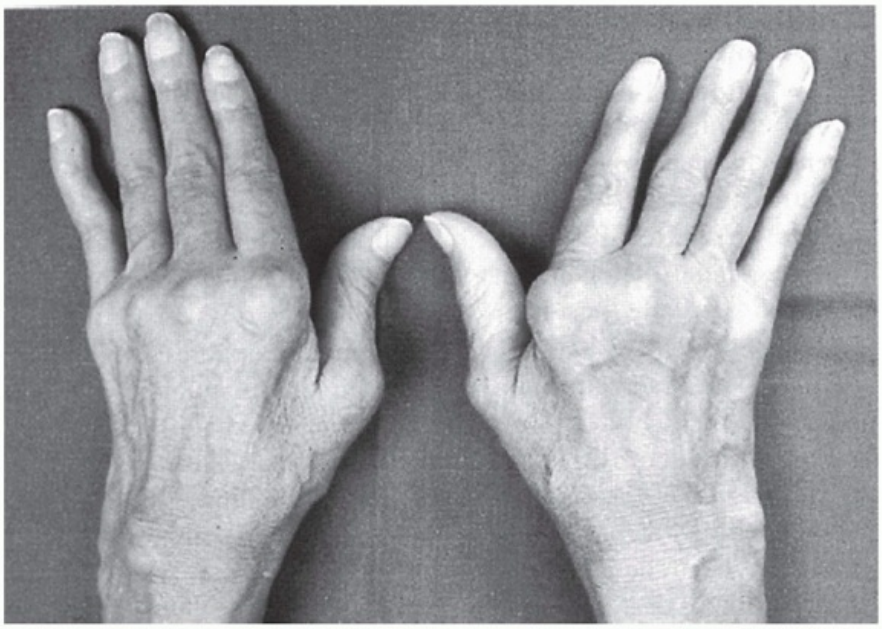

FIG. 44.15. Rheumatoid Arthritis. Typical ulnar deviation and swan's neck deformation of the hands in rheumatoid arthritis.

The etiology of RA remains ill-defined. As there is a possible overlap between infection-associated arthropathies (eg, Reiter syndrome), bacteria have always been good candidates as a cause for arthritis, and molecular mimicry has been suggested as a cause, as cross-reactivities of autoantibodies between self-protein and bacterial proteins have been detected.[123] However, while it is quite possible to demonstrate cross-reactive antibodies and T cells in human blood, proof of their pathogenetic involvement is exceedingly difficult to

obtain. The immune system is likely to have developed to tolerate a low level cross-reactivity, which might be necessary for its proper function, and it remains difficult to prove a mechanistic link between autoreactive cells and pathologic autoimmunity in humans.

Autoimmune Features

The evidence that RA is an autoimmune disease stems, as in the case of MS and T1D, from the observation that T lymphocytes in a patient's blood can react with joint-derived autoantigens and that IgM autoantibodies, as well as ANAs, are readily found in humans.[503] Inflammatory signs in the serum include complement activation and increased erythrocyte sedimentation rate, which indicates that the antibodies could possibly play a role in the disease process. Similarly, immunization with collagen can induce arthritis in susceptible mouse strains (Fig. 44.16). Figure 44.17 summarizes the major pathogenetic pathways leading to progressive joint destruction. As in many other autoimmune disease animal models, collagen-induced arthritis models require strong immunization with autoantigens and adjuvant, supporting the concept that breaking of self-tolerance, even on a genetically susceptible background, requires a rather pronounced inflammatory stimulus. In contrast, how disease is initiated in humans is unclear. The immunopathogenesis of RA has been redefined since the discovery of the Th17 lineage, which is now firmly established as a principal instigator of joint inflammation in animal models and has been repeatedly found in joints from affected patients. Current treatment modalities were generally developed based on the classification of RA as a prototypical Th1-driven condition. Potential future treatments, including anti-IL-17 and anti-IL-6 therapy, may be effective by dampening this Th17-driven disease component.

Treatment and Prevention

Treatment of RA was historically regarded as a difficult task because the medications that adequately suppress joint inflammation also had strong systemic side effects. Therefore, a delicate balance between different therapeutic approaches that target different stages of the inflammatory process needs to be established. Corticosteroids and nonsteroidal anti-inflammatory drugs can provide some baseline relief but are not able to halt progressive joint destruction. Gold compounds, methotrexate, and cyclophosphamide are more effective but also have profound systemic side effects. For this reason, progress with novel immunomodulatory interventions is of important benefit for patients with RA.

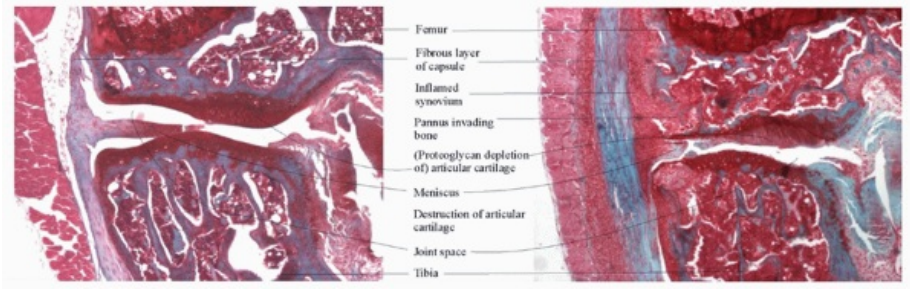

FIG. 44.16. Immunopathology of Collagen-Induced Arthritis in Mice. All features that are characteristic to rheumatoid arthritis can be found in sections from knee joints,

here stained for proteoglycans with Safranin O. *Left* is a joint from a healthy control animal, while *right* is from a mouse with full-blown arthritis. Both inflammation and structural damage to cartilage and bone are evident.

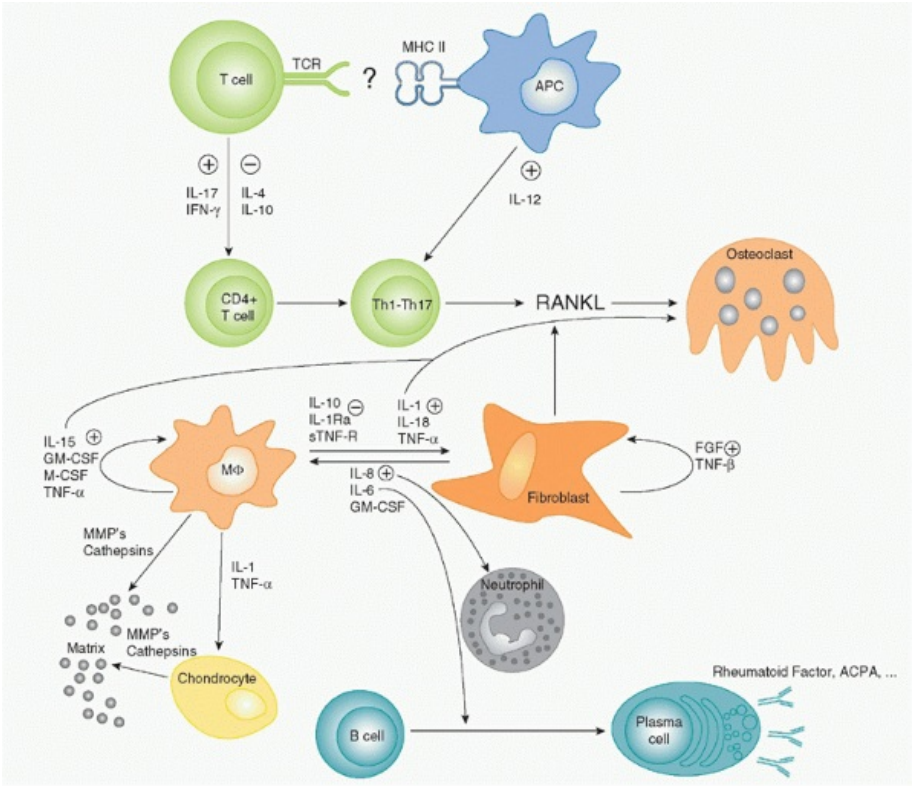

FIG. 44.17. Pathogenic Pathways Leading to Joint Destruction in Rheumatoid Arthritis (RA). T cells are thought to be essential in the etiology of RA, but it is unknown what triggers their activation. Once activated, T cells are skewed to a helper T (Th)1 or Th17 phenotype and release factors such as RANKL that activate bone resorption through osteoclasts. Macrophages are major effectors and are believed to account for the high levels of tumor necrosis factor (TNF) found in rheumatoid synovial tissue, resulting in cartilage destruction via effects on chondrocytes. Finally, pathogenic antibodies are produced such as rheumatoid factor as a consequence of B-cell activation. Note that the introduction of biologics such as anti-TNF, cytotoxic T-lymphocyte antigen-4Ig or anti-cluster of differentiation 20 intervene at distinct levels within the pathogenic process.

Application of cyclosporin A was shown to be effective by suppressing proliferation and activation of T lymphocytes. A more selective intervention has also been established based on research by Feldman et al.[171] TNF-α was found to play a key role in an in vitro model of synovial destruction using human cells, and numerous TNF-α blocking agents were subsequently developed. After promising preclinical results in animal models, this intervention was tested in clinical trials and is now licensed for treatment of RA and many other autoimmune conditions, as discussed previously. Although side effects do occur, as expected,

they appear tolerable and clinical improvement of disease is pronounced, although not all patients respond to treatment. In some patients, SLElike symptoms were observed, indicating that blocking a cytokine beneficial for one autoimmune disorder might be detrimental for another one. Indeed, TNF blockade appears to be less effective in MS because this cytokine exhibits complex dual functions depending on the disease stage. In T1D, a small trial did suggest some effectiveness, although animal models previously indicated that time of administration crucially dictated outcomes in terms of β cell preservation.[504] Based on the success story with TNF blockade in RA, and more particularly the approach of determining the dominant cytokine(s) locally at the target site, it may

be possible to find similar key cytokines for T1D or MS treatment. It should be stressed that the successful intervention was developed based on human cell cultures and not on animal models, underlining the need for direct research on human materials, if and when possible. Other strategies based on therapeutic antibody blockade such as CTLA-4Ig, anti-IL-1, and anti-CD20 adminstration have recently expanded the therapeutic arsenal for RA and in combination have led to dramatic improved life quality of patients with RA. It is also well established that anti-TNF biologics are the first to halt structural joint damage, a feat not achieved by any of the earlier generation disease-modifying antirheumatic drugs. Future improvements likely lie in this aspect of disease, which principally accounts for long-term disability in RA. Finally, high hopes were raised by the successful trials with Janus kinase inhibitors, which are small molecular compounds that can be orally administered and act at signaling pathways common to a variety of cytokines.

Scleroderma (Systemic Sclerosis)

Introduction and Disease Description

The term scleroderma, also known as systemic sclerosis (SSc), is derived from the Greek words *skleros* (hard) and *derma* (skin). Apart from thickening and tightening of the skin, this chronic condition often involves damage to internal body organs. The skin thickening in patients with SSc typically starts with the fingers and can proceed to affect the whole body. The thickening results from vasculopathy and sclerosis or stiffening of structures due to an increased collagen content of the connective tissue.[505] Depending on the degree of skin thickening, SSc can be divided into limited SSc (vascular and/or SSc-associated antibodies but no skin involvement), limited cutaneous SSc (typically involving forearms, hands, legs, feet, and face) or diffuse SSc (can affect almost any area of the body).[506,507] SSc is a relatively rare disorder—about 20 new cases are diagnosed annually per million in the Unites States—which primarily targets adult women.[508] While symptoms may initially be rather benign, serious complications can develop over time and may involve various organs such as the gastrointestinal tract, lung, kidney, heart, and musculoskeletal system.

Autoimmune Features

The pathophysiology of SSc is thought to have a vascular, immunologic, and fibrotic component.[505] A prominent diagnostic marker in SSc is the presence of ANAs while also nonnuclear antibodies such as antifibrillin and anti-MMP-1/3 are commonly found.[509] B cells in general seem to have a chronically activated phenotype,[510] while an oligoclonal T-cell

subset has also been found in skin lesions.[511] The fibrotic aspect is caused by the overproduction of cellular matrix by activated fibroblasts, which in turn induces contraction of the affected tissue.[512] The vascular component encompasses reversible vasospasms (as in Raynaud phenomenon) as well as structural changes in vessel architecture sometimes leading to obliteration of the vessel lumen. The elevation of endothelium-derived serum factors in SSc such as von Willebrand factor and endothelin-1 but also circulating endothelial cells point toward the existence of vascular abnormalities.[513,514,515] Moreover, vasculogenesis appears to be impaired in SSc.[516] It is still largely unresolved how these three mechanisms act in concert to culminate in the characteristic SSc disease phenotype.

Genetic Features and Environmental Factors

Family history constitutes a strong risk factor for SSc, although the low disease concordance rates in twin studies suggest that environmental factors play a role.[517] The advent of GWAS has offered more robust datasets in recent years and has added new susceptibility loci.[518,519] In accordance with our present understanding of the pathophysiology of SSc, the associated polymorphisms can be categorized in relation to either immunity, fibrosis, or vascular disease (Table 44.5). Possible environmental factors that may act in concert with a susceptible genetic constitution include viral or bacterial

agents,[520] vitamin D levels,[521] and a wide host of occupational factors.[522] The vast range of potential interactions between the many susceptibility genes and environmental factors may account for the disease's notable heterogeneity.

TABLE 44.5 Systemic Sclerosis Susceptibility Genes Organized According to Possible Involvement in One of Three Pathophysiologic Pillars of the Disease

Immunity	Vascular Disease	Fibrosis
Transcription factors	*Angiogenesis*	*Fibroblast proliferation, ECM production*
STAT4; TBX21; IRF5	CXCL12; VGEF	CTGF
Lymphocyte (co-)stimulation	*Vascular integrity, survival*	*Platelet aggregation*
CD247 (CD3ζ); CD226; TNFSF4 (OX40L)	CD105; HIF1A	Serotonin 5-HT2A receptor

Phospatases/kinases	*Vasoconstriction*	*Fibrogenic phenotype*
PTPN22; BLK	ACE; eNOS and iNOS; ET-1 and receptors	IL-1α/β
B cell-specific scaffold protein	*Voltage-gated K+ channel*	*ECM degradation*
BANK1	KCNA5	MMP
Ubiquitin-modifying enzyme	*GPI-anchored surface receptor*	
TNFAIP3	CD87	
Apoptosis	*Blood clotting*	
FAS	Fibrinogen	

CD, cluster of differentiation; CTGF, connective tissue growth factor; ECM, extracellular matrix; FAS, TNF receptor superfamily, member 6 or CD95; GPI, glucose-6-phosphate isomerase; IL, interleukin; MMP, matrix metalloproteinases; TNF, tumor necrosis factor; VGEF, vascular endothelial growth factor.

Principal gene type/function is listed but does not exclude other functions in systemic sclerosis. References to all original studies can be found in Romano et al.560

In italics: gene category according to function. Regular text: respective genes within this category related to sclerosis.

Animal Models

Multiple genetic and inducible animal models each cover some of the three aspects of SSc to a varying degree (see the excellent review in Beyer et al.[523]). We mention here three examples that demonstrate the partial spectrum of SSc characteristics mimicked by each model. A widely used inducible model is the bleomycin-induced skin fibrosis model. This

compound, originally isolated from *Streptomyces verticillus*, induces skin fibrosis localized to the injection area after repeated injections.[524] The model mimics the inflammatory changes in SSc that are thought to occur early in the disease course, usually before a patient seeks medical care. It is therefore useful for preclinical evaluation of therapeutics that target inflammation or early-stage cutaneous fibrosis. An obvious disadvantage is that fibrosis is restricted to the injection site, while SSc in humans typically presents as a systemic disease involving both skin and internal organs. Furthermore, vascular phenomena as seen in patients with SSc are absent, which is a serious shortcoming.

One of the genetically triggered models is the TSK-1 mouse,[525] where SSc-like skin tightening is caused by a tandem duplication of the fibrillin 1 gene (*Fbn1*).[526] Whereas the homozygous mouse dies before birth, heterozygous TSK-1 mice develop SSc-like disease, with skin fibrosis and autoimmunity. A remarkable parallel with human disease is the occurrence of autoantibodies against SSc-specific antigens (eg, topoisomerase I). The excessive release of extracellular matrix proteins by activated fibroblasts underlies the fibrosis seen in these animals. These mice do lack a vascular phenotype and are commonly used to study later stages of SSc associated with low inflammation.

One mouse model that does display characteristic SSclike microvasculopathy but lacks autoimmune phenomena is the *Fra2* (Fos-related AP1 transcription factor) transgenic mouse, which expresses Fra2 under the control of a class I MHC antigen promoter.[527] These examples serve to highlight the notion that a perfect SSc model does not exist, but rather that several models can be chosen to study one or more of the three pillars underlying the disease.

Treatment and Prevention

At present, most therapeutical approaches are organ-based, while systematic treatment modalities aimed at the vascular and inflammatory component may also be used. Drugs such as metothrexate (skin involvement[528]), cyclophosphamide (interstitial lung disease[529]), or dihydropyridine calcium blockers (Raynaud phenomenon[530]) have proven efficacy but clearly offer incomplete protection. No effective therapies, however, exist today that effectively block the fibrotic aspect.

Systemic Lupus Erythematosus

Introduction and Disease Description

SLE is a systemic autoimmune disease sometimes referred to as the “great imitator.” The wide array of clinical manifestations includes skin rashes, photosensitivity, oral ulcers, arthritis, pleuritis, pericarditis, kidney problems, neurologic symptoms, and hematologic abnormalities.[531] Clinical course is typically of the relapsing-remitting type. As a consequence, the disease is notoriously difficult to diagnose, and adequate treatment ideally involves a team approach due to the number of organ systems involved. SLE affects up to 10 times more women than men while estimated incidence ranges widely, likely due to inconsistencies in diagnosis, from 1.8 to 7.6 cases per 100,000 persons per year in the United States.[531a] Hormones are suspected to codetermine disease and thus account for the increased prevalence of SLE among women. Patients commonly present with general complaints such as malaise, fatigue, fever, or weight loss, and a correct diagnosis critically

depends on careful history and physical examination combined with appropriate laboratory testing.

One of the most characteristic skin manifestations is the “butterfly” rash, which shows a malar (across the cheeks) distribution and often develops in response to sunlight exposure. Arthritic symptoms are in fact the most common manifestation of SLE, affecting mostly the small joints of the hands, wrists, and knees but may involve any joint. Renal involvement is considered the single most common cause of morbidity and represents a major cause of mortality in SLE. Of particular diagnostic importance in SLE are autoantibodies against intranuclear nucleic acids, proteins and ribonucleoprotein complexes, commonly known as ANAs (see the next section). A general ANA test is performed by incubation of the patient's serum with a fixed, permeabilized cell line followed by fluorescently labeled antihuman IgG detection antibody. Specific autoantibodies that are associated with SLE and are used for diagnosis include anti-dsDNA, anti-Smith (part of small nuclear ribonucleoproteins), anti-U1-ribonucleoprotein, anti-Ro/SSA (largely unknown location and function), and anti-La/SSB (a nuclear phosphoprotein). This list is not exhaustive, and it has become clear that SLE sera contain antibodies against extranuclear components also, such as antiribosomal antibodies and various cell membrane components. None of these antibodies, however, represents a reliable diagnostic tool that covers the entire spectrum of SLE manifestations. Anti-Smith, for instance, is highly specific but is detected in only 10% of Caucasian patients with SLE.

Genetics

As expected from the heterogenous disease phenotype, genetic predisposition in SLE is extremely complex. Before the GWAS era, only a limited set of genes were known to be associated with SLE. As in most autoimmune diseases discussed here, HLA genes contained SLE-associated polymorphisms in addition to genes involved in the complement cascade (C2, C4), Fcγ receptors (FCGR2A, FCGR3A), B-cell differentiation (PDC1), a tyrosine phosphatase (PTPN22), and factors involved in cytokine signaling (TNF-α, IRF5).

At the time of writing, the combined results from seven GWAS are published, drastically adding to the number of SLE genes. While a complete summary of all associations is beyond the scope of this chapter (there is a full list in Sestak

et al.[532]), it can be concluded that many aspects of SLE genetics show overlap with other autoimmune diseases. For instance, the SLE susceptibility genes BANK1, BLK, IRF5, PTPN22, and STAT4 are all included in Table 44.5 detailing the genetic landscape of systemic sclerosis, while most are well-known autoimmune genes found in several other autoimmune conditions (eg, PTPN22, STAT4, and the HLA genes). Overall, the genetic data indicate that SLE results from interference with a diverse array of pathways such as antigen presentation, clearance of apoptotic material and immune complexes, immune cell function, innate immunity, cytokines, cellular energy machinery and leukocyte/endothelial adhesion, lymphocyte signaling, IFN response, complement activation, and apoptosis. Therefore, it remains to be seen to what extent this wealth of data will translate to conceptual and in particular translational insight. Although it is interesting to see that some of the usual suspects underlie SLE genetics, it is hoped that a thorough genetic understanding will explain which specific gene set provokes the SLE disease phenotype.

Autoimmune Features

A myriad of immunologic mechanisms is thought to underlie SLE, mirroring the disease's complex genetic and phenotypic profile (Fig. 44.18). How all these individual immune pathways culminate in clinical SLE is still unclear, and it is anticipated that different combinations of mechanisms might affect individual patients. As in many other autoimmune disorders, inadequate thymic selection is hypothesized to be responsible for the release of naive autoreactive T and B cells into the periphery.[533] What triggers their activation is still elusive, although support for some of the environmental factors mentioned before exists. The Epstein-Barr virus, for instance, has received a considerable degree of attention, and higher loads were found in patients with SLE in comparison with controls.[534] Molecular similarities between the Epstein-Barr virus nuclear antigen 1 and the common lupus autoantigen Ro were identified, fueling speculation about the role of mimicry in SLE.[535] A feature that further confounds our interpretation of the disease is the existence of autoantibodies up to 10 years prior to disease onset. Thus, whatever the inciting antigen may be, it is likely that the immune system generally encounters it long before presentation of clinical symptoms.[536]

Altered T-cell signaling of potentially autoreactive T cells may then lead to a lower activation threshold (ie, a relatively weak signal from presented self-antigen suffices to trigger activation). T cells are thought to be directly responsible for increased levels of proinflammatory cytokines, notably IL-17, which is found in the blood and kidneys of patients with SLE. Another notable feature is a reduced ability of the T cells to secrete IL-2, which could be the cause of an impaired T_{reg}-cell compartment or reduced activation-induced cell death followed by increased longevity of autoreactive effectors.

Increased turnover from B cells to plasma cells is a hallmark and is a consequence of their encounter with self-antigen in the presence of T-cell help.[537] Follicular Th cells are a specialized subset important in the formation of germinal centers and development of T cell-dependent B-cell responses. Emerging evidence underscores the potential pathogenic role in the germinal centers during SLE development. Plasma cells in turn produce autoantibodies that upon binding to self-molecules cause activation of the complement cascade. A first wave of apoptotic cell death is thought to be ineffectively cleared by macrophages leading to the presentation of intracellular self-antigens instead of the default pathway of “silent” removal. This process leads to variable patterns of “antigen spreading” and subsequent rounds of uncontrolled apoptosis. Other innate cell types such as dendritic cells further augment inflammation by type I IFN release and upregulation of activation markers. In addition, surface markers such as CD44 that are important in homing appear to be altered on T cells, leading to increased migration to sites of inflammation.

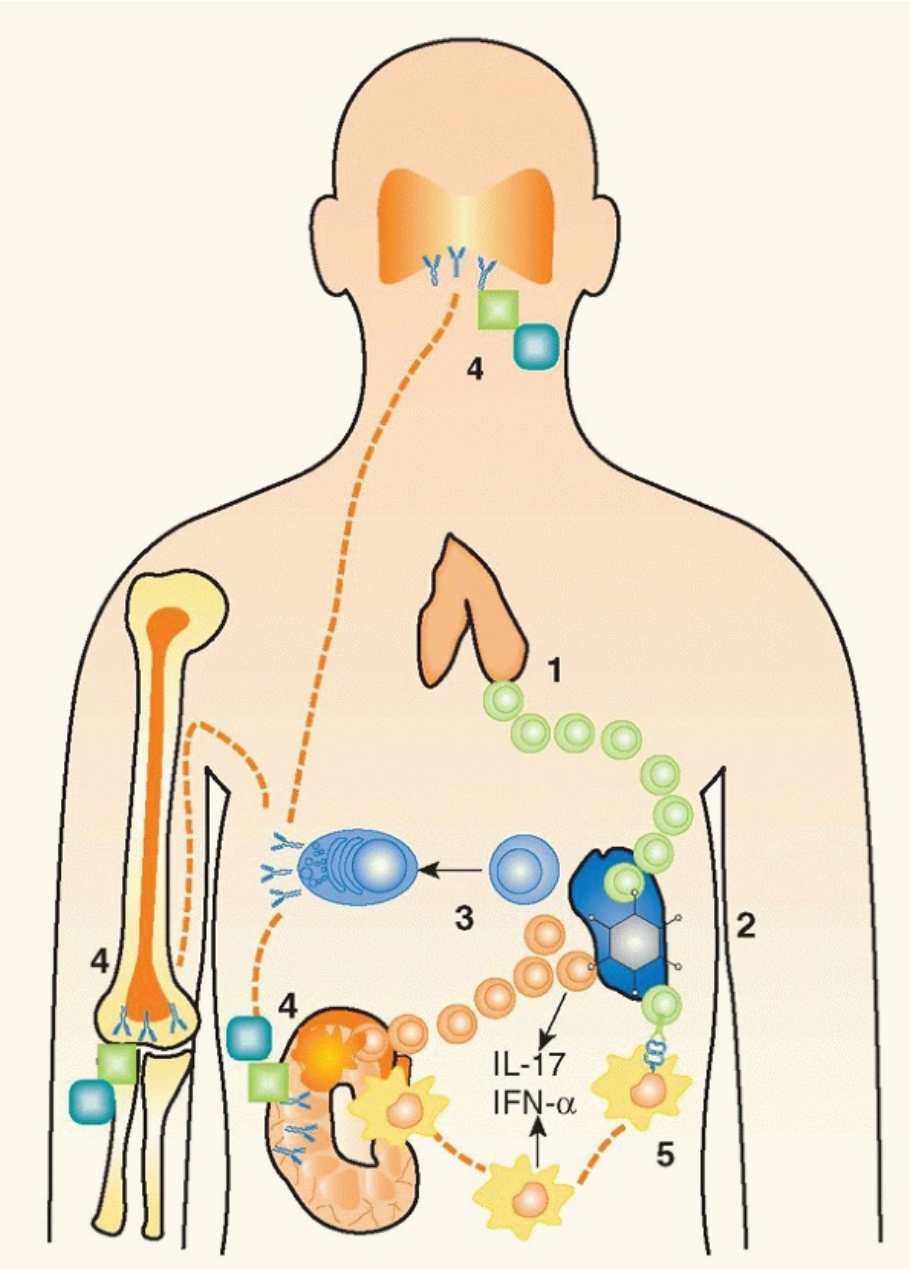

FIG. 44.18. Pathogenic Immune Mechanisms in Systemic Lupus Erythematosus. **1:** As in many autoimmune diseases, defective thymic selection is thought to contribute. **2:** Naive autoreactive cells reach the periphery and are activated by an unknown stimulus, possibly virus. **3:** These activated T helper cells in turn aid in generating plasma cells that produce copious amounts of autoantibodies. **4:** These autoantibodies bind components in various organs, here shown as joints, skin, and kidney. Complement activation and tissue inflammation initiates. T cells and other leukocytes may also infiltrate. **5:** Due to inadequate processing of apoptotic cells, a new round of autoimmunity is initiated by antigen presentation.

Animal Models

We will, without being exhaustive, discuss here two spontaneous and two induced mouse models that have been

instrumental in delineating SLE pathophysiology (see full reviews in Kippel and Dieppe,[531] and Perry et al.[538]). Numerous genetically modified strains were found to exhibit some SLE-like features and are thus informative in the identification of gene function but are not commonly used to study SLE per se.

One of the most widely adopted models is the New Zealand Black/White F1 mouse. This strain originates from a cross between the New Zealand Black and New Zealand White strains, of which only the former develops mild clinical signs of autoimmunity. The F1 hybrids, however, display a lupus-like phenotype including B-cell hyperactivity, development of ANA, and immune complex-mediated glomerulonephritis around 5 to 6 months of age. Mice typically suffer kidney failure and die around 10 to 12 months of age. As in humans, the disease develops more frequently in females and is hormone dependent. Extensive genetic analysis has been performed on related inbred strains that resulted from accidental backcrossing (New Zealand mixed strains) and led to the identification of multiple susceptibility loci. Several of these were also identified in human SLE subjects, including MHC class II alleles, Fcγ receptors, and components of the complement system.

The MRL lpr/lpr strain is another spontaneous model that develops massive lymphadenopathy, high concentrations of autoantibodies such as ANAs, anti-dsDNA and anti-Sm, and renal disease. In this strain, both males and females are equally affected. The *lpr* mutation interferes with expression of the Fas receptor (CD95), an important mediator of apoptosis. As discussed previously, defective apoptosis may in part underlie SLE in humans, and it is known that aberrant Fas signaling can lead to the development of autoimmune lymphoproliferative syndrome, which shares many symptoms with SLE.

SLE-like symptoms can be induced by the repeated injection of pristine, a compound found in mineral oil. These features include the development of ANAs, immune-complex deposition, and severe disease of the kidneys. This model can be reproduced in most mouse strains, although the extent of susceptibility varies. In strains such as the SJL/J mouse, females are more prone to disease development.

Alternatively, some aspects of SLE can be mimicked by the experimental induction of graft-versus-host disease. Disease aspects consist of lymphoid hyperplasia, formation of ANAs, and development lupus nephritis leading to severe kidney dysfunction and mortality. Only certain strains are prone to induction, once again illustrating the requirement for a genetic contribution in addition to an environmental stimulus. Disease severity can be tailored by adjusting the number of allografted cells, and onset occurs from a known starting point relative to injection. ANA sare usually detectable only 2 weeks after injection, which makes it a suitable model for the more rapid assessment of pathogenic pathways and experimental therapies.[539]

Therapy

The multiorgan nature of the condition and its course of relapses and remissions dictates that therapeutic management is in accordance with disease activity. In addition to diverse clinical scoring systems, active disease often correlates with altered levels of immune markers such as increased antidsDNA and decreased serum complement. Nonsteroidal anti-inflammatory drugs are prescribed to treat musculoskeletal symptoms or SLE-associated fever. Acute symptoms are generally treated by some form of corticosteroid treatment, either topical in

case of cutaneous flares or injected when major organ disease develops. Immunosuppressive agents such as azathioprine are used mostly when major organ disease threatens to considerably worsen the patient's condition. The antimalarial agent hydroxychloroquine is known to ameliorate disease by inhibiting the function of TLRs. It is thus clear that, in line with the disease's protean pattern, no disease-specific treatment options exist, and that the therapeutic goal often is to prevent where flares where possible (eg, avoid sun exposure) and tackle them in a symptomatic fashion as they occur. Since the approval of an antimalarial and corticosteroids for treatment of SLE in 1955, no new agents have been introduced.

After more than 50 years and many failed trials, the U.S. Food and Drug Administration finally approved a new drug for SLE. Belimumab (marketed as Benlysta® [GlaxoSmithKline, Brentford, UK]) is a fully human monoclonal antibody that binds to soluble human B-lymphocyte stimulator and inhibits its biologic activity.[540] B-lymphocyte stimulator was shown to be a key survival cytokine for B lymphocytes which, as argued previously, are a major pathogenic immune subset in SLE. In patients with SLE, B-lymphocyte stimulator concentrations reflect disease activity and correspond with anti-dsDNA antibody titers.[541,542] It was found that the drug selectively decreases B-cell numbers and shortlived plasma cells as well as SLE-associated anti-dsDNA antibody titers.[543] In two phase III trials that had enrolled patients with serologically active disease, a modest clinical effect was demonstrated combined with a safety profile similar to that of placebo.[544] It was argued that both inclusion criteria (patients with SLE activity that actually stands to be improved) and optimized disease-assessment indices contributed significantly to the successful outcome of these studies.[545] Initial studies with a humanized anti-CD22 monoclonal also had an encouraging initial outcome, further corroborating the idea of B cells as a suitable target cell in SLE.[546] The fact that not just any therapy that targets B cells has therapeutic potential was underscored by two recent phase III trials that failed to show efficacy of rituximab (anti-CD20) in SLE.[547,548] These recent advances, however, represent a glimmer of hope for patients affected by this particularly heterogenic and pathophysiologically complex condition.

CONCLUSION

A common trait among diverse animal models for autoimmune diseases and the human conditions they aim to model appears to be the difficulty with which experimental autoimmunity is achieved. Breaking of tolerance to self-antigens requires in most instances strong inflammatory (eg, pertussis toxin and adjuvant in EAE or collagen-induced arthritis) or infectious (eg, RIP-LCMV model for T1D or TMEV/mouse hepatitis virus models for MS) stimuli. In addition,

many animal models require a genetically susceptible background or have to rely on artificial autoantigen expression by means of transgenic technology. In contrast to the relatively easy detection of autoreactive lymphocytes in antigeninduced animal models, it has been a daunting endeavor in humans (as well as spontaneous disease models such as the NOD mouse) and data obtained frequently vary considerably between different patients as well as laboratories. What are the implications for our understanding of autoimmune diseases in humans given these challenges?

First, it is unlikely that strong inflammatory stimuli can be provided under natural conditions in the absence of infectious disease. Thus, viral and bacterial infections remain prime candidates for causing secondary autoimmunity (see Miller TMEV model of APC-mediated determinant spreading to autoantigens). Infectious pathogens can provide the "danger signals"[548a] needed for propagation of extended inflammation leading to clinical disease. Nevertheless, proof of a causal relationship is exquisitely difficult (if not impossible), as traces of pathogens detected may have no relation to the underlying disease process or may be cleared from the system by the time of secondary clinical disease. In addition, disease is likely dependent on individual pathogen strains, making a very detailed immunologic profiling in prospective clinical trials necessary. It is possible that the introduction of new antivirals might unmask such an association in the future. Finally, the importance of the gut microbiome has attracted some attention in the context of autoimmune disease.[549] This is not unexpected because the gut harbors the major part of our immune system, and immune tolerance can often be induced by passage of an antigen through the gastrointestinal system. We now have gained a more precise view on the vast diversity of commensals inside our bodies,[550,551] and it appears that this composition affects the likelihood of a genetically susceptible subject to develop autoummunity.[261] For instance, generation of the pathogenic Th17 subset described previously in many autoimmune conditions critically depends on the composition of microbiota.[552]

Second, a genetically susceptible background will likely be required to provide a "fertile field" for initiating a chronic inflammation involving autoantigens. This probably occurs via a multifaceted network of multiple susceptibility and protective genes, and it will be impossible to treat a respective disease just by analyzing the background genes involved. Last, autoreactive lymphocytes might predominantly be present in the affected organ or site and not in the peripheral blood, which makes their identification and characterization in humans rather difficult.

A word of caution should be devoted to our interpretation of specific findings obtained in individual animal models. Animal models should serve to teach us paradigms of how a disease *could* develop kinetically in vivo. The precise parameters, targeted antigens, susceptibility genes, and effector molecules may be considerably different in humans. Thus, for example, if there is indication that insulin is a primary antigen in the NOD mouse, this may or may not have direct relevance to the human disease. Some evidence exists that leads us to believe that insulin, indeed, maybe also a primary antigen in human autoimmune diabetes, but other antigens such as GAD might also play a role. The opportunities as well as limits of each animal model have to be delineated. Again, the NOD mouse appears to be prone toward multiorgan autoimmunity (induction of EAE,[553] neuritis,[554] arthritis, and hepatitis have all been observed in NOD or NOD-congenic mouse strains) and exhibits, in addition to diabetes, thyroiditis, sialitis, and orchitis. Thus, diabetes in the NOD mouse is clearly different from typical human T1D. Therefore, treatments capable of correcting the systemic immune dysregulation that predominates in the NOD model may not directly apply to human T1D where no such pronounced systemic dysregulation is present. Based on these considerations, it is not surprising that out of the more than 140 therapeutic strategies that prevent diabetes in the NOD mouse, only a handful have made it to phase I human trials. An appealing solution that has shown promise in T1D epitope discovery is the development of humanized NOD mice expressing HLA class I transgenic molecules for antigen presentation.

Remarkably, several of the islet epitopes that become targeted under these conditions correspond to the disease-associated specificities found in peripheral blood mononuclear cells from patients with T1D.[555] When those epitopes are administrated in a tolerogenic fashion, the disease course can be significantly altered.[556] While still animal data in principal, these results may have higher translational value when tolerogenic strategies are to be envisioned for humans. Experiments with human cells or materials should be undertaken in order to solidify the choice of molecules or target antigens. The successful story in respect to blockade of TNF-α to treat RA underlines the importance of this step. Employment of a multiplicity of models thus becomes imperative to evaluate potential candidate interventions, as does a careful proceeding, objective evaluation, and avoidance of premature conclusions. In addition to the researchers and clinicians, publishers as well as news media will have to share this responsibility. Continued research will undoubtedly provide us eventually with sufficient insight into the complexities of autoimmunity and autoimmune disorders, but patience and perseverance coupled with experimental objectivity will be required.

Therapeutic progress remains variable using monoclonal antibodies to dampen autoimmune processes in humans. Succesful examples include anti-TNF, CTLA-4Ig, anti-CD20, and anti-IL-1 treatments, while a highly anticipated non-Fc binding anti-CD3 antibody recently failed to clear phase III trials in recent-onset T1D.[557,558] As discussed previously, many of the successful therapies have also shown efficacy in a diverse array of other autoimmune diseases such as in psoriasis, Crohn disease, RA, MS, and uveitis. Thus, the road map to therapeutic success might lie in a well-tuned combination of such therapeutics, likely in conjunction with autoantigenspecific approaches that mediate more long-term and lasting tolerance. Augmentation of adaptive as well as intrinsic T_{reg}s might be, in the end, the key for achieving site-specific tolerance (involving bystander suppression of effector responses and “infectious” induction of more T_{reg}s) and is being explored with encouraging developments in many preclinical models.

REFERENCES

1. Lacan J. The field of the other and back to the transference. In: Miller JA, ed. *The Four Fundamental Concepts of Psycho-Analysis*. New York, NY: W.W. Norton & Company; 1981:203-260.

2. Tauber A. The biological notion of self and non-self. In: *The Stanford Encyclopedia of Philosophy*. The Metaphysics Research Lab; 2012. http://plato.stanford.edu/entries/biology-self/

3. Littré E, Robin C. Dictionnaire de médecine de chirurgie, de pharmacie de l'art vétérinaire et des sciences qui s'y rapportent. Paris: J. -B. Baillière et Fils. 1878.

4. Pasteur L. De l'attenuation du virus cholera des poules. *Compt Rend Acad Sci* . 1880;91:673-680.

5. von Behring E, Kitasato S. Über das Zustandekommen der Diphtherie-Immunität und

der Tetanus-Immunität bei Thieren. *Dtsch Med Wochenschr*. 1890;16:1113-1114.

6. Ehrlich P. Die Schutzstoffe des Blutes. In: Wangerin A, ed. *Verhandlungen der Gesellschaft Deutscher Naturforscher und Ärzte - 73. Versammlung zu Hamburg*. Leipzig: Verlag von F.C.W. Vogel; 1901:250-275.

7. Römer P, Gebb H. Beiträge zur Frage der Anaphylaxie durch Linseneiweiss und Eiweiss aus andern Geweben des Auges. *Albrecht von Graefes Arch Ophtalmol*. 1912;81:367-402.

8. Silverstein A. *A History of Immunology*. San Diego, CA: Academic Press; 1989.

9. Portier P, Richet C. De l'action anaphylactique de certain venins. *CR Soc Biol (Paris)*. 1902;54:170-172.

10. Arthus M. Injections repetees de serum du cheval chez le lapin. *C R Soc biol (Paris)*. 1903;55:817-820.

11. Donath J, Landsteiner K. Uber paroxysmale Hamoglobinurie. *Munch Med Wochenschr*. 1904;51:1590-1601.

12. von Pirquet C, Schick B. *Die Serumkrankeit*. Leipzig, Germany: Deuticke; 1905.

13. Weil E, Braun H. Ueber Antikörperbefunde bei Lues, Tabes und Paralyse. *Berl Klin Wochenschr*. 1907;49:1570-1574.

14. Uhlenhuth P. *Festschrift zum 60*. Jena, Germany: Geburtstag von Robert Koch; 1903.

15. Traub E. Persistence of lymphocytic choriomeningitis vieus in immune animals and its relation to immunity. *J Exp Med*. 1936;63:847-861.

16. Owen RD. Immunogenetic consequences of vascular anastomoses between bovine cattle twins. *Science*. 1945;102:400-401.

17. Medawar PB. The behaviour and fate of skin autografts and skin homografts in rabbits. *J Anat*. 1944;78:176-199.

18. Medawar PB. A second study of the behaviour and fate of skin homografts in rabbits. *J Anat*. 1945;79:157-176.

19. Medawar PB. Immunity to homologous grafted skin. I. The suppression of cell division in grafts transplanted to immunized animals. *Brit J Exp Pathol*. 1946;27:9-14.

20. Medawar PB. Immunity to homologous grafted skin. II. The immunity relationship between the antigens of blood and skin. *Brit J Exp Pathol*. 1946;27:15-24.

21. Burnet FM, Fenner F. *The Production of Antibodies*. 2nd ed. New York, NY: Macmillan; 1949.

22. Burnet FM. *The Clonal Selection Theory of Acquired Immunity*. Cambridge, United Kingdom: Cambridge University Press; 1957.

23. Witebsky E, Rose NR. Studies on organ specificity. V. Changes in the thyroid glands of rabbits following active immunization with rabbit thyroid extracts. *J Immunol*. 1956;76:417-427.

24. Fleck L. *Entstehung und Entwicklung einer wissenschaftlichen tatsache. Einfurung in die Lehre vom Dekstil und Denkkollektiv*. Basel, Switzerland: B. Schwabe und Co. Verlagsbuchhandlung; 1935.

25. Jerne NK. Towards a network theory of the immune system. *Ann Immunol*. 1974:373-389.

26. Coutinho A, Forni L, Holmberg D, Ivars F, Vaz N. From an antigen-centered, clonal perspective of immune responses to an organism-centered, network perspective of autonomous activity in a self-referential immune system. *Immunol Rev*. 1984;79:151-168.

27. Grabar P. Hypothesis. Auto-antibodies and immunological theories: an analytical review. *Clin Immunol Immunopathol*. 1975;4:453-466.

28. Wigzell H. Positive autoimmunity. In: Talal N, ed. *Autoimmunity: Genetic, Immunologic, Virologic and Clinical Aspects*. New York, NY: Academic Press; 1997:693-707.

29. Schwartz M, Cohen IR. Autoimmunity can benefit self-maintenance. *Immunol Today*. 2000;21:265-268.

30. Matzinger P. Tolerance, danger, and the extended family. *Ann Rev Immunol*. 1994;12:991-1045.

31. Dembic Z. Immune system protects integrity of tissues. *Mol Immunol*. 2000;37(10):563-569.

32. Pradeu T, Carosella ED. On the definition of a criterion of immunogenicity. *Proc Natl Acad Sci U S A*. 2006;103(47):17858-17861.

33. Pradeu T, Carosella ED. Critical analysis of the immunological self/non-self model and of its implicit metaphysical foundations [in French]. *Comptes rendus biologies*. 2004;327(5):481-492.

34. Cohn M. A commentary on the Zinkernagel-Hengartner “Credo 2004.” *Scand J Immunol*. 2005;62(3):187-194.

35. Sercarz EE, Maverakis E. Recognition and function in a degenerate immune system. *Mol Immunol*. 2004;40(14-15):1003-1008.

36. Tada T. The immune system as a supersystem. *Annu Rev Immunol*. 1997;15:1-13.

37. Hedrick SM. The acquired immune system: a vantage from beneath. *Immunity*. 2004;21(5):607-615.

38. Rose NR, Mackay IR. Prelude. In: Rose NR, Mackay IR, eds. *The Autoimmune Diseases*. San Diego, CA: Academic Press; 1998:1-4.

39. Schwartz R. Foreword. In: Lahita RG, Chiorazzi N, Reeves WH, eds. *Textbook of the Autoimmune Diseases*. Philadelphia, PA: Lippincott Williams & Wilkins; 2000:XIX-XX.

40. MedlinePlus. Autoimmune disorders. www.nlm.nih.gov/medlineplus/ency/article/000816.htm. Accessed December 22, 2011.

41. Zinkernagel RM. Anti-infection immunity and autoimmunity. *Ann NY Acad Sci* . 2002;958:3-6.

42. Witebsky E, rose NR, Terplan K, Paine JR, Egan RW. Chronic thyroiditis and autoimmunization. *JAMA*. 1957;164:1439-1447.

43. Rose NR, Bona C. Defining criteria for autoimmune disease (Witebsky's postulates revisited). *Immunol Today*. 1993;114:426-430.

44. Alvarez F, Berg PA, Bianchi FB, et al. International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol*. 1999;31(5):929-938.

45. Jacobson DL, Gange SJ, Rose NR, Graham NM. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. *Clin Immunol Immunopathol*. 1997;84(3):223-243.

46. Eaton WW, Rose NR, Kalaydjian A, Pedersen MG, Mortensen PB. Epidemiology of autoimmune diseases in Denmark. *J Autoimmunity*. 2007;29(1):1-9.

47. Cooper GS, Bynum ML, Somers EC. Recent insights in the epidemiology of autoimmune diseases: improved prevalence estimates and understanding of clustering of diseases. *J Autoimmunity*. 2009;33 (3-4):197-207.

48. Walsh SJ, Rau LM. Autoimmune diseases: a leading cause of death among young

and middle-aged women in the United States. *Am J Pub Health*. 2000;90(9):1463-1466.

49. Grimaldi LM, Palmeri B, Salemi G, et al. High prevalence and fast rising incidence of multiple sclerosis in Caltanissetta, Sicily, southern Italy. *Neuroepidemiology*. 2007;28(1):28-32.

50. Catassi C, Kryszak D, Bhatti B, et al. Natural history of celiac disease autoimmunity in a USA cohort followed since 1974. *Ann Med*. 2010;42(7):530-538.

51. Patterson CC, Dahlquist GG, Gyurus E, Green A, Soltesz G. Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study. *Lancet*. 2009;373(9680):2027-2033.

52. Harjutsalo V, Sjoberg L, Tuomilehto J. Time trends in the incidence of type 1 diabetes in Finnish children: a cohort study. *Lancet*. 2008;371(9626):1777-1782.

53. Matsumoto I, Maccioni M, Lee DM, et al. How antibodies to a ubiquitous cytoplasmic enzyme may provoke joint-specific autoimmune disease. *Nat Immunol*. 2002;3(4):360-365.

54. Binstadt BA, Patel PR, Alencar H, et al. Particularities of the vasculature can promote the organ specificity of autoimmune attack. *Nat Immunol*. 2006;7(3):284-292.

55. Shoda LK, Young DL, Ramanujan S, et al. A comprehensive review of interventions in the NOD mouse and implications for translation. *Immunity*. 2005;23(2):115-126.

56. Anderson MS, Bluestone JA. The NOD mouse: a model of immune dysregulation. *Annu Rev Immunol*. 2005;23:447-485.

57. Chaparro RJ, Konigshofer Y, Beilhack GF, Shizuru JA, McDevitt HO, Chien YH. Nonobese diabetic mice express aspects of both type 1 and type 2 diabetes. *Proc Natl Acad Sci U S A*. 2006;103(33):12475-12480.

58. McGonagle D, McDermott MF. A proposed classification of the immunological diseases. *PLoS Med*. 2006;3(8):e297.

59. Gershon RK, Kondo K. Infectious immunological tolerance. *Immunology*. 1971;21(6):903-914.

60. Schubert LA, Jeffery E, Zhang Y, Ramsdell F, Ziegler SF. Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. *J Biol Chem*. 2001;276(40):37672-37679.

61. Ziegler SF. FOXP3: of mice and men. *Annu Rev Immunol*. 2006;24: 209-226.

62. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003;299(5609):1057-1061.

63. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol*. 2003;4(4):337-342.

64. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*. 2003;4(4):330-336.

65. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity*. 2005;22(3):329-341.

66. Wan YY, Flavell RA. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proc Natl Acad Sci U S A* . 2005;102(14): 5126-5131.

67. Haase C, Ejrnaes M, Juedes AE, Wolfe T, Markholst H, von Herrath MG. Immunomodulatory dendritic cells require autologous serum to circumvent nonspecific immunosuppressive activity in vivo. *Blood*. 2005;106(13):4225-4233.

68. Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev*. 2006;212:28-50.

69. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol*. 2006;24:99-146.

70. Shevach EM. Regulatory T cells in autoimmmunity. *Annu Rev Immunol*. 2000;18:423-449.

71. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol*. 2002;2(6):389-400.

72. Zhou X, Bailey-Bucktrout SL, Jeker LT, et al. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol*. 2009;10(9):1000-1007.

73. Murai M, Turovskaya O, Kim G, et al. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat Immunol*. 2009;10(11): 1178-1184.

74. Brusko TM, Putnam AL, Bluestone JA. Human regulatory T cells: role in autoimmune disease and therapeutic opportunities. *Immunol Rev*. 2008;223:371-390.

75. Ashton-Rickardt PG, Bandeira A, Delaney J, et al. Evidence for a differential avidity

model of T-cell selection in the thymus. *Cell*. 1994;76:651-663.

76. Hoffmann MW, Heath WR, Ruschmeyer D, Miller JF. Deletion of high-avidity T cells by thymic epithelium. *Proc Natl Acad Sci U S A* . 1995;92(21):9851-9855.

77. Jameson SC, Bevan MJ. T-cell selection. *Curr Opin Immunol* . 1998;10(2):214-219.

78. Sebzda E, Mariathasan S, Ohteki T, Jones R, Bachmann MF, Ohashi PS. Selection of the T cell repertoire. *Annu Rev Immunol*. 1999;17:829-874.

79. Kishimoto H, Sprent J. The thymus and negative selection. *Immunol Res*. 2000;21(2-3):315-323.

80. Nikolic-Zugic J, Bevan MJ. Role of self-peptides in positively selecting the T-cell repertoire. *Nature*. 1990;344(6261):65-67.

81. Surh CD, Lee DS, Fung-Leung WP, Karlsson L, Sprent J. Thymic selection by a single MHC/peptide ligand produces a semidiverse repertoire of CD4+ T cells. *Immunity*. 1997;7(2):209-219.

82. Steinman L. A few autoreactive cells in an autoimmune infiltrate control a vast population of nonspecific cells: a tale of smart bombs and the infantry. *Proc Natl Acad Sci U S A*. 1996;93:2253-2256.

83. van den Elzen P, Menezes JS, Ametani A, et al. Limited clonality in autoimmunity: drivers and regulators. *Autoimmun Rev*. 2004;3(7-8): 524-529.

84. Han B, Serra P, Amrani A, et al. Prevention of diabetes by manipulation of anti-IGRP autoimmunity: high efficiency of a low-affinity peptide. *Nat Med*. 2005;11(6):645-652.

85. Allison JP. CD28-B7 interactions in T-cell activation. *Curr Opin Immunol* . 1994;6(3):414-419.

86. Harding FA, Allison JP. CD28-B7 interactions allow the induction of CD8+ cytotoxic T-lymphocytes in the absence of exogenous help. *J Exp Med*. 1993;177(6):1791-1796.

87. Schwartz RH. A cell culture model for T lymphocyte clonal anergy. *Science*. 1990;248(4961):1349-1356.

88. Lenardo M, Chan KM, Hornung F, et al. Mature T lymphocyte apoptosis —immune regulation in a dynamic and unpredictable antigenic environment. *Annu Rev Immunol*. 1999;17:221-253.

89. Abbas AK. Die and let live: eliminating dangerous lymphocytes. *Cell*. 1996;84:655-657.

90. Van Parijs L, Abbas AK. Homeostasis and self-tolerance in the immune system: turning lymphocytes off. *Science*. 1998;280(5361):243-248.

91. Chambers CA, Kuhns MS, Egen JG, Allison JP. CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. *Annu Rev Immunol*. 2001;19:565-594.

92. Salomon B, Bluestone JA. Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. *Annu Rev Immunol*. 2001;19:225-252.

93. Weiner HL, Friedman A, Miller A, et al. Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. *Annu Rev Immunol*. 1994;12:809-837.

94. Seewaldt S, Thomas HE, Ejrnaes M, et al. Virus-induced autoimmune diabetes: most beta-cells die through inflammatory cytokines and not perforin from autoreactive (anti-viral) cytotoxic T-lymphocytes. *Diabetes*. 2000;49(11):1801-1809.

95. Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*. 2006;441(7090):235-238.

96. Marth T, Strober W, Seder RA, Kelsall BL. Regulation of transforming growth factor-beta production by interleukin-12. *Eur J Immunol* . 1997;27(5):1213-1220.

97. Seder RA, Marth T, Sieve MC, et al. Factors involved in the differentiation of TGF-b-producing cells from naive CD4+ T cells: IL-4 and IFN-g have opposing effects, while TGF-b positively regulates its own production. *J Immunol*. 1998;160:5719-5728.

98. King C, Davies J, Mueller R, et al. TGF-b1 alters APC preference, polarizing islet antigen responses toward a Th2 phenotype. *Immunity*. 1998;8:601-613.

99. Rizzo LV, Morawetz RA, Miller-Rivero NE, et al. IL-4 and IL-10 are both required for the induction of oral tolerance. *J Immunol*. 1999;162(5):2613-2622.

100. Homann D, Holz A, Bot A, et al. Autoreactive CD4+ lymphocytes protect from autoimmune diabetes via bystander suppression using the IL-4/STAT6 pathway. *Immunity*. 1999;11:463-472.

101. King C, Hoenger RM, Cleary MM, et al. Interleukin-4 acts at the locus of the antigen-presenting dendritic cell to counter-regulate cytotoxic CD8+ T-cell responses. *Nat Med*. 2001;7(2):206-214.

102. Tree TI, Lawson J, Edwards H, et al. Naturally arising human CD4 T-cells that

recognize islet autoantigens and secrete interleukin-10 regulate proinflammatory T-cell responses via linked suppression. *Diabetes*. 2010;59(6):1451-1460.

103. Zhang ZY, Lee CS, Lider O, Weiner HL. Suppression of adjuvant arthritis in Lewis rats by oral administration of type II collagen. *J Immunol* . 1990;145(8):2489-2493.

104. Maron R, Blogg NS, Polanski M, Hancock W, Weiner HL. Oral tolerance to insulin and the insulin B-chain: cell lines and cytokine patterns. *Ann N Y Acad Sci* . 1996;778(346):357.

105. Hancock WW, Polanski M, Zhang J, Blogg N, Weiner HL. Suppression of insulitis in non-obese diabetic (NOD) mice by oral insulin administration is associated with selective expression of interleukin-4 and -10, transforming growth factor-beta, and prostaglandin-E. *Am J Pathol* . 1995;147(5):1193-1199.

106. Bergerot I, Arreaza GA, Cameron MJ, et al. Insulin B-chain reactive CD4+ regulatory T-cells induced by oral insulin treatment protect from type 1 diabetes by blocking the cytokine secretion and pancreatic infiltration of diabetogenic effector T-cells. *Diabetes*. 1999;48(9): 1720-1729.

107. Charlton B, Lafferty KJ. The Th1/Th2 balance in autoimmunity. *Curr Opin Immunol* . 1995;7(6):793-798.

108. Paul WE. Interleukin-4: a prototypic immunoregulatory lymphokine. *Blood*. 1991;77:1859-1870.

109. Lepault F, Gagnerault MC. Characterization of peripheral regulatory CD4+ T cells that prevent diabetes onset in nonobese diabetic mice. *J Immunol* . 2000;164:240-247.

110. Sercarz EE. Processing creates the self. *Nat Immunol* . 2002;3(2): 110-112.

111. Anderton SM, Viner NJ, Matharu P, Lowrey PA, Wraith DC. Influence of a dominant cryptic epitope on autoimmune T cell tolerance. *Nat Immunol* . 2002;3(2):175-181.

112. Moudgil KD, Sekiguchi D, Kim SY, Sercarz EE. Immunodominance is independent of structural constraints: each region within hen eggwhite lysozyme is potentially available upon processing of native antigen. *J Immunol* . 1997;159(6):2574-25759.

113. Bhardwaj V, Kumar V, Geysen HM, Sercarz EE. Degenerate recognition of a dissimilar antigenic peptide by myelin basic protein-reactive T cells. *J Immunol* . 1993;151:5000-5010.

114. Zehn D, Bevan MJ. T cells with low avidity for a tissue-restricted antigen routinely evade central and peripheral tolerance and cause autoimmunity. *Immunity*. 2006;25(2):261-270.

115. Ashton-Rickardt PG, Bandeira A, Delaney JR, et al. Evidence for a differential avidity model of T cell selection in the thymus. *Cell*. 1994;76(4):651-663.

116. Moudgil KD, Sercarz EE. Understanding crypticity is the key to revealing the pathogenesis of autoimmunity. *Trends Immunol*. 2005;26(7):355-359.

117. Carbone FR, Kurts C, Bennett SRM, Miller JFAP, Heath WR. Crosspresentation: a general mechanism for CTL immunity and tolerance. *Immunol Today*. 1998;19(8):368-373.

118. van Stipdonk MJ, Lemmens EE, Schoenberger SP. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat Immunol*. 2001;2(5):423-429.

119. Blattman JN, Sourdive DJ, Murali-Krishna K, Ahmed R, Altman JD. Evolution of the T cell repertoire during primary, memory, and recall responses to viral infection. *J Immunol*. 2000;165(11): 6081-6090.

120. Heath WR, Kurts C, Miller JFAP, Carbone FR. Cross-tolerance: a pathway for inducing tolerance to peripheral tissue antigens. *J Exp Med*. 1998;187(10):1549-1553.

121. Savinov AY, Wong FS, Stonebraker AC, Chervonsky AV. Presentation of antigen by endothelial cells and chemoattraction are required for homing of insulin-specific CD8+ T cells. *J Exp Med*. 2003;197(5):643-656.

122. Taurog JD, Maika SD, Satumtira N, et al. Inflammatory disease in HLA-B27 transgenic rats. *Immunol Rev*. 1999;169:209-223.

123. Schwimmbeck P, Yu DT, Oldstone MB. Autoantibodies to HLA-B27 in sera of patients with ankylosing spondylitis and Reiter's syndrome. *J Exp Med*. 1987;166:173-181.

124. McDevitt HO. The role of MHC class II molecules in susceptibility and resistance to autoimmunity. *Curr Opin Immunol*. 1998;10:677-681.

125. Sonderstrup G, McDevitt HO. DR, DQ, and you: MHC alleles and autoimmunity. *J Clin Invest*. 2001;107(7):795-796.

126. Molberg O, Kett K, Scott H, Thorsby E, Sollid LM, Lundin KE. Gliadin specific, HLA DQ2-restricted T cells are commonly found in small intestine biopsies from coeliac disease patients, but not from controls. *Scand J Immunol*. 1997;46(1):103-108.

127. von Herrth M, Wolfe T, Mohrle U, Coon B, Hughes AC. MHC nonlinked factors can prevent virally induced type 1 diabetesin SV129 (H-2Db) mouse strains. *J Immunol*.

2001; Submitted.

128. de Bakker PI, McVean G, Sabeti PC, et al. A high-resolution HLA and SNP haplotype map for disease association studies in the extended human MHC. *Nat Genet*. 2006;38(10):1166-1172.

129. Vijayakrishnan L, Slavik JM, Illes Z, et al. An autoimmune disease-associated CTLA-4 splice variant lacking the B7 binding domain signals negatively in T cells. *Immunity*. 2004;20(5):563-575.

130. Bach JF. Organ-specific autoimmunity. *Immunol Today*. 1995;16: 353-355.

131. Bach JF. Insulin dependent diabetes mellitus as an autoimmune disease. *Endocrine Rev*. 1994;15(4):516-542.

132. Bach JF. Predictive medicine in autoimmune diseases: from the identification of genetic predisposition and environmental influence to precocious immunotherapy. *Clin Immunol Immunopath*. 1994;72:156-161.

133. Todd JA. The role of MHC class II genes in susceptibility to insulin-dependent diabetes mellitus. *Curr Top Microbiol Immunol* . 1990;164(5):17-40.

134. Vyse TJ, Todd JA. Genetic analysis of autoimmune disease. *Cell*. 1996;85:311-318.

135. Redondo MJ, Rewers M, Yu L, et al. Genetic determination of islet cell autoimmunity in monozygotic twin, dizygotic twin, and non-twin siblings of patients with type 1 diabetes: prospective twin study. *BMJ*. 1999;318:698-702.

136. Aly TA, Ide A, Jahromi MM, et al. Extreme genetic risk for type 1A diabetes. *Proc Natl Acad Sci U S A*. 2006;103(38):14074-14079.

137. Hirschhorn JN, Gajdos ZK. Genome-wide association studies: results from the first few years and potential implications for clinical medicine. *Annu Rev Med*. 2011;62:11-24.

138. Hakonarson H, Grant SF. Genome-wide association studies in type 1 diabetes, inflammatory bowel disease and other immune-mediated disorders. *Sem Immunol*. 2009;21(6):355-362.

139. Sevilla N, Homann D, von Herrath MG, et al. Virus-induced diabetes in a transgenic model: role of cross-reacting viruses and quantitation of effector T cells needed to cause disease. *J Virol*. 2000;74(7):3284-3292.

140. Raine T, Zaccone P, Mastroeni P, Cooke A. Salmonella typhimurium infection in nonobese diabetic mice generates immunomodulatory dendritic cells able to prevent type 1 diabetes. *J Immunol*. 2006;177(4):2224-2233.

141. Christen U, Benke D, Wolfe T, et al. Cure of prediabetic mice by viral infections involves lymphocyte recruitment along an IP-10 gradient. *J Clin Invest*. 2004;113(1):74-84.

142. Filippi CM, Estes EA, Oldham JE, von Herrath MG. Immunoregulatory mechanisms triggered by viral infections protect from type 1 diabetes in mice. *J Clin Invest*. 2009;119(6):1515-1523.

143. Zhao ZS, Granucci F, Yeh L, Schaffer PA, Cantor H. Molecular mimicry by herpes simplex virus-type 1: autoimmune disease after viral infection. *Science*. 1998;279(5355):1344-1347.

144. Oldstone MBA. Molecular mimicry and autoimmune disease. *Cell*. 1987;50:819-820.

145. Horwitz MS, Bradley LM, Harbertson J, Krahl T, Lee J, Sarvetnick N. Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. *Nat Med*. 1998;4:781-785.

146. Olson JK, Girvin AM, Miller SD. Direct activation of innate and antigen-presenting functions of microglia following infection with Theiler's virus. *J Virol*. 2001;75(20):9780-9789.

147. Miller SD, Vanderlugt CL, Begolka WS, et al. Persistent infection with Theiler's virus leads to CNS autoimmunity via epitope spreading. *Nat Med*. 1997;3(10):1133-1136.

148. Katz-Levy Y, Neville KL, Girvin AM, et al. Endogenous presentation of self myelin epitopes by CNS-resident APCs in Theiler's virus-infected mice. *J Clin Invest*. 1999;104(5):599-610.

149. Oldstone MB. Prevention of type I diabetes in nonobese diabetic mice by virus infection. *Science*. 1988;239:500-502.

150. Blumberg RS, Saubermann LJ, Strober W. Animal models of mucosal inflammation and their relation to human inflammatory bowel disease. *Cur Opin Immunol* . 1999;11:648-656.

151. Kelsall BL, Strober W. Dendritic cells of the gastrointestinal tract. *Springer Semin Immunopathol*. 1997;18:409-420.

152. La Cava A, Sarvetnick N. The role of cytokines in autoimmunity. *Curr Dir Autoimmun*. 1999;1:56-71.

153. von Andrian UH, Mackay CR. T-cell function and migration. Two sides of the same coin. *N Engl J Med* . 2000;343(14):1020-1034.

154. Godessart N, Kunkel SL. Chemokines in autoimmune disease. *Curr Opin Immunol* . 2001;13(6):670-675.

155. O'Shea JJ, Ma A, Lipsky P. Cytokines and autoimmunity. *Nat Rev Immunol* . 2002;2(1):37-45.

156. Langrish CL, Chen Y, Blumenschein WM, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med*. 2005;201(2):233-240.

157. Harrington LE, Hatton RD, Mangan PR, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol*. 2005;6(11):1123-1132.

158. Park H, Li Z, Yang XO, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol*. 2005;6(11):1133-1141.

159. Fouser LA, Wright JF, Dunussi-Joannopoulos K, Collins M. Th17 cytokines and their emerging roles in inflammation and autoimmunity. *Immunol Rev*. 2008;226:87-102.

160. Manoury-Schwartz B, Chiocchia G, Bessis N, et al. High susceptibility to collagen-induced arthritis in mice lacking IFN-gamma receptors. *J Immunol*. 1997;158(11):5501-5506.

161. Vermeire K, Heremans H, Vandeputte M, Huang S, Billiau A, Matthys P. Accelerated collagen-induced arthritis in IFN-gamma receptor-deficient mice. *J Immunol* . 1997;158(11):5507-5513.

162. Nakae S, Nambu A, Sudo K, Iwakura Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. *J Immunol*. 2003;171(11):6173-6177.

163. Lubberts E, Koenders MI, Oppers-Walgreen B, et al. Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. *Arthritis Rheumat*. 2004;50(2):650-659.

164. Sarkar S, Fox DA. Targeting IL-17 and Th17 cells in rheumatoid arthritis. *Rheum Dis Clin North Am*. 2010;36(2):345-366.

165. Kim S, Kim KA, Hwang DY, et al. Inhibition of autoimmune diabetes by Fas ligand: the paradox is solved. *J Immunol*. 2000;164(6):2931-2936.

166. Hugues S, Mougneau E, Ferlin W, et al. Tolerance to islet antigens and prevention from diabetes induced by limited apoptosis of pancreatic beta cells. *Immunity*. 2002;16(2):169-181.

167. Pope RM. Apoptosis as a therapeutic tool in rheumatoid arthritis. *Nat Rev Immunol* . 2002;2(7):527-535.

168. Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. *Nat Med*. 1999;5(11):1249-1255.

169. Delovitch TL, Singh B. The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. *Immunity*. 1997;7:727-738.

170. Christen U, Wolfe T, Mohrle U, et al. A dual role for TNF-alpha in type 1 diabetes: islet-specific expression abrogates the ongoing autoimmune process when induced late but not early during pathogenesis. *J Immunol* . 2001;166(12):7023-7032.

171. Maini RN, Elliott M, Brennan FM, Williams RO, Feldmann M. TNF blockade in rheumatoid arthritis: implications for therapy and pathogenesis. *APMIS*. 1997;105(4):257-263.

172. Feldmann M, Brennan FM, Maini RN. Rheumatoid arthritis. *Cell*. 1996;85(3):307-310.

173. Garber K. Pfizer's JAK inhibitor sails through phase 3 in rheumatoid arthritis. *Nat Biotechnol*. 2011;29(6):467-468.

174. Tisch R, McDevitt H. Antigen specific immunotherapy: is it a real possibility to combat T-cell medicated autoimmunity? *Proc Natl Acad Sci U S A* . 1994;91:437-438.

175. Tisch R, Wang B, Weaver DJ, et al. Antigen-specific mediated suppression of beta cell autoimmunity by plasmid DNA vaccination. *J Immunol* . 2001;166(3):2122-2132.

176. Shevach EM, McHugh RS, Piccirillo CA, Thornton AM. Control of T-cell activation by CD4+ CD25+ suppressor T cells. *Immunol Rev*. 2001;182:58-67.

177. Bresson D, Togher L, Rodrigo E, et al. Anti-CD3 and nasal proinsulin combination therapy enhances remission from recent-onset autoimmune diabetes by inducing Tregs. *J Clin Invest*. 2006;116(5):1371-1381.

178. Sicotte NL, Voskuhl RR. Onset of multiple sclerosis associated with anti-TNF therapy. *Neurology*. 2001;57(10):1885-1888.

179. Riminton DS, Korner H, Strickland DH, Lemckert FA, Pollard JD, Sedgwick JD. Challenging cytokine redundance: inflammatory cell movement and clinical course of experimental autoimmune encephalomyelitis are normal in lymphotoxin-deficient, but not tumor necrosis factor-deficient, mice. *J Exp Med*. 1998;187(9):1517-1528.

180. Sedgwick JD, Riminton DS, Cyster JG, Korner H. Tumor necrosis factor: a master-regulator of leukocyte movement. *Immunol Today*. 2000;21(3):110-113.

181. Coon B, An L-L, Whitton JL, von Herrath MG. DNA immunization to prevent autoimmune diabetes. *J Clin Invest*. 1999;104:189-194.

182. Tang Q, Henriksen KJ, Bi M, et al. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med*. 2004;199(11):1455-1465.

183. Dayan CM, Daniels GH. Chronic autoimmune thyroiditis. *N Engl J Med*. 1996;335(2):99-107.

184. Weetman AP. Graves' disease. *N Engl J Med*. 2000;343(17):1236-1248.

185. Seddon B, Mason D. Peripheral autoantigen induces regulatory T cells that prevent autoimmunity. *J Exp Med*. 1999;189(5):877-881.

186. Vrbikova J, Janatkova I, Zamrazil V, Tomiska F, Fucikova T. Epstein-Barr virus serology in patients with autoimmune thyroiditis. *Exp Clin Endocrinol Diabetes*. 1996;104(1):89-92.

187. Tomer Y, Davies TF. Infections and autoimmune endocrine disease. *Baillieres Clin Endocrinol Metab*. 1995;9(1):47-70.

188. Sellmeyer DE, Grunfeld C. Endocrine and metabolic disturbances in human immunodeficiency virus infection and the acquired immune deficiency syndrome. *Endocr Rev*. 1996;17(5):518-532.

189. Grinspoon SK, Bilezikian JP. HIV disease and the endocrine system. *N Engl J Med*. 1992;327:1360-1365.

190. Hellerstein MK. Endocrinology abnormalities. In: Cohen PT, Sande M, Volberding P, eds. *The AIDS Knowledge Base*. Boston, MA: Massachusetts Medical Society; 1995:1-10.

191. Dluhy RG. The growing spectrum of HIV-related endocrine abnormalities. *J Clin Endocrinol Metab*. 1990;70:563-565.

192. Heufelder AH, Hofbauer LC. Human immunodeficiency virus infection and the thyroid gland. *Eur J Endocrin*. 1996;134:669-674.

193. Friedman ND, Spelman DW. Subacute thyroiditis presenting as pyrexia of unknown origin in a patient with human immunodeficiency virus infection. *Clin Infect Dis*. 1999;29(5):1352-1353.

194. Jaeger P, Otto S, Speck RF, et al. Altered parathyroid gland function in severely immunocompromised patients infected with human immunodeficiency virus. *J Clin Endocrinol Metab*. 1994;79(6):1701-1705.

195. Burch HB, Nagy EV, Lukes YG, et al. Nucleotide and amino acid homology between the human thyrotropin receptor and HIV-1 nef protein: identification and functional analysis. *Biochem Biophys Res Comm*. 1991;181:498-505.

196. Wick G, Trieb K, Aguzzi A, Recheis H, Anderl H, Grubeck-Loebenstein B. Possible role of human foamy virus in Graves' disease. *Intervirology*. 1993;35:101-107.

197. Lagaye S, Vexiau P, Morozov V, et al. Human spumaretrovirus-related sequences in the DNA of leukocytes from patients with Graves disease. *Proc Natl Acad Sci U S A* . 1992;89(21):10070-10074.

198. Mine H, Kawai H, Yokoi K, Akaike M, Saito S. High frequencies of human T-lymphotropic virus type I (HTLV-I) infection and presence of HTLV-II proviral DNA in blood donors with anti-thyroid antibodies. *J Mol Med* . 1996;74(8):471-477.

199. Yokoi K, Kawai H, Akaike M, Mine H, Saito S. Presence of human T-lymphotropic virus type II-related genes in DNA of peripheral leukocytes from patients with autoimmune thyroid diseases. *J Med Virol* . 1995;45(4):392-398.

200. Mizokami T, Okamura K, Ikenoue H, et al. A high prevalence of human T-lymphotropic virus type I carriers in patients with antithyroid antibodies. *Thyroid*. 1994;4(4):415-419.

201. Akamine H, Takasu N, Komiya I, et al. Association of HTLV-1 with autoimmune thyroiditis in patients with adult T-cell leukemia (ATL) and in HTLV-I carriers. *Clin Endocrinol (Oxf)*. 1996;45(4):461-466.

202. McMurray RW, Elbourne K. Hepatitis C virus infection and autoimmunity. *Semin Arthritus Rheum*. 1997;26(4):689-701.

203. Cosserat J, Cacoub P, Bletry O. Immunological disorders in C virus chronic hepatitis. *Nephrol Dial Transplant* . 1996;11(4):31-35.

204. Degos F. Natural history of hepatitis C virus infection. *Nephrol Dial Transplant* . 1996;4:16-18.

205. Ganne-Carrie N, Medini A, Coderc E, et al. Latent autoimmune thyroiditis in untreated patients with HCV chronic hepatitis: a casecontrol study. *J Autoimmun*. 2000;14(2):189-193.

206. Manns MP, Rambusch EG. Autoimmunity and extrahepatic manifestations in hapatitis

C virus infection. *J Hepatol*. 1999;1:39-42.

207. Preziati D, La Rosa L, Covini G, et al. Autoimmunity and thyroid function in patients with chronic active hepatitis treated with recombinant interferon alpha-2a. *Eur J Endocrinol*. 1995;132(5):587-593.

208. Gregorio GV, Jones H, Choudhuri K, et al. Autoantibody prevalence in chronic hepatitis B virus infection: effect in interferon alfa. *Hepatology*. 1996;24(3):520-523.

209. Fernandez-Soto L, Gonzalez A, Escobar-Jimenez F, et al. Increased risk of autoimmune thyroid disease in hepatitis C vs hepatitis B before, during, and after discontinuing interferon therapy. *Arch Intern Med*. 1998;158:1445-1448.

210. Sever JL, South MA, Shaver KA. Delayed manifestations of congenital rubella. *Rev Infect Dis*. 1985;7:S164-S169.

211. Klavinskis LS, Notkins AL, Oldstone MBA. Persistent viral infection of the thyroid gland: alteration of thyroid function in the absence of tissue injury. *Endocrinology*. 1988;122:567-575.

212. Srinivassapa J, Gazelli C, Onodera T, et al. Virus-induced thyroiditis. *Endocrinology*. 1988;122:563-566.

213. Onodera T, Awaya A. Anti-thyroglobulin antibodies induced with recombinant reovirus infection in BALB/c mice. *Immunology*. 1990;71:581-585.

214. Ziemiecki A, Kromer G, Muller RG, et al. ev 22, a new endogenous avian leukosis virus locus found in chickens with spontaneous autoimmune thyroiditis. *Arch Virol*. 1988;100:267-271.

215. Carter JK, Smith RE. Rapid induction of hypothyroidism by an avian leukosis virus. *Infect Immunity*. 1983;40:795-805.

216. Kuhr T, Hala K, Dietrich H, Herold M, Wick G. Genetically determined target organ susceptibility in the pathogenesis of spontaneous autoimmune thyroiditis: aberrant expression of HMC-class II antigens and the possible role of virus. *J Autoimmun*. 1994;7(1):13-25.

217. Mason LJ, Timothy LM, Isenberg DA, Kalsi JK. Immunization with a paptide of Sm B/B' results in limited epitope spreading but not autoimmune disease. *J Immunol*. 1999;162:5099-5105.

218. von Mering J, Minkowski O. Diabetes mellitus nach Pankreasextirpation. *Arch Exp Pathol Pharmakol*. 1890;26:371-374.

219. Schmidt MB. Uber die Beziehung der Langerhansschen Insein des Pankreas zum Diabetes Mellitus. *Munch Med Wochenschr*. 1902;49:51.

220. Banting FG, Best CH. The internal secretion of the pancreas. *J Lab Clin Med*. 1922;7:251-266.

221. Gepts W. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes*. 1965;14(10):619-633.

222. Bottazzo GF, Florin-Christensen A, Doniach D. Islet cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. *Lancet*. 1974;2:1279-1281.

223. MacCuish AC, Irvine WJ, Barnes EW, Duncan LJ. Antibodies to pancreatic islet cells in insulin-dependent diabetics with coexistent autoimmune disease. *Lancet*. 1974;2:1529-1531.

224. Nerup J, Platz P, Andersen OO, et al. HL-A antigens and diabetes mellitus. *Lancet*. 1974;2(7885):864-866.

225. Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet*. 2001;358(9277):221-229.

226. Atkinson MA, Maclaren NK. The pathogenesis of insulin-dependent diabetes mellitus. *N Engl J Med*. 1994;331(21):1428-1436.

227. Jackson RA, Soeldner JS, Eisenbarth GS. Predicting insulin-dependent diabetes. *Lancet*. 1988;10(2):627-628.

228. Eisenbarth GS, Gianani R, Yu L, Pietropaolo M, Harrison C, Jackson R. Dual-parameter model for prediction of type I diabetes mellitus. *Proc Assoc Am Physicians*. 1998;110(2):126-135.

229. Wong FS, Karttunen J, Dumont C, et al. Identification of an MHC class I-restricted autoantigen in type 1 diabetes by screening an organ-specific cDNA library. *Nature Med*. 1999;5(9):1026-1031.

230. Lieberman SM, Evans AM, Han B, et al. Identification of the beta cell antigen targeted by a prevalent population of pathogenic CD8+ T cells in autoimmune diabetes. *Proc Natl Acad Sci U S A*. 2003;100(14):8384-8388.

231. Trudeau JD, Kelly-Smith C, Verchere CB, et al. Prediction of spontaneous autoimmune diabetes in NOD mice by quantification of autoreactive T cells in peripheral blood. *J Clin Invest*. 2003;111(2):217-223.

232. Yoon J-W, Yoon C-S, Lim H-W, et al. Control of autoimmune diabetes in NOD mice

by GAD expression or suppression in betta cells. *Science*. 1999;284:1183-1187.

233. Nakayama M, Abiru N, Moriyama H, et al. Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. *Nature*. 2005;435(7039):220-223.

234. von Herrath MG, Dockter J, Oldstone MBA. How virus induces a rapid or slow onset insulin-dependent diabetes mellitus in a transgenic model. *Immunity*. 1994;1:231-242.

235. DiLorenzo TP, Graser RT, Ono T, et al. Major histocompatibility complex class I-restricted T cells are required for all but the end stages of diabetes development in nonobese diabetic mice and use a prevalent T cell receptor alpha chain gene rearrangement. *Proc Natl Acad Sci U S A* . 1998;95(21):12538-12543.

236. Graser RT, DiLorenzo TP, Wang F, et al. Identification of a CD8 T cell that can independently mediate autoimmune diabetes development in the complete absence of CD4 T cell helper functions. *J Immunol* . 2000;164:3913-3918.

237. Eisenbarth GA. Type 1 diabetes mellitus. A chronic autoimmune disease. *N Engl J Med*. 1986;314:1360-1368.

238. Durinovic-Bello I, Hummel M, Ziegler AG. Cellular immune response to diverse islet cell antigens in IDDM. *Diabetes*. 1996;45(6):795-800.

239. Fuchtenbusch M, Kredel K, Bonifacio E, Schnell O, Ziegler A-G. Exposure to exogenous insulin promotes IgG1 and the T-helper 2-associated IgG4 responses to insulin but not to other islet autoantigens. *Diabetes*. 2000;49:918-925.

240. Roep BO, Stobbe I, Duinkerken G, et al. Auto- and alloimmune reactivity to human islet allografts transplanted into type 1 diabetic patients. *Diabetes*. 1999;48(3):484-490.

241. Martin S, Wolf-Eichbaum D, Duinkerken G, et al. Development of type 1 diabetes despite severe hereditary B-lymphocyte deficiency. *N Engl J Med* . 2001;345(14):1036-1040.

242. Hiemstra HS, Drijfhout JW, Roep BO. Antigen arrays in T cell immunology. *Curr Opin Immunol* . 2000;12:80-84.

243. Noorchashm H, Lieu YK, Noorchashm N, et al. I-Ag7-mediated antigen presentation by B lymphocytes is critical in overcoming a checkpoint in T cell tolerance to islet beta cells of nonobese diabetic mice. *J Immunol* . 1999;163(2):743-750.

244. Kaufman DL, Tisch R, Sarvetnick N, et al. Report from the 1st International NOD Mouse T-Cell Workshop and the follow-up miniworkshop. *Diabetes*. 2001;50(11):2459-2463.

245. van de Linde P, Roep BO. T-cell assays to determine disease activity and clinical efficacy of immune therapy in type 1 diabetes. *Am J Ther*. 2005;12(6):573-579.

246. Herold KC, Brooks-Worrell B, Palmer J, et al. Validity and reproducibility of measurement of islet autoreactivity by T-cell assays in subjects with early type 1 diabetes. *Diabetes*. 2009;58(11):2588-2595.

247. Velthuis JH, Unger WW, Abreu JR, et al. Simultaneous detection of circulating autoreactive CD8+ T-cells specific for different islet cellassociated epitopes using combinatorial MHC multimers. *Diabetes*. 2010;59(7):1721-1730.

248. Arif S, Tree TI, Astill TP, et al. Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health. *J Clin Invest*. 2004;113(3):451-463.

249. Redondo MJ, Jeffrey J, Fain PR, Eisenbarth GS, Orban T. Concordance for islet autoimmunity among monozygotic twins. *N Engl J Med*. 2008;359(26):2849-2450.

250. Concannon P, Rich SS, Nepom GT. Genetics of type 1A diabetes. *N Engl J Med*. 2009;360(16):1646-1654.

251. Wicker LS, Todd JA, Peterson LB. Genetic control of autoimmune diabetes in the NOD mouse. *Annu Rev Immunol*. 1995;13:179-200.

252. McDevitt H, Singer S, Tisch R. The role of MHC class II genes in susceptibility and resistance to type I diabetes mellitus in the NOD mouse. *Horm Metab Res*. 1996;28(6):287-288.

253. Serreze DV, Leiter EH. Genes and cellular requirements for autoimmune diabetes susceptibility in nonobese diabetic mice. In: von Herrath MG, ed. *Curr Dir Autoimmun - Molecular Pathology of Type 1 Diabetes Mellitus*. Basel, Switzerland: S. Karger; 2001;4:31-67.

254. Van Houten N, Blake SF. Direct measurement of anergy of antigen-specific T cells following oral tolerance induction. *J Immunol*. 1996;157(4):1337-1341.

255. Weiner HL. Oral tolerance: mobilizing the gut. *Hosp Pract (Off Ed)*. 1995;30(9):53-58.

256. Benson JM, Whitacre CC. The role of clonal deletion and anergy in oral tolerance. *Res Immunol*. 1997;148:533-541.

257. Strobel S, Mc Mowat A. Immune responses to dietary antigens: oral tolerance. *Immunol Today*. 1998;19(4):173-181.

258. Viney JL, Mowat AM, O'Malley JM, Williamson E, Fanger NA. Expanding dendritic cells in vivo enhances the induction of oral tolerance. *J Immunol*. 1998;160:5815-5825.

259. Maeda Y, Noda S, Tanaka K, et al. The failure of oral tolerance induction is functionally coupled to the absence of T cells in Peyer's patches under germfree conditions. *Immunobiology*. 2001;204(4):442-457.

260. Atkinson MA, Maclaren NK. The pathogenesis of insulin-dependent diabetes mellitus. *N Engl J Med*. 1994;331(21):1428-1436.

261. Wen L, Ley RE, Volchkov PY, et al. Innate immunity and intestinal microbiota in the development of type 1 diabetes. *Nature*. 2008;455(7216):1109-1113.

262. Paronen J, Knip M, Savilahti E, et al. Effect of cow's milk exposure and maternal type 1 diabetes on cellular and humoral immunization to dietary insulin in infants at genetic risk for type 1 diabetes. Finnish trial to reduce IDDM in the Genetically at Risk Study Group. *Diabetes*. 2000;49(10):1657-1665.

263. Karjalainen J, Martin JM, Knip M, et al. A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus. *N Engl J Med*. 1992;327(5):302-307.

264. Honeyman MC, Coulson BS, Stone NL, et al. Association between rotavirus infection and pancreatic islet autoimmunity in children at risk of developing type 1 diabetes. *Diabetes*. 2000;49(8):1319-1324.

265. Makela M, Oling V, Marttila J, et al. Rotavirus-specific T cell responses and cytokine mRNA expression in children with diabetes-associated autoantibodies and type 1 diabetes. *Clin Exp Immunol*. 2006;145(2):261-270.

266. Yeung WC, Rawlinson WD, Craig ME. Enterovirus infection and type 1 diabetes mellitus: systematic review and meta-analysis of observational molecular studies. *BMJ*. 2011;342:d35.

267. Lonnrot M, Korpela K, Knip M, et al. Enterovirus infection as a risk factor for beta-cell autoimmunity in a prospectively observed birth cohort: the Finnish Diabetes Prediction and Prevention Study [In Process Citation]. *Diabetes*. 2000;49(8):1314-1318.

268. Notkins AL, Yoon JW. Virus-induced diabetes mellitus. In: Notkins AL, Oldstone MBA, eds. *Concepts in Viral Pathogenesis*. New York, NY: Springer-Verlag; 1984:241-247.

269. Jenson AB, Rosenberg HS, Notkins AL. Pancreatic islet cell damage in children with fatal viral infections. *Lancet*. 1980;2:354-358.

270. Mena I, Fischer C, Gebhard JR, Perry CM, Harkins S, Whitton JL. Coxsackievirus infection of the pancreas: evaluation of receptor expression, pathogenesis, and

immunopathology. *Virology*. 2000;271(2): 276-288.

271. Serreze DV, Ottendorfer EW, Ellis TM, Gauntt CJ, Atkinson MA. Acceleration of type 1 diabetes by a coxsackievirus infection requires a preexisting critical mass of autoreactive T-cells in pancreatic islets. *Diabetes*. 2000;49(5):708-711.

272. Stene LC, Oikarinen S, Hyoty H, et al. Enterovirus infection and progression from islet autoimmunity to type 1 diabetes: the Diabetes and Autoimmunity Study in the Young (DAISY). *Diabetes*. 2010;59(12): 3174-3180.

273. Oikarinen S, Martiskainen M, Tauriainen S, et al. Enterovirus RNA in blood is linked to the development of type 1 diabetes. *Diabetes*. 2011;60(1):276-279.

274. Richardson SJ, Willcox A, Bone AJ, Foulis AK, Morgan NG. The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes. *Diabetologia*. 2009;52(6):1143-1151.

275. Nejentsev S, Walker N, Riches D, Egholm M, Todd JA. Rare variants of IFIH1, a gene implicated in antiviral responses, protect against type 1 diabetes. *Science*. 2009;324(5925):387-389.

276. Smyth DJ, Cooper JD, Bailey R, et al. A genome-wide association study of nonsynonymous SNPs identifies a type 1 diabetes locus in the interferon-induced helicase (IFIH1) region. *Nat Genet*. 2006;38(6):617-619.

277. von Herrath M. Diabetes: A virus-gene collaboration. *Nature*. 2009;459(7246):518-519.

278. Babu PG, Huber SA, Craighead JE. Contrasting features of T-lymphocytemediated diabetes in encephalomyocarditis virus-infected Balb/cBy and Balb/cCum mice. *Am J Pathol*. 1986;124:193-198.

279. Baum H. Mitochondrial antigens, molecular mimicry and autoimmune disease. *Biochimicaet Biophysica Acta*. 1995;1271:111-121.

280. Gianani R, Sarvetnick N. Viruses, cytokines, antigens, and autoimmunity. *Proc Natl Acad Sci U S A*. 1996;93(6):2257-2259.

281. Foulis AK, McGill M, Farquharson MA, Hilton DA. A search for evidence of viral infection in pancreases of newly diagnosed patients with IDDM. *Diabetologia*. 1997;40(1):53-61.

282. Shrinivasappa J, Saegusa J, Prabhakar B, et al. Frequency of reactivity of monoclonal antiviral antibodies with normal tissues. *J Virol*. 1986;57:397-401.

283. Oldstone MBA. Molecular mimicry as a mechanism for the cause and as a probe uncovering etiologic agent(s) of autoimmune disease. *Curr Top Microbiol Immunol* . 1989;145:127-135.

284. Horwitz MS, Sarvetnick N. Viruses, host responses, and autoimmunity. *Immunol Rev*. 1999;169:241-253.

285. Buschard K. Diabetic animal models. *APMIS*. 1996;104(9):609-614.

286. Katz J, Benoist C, Mathis DX. T helper cell subsets in IDDM. *Science*. 1995;268:1185-1188.

287. Wilberz S, Partke HJ, Dagnaes-Hansen F, Herberg L. Persistent MHV (mouse hepatitis virus) infection reduces the incidence of diabetes mellitus in non-obese diabetic mice. *Diabetologia*. 1991;34:2-5.

288. Oldstone MBA. Viruses as therapeutic agents. I. Treatment of nonobese insulin-dependent diabetes mice with virus prevents insulin-dependent diabetes mellitus while maintaining general immune competence. *J Exp Med*. 1990;171:2077-2089.

289. Toshiyuki M, Ogawa M, Kobayashi F, Yoshihiro T, Hirotsugu U. Electron microscope studies on the interaction of pancreatic islet cells and splenic lymphocytes in non-obese diabetic (NOD) mice. *Biomed Res*. 1988;9:67-73.

290. Morgan D, Liblau R, Scott B, et al. CD8+ T cell-mediated spontaneous diabetes in neonatal mice. *J Immunol* . 1996;157:978-984.

291. Kurts C, Cannarile M, Klebba I, Brocker T. Cutting edge: dendritic cells are sufficient to cross-present self-antigens to CD8 T cells in vivo. *J Immunol* . 2001;166(3):1439-1442.

292. Kurts C, Heath WR, Carbone FR, Kosaka H, Miller JF. Crosspresentation of self antigens to CD8+ T cells: the balance between tolerance and autoimmunity. *Novartis Found Symp*. 1998;215:172-181.

293. Kurts C, Heath WR, Kosaka H, Miller JF, Carbone FR. The peripheral delection of autoreactive CD8+ T cells induced by cross-presentation of self-antigens involves signaling through CD95 (Fas, Apo-1). *J Exp Med* . 1998;188(2):415-420.

294. Oldstone MB, Nerenberg M, Southern P, Price J, Lewicki H. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. *Cell* . 1991;65: 319-331.

295. Ohashi P, Oehen S, Buerki K, et al. Ablation of “tolerance” and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell* . 1991;65:305-317.

296. Berdanier CD. Diet, autoimmunity, and insulin-dependent diabetes mellitus: a controversy. *PSEBM*. 1995;209:223-230.

297. Hiltunen M, Hyoty H, Knip M, et al. Islet cell antibody seroconversion in children is temporally associated with enterovirus infections. Childhood Diabetes in Finland (DiMe) Study Group. *J Infect Dis*. 1997;175(3):554-560.

298. Peterson JS, Dyrberg T, Karlsen AE, et al. Glutamic acid decarboxylase (GAD_{65}) autoantibodies in prediction of b-cell function and remission in recent onset IDDM after cyclosporin treatment. *Diabetes*. 1994; 43:1291-1296.

299. Peterson JS, Hejnaes KR, Moody A, et al. Detection of GAD_{65} antibodies in diabetes and other autoimmune diseases using a simple radioligand assay. *Diabetes*. 1994;43:459-467.

300. Wucherpfennig KW, Strominger JL. Molecular mimicry in T-cell mediated autoimmunity: viral peptides activate human T-cell clones specific for myelin basic protein. *Cell*. 1995;80:695-705.

301. von Herrath MG, Dockter J, Oldstone MB. How virus induces a rapid or slow onset insulin-dependent diabetes mellitus in a transgenic model. *Immunity*. 1994;1(3):231-242.

302. Ludewig B, Odermatt B, Landmann S, Hengartner H, Zinkernagel RM. Dendritic cells induce autoimmune diabetes and maintain disease via de novo formation of local lymphoid tissue. *J Exp Med*. 1998;188(8):1493-1501.

303. Ludewig B, Odermatt B, Ochsenbein AF, Zinkernagel RM, Hengartner H. Role of dendritic cells in the induction and maintenance of autoimmune diseases. *Immunol Rev*. 1999;169:45-54.

304. Ludewig B, Junt T, Hengartner H, Zinkernagel RM. Dendritic cells in autoimmune diseases. *Curr Opin Immunol* . 2001;13(6):657-662.

305. von Herrath MG, Guerder S, Lewicki H, Flavell RA, Oldstone MB. Coexpression of B7-1 and viral (“self”) transgenes in pancreatic beta cells can break peripheral ignorance and lead to spontaneous autoimmune diabetes. *Immunity*. 1995;3(6):727-738.

306. Kagi D, Odermatt B, Ohashi PS, Zinkernagel RM, Hengartner H. Development of insulitis without diabetes in transgenic mice lacking perforin-dependent cytotoxicity. *J Exp Med*. 1996;183(5):2143-2152.

307. Chatenoud L, Salomon B, Bluestone JA. Suppressor T cells—they're back and critical for regulation of autoimmunity! *Immunol Rev*. 2001;182:149-163.

308. Herold KG, Lenschow DJ, Bluestone JA. CD28/B7 regulation of autoimmune

diabetes. *Immunol Res*. 1997;16(1):71-84.

309. Green EA, Flavell RA. The temporal importance of TNF alpha expression in the development of diabetes. *Immunity*. 2000;12:459-469.

310. Brod SA, Atkinson M, Lavis VR, et al. Ingested IFN-alpha preserves residual beta cell function in type 1 diabetes. *J Interferon Cytokine Res*. 2001;21(12):1021-1030.

311. Stauffer Y, Marguerat S, Meylan F, et al. Interferon-alpha-induced endogenous superantigen. a model linking environment and autoimmunity. *Immunity*. 2001;15(4):591-601.

312. Bosi E, Minelli R, Bazzigaluppi E, Salvi M. Fulminant autoimmune Type 1 diabetes during interferon-alpha therapy: a case of Th1-mediated disease? *Diabet Med*. 2001;18(4):329-332.

313. von Herrath MG, Allison J, Miller JF, Oldstone MB. Focal expression of interleukin-2 does not break unresponsiveness to “self” (viral) antigen expressed in beta cells but enhances development of autoimmune disease (diabetes) after initiation of an anti-self immune response. *J Clin Invest*. 1995;95(2):477-485.

314. Lee MS, Wogensen L, Shizuru J, Oldstone MB, Sarvetnick N. Pancreatic islet production of murine interleukin-10 does not inhibit immune-mediated tissue destruction. *J Clin Invest*. 1994;93:1332-1338.

315. Lee M-S, Mueller R, Wicker LS, Peterson LB, Sarvetnick N. IL-10 is necessary and sufficient for autoimmune diabetes in conjunction with NOD MHC homozygosity. *J Exp Med*. 1996;183:2663-2668.

316. King C, Mueller Hoenger R, Malo Cleary M, et al. Interleukin-4 acts at the locus of the antigen-presenting dendritic cell to counter-regulate cytotoxic CD8+ T-cell responses. *Nat Med*. 2001;7(2):206-214.

317. von Herrath MG, Whitton JL. DNA vaccination to treat autoimmune diabetes. *Ann Med*. 2000;32:285-292.

318. Bot A, Smith D, Bot S, et al. Plasmid vaccination with insulin b chain prevents autoimmune diabetes in nonobese diabetic mice. *J Immunol*. 2001;167(5):2950-2595.

319. Garren H, Ruiz PJ, Watkins TA, et al. Combination of gene delivery and DNA vaccination to protect from and reverse Th1 autoimmune disease via deviation to the Th2 pathway. *Immunity*. 2001;15(1):15-22.

320. Tisch R, Wang B, Atkinson MA, Serreze DV, Friedline R. A glutamic acid decarboxylase 65-specific th2 cell clone immunoregulates autoimmune diabetes in

nonobese diabetic mice. *J Immunol*. 2001;166(11): 6925-6936.

321. Wherrett DK, Bundy B, Becker DJ, et al. Antigen-based therapy with glutamic acid decarboxylase (GAD) vaccine in patients with recent-onset type 1 diabetes: a randomised double-blind trial. *Lancet*. 2011;378(9788):319-327.

322. von Herrath M. Combination therapies for type 1 diabetes: why not now? *Immunotherapy*. 2010;2(3):289-291.

323. Gaur A, Boehme SA, Chalmers D, et al. Amelioration of relapsing experimental autoimmune encephalomyelitis with altered myelin basic protein peptides involves different cellular mechanisms. *J Neuroimmunol*. 1997;74(1-2):149-158.

324. Pedotti R, Mitchell D, Wedemeyer J, et al. An unexpected version of horror autotoxicus: anaphylactic shock to a self-peptide. *Nat Immunol*. 2001;2(3):216-222.

325. Kappos L, Comi G, Panitch H, et al. Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial. The Altered Peptide Ligand in Relapsing MS Study Group. *Nat Med*. 2000;6(10):1176-1182.

326. Walter M, Philotheou A, Bonnici F, Ziegler AG, Jimenez R. No effect of the altered peptide ligand NBI-6024 on beta-cell residual function and insulin needs in new-onset type 1 diabetes. *Diabetes Care*. 2009;32(11):2036-2040.

327. Skyler JS, Krischer JP, Wolfsdorf J, et al. Effects of oral insulin in relatives of patients with type 1 diabetes: the diabetes prevention trial— type 1. *Diabetes Care*. 2005;28(5):1068-1076.

328. Sherry N, Hagopian W, Ludvigsson J, et al. Teplizumab for treatment of type 1 diabetes (Protege study): 1-year results from a randomised, placebo-controlled trial. *Lancet*. 2011;378(9790):487-497.

329. Orban T, Bundy B, Becker DJ, et al. Co-stimulation modulation with abatacept in patients with recent-onset type 1 diabetes: a randomised, double-blind, placebo-controlled trial. *Lancet*. 2011;378(9789):412-419.

330. Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, et al. Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. *N Engl J Med*. 2009;361(22):2143-2152.

331. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med*. 2000;343(4):230-238.

332. Kroon E, Martinson LA, Kadoya K, et al. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nat Biotechnol*. 2008;26(4):443-452.

333. D'Amour KA, Bang AG, Eliazer S, et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol*. 2006;24(11):1392-1401.

334. Ettinger RA, Nepom GT. Molecular aspects of HLA class II alphabeta heterodimers associated with IDDM susceptibility and protection. *Rev Immunogenet*. 2000;2(1):88-94.

335. Coulombe M, Yang H, Wolf LA, Gill RG. Tolerance to antigen-presenting cell-depleted islet allografts is CD4 T cell dependent. *J Immunol*. 1999;162:2503-2510.

336. Parker DC, Greiner DL, Phillips NE, et al. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. *Proc Natl Acad Sci U S A* . 1995;92:9560-9564.

337. Rossini AA, Greiner DL, Mordes JP. Induction of immunologic tolerance for transplantation. *Physiolog Rev*. 1999;79(1):99-141.

338. Welsh RM, Markees TG, Woda BA, et al. Virus-induced abrogation of transplantation tolerance induced by donor-specific transfusion and anti-CD154 antibody. *J Virol*. 2000;74(5):2210-2218.

339. Wilson SB, Kent SC, Patton KT, et al. Extreme Th1 bias of invariant Valpha24JalphaQ T cells in type 1 diabetes. *Nature*. 1998;391(6663): 177-181.

340. Utz U, Biddison WE, McFarland HF, McFarlin DE, Flerlage M, Martin R. Skewed T-cell receptor repertoire in genetically identical twins correlates with MS. *Nature*. 1993;364:243-247.

341. Muraro PA, Vergelli M, Kalbus M, et al. Immunodominance of a low-affinity major histocompatibility complex-binding myelin basic protein epitope (residues 111-129) in HLA-DR4 (B1*0401) subjects is associated with a restricted T cell receptor repertoire. *J Clin Invest*. 1997;100(2):339-349.

342. Scholz C, Patton KT, Anderson DE, Freeman GJ, Hafler DA. Expansion of autoreactive T cells in multiple sclerosis is independent of exogenous B7 costimulation1. *J Immunol*. 1998;160:1532-1538.

343. de Seze J, Dubucquoi S, Lefranc D, et al. IgG reactivity against citrullinated myelin basic protein in multiple sclerosis. *J Neuroimmunol*. 2001;117(1-2):149-155.

344. Mason JL, Suzuki K, Chaplin DD, Matsushima GK. Interleukin-1beta promotes repair

of the CNS. *J Neurosci*. 2001;21(18):7046-7052.

345. Arnett HA, Mason J, Marino M, Suzuki K, Matsushima GK, Ting JP. TNF alpha promotes proliferation of oligodendrocyte progenitors and remyelination. *Nat Neurosci*. 2001;4(11):1116-1122.

346. Ebers G, Bulman D, Sadovnik A, et al. A population based study of multiple sclerosis in twins. *New Engl J Med*. 1987;315:1638-1642.

347. Vergelli M, Mazzanti B, Traggiai E, et al. Short-term evolution of autoreactive T cell repertoire in multiple sclerosis. *J Neurosci Res*. 2001;66(3):517-524.

348. Anderson DE, Sharpe AH, Hafler DA. The B7-CD28/CTLA-4 costimulatory pathways in autoimmune disease of the central nervous system. *Curr Opin Immunol*. 1999;11(6):677-683.

349. Kanter JL, Narayana S, Ho PP, et al. Lipid microarrays identify key mediators of autoimmune brain inflammation. *Nat Med*. 2006;12(1):138-143.

350. Iglesias A, Bauer J, Litzenburger T, Schubart A, Linington C. T- and B-cell responses to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis and multiple sclerosis. *Glia*. 2001;36(2):220-234.

351. Challoner PB, Smith KT, Parker JD, et al. Plaque-associated expression of human herpes virus 6 in multiple sclerosis. *Proc Natl Acad Sci U S A*. 1995;92(16):7440-7444.

352. Miller S, Karpus WJ. The immune pathogenesis and regulation of T-cell mediated demyelinating diseases. *Immunol Today*. 1994;15:358-362.

353. Neumann H, Schmidt H, Cavalie A, Jenne D, Wekerle H. Major histocompatibility complex (MHC) class 1 gene expression in single neurons of the central nervous system: differential regulation by interferon (IFN-gamma) and tumor necrosis factor (TNF)-a. *J Exp Med*. 1997;185(2):305-316.

354. Ruiz PJ, Garren H, Ruiz IU, et al. Suppressive immunization with DNA encoding a self-peptide prevents autoimmune disease: modulation of T cell costimulation. *J Immunol*. 1999;162(6):3336-3341.

355. Katz-Levy Y, Neville KL, Girvin AM, et al. Endogenous presentation of self myelin epitopes by CNS-resident APCs in Theiler's virus-infected mice. *J Clin Invest*. 1999;104:599-610.

356. Olson JK, Croxford JL, Calenoff MA, Dal Canto MC, Miller SD. A virus-induced molecular mimicry model of multiple sclerosis. *J Clin Invest*. 2001;108(2):311-318.

357. Buchmeier MJ, Lane TE. Viral-induced neurodegenerative disease. *Curr Opin Microbiol*. 1999;2(4):398-402.

358. Evans CF, Horwitz MS, Hobbs MV, Oldstone MB. Viral infection of transgenic mice expressing a viral protein in oligodendrocytes leads to chronic central nervous system autoimmune disease. *J Exp Med*. 1996;184(6):2371-284.

359. Garren H, Robinson WH, Krasulova E, et al. Phase 2 trial of a DNA vaccine encoding myelin basic protein for multiple sclerosis. *Ann Neurol*. 2008;63(5):611-620.

360. Martin R, Sturzebecher CS, McFarland HF. Immunotherapy of multiple sclerosis: where are we? Where should we go? *Nat Immunol*. 2001;2(9):785-788.

361. McFarland HF. Complexities in the treatment of autoimmune disease. *Science*. 1996;274(5295):2037-2038.

362. Weiner HL, Mackin GA, Matsui M, et al. Double-blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis. *Science*. 1993;259(5099):1321-1324.

363. Jacobs LD, Beck RW, Simon JH, et al. Intramuscular interferon beta-1a therapy initiated during a first demyelinating event in multiple sclerosis. CHAMPS Study Group. *N Engl J Med*. 2000;343(13):898-904.

364. Rice GP, Woelfel EL, Talbot PJ, et al. Immunological complications in multiple sclerosis patients receiving interferon. *Ann Neurol*. 1985;18(4):439-442.

365. Chen M, Gran B, Costello K, Johnson K, Martin R, Dhib-Jalbut S. Glatiramer acetate induces a Th2-biased response and crossreactivity with myelin basic protein in patients with MS. *Mult Scler*. 2001;7(4):209-219.

366. Bielekova B, Lincoln A, McFarland H, Martin R. Therapeutic potential of phosphodiesterase-4 and -3 inhibitors in Th1-mediated autoimmune diseases. *J Immunol*. 2000;164(2):1117-1124.

367. Bielekova B, Goodwin B, Richert N, et al. Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. *Nat Med*. 2000;6(10):1167-1175.

368. Liossis SN, Sfikakis PP. Costimulation blockade in the treatment of rheumatic diseases. *Bio Drugs*. 2004;18(2):95-102.

369. Smith CE, Miller SD. Multi-peptide coupled-cell tolerance ameliorates ongoing relapsing EAE associated with multiple pathogenic autoreactivities. *J Autoimmunity*. 2006;27(4):218-231.

370. Viglietta V, Bourcier K, Buckle GJ, et al. CTLA4Ig treatment in patients with multiple sclerosis: an open-label, phase 1 clinical trial. *Neurology*. 2008;71(12):917-924.

371. Lopez de Castro JA. The pathogentic role of HLA-B27 in chronic arthritis. *Curr Opin Immunol*. 1998;10(1):59-66.

372. Wildner G, Diedrichs-Mohring M, Thurau SR. Induction of arthritis and uveitis in Lewis rats by antigenic mimicry of peptides from HLAB27 and cytokeratin. *Eur J Immunol*. 2002;32(1):299-306.

373. Lee SJ, Im HY, Schueller WC. HLA-B27 positive juvenile arthritis with cardiac involvement preceding sacroiliac joint changes. *Heart*. 2001;86(6):E19.

374. Sun B, Rizzo LV, Sun S-H, et al. Genetic susceptibility to experimental autoimmune uveitis involves more than a predisposition to generate a T helper-1-like or a T helper-2-like response. *J Immunol*. 1997;159: 1004-1011.

375. Tarrant TK, Silver PB, Chan CC, Wiggert B, Caspi RR. Endogenous IL-12 is required for induction and expression of experimental autoimmune uveitis. *J Immunol*. 1998;161(1):122-127.

376. Rizzo LV, Morawetz RA, Miller-Rivero NE, et al. IL-4 and IL-10 are both required for the induction of oral tolerance. *J Immunol*. 1999;162:2613-2622.

377. Wildner G, Thurau SR. Orally induced bystander suppression in experimental autoimmune uveoretinitis occurs only in the periphery and not in the eye. *Euro J Immunol*. 1995;25(5):1292-1297.

378. Wildner G, Thurau SR. Orally induced bystander suppression in experimental autoimmune uveoretinitis occurs only in the periphery and not in the eye. *Eur J Immunol*. 1995;25(5):1292-1297.

379. Yip L, Su L, Sheng D, et al. Deaf1 isoforms control the expression of genes encoding peripheral tissue antigens in the pancreatic lymph nodes during type 1 diabetes. *Nat Immunol*. 2009;10(9):1026-1033.

380. Gangappa S, Sam Babu J, Thomas J, Daheshia M, Rouse BT. Virusinduced immunoinflammatory lesions in the absence of viral antigen recognition. *J Immunol*. 1998;161:4289-4300.

381. Chun S, Daheshia M, Lee S, Rouse BT. Immune modulation by IL-10 gene transfer via viral vector and plasmid DNA: implication for gene therapy. *Cell Immunol*. 1999;194(2):194-204.

382. Panoutsakopoulou V, Sanchirico ME, Huster KM, et al. Analysis of the relationship

between viral infection and autoimmune disease. *Immunity*. 2001;15(1):137-147.

383. Kosiewicz MM, Alard P, Streilein JW. Alterations in cytokine production following intraocular injection of soluble protein antigen: impairment in IFN-g and induction of TGF-b and IL-4 production. *J Immunol*. 1998;161:5382-5390.

384. Takeuchi M, Alard P, Streilein JW. TGF-b promotes immune deviation by altering accessory signals of antigen-presenting cells. *J Immunol*. 1998;160:1589-1597.

385. Thurau SR, Diedrichs-Mohring M, Fricke H, Burchardi C, Wildner G. Oral tolerance with an HLA-peptide mimicking retinal autoantigen as a treatment of autoimmune uveitis. *Immunol Lett*. 1999;68(2-3): 205-212.

386. Nussenblatt RB, Peterson JS, Foster CS, et al. Initial evaluation of subcutaneous daclizumab treatments for noninfectious uveitis: a multicenter noncomparative interventional case series. *Ophthalmology*. 2005;112(5):764-770.

387. Bhat P, Castañeda-Cervantes RA, Doctor PP, et al. Intravenous daclizumab for recalcitrant ocular inflammatory disease. *Graefes Arch Clin Exp Ophthalmol*. 2009;247(5):687-692

388. Yamanaka T, Straumfors A, Morton H, Fausa O, Brandtzaeg P, Farstad I. M cell pockets of human Peyer's patches are specialized extensions of germinal centers. *Eur J Immunol*. 2001;31(1):107-117.

389. Iwasaki A, Kelsall BL. Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3alpha, MIP-3beta, and secondary lymphoid organ chemokine. *J Exp Med*. 2000;191(8):1381-1394.

390. Harrison LC, Dempsey-Collier M, Kramer DR, Takahashi K. Aerosol insulin induces regulatory CD8 gamma delta T cells that prevent murine insulin-dependent diabetes. *J Exp Med*. 1996;184(6):2167-2174.

391. Hanninen A, Harrison LC. Gamma delta T cells as mediators of mucosal tolerance: the autoimmune diabetes model. *Immunol Rev*. 2000;173:109-119.

392. Chen Y, Inobe J, Kuchroo VK, Baron JL, Janeway CA Jr, Weiner HL. Oral tolerance in myelin basic protein T-cell receptor transgenic mice: suppression of autoimmune encephalomyelitis and dose-dependent induction of regulatory cells. *Proc Natl Acad Sci U S A*. 1996;93(1):388-391.

393. Strickland RG, Mackay IR. A reappraisal of the nature and significance of chronic atrophic gastritis. *Am J Dig Dis*. 1973;18(5):426-440.

394. Carmel R. Prevalence of undiagnosed pernicious anemia in the elderly. *Arch Intern*

Med. 1996;156(10):1097-1100.

395. Toh BH, van Driel IR, Gleeson PA. Pernicious anemia. *N Engl J Med*. 1997;337(20):1441-1448.

396. Alderuccio F, Sentry JW, Marshall AC, Biondo M, Toh BH. Animal models of human disease: experimental autoimmune gastritis-a model for autoimmune gastritis and pernicious anemia. *Clin Immunol*. 2002;102(1):48-58.

397. Thornton AM, Shevach EM. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J Immunol*. 2000;164:183-190.

398. Itoh M, Takahashi T, Sakaguchi N, et al. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic selftolerance. *J Immunol*. 1999;162:5317-5326.

399. Hanauer SB. Inflammatory bowel disease. *N Engl J Med*. 1996;334(13): 841-848.

400. Crispe IN. Hepatic T cells and liver tolerance. *Nat Rev Immunol*. 2003;3(1):51-62.

401. Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. *Nat Rev Immunol*. 2005;5(3):215-229.

402. Good MF. Development of immunity to malaria may not be an entirely active process. *Parasite Immunol*. 1995;17(2):55-59.

403. Calne R, Davies H. Organ graft tolerance: the liver effect. *Lancet*. 1994;343(8889):67-68.

404. Calne RY. Immunological tolerance—the liver effect. *Immunol Rev*. 2000;174:280-282.

405. Mazariegos GV, Reyes J, Marino IR, et al. Weaning of immunosuppression in liver transplant recipients. *Transplantation*. 1997;63(2): 243-249.

406. Klein I, Crispe IN. Complete differentiation of CD8+ T cells activated locally within the transplanted liver. *J Exp Med*. 2006;203(2):437-447.

407. Manns MP, Vogel A. Autoimmune hepatitis, from mechanisms to therapy. *Hepatology*. 2006;43(2)(suppl 1):S132-S144.

408. Krawitt EL. Autoimmune hepatitis. *N Engl J Med*. 2006;354(1):54-66.

409. Czaja AJ. Autoimmune liver disease. *Curr Opin Gastroenterol*. 2006;22(3):234-240.

410. Kaplan MM, Gershwin ME. Primary biliary cirrhosis. *N Engl J Med*. 2005;353(12):1261-1273.

411. Leung PS, Coppel RL, Gershwin ME. Etiology of primary biliary cirrhosis: the search for the culprit. *Semin Liver Dis*. 2005;25(3):327-336.

412. Kouroumalis E, Notas G. Pathogenesis of primary biliary cirrhosis: a unifying model. *World J Gastroenterol*. 2006;12(15):2320-2327.

413. MacFaul GR, Chapman RW. Sclerosing cholangitis. *Curr Opin Gastroenterol*. 2006;22(3):288-293.

414. O'Mahony CA, Vierling JM. Etiopathogenesis of primary sclerosing cholangitis. *Semin Liver Dis*. 2006;26(1):3-21.

415. Neuberger J. Halothane hepatitis. *Eur J Gastroenterol Hepatol*. 1998;10(8):631-633.

416. Waldenström J. Leber, Blutproteine und Nahrungseiweiss. *Dtsch Z Verdau Stoffwechselkrankh*. 1950;2:113-119.

417. Kunkel HG, Ahrens EH, Eisenmemger WJ, et al. Extreme hypergammaglobulinemia in young women with liver disease of unknown etiology (abstract). *J Clin Invest*. 1951;30:654.

418. Bearn AG, Kunkel HG, Slater R. The problem of chronic liver disease in young women. *Am J Med*. 1956;21:3-15.

419. Mackay IR, Taft CO, Cowling DS. Lupoid hepatitis. *Lancet*. 1956;2:1323-1326.

420. Johnson PJ, McFarlane IG. Meeting report: International Autoimmune Hepatitis Group. *Hepatology*. 1993;18(4):998-1005.

421. Manns MP, Obermayer-Straub P. Viral induction of autoimmunity: mechanisms and examples in hepatology. *J Viral Hepat*. 1997;4(suppl 2):42-47.

422. Moritoki Y, Lian ZX, Ohsugi Y, Ueno Y, Gershwin ME. B cells and autoimmune liver diseases. *Autoimmun Rev*. 2006;5(7):449-457.

423. Longhi MS, Ma Y, Bogdanos DP, Cheeseman P, Mieli-Vergani G, Vergani D. Impairment of CD4(+)CD25(+) regulatory T-cells in autoimmune liver disease. *J Hepatol*. 2004;41(1):31-37.

424. Ruiz-Moreno M, Rua MJ, Carreno V, et al. Autoimmune chronic hepatitis type 2 manifested during interferon therapy in children. *J Hepatol*. 1991;12(2):265-266.

425. Obermayer-Straub P, Strassburg CP, Manns MP. Target proteins in human autoimmunity: cytochromes P450 and UDP-glucuronosyltransferases. *Can J Gastroenterol*. 2000;14(5):429-439.

426. Cerasoli DM, McGrath J, Carding SR, Shih FF, Knowles BB, Caton AJ. Low avidity recognition of a class II-restricted neo-self peptide by virus-specific T cells. *Intl Immunol*. 1995;7:935-945.

427. Jaeckel E. Animal models of autoimmune hepatitis. *Semin Liver Dis*. 2002;22(4):325-338.

428. Heneghan MA, McFarlane IG. Of mice and women: toward a mouse model of autoimmune hepatitis. *Hepatology*. 2005;42(1):17-20.

429. Guidotti LG, Ishikawa T, Hobbs MV, Matzke B, Schreiber R, Chisari FV. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. *Immunity*. 1996;4(1):25-36.

430. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. *Annu Rev Immunol*. 1995;13:29-60.

431. Alferink J, Aigner S, Reibke R, Hammerling GJ, Arnold B. Peripheral T-cell tolerance: the contribution of permissive T-cell migration into parenchymal tissues of the neonate. *Immunol Rev*. 1999;169: 255-261.

432. Voehringer D, Blaser C, Grawitz AB, Chisari FV, Buerki K, Pircher H. Break of T cell ignorance to a viral antigen in the liver induces hepatitis. *J Immunol*. 2000;165(5):2415-2422.

433. Yamauchi K, Yamaguchi N, Furukawa T, et al. A murine model of acute liver injury induced by human monoclonal autoantibody. *Hepatology*. 2005;42(1):149-155.

434. Lapierre P, Djilali-Saiah I, Vitozzi S, Alvarez F. A murine model of type 2 autoimmune hepatitis: xenoimmunization with human antigens. *Hepatology*. 2004;39(4):1066-1074.

435. Lapierre P, Beland K, Djilali-Saiah I, Alvarez F. Type 2 autoimmune hepatitis murine model: the influence of genetic background in disease development. *J Autoimmunity*. 2006;26(2):82-89.

436. Holdener M, Hintermann E, Bayer M, et al. Breaking tolerance to the natural human liver autoantigen cytochrome P450 2D6 by virus infection. *J Exp Med*. 2008;205(6):1409-1422.

437. Mackay IR. Chronic hepatitis: effect of prolonged suppressive treatment and

comparison of azathioprine with prednisolone. *Q J Med*. 1968;37(147):379-392.

438. Al-Khalidi JA, Czaja AJ. Current concepts in the diagnosis, pathogenesis, and treatment of autoimmune hepatitis. *Mayo Clin Proc*. 2001;76(12):1237-1252.

439. Kirk AP, Jain S, Pocock S, Thomas HC, Sherlock S. Late results of the Royal Free Hospital prospective controlled trial of prednisolone therapy in hepatitis B surface antigen negative chronic active hepatitis. *Gut*. 1980;21(1):78-83.

440. Sanchez-Urdazpal L, Czaja AJ, van Hoek B, Krom RA, Wiesner RH. Prognostic features and role of liver transplantation in severe corticosteroid-treated autoimmune chronic active hepatitis. *Hepatology*. 1992;15(2):215-221.

441. Neuberger J, Portmann B, Calne R, Williams R. Recurrence of autoimmune chronic active hepatitis following orthotopic liver grafting. *Transplantation*. 1984;37(4):363-365.

442. Jones DE. Pathogenesis of primary biliary cirrhosis. *J Hepatol*. 2003;39(4):639-648.

443. Lang KS, Recher M, Junt T, et al. Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. *Nat Med*. 2005;11(2):138-145.

444. Hirschfield GM, Liu X, Xu C, et al. Primary biliary cirrhosis associated with HLA, IL12A, and IL12RB2 variants. *N Engl J Med*. 2009; 360(24):2544-2555.

445. Mells GF, Floyd JA, Morley KI, et al. Genome-wide association study identifies 12 new susceptibility loci for primary biliary cirrhosis. *Nat Genet*. 2011;43(4):329-332.

446. Hirschfield GM, Liu X, Han Y, et al. Variants at IRF5-TNPO3, 17q12-21 and MMEL1 are associated with primary biliary cirrhosis. *Nat Genet*. 2010;42(8):655-657.

447. Irie J, Wu Y, Wicker LS, et al. NOD.c3c4 congenic mice develop autoimmune biliary disease that serologically and pathogenetically models human primary biliary cirrhosis. *J Exp Med*. 2006;203(5): 1209-1219.

448. Oertelt S, Lian ZX, Cheng CM, et al. Anti-mitochondrial antibodies and primary biliary cirrhosis in TGF-beta receptor II dominantnegative mice. *J Immunol*. 2006;177(3):1655-1660.

449. Karlsen TH, Franke A, Melum E, et al. Genome-wide association analysis in primary sclerosing cholangitis. *Gastroenterology*. 2010;138(3):1102-1111.

450. Melum E, Franke A, Schramm C, et al. Genome-wide association analysis in primary sclerosing cholangitis identifies two non-HLA susceptibility loci. *Nat Genet*. 2011;43(1):17-19.

451. Hsu W, Zhang W, Tsuneyama K, et al. Differential mechanisms in the pathogenesis of autoimmune cholangitis versus inflammatory bowel disease in interleukin-2Ralpha(-/-) mice. *Hepatology*. 2009;49(1):133-140.

452. Janse M, Lamberts LE, Franke L, et al. Three ulcerative colitis susceptibility loci are associated with primary sclerosing cholangitis and indicate a role for IL2, REL, and CARD9. *Hepatology*. 2011;53(6): 1977-1985.

453. Vierling JM. Animal models for primary sclerosing cholangitis. *Best Pract Res Clin Gastroenterol*. 2001;15(4):591-610.

454. Cullen SN, Chapman RW. The medical management of primary sclerosing cholangitis. *Semin Liver Dis*. 2006;26(1):52-61.

455. Lo SK, Wendon J, Mieli-Vergani G, Williams R. Halothane-induced acute liver failure: continuing occurrence and use of liver transplantation. *Eur J Gastroenterol Hepatol*. 1998;10(8):635-639.

456. Gut J, Christen U, Frey N, Koch V, Stoffler D. Molecular mimicry in halothane hepatitis: biochemical and structural characterization of lipoylated autoantigens. *Toxicology*. 1995;97(1-3):199-224.

457. Kosuda LL, Bigazzi PE. Chemical-induced autoimmunity. In: Smialowicz RJ, Holsapple MP, eds. *Experimental Immunotoxicology*. Boca Raton, FL: CRC Press; 1996:419-465.

458. Kita H, Mackay IR, Van De Water J, Gershwin ME. The lymphoid liver: considerations on pathways to autoimmune injury. *Gastroenterology*. 2001;120(6):1485-1501.

459. Tipping PG, Kitching AR. Glomerulonephritis, Th1 and Th2: what's new? *Clin Exp Immunol*. 2005;142(2):207-215.

460. Robson MG, Walport MJ. Renal autoimmunity. In: Lahita RG, Chiorazzi N, EReeves WH, eds. *Textbook of the Autoimmune Diseases* . Philadelphia, PA: Lippincott Williams & Wilkins; 2000:323-349.

461. Wilson CB, Borza DB, Hudson BG. Autoimmune renal disease involving basement membrane antigens. In: Theofilopoulos AN, Bona CA, eds. *The Molecular Pathology of Autoimmune Diseases*. 2nd ed. New York, NY: Taylor & Francis; 2002:999-1028.

462. Racusen LC. Autoimmune diseases in the kidney. In: Rose NR, Mackay CR, eds. *The Autoimmune Diseases*. 3rd ed. San Diego, CA: Academic Press; 1998:603-621.

463. Schena FP. Survey of the Italian Registry of Renal Biopsies. Frequency of the renal

diseases for 7 consecutive years. The Italian Group of Renal Immunopathology. *Nephrol Dial Transplant*. 1997;12(3): 418-426.

464. Gesualdo L, Di Palma AM, Morrone LF, Strippoli GF, Schena FP. The Italian experience of the national registry of renal biopsies. *Kidney Int*. 2004;66(3):890-894.

465. Borza DB, Neilson EG, Hudson BG. Pathogenesis of Goodpasture syndrome: a molecular perspective. *Semin Nephrol*. 2003;23(6): 522-531.

466. Lin M-S, Liu Z, Drolet BA, Diaz LA. Cutaneous autoimmune diseases. In: Rose NR, Mackay IR, eds. *The Autoimmune Diseases*. 3rd ed. San Diego, CA: Academic Press; 1998:545-570.

467. Lin M-S, Arteaga LA, Warren SJP, Diaz LA. Pemphigus. In: Theofilopoulos AN, Bona CA, eds. *The Molecular Pathology of Autoimmune Diseases* . Ann Arbor, MI: Taylor & Francis; 2002:799-816.

468. Bystryn JC, Rudolph JL. Pemphigus. *Lancet*. 2005;366(9479):61-73.

469. Humbert P, Dupond JL, Vuitton D, Agache P. Dermatological autoimmune diseases and the multiple autoimmune syndromes. *Acta Derm Venereol Suppl (Stockh)*. 1989;148:1-8.

470. Korman N. Pemphigus. *J Am Acad Dermatol* . 1988;18(6):1219-1238.

471. Hertl M, Eming R, Veldman C. T cell control in autoimmune bullous skin disorders. *J Clin Invest*. 2006;116(5):1159-1166.

472. Takahashi H, Kouno M, Nagao K, et al. Desmoglein 3-specific CD4+ T cells induce pemphigus vulgaris and interface dermatitis in mice. *J Clin Invest*. 2011;121(9):3677-3688.

473. Veldman C, Hohne A, Dieckmann D, Schuler G, Hertl M. Type I regulatory T cells specific for desmoglein 3 are more frequently detected in healthy individuals than in patients with pemphigus vulgaris. *J Immunol*. 2004;172(10):6468-6475.

474. Yamagami J, Payne AS, Kacir S, Ishii K, Siegel DL, Stanley JR. Homologous regions of autoantibody heavy chain complementaritydetermining region 3 (H-CDR3) in patients with pemphigus cause pathogenicity. *J Clin Invest*. 2010;120(11):4111-4117.

475. Camisa C, Helm TN. Paraneoplastic pemphigus is a distinct neoplasiainduced autoimmune disease. *Arch Dermatol*. 1993;129(7):883-886.

476. Anhalt GJ, Labib RS, Voorhees JJ, Beals TF, Diaz LA. Induction of pemphigus in neonatal mice by passive transfer of IgG from patients with the disease. *N Engl J Med*.

1982;306(20):1189-1196.

477. Koch PJ, Mahoney MG, Ishikawa H, et al. Targeted disruption of the pemphigus vulgaris antigen (desmoglein 3) gene in mice causes loss of keratinocyte cell adhesion with a phenotype similar to pemphigus vulgaris. *J Cell Biol* . 1997;137(5):1091-1102.

478. Amagai M, Tsunoda K, Suzuki H, Nishifuji K, Koyasu S, Nishikawa T. Use of autoantigen-knockout mice in developing an active autoimmune disease model for pemphigus. *J Clin Invest*. 2000;105(5):625-631.

479. Tsunoda K, Ota T, Suzuki H, et al. Pathogenic autoantibody production requires loss of tolerance against desmoglein 3 in both T and B cells in experimental pemphigus vulgaris. *Eur J Immunol* . 2002;32(3):627-633.

480. Joly P, Mouquet H, Roujeau JC, et al. A single cycle of rituximab for the treatment of severe pemphigus. *N Engl J Med* . 2007;357(6):545-552.

481. Passeron T, Ortonne JP. Physiopathology and genetics of vitiligo. *J Autoimmunity*. 2005;25 Suppl:63-68.

482. Spritz RA. The genetics of generalized vitiligo and associated autoimmune diseases. *J Dermatol Sci* . 2006;41(1):3-10.

482a. Boissy RE, Nordlund JJ. Vitiligo: current medical and scientific understanding. *G Ital Dermatol Venereol*. 2011;146(1):69-75.

483. Alkhateeb A, Fain PR, Thody A, Bennett DC, Spritz RA. Epidemiology of vitiligo and associated autoimmune diseases in Caucasian probands and their families. *Pigment Cell Res*. 2003;16(3):208-214.

484. Eisenbarth GS. Banting Lecture 2009: an unfinished journey: molecular pathogenesis to prevention of type 1A diabetes. *Diabetes*. 2010; 59(4):759-774.

485. Le Poole IC, Wankowicz-Kalinska A, van den Wijngaard RM, Nickoloff BJ, Das PK. Autoimmune aspects of depigmentation in vitiligo. *J Investig Dermatol Symp Proc* . 2004;9(1):68-72.

486. Ogg GS, Rod Dunbar P, Romero P, Chen JL, Cerundolo V. High frequency of skin-homing melanocyte-specific cytotoxic T lymphocytes in autoimmune vitiligo. *J Exp Med*. 1998;188(6):1203-1208.

487. Lang KS, Caroli CC, Muhm A, et al. HLA-A2 restricted, melanocytespecific CD8(+) T lymphocytes detected in vitiligo patients are related to disease activity and are predominantly directed against MelanA/MART1. *J Invest Dermatol* . 2001;116(6):891-897.

488. Kroll TM, Bommiasamy H, Boissy RE, et al. 4-Tertiary butyl phenol exposure sensitizes human melanocytes to dendritic cell-mediated killing: relevance to vitiligo. *J Invest Dermatol*. 2005;124(4):798-806.

489. Hara I, Takechi Y, Houghton AN. Implicating a role for immune recognition of self in tumor rejection: passive immunization against the brown locus protein. *J Exp Med*. 1995;182(5):1609-1614.

490. Laberge G, Mailloux CM, Gowan K, et al. Early disease onset and increased risk of other autoimmune diseases in familial generalized vitiligo. *Pigment Cell Res*. 2005;18(4):300-305.

491. Spritz RA, Chiang PW, Oiso N, Alkhateeb A. Human and mouse disorders of pigmentation. *Curr Opin Genet Dev* . 2003;13(3):284-289.

492. Birlea SA, Jin Y, Bennett DC, et al. Comprehensive association analysis of candidate genes for generalized vitiligo supports XBP1, FOXP3, and TSLP. *J Invest Dermatol* . 2011;131(2):371-381.

493. Jin Y, Mailloux CM, Gowan K, et al. NALP1 in vitiligo-associated multiple autoimmune disease. *N Engl J Med* . 2007;356(12):1216-1225.

494. Spritz RA. Recent progress in the genetics of generalized vitiligo. *J Genetics Genomics*. 2011;38(7):271-278.

495. Birlea SA, Gowan K, Fain PR, Spritz RA. Genome-wide association study of generalized vitiligo in an isolated European founder population identifies SMOC2, in close proximity to IDDM8. *J Invest Dermatol* . 2010;130(3):798-803.

496. Jin Y, Birlea SA, Fain PR, et al. Variant of TYR and autoimmunity susceptibility loci in generalized vitiligo. *N Engl J Med* . 2010;362(18): 1686-1697.

497. Jin Y, Birlea SA, Fain PR, et al. Common variants in FOXP1 are associated with generalized vitiligo. *Nat Genet*. 2010;42(7):576-578.

498. Quan C, Ren YQ, Xiang LH, et al. Genome-wide association study for vitiligo identifies susceptibility loci at 6q27 and the MHC. *Nat Genet*. 2010;42(7):614-618.

499. Wang X, Erf GF. Apoptosis in feathers of Smyth line chickens with autoimmune vitiligo. *J Autoimmunity*. 2004;22(1):21-30.

500. Erf GF, Bersi TK, Wang X, Sreekumar GP, Smyth JR Jr. Herpesvirus connection in the expression of autoimmune vitiligo in Smyth line chickens. *Pigment Cell Res*. 2001;14(1):40-46.

500a. Rose NR. Autoimmunity in coxsackievirus infection. *Curr Top Microbiol Immunol* . 2008;323:293-314.

501. Wicks I, McColl G, Harrison L. New perspectives on rheumatoid arthritis. *Immunol Today*. 1994;15(12):553-556.

502. Suzuki A, Kochi Y, Okada Y, Yamamoto K. Insight from genome-wide association studies in rheumatoid arthritis and multiple sclerosis. *FEBS Lett*. 2011;585(23):3627-3632.

503. Rudolphi U, Hohlbaum A, Lang B, Peter HH, Melchers I. The B cell repertoire of patients with rheumatoid arthritis. Frequencies and specificities of peripheral blood B cells reacting with human IgG, human collagens, a mycobacterial heat shock protein and other antigens. *Clin Exp Immunol*. 1993;92(3):404-411.

504. Mastrandrea L, Yu J, Behrens T, et al. Etanercept treatment in children with new-onset type 1 diabetes: pilot randomized, placebo-controlled, double-blind study. *Diabetes Care*. 2009;32(7):1244-1249.

505. Denton CP, Black CM, Abraham DJ. Mechanisms and consequences of fibrosis in systemic sclerosis. *Nat Clin Pract Rheumatol* . 2006;2(3): 134-144.

506. LeRoy EC, Black C, Fleischmajer R, et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J Rheumatol* . 1988;15(2):202-205.

507. LeRoy EC, Medsger TA Jr. Criteria for the classification of early systemic sclerosis. *J Rheumatol*. 2001;28(7):1573-1576.

508. Mayes MD. Scleroderma epidemiology. *Rheum Dis Clin North Am*. 2003;29(2):239-254.

509. Senecal JL, Henault J, Raymond Y. The pathogenic role of autoantibodies to nuclear autoantigens in systemic sclerosis (scleroderma). *J Rheumatol* . 2005;32(9):1643-1649.

510. Sato S, Fujimoto M, Hasegawa M, Takehara K. Altered blood B lymphocyte homeostasis in systemic sclerosis: expanded naive B cells and diminished but activated memory B cells. *Arthritis Rheum*. 2004;50(6):1918-1927.

511. Sakkas LI, Xu B, Artlett CM, Lu S, Jimenez SA, Platsoucas CD. Oligoclonal T cell expansion in the skin of patients with systemic sclerosis. *J Immunol* . 2002;168(7):3649-3659.

512. Abraham DJ, Eckes B, Rajkumar V, Krieg T. New developments in fibroblast and myofibroblast biology: implications for fibrosis and scleroderma. *Curr Rheumatol Rep* . 2007;9(2):136-143.

513. Yamane K, Kashiwagi H, Suzuki N, et al. Elevated plasma levels of endothelin-1 in systemic sclerosis. *Arthritis Rheum*. 1991;34(2):243-244.

514. Del Papa N, Colombo G, Fracchiolla N, et al. Circulating endothelial cells as a marker of ongoing vascular disease in systemic sclerosis. *Arthritis Rheum*. 2004;50(4):1296-1304.

515. Kahaleh MB, Osborn I, LeRoy EC. Increased factor VIII/von Willebrand factor antigen and von Willebrand factor activity in scleroderma and in Raynaud's phenomenon. *Ann Int Med*. 1981;94(4 pt 1):482-484.

516. Kuwana M, Okazaki Y, Yasuoka H, Kawakami Y, Ikeda Y. Defective vasculogenesis in systemic sclerosis. *Lancet*. 2004;364(9434):603-610.

517. Feghali-Bostwick C, Medsger TA Jr, Wright TM. Analysis of systemic sclerosis in twins reveals low concordance for disease and high concordance for the presence of antinuclear antibodies. *Arthritis Rheum*. 2003;48(7):1956-1963.

518. Zhou X, Lee JE, Arnett FC, et al. HLA-DPB1 and DPB2 are genetic loci for systemic sclerosis: a genome-wide association study in Koreans with replication in North Americans. *Arthritis Rheum*. 2009;60(12):3807-3814.

519. Radstake TR, Gorlova O, Rueda B, et al. Genome-wide association study of systemic sclerosis identifies CD247 as a new susceptibility locus. *Nat Genet*. 2010;42(5):426-429.

520. Grossman C, Dovrish Z, Shoenfeld Y, Amital H. Do infections facilitate the emergence of systemic sclerosis? *Autoimmun Rev*. 2011;10(5): 244-247.

521. Caramaschi P, Dalla Gassa A, Ruzzenente O, et al. Very low levels of vitamin D in systemic sclerosis patients. *Clin Rheumatol*. 2010;29(12):1419-1425.

522. Mora GF. Systemic sclerosis: environmental factors. *J Rheumatol*. 2009;36(11):2383-2396.

523. Beyer C, Schett G, Distler O, Distler JH. Animal models of systemic sclerosis: prospects and limitations. *Arthritis Rheum*. 2010;62(10):2831-2844.

524. Yamamoto T, Takagawa S, Katayama I, et al. Animal model of sclerotic skin. I: local injections of bleomycin induce sclerotic skin mimicking scleroderma. *J Invest Dermatol*. 1999;112(4):456-462.

525. Green MC, Sweet HO, Bunker LE. Tight-skin, a new mutation of the mouse causing excessive growth of connective tissue and skeleton. *Am J Pathol*. 1976;82(3):493-512.

526. Siracusa LD, McGrath R, Ma Q, et al. A tandem duplication within the fibrillin 1 gene is associated with the mouse tight skin mutation. *Genome Res*. 1996;6(4):300-313.

527. Eferl R, Hasselblatt P, Rath M, et al. Development of pulmonary fibrosis through a pathway involving the transcription factor Fra-2/AP-1. *Proc Natl Acad Sci U S A* . 2008;105(30):10525-10530.

528. van den Hoogen FH, Boerbooms AM, Swaak AJ, Rasker JJ, van Lier HJ, van de Putte LB. Comparison of methotrexate with placebo in the treatment of systemic sclerosis: a 24 week randomized doubleblind trial, followed by a 24 week observational trial. *Br J Rheumatol* . 1996;35(4):364-372.

529. Tashkin DP, Elashoff R, Clements PJ, et al. Cyclophosphamide versus placebo in scleroderma lung disease. *N Engl J Med* . 2006;354(25): 2655-2666.

530. Thompson AE, Shea B, Welch V, Fenlon D, Pope JE. Calcium-channel blockers for Raynaud's phenomenon in systemic sclerosis. *Arthritis Rheum*. 2001;44(8):1841-1847.

531. Klippel JH, Dieppe P. *Rheumatology*. 2nd ed. London, England: Mosby; 1998.

531a. Centers for Disease Control and Prevention. http://www.cdc.gov/arthritis/basics/lupus.htm/. Accessed July 31, 2012.

532. Sestak AL, Furnrohr BG, Harley JB, Merrill JT, Namjou B. The genetics of systemic lupus erythematosus and implications for targeted therapy. *Ann Rheum Dis*. 2011;70(Suppl 1):i37-i43.

533. Tsokos GC. Systemic lupus erythematosus. *N Engl J Med* . 2011;365(22): 2110-2121.

534. Kang I, Quan T, Nolasco H, et al. Defective control of latent Epstein-Barr virus infection in systemic lupus erythematosus. *J Immunol* . 2004;172(2):1287-1294.

535. Poole BD, Scofield RH, Harley JB, James JA. Epstein-Barr virus and molecular mimicry in systemic lupus erythematosus. *Autoimmunity*. 2006;39(1):63-70.

536. Arbuckle MR, McClain MT, Rubertone MV, et al. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *N Engl J Med* . 2003;349(16):1526-1533.

537. Carroll MC. A protective role for innate immunity in systemic lupus erythematosus. *Nat Rev Immunol* . 2004;4(10):825-831.

538. Perry D, Sang A, Yin Y, Zheng YY, Morel L. Murine models of systemic lupus

erythematosus. *J Biomed Biotechnol* . 2011;2011:271694.

539. Bruijn JA, van Elven EH, Hogendoorn PC, Corver WE, Hoedemaeker PJ, Fleuren GJ. Murine chronic graft-versus-host disease as a model for lupus nephritis. *Am J Pathol* . 1988;130(3):639-641.

540. Baker KP, Edwards BM, Main SH, et al. Generation and characterization of LymphoStat-B, a human monoclonal antibody that antagonizes the bioactivities of B lymphocyte stimulator. *Arthritis Rheum*. 2003;48(11):3253-3265.

541. Cheema GS, Roschke V, Hilbert DM, Stohl W. Elevated serum B lymphocyte stimulator levels in patients with systemic immunebased rheumatic diseases. *Arthritis Rheum*. 2001;44(6):1313-1319.

542. Petri M, Stohl W, Chatham W, et al. Association of plasma B lymphocyte stimulator levels and disease activity in systemic lupus erythematosus. *Arthritis Rheum*. 2008;58(8):2453-2459.

543. Wallace DJ, Stohl W, Furie RA, et al. A phase II, randomized, double-blind, placebo-controlled, dose-ranging study of belimumab in patients with active systemic lupus erythematosus. *Arthritis Rheum*. 2009;61(9):1168-1178.

544. Navarra SV, Guzman RM, Gallacher AE, et al. Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial. *Lancet*. 2011;377(9767): 721-731.

545. Furie R, Petri M, Zamani O, et al. A phase III, randomized, placebocontrolled study of belimumab, a monoclonal antibody that inhibits B lymphocyte stimulator, in patients with systemic lupus erythematosus. *Arthritis Rheum*. 2011;63(12):3918-3930.

546. Dorner T, Kaufmann J, Wegener WA, Teoh N, Goldenberg DM, Burmester GR. Initial clinical trial of epratuzumab (humanized anti-CD22 antibody) for immunotherapy of systemic lupus erythematosus. *Arth Res Therapy*. 2006;8(3):R74.

547. Merrill JT, Neuwelt CM, Wallace DJ, et al. Efficacy and safety of rituximab in moderately-to-severely active systemic lupus erythematosus: the randomized, double-blind, phase II/III systemic lupus erythematosus evaluation of rituximab trial. *Arthritis Rheum*. 2010;62(1):222-233.

548. Furie R, Looney RJ, Rovin B, et al. Efficacy and safety of rituximab in subjects with active proliferative lupus nephritis (LN): results from the randomized, double-blind phase III LUNAR Study [abstract]. *Arthritis Rheum*. 2009;60:S429.

548a. Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol* . 1994;12:991-1045.

549. Chervonsky AV. Influence of microbial environment on autoimmunity. *Nat Immunol*. 2010;11(1):28-35.

550. Gill SR, Pop M, Deboy RT, et al. Metagenomic analysis of the human distal gut microbiome. *Science*. 2006;312(5778):1355-1359.

551. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. *Nature*. 2007;449(7164): 804-810.

552. Ivanov, II, Frutos Rde L, Manel N, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe*. 2008;4(4):337-349.

553. Winer S, Astsaturov I, Cheung RK, et al. Type I diabetes and multiple sclerosis patients target islet plus central nervous system autoantigens; nonimmunized nonobese diabetic mice can develop autoimmune encephalitis. *J Immunol*. 2001;166(4):2831-2841.

554. Salomon B, Rhee L, Bour-Jordan H, et al. Development of spontaneous autoimmune peripheral polyneuropathy in B7-2-deficient NOD mice. *J Exp Med*. 2001;194(5):677-684.

555. Takaki T, Marron MP, Mathews CE, et al. HLA-A*0201-restricted T cells from humanized NOD mice recognize autoantigens of potential clinical relevance to type 1 diabetes. *J Immunol*. 2006;176(5):3257-3265.

556. Niens M, Grier AE, Marron M, Kay TW, Greiner DL, Serreze DV. Prevention of “humanized” diabetogenic CD8 T-cell responses in HLA-transgenic NOD mice by a multipeptide coupled-cell approach. *Diabetes*. 2011;60(4):1229-1236.

557. Herold KC, Hagopian W, Auger JA, et al. Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. *N Engl J Med*. 2002;346(22):1692-1698.

558. Keymeulen B, Vandemeulebroucke E, Ziegler AG, et al. Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. *N Engl J Med*. 2005;352(25):2598-2608.

559. Coppieters K, Amirian N, von Herrath M. Intravital imaging of CTLs killing islet cells in diabetic mice. *J Clin Invest*. 2012;122(1):119-131.

560. Romano E, Manetti M, Guiducci S, Ceccarelli C, Allanore Y, Matucci-Cerinic M. The genetics of systemic sclerosis: an update. *Clin Exp Rheumatol*. 2011;29(2)(suppl 65):S75-S86.

561. Miller A, Lider O, Weiner HL. Antigen-driven bystander suppression after oral administration of antigens. *J Exp Med*. 1991;174(4):791-798.

562. Waldmann H. Tolerance can be infectious. *Nat Immunol*. 2008;9(9): 1001-1003.

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Chapter 45

Immunologic Mechanisms of Allergic Disorders

Marsha Wills-Karp

Ian Lewkowich

INTRODUCTION

This chapter will provide an overview of our current understanding of diseases gathered under the rubric of "allergy." These diverse diseases (anaphylaxis, asthma, allergic rhinitis, atopic dermatitis, food allergy) are united at least superficially by the facts that a) these conditions all result from the expression of harmful immune responses; b) the implicated immune responses are all associated with the generation of immunoglobulin (Ig)E (whether or not IgE is integral to pathogenesis); and c) the antigens driving such immune responses are not derived from infectious pathogens. The basic cellular and molecular mechanisms that underlie the pathogenesis of allergic disorders, as well as the environmental and genetic substrates for their generation, will be closely considered. Although the study of allergic diseases has focused on the adaptive immune response in the last decade, in the period since the last edition of this book, the focus has shifted to dissecting the role of innate immune mechanisms in regulating susceptibility to the development of aberrant immune responses in allergic diseases. Thus, this edition will reflect this change in perspective when possible. Although much of our current understanding of mechanism in allergic disease has derived from the study of animal models, mechanistic data on human disease will be discussed wherever possible. The chapter finishes with a brief survey of the clinical and therapeutic characteristics of the major human allergic disorders. Readers are referred to clinically oriented texts for a fuller discussion of such issues.

HISTORICAL PERSPECTIVE

The term allergy was coined in 1906 by the astute pediatrician Clemens von Pirquet, who argued that antigenic stimuli led to two distinct categories or patterns of response: immunity and allergy.[1] The former, an old concept, referred to those responses leading to protection from infectious challenge. The latter, a novel theoretical construct, referred to "altered reactivity" that itself led to host damage. This idea of allergy, that is, the notion that the immune response can itself be a cause of disease, was a powerful conceptual advance that led to novel insights into the pathogenesis of a variety of diseases. Quite naturally, this concept of allergy initially included autoimmune diseases in addition to those conditions that find classification as allergic diseases today. As noted previously, current usage largely restricts allergy to diseases caused by the subset of harmful immune responses (to pathogen-unrelated antigens) that is associated with the generation of IgE. There is some artificiality to this. Very similar patterns of immune response can drive pathology in response

to infectious pathogens such as tissue helminths. Further, IgE may be more a marker of an underlying pattern of immune response than a mechanistic participant in the immunopathogenesis of at least some subtypes of allergic disease. As long as these caveats are kept in mind, however, this concept of allergic disease long enshrined by clinical subspecialists has considerable theoretical and practical utility.

The trail leading to the specific identification of IgE began with demonstration by Prausnitz and Kuster in 1921 that hypersensitivity to an antigen could be passively transferred in serum from one individual to another.[2] The instigating antigens (allergens in contemporary parlance) were known as atopens, and the mysterious plasma factor that conferred sensitivity was called atopic reagin. It was not until 1966 that Teruko and Kimishige Ishizaka demonstrated that reaginic activity was carried by a novel class of Ig, IgE.[3,4,5] The word atopy has since come to denote the propensity for developing allergic reactions to common environmental antigens (allergens), a propensity defined operationally by elevations in serum levels of IgE reactive with, or by skin test reactivity to, such antigens. Definitions of other key terms are given in Table 45.1.

General Features of Atopic Disorders

Allergic disorders are categorized by the anatomic site where disease is manifested: atopic dermatitis (skin), atopic rhinitis (nasal passages), atopic asthma (lung), food allergy (gut), and anaphylaxis (systemic) (Table 45.2). These clinical entities all involve a similar allergic effector cascade, at least superficially, with differences in presentation likely reflecting variation in the physiochemical characteristics of the allergen, the site of initial sensitization to the allergen, the route and dose of allergen exposure, and the programmed response of resident cells (eg, epithelial cells) to injury and inflammation. Anaphylaxis aside, there is often a stereotypical sequence in the development of allergic manifestations of disease in patients with atopy, with early expression of food sensitivities or atopic dermatitis and the subsequent development of either atopic rhinitis or asthma. Many individuals will have all three of the latter clinical entities, which form the “atopic triad.”

Atopic disorders represent a major health problem worldwide, affecting 5% to 30% of the population. The incidence of atopic diseases, including asthma, atopic rhinitis, and atopic dermatitis, has increased dramatically in recent

years in westernized countries.[6,7,8] In such countries, 30% of the population manifest some form of atopic disease at some time in their lives. Interestingly, the low baseline level of atopic diseases in developing countries has not changed over the same time period, suggesting that factors associated with the westernized lifestyle predispose to atopic disease. The widespread prevalence and morbidity of atopic diseases imposes a heavy burden on society.

TABLE 45.1 Definitions of Key Terms

Term	Definition
Allergen	An environmental antigen that typically elicits allergic responses in

	susceptible individuals. These antigens ordinarily have little or no intrinsic toxicity.
Allergy	Clinically adverse reactions to environmental antigens reflecting acquired immune responses that are marked phenotypically, by the presence of allergen-specific IgE, along with mast cell and eosinophil recruitment and/or activation. CD4+ T cells that produce a Th2 profile of cytokines (IL-4, IL-5, and IL-13) are thought to be central to the development of allergic responses.
Atopy	The propensity for developing immediate hypersensitivity reactions to common environmental allergens, defined operationally by elevations in serum levels of IgE reactive with allergens or by skin-test reactivity to allergens.
Allergic diseases	The group of clinical disorders (such as allergic asthma, allergic rhinitis [hay fever], and atopic dermatitis) in which IgE-associated immune responses, typically directed against otherwise innocuous environmental allergens, are thought to have a pathogenic role.

CD, cluster of differentiation; Ig, immunoglobullin; IL, interleukin.

The defining feature of atopy is the production of IgE in response to exposure (via muocosa or the skin) to a variety of ubiquitous, and otherwise innocuous, antigens. Such IgE production is a tightly regulated process, part of a complex network of cellular and molecular events necessary for the development of the allergic response. Initiation of this response appears to occur with presentation of the allergen by antigen-presenting cells (APCs) residing in the mucosa to cluster of differentiation (CD)4+ T cells in the draining lymph nodes (a process referred to as sensitization). In atopic individuals, responding allergen-specific T cells polarize to a Th2 pattern of production, with the elaboration of cytokines such as interleukin (IL)-4, IL-13, IL-5, and IL-9 (vide infra). Although T cells from nonatopic individuals clearly recognize these same environmental antigens, the expansion and differentiation of such T cells does not involve Th2-deviation. Instead, it is thought that nonatopic individuals develop “tolerance” to these innocuous environmental antigens. The mechanisms controlling allergen-associated Th2 polarization in atopic individuals or tolerance in nonaffected individuals are not completely understood. It appears likely that genetic and environmental factors impacting on the antigen-presenting process play a key role.

TABLE 45.2 Major Features of Allergic Immune Responses

1. Responses are elicited by certain groups of environmental allergens such as foods, drugs, and proteins derived from pollens, insects (house dust mite), and animal dander.

2. In susceptible individuals, allergens are sensed by a variety of pattern recognition receptors in the mucosal epithelium resulting in the elaboration of cytokines (TSLP, IL-15, IL-33) and chemokines that recruit, activate, and instruct antigenpresenting cells residing in the mucosa of the skin, gastrointestinal tract, or respiratory tract to drive naïve T cells to undergo differentiation to a Th2 cytokine producing pattern.

3. Elaboration of Th2 cytokines (IL-4, IL-5, IL-3, IL-9) initiates the allergic cascade via their combined ability to regulate IgE production, FcεRI expression, mast cell phenotype, and development, recruitment, and activation of eosinophils.

4. Under the control of Th2 cell-derived signals (IL-4, IL-13, and CD40L), B cells undergo class switching to production of the IgE subclass.

5. Upon reexposure to the offending allergen, acute responses occurring within minutes of allergen exposure result from release of preformed mediators (histamine, tryptase) from FcεRI-bearing cells via the cross-linking of allergen and IgE on their surface. Cells activated during the acute phase also release cytokines and mediators that perpetuate the Th2-driven response.

6. Late-phase responses are due to the combined effects of inflammatory cells (eosinophils and T cells) recruited to the tissues within 6 to 24 hours after the initial allergen exposure.

7. Repeated allergen exposures in the context of an already inflamed tissue results in structural changes (remodeling) such as smooth muscle thickening, tissue fibrosis, and mucus cell hyperplasia.

CD, cluster of differentiation; Ig, immunoglobulin; IL, interleukin; TSLP, thymic stromal lymphopoietin.

The elaboration of Th2 cytokines sets into motion a complex series of events leading to IgE production; the development, recruitment, and activation of effector cells such as mast cells, basophils, eosinophils, and effector T cells; and a variety of downstream effector cascades. Once an atopic individual is sensitized, the manifestations of allergy are readily induced upon reexposure to the allergen (the elicitation phase). Although the effector phases of IgE-associated atopic disorders generally appear as a continuum, it is useful to define three temporal patterns: 1) acute reactions (developing within seconds to minutes of allergen exposure), 2) delayed or late reactions (developing hours after allergen exposure), and 3) chronic reactions (developing over days to years). Acute reactions result from cross-linking of high-affinity FcεR (FcεRI) on the surface of mast cells/basophils, induced by the interaction of allergen with cell-bound IgE. Such cross-linking results in the release of vasoactive mediators, chemotactic factors, and cytokines that initiate the so-called allergic cascade. This early reaction may resolve within minutes but is often followed by late-phase responses that begin 3 to 6 hours after antigen challenge and

may persist for days in the absence of therapy. The pathophysiologic consequences of chronic reactions are associated with the migration of eosinophils and lymphocytes from the blood into affected tissues.

ALLERGENS

Definition and General Characteristics of Allergens

Allergens are, by definition, antigens that can elicit specific IgE responses in genetically susceptible individuals. The list of structures that have been identified as allergens represents a tiny subset of the antigenic universe to which humans are routinely exposed. Allergens are generally subdivided by route of exposure and source. Such allergens include aeroallergens (pollens, mold spores, animal dander, fecal material excreted by mites and cockroaches), food allergens, stinging insects, pharmaceuticals, and latex.

Allergen Classification

Purified allergens are named in accordance with guidelines published in 1994 by the World Health Organization International Union of Immunologic Societies Allergen Nomenclature Sub-Committee, based on their source and the order in which they were discovered.[9] The names incorporate the first three letters of the genus and the first letter of the species from which the allergen is derived, plus an Arabic numeral that is used to denote structurally homologous allergens from the same species. For example, the two major species of dust mite (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) are designated as Der p (Der p 1, Der p 2) and Der f (Der f 1, Der f 2). Other major allergens include Fel d 1 from the cat, Bet v 1, from birch pollen, Amb a 1 from ragweed pollen, Phl p 1 from the pollen of timothy grass, and Bla g 2 from cockroach.

Specific Allergens

Aeroallergens

Aeroallergens are airborne proteins or glycoproteins derived from a variety of different sources, including pollinating trees and grasses, mold spores, animal dander (cat, dog, and rodent), and particulates secreted by dust mites and cockroaches. Factors that affect the growth or accumulation of these latter organisms (high humidity, well-insulated homes, fitted carpets) increase the levels of these allergens in the indoor environment. Exposure to such indoor allergens is also dependent on a variety of geographical, climatic, and socioeconomic factors. Interestingly, whereas indoor allergens are more closely associated with development of asthma, outdoor allergens (eg, ragweed pollen) appear to be more important to the development of allergic rhinitis. The mechanisms underlying such associations remain obscure. Speculation has focussed on the physiochemical nature (size, chemical structure) and pattern of exposure (acute versus chronic).

Food Allergens

Although hundreds of different foods are ingested, only a small number account for the vast majority of food allergy. The most common foods responsible for childhood food allergy are milk, egg, peanut, soy, and wheat. Responses to food allergens are relatively common in children under the age of 2 but usually disappear as the child ages. In contrast, in adult food

allergy, the most common offending foods are peanuts, tree nuts, fish, and shellfish. Most food allergens have been found to be water-soluble glycoproteins ranging in size from 10 to 40 kD that are heat and acid stable and resistant to proteolytic degradation. Exceptions to this are fruit and vegetable allergens. Reactions to food allergens can be fatal, with one of the most severe food reactions occurring in response to peanut allergens.

Latex Allergens

A new class of antigens associated with immediate hypersensitivity reactions to latex rubber has been identified in the last few years. Latex allergy is frequently seen in health care workers, rubber industry workers, and subjects undergoing multiple surgical procedures in early infancy.[10,11] Symptoms manifest as contact urticaria, rhinoconjunctivitis, asthma, and mucosal swelling. However, severe reactions and death have occurred upon exposure of patients to latex balloons on the rectal mucosa, especially in children with spina bifida. Multiple individual latex allergens have been identified, eight of which have received an international nomenclature designation. These include Hev b1, rubber elongation factor; Hev b2, B-1,3-glucanase; Hev b3, homologous to Hev b1; Hev b4, a microhelix component; Hev b6, prohevein/hevein; and Hev b7, a patatin-like protein.[12] It has recently been appreciated that individuals with allergies to certain fruits such as banana, avocado, kiwi, and chestnut develop clinical symptoms upon initial contact with latex.[13] This phenomenon has been coined “the latex-fruit syndrome.” It is thought to occur as a result of the presence of IgE reactive to enzymes such as β-glucanase and chitinases that are present in both fruits and rubber. Interestingly, mammalian chitinases have been identified as inducible by Th2 cytokines such as IL-13.

Pharmaceuticals

Adverse drug reactions are relatively common clinical problems. Most conventional pharmaceutical agents are relatively low-molecular-weight compounds that become allergens only after their haptenization to endogenous proteins. The penicillins are classic instigators of allergic reactions. Penicillin is associated with a relatively high incidence of allergic reactions because of the chemical reactivity of penicillin and its metabolites. Although penicillin itself is the major allergen, its metabolic products, penicilloate and penilloate, are minor allergens but are responsible for a disproportionate share of severe, life-threatening reactions. Moreover, the drug is often administered parenterally, which greatly increases the probability that an adverse IgE-associated response will be fatal. Other agents, such as quaternary ammonium compounds (neuromuscular blocking agents) and sulfonamides (antibiotics) are relatively common stimuli of allergic reactions.

Insect Venom Allergens

Stinging insect hypersensitivity develops in both nonatopic and atopic individuals. Individuals are sensitized when

relatively high levels of proteins (approximately 50 µg) in venom are injected subcutaneously during a sting. The venom-associated allergens of several vespids (yellow jacket, wasp, fire-ants, and white faced hornet) are cross-reactive and include antigen 5, phospholipase, and hyaluronidase. The honeybee venom contains distinct allergens, including two major ones,

phospholipase A_2 and hyaluronidase, and a less important one, melittin. Many of these allergens have proteolytic activity.

Biological Properties of Allergens

The major allergens are a diverse group of proteins in which no one biologic property appears to be dominant. Allergens constitute a diverse range of molecules that derive from a variety of environmental sources, such as plants (trees, grasses), fungi (*Alternaria alternata*), arthropods (mites, cockroaches), and other mammals (cats, dogs, cows). As they are derived from complex living organisms, they serve a broad range of functions in their respective hosts, from structural to enzymatic. For example, the common house dust mite allergens include several cysteine proteases (Der p 1, Der p 3), serine proteases (Der p 3, Der p 6, Der p 9), chitinases (Der p 15, Der p 18), lipid-binding molecules (Der p 2), and structural molecules such as tropomyosin (Der p 10). Some are species specific; others are molecules with broad biochemical homology that are found in many species. The fact that there does not appear to be a common strucutural motif or conformational sequence associated with allergenic potential leaves open the possibility that proteins with allergenic potential exhibit a necessary commonality of biologic function. Indeed, it has been recently proposed that allergens are linked by their ability to activate the innate immune system at mucosal surfaces, triggering the activation of structural cells and initiating the influx of innate immune cells that subsequently promote Th2-polarized adaptive immune responses.

Recent studies suggest that allergens may activate the innate immune response either through their intrinsic enzymatic activity or through activation of pattern recognition receptors (PRRs) on mucosal epithelial cells or APCs directly. Allergens from diverse sources have enzymatic activity that may bias the immune response toward a Th2 phenotype. Several allergens have cysteine or serine protease activity, including diverse allergens from arthopods (eg, house dust mites[14,15]), German cockroaches,[16] fungi,[17] mammals (eg, *Felis domesticus*[18]), and plants (eg, pollens from ragweed[19]). In addition, many forms of occupational allergy are associated with encounters with proteolytic enzymes such as those used in the manufacture of detergents (alkaline detergents)[20] or in the food industry (papain).[21] Moreover, the potent peanut allergen, Ara h 2, is homologous to and functions as a trypsin inhibitor.[22] Other allergens, such as ragweed pollen, have been shown to contain intrinsic nicotinamide adenine dinucleotide phosphateoxidases. Pollen-derived nicotinamide adenine dinucleotide phosphate-oxidases have been shown to rapidly increase the levels of reactive oxygen species (ROS) in lung epithelium as well as the amount of oxidized glutathione and 4-hydroxynonenal in airway-lining fluid.[23] These oxidases, as well as products of oxidative stress (such as oxidized glutathione and 4-hydroxynonenal) generated by these enzymes, are thought to play a pivotal role in the development of lung inflammation.[24]

Alternatively, it has recently been recognized with the discovery of PRRs that allergens and their soluble components may contain pathogen-associated molecular patterns (PAMPs) that actively interact with innate recognition systems present in the mucosal layer of various tissues. Specifically, it has long been appreciated that allergens contain substances such as endotoxin, chitin, or β-glycans, which are recognized by PRRs (toll-like receptor [TLR]4, TLR2, dectin-1, mannose receptor) on cells lining mucosal surfaces such as the epithelium or

on underlying dendritic cells (DCs). Activation of these PRRs both directly and indirectly provides signals required for productive DC:T cell interactions at mucosal surfaces. The ability to provide these danger signals to DCs likely underlies the unique ability of allergens to initiate allergic responses. The best example of this comes from the recent observation that the house dust mite allergen Der p 2 has been shown to be a molecular and functional mimic of the TLR4 receptor adapter molecule MD-2[25] and to drive allergic inflammation through activation of the TLR4 complex directly in the absence of MD-2. Several other members of the MD-2-like lipid-binding family are major allergens, suggesting generality for these findings.

Many allergens also contain carbohydrate moieties that can stimulate the C-type lectin receptors that recognize complex carbohydrates. For example, the peanut allergen Ara h 1[26] and the dust mite allergen Der p 1[27] have both been shown to bind to C-type lectin receptors on DCs, thereby enhancing their uptake and activation of a Th2 immune response. Evidence for the importance of these biologic properties to allergenicity will be discussed further in the following. Nonetheless, although many of these allergens can potentially create a microenvironment conducive to Th2-cell differentiation and expansion, normal individuals do not mount Th2 responses when exposed to these allergens, which suggests that despite the nature of these antigens, other factors are necessary for the development of allergic outcomes in susceptible individuals.

TYPE 2 POLARIZED IMMUNE RESPONSES IN ATOPY

As the primary orchestrator of specific immune responses to foreign antigens, the T-lymphocyte has been implicated in the pathogenesis of allergic diseases. Several lines of evidence support a causal role for T-lymphocytes in allergic disorders. Increased numbers of T-lymphocytes are found in the bronchial mucosa, nasal mucosa, and skin of patients with allergic asthma, rhinitis, and dermatitis, respectively, when compared with nonatopic controls.[28,29,30] In asthma and allergic rhinitis, CD4+ T cells predominate. In atopic dermatitis, however, excess CD4+ and CD8+ T populations are both present in skin lesions.[30] Further, there is a generalized increase in T-cell activation in allergic individuals both at the site of disease and systemically. Experimental data support

a generalized increase in T-cell activation in all disorders of the atopic triad, with increased expression of the IL-2 receptor, class II histocompatibility antigens (human leukocyte antigen [HLA]-DR), and very late activation antigen-1.[28,30] These activated T cells have the capacity to rapidly expand in response to specific stimuli, and through the release of a variety of cytokines they recruit and activate other immune cells (B cells, CD8 T cells, macrophages, mast cells, neutrophils, eosinophils, basophils) thereby initiating a complex series of events resulting in the symptoms of allergic diseases. Following initial activation, CD4+ T cells remain in lymphoid tissues as memory cells. These memory cells retain the ability to respond to specific antigens upon reexposure throughout the lifetime of the individual.

As has been covered in detail in other sections of this text, functional subsets of CD4+ T cells have been distinguished at both clonal and population levels by the unique profiles of cytokines that they produce.[31,32] The differential presence of these cytokine phenotypes in a variety of allergic and infectious diseases both in mice and in humans has provided

descriptive power and theoretical insight into disease pathogenesis.[33,34] Th1 cells producing tumor necrosis factor (TNF)-β, and interferon (IFN)γ, are critical in the development of cell-mediated immunity, macrophage activation, and the production of complement fixing antibody isotypes. Th2 cells producing IL-4, IL-13, IL-5, IL-9, and IL-6 are important in the stimulation of IgE production, mucosal mastocytosis, eosinophilia, and macrophage deactivation. Another subpopulation of T cells, referred to as regulatory T (T_{reg}) cells,[35] have immunosuppressive functions and cytokine profiles distinct from either Th1 or Th2 cells. These cells are thought to play an important role in limiting immune responses to self- or exogenous antigens by preventing the activation and function of nonregulatory effector T cells through through cell-to-cell interactions or through elaboration of IL-10 and/or transforming growth factor (TGF)-β.

Until recently, the development of allergies has been thought to be due solely to an imbalance between allergenspecific Th1 and Th2 cells with a skew toward Th2 immune responses. Several lines of evidence suggest that allergic diseases may arise as a result of an imbalance between allergen-specific T_{reg}s and Th2 cells, resulting in a loss of tolerance mediated via T_{reg}s. Whether this imbalance occurs due to overzealous Th2 immune responses, to impaired T_{reg} responses, or a combination of both is an open question. Recent evidence also suggests a role for the Th17 subset, which uniquely produces IL-17A and IL-17F.[36] This subset has been generally shown to be important in neutrophil development, activation, and recruitment, and the induction of inflammatory mediators from a variety of tissue resident cells such as epithelial cells. In the context of allergic disorders, Th17 cells are likely to enhance Th2-dependent immune responses. Evidence for each will be discussed subsequently.

Type 2 Polarized Immune Responses in Atopy Disorders

Several lines of evidence support the involvement of Th2 cytokines in the pathogenesis of allergic disorders. Firstly, T cells at the site of disease (bronchoalveolar lavage, bronchial biopsies, nasal biopsies) in allergic individuals (allergic asthma, atopic rhinitis) express elevated levels of messenger ribonucleic acid (mRNA) for IL-4, IL-13, granulocyte macrophage-colony stimulating factor (GM-CSF), and IL-5.[37,38,39,40,41] In atopic dermatitis patients, elevated Th2 cytokines (IL-4, IL-13, IL-5) and their receptors (IL-4R, IL-5R) are found in skin lesions in acute disease,[38] while cytokine patterns in chronic lesions are mixed, with both Th2 cytokines (IL-5 and IL-13) and Th1 cytokines (IFNγ) being expressed.[39] Secondly, it has been shown that successful therapeutic treatment of these disorders is associated with a reduction in the Th2 cytokine pattern. For example, both steroid treatment and immunotherapeutic regimes result in reductions in Th2 cytokine levels in the nasal mucosa[40] of patients with allergic rhinitis and in bronchoalveolar lavage (BAL) of asthmatic patients.[41]

Although considerable descriptive evidence suggests that CD4+ T-lymphocytes and Th2 cytokines are important in the pathogenesis of atopic disorders in humans, definitive proof is, of course, difficult to obtain. As a result, experimental animal models have been extremely useful in mechanistic delineation of the role of CD4+ T cells and T cell-derived cytokines in the pathogenesis of allergic disorders. Murine models of antigen-driven asthma have

consistently revealed a causal role for CD4+ T cells in the development of the signs of allergic airway disease.[42,43] In these models, sensitization with various allergens (ovalbumin, house dust mite, ragweed, aspergillius) by either intraperitoneal injections or airway installation, followed by direct airway challenge, induces a phenotype closely resembling that observed in human asthmatics. Specifically, allergen sensitization and challenge results in airway hyperresponsiveness (AHR), eosinophilic inflammation, elevations in allergen-specific IgE levels, and mucus hypersecretion. Regardless of mouse strains or exposure protocols, an absolute requirement for CD4+ T cells for the development of allergic responses is clear in such models. A lack of CD4+ T cells, achieved either by antibody depletion[42] or gene targeting,[43] is associated with prevention of the development of allergen-induced airway responses. Furthermore, adoptive transfer of Th2 clones into the mouse lung is sufficient for the development of allergic airway symptoms.[44] On the other hand, transfer of Th1 clones[44] or administration of agents such as IL-12 and IFNγ that inhibit Th2 cytokine production and stimulate Th1 pathways prevent the development of allergen-induced AHR and eosinophilic inflammation in murine models.[45,46,47] Conversely, mice deficient in T-bet, a transcription factor important in IFNγ secretion, spontaneously develop Th2-mediated allergic airway responses.[48] Several studies have shown evidence of association between polymorphisms in the T-bet gene and asthma phenotypes in humans.[49] The involvement of each of the specific Th2 cytokines in atopic airway responses has been demonstrated in studies in which IL-4, IL-5, IL-13, and IL-9 have been manipulated through either antibody blockade[50,51] or gene targeting.[52,53,54] Similar roles for CD4+ T cells and Th2 cytokines have been demonstrated in experimental mouse models of each of the atopic disorders including atopic rhinitis,[55,56] food allergy,[57]

and atopic dermatitis.[58] Collectively, the Th2 cytokines orchestrate the elicitation of the allergic response via their ability to regulate IgE production and recruitment and activation of various effector cells (eg, mast cells, eosinophils). In particular, IL-4, through its critical role in Th2 differentiation, has been shown to be essential in the initiation of allergic airway responses.[59,60] IL-5 clearly plays a role in eosinophil development, recruitment, and activation at the site of Th2-inflammatory responses,[61] whereas IL-9 appears to be an important regulator of mast cell activation.[62] IL-13 has been shown to have a singular role in the effector phase of the allergic response.[63,64] Specifically, it is sufficient to induce many of the manifestations of allergic disease including airway inflammation, AHR, and mucus cell hypersecretion in allergic airway diseases.

ROLE OF NATURAL KILLER T CELLS IN ALLERGIC DISEASE

Although allergic diseases have clearly been associated with polarized Th2 cytokine production in tissues, the source of these Th2 cytokines has been recently debated. Specifically, studies have implicated invariant T-cell receptor (TCR)+ CD1d-restricted CD4+ natural killer (NK) T cells as one of the major sources of Th2 cytokines.[65,66,67] Akbari et al.[65] demonstrated that NKT-deficient mice do not develop AHR in response to systemic ovalbumin priming in the presence of adjuvants and subsequent allergen aerosol challenge.

The resistance of these mice to the development of allergen-specific AHR can be overcome by adoptive transfer of tetramer purified NKT cells producing IL-4 and IL-13 or by rIL-13 treatment. In support of a role for NKT cells, Morishima et al.[68] have shown that priming of mice with ovalbumin plus the NKT cell ligand α-GalCer overcomes the tolerance that is normally seen with airway exposure to ovalbumin (OVA) alone. In this model, the development of Th2-mediated AHR is CD1d+ dependent but it does not occur in mice deficient in major histocompatibility complex (MHC) class II, suggesting that NKT cells are necessary but not sufficient to induce Th2-mediated responses. Interestingly, a single administration of α-GalCer to the mouse airway induces AHR, suggesting that NKT cells may serve as effector cells in the absence of conventional Th2 responses through their ability to produce Th2 cytokines such as IL-13. The effect of NKT-cell activation appears to be dependent upon the timing and context of activation as other studies have shown that activation of NKT cells with α-GalCer + OVA in OVA-primed mice actually inhibits AHR, and Th2 cytokine production in a NKT cell and IFNγ-dependent manner.[69] In support of a role for NKT cells in human asthma, Akbari et al.[70] demonstrated that a large fraction of CD4+CD3+ cells in the lungs of allergic asthmatic individuals are not MHC class II-restricted, but rather NKT cells. Similarly, Sen et al.[71] found that Vα24+ invariant NKT cells in the blood of patients with asthma selectively expressed CCR9, and that large numbers of CCR9+ and Vα24+ cells were present in bronchial-biopsy samples from patients with asthma, but not from control subjects. They also showed that conventional CD3+α/β T cells could be polarized to a Th2 phenotype by cell-to-cell contact with Vα24+ invariant NKT cells, with enhanced expression of CCR9, from patients with asthma. The induction of Th2 cytokine production requires CCL25 and CCR9 to activate adjacent membrane signaling by CD226, a leukocyte-adhesion molecule, that is expressed on monocytes. CD226 appears to be critical for activating Vα24+ invariant NKT cells for the induction of a Th2 bias in conventional T cells. DCs and epithelial cells are major sources of CCL25. They also showed that the numbers of CCR9+Vα24+ NKT cells in the peripheral blood of symptomatic patients with asthma decreased after steroid treatment and when asthma was clinically silent. Taken together, these studies suggest that NKT cells play a prominent role in the development of some Th2 immune allergic responses; however, the exact nature of their role is dependent upon both the timing and dose of the activating ligand. Thus, NKT cells can function as an adjuvant when iNKT cells are activated during adminstration of a protein antigen, can direct induction of AHR when they are activated in the absence of other signals, or can function to prevent the development of AHR when they are activated by strong NKT cell activating agents once inflammation is established. Recent studies demonstrate that house dust extracts collected from different homes contain antigens capable of activating both mouse and human iNKT cells. These extracts enhanced OVA-induced AHR in an iNKT-dependent manner, suggesting that they have adjuvant properties. Interestingly, administration of these extracts together with OVA augmented the synthesis of cytokines from both Va14i NKT cells and conventional CD4+ T cells in the lung.[72] These results suggest a scenario in which NKT cells and conventional CD4+ T cells responding to their respective glycolipid and peptide antigens work in concert to mediate the development of allergic responses. Moreover, human CD1d-restricted T cells recognize lipids in pollens.[73] Interestingly, iNKT cells do not appear to be required for the development of another allergic disorder, namely atopic dermatitis.[74] Further studies will be required to determine the extent of the contribution of NKT cells in various allergic disorders and to define the specific

endogenous or exogenous glycoplipid antigens that are activating NKT cells in allergic patients.

TH17 CELLS IN ALLERGIC DISEASE

Although allergic diseases are typically thought to be Th2-dominated, recent studies suggest that certain forms of disease may also be associated with elevations in the cytokines IL-17A, IL-17F, IL-21, and IL-22 produced by the newly described Th17 subset.[75] These cytokines are strongly associated with neutrophil responses and protection from bacterial or fungal pathogens. Evidence for a pathogenic role for Th17 cells in severe forms of asthma include the observations that elevated levels of IL-17A in serum, sputum, or bronchial biopsy specimens,[76,77,78] and polymorphisms in the IL17A or IL17F promoters[79,80] are significant risk factors for the development of severe asthma. Similarly, single nucleotide polymorphisms (SNPs) in the gene that encodes the p40 subunit of IL-23 (also shared by IL-12) are associated with

severe asthma.[81,82] Interestingly, exposure to many known triggers of asthma exacerbations, including organic dust, inhaled particulate matter, and ozone, have been shown to trigger release of Th17 cytokines.[83,84,85]

Evidence from animal studies also supports a role for Th17 cells in more severe allergic disease. A number of studies demonstrate that mice lacking IL-17A (either through genetic manipulation, following anti-IL-17A treatment or pharmacologic intervention) display reduced airway inflammation and AHR.[86,87,88,89] Similarly, IL-23-deficient mice have decreased airway inflammation and Th2 immune responses.[90] Moreover, mouse strains that are genetically predisposed to mount a mixed Th2/Th17 response (A/J mice) demonstrate increased IL-23 production and more severe AHR than those that mount a pure Th2 response (C3H/HeJ mice).[76,91] Blockade of IL-17A reduced severity of AHR in A/J mice, while exogenous IL-17A exacerbated AHR in C3H/HeJ mice.[76] Similarly, simultaneous transfer of both Th2- and Th17-cytokine-producing T cells induces a significantly more intense AHR.[92,93,94]

Conflicting reports do exist however, with some reports arguing for either no role for IL-17A in promoting the development of allergic asthma, or a protective role for this cytokine.[95,96] While the reasons for these discrepancies remain unclear, a recent report by Besnard et al.[97] suggest that the pathogenicity of IL-17A may in turn be regulated by an additional cytokine, IL-22. In the presence of IL-22, the authors demonstrate that IL-17A is pathogenic, while in its absence, IL-17A appears to play a protective role. Thus, the presence of IL-22, a cytokine coproduced by Th17 cells, may regulate the pathogenicity of IL-17A. It is interesting that recent evidence suggests that different “subsets” of Th17 cells develop in the presence of different extrinsic signals,[98,99] suggesting the possibility that different Th17 cell subsets may play protective or pathogenic roles based on which signals they are exposed to during development.

While the role of Th17 cells has been studied most extensively in allergic asthma, there is also evidence that IL-17A plays a role in other allergic diseases. Increased IL-17A mRNA

expression is associated with the presence of nasal polyps in patients with both chronic rhinosinusitis and allergic rhinitis but not in those with allergic rhinitis alone.[100,101] These findings suggest that Th17 cells may be involved in particular subtypes of allergic rhinitis. In atopic dermatitis, increased IL-17A expression is found in biopsies of acute skin lesions, and there is a correlation between the number of Th17 cells found in the peripheral blood and the severity of acute atopic dermatitis (AD) lesions.[102,103] Filaggrin-deficient mice also display increased IL-17A expression in skin lesions after epicutaneous exposure to an allergen.[104] Interestingly, the association between IL-17A and AD lesions does not extend to chronic lesions, as very little IL-17A expression is detected in chronic lesions in patients with AD patients.[105,106] Rather, chronic AD lesions appear to be associated with infiltration of "Th22" cells—a subset of T cells that produce high levels of IL-22, but limited IL-4, IL-17A, or IFNγ.[106,107] Thus, the interplay of IL-17A and IL-22 may also play an important role in regulating pathogenicity of Th17 cells in AD.

While the mechanisms through which Th17 cells enhance the severity of allergic diseases remain unclear, a number of potential mechanisms have been proposed. The ability of IL-17A to regulate disease severity may be related to its capacity to support neutrophil accumulation, as asthma and allergic rhinitis in some patients are associated with neutrophilia.[108,109] Although the role of neutrophils in these diseases is not entirely clear, mice lacking neutrophils have been demonstrated to develop less severe allergen-induced AHR. However, enhancing neutrophil recruitment was not sufficient to trigger enhanced disease.[110] IL-17A also directly induces the production of the mucins Muc5AC and Muc5B by tracheal epithelial cells,[111] and overexpression of IL-17A by pulmonary epithelial cells is accompanied by increased mucus staining,[112] suggesting that IL-17A may be involved in mucus hypersecretion or goblet cell hyperplasia. Finally, of relevance for all models of allergic disease, IL-17A has also been shown to directly enhance IL-13-driven signaling both in vitro and in vivo,[76,113] suggesting that the presence of Th17 cytokines may serve to exacerbate Th2-driven immunopathology.

It has been recently postulated that Th17-derived cytokines may directly contribute to the steroid resistance observed in individuals with severe asthma. McKinley et al.[92] have reported that while in vitro culture of Th2 cells in the presence of the steroid dexamethasone completely abrogated Th2 cytokine production, culture of Th17 cells with dexamethasone failed to suppress their cytokine-producing capacity. Similarly, transfer of Th2 cells into naïve mice induced the development of steroid-sensitive AHR whereas transfer of Th17 cells triggered AHR that was resistant to steroid treatment. While the mechanisms of IL-17-driven steroid resistance are unclear, a recent report showed that IL-17-driven inhibition of histone deacetylase 2 activity was responsible for promoting steroid-insensitive production of IL-8 by human bronchial epithelial cells, suggesting that epigenetic mechanisms may be at play.[114]

IMPAIRED TREGULATORY CELL FUNCTION IN ATOPIC INDIVIDUALS

It has recently been hypothesized that under noninflammatory conditions, the outcome of immune responses to innocuous environmental allergens is the development of immunologic tolerance. Moreover, it is thought that a loss of tolerance results in Th2-biased immune responses at mucosal surfaces. Although the specific immunologic events that mediate

tolerance in this setting are not well understood, recent studies have suggested that T_{reg} cells protect against the development of allergic disease and that their function is impaired in genetically susceptible individuals. As is described in depth in other chapters within this text, T_{reg}s are cells that inhibit the development and function of other nonregulatory T cells. To date, two major categories of T_{reg} cells have been described. The first is the naturally occurring, thymically derived CD4+CD25+ T_{reg} cells that express high levels of the transcription factor Foxp3, which is essential for their development and function. The other major category is the antigen-specific T_{reg}, which can be induced in vitro and in vivo under particular conditions

of antigen stimulation. These antigen-specific T_{reg}s secrete anti-inflammatory cytokines such as IL-10 and TGF-β and regulate immune responses and inflammatory pathologies. Antigen-induced T_{reg}s that secrete IL-10 are often referred to as IL-10-T_{reg} cells, or Tr1 cells, whereas those that secrete TGF-β have been referred to as Th3 cells.

Several lines of evidence from both human and animal studies have suggested that alterations in T_{reg} populations and function may contribute to susceptibility to allergic disease. In animal models, several studies show that adoptive transfer of CD4+CD25+ T_{reg}s can reverse established airway inflammation through an IL-10-dependent process. Conversely, reduced expression of membrane-bound TGF-β exacerbates airway pathology in an asthma model.[115] Furthermore, environmental exposures to agents that induce T_{reg} cell expansion have been shown to ameliorate the development of asthma. In particular, exposure of mice early in life to lipopolysaccharide (LPS) resulted in the expansion of T cells expressing CD25 and IL-10 concomitant with reduced allergic manifestations upon sensitization and challenge as compared to those mice that were not exposed to LPS.[116] Treatment of mice with mycobacterium-induced allergen-specific T_{reg} cells producing IL-10 and TGF-β protected against airway inflammation.[117] Similarly, heat-killed *Listeria monocytogenes* treatment induced allergen-specific T_{reg}, producing IFN-γ and IL-10, which protected against food allergy in dogs.[118]

Although fewer conclusive studies have been conducted in humans, several support the hypothesis that impaired T_{reg} function may contribute to development of Th2-dependent pathologies in humans. For example, the frequency of allergen-specific IL-10-secreting cells was significantly decreased in allergic patients as compared to nonatopic individuals.[119] Whether the IL-10 is derived from T_{reg}s or activated effector T cells is unknown. In addition to a relative lack of T_{reg}s in atopic individuals, Ling et al.[120] have shown that CD4+CD25+ cells from atopic individuals have impaired ability to suppress effector T-cell cytokine production. Another interesting observation regarding the role of T_{reg}s in susceptibility to atopy is the finding that children who outgrew milk allergy had higher numbers of CD4+CD25+ cells in their blood.[121] Furthermore, therapies shown to be beneficial in the treatment of allergy and asthma, such as allergen immunotherapy or glucocorticoid therapy, have been shown to increase the induction of allergen-specific IL-10-secreting T_{reg} cells, concomitant with a reduction in allergen-specific Th2 responses.[122]

Although the mechanisms by which T_{reg}s regulate the development of allergic inflammation are not completely understood, they are thought to prevent allergic symptoms by suppression of Th2 cell effector function through the elaboration of the suppressive cytokines, IL-10 and TGF-β. However, recent studies now support the concept that T_{reg}s may alter sensitization and ongoing Th2 immune responses via regulating airway DC function. Mice lacking the transcription factor RunX3, which is involved in downstream TGF-β signaling, spontaneously develop symptoms of asthma concomitant with increased numbers of lung DCs displaying a mature phenotype with increased expression of MHC II, OX40 ligand, and CCR7.[123] In further support of a role for T_{reg} cell regulation of DCs, it has been shown that in mice (C3H/HeJ) that are resistant to house dust mite-induced asthma and ARH, T_{reg} depletion with the CD25-depleting antibody similarly led to increased numbers of pulmonary myeloid DCs with increased expression of MHC II, CD80, and CD86 and an increased capacity to stimulate T-cell proliferation and Th2 cytokine production.[124] In contrast, T_{reg}s from normally susceptible A/J mice do not suppress inflammation and AHR. These data suggest that resistance to allergen-driven AHR is mediated in part by CD4+CD25+ T_{reg} suppression of DC activation and that the absence of this regulatory pathway contributes to susceptibility.

The lack of allergen-specific tolerance in allergic individuals has been hypothesized to be related to improved hygiene in industrialized countries, possibly due to reduced infections, alterations in commensal microflora in the intestinal tract and/or reduced TLR signaling. This will be discussed in more detail in the following sections. Taken together, these studies suggest that T_{reg}s may induce tolerance to or provide protection against inhaled allergens in healthy individuals and that an imbalance between allergen-specific T_{reg}s and Th2 cytokine-producing cells may underlie susceptibility to the development of atopic diseases.

Determinants of Susceptibility to Type 2 Immune Responses in Atopic Individuals

Overview

Very little is known conclusively about the underlying causes of the aberrant expansion of Th2 cytokine-producing cells in atopic humans. However, the skewed Th2 immune responses in atopic disorders may be due to either overzealous Th2 immune responses, impaired ability to generate either Th1 or T regulatory responses, a lack of exposure to Th1- and T_{reg}-promoting agents, or a combination of each of these. This balance is influenced by a number of genetic, environmental, and epigenetic factors that control both the innate and adapative immune responses to allergens at mucosal surfaces. There is supportive data for each of these determinants in atopic diseases.

Genetic Influences on Allergen Sensitization

There is substantial evidence suggesting that the development of atopic diseases is genetically controlled.[125,126,127,128,129] Familial aggregation and twin studies have confirmed a fundamental contribution of genetic factors to the development of atopy and specific clinical atopic phenotypes.[125] Specifically, individuals with a first-degree relative with atopy are at a significantly greater risk of developing atopy. Moreover, individuals with two “atopic” parents are at a greater risk of developing an allergic disease than those with only

one atopic parent. Further support for a genetic basis of atopy is the fact that there is a greater concordance for atopic disease between monozygotic twins compared to dizygotic twins.[126] The concordance is not 100%, suggesting that atopy is a polygenic disorder with strong environmental influences. Despite the

complex genetic nature of atopic disorders, multiple studies have reported evidence of linkage of atopic related traits with five primary chromosomal regions including 1) the Th2 cytokine gene cluster on 5q31-q33 including the IL-4, IL-13, CD14, SPINK5, LTC4S, and CYFIP2 genes; 2) the HLA region on 6p21 including both classical (HLA-DRB1, HLADQB1, HLA-G) and nonclassical HLA genes (TNF, LTA); 3) the region that contains the high-affinity IgE receptor gene (FcεRIb) on 11q13; 4) a large region on chromosome 12q14 that spans several candidate genes (Stat6, IFNγ, stem cell factor, nitric oxide synthase 1); and 5) a region of 16q containing the IL4RA.[127] Disease-specific genes have also been identified through candidate gene approaches and genome-wide association studies: asthma (IL33, IL18R1, IL2RB, SMAD3, ORMDL3, HLA-DAQ),[128] AD (the epithelial barrier gene, filaggrin),[129] and eosinophilic eosphagitis (thymic stromal lymphopoietin [TSLP]).[130]

Environmental Influences on Development of Atopic Disease

Despite the clear heritable component of allergies, the incomplete concordance of disease in twin studies and the rapid shift in the rate at which the incidence of atopic disorders is rising suggests a recent change in environmental influences on this process. Changes in exposures to allergens, infectious agents or their byproducts, and/or pollutants have all been suspected. It is likely that multiple environmental factors influence the development of allergic diseases, and that there may be complex interactions between these individual factors (Fig. 45.1).

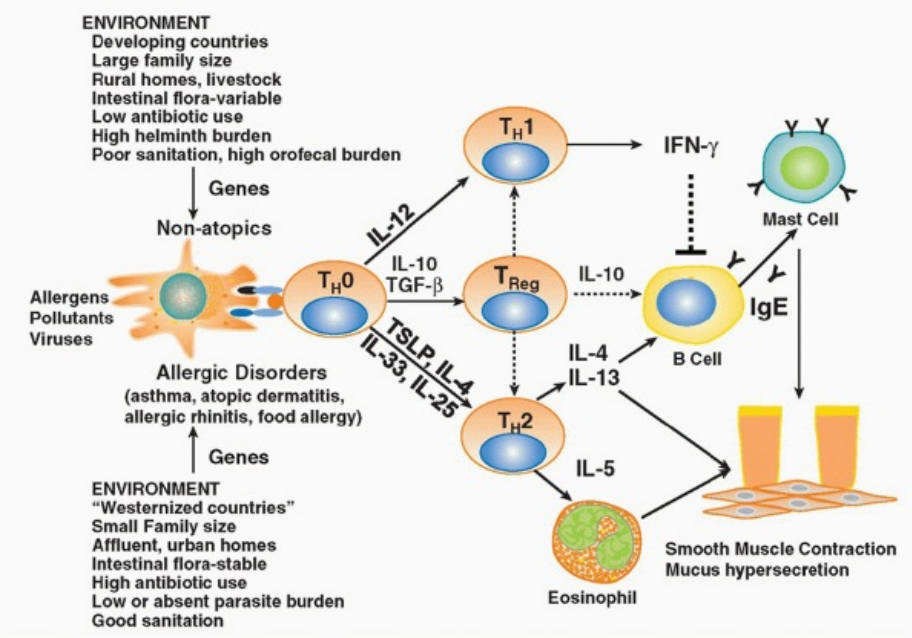

FIG. 45.1. Allergic Diseases are Associated with Aberrant Adaptive Immune Responses to Innocuous Environmental Antigens. The development of allergic responses to harmless antigens is influenced both by environmental factors and the

genetic background of the individual. Under conditions in which microbial exposure is minimal such as in developed countries, T cells differentiate along a Th2 pattern with elaboration of interleukin (IL)-4, IL-13, IL-5, and IL-9. In contrast, microbial exposure in early life protects against the development of harmful Th2 immune responses via the induction of regulatory T cells.

Timing of Allergen Exposure

The spectrum of antigens an atopic individual is sensitized to is dependent on their environment in early life. This tenet is based on the positive correlations observed between allergen exposures present during the month of birth and the development of sensitization to the same allergens later in childhood (eg, birch, grass, and dust mite allergens).[131,132,133] For example, Scandinavian babies born in late winter or early spring are more likely to develop IgE antibody to birch pollen, which is prevalent during the spring, than those born at other times of the year.[131] In addition, avoidance or withholding certain allergenic foods during the first few months of life tends to prevent sensitization and subsequent allergic responses to these particular foods.

It has recently been hypothesized that exposure to environmental antigens occurs both prenatally and postnatally. In this regard, several independent studies have provided support for the postulate that prenatal events may influence susceptibility to allergic diseases.[134,135] For example, it has been shown that cord blood mononuclear cells respond to inhalant and food allergens, suggesting that initial priming

of allergen-specific T-cell responses may occur before birth.[134,135] It has been demonstrated that the responding cord blood T cells were indeed of fetal origin, as they did not respond to common vaccine antigens such as tetanus toxoid, which the majority of adults in the study population expressed active immunity against.[134] These findings suggest that transfer of substances from mother to child (eg, allergens, antibodies, toxins, hormones, or other immune mediators via the placenta) may occur during gestation, which might prime or sensitize the developing infant to environmental antigens. In addition, factors present in the in utero environment may serve to influence the nature of the immune response of the fetus to allergens that cross the placenta. Indeed, mediators such as PGE2 and high levels of progesterone, which has been shown to alter the Th1/Th2 balance by either suppressing Th1 cytokine production or inducing a Th2 pattern, are present during pregnancy. Th2 skewing is thought to take place to suppress IFNγ production that is toxic to the fetus. Accordingly, exogenous antigens that leak across the placenta are likely to be presented to the fetal immune system within a milieu conducive to selection for Th2 immunity. This contention is supported by studies showing that cord blood cells from both allergic and noallergic infants both produce high levels of Th2 cytokines and low IFNγ levels.[135,136] Although immune responses in neonates are skewed toward a Th2 phenotype, studies of human infants indicate that this Th2 skew gradually diminishes during the first 2 years of life in nonallergic individuals.[137] In allergic infants, the reverse occurs, with the strength of neonatal Th2 responses increasing over a similar period. This has been supported by Holt and colleagues,[138] who showed that although the levels of IgE vary considerably during

early childhood, atopic subjects eventually lock into a stable pattern of increasing antibody production and Th2-polarized cellular immunity that is associated with stable expression of the IL-4 receptor in allergen-specific Th2 memory cells, which is absent during infancy. The persistence of this neonatal bias and the failure to produce Th1 or T_{reg}-type responses may be an important feature of the atopic disease state.

The redirection of Th2 responses is thought to occur simultaneously with childhood bacterial or viral infections in early life. This relationship has been capsulated in the “hygiene hypothesis,” which argues that early childhood infections inhibit the tendency to develop allergic disease.[139,140] Several epidemiologic studies have provided support for this hypothesis by demonstrating an inverse relationship between farm living,[141] pet ownership,[142] or drinking unprocessed cow's milk[143] in early life and atopy. Although the general implication of these studies is that protection from allergy seems to be associated with an increase in microbial exposure, the link has remained fairly indirect.

The complex interface with the microbial world provided by the gastrointestinal tract might well be important to the interrelationship between infection and allergy. The endogenous flora of the gut provides a wealth of stimuli for the developing immune system at birth. There seem to be both quantitative and qualitative differences in early childhood patterns of bacterial colonization between the developed and developing world. For example, a study of Swedish and Pakistani infants indicates that intestinal colonization with aerobic gram-negative bacteria tends to occur later in developed than developing countries.[144] Once they are colonized, infants in developed countries tend to carry the same enterobacterial strains, whereas infants in developing countries are often colonized serially with different strains. Differences in intestinal flora have also been found between allergic and nonallergic children in Europe.[145,146] Allergic children seem to be colonized less often with lactobacilli in Sweden and Estonia than nonallergic controls. This idea is consistent with the observation that oral tolerance cannot be induced in germ-free mice.[147] Furthermore, elimination of commensal intestinal microflora with broadspectrum antibiotics also prevented oral tolerance from developing and resulted in susceptibility to intestinal inflammation. These observations suggest that TLR signaling by commensal bacteria under normal steady-state conditions is required for maintenance of intestinal epithelial homeostatsis, and possibly for the induction of some forms of T_{reg}s. The intestine has been shown to affect immune responses at other mucosal surfaces including the respiratory tract and lacrimal, salivary, and mammary glands through secretory IgA antibodies.[148,149] A recent study suggests that not only are there differences in gut flora between individuals living in developed versus developing countries, but that allergic asthmatic individuals display a different spectrum of bacterial species in their lungs than do healthy control individuals.[150] In both compartments, a shift from protective bacteriodes to clostridial species has been observed. Recent studies demonstrating the role of a specific clostridial species, segmented filamentous bacteria, in the generation of Th17 responses further support a role for an altered microbiome in the pathogenesis of allergic disorders.[151] Further studies on the influence of the microbiome are likely to provide tremendous insight into the mechanisms underlying the hygiene hypothesis.

Further support for the hygiene hypothesis is the striking association between polymorphisms in genes in the TLR pathways and atopic phenotypes. Specifically, an association has been

consistently reported between a polymorphism in the CD14 gene, increased levels of sCD14, and decreased levels of IgE, reflecting the importance of bacterial LPS in downregulating Th2 responses.[152] Moreover, a polymorphism in the coding region of the TLR4 gene (A/G 896) resulting in reduced cell surface expression of TLR4 and subsequent disruption of LPS-mediated signaling, has been associated with atopy in children.[153] However, the data in asthma is conflicting with some studies showing an association between asthma and TLR4 polymorphisms[153] while others do not.[154] Interestingly, inheritance of both the TLR4 SNP (A/G+896) and the IL-4 SNP (-590) confers greater risk of asthma pathogenesis in females.[155] Similarly, a polymorphism in the TLR2 gene (TLR2-16934) was shown to be a major susceptibility gene for children living on farms.[156] Clearly these innate immune pathways play a critical role in determining the inflammatory reactions of the airways and the outcome of T-cell responses to inhaled allergens.

Although the hygiene hypothesis is likely oversimplified, it is theoretically possible that APCs at mucosal surfaces in atopic individuals receive less innate immune stimulation or stimulation of a different type in the form of microbial stimuli (LPS, peptidoglycans, mycobacterial antigens) and therefore they fail to redirect weak Th2 responses into protective Th1 responses or T_{reg} responses. This hypothesis may provide a plausible explanation for the rising prevalence of these disorders in developed countries.

Exposure to other environmental agents such as diesel particles, ozone, secondhand tobacco smoke, and viruses (respiratory syncytial virus [RSV], rhinoviruses) can also enhance sensitization to allergens in young children. Numerous epidemiologic studies illustrate clear associations between exposures to these agents and enhanced antigenic sensitization and worsening of disease.[157,158] Nasal challenge with diesel alone increased IgE production in both atopic and nonatopic individuals, suggesting that indeed diesel may be a sensitizer.[157] When evaluated together with allergen, diesel exposure of ragweed-sensitive subjects resulted in a significant increase in allergen specific IgE with an increase in Th2 cytokine production. Ozone can alter both immediate and late-phase responses of asthmatics and allergic rhinitics to inhaled allergen.[158] Epidemiologic studies have shown that exposure to environmental tobacco smoke in childhood is associated with increased skin test reactivity, serum IgE, and prevalence of eosinophilia.[159,160] Recently, it has been shown that controlled chamber exposures of ragweed-sensitive subjects to environmental tobacco smoke resulted in enhanced production of allergen-specific IgE, Th2 cytokines, and decreased IFNγ levels in nasal lavage fluids.[161] Although there is controversy regarding whether RSV is an inducer of allergic asthma or rather a predictor of aberrant immune responses, severe RSV infections are strongly associated with asthma.[162] Similarly, rhinoviral infections are strongly associated with exacerbations in atopic airway disease.[163] In summary, the complex interplay between genetic and environmental factors likely governs susceptibility to the development of atopic disorders.

Epigenetic Influences on Development of Atopy

The inheritance of atopic disorders appears to be preferentially associated with the atopic status of the mother. As asthma risk alleles are inherited from both parents, the preferential

link of atopy with the atopic status of the mother suggests that factors present in the in utero environment contribute to subsequent risk of atopy in the offspring[164] (Fig. 45.2). These modifications in gene expression in response to environmental cues can result from modifications of deoxyribonucleic acid (DNA; eg, by methylation) or of proteins that intimately associate with DNA (eg, acetylation, methylation, or phosphorylation of histones). Such differences could result in differential "prenatal programming" of immune cells in the fetus depending on the asthma status of the mother or from exposure to asthma medications taken by asthmatic mothers during pregnancy.

The first evidence that fetal genotype interacts with "maternal asthma" to determine risk for asthma in the child was provided by a positional cloning study that identified HLA-G as an asthma susceptibility gene.[165] In that study, the -964G allele was associated with asthma only in families with an affected mother, and the -964A allele was associated with asthma in families with an unaffected mother. Subsequent studies implicated a SNP (+3142C/G; rs1063320) that resides in the 3′ untranslated region within a target site for three micro ribonucleic acids (miRNAs).[166] In the presence of any of these miRNAs, expression of the G allele is suppressed whereas expression levels are unchanged for the C allele. The GG genotype, which is suppressed in the presence of the miRNAs, was associated with significant protection from asthma

among children of mothers with asthma but with modest risk for asthma among children of mothers without asthma. The mechanism through which the "maternal asthma-fetal HLA-G genotype" interaction influences subsequent risk for asthma in the child is still unknown, but modulation of expression via miRNA targeting could be involved. Because asthma in the mother is such a strong predictor of asthma in her children, it is likely that other, as yet undiscovered, genes also play a role in modulating this effect.

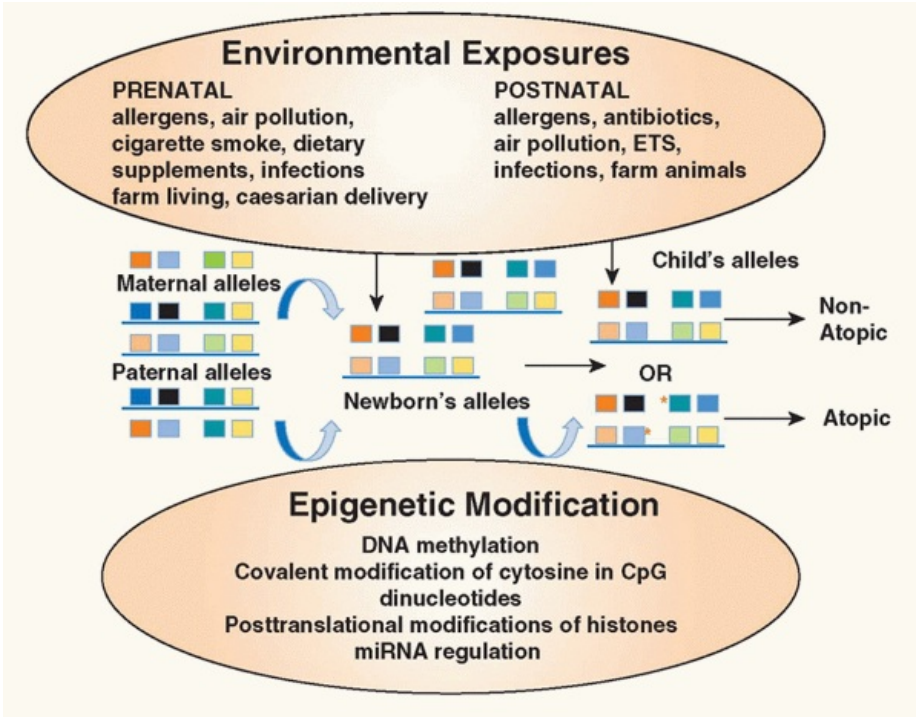

FIG. 45.2. Environmental Epigenetics and Atopy. Exposure of the mother to environmental factors (environmental tobacco smoke, air pollution, allergens, antibiotics, dietary supplements) during pregnancy may induce epigenetic changes (deoxyribonucleic acid methylation, covalent modification of cytosine in cytosin-phosphatidyl-guanosin dinucleotides, posttranslational modifications of histones, micro ribonucleic acid regulation) in gene expression in the offspring providing an explanation for the heretofore unexplained maternal influence on the inheritance of atopic diseases. In addition, exposure of the infant in early childhood to environmental factors may also alter expression of genes important in allergic inflammation. Collectively, these influences contribute to the risk of the child developing atopic diseases. *Red asterisks* refer to sites of epigenetic changes. Adapted from Miller and Ho.[164]

Recent evidence suggests that prenatal and postnatal exposures to the key environmental triggers of allergic disease described previously (ie, allergens, microbial infections, tobacco smoke, other pollutants, dietary supplements) can induce epigenetic changes in gene expression and alter disease risk through activating or silencing immune-related genes with subsequent effects on immune programming. For example, Fedulov and Kobzik et al.[167] demonstrated that DNA from splenic DCs harvested from pups born to mothers previously sensitized and challenged to OVA was hypermethylated. Moreover, adoptive transfer of these DCs from pups of allergic mothers into offspring from nonatopic mothers transferred increased susceptibility to allergic disease to recipients despite there being no differences in DNA sequences. The specific genes conferring this susceptibility remain to be determined.

There is also strong evidence that the association of maternal smoking in pregnancy with asthma risk may be due to epigenetic changes.[164] Indeed, altered DNA methylation patterns have been observed in several genes in buccal cells from children exposed in utero to tobacco smoke.[168] Likewise, exposure to diesel exhaust partially augmented the production of IgE following allergen sensitization through hypermethylation of IFNγ cytosin-phosphatidyl-guanosins (CpGs) and hypomethylation of CpGs in the IL-4 locus.[169] Further evidence for epigenetic contributions to disease risk is provided by the observation in mouse models of asthma that a maternal diet rich in methyl donors (folate) can increase susceptibility to allergic disease in offspring that is mediated through increased DNA methylation.[170] Studies in humans examining the role of folate have been conflicting, with one study showing no association,[171] whereas another showed an association between folate supplementation in late pregnancy and a physician's diagnosis of asthma in the offspring.[172]

Several lines of evidence suggest that the protective effects of maternal exposure to farm environments, which increase microbial burden prenatally, may occur through epigenetic changes. Specifically, studies have shown that nonpathogenic microbial strains isolated from farm environments can induce epigenetic effects when administered to pregnant animals and protect the offspring from experimental asthma.[173] The protective effects were IFNγ-dependent and associated with protection against the loss of histone 4 acetylation of the IFNγ gene in the CD4+ T cells of the offspring. In contrast, a decrease in H4 acetylase was observed in the IL-4 promoter. Moreover, pharmacologic inhibition of H4 acetylation in offspring abolished the protection. Further examination of epigenetic changes in allergy will

undoubtedly enhance our understanding of the ontogeny of allergic diseases.

FACTORS REGULATING TYPE 2 IMMUNE RESPONSES IN ALLERGIC INDIVIDUALS

Differentiation of naïve CD4+ T cells into the various subsets requires several signals derived from either APCs or accessory cells. These include presentation of antigen in the context of MHC II, signals conveyed through costimulatory ligand-receptor interactions, and signals conveyed through the actions of specific cytokines. Although the factors driving Th1, Th17, and T_{reg} subsets are well defined, the factors critical for the initiation of the type-2 response have long eluded investigators. In contrast to the steps involved in the differentiation of other T subsets, traditional antigen loaded APCs themselves are not sufficient to drive the early IL-4 required for Th2 differentiation or for full development of Th2 effector functions. Recently, however, several critical observations have led to a more detailed understanding of the type 2 response. First, three type 2-inducing cytokines, TSLP, IL-25, and IL-33, were identified as being necessary for the upregulation of type 2 effector cytokines, mirroring the role of IL-12 in the type 1 response. Secondly, recent evidence suggests that these type 2-promoting cytokines are produced by resident cells in mucosal tissues in response to innate immune receptor stimulation, not by the APCs themselves. Thirdly, exciting evidence suggests that type 2 effector cytokines can be produced by the newly described lineage negative innate lymphoid cell (ILC) populations in response to the type 2-promoting cytokines (IL-25, IL-33). Thus the overall emerging scenario is one in which allergens uniquely interact with mucosal epithelial cells and resident cells to produce these Th2-promoting cytokines through stimulation of various PRRs or danger associated molecular patterns expressed on resident mucosal cells. These cytokines (IL-33, IL-25) can themselves drive type 2 effector cytokines in innate cells (mast cells, basophils, ILCs) independently of APCs. In parallel, they also license traditional APCs to present allergenic peptides to conventional naïve T cells in draining lymph nodes, driving Th2 differentiation and T-cell memory development. The relevance of each of these steps to the development of allergic diseases will be discussed in the following sections (Fig. 45.3).

Innate Immune Responses to Environmental Antigens

Allergens interact with innate recognition systems present on a diversity of cells at the mucosal surface of the respiratory tract, skin, and gastrointestinal tract, including epithelial cells and phagocytic cells (DCs, macrophages). As the primary interface with the environment, the epithelial cell layer represents the front-line defense against injury by pathogens or environmental irritants. Emerging evidence suggests that epithelial cells play a critical role in both antigen recognition and danger recognition. Allergenic particles and their associated soluble components interact directly with epithelial cells through a variety of diverse mechanisms including 1) binding of PRRs including various members of the TLR family, the C-type lectin receptor (CLR) family, and nucleotide oligomerization domain (NOD) receptor family; 2) the activation of protease-activated receptors (PARs); and

3) activation of various danger sensing pathways (extracellular nucleotides, uric acid).

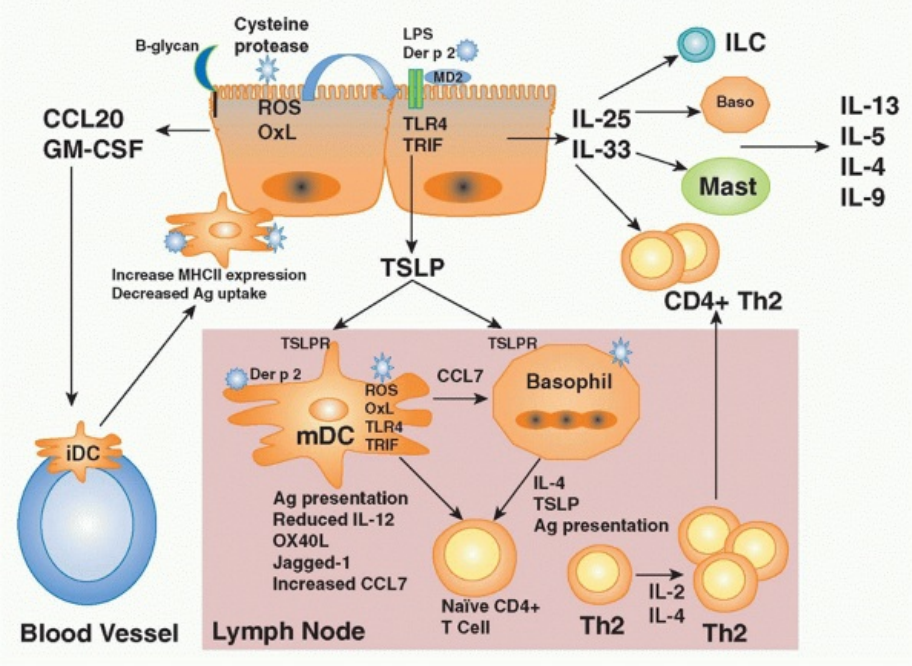

FIG. 45.3. Schematic of Events Leading to the Initiation of Th2-Mediated Immune Responses. Upon encounter with allergen, epithelial cells of the respiratory tract, the intestinal tract, and the skin release mediators that recruit (CCL20), activate (granulocyte macrophage-colony stimulating factor [GM-CSF]), and/or drive Th2 cell differentiation (thymic stromal lymphopoietin [TSLP], interleukin [IL]-25, IL-33). IL-25 and IL-33 recruit and activate a variety of innate immune cells such as basophils, mast cells, and innate lymphoid cells, which can produce the protypical type 2 cytokines IL-4, IL-5, and IL-13 driving the allergic phenotype. The elevations in IL-25 and IL-33 can perpetuate the allergic response by further type 2 cytokine production from basophils, eosinphils, or by initiating conventional Th2-cell expansion through activating dendritic cells (DCs) during encounters with allergens. In parallel, immature myeloid DCs are recruited to mucosal surfaces following the release of chemokines (ie, CCL20) and cytokines (GM-CSF) by epithelial cells in response to inflammatory stimuli such as allergens, pollutants, irritants, and/or viral infections. Once in the tissues, DCs mature and express costimulatory molecules (ie, CD86, OX40L, Jagged-1) under the influence of cytokines (TSLP, IL-33, IL-25) derived from surrounding tissues through stimulation of pattern recognition receptors (likely TLR4, -2; C-type lectins) expressed on their surface. In atopic individuals, it has been postulated that myeloid DCs appear to be the predominant population of DCs taking up antigen and driving the differentiation of naïve T cells toward a Th2 profile. They then migrate to the draining lymph node to stimulate naïve T-cell differentiation and proliferation. Proteasecontaining allergens induce the production of TSLP by epithelial cells through a series of steps including generation of reactive oxygen species, production of oxidized lipids, which in turn stimulate TLR4-TRIF signaling pathways leading to TSLP expression. These same pathways induce the production of the chemokine CCL7 by DCs, which recruits basophils to the lymph node. In the lymph node, they can also directly stimulate basophils to produce TSLP and IL-4. Basophils and DCs drive Th2 cell differentiation under the influence of various cytokines such as TSLP, IL-4, and IL-25. These Th2 cells proliferate in response to IL-2 and IL-4 in an autocrine fashion. Activated Th2 cells in turn migrate back to the site of antigen

stimulation under the influence of Th2 selective chemokines (CCL17, CCL12). Once in the tissues, Th2 cells secrete a profile of Th2 cytokines (IL-4, IL-5, IL-13, IL-9) that initiate a cascade of downstream pathways that collectively lead to the development of the allergic phenotype. Not shown on the figure is the fact that through their effects on the airway epithelium, Th2 cytokines may further amplify Th2 immune responses through the induction of Th2-selective chemokines (CCL17, CCL12) and/or IL-25 and IL-33 initiating a positive feedback loop. baso, basophil; iDC, immature dendritic cell; ILC, innate lymphoid cell; mDC, myeloid dendritic cell; ROS, reactive oxygen species.

Activation of epithelial cells through these diverse pathways can induce the release of cytokines (TSLP, IL-25, IL-33), chemokines, and growth factors that both facilitate entry of antigens into the mucosa; recruit APCs; and provide instructive signals to antigen APCs. As the absence of PRR activation and instruction of APCs results in the development of tolerance to exogenous antigens, alterations in these innate immune responses occurring at the epithelial-environmental interface may be important determinants of susceptibility or resistance to the development of allergic diseases (Fig. 45.4).

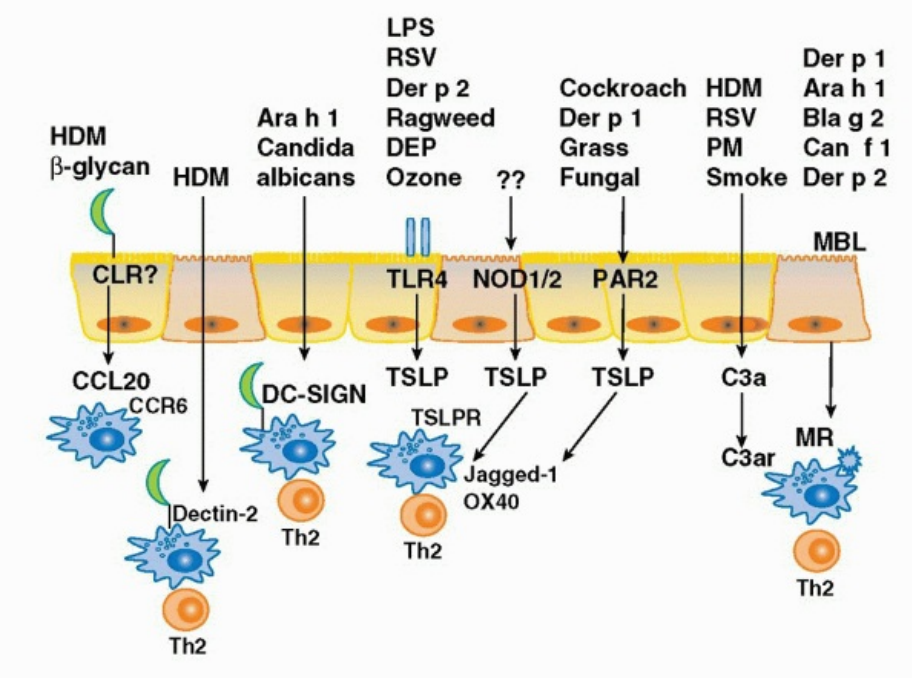

FIG. 45.4. Innate Immune Pathways Activated by Allergens. Allergenic peptides are recognized by several traditional pattern receptors including toll-like receptors, C-type lectin receptors, and nucleotide oligomerization domain receptors. In addition, other classes of allergens initiate Th2-mediated immune responses through the activation of protease-activated receptors and the cleavage of complement pathways. Activation of these innate immune pathways on mucosal epithelial cells leads to the production of chemokines which recruit immature demdritic cells (DCs), the production of Th2-promoting cytokines such as thymic stromal lymphopoietin, the enhancement of DC internalization of allergens, and the upregulation of expression of costimulatory molecules associated with Th2 differentiation such as OX40L and jagged-1. DEP, diesel

exhaust particles; HDM, house dust mite; MBL, mannan-binding lectin; MR, mannose receptor; PM, particulate matter. Der p 1 and Der p 2 are house dust mite-derived allergens; Ara h 1 is the major peanut allergen; Blag 2 is the major cockroach allergen; Can f 1 is the major canine allergen.

Role of Toll-Like Receptor Stimulation in Allergic Inflammation

The most well-studied family of PRRs in allergic inflammation is the TLR family. Epidemiologic studies have consistently reported an inverse correlation between high levels of bacterial products such as LPS in the ambient environment during very early life and the subsequent development of atopy and allergic disease.[174,175] It has been postulated that such exposures drive counterregulatory immune responses in the developing immune system.[176] On the other hand, controlled human challenge studies have shown that LPS exposure of sensitized individuals can exacerbate existing disease.[177] Although the mechanisms underlying this apparent paradox are not entirely clear, the complexity of the responses to TLR agonists may be due to several factors including the array of TLR receptors activated by complex allergens (TLR9 versus TLR4), their relative abundance, and the timing of exposure during the life of the individual. For example, TLR9 stimulation clearly prevents and inhibits the development of experimental allergic inflammation at all doses,[178] whereas TLR2 and TLR4 pathway stimulation has been shown to both drive[179,180] and inhibit[180,181] the development of Th2-mediated allergic inflammation in experimental mouse models. Bottomly and colleagues[180] have shed some light on this complexity, demonstrating that the impact of TLR4 stimulation on allergic inflammation is highly dependent upon the dose of TLR4 agonist. Specifically, they showed that although airway sensitization with the normally tolerizing antigen, OVA along with 1 ng of LPS (LPS-depleted) was reported to induce tolerance, sensitization in the presence of “low-dose” (0.1 μg) LPS promoted TLR4-dependent, Th2 inflammation, and sensitization in the presence of “high-dose” (100 μg) LPS led to a Th1 response. Although these studies provided a plausible explanation for the LPS dose effects observed in epidemiologic studies, they did not explain how stimulation through the same receptor could result in two distinct biologic outcomes. To address this issue, Tan and colleagues[182] examined allergic responses in a series of bone marrow chimeric mice expressing TLR4 in specific compartments. They show that strong (high-dose LPS) TLR4 signaling always results in a Th1 response despite the fact that high LPS stimulation of mice expressing TLR4 only in the stromal compartment drives Th2 responses, as a result of the dominant influence of the hematopoietic cell compartment under these conditions. Surprisingly, they found that at very low LPS levels, mice expressing TLR4 only in the stromal compartment did not mount Th2 or Th1 immune responses. However, when mice that had competent TLR4 signaling in both the stromal and hematopoietic compartments were exposed to low levels of LPS+OVA, they mounted Th2 immune responses, suggesting that once a threshold level of TLR4 stimulation is reached in the stromal compartment, Th2 responses ensue. The authors propose that the ability of stromal cells (presumably epithelial cells) to drive Th2 responses is likely through their ability to secrete TSLP and to promote the maturation of Th2-inducing DCs that express the Notchligand Jagged-1, but not the Th1-inducing ligand, Delta-4. As Jagged-1 has been shown to induce the expression of the critical

Th2 transcription factor, GATA3 in CD4+ T cells through binding Notch ICD, these findings may explain the association between low-level activation of TLR4 pathways and Th2-cell differentiation.[183] In contrast to Tan's findings, another group[184] showed that stromal cell TLR4 signaling was sufficient to drive Th2 immune responses when mice were exposed to dust mite extracts containing low levels of LPS, suggesting that the dust mite extracts might contain endogenous TLR4 agonists that shift the dose response of the stromal compartment to TLR4 stimulation into the Th2-inducing range.

A recent study has provided a compelling mechanism by which endogenous components of dust mites may drive TLR4 signaling. Based on the recent discovery of a structural homology between Der p 2, one of the major house dust mite allergens, and MD-2, a member of the lipid-recognition (ML) domain family of proteins, which is the LPS-binding member

of the TLR4 signaling complex, Trompette and colleagues[25] asked the question whether Der p 2 and MD-2 exhibited functional homology as well. Indeed, they reported that Der p 2 facilitates TLR4 signaling through direct interactions with the TLR4 complex, reconstituting LPS-promoted TLR4 signaling in the absence of MD-2. They also demonstrated that Der p 2 could facilitate LPS signaling in primary APCs, with or without MD-2 being present. Finally, they reported that in vivo delivery of Der p 2 drives the development of experimental allergic asthma in a TLR4-dependent manner, retaining this property in mice with a genetic deletion of MD-2. These data suggest the possibility that Der p 2-mediated activation of TLR4 signaling in the airway epithelium under conditions of low bacterial product exposure—those associated with increasing rates of aeroallergy in the urban, developed world— may shift the LPS/TLR4 response curve from the tolerizing into the Th2-inducing range. Der p 2 exposure may serve to facilitate TLR4 signaling by airway epithelial cells as they have been reported to express TLR4, but little or no MD-2, in the basal state.[185]

The fact that the major dust mite allergen, Der p 2, is a molecular mimic of an endogenously expressed mammalian lipid binding family member has several important implications for our understanding of allergenicity. As numerous other members of the MD-2-like lipid binding family are major allergens, the activation of innate immune pathways via lipid binding is likely to be a common feature of allergens. Indeed, the recently solved structures of several allergens including Der p 5 and Der p 7 suggest that they possess the propensity to bind hydrophobic compounds.[186,187] Of note, Der p 7 has been shown to resemble the LPS binding protein and to bind to the lipopeptide polymyxin B from gram-positive bacteria. More broadly, a wide range of allergens are lipid binding proteins (ie, lipid transfer proteins [peach allergen Pru p 3], steroid-like molecules [cat allergen Fel d 1], lipocalins [horse allergen Equ c 1, mouse allergen Mus m 1]). Further studies are clearly needed to define the lipids naturally bound by these allergens, the receptors activated by such lipids, and the precise pathways of innate and adaptive immune responses driven by such activation.

Despite our rudimentary understanding of the role of PAMPs in allergic responses, one may speculate that either differences in exposures to microbial products or polymorphisms in genes of the TLR pathway may both be important determinants of the risk of developing allergic disease through altering the phenotype and function of DCs. Along these lines, as discussed previously, genetic studies show that polymorphisms of different components of the TLR pathways (CD14, TLR4, TLR2) may partly explain susceptibility to

atopy.[152,153,154,155,156]

Role of Carbohydrate Recognition in Th2 Immunity

Just as the mammalian immune system has evolved mechanisms to recognize bacterial proteins in association with PAMPs that induce appropriate Th1 responses, recent studies suggest an important role for complex carbohydrates in driving Th2 immune responses to allergens. In particular, fucosylated glucans are a diverse class of naturally occurring glucose polymers that are widely expressed in the cell walls of fungi, helminths, pollens, and certain bacteria, but they are not found in mammalian cells. Evidence is emerging that these carbohydrates drive strong Th2-biased immune responses through their interaction(s) with a large array of CLRs.

The C-type lectin family of soluble and transmembrane receptors demonstrate unique specificity for carbohydrate residues via distinct clustering of carbohydrate recognition domains.[188] Transmembrane members of the CLR family include collectins, the mannose receptor family, selectins, dectin 1 and 2, and DC-specific intercellular adhesion molecule-3-grabbing nonintegrin. Ligand engagement of these receptors is involved in processes ranging from cellular trafficking to cell signaling and pathogen recognition mediated through FcRγ and Syk activation.[188]

Several studies suggest the involvement of various C-type lectins in regulating allergic inflammation. For example, it has been reported that β-glucan structures present in the peanut glycoallergen Ara h 1 have Th2-inducing characteristics; native, but not deglycosylated, Ara h 1 has been shown to activate human monocyte-derived DCs and induce IL-4- and IL-13-secreting Th2 cells.[26] The Th2-promoting actions of Ara h 1 were mediated through binding of DC-specific intercellular adhesion molecule-3-grabbing nonintegrin and activation of ERK/MAPK signaling in DCs. Consistent with a role for lectins in promoting Th2 immune responses, a recent study showed that the CLR dectin-2 was required for the development of house dust mite-induced allergic inflammation.[189] Consistent with a major role for CLRs in initiation of Th2 immune responses, β-glucans have been shown to mediate several aspects of DC function including antigen uptake, recruitment and activation of DCs, and instruction for Th2 differentiation. For example, blockade of the mannose receptor, an endocytic CLR, significantly reduced Der p 1 uptake by DCs.[190] Moreover, recent studies have shown that β-glucans contained in house dust mite extracts and in molds may initiate immune responses through the induction of the chemokine, CCL20, which recruits immature DCs. The induction of CCL20 was shown to occur through β-glucan and Syk-dependent signaling pathways.[191] Although the study of the role of carbohydrates as Th2-inducing PAMPs is only in its infancy, the evidence thus far suggest that carbohydrate moieties contained in common allergens function as strong Th2 inducers through activation of variety of CLRs on DCs.

Allergens containing lectins may also activate the lectin pathway of complement pathway activation, a highly conserved component of innate immunity. The complement system is a sophisticated network of soluble and membranebound proteins, which serve as both immune sensors and immune activators. Like other PRR pathways, the complement system can be activated by “hardwired” PRRs that have elvolved to recognize both endogenous and exogenous “danger” motifs. Lectin recognition receptors in the complement system include:

mannan-binding lectin, and ficolins. Recognition of pathogen-associated carbohydrate residues by the soluble mannan-binding lectin triggers the serine protease-mediated cleavage of complement components C4 and C2 resulting in the generation of C3 convertase that carries out the rest of the complement cascade. Recognition by mannan-binding lectin is an integral part of the first line

of defense against a large number of microbial pathogens including bacteria, fungi, protozoa, and viruses. Indeed, it has recently been shown that the major cat allergen, Fel d 1, is a novel ligand of the cysteine-rich domain of the mannose receptor and the development of allergic inflammation induced by Fel d 1 was attenuated in mannose receptor-deficient mice.[192] Likewise the mannose receptor has been shown to mediate the internalization of a diverse range of allergens (Der p 1, Der p 2, Can f 1, Bla g 2, Ara h 1) into monocyte-derived DCs through their carbohydrate moieties.[193] Alterations in these pathways may lead to enhanced recognition of allergens as sequence variations in the MRC1 gene have recently been associated with the development of asthma in two independent and ethnically diverse populations (Japanese and African American).[194]

Nucleotide Oligomerization Domain Receptors in Allergic Inflammation

Although NOD receptors have not been as well studied as other PRRs, it has recently been shown that NOD1 (which binds cell wall peptidoglycans of gram-negative bacteria) and NOD2 signaling in stromal cells drives Th2 immune responses through the TSLP-dependent induction of OX40L.[195] Studies demonstrating that polymorphisms in the intracellular NOD1 protein were associated with atopic eczema and asthma[196] suggest that alterations in these pathways may play an important role in susceptibility to some atopic disorders.

Proteases in Allergic Inflammation

Several of the common allergens (house mite-derived, Der p 1, and Der p 3; Fed d 1 from domestic cats; cockroach allergens; fungal allergens) possess intrinsic protease activity. While the link between protease activity and type 2 inflammation is well documented, the mechanistic basis for these observations has been unclear. Allergens containing protease activity, such as Der p 1, a cysteine protease, have been demonstrated to produce changes in the barrier function of the epithelium, probably by disrupting the epithelial tight junctions by degrading the tight junction proteins ZO-1 and desmoplakin.[197] Disruption of the tight junctions may both facilitate access of allergens to the underlying cells including DCs and may also directly initiate inflammatory cascades (modulate the function or immune and structural cells) in DCs and/or airway epithelium. For example, Der p1 cleaves several important immunomodulatory molecules including CD25, CD23, and various complement components.[198,199] As a result of cleavage of CD25, peripheral blood T cells show markedly diminished proliferation and IFNγ secretion in response to potent stimulation by anti-CD3 mAb. These findings suggest that Der p1 decreases the growth and expansion of antigen-specific Th1 cells augmenting expansion of the antigen-specific Th2 cells that favor a proallergic response. Der p 1 cleavage of CD23 on murine B cells that would normally serve to inhibit IgE synthesis would further potentiate allergic responses by disrupting an important negative regulator of IgE production.[199]

Although the cellular mechanisms by which protease activity induces cytokine release remain unclear, members of a recently identified G protein-coupled family of cell surface receptors, designated PARs, have been implicated. Indeed, allergens such as the house dust mite serine proteases, Der p 3, Der p 9, cockroach extracts, and pollens activate PARs. Asokananthan et al.[200] have shown that endogenous peptidases caused the release of cytokines through the activation of PARs on the respiratory epithelium, and that all four members of the PAR family were expressed on respiratory epithelial cells. In vivo experiments in experimental allergen models support the contention that activation of PAR pathways is associated with enhanced allergic responses. Specifically, PAR-2 overexpression enhanced OVA-induced eosinophilia and bronchial hyperresponsiveness, whereas PAR-2 deficiency was associated with reduced allergendriven eosinophilia and IgE production.[201] Although the exact mechanisms by which PAR-2 mediates Th2 induction is unknown, PAR-2 was shown to mediate, in part, the induction of TSLP from airway epithelial cells in vitro in response to the protease activity of the common environmental fungus *Alternaria alternata*, as well as in response to the cysteine protease papain.[202]

Role of Danger-Associated Molecular Patterns in Regulation of Th2 Immunity

Several sensors of cellular damage have recently been implicated in the development of allergic inflammation. First, Eisenbarth and colleagues[203] showed that the adjuvant alum promotes humoral responses by activating the nucleotide binding domain-leucine rich repeat (LRR)-containing receptor (NLR) inflammasome. Moreover, it has been shown that allergen stimulation of airway epithelial cells results in an upregulation of extracellular adenotriphosphate levels that trigger the migration of myeloid DCs (mDCs) into the airways and mediate the development of allergic inflammation in vivo.[204] Idzko and colleagues[205] have recently shown that these adenotriphosphate-triggered effects on airway inflammation are regulated via the P2Y(2)R. Another sign of cellular damage is the release of the potent antioxidant uric acid. Recent studies show that administration of uric acid crystals together with protein antigen was sufficient to promote Th2 cell immunity and the features of asthma.[206] Surprisingly, the adjuvant effects of uric acid did not require the inflammasome (Nlrp3, Pycard) or the IL-1 (Myd88, IL-1r) axis, but promoted Th2 cell immunity by activating DCs through spleen tyrosine kinase (syk) and PI3-kinase δ signaling. Recently, a member of the trefoil factor family (TFF2), which has previously been shown to be a sensor of damage to the gut epithelium, has been shown to regulate Th2 immune responses to both allergens and helminth infections via induction of IL-33 by the epithelium and alveolar macrophages.[207]

These studies demonstrated that in contrast to Th1-inducing factors, which directly license DCs through PRRs, allergens license antigen-loaded DCs and accessory cells through a variety of innate signals derived from the mucosal epithelium to induce Th2 immunity. Thus, licensing of APCs in trans by signals emanating from the stromal compartment appears to be a unique feature of Th2 immune responses. As will be discussed subsequently, this concept is entirely consistent with studies showing that the primary cytokines driving Th2 differentiation are stromal cell (epithelial cell)-derived.

Cytokine Regulation of Th2 Differentiation in Allergic Disease

One of the most important variables in instruction of T-cell differentiation comes from the local cytokine milieu at the time of antigen presentation. Recently, Th2 cell-polarizing factors have been finally identified, these include TSLP, IL-25, and IL-33 (Fig. 45.5). Alterations in either the production of or responsiveness to these signals could lead to the development of aberrant Th2 immune responses in atopic individuals. Indeed, there is evidence to support each of these possibilities.

Thymic Stromal Lymphopoietin Initation of Th2 Immune Responses

TSLP is an IL-7-like cytokine originally characterized by its ability to promote the activation of B cells and DCs.[208] The TSLP receptor is comprised of the IL-7Rα chain and the unique TSLPR chain. TSLP is expressed by epithelial cells with the highest levels being found in lung- and skin-derived epithelial cells. TSLP has been shown to activate DCs such that they acquire the ability to prime naïve T cells for the production of Th2 cytokines, while downregulating IFNγ and IL-10. In support of a role for TSLP in Th2 responses, TSLP levels have been shown to be elevated in the airways of asthmatic individuals[209] and in animal models[210,211] of atopic disease. Overexpression of TSLP in the lungs of mice has been shown to lead to the development of Th2 immune responses, whereas TSLPR knockout mice develop strong Th1 responses to allergen and are protected against the development of allergen-driven asthmatic responses.[210,211] Finally, overexpression of TSLP in the skin of mice results in an atopic eczema-like phenotype.[212] In humans, the gene encoding TSLP is located on chromosome 5q22 in a strong linkage region for asthma. Polymorphisms in TSLP have been associated both with asthma[213] and eosinophilic eosophagitis.[130] Although the IL-7Rα gene is located on chromosome 5p13, it remains to be determined whether polymorphisms in the IL-7Rα receptor influence TSLP signaling. Although the precise mechanisms underlying the ability of TSLP to prime DCs is not known, one mechanism that has been postulated is that TSLP induces DCs to express OX40 ligand (OX40L) and decrease IL-12 production.[214] In support of this contention, TSLP-induced OX40L on DCs was required for triggering naive CD4+ T cells to produce IL-4, -5, and -13.[215]

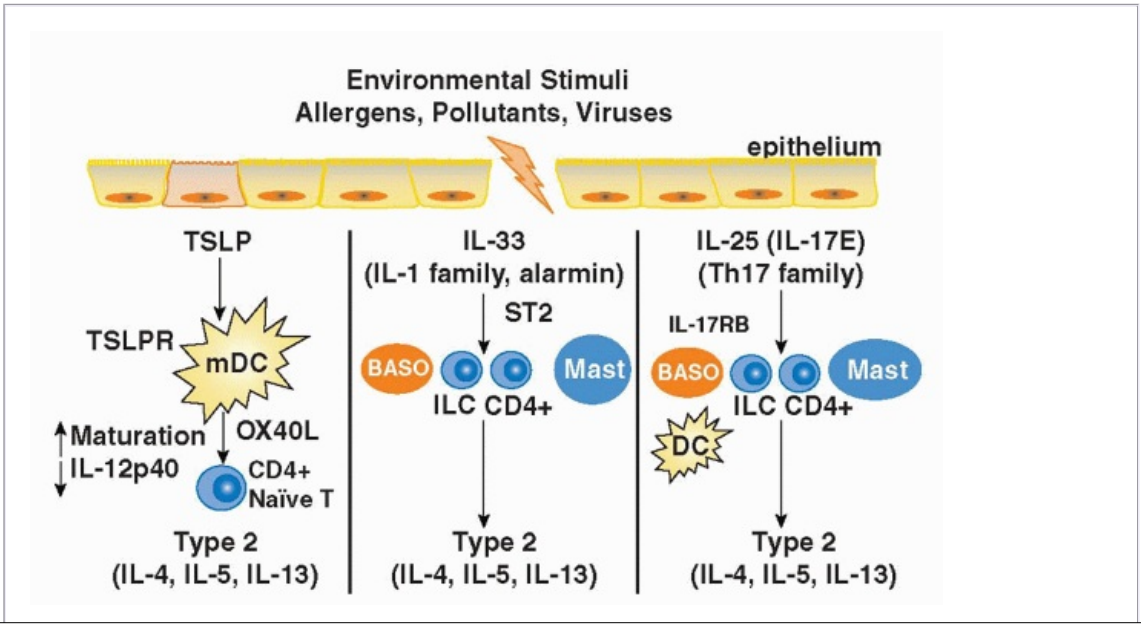

FIG. 45.5. Members of Three Distinct Cytokine Families Promote Th2 Differentiation. Thymic stromal lymphopoietin (TSLP), an interleukin (IL)-7-like cytokine released from mucosal epithelial cells, binds its receptor TSLPR on dendritic cells and induces the expression of OX40L and the suppression of IL-12 production, thus driving the differentiation of naïve T cells toward a Th2 phenotype. IL-33 is an IL-1 family member that binds its unique receptor ST2 on a variety of innate immune cells including basophils, innate lymphoid cells, conventional CD4+ T cells, and mast cells driving the production of type 2 cytokines. IL-25, a member of the IL-17 cytokine family, binds its receptor IL-17RB on a variety of innate immune cells including basophils, innate lymphoid cells, mast cells, and conventional CD4+ T cells driving their production of type 2 cytokines. IL-25 and IL-33 can also stimulate type 2 cytokine production indirectly by activating dendritic cells to promote Th2 differentiation.

Interleukin-25 in Allergic Inflammation

IL-25 is a novel member of the IL-17 cytokine family (IL-17E) that promotes CD4+ T-helper 2 lymphocyte-like (Th type-2) inflammatory responses. IL-25 is produced by epithelial cells, activated human basophils, eosinophils, and mast cells. Recombinant IL-25 administration to mice has been shown to induce IL-4, IL-5, and IL-13 production, and systemic Th2-cell responses, characterized by elevated IgE production, eosinophilia, and remodeling in several organs including the lungs, gastrointestinal tract, and skin of naïve mice.[216] Interestingly, IL-25 can induce these Th2-mediated immune responses in mice lacking T and B cells, suggesting that IL-25 acts upon another cell type besides T cells to drive IL-13 production.[217] A search for the cellular targets of IL-25 has led to the discovery of a family of ILCs that produce type 2 cytokines (ILC2 cells).[217] These ILC2 cells belong to a heterogeneous family of innate cells that do not express surface markers of adaptive immunity (non-T, non-B cells) and are not antigen restricted like their adaptive counterparts. However, as they express CD45 and are dependent on traditional T-cell growth factor signaling pathways such as those mediated through the common γ-chain and IL-7 receptor α-chain (CD127), they have been called ILCs. The population of ILC2s that produce type 2 cytokines in response to IL-25 and IL-33 are lineage-negative (CD4-CD127+IL-1RL1+IL-17RB+, RORγt-). Several ILC2 cells have been discovered in the mouse by independent groups and have been designated natural helper cells,[218] multipotent progenitor type 2 cells,[219] nuocytes,[217] and innate helper type 2 cells.[220] Recently, it has been shown that adoptive transfer of wild-type nuocytes producing IL-13, but not IL-13-/-nuocytes, into IL-13-deficient mice was sufficient to induce allergic inflammation.[221] Although there is much to learn about these populations and the mechanisms regulating their function, increased IL-25 and IL-25R expression has been detected in patients with asthma, suggesting that overproduction of IL-25 may be associated with aberrant type 2 immune responses.[222]

Interleukin-33 and Nuocytes in Atopy

IL-33 is a member of the IL-1 family that stimulates type 2 cytokine production through binding its receptor composed of the IL-33 specific receptor chain, IL-1RL1 (ST2) and the

IL-1 receptor accessory protein (IL-1RAcP),[223] both of which are widely expressed, particularly by innate immune cells (mast cells, basophils, DCs), Th2 cells, and innate lymphoid populations described previously.[217,223] IL-33 is thought to function as an "alarmin" released from stromal cells (epithelial cells, endothelial cells, and airway smooth muscle) following cellular injury to alert the immune system to tissue damage or stress. A role for IL-33 in type 2-cytokine dependent immune responses in vivo is supported by the finding that administration of IL-33 to naive mice induces eosinophilia, increased serum IgE levels, IL-5 and IL-13 production, and mucus cell changes in the respiratory and gastrointestinal tracts.[224] Moreover, blocking IL-33 with an ST2 fusion protein decreases eosinophilic airway inflammation, concomitant with a decrease in IL-4 and IL-5 expression.[225] Notably, the levels of soluble ST2 protein and IL-33 mRNA protein are increased in sera and tissues from patients with asthma.[226,227] Genome-wide association studies have identified polymorphisms in the genes encoding Il1rl1 and Il33 in patients with asthma, hypereosinophilic diseases, and allergic rhinitis, suggesting an association with atopic disorders in general.[228,229,230]

Although the exact mechanisms by which IL-33 induces type 2 cytokine production are unknown, it has been shown to induce Th2 cytokines from a number of innate immune cells including basophils, mast cells, and the newly described ILC populations. In support of a role for the activation of ILCs by IL-33, it has recently been shown that administration of rIL-33 induces AHR and eosinophilic inflammation in mice independently of adaptive immune cells.[224] Interestingly, it was initially reported that OVA-induced AHR was not dependent on ILCs,[231] whereas another study suggest that fungal antigen-induced allergic inflammation was dependent upon IL-33 and innate lymphoid populations.[232] Collectively, these results suggested that antigens, which contain PAMPs that activate or injure the epithelium, recruit and/or activate ILCs. Consistent with this hypothesis, it has recently been shown that IL-13-producing ILCs are important in protection of the mucosal epithelium following viral infections, despite the fact that the type 2 cytokines do not play a role in clearance of the virus.[233] In support of a role for ILCs in allergic disorders, Mjosberg et al.[234] recently reported that a lineage-negative CD127(+)CD161(+) ILC population that responded to both IL-25 and IL-33 by producing IL-13 was enrichment in nasal polyps of patients with chronic rhinosinusitis. Although much remains to be learned about the role of ILC2 cells in type 2-mediated immunity, these new studies raise the possibility that expansion of these unique ILCs in response to exposure to allergens, infectious agents, or environmental toxins may unwittingly lead to the establishment of type 2-mediated diseases.

Antigen-Presenting Cells in Atopic Individuals

Although as discussed previously, type 2 immune responses can arise independently of conventional CD4+ T cells, it is clear that sensitization to allergens involves the development of memory T cells directed against these proteins, which requires the presentation of antigen to CD4+ T cells. A number of professional APCs such as DCs, Langerhans cells, B cells, and macrophages are present at mucosal surfaces and have the cellular specialization to capture and process antigen for presentation to T cells, it has become increasingly clear over the last

decade that DCs and Langerhans cells are the most important professional APCs at mucosal surfaces. However, recent evidence suggests that in specific instances, basophils may also serve as an important APC.

Dendritic Cells in Allergic Inflammation

DCs migrate as precursor cells to sites of the body where antigen entry occurs (ie, lung, skin, gastorintestinal tract). At these sites, they form an extensive network of cells, extending cell projections between resident cell types that ensure accessibility to allergens. DCs that reside in the periphery have an immature phenotype, specialized for uptake and recognition of antigens, but not yet capable of stimulating naive T cells, because they lack costimulatory molecules on their surface (CD80, CD86). Normally, DCs present antigens quite inefficiently; however, when antigen is encountered in an inflammatory context (eg, in the presence of antigen-derived danger signals) or through indirect sensing of “danger” from surrounding cells via the release of inflammatory cytokines, DCs undergo a developmental program referred to as maturation. During maturation, they lose their ability to take up antigen and acquire a phenotype of professional APCs expressing all the costimulatory molecules and chemokines required to attract and stimulate naïve T cells. Once DCs have acquired antigens at the mucosal surface, they migrate to T-cell areas of draining lymph nodes and present their antigen cargo to naive T cells. During this interaction with naive T cells in the lymph nodes, DCs have an opportunity to influence T-cell differentiation along a Th1, Th2, Th17, or T_{reg} cell pattern by secretion of a specific cytokine pattern, depending upon their lineage, their maturation status, and the consequent costimulatory molecule pattern. Thus, alterations in the signals provided by DCs to T cells may underlie the development of polarized Th2-cell responses in the context of allergic disease. Considerable evidence is accumulating to support this tenet.

Indeed, several lines of evidence in murine models of allergic disease as well as in human atopic support a pivotal role for DCs in the development of allergic responses. For example, Lambrecht et al.[235] have demonstrated that infusion of antigen-pulsed bone marrow-derived DCs into the respiratory tract of mice primed them for development of Th2-mediated immune responses upon subsequent antigen challenge. Conversely, using a suicide gene approach to specifically deplete DCs, it was demonstrated that antigen exposure did not induce allergic responses in the absence of DCs. In humans, a critical role for DCs in generation of allergic responses is the demonstration that the numbers of HLA-DR-expressing DCs are increased in the mucosal tissues of patients with asthma, allergic rhinitic, or AD when compared to the low level of DCs found in specimens from normal controls.[236,237,238,239] Moreover, the improvement in lung function associated with regular use of steroids is associated with a reduction in the number of CD1a+HLA-DR+DCs in the airways of patients with asthma.[240]

DCs play a major role in the surveillance of peripheral tissue sites for incoming antigens, a function that is dependent on their capacity for tightly controlled migration between different compartments. Under steady state conditions, there is a continuous turnover of DCs at mucosal surfaces, with the recruitment of immature DCs from circulating precursors, balanced by the exit of mature, antigen-loaded cells to regional lymph nodes. In both humans and mice, DCs are not constitutively present in the tracheal-bronchial mucosa in the first year of life[241]; their occurrence appears to be dependent on exposure to inflammatory stimuli from

the local environment.[242,243] Although the exact signals that regulate the rapid recruitment of immature DCs from the blood into the mucosal tissues are not fully understood, exposure to a number of environmental triggers (allergens, particulate matter, smoke, RSV, ozone) have been shown to dramatically increase the recruitment of DCs in both animal models of atopic disease and atopic humans.[243,244] These triggers likely attract DCs to mucosal surfaces through their ability to induce a set of chemokines that bind to receptors exclusively expressed on immature DCs. Indeed, it has been shown that epithelial cells synthesize and secrete the CCR6 ligand, MIP-3α/CCL20, in response to allergens and particulate matter.[245] Interestingly though, Der p1 stimulation of bronchial epithelial cells from patients with asthma results in CCL20 production and migration of Langerhans cell precursors; however, this is not observed in cells from healthy individuals.[246] The ability of these triggers to induce DC chemokines may explain the adjuvant effect of each of these irritants in induction of allergic responses. These studies suggest that responsiveness of mucosal epithelial cells to certain pathogens and/or environmental stimuli early in childhood may enhance or inhibit sensitization to allergens by affecting DC recruitment.

The maturational state of DCs is thought to be an important determinant of the development of tolerance or immunity. It has been suggested that DC immaturity may contribute to the development of tolerance. Indeed, studies from both animal models and atopic humans suggest that the magnitude and pattern of costimulatory molecule expression on DCs is strongly associated with susceptibility to atopic disease. In this regard, DCs influence the nature of the immune response to antigen stimulation through the balance of positive signals provided by CD28 engagement of B7-1, B7-2, B7-H3, and B7-H4 on resting T cells and negative signals provided through ligation of CTLA4 and PD-1 on resting T cells. Indeed, differences in the relative expression of these positive and negative signals have been shown to be important in various murine models of allergic disease. Specifically, it has been reported in numerous studies that Th2-mediated immune responses are predominantly dependent upon DC expression of CD86, not CD80.[247] In contrast, stimulation of CTLA4 and BTLA have been shown to decrease allergic responses.[248] CTLA4 in particular is predominantly expressed on T_{reg}s and is thought to mediate its inhibitory effects through T_{reg} suppressive functions. While studies have shown that B7RPICOS interactions mediate tolerance through the induction of IL-10-secreting Tr1 cells,[249,250] another study has reported that ICOS-ICOSL interactions positively regulates allergic responses.[251]

Several studies have suggested that DCs from atopic individuals have a different phenotype from those of nonatopic individuals.[252,253,254,255] First, the functional capacity of DCs from atopic individuals has been elegantly studied in a humanized severe combined immune deficiency mouse model.[254] In this model, it has been demonstrated that adoptive transfer of DCs from atopic individuals reconstitutes Th2-polarized immunity associated with allergic inflammation in the lungs of mice. In contrast, transfer of DCs from nonatopics resulted in combined Th1/Th2 responses. Purified T cells from house dust mite-sensitive patients stimulated by autologous Der p1-pulsed DC preferentially produced IL-4.[255] Interestingly, DCs from nonatopic individuals stimulated with Der p 1 expressed CD80, produced IL-12, and stimulated IFNγ production in T cells. Another intriguing finding of this study was that the effects of Der p1 on costimulatory molecule expression in DCs from allergic patients was

dependent on the enzymatic activity of Der p1, as a cysteine protease inhibitor prevented these effects. It has also been shown that differences in inhibitory molecule expression may play a role in susceptibility to allergic disease. Specifically, Akdis et al.[255] showed that allergenspecific (Der p1) T cells from nonatopic individuals produced high levels of IL-10 that were associated with elevated T-cell surface expression of IL-10R, CTLA4, TGF-βR1 and Il, CD25, and PD-1 as compared to those of atopic individuals. Neutralization of these suppressor pathways led to increased production of Th2 cytokines, suggesting that ligation of these inhibitory receptors on T cells by their DC ligands is critical in maintaining the balance between Th2 and tolerogenic T-cell responses. These studies suggest that DCs from atopic individuals display altered costimulatory molecule patterns as compared to normal individuals in response to direct stimulation with allergens and the PAMPs they may contain. Although the pattern and level of expression of costimulatory molecules on DCs and their ligands on T cells may be controlled by exogenous signals, polymorphisms in several costimulatory molecules have been shown to be associated with increased risk of development of Th2-mediated immune responses and atopic diseases such as asthma and atopic dermatitis. Specifically, polymorphisms in ICOS (1413G/A) are associated with allergic sensitization and Th2 cytokine production,[256] and polymorphisms in the CTLA4 gene are associated with infant atopic dermatitis[257] and asthma.[258,259]

Several lines of evidence suggest that alterations in the relative proportion of specific DC subsets taking up antigen at mucosal surfaces may play an important role in the development of immunity or tolerance. For example, adoptive transfer of mDC to the murine airway prior to antigen exposure has been shown to induce Th2-mediated immune responses to a tolerogenic antigen,[235] whereas depletion of plasmacytoid DC (pDC) in mice during inhalation of the normally inert antigen OVA via the airways without systemic priming led to a Th2 response and all the features of asthma.[260] These results strongly suggested that pDCs might provide intrinsic protection against inflammatory responses

to harmless antigens. Although the precise mechanisms by which pDCs promote tolerance are unknown, several possibilities exist. First, pDCs can produce the tryptophanmetabolizing enzyme indoleamine 2,3-dioxygenase, which has a strong inhibitory activity on T-cell proliferation.[261] Moreover, human pDCs activated by CpG oligonucleotides induce the generation of T_{reg}s.[262] Although the exact factors regulating the recruitment and/or activation of different DC subsets is unknown, Kohl et al.[263] have shown that complement activation at the airway surface is an important regulator of DC subset recruitment and activation. Specifically, they demonstrate that C5a preferentially results in the recruitment of pDCs to the lung, which is associated with protection against the development of Th2-mediated allergic airway responses. Taken together, these studies suggest that the balance between mDCs and pDCs may play a pivotal role in T-cell responses to antigen encounter at mucosal surfaces with myeloid cells driving immunity, while pDCs mediate tolerance to mucosal antigens.

Due to the difficulty in sampling DC populations in tissues from atopic individuals, the role of mDCs and pDCs in atopic individuals remains controversial. For example, Hagendorens and colleagues[264] compared DC populations in cord blood from children at low versus high risk of atopy and did not detect any differences. In contrast, a moderate reduction in pDC

numbers in the blood was reported in atopic asthmatic children compared with healthy controls, which the authors suggested might be due to increased migration of pDCs to the target organ. In contrast, Matsuda et al.[265] showed that adult patients with asthma have increased numbers of pDCs in the blood as compared to healthy controls. In patients with atopic dermatitis, Hashizume et al.[266] reported an overall increase in the numbers of DCs in the blood with a relative increase in pDCs over mDCs. However, when DCs were evaluated in the nasal epithelium of atopic rhinitis patients, the number of pDCs was reduced. Although there is controversy as to the relative proportion of individual DC subsests present in the blood and tissues of atopic individuals, there is evidence that DC subsets may be functionally different between atopic and healthy individuals. Specifically, Der p1-pulsed mDCs from healthy donors produced IL-10, whereas mDCs from allergic patients did not.[267] Although the study of DC subsets in human disease is in its infancy, these studies suggest that differences in the balance of DC subsets and their activation state may play an important role in determining the development of tolerance or immunity to environmental antigens. However, additional studies of DC subsets in relevant tissues are needed. Collectively, these studies suggest that altered DC function may underlie the propensity of atopic individuals to mount Th2-biased immune responses to environmental allergens.

Basophils as Drivers of Th2 Immunity

Although there is a wealth of evidence suggesting a pivotal role for DC in driving Th2 immunity, recent evidence suggests that they may not be sufficient to induce Th2 immune response to certain allergens. Medzhitov and colleagues recently provided surprising evidence that MHCII-positive IL-4-producing basophils were both necessary and sufficient for the generation of type 2 immunity to protease antigens.[268,269] Specifically, they demonstrated that immunization with the cysteine protease allergen papain resulted in the transient recruitment of basophils to lymph nodes that peaked 1 day prior to the peak of IL-4-producing CD4+ T cells. These papain-elicited basophils within the lymph node were shown to express IL-4 and TSLP. In vivo depletion of TSLP or basophils correlated with impaired Th2 cell differentiation following papain immunization, suggesting a role for basophil-derived TSLP in papain-mediated Th2 cytokine responses. Of note, treatment of basophils with papain in vitro also resulted in the production of IL-4 independently of IgE. These findings were unexpected as basophils had been thought to be MHC class II negative. However, the authors showed in a subsequent study that papain stimulation upregulated the basophil expression of MHCII and costimulatory molecules.[268] Moreover, papain stimulation of basophils induced the production of IL-2, IL-4, IL-6, and IL-13. They formally demonstrated the antigen-presenting capability of basophils in an adoptive transfer experiment in which antigen-loaded MHCII basophils transferred into Ciita-deficient mice induced Th2-mediated responses following papain exposure. Quite surprising, the authors found no role for traditional DCs in papain-induced Th2 immune responses in vivo. These results suggested that through their ability to rapidly secrete IL-4 and TSLP in response to both proteolytic allergens and IgE stimulation, basophils are uniquely poised at the interface between the innate sensing of antigens and the initiation of adaptive Th2 cytokine responses.

Lambrecht and colleagues[270] subsequently examined the role of basophils to dust mite allergens and found that IL-4 competent basophils and inflammatory DCs were recruited to the draining mediastinal lymph nodes in a TLR4-/MyD88-dependent manner. They also

demonstrated that depletion of basophils only partially reduced Th2 immunity and that they did not take up inhaled dust mite antigens, present them to T cells, or express antigen-presentation machinery, whereas a population of FcεRI+DCs readily did. Inflammatory DCs were necessary and sufficient for induction of Th2 immunity and features of asthma, whereas basophils were not required. The discrepancies in these two studies may reflect differing requirements for Th2-inducing pathways depending on the nature of the allergen and its adjunct properties or the fact that a population of FcεR1-positive cells contributed to the results of the Medzhitov study.

In an elegant series of studies, Tang et al.[271] resolved the conflict between the DC and basophil camps by showing that neither DCs nor basophils were sufficient to drive Th2 immune responses to papain immunization, but that they worked in concert to drive robust Th2 immune responses. Mechanistically, they found that DCs were essential for providing sufficient antigen presentation and costimulation to drive T-cell proliferation, but were unable to induce sufficient IL-4 production to drive Th2 differentiation. In contrast, basophils provided the critical IL-4 production, but were unable to drive T-cell proliferation. Thus, together they synergized to drive robust Th2 immune responses to papain. Their studies also provided a putative scheme by

which papain may activate DCs and basophils. Papain plus antigen exposure initially induced ROS generation in lymph node DCs, dermal DCs, and epithelial cells of the skin. Subsequently, ROS orchestrated Th2 responses by inducing oxidized lipids that triggered the induction of TLR4-TRIF signaling and the upregulation of TSLP production by keratinocytes. TSLP subsequently drove Th2-cell differentiation by suppressing production of the Th1-inducing molecules IL-12 and CD70 in lymph node DCs and by inducing the chemokine CCL7, which mediated recruitment of IL-4+ basophils to the lymph node. Whether basophils contribute to all allergen-driven Th2 immune responses remains unknown. These results suggest that the diversity of Th2 inducing pathways presumably reflects the functional diversity of allergens that can trigger them (Fig. 45.5).

Alterations in Molecular Mechanisms of Th2 Cell Differentiation

Although the exact molecular events that drive Th2 differentiation have not been fully elucidated, a picture is beginning to emerge.[272] A model has been put forth in which following presentation of antigen in the context of MHC II and TCR stimulation of naïve CD4+ T cells, a modest increase in the mRNA levels of GATA3, the master regulator of Th2 differentiation,[273] occurs that steadily accumulates over the first 24 hours. Once produced, GATA-3 remodels chromatin (demethylation) around the Th2 cytokine locus resulting in opening of the Th2 locus and marked upregulation of IL-4.[272] GATA-3 also enhances Th2 differentiation by inhibiting expression of the IL-12 receptor B2 chain and the Stat4 genes, which are required for Th1 differentiation programming. In parallel, TCR stimulation induces IL-2 production, which in turn activates STAT5.[274,275] TSLP and IL-7 may also contribute to STAT5 activation. The combination of STAT5 activation and GATA3 induction stabilizes Il4 expression in developing Th2 cells.[276] Another potential signal involved in early IL-4 production is the binding of Jagged-1 on APCs with Notch ICD expressed on naive CD4 T cells.[183] The induction of IL-4 by Notch is thought to be partially due to its ability to increase

expression of GATA3.[277]

Once the early IL-4 accumulates, it binds its receptor composed of the IL-4Rα and the common gamma chain (γc) and activates STAT6, which further enhances GATA-3 and induces the expression of other Th2-specific factors such as c-maf,[278] which together in combination with continued STAT5 activation results in further upregulation of IL-4, initiating a positive feedback loop that drives commitment to a Th2 phenotype. The expression of specific Th2 cytokine genes (IL-4, IL-5, IL-13, IL-9) is controlled by the synergistic actions of the GATA-3 and c-Maf, and by the more widely expressed and transiently induced transcription factors such as NF-ATc, NF-κB, AP-1, and c-EBPb, respectively.[277] NFATc, unlike GATA3, has been shown to directly activate the IL-4 promoter.[279] In contrast, NF-κB appears to be more important for antigen-induced IL-5 gene expression than for IL-4 gene expression.[280] During subsequent antigenic stimulation of the effector/memory cells, which maintain an open chromatin structure with high levels of GATA-3 and c-Maf expression, they are able to rapidly induce Th2 cytokine gene expression.

Altered regulation of one or more of the primary transcription factors involved in Th2 differentiation may drive susceptibility to the development of allergic diseases. This is supported by several lines of evidence including the fact that GATA-3 expression has been shown to be elevated in bronchoalveolar lavage fluids and bronchial biopsies of asthmatics when compared to normals.[281] There are several lines of evidence that support a role for altered IL-4R signaling in Th2 polarization in atopic disorders including the following 1) a specific polymorphism in the IL-4 gene itself has been shown to correlate with high serum IgE levels and enhanced IL-4 gene expression[282]; 2) a similar association has been reported between the T allele of the -590C/T polymorphism of the IL-4 gene promoter region and atopic dermatitis[283]; and lastly 3) a GT repeat polymorphism in the first exon of the STAT6 gene has recently been associated with increased prevalence of several atopic disorders (bronchial asthma, atopic dermatitis, food allergies).[284] Although it is not currently known whether inheritance of a single gene variant or a combination of genetic variants is required to drive Th2-cell commitment in atopic individuals, considerable evidence is mounting to suggest that genetic differences in factors important in Th2-cell commitment may underlie susceptibility to development of atopic disorders.

Altered Tregulatory Cell-Promoting Cytokines in Atopic Disorders

Several lines of evidence suggest that the production of cytokines by DCs leading to T_{reg} generation is altered in atopic individuals. The DC cytokines that are known to regulate T_{reg} differentiation are IL-10 and TGF-β. Although altered levels of IL-10 have been observed in atopic disorders, the relationship is not straightforward. For example, there are conflicting reports about the levels of IL-10 in atopic patients, with some studies demonstrating elevations in IL-10 levels in bronchial biopsies,[285] peripheral blood mononuclear cells,[286] BAL fluids,[287] and in the gut mucosa of patients with asthma,[288] while others report diminished IL-10 production in sputum from patients with asthma[289] and isolated T cells from children with asthma or atopic dermatitis.[290] Results from animal studies show similar disparities. IL-10 knockout mice have been shown to have both reduced allergic

responses[291] as well as enhanced responses to allergen challenge.[292] Investigators have suggested that the discrepancies in the results of studies with IL-10-deficient mice may be dependent on the genetic background of the strain.[292] In contrast, studies in which animals have been treated with recombinant IL-10[293] or overexpress the IL-10 gene[294] uniformly demonstrate that IL-10 suppresses inflammation and decreases development of Th2-mediated immune responses. Moreover, adoptive transfer of IL-10-expressing DCs induced tolerance to allergen exposure.[295] The variability in the results may reflect the pleiotrophic actions of IL-10 during the course of allergic reactions. IL-10 can clearly influence T-cell differentiation through its inhibitory actions on IL-12 as well as its ability to reduce antigen presentation to

T cells by limiting class II, CD80, and CD86 expression on APCs. IL-10 not only generates tolerance in T cells, but it is a potent anti-inflammatory cytokine that can serve to dampen inflammation once initiated.[296] In this regard, it has been shown to reduce total and specific IgE production while increasing protective IgG4 levels.[297] The fact that steroids,[298] lactobacillus,[299] and standard immunotherapy regimes,[300] all of which successfully resolve symptoms in atopic diseases, induce IL-10 production suggests that perhaps IL-10 is protective and that IL-10 production may be impaired in atopic individuals. Diminished IL-10 production may lead both to loss of tolerance to environmental antigens as well as an inability to control inflammation once initiated. The elevations in IL-10 tissue levels observed during challenge with allergens may reflect compensatory mechanisms designed to suppress harmful inflammatory responses. In this regard, genetic polymorphisms in the promoter of the IL-10 gene (C to A change at position 571) have been associated with asthma and elevated serum IgE levels.[301]

Another DC-derived factor that regulates T_{reg} differentiation is TGF-β. Although TGF-β is a pleiotrophic cytokine, it has been shown to play an important role in T_{reg} differentiation and function. Specifically, it plays a role in the conversion of naïve CD4 T cells into the regulatory phenotype and has been reported to mediate the cell-contact dependent suppressive activity of T_{reg}s. Elemental to this conversion process is induction of expression of the forkhead transcription factor, Foxp3 in CD4+CD25- T cells by TGF-β. Importantly, in a murine asthma model, administration of these TGF-β-induced suppressor T cells prevented house dust mite-induced allergic responses.[302] More recent studies show that the induction of T_{reg} suppressor function and FoxP3 expression by TGF-β is dependent upon TGF-β's ability to enhance CD80:CTLA-4 interactions on T cells.[303] Consistent with an important role for TGF-β in regulation of T-cell differentiation, several studies,[304] but not all,[305] suggest that SNPs in the TGF-β1 gene may be associated with atopic and asthma phenotypes.[255] Collectively, these studies suggest that dysregulation of factors controlling T_{reg} cell differentiation and/or function may predispose to the development of deleterious Th2-mediated allergic responses to environmental antigens.

TH2 CYTOKINE REGULATION OF ALLERGIC INFLAMMATION

The dependence of the immunopathogenic consequences of allergic immune responses on

Th2 cells likely stems from their pivotal role in regulating the primary effectors of both the acute- and late-phase reactions, namely, IgE and eosinophils. In addition, as will be discussed in a later section, Th2 cell-derived cytokines themselves also serve as effector cells of the allergic response.

One of the major roles of IL-4 in allergic inflammation is as the primary inducer of Ig-class switching in B cells that leads to the synthesis and secretion of IgE.[306] The importance of IL-4 to IgE synthesis has been demonstrated by the fact that STAT6-deficient mice do not produce IgE.[307] The exact mechanisms by which IL-4 regulates IgE class switching will be discussed in the following. IL-13 is also able to regulate IgE synthesis in humans, but its role in IgE synthesis in mice is controversial.[308] The combination of the effects of IL-4 on IgE synthesis and mast cell growth suggests a primary role for IL-4 in the development of the early-phase response. As will be discussed in greater detail in a later section, IgE activation of mast cells leads to the synthesis and release of a number of inflammatory mediators that may contribute to the vascular, smooth muscle, and mucus changes observed in the early-phase response to allergen challenge.

Through their unique and overlapping actions, IL-4, IL-13, and IL-5 coordinately regulate the development, recruitment, and activation of eosinophils. IL-4 and IL-13 have both been shown to contribute to the recruitment of eosinophils into sites of inflammation as evidenced by the fact that inhibition of either IL-4 or IL-13 by antibody blockade or gene deletion eliminates allergen-driven increases in tissue eosinophils.[50,52] Because IL-5 has been shown to be the primary determinant of eosinophil differentiation, activation, and survival, it plays a critical role in eosinophil regulation in allergic responses. The importance of IL-5 in antigen-induced eosinophilia has been examined in numerous animal studies.[51,53] For example, blockade of endogenous IL-5 levels in antigen-sensitized mice has resulted in significant suppression of both BAL and tissue eosinophilia.[51] More definitively, mice in which the IL-5 gene has been disrupted do not develop eosinophilic inflammation.[53] Reconstitution of these mice with IL-5 completely restored aero-allergen-induced eosinophilia. Thus through the coordinate regulation of IgE and eosinophilia, Th2 cytokines orchestrate the elicitation phase of allergic immune responses. In the next section, we discuss in more detail the steps involved in IgE regulation.

REGULATION OF IMMUNOGLOBULIN E SYNTHESIS

Ishizaka and colleagues purified IgE in 1966.[4,309] IgE has the shortest half-life (2.5 days) of all classes of Igs. In addition, it is present in serum at levels considerably lower than other Ig classes such as IgG. There is considerable heterogeneity in the levels of IgE among individuals. For example, levels of <100 ng/mL are observed in most normal individuals, whereas in parasitized or atopic individuals IgE levels can reach as high as 1000 ng/mL. The high variability in serum levels contrasts strikingly to other Ig isotypes and suggests that tight control of IgE may therefore be important to prevent the potentially lethal consequences of IgE-mediated inflammation. Indeed, synthesis of IgE and its receptor expression appear to be regulated by a series of steps involving both cell-cell contact with CD4+ Th2 cells and activation by cytokines secreted by these cells. In this section, we will discuss our current knowledge of the complex steps involved in regulation of IgE synthesis and expression of its receptors.

Isotype Class Switching to Immunoglobulin E Production

The production of IgE antibodies by B cells is triggered by a complex series of secreted signals and cell surface

interactions, followed by molecular genetic rearrangements at the Ig heavy chain locus, IgH (Figs. 45.6 and 45.7). The first step in IgE production is the binding of allergen to allergen-specific B cells via their membrane-bound Ig receptor. The B cells then internalize and process the allergen, and present the processed allergen to T cells as peptide fragments in association with MHC II molecules. The peptide-MHC II complex is then recognized by the TCR on Th2 cells. Initially, all B cells produce IgM antibodies. At this point, a $V_H(D)J_H$ cassette of sequences encoding the variable domain is immediately adjacent to the Cμ exons, which encode the IgM constant regions at the 5′ end of the IgH locus. Further downstream in IgH are several widely spaced clusters of exons. C regions encode the constant region domains of the IgG, IgE, and IgA heavy chain isotypes. Upon stimulation by cytokines, along with critical cell-cell interactions with CD4+ T cell surface accessory molecules, B cells can change the isotype of the antibodies they produce while retaining their original antigenic specificity. This process requires that genomic DNA be spliced and rejoined to move the VDJ elements from their location proximal to Cμ to a position many kilobases downstream next to the C-region exons encoding the heavy chains of other isotypes. A large amount of intervening DNA is excised and discarded in this irreversible process, and therefore the mechanism is referred to as deletional switch recombination.

Isotype switching to IgE production requires two signals.[310] The first signal is provided by the Th2 cytokines, IL-4, and IL-13, and is IgE isotype specific. IL-4 and IL-13 stimulate transcription at the Cε gene locus, which contains the exons encoding the constant-region domains of the IgE ε heavy chain. This transcription is initiated at a site upstream the ε switch region designated Iε. The second signal is a B cell-activating signal provided through CD40/CD40L interactions. Together, these interactions result in induction of the necessary deletional switch recombination that brings into proximity all of the elements of a functional ε heavy chain.

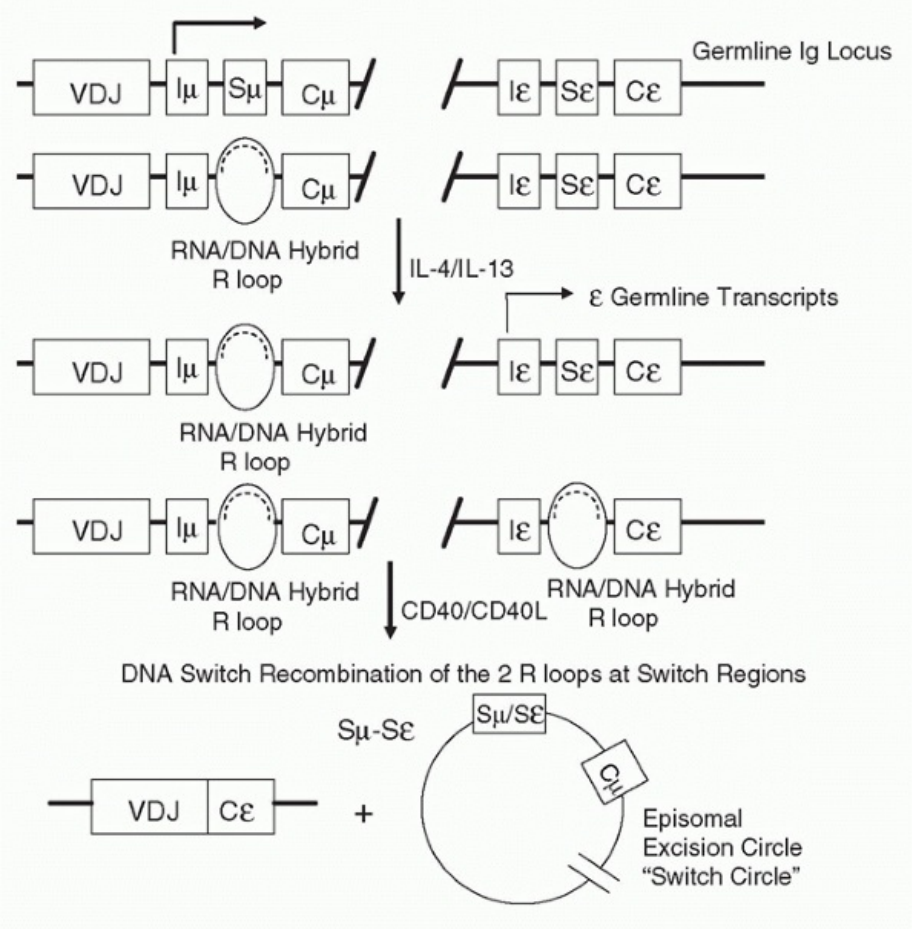

FIG. 45.6. Schematic Representation of the Molecular Steps Involved in Immunoglobulin E Class Switching. In naïve resting B cells, the VDJ sequences encoding the variable region are located at the 5′ end of the Ig locus. After stimulation by interleukin (IL)-4 or IL-13, transcription is initiated at the Iε promotor to produce ε germline transcripts. Sε ribonucleic acid (RNA) remains hybridized to the Sε deoxyribonucleic acid (DNA) forming an RNA-DNA hybrid structure, called an R loop. The R loop serves as a substrate for nucleases that result in doublestranded DNA breaks. Switch recombination and joining is dependent on a second signal provided by the CD40/CD40L interaction. This process results in the formation of an episomal excision circle that is eventually lost during cell division.

The first signal for IgE class switching is provided by the T cell-derived cytokines, IL-4/IL-13. IL-4 induces ribonucleic acid (RNA) transcription at the Cε locus via stimulation of STAT6 through binding the type I IL-4 receptor composed of the IL-4Rα chain and the γ c chain or, if expressed, the type II receptor composed of the IL-4Rα and IL-13Rα1 chains. IL-13 has also been shown to regulate IgE class switching in humans through binding the type II receptor. However, there is considerable controversy about the role of IL-13 in IgE class switching in mice. Following either IL-4 or IL-13 binding to its respective receptor complex, JAK kinases phosphorylate tryrosine residues in the intracellular domains of the receptor chains, providing docking sites for STAT6. These STAT6 molecules become phosphorylated and then form homodimers that translocate to the nucleus. In the nucleus, they bind to specific sequences (TTCN(N)GAA) in the promoter of IL-4/IL-13 responsive genes, including Iε. The importance

of STAT6 in IL-4-induced isotype switching is supported by the fact that germline transcription and IgE class switching are markedly impaired in STAT6-deficient mice.[311] Other transcription

factors are also important in induction of germline transcription of Cε locus. The importance of NF-κB for the induction of germline transcripts has been recently confirmed by the finding that expression of germ line transcripts for Cε are severely diminished in NF-κB p50 knockout mice.[312]

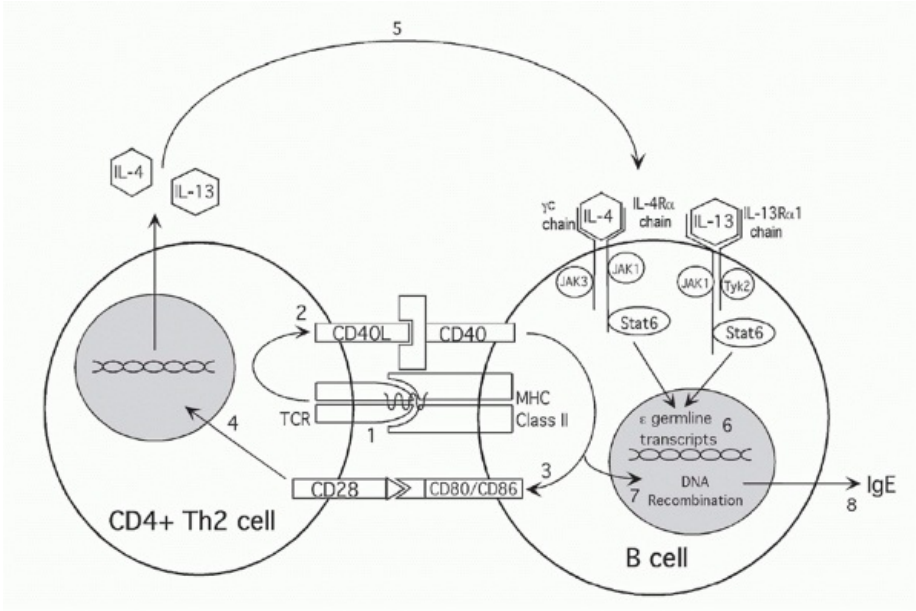

FIG. 45.7. The regulation of immunoglobulin E synthesis involves many sequential steps: 1) recognition of antigen/major histocompatibility complex class II by the T-cell receptor (TCR); 2) TCR activation leads to the induction of cluster of differentiation (CD)40 ligand on the T cell, which is then recognized by CD40 on the B cell; 3) CD40/CD40L cognate interaction leads to induction of CD80/CD86 on the B cell, which can then interact with CD28; 4) the Th2 cell secretes IL-4 and IL-13; 5) IL-4 and IL-13 bind their cognate receptors on B cells and result in activation of Stat6; 6) IL-4 and IL-13 signaling events result in initiation of transcription at the Iε promotor and the production of immature germline ε transcripts; 7) the CD40/CD40L interaction provides the necessary second signal required for recombination of the germline locus; and 8) transcription of mature ε transcripts from the rearranged locus followed by translation and secretion of IgE.

IL-4- and/or IL-13-induced transcription factor binding to the Cε locus results in germline transcription of the Cε locus. Germline transcripts originate from a 5′ promoter of the Iε exon, which is located just upstream of the 4 Cε exons. IL-4 induces the appearance of 1.7 to 1.9 kb germ-line Cε transcripts that contain an Iε exon, located 2 kb upstream of Sε, spliced to the Cε1-Cε4 exons. After processing, the mature germline mRNAs include the 140-bp Iεexon and exons Cε1-Cε4. These transcripts have been referred to as sterile because of the presence of stop codons in each of the three reading frames of Iε. However, it is the process of transcription itself that appears to facilitate the deletional switch recombination event. For

example, Harriman et al.[313] analyzed IgA switching using mice retaining a normal Iα promoter in which the Iα exon was replaced by an HPRT minigene and found that switching can occur to the locus despite the absence of complete Iα-containing transcripts. This has been demonstrated at the Cε locus in that switch recombination occurred at the Cε locus when the Iε exon and promoter were intact, but Cε exons were absent.[314]

The second signal for IgE class switching is dependent on cell-to-cell contact between T and B cells. Specifically, the interaction between CD40 on the surface of B cells with CD40L on the T-cell surface is critical to drive the IgE switch to completion and lead to IgE production. CD40 is a 50kd surface glycoprotein that is constitutively expressed on all human B-lymphocytes. CD40L is transiently induced on T cells after stimulation of the TCR by antigen/MHC complexes. Binding of newly expressed CD40L with CD40 on B cells provides the second signal for induction of deletional switch recombination to IgE.

Several lines of evidence support a critical role for CD40/CD40L interactions in isotype switching. First, it had been shown by numerous groups that isotype switching required the presence and contact with T cells. After an extensive search for the T-cell contact signal, it was discovered that CD40/CD40L interactions were responsible for the T-cell dependency of this process. Proof of this was provided by the observation that activation of CD40L could completely

substitute for T-cell help.[315] Furthermore, a soluble form of CD40 inhibits its interactions with CD40L, blocking IL-4 driven IgE synthesis in human B cells.[316] Lastly, genetic deficiencies in CD40L or CD40 in humans and mice, respectively, disrupt IgE synthesis. In humans, CD40L is encoded on the X chromosome and individuals with the X-linked hyper-IgM syndrome are deficient in CD40L. Their B cells are unable to produce IgG, IgA, or IgE.[317] Similarly, mice deficient in either the CD40L or CD40 genes have the same defect in antibody production.[318,319]

CD40/CD40L interactions are thought to provide a second signal via stimulation of a number of signaling pathways that likely synergize with those initiated by IL-4 and IL-13 to achieve ε-germline transcription. Specifically, after interaction with CD40L on the B-cell surface, CD40 aggregation triggers signal transduction through four intracellular proteins, which belong to the family of TNF receptor-associated factors (TRAFs). TRAFs-2, -5, and -6 are known to associate with the intracytoplasmic domain of CD40 after its multimerization by interaction with CD40L. TRAF-2, TRAF-5, and TRAF-6 promote the dissociation of NF-κB from its inhibitor, IκB. In turn, NF-κB can synergize with STAT6 induced by IL-4/IL-13 signaling to activate the Iε promoter, as described previously. In addition to triggering TRAF associations, engagement of CD40 activates protein tyrosine kinases, such as JAK kinases.

Another mechanism by which cytokine and CD40L activation may induce class switching is via induction of expression of proteins required in deletional recombination. Specifically, both cytokine and CD40L induction of class switching has been shown to require the synthesis of new proteins. One of these proteins is activation-induced cytidine deaminase (AID).[320] AID is expressed in activated B cells and in germinal centers of lymph nodes. Mice deficient in AID have a dramatic impairment in isotype switching, with elevated IgM levels and low or absent IgE, IgG, and IgA isotypes.[321] Interestingly, a rare autosomal form of hyper-IgM syndrome

has now been attributed to mutations in this gene.[322] These studies indicate that this protein is critical to isotype switching.

Although not completely elucidated, the mechanisms through which AID is recruited to the appropriate S region and facilitates class switch recombination are becoming clearer. It appears that regulation of AID activity occurs in two distinct phases: recruitment of AID to the S regions at both the donor and acceptor sites, and then local control of AID activity. In the first phase, once chromatin is opened at a given germline Ig locus (in the case of Cε, triggered by stimulation through CD40 in the presence of IL-4), gene transcription begins through the Iε and Sε regions. Due to the highly repetitive nature of the S regions, RNA Pol II stalls during transcription. Stalled RNA Pol II is known to be bound by Spt5. RNA Pol II-bound Spt5 then recruits AID to the complex, effectively targeting the S regions (both Sε and Sμ) for AID recruitment. Although Spt5 is a critical factor, there is additional evidence that both the scaffold protein 14-3-3 (perhaps through direct interactions with ACGT sequence in the S region) and PTBP2 are also involved in recruiting AID to the switch regions.

Once recruited, AID is activated by phosphorylation of S38 by the protein kinase PKA, which associates independently with the S region. Also involved in activating AID is PP2A, which removes an inhibitory phosphate at S3. Phosphorylation S3 appears to limit the ability of AID to associate with the Sμ region. Once activated, AID is then able to deaminate cytidines in both strands of DNA (partly through interactions with RPA and the RNA exosome), converting them to uracils. These uracil bases are subsequently removed by uracil DNA glycosylase, creating multiple abasic sites, in both DNA strands. Two enzymes, AP endonuclease 1 and AP endonuclease 2 then create breaks at the abasic sites, resulting in double-stranded breaks in both donor (in this case, Sμ) and acceptor (Sε) sites. The intervening DNA is spliced out, and the two switch regions are joined together, placing the Cε locus immediately adjacent to the VDJ regions.[323]

Negative Regulation of Immunoglobulin E Synthesis

Several cytokines including TGF-β, IFNα, and IFNγ can inhibit IL-4-dependent IgE synthesis in both mice[324] and humans.[325] IFNγ suppresses the expression of ε germline transcripts in murine B cells stimulated with IL-4 and LPS. IFNγ may affect recombination events without affecting the expression of ε germ-line transcripts. Other agents, including IL-12[326] and IL-10,[297] have been reported to inhibit IgE while enhancing IgG4.[326] Interestingly, IL-21 has recently been shown to inhibit IgE isotype class switching and IgE+ cell clonal expansion.[327] Although the exact mechanism by which IL-21 regulates IgE production is not entirely understood, it is thought that IL-21 directly antagonizes IL-4- and LPS-induced Iε switch recombination.[328] Because IL-21 does not alter proliferation or STAT6 phosphorylation of murine B cells in response to IL-4, the mechanism is unknown. Recent studies suggest that IL-21 regulates IgE synthesis through STAT3-mediated processes,[329] and indeed patients with mutations in STAT3 do not respond to IL-21.[330] Mutations in STAT3 are associated with hyper-IgE syndrome, a condition in which patients have extremely elevated serum IgE levels and recurrent eczema.[330] Another mechanism of regulation of IgE is through the competition with IL-4-driven IgG4 blocking antibodies. IgE mediated-hypersensitivity reactions are rare in patients with chronic helminth infections even though FcεRI-bearing cells are sensitized with

antiparasite IgE. The inhibition of allergic reactivity is mainly due to IgG4-blocking antibodies as evidenced by the fact that the depletion of IgG4 specifically removes the blocking activity from the sera of microfilaremic patients. IgG4-blocking activity has also been detected in patients receiving immunotherapy for insect venom[331] and house dust mite hypersensitivity.[332] Blocking IgG4 competes with cell-bound IgE for allergen binding. Because the IgG4 molecule is functionally monovalent, does not fix complement, and binds weakly to Fcγ receptors, antigen binding to IgG4 has no harmful consequences. However, competition between IgG4 and IgE can occur only if the two antibody isotypes have at least in part the same antigen specificity. A molecular mechanism for this overlap in specificity was provided by the demonstration that isotype switching can occur sequentially from IgM to IgE through IgG4. This has been formally demonstrated in that IgG antibody in mice can completely suppress IgE-mediated anaphylaxis.[333]

Immunoglobulin E Receptors

The two major Fc receptors for IgE are called FcεRI and FcεRII (CD23).[334] They are distinguished by their structure and their relative affinities for IgE. The high affinity IgE receptor FcεRI binds monomeric IgE with an affinity constant of $10^{10}M^{-1}$ while CD23 binds with a much lower affinity ($10^{8}M^{-1}$). The high-affinity receptors are constitutively expressed at high levels on mast cells and basophils. They are also found, albeit at lower levels, on peripheral blood DCs, monocytes, and human Langerhans cells. The low-affinity receptor is also expressed on a wide variety of cells including B cells, T cells, Langerhans cells, monocytes, macrophages, platelets, and eosinophils.

FcεRI-Mediated Signal Transduction

FcεRI is a member of the multisubunit immune response receptor family of cell surface receptors that lack intrinsic enzymatic activity but transduce intracellular signals through association with cytoplasmic tyrosine kinases.[335] In rodents, FcεRI is expressed on mast cells and basophils as a heterotetramer consisting of a single IgE binding α subunit, a β subunit, and two disulfide-linked γ subunits. The α chain consists of two extracellular Ig-like loops, a single transmembrane region containing an aspartic acid residue, and a short cytoplasmic domain that lacks signal transduction motifs. The charged amino acid within the transmembrane domain mediates the association of the α subunit with the signaling component of the γ subunit. The β subunit consists of four membrane-spanning domains and a cytoplasmic tail capable of transducing intracellular signals that amplify γ-mediated signaling events. In rodents, all three subunits are required for the cell surface expression of FcεRI.[334,335] In contrast, in humans the receptor can be expressed as two different isoforms, a tetramer (αβγ2) or a trimer (αγ2). The tetrameric complex (αβγ2) is expressed on mast cells and basophils, whereas the trimer is expressed on Langerhans cells, DCs, monocytes and macrophages, and eosinophils.

FcεRI signaling occurs upon IgE binding to the receptor. This requires two sequential events: 1) binding of IgE antibody to the FcεRI, and 2) cross-linking of IgE antibody by bivalent or multivalent antigen. Cross-linking of the receptor initiates a coordinated sequence of biochemical and morphologic events that result in 1) exocytosis of secretory granules

containing histamine and other performed mediators, 2) synthesis and secretion of newly formed lipid mediators such as prostaglandins and leukotrienes, and 3) synthesis and secretion of cytokines. Although the exact signaling pathways governing each of these functions are not known, the following model has been proposed. The β and γ subunits each contain a conserved immunoreceptor tyrosine-based activation motif (ITAM) within their cytoplasmic tails that is rapidly phosphorylated on tyrosine after FcεRI aggregation.[335,336] Tyrosine phosphorylation of the β and γ ITAMs is mediated by Lyn, which is constitutively associated with the β subunit and activated after antigen mediated FcεRI aggregation. FcεRI cross-linking leads to recruitment and activation of the tyrosine kinase Syk, which binds to the tyrosine phosphorylated γ ITAMs through its tandem SH2 domains.[334] Recruitment of Syk occurs upstream of several signal transduction pathways. The importance of Syk is demonstrated by using Syk-deficient mast cells, which fail to degranulate, synthesize leukotrienes, and secrete cytokines after FcεRI stimulation.[337] Ultimately this pathway leads to the activation of mitogen-activated protein kinases and activation of protein kinase C pathways. Protein kinase C pathways are thought to be important in exocytosis and granule content release and gene expression. Activation of mitogen-activated protein kinases also regulates the enzymatic activity of PLA_2, leading to generation of a variety of lipid mediators (platelet-activating factor [PAF], PGD_2, and LTC_4). Antigen-mediated aggregation of FcεRI also stimulates the recruitment and activation of p21ras, which has been implicated in FcεRI induced cytokine transcription and secretion.[336]

Regulation of FcεRI Surface Expression

Although the expression of FcεRI on the surface of mast cells appears to occur early in their differentiation and/or maturation in vivo, the levels are known to be regulated by several factors post maturation. Studies in both mice and humans have revealed that the levels of FcεRI on mast cells can be regulated by IgE itself as well as by Th2 cytokines. Indeed, atopic individuals with high serum IgE levels show markedly upregulated mast cell and basophil FcεRI levels.[338,339] Moreover, anti-IgE treatment of atopic individuals results in downregulation of FcεRI expression on human basophils.[340] Further evidence is provided by murine studies in which IgE-deficient mice exhibit dramatically reduced levels of receptors on mast cells and basophils. As IgE-deficient mice still express receptors, albeit at lower levels, other mechanisms of regulation are thought to exist. In fact, cytokines such as IL-4 and IL-13 have been shown to upregulate FcεRIα expression on mast cells, basophils, and monocytes. Glucocorticoids have been shown to inhibit IL-4- and IL-13-induced upregulation of the FcεRIα chain on monocytes.[341] These results suggest that through a multistep positive feedback process, Th2 cytokines enhance both the production of IgE and the expression of its receptor, which leads to further mast cell activation and release of Th2 cytokines in the local microenvironment, serving to perpetuate the allergic response.

Mast cell activation is subject to negative regulation by a growing family of structurally and functionally related inhibitory receptors. These include FcγRIIB, CTLA4, killer cell inhibitory receptors, and gp^{49b1} on mast cells. Indeed, gp^{45b1}-deficient mice exhibit more severe anaphylactic reactions than their normal counterparts.[342] Each of these receptors possesses an immunoreceptor tyrosine-based inhibitory motif. Coaggregation of FcεRI and these inhibitory receptors on the surface of mast cells results in inhibition of FcεRI-induced

mast cell activation.[343,344] In general, inhibitory receptors are thought to inhibit the actions of activation receptors containing ITAMs by recruiting phosphatases through an immunoreceptor tyrosine-based inhibition motif.[345]

FcεRII (CD23)

In humans, CD23 (FcεRII, B cell differentiation antigen) is a calcium-dependent C-type lectin of 45 kDa. It has wide distribution among hematopoietic and structural cells and exists in two forms, CD23a and CD23b, resulting from alternative splicing at the N terminus and differing by five amino acids in the cytoplasmic domain. The isoforms of CD23 are found on B cells; one is constitutively expressed (CD23a), whereas the other form, CD23b, is induced by factors such as IL-4[346] and CD40L in conjunction with IL-4. CD23b is also found on non-B cells such as T cells, Langerhans cells, monocytes, macrophages, platelets, and eosinophils,[347,348] and mediates different biologic functions. The b form has been shown to be associated with phagocytosis of soluble IgE complexes, while the a form is associated with endocytosis of IgE-coated particules.[349]

Structurally, CD23 presents a single membrane-spanning domain followed by an extracellular domain that consists of three regions: the alpha-helical coiled coil stalk region, which mediates the formation of trimers, followed by the lectin head, which binds IgE, and at the C-terminus a short tail containing an inverse arginine-glycine-aspartate (RGD) sequence, a common recognition site of integrins.[350,351] CD23 is cleaved at the membrane to yield a series of soluble fragments. sCD23s of varying molecular weights arise by an autocatalytic process involving matrix metalloproteinase cleavage of membrane bound CD23. The endogenous proteases that participate in CD23 shedding have not been identified. Recently ADAM10 and metalloproteinase 9 have been shown to be CD23 sheddases.[352] CD23 expression is upregulated by several factors including by its ligand, IgE,[353] and by IL-4.[354] On the other hand, IFNγ counteracts the inducing effect of IL-4 on CD23 expression.

CD23 is thought to mediate a number of effects including regulation of IgE synthesis, antigen presentation, proliferation and differentiation of B cells, and activation of monocytes, effects that can be ascribed to the membrane and soluble forms of CD23.[354] Binding of IgE to the membrane bound form of the receptor transduces an inhibitory signal that prevents further IgE synthesis.[355] In contrast, the soluble forms described previously upregulate IgE production; their release has been found to be inhibited by IgE binding.[356] Soluble CD23 also ligates CD11b/CD11c to promote release of proinflammatory mediators such as IL-1β, IL-6, and TNF-α.[357]

CD23 expression on B cells and monocytes and sCD23 production is markedly increased in allergic disorders.[358,359] Moreover, reduction of allergen-induced CD23 expression on B cells has been observed after successful desensitization therapy.[359] A recent clinical trial using anti-CD23 (lumiliximab) directed against the lectin part of the molecule showed that while lumiliximab treatment of allergic patients reduced serum IgE levels by two-thirds, this reduction was insufficient to reduce disease symptoms.[360] This antibody is thought to work by mimicking IgE binding and stabilizing CD23 resulting in less cleavage by ADAM10.

Dysregulation of the CD23 pathway in atopic patients might be part of their propensity to develop IgE antibodies and may cause an enhancement of the inflammatory reaction through the action of sCD23 as well as through IgE-dependent triggering of CD23 on non B cells.

EFFECTOR CELLS OF THE ALLERGIC RESPONSE

Overview

Once a genetically susceptible individual is sensitized to a given allergen and IgE antibody has been formed, subsequent exposure to allergens readily induces the manifestations of atopic disease. Although these responses are generally a continium, they have been categorized into three types based on their temporal sequence: 1) acute or immediate responses, 2) late-phase reactions, and 3) chronic allergic inflammation.

Exposure of a sensitized individual to allergens results in immediate reactions, the characteristics of which are dependent upon the site of entry of the allergen. In the nasal mucosa, allergen provocation of sensitized individuals results in sneezing, itching, and nasal discharge. Acute allergic reactions, elicited in the skin at the sites of allergen injection, are characterized by intense itching, redness, and edema. In patients with asthma subjected to allergen inhalation, these mediators rapidly elicit bronchial mucosa edema, mucus production, and smooth muscle constriction. Acute or immediate responses are thought to be due to the release of preformed mediators released by antigen interaction with Fc receptors and IgE on IgE-bearing cells (mast cells and basophils). The release of mast cell products produce multiple local effects, including enhanced local vascular permeability (leading to leakage of plasma proteins, including fibrogen, resulting in local deposition of cross-linked fibrin and tissue swelling), increased cutaneous blood flow with intravascular fluid from postcapillary venules producing erythema, and other effects, such as itching, due to the stimulation of cutaneous sensory nerves by histamine. Typically, these reactions are detectable within a few minutes of allergen challenge, reach a maximum in 30 to 60 minutes, then rapidly wane.

In many individuals, the acute phase is followed by what has been termed a late-phase reaction that occurs within 6 to 48 hours after allergen exposure and can persist for several days without therapy. The characteristic signs and symptoms of late-phase reactions are reddening and swelling of the skin, sneezing and nasal discharge, and wheezing and cough upon lower airways challenge. Late-phase reactions are thought to occur as a result of recruitment of circulating leukocytes to the site of allergen exposure following antigen presentation to T cells. Both eosinophils and T cells are assumed to mediate the late-phase response. However, mast cells may also contribute to the late-phase response. The importance of leukocyte recruitment is supported by the fact that a variety of treatments that are associated with a reduction in the leukocyte recruitment elicited at sites of late-phase reactions can also reduce the signs and symptoms of these responses.

In naturally occurring allergic diseases, patients typically experience repeated exposure to the offending allergens over a period of weeks to years. Although the specific features of pathology of each of these diseases vary according to the anatomic site affected, it has been generally recognized that the structural changes that occur in each tissue are due

to the persistence of inflammation. These tissue changes range from thickened skin and fibrotic papules to extensive remodeling of the airway wall with smooth muscle hypertrophy, subepithelial fibrosis, and mucus cell hypertrophy. In each case, these structural changes are

associated with significant alterations in their function.

Development of Mast Cells and Basophils

History and Overview

Mast cells and basophils were discovered by Paul Ehrlich in the late 1800s based on staining of their cytoplasmic granules with aniline and basophilic dyes, respectively.[361] It was once thought that basophils might be circulating precursors of mast cells or that mast cells were "tissue basophils," but current evidence suggest that they are indeed distinct cell types. Although these cell types have unique functions and release a unique profile of mediators, they also produce an overlapping array of mediators that are known to contribute to the allergic diathesis. It is hypothesized that IgE produced by allergen-reactive B cells binds to FcεR receptors present on the surface of mast cells and basophils, and that when challenged with allergen, these cells release vasoactive mediators as well as chemotactic factors and cytokines that promote leukocyte infiltration and exacerbate the inflammatory response. Through the production and release of these proinflammatory molecules, mast cells and basophils set into motion a series of events that result in immediate responses to allergens in the skin, lungs, and nose of atopic individuals and may also contribute to the late-phase response (Fig. 45.8).

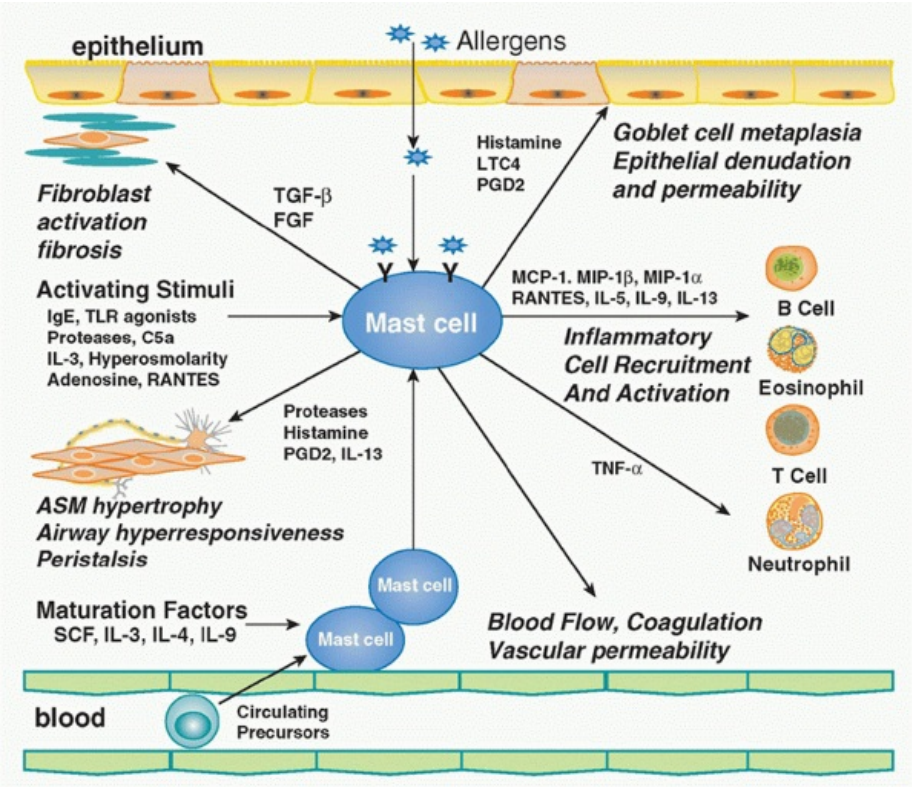

FIG. 45.8. Mast Cells are Produced in Bone Marrow and Released into Circulation as Immature Precursors. They mature in tissues under the influence of stem cell factor, interleukin (IL)-3, IL-4, and IL-9. Once activated by either antigen cross-linking of surface immunoglobulin E or via a variety of other factors (toll-like receptor ligands, proteases, C5a, IL-3, hyperosmolarity, adenosine, RANTES), mast cells degranulate and release a variety of mediators including vasoactive and bronchoactive mediators

(histamine, LTC4, PGD_2, proteases), chemokines (MCP-1, MIP-1α, MIP-1β, RANTES), cytokines (IL-9, IL-13, IL-5, tumor necrosis factor-α), and growth factors (transforming growth factor-β and FGF). Through the release of this vast array of mediators, mast cells induce many features of the early-phase allergic response including enhanced vascular and epithelial permeability, mucus secretion, and airway hyperresponsiveness. They also contribute to the last-phase allergic response via the recruitment and activation of a number of inflammatory cells including neutrophils, eosinophils, T cells, and B cells. Through the activation of inflammatory cells as well as direct effects on mucosal cells, mast cells also contribute to the remodeling (goblet cell metaplasia, tissue fibrosis, and smooth muscle hypertrophy) of mucosal tissues observed in chronic stages of the allergic response.

Basophils

Basophils are a small population of peripheral blood leukocytes containing cytoplasmic granules that stain with basophilic dyes. They typically exhibit a segmented nucleus with marked condensation of nuclear chromatin and contain round or oval cytoplasmic granules. Basophils are thought to arise from pluripotent CD34+ progenitors found in cord blood, peripheral blood, and bone marrow. They have been suggested to evolve from CD34+/IL-3Rα+/IL-5Rα+ eosinophil/basophil progenitors, as supported by the occurrence of granulocytes with a hybrid eosinophil/basophil phenotype in patients with chronic or acute myelogenous leukemia or in cell culture.[362,363,364] Unlike mast cells, basophils differentiate and mature in the bone marrow and then circulate in the blood where they constitute < 1% of circulating leukocytes. IL-3 appears to be an important developmental factor for basophils,[365] although many other growth factors such as IL-5, GM-CSF, TGF-α, and nerve growth factor likely influence their development.[366,367,368] Like mast cells, basophils possess high affinity IgE receptors (FcεR1) that are cross-linked upon engagement of receptor-bound IgE by their cognate antigens, resulting in release of a number of mediators that are in part common for both cell types.

Until recently, the lack of specific markers for detection of basophils in tissues and the inability to isolate significant numbers of these cells from peripheral blood had hampered the study of basophil biology. Participation of basophils in allergic reactions has traditionally been documented by indirect means such as by determining the pattern of mast cell- or basophil-specific mediators like histamine (derived from both), PGD_2 (mast cells only), and LTC_4 (primarily from basophils). Despite the difficulties in studying basophils, recent studies suggest that they rapidly produce large amounts of the immunoregulatory cytokines IL-4 and IL-13 and constitutively express CD40L and chemokine receptor 3 (CCR3) on their surface. These findings taken together with the demonstration that they are rapidly recruited to the skin,[369] lung,[370] and nose[371] after allergen challenge suggests that they likely play an important role in allergic diseases. However, as much more is known about the role of mast cells in the immune response, we will confine our discussion to mast cells except where specific information about basophils is available.

Mast Cells

Mast cells typically appear as round or elongated cells with a nonsegmented or occasionally bi- or multinucleated nucleus. Their intracellular granules stain purple with aniline blue dyes. This change in color represents the interaction of the dyes with the highly acidic heparin contained in the mast cell granules. Mast cells like other granulocytes are derived from CD34+ hematopoietic progenitor cells; however, they are distinct from other granulocytes in that they mature in the periphery. Several lines of evidence suggest that interactions between the tyrosine kinase receptor c-kit, which is expressed on the surface of mast cells, and the c-kit ligand stem cell factor (SCF) are essential for normal mast cell development and survival. For example, mice with mutations that either result in markedly impaired c-kit function or a reduction in c-kit virtually lack mast cells. rSCF can induce mast-cell hyperplasia in vivo in mice, rats, primates, and humans.[372]

Mast cells are distributed throughout normal connective tissues where they often lie adjacent to blood and lymphatic vessels, near or within nerves, or beneath epithelial surfaces that are exposed to the external environment, such as those of the respiratory tract, gastrointestinal tract, and skin (see Fig. 45.6). At these locations, they are ideally situated to encounter foreign antigens and to release their products in close proximity to their respective target cells (ie, epithelial cells, vascular endothelium, smooth muscle, and fibroblasts). In humans and mice, the number of mast cells in a tissue varies markedly depending on the anatomic site and the immunologic status of the host.

The mast cell population is composed of a heterogeneous group of cells with respect to their structure and function.[373] On the basis of their content of neutral serine proteases, they had previously been divided into two phenotypes. One subset, designated MC_{TC}, contains tryptase, chymase, cathespsin G, and carboxypeptidase, whereas the other phenotype, designated MC_T, contains only tryptase. MC_{TC} are found predominantly in skin and at subepithelial locations in the bronchial, nasal, and gastrointestinal mucosa, whereas MC_T are located predominantly in alveolar walls, intestinal epithelium, and in airway epithelium in patients with allergic disease. However, more recent studies in both humans and animal models suggest that mast cells in different anatomic sites and even within a single site can vary in several aspects of their phenotype including morphology, responsiveness to various stimuli and activation, and mediator content.

The tissue levels of mast cells and their specific phenotypes are likely controlled by a complex interplay between SCF, other growth factors, and cytokines. SCF is necessary to elicit the c-kit-mediated signaling that ensures the expansion of cells of the mast cell lineage, whereas the development of different phenotypes appears to be determined by their responsiveness to signals from T cells. Specifically, MC_T-cell expansion appears to be T cell dependent, while expansion of the MC_{TC} population is T cell independent. This contention is supported by the fact that humans with T-cell deficiencies have a lack of intraepithelial intestinal mast cells while maintaining submucosal mast cell populations.[374] Similarly, athymic mice lack intraepithelial cells and are unable to expand this population in the gastrointestinal tract in response to helminthic infections.[375] IL-3, IL-4, and IL-9, in particular, are among the T cell-derived cytokines known to influence mast cell development and phenotypic characteristics. A role for IL-3 in mast cell hyperplasia has been demonstrated in intestinal helminthic infections in which IL-3 depletion inhibits the intraepithelial mast cell

hyperplasia normally observed in the jejunum of infected mice.[376] A similar case can be made for IL-9 as IL-9 transgenic mice spontaneously develop intraepithelial mast cell hyperplasia in the jejunum.[377] The action of IL-9 can be blocked by depletion of SCF. Moreover, IL-9 and IL-10 reversibly induce MMCP-1 and MMCP-2 expression in mast cells through transcript stabilization, suggesting that T cell-derived cytokines can influence the spectrum of mediators produced by a given

mast cell. These studies suggest that the regulation of mast cell phenotype in the microenvironment is dynamic and that T cell-derived cytokines are major determinants of both the numbers, distribution, and phenotype of mast cells in tissues.

Mast Cell-Derived Mediators

Mast cells and basophils release a wide array of potent biologically active mediators that have both unique and overlapping activities on various target cells. Some of these products are stored preformed in the cytoplasmic granules, whereas others are synthesized upon activation of the cell by IgE-dependent processes or non-IgE-dependent stimuli. These mediators can be categorized into three main groups: 1) performed secretory granule-associated mediators, 2) lipid-derived mediators, and 3) cytokines.

Preformed Mediators

The secretory granules of human mast cells contain a crystalline complex of preformed inflammatory mediators ionically bound to a matrix of proteoglycan. When mast cell activation occurs, the granules swell and lose their crystalline nature, and the individual mediators are released by exocytosis. The mediators stored in mast cells include histamine, proteoglycans, serine proteases, carboxypeptidase A, and small amounts of sulphatases. In mouse and rat mast cells, the granules also contain serotonin.[378,379,380]

The mediator most associated with the mast cell is histamine. Histamine is a biogenic amine formed in mast cells and basophils by the decarboxylation of histidine. It is present in the granules at approximately 100 mmol/L or 1 pg/cell. Histamine has many potent activities that are pertinent to the early phase of the allergic response including vasodilatation, increased vasopermeability, smooth muscle contraction, and increased mucus production. Histamine exerts its biologic and pathologic effects via specific receptors on various cells such as smooth muscle, endothelial cells, and nerves. At least three types of histamine receptors have been identified: H1, H2 and H3. Histamine is very rapidly metabolized with a half-life of about 1 minute by histamine-N methyltransferase and histaminase. Increased levels of histamine have been found in BAL fluids of patients with asthma, atopic dermatitis, and allergic rhinitis.[381] Interestingly, although antihistamines inhibit the immediate allergic responses, they do not seem to inhibit late-phase responses.

Heparin is the predominant proteoglycan in human mast cells. It constitutes about 75% of the total. The remainder is comprised of chondroitin sulphates. The proteoglycan is the storage matrix inside the granule, and the acid sulfate groups of the glycosaminoglycans provide binding sites for the other preformed mediators. Proteoglycans also have anticoagulant, anticomplement, and antikallikrein effects. In addition to regulating the kinetics of release of mediators from the granule matrices, proteoglycans can also regulate the activity of some of the associated mediators.

The major mast cell protease, which is present in all types of mast cells, is tryptase. Tryptase is a serine protease, and it is stored fully active in the granule. There are two distinct forms of tryptase with 90% amino acid sequence homology: α-tryptase and β-tryptase.[382,383] The β-tryptase form is a useful clinical biomarker for anaphylaxis. Tryptase levels measured within 4 hours of a presumed anaphylactic reaction are more sensitive than serum or urine histamine in implicating mast cell activation and degranulation.[384,385] By weight, tryptase is the major enzyme stored in the cytoplasmic granules of human mast cells and occurs in all human mast cell populations. It has many activities, including cleavage of peptides such as vasoactive intestinal peptide, bronchodilator peptides, and calcitonin gene-related peptid, but not substance P. It mediates sensitization of smooth muscle; cleavage of type IV collagen, fibronectin, and type VI collagen; upregulation of intercellular adhesion molecule-1 on epithelial cells; and mitogenic activity for fibroblasts and epithelial cells.[380] Some of these activities have led to speculation that mast cells may be involved in chronic inflammation and tissue remodeling.[386]

The other major neutral protease in mast cells is chymase. It is also a serine protease that is stored in the active form in the granules of some human mast cells. Unlike tryptase, chymase is only present in a subset of mast cells. Chymase can cleave angiotensin I and neurotensin, but has no activities on vasoactive intestinal peptide (VIP) or substance P. Importantly, it can degrade IL-4. Some subsets of mast cells also contain other proteinases such as carboxypeptidase and cathepsin G.

Newly Synthesized Mediators

Lipid Mediators

Activation of mast cells not only results in release of preformed granule-associated mediators but can also initiate the de novo synthesis of certain lipid-derived substances. Of particular importance are the cycloxygenase and lipoxygenase metabolites of arachidonic acid. These products possess potent inflammatory activity. Lipoxygenases generate leukotrienes, hydroperoxyeicosatetraenoic acids, and the reduced products of hydroperoxyeicosatetraenoic acids, hydroxyeicosatetrenoic acids, whereas cycloxygenase products include prostaglandins and thromboxanes.

Leukotrienes are produced by the activity of 5-lipoxygenase on arachidonic acid. Arachidonic acid is converted to 5-hydroperoxyeicosatetraenoic acid, which can then be converted to LTB_4 through the action of the LTA_4 hydrolase or to LTC_4 via LTC_4 synthase. LTC_4 can then be converted to LTD_4 and LTE_4. Human mast cells generally produce more LTC_4 than LTB_4. The leukotrienes were originally discovered in 1938 and referred to as the slow-reacting substance of anaphylaxis until their structural elucidation in 1979.[387]

Leukotrienes induce a prolonged cutaneous wheal-and-flare response, stimulate prolonged bronchoconstriction (10 to 1000 times more potently than histamine), enhance vascular permeability, promote bronchial mucus secretion, and induce constriction of arterial and pulmonary smooth muscle.[388] Cysteinyl leukotrienes have been detected in the BAL fluid of patients with asthma and in the urine of patients following inhaled allergen challenge or aspirin challenge in sensitive individuals.[389] Cysteinyl leukotriene receptor antagonists and

leukotriene synthesis blockers have been introduced into clinical practice as novel therapies for asthma.[390,391,392,393]

In patients with asthma, clinical trials have demonstrated that approximately 80% of the early bronchoconstrictor response can be eliminated with cysteinyl leukotriene antagonists.[393] These blockers also inhibit 50% of the late-phase response to inhaled allergens, supporting the importance of cysteinyl leukotrienes in the late phase of the allergic response. However, the fact that PGD_2 and tryptase are not found in the BAL fluid during the late-phase reaction suggests that the cysteinyl leukotrienes noted in the late phase are the product of basophils or eosinophils and not mast cells.

In contrast to the cysteinyl leukotrienes, mast cells produce very little LTB_4. LTB_4 is a potent chemotactic factor for neutrophils and to a lesser extent for eosinophils.[394] Certain mast cell types, including bone marrow-derived murine mast cells and human lung mast cells, may also secrete PAF.[395,396] PAF has several actions that suggest that it is an important mediator of anaphylaxis including 1) its ability to induce aggregation and degranulation of platelets, 2) its induction of wheal and flare reactions in human skin, 3) its ability to increase lung resistance, and 4) induction of systemic hypotension.

The major cyclooxygenase-derived product in mast cells is PGD_2, which is a potent bronchoconstrictor. PGD_2 is rapidly degraded to 9α,11β-PGF_2, producing another potent bronchoconstrictor. Maximal activation of mast cells yields 50 to 100 ng PGD_2 per 10^6 mast cells.[397] Although PGD_2 is 100-fold less potent than cysteinyl leukotrienes, it is released in larger molar amounts and, thus, is important in the early bronchoconstrictor response to airway allergen challenge.[398] PGD_2 exerts its effects by interacting with the thromboxane receptor on airway smooth muscle.[399] PGD_2 is also chemotactic for neutrophils and is an inhibitor of platelet aggregation. There is considerable evidence that prostanoids are generated during allergic reactions in vivo. PGD_2 is elevated in the BAL of asthmatic subjects following inhaled allergen challenge.[400] Furthermore, the metabolite of PGD_2, 9α,11β-PGF_2, rises markedly in the urine after allergen or aspirin challenge of sensitive asthmatics.[401] Significantly raised PGF2α and PGE_2 levels are reported in the serum of asthmatics.[402] These findings are confused by the fact that there is no therapeutic benefit from cyclooxygenase inhibitors and, in fact, cyclooxygenase inhibitors often exacerbate underlying asthma.[403,404]

Mast Cell-Derived Cytokines

Mast cells may be important initiators of both the early- and late-phase allergic reaction by their ability to synthesize and secrete cytokines. Mast cells express messages for a number of cytokines including 1) proinflammatory cytokines, 2) immunoregulatory cytokines, and 3) chemokines.

Mast cells contain preformed stores of several proin-flammatory cytokines including TNF-α, IL-8, IL-6, and IL-1α. Release of these cytokines early in the immune response likely contributes to the recruitment of leukocytes during the late-phase response via their ability to

increase expression of adhesion molecules such as P- and E-selectin, vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule-1 on vascular endothelial cells. Mast cells appear to be an important initial source of TNF-α during allergic responses. Mast cells produce a number of immunoregulatory cytokines such as IL-3, IL-4, IL-5, GM-CSF, IL-13, and IL-16. In the human bronchial mucosa, IL-4 immunoreactivity is seen in approximately 80% of mast cells.[405,406] Indeed, it has been suggested that mast cells are the primary source of IL-4 protein in inflamed airways. Although cytokine production by basophils has not been as extensively studied as that in mast cells, they have been shown to produce large quantities of IL-4 and IL-13. The rapid and perhaps sustained production of cytokines by these cells at sites of allergic inflammation may intensify or perpetuate IgE production and Th2 cell differentiation. Mast cells have also been shown to produce a number of chemokines such as MCP-1, MIP-1β, MIP-1α, and RANTES. Elaboration of these chemokines may contribute to the cellular component of the late-phase response.

Mast Cell Activation

Mast cell activation may be initiated upon interaction of a multivalent antigen with its specific IgE antibody attached to the cell membrane via its high affinity receptor, FcεR1. Cross-linking of IgE by the interaction of allergen with specific determinants on the Fab portion of the molecule brings the receptors into juxtaposition and initiates mast cell activation and mediator generation and release. Mast cells may also be activated by non-IgE-mediated stimuli such as neuropeptides, complement components, and drugs such as opiates. In addition to IgE-dependent stimuli, several non-IgE-dependent stimuli activate mast cells and basophils. C5a, IL-3, fMLP, certain cytokines (IL-33), and chemokines (RANTES) are known to induce histamine and mediator release in mast cells and basophils. Hyperosmolarity itself also stimulates mediator secretion from these cells. Degranulation produced by both IgE-dependent and non-IgE-dependent stimuli appear similar. However, the biochemical processes that lead to mediator release may differ.

Role of Mast Cells in Acute-Phase Responses

Several lines of evidence suggest that mast cells and basophils play a pivotal role in the generation of acute-phase responses. First, allergen provocation of atopic individuals is associated with extensive activation of mast cells as judged by the detection of the release of mast cell-associated mediators (histamine, tryptase) at the site of allergen challenge. Secondly, therapeutics that inhibit the release of these mediators (mast cell stabilizers) or their actions (antihistamines) effectively attenuate acute allergic responses. Despite this evidence in humans, the data in animal models is conflicting. While the preponderance of studies in mice suggests that acute responses are mast cell- and IgE-dependent, others suggest that these responses are IgE-independent. For example, it has been shown that responses induced in mice by passive transfer of allergen-specific IgE antibodies and subsequent intravenous allergen challenge are inhibited in genetically mast cell-deficient $Kit^{W}/Kit^{W\text{-}v}$ mice. When these mice were reconstituted with mast cells by adoptive transfer, their acute-phase responses were restored.[407] In contrast, some investigators have reported that anaphylactic responses

develop normally in IgE-deficient mice.[408] Although these conflicting results may reflect inherent differences in mast cell biology between humans and mice, they imply that both mast

cell- and IgE-dependent and -independent processes are likely involved in the expression of the physiologic features of the acute-phase reaction.

Role of Mast Cells in Late-Phase Responses

Although it has long been thought that mast cells only contribute to the early acute response, recent evidence demonstrates their potential contribution to the late-phase response. First, through the elaboration of proinflammatory cytokines, chemokines, and immunoregulatory cytokines as discussed previously, mast cells may contribute to the cellular component of the late-phase response as well as favor the acquisition of the Th2 phenotype by providing a continuously high concentration of IL-4. Secondly, mast cell-derived mediators may also contribute to the chronic remodeling of mucosal tissues as many of the mediators they release influence connective tissue turnover. Specifically, histamine and tryptase have been shown to stimulate fibroblast growth and collagen synthesis in vitro and in vivo. Lastly, studies in humans have shown that stabilization of mast cells with sodium nedocromil effectively inhibits both the early- and late-phase response to allergen exposure.[409] Furthermore, recent clinical trials utilizing a monoclonal antibody against IgE resulted in reductions in symptoms and improvement in lung function in asthmatics.[410] Although these reports support a role for mast cells in the late-phase response, studies of animal models of AD and asthma suggest that late-phase reactions are similar in wild-type and IgE-deficient mice,[411] suggesting that although mast cells are capable of inducing late phase responses, additional mast cell-independent processes also contribute to the development of late-phase responses.

EOSINOPHIL BIOLOGY

Eosinophil blood and tissue levels are generally quite low in the absence of parasitic infection or atopy. One of the hallmarks of allergic disorders is heightened production of eosinophils in bone marrow and the accumulation of eosinophils in tissues and blood. Eosinophil differentiation, recruitment, and activation are under the regulation of a series of molecular events orchestrated by Th2 cytokines. Although the exact role of eosinophils in the pathology of allergic responses is not known, eosinophils are known to release a myriad of mediators and cytokines that have the potential to induce the symptoms of allergy as well as amplify the allergic response through the release of immunoregulatory and proinflammatory cytokines.

Eosinophils are bone marrow-derived granulocytes that are characterized by their bilobed nuclei and their distinctive cytoplasmic granules (Fig. 45.9).[412,413] They contain three distinct types of cytoplasmic granules: 1) eosinophil specific granules, which contain electron-dense crystalloid cores; 2) primary granules, which lack a crystalloid core and develop early in eosinophil maturation; and 3) smaller granules, which contain arylsulfatase and other enzymes. Eosinophils also contain varying numbers of lipid bodies. Lipid bodies are nonmembrane-bound, lipid-rich inclusions that are also found in macrophages and mast cells and are thought to contribute to the formation of eicosanoid mediators. Eosinophils are generally identified in blood and tissues by the affinity of their cytoplasmic granules for acid aniline dyes such as eosin and in tissues by immunostaining for eosinophil-specific proteins such as major basic protein (MBP).

Eosinophil-Derived Mediators

Eosinophil-Specific Cationic Proteins

Eosinophils store four highly basic, low-molecular-weight proteins in their cytoplasmic granules: MBP, eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPO), and eosinophil cationic protein (ECP). MBP, EPO, and ECP are potent toxins for helminths and bacteria; they are strongly implicated as mediators of allergic diseases such as asthma, atopic dermatitis, and allergic rhinitis. MBP is potently toxic for mammalian cells in vitro, and high levels of MBP are found in the body fluids of patients with asthma and other allergic disorders.[414] Both MBP and ECP exert their toxicity by damaging target cell membranes through charge-mediated interactions. In addition to its toxic properties, MBP activates platelets, mast cells, and basophils, which in turn release histamine. Furthermore, MBP administration to primates induces AHR. MBP may induce AHR through its demonstrated ability to competitively inhibit binding to cholinergic muscarinic M2 receptors on parasympathetic nerves.[415] These receptors normally function as autoreceptors that inhibit the release of acetylcholine from the nerve ending. Thus, inhibition of these receptors by MBP would enhance the release of acetylcholine in the airway wall, resulting in heightened contractile responses. Both ECP and EDN have partial sequence identity with pancreatic ribonucleases; however, EDN is more potent as a ribonuclease than is ECP.

EPO, which is distinct from the myeloperoxidase of neutrophils and monocytes, consists of two polypeptides of about 15 and 55 kDa. It catalyzes the formation of hypobromous acid from hydrogen peroxidase and halide ions (preferentially bromide). HOBr reacts with primary amines to form bromamines, and it converts tyrosine to 3-bromotyrosine. Increases in the levels of 3-bromotyrosine in BAL proteins have been observed in asthmatics following allergen provocation.[416] Thus, the oxidative pathways induced in activated eosinophils may damage biomolecules in vivo.

Another prominent component of eosinophil primary granules are Charcot-Leyden crystals (CLCs). This protein comprises up to 10% of the total cellular protein in human eosinophils. Although the CLC protein possesses lysophospholipase activity, structural analysis suggests that the CLC protein is similar to that of galectins 1 and 2, members of the “S-type” lectin superfamily.[417] Recent studies in the mouse lung suggest that CLCs may contain a protein called YM-1 (T-lymphocyte-derived eosinophil chemotactic factor), which has sequence homology to chitinase.[418] Chitinase activity of these proteins may explain the strong association between eosinophilia and parasite infestations. These crystals are often found in sputum, feces, and tissues in patients

with allergic asthma and other eosinophil-related diseases characterized by significant eosinophilia.

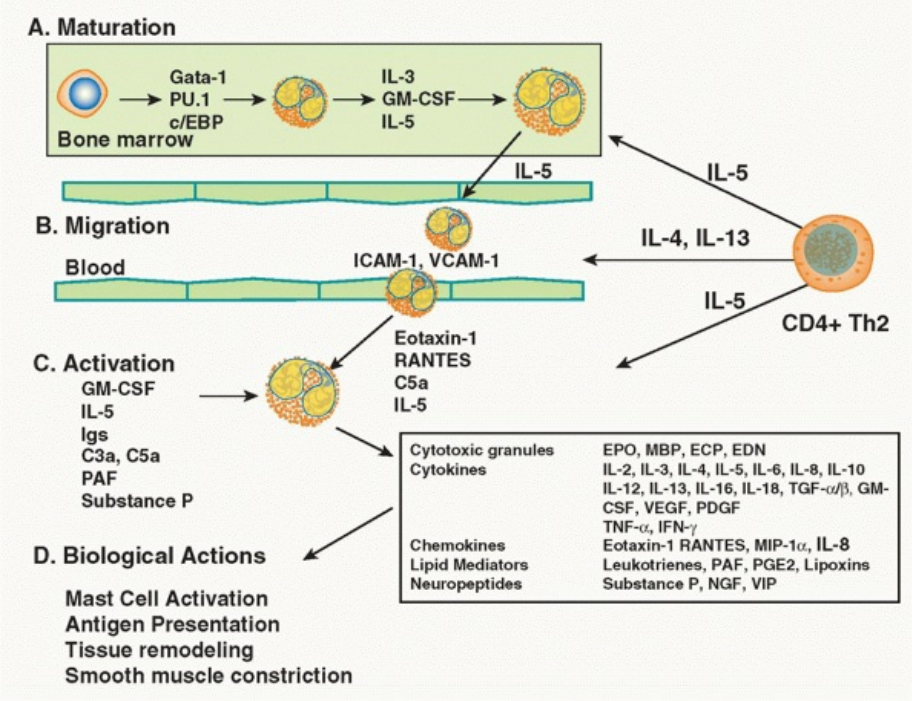

FIG. 45.9. Schematic Representation of Eosinophil Actions During Allergic Immune Responses. A: Maturation. **B:** Migration. **C:** Activation. **D:** Pleiotrophic Actions. Eosinophils develop in bone marrow where they differentiate from hematopoietic progenitor cells into mature eosinophils under the control of several transcription factors including GATA-1, PU.1, and c/EBP. Th2 cytokines coordinately regulate eosinophil recruitment, activation, and accumulation at the site of antigen exposure. Following allergen-specific induction of Th2 cytokine production (interleukin [IL]-5, IL-4, IL-13, IL-3, granulocyte macrophage-colony stimulating factor [GM-CSF]), IL-5 rapidly induces differentiation of eosinophils from myeloid precursors in bone marrow to stimulate their release into the bloodstream. IL-4 and/or IL-13 promote eosinophil egress from the vascular compartment by upregulating vascular cell adhesion molecule-1 expression on vascular endothelial cells. Subsequently, IL-4 and IL-13 guide eosinophils to the site of allergen exposure by regulating the production of various chemokines (eg, RANTES, eotaxin) by local cells such as macrophages and epithelial cells. Once these cells accumulate in tissues, locally produced IL-5 along with other cytokines, such as GM-CSF and IL-3, promote their actions by prolonging their survival in tissues. Eosinophils store and release a number of proteins in their specific granules. Specifically, major basic protein is stored in the core of the granule, while eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase are found in the matrix of the granules. Eosinophils also contain lipid bodies in which products of lipoxygenase and cycloxygenase are formed. Eosinophils also release a number of cytokines, chemokines, and neuropeptides that are important in the allergic diathesis.

Lipid Mediators

Upon stimulation, eosinophils elaborate several bioactive lipids, including products of the 5- and 15-lipooxygenase pathway, products of the cyclooxygenase pathway, and PAF. Lipid bodies, or intracellular lipid-rich domains, are induced to develop in many activated

eosinophils in vivo and are sites for enhanced synthesis of both lipoxygenase- and cyclooxygenase-derived eicosanoids. The activities of eosinophil-derived lipids, which are generally proinflammatory, are multiple and include potent smooth muscle contraction, vasoactivity, and mucus secretion activities.

Cytokines

In recent years, it has been recognized that eosinophils are a major source of cytokines and appear to store some if not all of these cytokines in cytoplasmic-specific granules. Triggering of eosinophils by engagement of receptors for cytokines, Ig, and C3 can lead to secretion of an array of proinflammatory and immunoregulatory cytokines, chemokines, and growth factors including IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IFNγ, TNF-α, GM-CSF, TGF-β, TGF-α, RANTES, MIP-1α, eotaxin, VEGF, platelet-derived growth factor-β, and heparin-binding epidermal growth factor.[61] Through the quick release of this diverse array of cytokines at the inflammatory loci, the eosinophil is poised to perpetuate and/or intensify the eosinophil-mediated

inflammatory response, both by enhancing its own activation and through the release of Th2 cytokines.

Recruitment of Eosinophils to Sites of Inflammation

Preferential accumulation of eosinophils at sites of allergic inflammation involves multiple molecular events that are integrated and controlled by Th2 cytokines.[419] These include a) differentiation and release of mature eosinophils from bone marrow into the bloodstream,; b) upregulation of specific adhesion molecules on eosinophils and endothelium, c) stimulation of C-C chemokine production and egress of eosinophils from the blood into the tissues, and d) production of eosinophil-active cytokines that increase eosinophil survival in tissues.

Eosinophils are terminally differentiated granulocytes that develop from CD34+ hematopoietic progenitor cells in the bone marrow. Under the influence of GM-CSF, IL-3, and IL-5, they differentiate into mature eosinophils. These cytokines likely promote eosinophilopoiesis via induction of GATA-1, PU.1, or C/EPS through their shared common β-chain.[420,421] Of these, IL-5 has the most specific effects on eosinophil differentiation and production. In addition to its effects on eosinophil differentiation, IL-5 can rapidly induce the release of developed eosinophils from bone marrow into peripheral circulation. The importance of IL-5 to blood and tissue eosinophilia has been repeatedly shown in in vitro and in vivo studies of gene targeted and overexpressing mouse lines.[422]

Circulating eosinophils are recruited to sites of inflammation by the combined actions of inflammatory mediators, adhesion molecules, and chemoattractants. Several cell adhesion molecules have been implicated in eosinophil adherence to cytokine-stimulated vascular endothelium, including intercellular adhesion molecule-1, E-selectin, L-selectin, and the very late antigen-4/VCAM-1. The importance of VCAM-1 to eosinophil recruitment has been shown in primate and mouse studies in which blockade of VCAM-1/very late activation antigen-4 interactions inhibits eosinophil infiltration of tissues.[423] IL-4 and IL-13 induce VCAM-1 expression on the surface of vascular endothelial cells, leading to preferential recruitment of eosinophils into sites of allergic inflammation.

Eosinophil migration to the sites of inflammatory loci is mediated by a number of chemoattractants. Until the last decade, mediators such as PAF, C5a, and LTB_4 were considered to be the most important eosinophil chemoattractants. Although potent chemoattractants, they do not show any specificity for eosinophils. However, there are a number of C-C chemokines that are eosinophil chemoattractants (RANTES [CCL5], eotaxin [CCL11], monocyte chemoattractant protein, MCP-4 [CCL13], MCP-3 [CC17], and MIP-α [CCL3]). The levels of most of these chemokines have been shown to be upregulated in the mucosal tissues of patients with asthma, atopic rhinitics, and atopic dermatitis. Although each of these chemokines is implicated in eosinophil chemotaxis, it was originally hypothesized that eotaxin would play a pivotal role in eosinophilic inflammation in that it binds specifically to the CCR3, while the other C-C chemokines bind multiple receptors. The fact that CCR3 is highly expressed on eosinophils, basophils, and Th2 cells but not on neutrophils suggests that it plays an important role in Th2-mediated inflammatory responses.[424,425] Surprisingly though, eotaxin-deficient mice have shown only minor defects in eosinophil accumulation,[426,427] indicating that eotaxin is not uniquely required for eosinophil accumulation. Along these lines, complete abrogation of eosinophil migration has not been observed by inhibition of any one chemokine,[426,427,428] suggesting that multiple chemokines working in concert are required to direct leukocytes to specific sites of inflammation. Thus, it has been postulated that a panoply of chemokines may be required to establish the multiple chemical gradients required for eosinophils to migrate through several local compartments from the vascular spaces to the mucosal surfaces in the nose, airway, and skin.

Based on the independent and overlapping roles of IL-4, IL-13, and IL-5, discussed previously, we would envision the following paradigm for regulation of allergen-induced tissues eosinophilia. Following allergen-specific induction of Th2 cytokine production, IL-5 would rapidly induce differentiation of eosinophils from myeloid precursors in bone marrow and stimulate their release into the bloodstream. IL-4 and/or IL-13 would promote eosinophil egress from the vascular compartment by upregulating VCAM-1 expression on vascular endothelial cells. Once the cells accumulate in tissues, locally produced IL-5 along with other mediators such as eotaxin would promote their actions by prolonging their survival in tissues. Within this paradigm, allergendriven eosinophil recruitment into tissues is coordinately regulated by the Th2 cytokines IL-4, IL-13, and IL-5.

Mechanisms of Eosinophil Activation

The effector functions of eosinophils are mediated by stimuli that induce degranulation. Eosinophil degranulation can be regulated by multiple components including those that primarily stimulate the cells (eg, Igs and lipid mediators), priming agents (eg, cytokines), and chemokines. However, the precise mechanisms by which eosinophil degranulation occurs in vivo are still poorly understood.

Eosinophils express receptors for several Igs including IgG, IgA, and IgE. Surfaces coated with IgG, IgA, and secretory IgA stimulate eosinophil degranulation in vitro. Of these Igs, sIgA is the best for inducing eosinophil degranulation and does not stimulate neutrophil degranulation. As IgA is the most abundant Ig isotype in mucosal secretions of the respiratory and gastrointestinal tracts, it may be an important regulator of eosinophil activation at these sites. IgE may also be important for eosinophil activation as eosinophils can bind IgE via

three distinct structures: the S-type lectin galectin-3, FcεRII/CD23, and FcεRI. It was initially shown that eosinophils, isolated from patients with parasite-induced eosinophilia, degranulate in response to IgE antibody or IgE-coated parasites.[429,430] Subsequent studies showed that local allergen provocation induces expression of FcεRI by eosinophils infiltrating into the airways[431] and skin of patients with allergic diseases.[432,433] In studies using sera from ragweed-allergic patients with hay

fever, ragweed-specific IgG, but not IgE, induced allergendependent eosinophil degranulation in vitro.[434,435] Thus, it remains to be determined whether IgE is an important regulator of eosinophil degranulation in atopy.

Eosinophil degranulation may also be induced by soluble stimuli alone. Cytokines, especially those with eosinophilopoietic activity, such as GM-CSF and IL-5, are potent inducers of eosinophil granule protein release. Interestingly, eosinophil granule proteins themselves, including MBP and EPO, stimulate eosinophils and cause degranulation, suggesting an autocrine mechanism of eosinophil degranulation.[436] Other physiologic stimuli for eosinophil degranulation include PAF, the complement fragments, C5a and C3a, and the neuropeptide substance P.[437] As previously discussed briefly, chemokines may also activate eosinophils by binding CCR3. In this regard, eotaxin induces eosinophil degranulation and leukotriene C_4 (LTC_4) release.

Eosinophils as Effector Cells in Allergic Responses

Eosinophils through the elaboration of a plethora of preformed and lipid mediators described previously are postulated to play a role in several aspects of the allergic response including antigen presentation, T-cell proliferation and differentiation, as well as mast cell activation and airway remodeling. Although it remains controversial, it has recently been shown that eosinophils may function in antigen presentation.[438] Specifically, eosinophils have been shown to effectively present soluble parasitic antigens to CD4+ T cells, thereby promoting T-cell proliferation and polarization. They may also regulate T-cell proliferation, activation, and polarization via the array of cytokines they produce as well as through their ability to synthesize indoleamine 2,3 dioxygenase.[439]

Circumstantial evidence for a causative role of eosinophils in allergic inflammation as well as the clinical symptoms of atopic disorders comes from a variety of studies.[440] First, elevated levels of activated eosinophils and their protein products (MBP, ECP, EPO, EDN) have been consistently demonstrated in BAL fluids and airway biopsy tissues from patients with asthma,[440] in nasal biopsy specimens from patients with allergic rhinitis (nasal fluids and nasal biopsies),[441] and in skin lesions from patients with atopic dermatitis.[442] Secondly, in each of these diseases, increased levels of eosinophils and their proteins correlate with disease severity. Lastly, successful steroid treatment is associated with a marked reduction in both blood and tissue eosinophil levels in asthma, atopic rhinitis, and atopic dermatitis.[443,444]

Despite the substantial body of circumstantial evidence that supports a causative role for eosinophils in the pathophysiology of atopic disorders, the results of studies designed to

define a pathogenic role for eosinophils in atopic disorders are conflicting. Studies in IL-5 gene knockout[53] and IL-5 transgenic mice[422] show that both eosinophilia and AHR are IL-5-dependent. In contrast, other investigators have demonstrated that blockade of eosinophils by IL-5 ablation did not affect AHR in animal models.[445] A caveat of these experiments is that IL-5 depletion did not completely deplete eosinophils. To address this issue, several groups have undertaken the task of developing eosinophil-deficient mice.[446,447] One group generated an eosinophil-deficient mouse via insertion of a cytocidal protein (diphtheria toxin A) in the promoter of the EPO gene. These mice were reported to be completely devoid of eosinophils in all tissues. Moreover, all features of the allergic phenotype were absent (AHR, mucus, eosinophilia, Th2 cytokine production) in these mice following allergen challenge. The other eosinophil-deficient mouse line (BALB/c background) harbors a deletion of a high-affinity GATA-binding site in the GATA-1 promoter leading to the specific ablation of the eosinophil lineage.[446,447] In these mice, complete eosinophil depletion had no effect on AHR, mucus production, or Th2 cytokine production, but did reduce airway remodeling assessed by collagen deposition. In contrast, when the GATA1 mutation was bred onto the C57BL/6 background, mature eosinophils were found to contribute to the generation of a Th2 response by producing or promoting the production of chemokines that recruit T cells that subsequently produce a Th2 response.[448] The safest conclusions to draw from these observations are that eosinophils and IL-5 are sufficient to induce AHR and goblet cell hyperplasia in the C57BL/6 mice, but that redundant mechanisms may exist in BALB/c mice that

bypass the requirement for eosinophilic inflammation.

The results in human anti-IL-5 asthma trials mirror that observed in experimental asthma models. Specifically, an initial multiple center study showed that treatment of mild asthmatics with mepolizumab, a humanized mouse IgG1 monoclonal anti-IL-5 antibody, did not benefit patients with asthma despite significantly lowering blood eosinophilia.[449] The interpretation of this early study was that anti-IL-5 treatment was insufficient to completely suppress tissue eosinophilia. Other studies conducted on patients with mild asthma also failed, although analyses of bronchial biopsies showed that mepolizumab only reduced the numbers of eosinophils by about 50% and did not appreciably reduce the degree of eosinophil granule protein deposition.[450,451] However, more recent studies that have evaluated anti-IL-5 monoclonal antibody effects in patient populations with more severe disease have shown some benefit. In one study, intravenous injection of anti-IL-5 monoclonal antibody improved the ability to rapidly exhale air (volume of air expelled during the initial second of forced expiration) and decreased asthma exacerbations, steroid requirement, and blood and sputum eosinophilia in patients with steroid-resistant asthma with sputum eosinophilia.[451] In another study, it decreased severe exacerbations and blood and sputum eosinophilia, and increased quality of life without increasing volume of air expelled during the initial second of forced expiration, decreasing symptoms, or decreasing AHR.[452] Thus the exact contribution of IL-5 and eosinophils to asthma pathogenesis is complex and remains to be fully elucidated. Despite the complex picture in asthma, anti-IL-5 therapies have shown some efficacy in the treatment of patients with the hypereosinophilic syndrome.[453]

Th2 Cytokine-Producing Cells as Effector Cells

Our recent understanding of the role of Th2 cells in the allergic diathesis suggest that they are important effector cells in

the allergic response in addition to their well-accepted role as orchestrators of the inflammatory process. Specifically, the Th2 cytokine IL-13 has been implicated as an effector molecule in allergic disease. Evidence for this contention comes from studies in which blockade of endogenous levels of IL-13 in antigen-sensitized mice by administration of a soluble form of the IL-13Rα2 chain, which binds only IL-13, completely reversed AHR and pulmonary mucus cell hyperplasia.[63,64] Furthermore, it was shown that recombinant IL-13 was able to recreate the symptoms of asthma (AHR, mucus hypersecretion, eosinophilia) in the absence of functional T cells or B cells.[39] These effects were shown to be independent of eosinophils as well.[454] These results suggested that the effects of IL-13 were not due to its known role in regulating IgE or eosinophil recruitment. Interestingly, despite the similarities in function between IL-13 and IL-4, IL-4 blockade at the time of antigen challenge does not ablate AHR.[60] This was further supported by the finding that transfer of Th2 cells derived from IL-4-deficient mice was still able to confer AHR.[44] Moreover, chronic expression of IL-13 in the mouse lung results in development of these features as well as subepithelial fibrosis and the formation of CLCs.[455] In contrast, overexpression of the IL-4 gene in the murine lung does not result in AHR or subepithelial fibrosis.[456] These studies suggest that IL-4 is essential for the initiation of Th2 polarized immune responses to allergenic peptides, whereas IL-13 alone may mediate the main physiologic consequences of allergic airway disease, namely AHR, mucus hypersecretion, and subepithelial fibrosis.

As IL-4 and IL-13 share a functional receptor, one of the questions puzzling immunologists is how IL-13 may play a preferential role in the effector arm of the allergic response. Several explanations have been postulated including the possibility that IL-4 production at the site of inflammation is short lived and that IL-13 may persist, giving the illusion that IL-13 is the more important mediator of the effector phase of the response. This hypothesis is not without merit as IL-4 is difficult to measure at the site of inflammation and kinetic studies have shown sustained IL-13 production in the lungs of patients with asthma.[457] However, several in vitro studies have also claimed to observe unique functions of these cytokines in systems in which the level of cytokines is controlled. It can always be debated that any differences observed in these systems may be due to differences in degradation of these two proteins in culture or that the recombinant forms of these proteins do not have equivalent potency. Another potential hypothesis is that IL-13 may act through an as yet unidentified receptor complex. In support of this hypothesis, a recent study demonstrates that although AHR and eosinophilia induced by adoptive transfer of IL-13-sufficient T cells are STAT6 dependent, these effects appear to occur independently of the IL-4Rα chain.[458] These studies suggest that an as yet unidentified IL-13 binding chain may exist or that different configurations of the existing chains may mediate distinct effects of IL-13 stimulation. As will be discussed subsequently, the IL-13Rα2 chain may play a role in mediating processes unique to IL-13 under some circumstances. Alternatively, it has recently been shown that IL-4 may induce inhibitory pathways through the type I receptor, which limit its proallergic effects mediated through the type II receptor.[459] On the other hand, it has been shown that IL-13 induces a small set of

epithelial-specific genes that are not upegulated by IL-4 in vivo but are however STAT6 dependent. This apparent IL-13 selectivity either occurs via IL-13 stimulation of a unique receptor complex or as a result of IL-4 inhibition of these putative pathways. Although the mystery underlying the unique functions of IL-4 and IL-13 is far from solved, multiple explanations for their apparently unique functions are emerging.

IL-13 has several actions that implicate it as an effector in the allergic diathesis such as its role in mucus production, fibrotic processes, and perhaps bronchoconstriction (Fig. 45.10). First, it has been shown to play an important role in mucus hypersecretion. Mucus hypersecretion is a consistent feature of the allergic phenotype in both the upper and lower respiratory tract. In fact, extensive plugging of the airway lumen has been associated with fatal episodes of asthma. This response is a Th2 cell-dependent process as adoptive transfer of Th2 cells into the murine lung reconstitutes the effect of antigen challenge.[44] Several lines of evidence suggest that mucus cell metaplasia is an IL-13-, not IL-4-, dependent process. For example, transfer of Th2 cells devoid of IL-4 or IL-5 genes still induce extensive goblet cell metaplasia in the murine lung. However, blockade of the IL-4Rα chain or deficiency in STAT6 prevents the development of mucus cell metaplasia following allergen challenge suggesting that IL-13 may be the ligand for the IL-4/STAT6 pathway in mucus cell changes.[50,307] Indeed, administration of soluble IL-13Rα2 reversed the metaplastic response of goblet cells induced by allergen sensitization and challenge. Administration of rIL-13 in vivo or overexpression of the IL-13 gene recapitulates antigen effects on mucus production. Conversely, allergeninduced goblet cell metaplasia is significantly reduced in IL-13-deficient mice.[460] This was not further reduced when IL-4 was blocked with neutralizing antibodies, suggesting that indeed IL-13 is the primary regulator in vivo of mucus cell hyperplasia. The mechanisms by which IL-13 regulates mucus production are not entirely clear. However, IL-13 has been shown to coordinately regulate a number of processes in the airway epithelium that may contribute to mucus hypersecretion such as the induction of mucin gene expression and ion channel gene expression and activity.[461]

Another feature of the chronic inflammatory response in both the skin and the airways is the presence of a fibrotic process. Recent data suggests that IL-13 is an important regulator of fibrotic processes. For example, overexpression of IL-13 in the murine lung induces a dramatic fibrotic response in the airway wall.[455] Furthermore, IL-13 transgenic mice express matrix proteases such as metalloproteinase and cathepsins that are thought to be important in the fibrotic response.[462] A clear demarcation of function for IL-4 and IL-13 in fibrosis has been demonstrated in Th2-mediated pathology of *Schistosome mansoni* infection in mice. *Schistosome*-induced collagen deposition is reduced in the lungs of STAT6-deficient animals but not in IL-4-deficient mice.[463] Furthermore, sIL-13Rα2-Ig delivery completely prevented the fibrotic response in parasite-infected mice. Thus in several models of Th2-mediated fibrosis, blockade

of IL-13 selectively inhibited fibrotic remodeling processes, suggesting that this cytokine may be an important mediator of inflammation-induced tissue fibrosis.

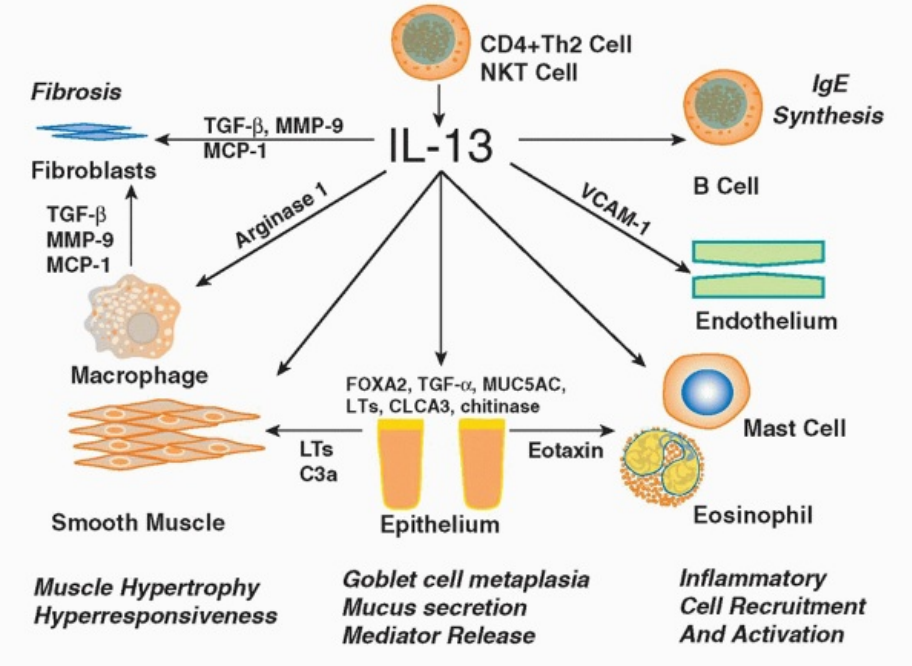

FIG. 45.10. Schematic Representation of the Role of Interleukin (IL)-13 in the Effector Phase of the Allergic Response. IL-13 is produced in the airway by a variety of cells including T cells (cluster of differentiation4+, natural killer T cells), eosinophils, and mast cells. IL-13 binds its receptor on multiple cell types and induces the expression of a variety of genes in target cells. The consequencecs of IL-13 receptor activation include immunoglobulin E synthesis, adhesion molecule expression on endothelial cells and egress of inflammatory cells into mucosal tissues, inflammatory cell activation (eosinophils and mast cells), epithelial cell activation leading to mucus hypersecretion (FOXA2, CLCA1/3, transforming growth factor [TGF]-α, MUC5AC) and mediator release (LTs, C3, eotaxin), smooth muscle activation and hypertrophy, airway hyperresponsiveness, activation of macrophages and fibroblasts leading to the production of various enzymes involved in collagen synthesis (arginase I, MMP-9, TGF-β, PDGF-AA), and deposition leading to tissue fibrosis. C3a, complement factor 3; CLCA1/2, $Ca+^{2}$ activating chloride channel gene 1/3; FOXA2, forkhead box a2 transcription factor; MMP9, metalloproteinase 9; MUC5AC, mucin gene 5AC; TGF-a, transforming growth factor alpha; VCAM-1, vascular cell adhesion molecule 1.

Although the mechanisms by which IL-13 induces tissue fibrosis are far from understood, several recent studies have begun to shed light on the sequence of events leading to IL-13-induced fibrosis. IL-13 likely regulates tissue fibrosis through a series of coordinated actions on a number of cells types within the lung. First, IL-13 has been shown by several investigators to upregulate the synthesis of arginase I.[464,465] Arginase I is an enzyme that hydrolyzes L-arginine to urea and L-ornithine, which is a necessary metabolite for the production of polyamines and prolines required for collagen synthesis by fibroblasts. Evidence of an in vivo role for this pathway in tissues fibrosis was provided by Hesse and colleagues.[464] Specifically, they showed that blockade of ornithine-aminodecarboxylase, which ultimately results in the generation of proline, markedly enhanced parasite-driven

tissue fibrosis. Consistent with a role for arginase I in asthma, Rothenberg and colleagues[465] demonstrated that the enzyme is upregulated in an ovalbumin model of asthma and in animals exposed to recombinant IL-13 as well as in lung tissues of asthmatic as compared to normal individuals. Secondly, the ability of IL-13 to induce the profibrotic mediator TGF-β also contributes to IL-13-dependent lung fibrosis.[466] In this regard, IL-13 induces the production of TGF-β both directly in epithelial cells and indirectly in monocytes/macrophages via its ability to recruit and activate these cells.[467] This is thought to occur via the interaction of MCP-1 with its receptor CCR2 on monocyte/macrophages, as both lung fibrosis and TGF-β production in IL-13 transgenic mice are diminished in mice devoid of CCR2.[468] Further support for this argument is the fact that MCP-1 has been shown to stimulate TGF-β1 production in lung fibroblasts.[469] In addition to stimulation of TGF-β synthesis, IL-13 also activates latent TGF-β through the induction of MPP9.[466]

Lastly, IL-13 may also have a direct effect on fibrotic processes by stimulating the proliferation of myofibroblasts via a STAT6-dependent process involving platelet-derived growth factor AA.[470] Lung fibrosis may also arise as a result of the ability of IL-13 to stimulate the accumulation of the nucleoside adenosine.[471] Specifically, it has been shown that IL-13-overexpressing mice have increased adenosine levels

and reduced adenosine deaminase gene expression and enzyme activity, while exhibiting marked increases in lung collagen. Furthermore, blockade of adenosine levels in the lung by adenosine deaminase enzyme therapy diminished IL-13-induced fibrosis concomitant with a reduction in elaboration of a selective set of chemokines (MCP-1, 2, 3, 5, and eotaxin). Collectively, these studies suggest that a number of IL-13-driven processes culminate in the generation of fibrogenic processes in the lung.

AHR or an exaggerated response to specific and nonspecific stimuli is a hallmark of asthma. The exact mechanisms by which IL-13 induces AHR are currently unknown. Several lines of evidence suggest that IL-13 can induce AHR in the absence of inflammatory cells. The initial IL-13Rα2Ig studies showed that IL-13 blockade inhibited AHR without affecting inflammatory cell recruitment or IgE synthesis.[61] Moreover, Grunig et al.[64] demonstrated that T and B cells were not essential for the effects of IL-13 on AHR. Support for this contention is provided by Vargaftig and colleagues,[472] who demonstrated that pretreatment of mice with vinblastine, a granulocyte inhibitor, failed to inhibit IL-13-induced AHR. In support of this theory, IL-13 has been shown to induce hyperresponsiveness as early as 6 hours after administration, long before significant inflammation has occurred.[473] More definitely, studies in eotaxin/IL-5 double knockout mice[454] confirm the contention that the traditional effector cells, B cells, mast cells, and eosinophils are not required for IL-13 to induce AHR. These results suggest that although IL-13 is able to direct the recruitment of inflammatory cells into the airways, they are likely not necessary for induction of AHR. Alternatively, IL-13 may induce AHR via direct effects on resident airway cells. Both airway epithelial cells and airway smooth muscle have been implicated by studies in driving AHR. However, whether AHR is mediated by direct effects on airway smooth muscle or indirectly via effects on resident airway cells such as the epithelium is currently a matter of debate. The importance of the IL-13-epithelial cell axis was illustrated by the demonstration that AHR develops in mice that

overproduce IL-13 in their lungs and express Stat6 only in airway epithelium.[474] However, a subsequent study showed that IL-13 also induced AHR in mice that selectively lack IL-4Rα in airway epithelial cells.[475] The suggestion that other cell types may contribute to IL-13-induced AHR led Finkelman and colleagues[476] to explore the role of the IL-4Rα in mice that express IL-4Rα only on smooth muscle cells or on all cell types other than smooth muscle. Studies with these mice demonstrate that direct effects of IL-13 on smooth muscle, as on airway epithelium, are sufficient but not necessary to induce AHR. Additional studies are required to determine whether IL-13-induced AHR can be totally accounted for by its effects on epithelial and smooth muscle cells or by as yet unidentified pathways.

The importance of IL-13 in allergic disorders in humans is supported by numerous reports of exaggerated IL-13 production in allergic diseases.[477,478,479] In asthma, in particular, message levels of IL-13 are elevated in bronchial biopsy specimens from allergic individuals when compared with those of control subjects.[477] Conversely, IL-13 levels are reduced in patients with asthma undergoing steroid treatment.[478] Moreover, a recent cohort study of neonates demonstrates that high IL-13 productive capacity of cord blood CD4+ T cells is a significant predictor of risk for subsequent development of atopic diseases.[479] In support of the notion that IL-13 is a central effector of allergic immune responses, several groups have reported strong associations of polymorphisms in the IL-13 promoter and coding region with various features of the asthmatic phenotype.[480,481] Of particular interest is the Arg130/Gln substitution in the coding region of IL-13 noted by multiple groups and detected across multiple ethnic groups. Moreover, Arima and colleagues have recently provided evidence that the Arg110Gln may be a functional variant.[482] Utilizing a mutant recombinant IL-13, they show that recombinant IL-13 containing the Gln110 variant bound the IL-13Rα2 chain with lower affinity than the wild-type IL-13, resulting in a lower clearance rate of the cytokine. Interestingly, they demonstrated that although the IL-13 variant did not alter any biologic properties of IL-13, patients with asthma homozygous for the Gln110 variant have higher serum levels of IL-13 than those without the variant. They postulated that impaired binding of IL-13 by the soluble IL-13Rα2 chain leads to higher serum levels and prolonged activity of IL-13 in vivo. Vercelli and colleagues[483] have shown that the -1112T variant of the IL-13 promoter is functional as expression of it in human and murine CD4+ Th2 lymphocytes enhanced IL-13 promoter activity. Interestingly, increased expression of IL-13-1112T in Th2 cells was associated with the creation of a Yin-Yang 1 binding site that overlapped a STAT motif involved in negative regulation of IL-13 expression and attenuated STAT6-mediated transcriptional repression. The enhanced IL-13 secretion in IL13-1112TT homozygotes suggests that expression of this allele may underlie its association with susceptibility to allergic inflammation.

Unifying Hypothesis

The elicitation of allergic airway responses in a sensitized individual upon reexposure to offending allergens is likely the culmination of a complex network of cellular and molecular events (Fig. 45.11). Following antigen challenge, cross-linking of antigen and IgE on IgE-bearing cells leads to the immediate release of substances such as histamine, leukotrienes, PGD_2, and tryptase. The actions of these mediators account for the immediate symptoms, such as smooth muscle constriction, vasodilation, and increased vascular permeability. It is

possible that the release of IL-13 by either mast cells or basophils can also induce many of these same symptoms. Secretion of chemokines (eotaxin) and cytokines (IL-4, IL-5) by basophils and mast cells also serve to attract more T cells, APCs, and effector cells such as eosinophils to the site of insult. Simultaneously, DCs within the mucosa are presenting allergen to T cells and within 6 to 48 hours, additional T cells are recruited to the area. The expression of FcεRI on the surface of DCs greatly facilitates their uptake, processing, and presentation of allergens to T cells during the elicitation phase of the response. Furthermore, the cytokine milieu created as a result of mast cell and basophil activation, namely a Th2

cytokine environment, most likely drives a polarized Th2 response. Further production of Th2 cytokines at the local site augments the recruitment and activation of additional eosinophils and T cells. During the late phase, release of mediators from both mast cells, basophils, eosinophils, and T cells act in concert to induce the vascular changes, bronchoconstrictor, and mucus changes observed during the late-phase response. Many repetitions of this sequence leads to persistent inflammation resulting in alterations in the structure and function of resident mucosal cells such as smooth muscle, fibroblasts, and epithelial cells. As many of the actions of these effector cells are redundant, it is not surprising that neither depletion of mast cells nor eosinophils has been sufficient to significantly ablate the physiologic consequences of allergen exposure. One point of convergence is the production of IL-13 by all of the likely suspects (eg, basophils, mast cells, eosinophils, and T cells). Given its ability to induce effector functions, elevations in IL-13 levels may not only provide a unifying explanation for the importance of multiple effector cells in allergic responses, but may also provide an ideal target for therapy.

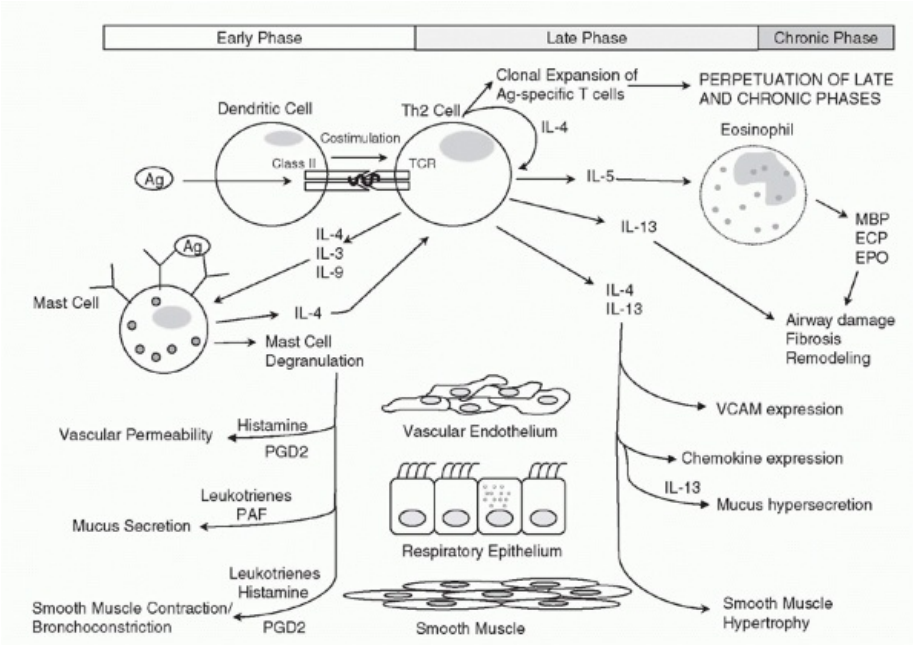

FIG. 45.11. Overview of the Acute, Late, and Chronic Phases of the Allergic Response. Cross-linking of surface immunoglobulin E on sensitized mast cells by antigen results in mast cell degranulation and release of numerous mediators including histamine, tryptase, PDG_2, and cytokines. These mediators are largely responsible for the early phase of the allergic response. Antigen presentation by dendritic cells to Th2

cells results in activation and clonal expansion of antigen-specific T cells and the subsequent release of Th2 cytokines, interleukin (IL)-4, IL-5, and IL-13. These cytokines result in recruitment of eosinophils, chemokine expression, smooth muscle hypertrophy, and mucus hypersecretion. Ultimately, long-term exposure to IL-13 and eosinophilic effector molecules leads to the structural changes observed in tissues of individuals with chronic disease.

CHARACTERISTICS OF SPECIFIC ATOPIC DISORDERS

In this section, we briefly discuss the clinical manifestations, pathogenesis, and therapeutic strategies for the treatment of the classical atopic syndromes including anaphylaxis, allergic rhinitis, atopic dermatitis, and asthma. Readers should consult clinical allergy textbooks for further details on these disorders.

Anaphylaxis

Anaphylaxis refers to a systemic, immediate hypersensitivity reaction that results from IgE-mediated release of vasoactive and inflammatory mediators from mast cells and basophils. Death from anaphylaxis is most often due to respiratory obstruction and/or cardiovascular collapse. The initial experimental description of the phenomenon dates to a paper published in 1902, in which Portier and Richet[484] described the sensitization of dogs to sea anemone venom, a process with fatal sequelae upon subsequent exposure to nonlethal doses of the venom. In opposition to prophylaxis, this was

termed anaphylaxis, meaning against or without protection. Common causes of anaphylaxis in humans include exposure to antibiotics and other drugs, radiocontrast media, latex, venom, and foods. The cause of anaphylaxis remains unidentified in up to two-thirds of patients. Whereas anaphylactic reactions involve IgE-mediated mast cell and basophil degranulation, anaphylactoid reactions result from mast cell and basophil degranulation by IgE-independent means. Underlying etiologies, where known, include drugs, biologic agents, and physical factors (eg, pressure, cold, sunlight); a substantial proportion of cases are idiopathic.

Recently anaphylaxis to ingestion of peanuts has become a common problem particularly in children.[485] The exact reasons for the rise in incidence of peanut-induced anaphylaxis are unknown. However, in a series of elegant experiments, Finkelman and colleagues[486] demonstrated that the high incidence, persistence, and severity of peanut allergy might result from a combination of properties that make it a perfect allergen: high content of several poorly digestible proteins that have strong B- and T-cell epitopes, the ability of at least one of these proteins to directly activate APCs, and the ability of soluble peanut molecules to rapidly activate complement with production of large amounts of the anaphylatoxin C3a. The rapid production of C3a through an antibody-independent pathway stimulates macrophages, basophils, and, to a lesser extent, mast cells to secrete PAF and histamine, which contribute to the induction of shock by increasing vascular permeability. Because activated complement is a potent adjuvant, peanut activation of complement might contribute to induction of the IgE response to peanut allergens in addition to the effector phase of peanutinduced shock. The fact that complement is activation by both hymenoptera venom[487] and metabolites of

penicillin[488] suggest that it may be a common mechanism of anaphylaxis.

Allergic Rhinitis

In 1819, John Bostock first described catarrus aestivus or hay fever.[489] In 1873, Charles Blackley recognized that pollen grains were the causative agents of hay fever. In the 1800s, hay fever was considered a rare disorder that was restricted to the privileged class. This is certainly no longer the case. According to recent estimates, up to 40% of children in the United States are affected by allergic rhinitis,[490] making it the most common atopic disorder in the industrial north. Allergic rhinitis is an IgE-mediated disease characterized by sneezing, rhinorrhea, nasal congestion, and nasal pruritis.[491,492] The seasonal form is caused by allergens released during tree, grass, or weed pollination; the perennial form is associated with allergies to animal dander, dust mites, and/or mold spores. Skin testing-the experimental interrogation of the ability of a panel of antigen extracts to induce cutaneous immediate type hypersensitivity responses—is often employed for diagnostic confirmation of atopy and to determine the allergens to which an individual is sensitized.

Treatment strategies include allergen avoidance, antihistamines, α-adrenergic agonists, intranasal steroids, topical ipratropium bromide, and immunotherapy regimes. Immunotherapy, introduced by Noon and Freeman in 1911 as a method for protecting patients against the effects of "pollen toxin,"[493] has been used since as a treatment for patients with allergic rhinitis and allergic asthma. Conventional allergen immunotherapy involves the subcutaneous injection of graded quantities of allergen. While such immunotherapy has been associated with therapeutic benefit, the relevant immunologic mechanisms remain obscure. In patients with venom anaphylaxis, allergen immunotherapy is the prophylactic treatment of choice.

Food Allergy

Food allergy needs careful distinction from the more common adverse reactions to ingested substances (eg, food intolerance, dose-related toxic reactions) that are not immune-mediated. True food allergy afflicts approximately 8% of children under the age of 3 years and 2% of the adult population. Such food hypersensitivity comprises several disorders that vary in time of onset, severity, and persistence. The most common type of food allergy is immediate gastrointestinal hypersensitivity. Symptoms, consisting of nausea, abdominal pain, colic, vomiting, and/or diarrhea, develop within minutes to 2 hours of antigen exposure. Infants with this syndrome may present with intermittent vomiting and poor weight gain. The predominant response to orally ingested antigens is the induction of tolerance. Food allergy represents an aberration of this process. Only a small number of foodstuffs account for the vast majority of offending allergens. In childhood, the most common allergens derive from milk, egg, peanut, soy, and wheat. In adults, the most common foods implicated are peanuts, tree nuts, fish, and shellfish. Most food allergens are relatively small, watersoluble, heat- and acid-stable glycoproteins that are resistant to proteolytic degradation. The higher incidence of disease in childhood is presumably related to factors regulating the ontogeny of the gut and immune system development.[494,495] Therapy revolves around avoidance of the offending allergen. Interestingly, patients with AD commonly have subclinical food hypersensitivity, with ingestion of the relevant allergen leading to worsening of their dermatitis.

Oral allergy syndrome represents a second type of food hypersensitivity. This is an

immediate type contact allergy that leads to pruritus, tingling, and swelling of the lips, palate, and throat following ingestion of the offending allergen, usually in fruits or vegetables. Oral allergy syndrome affects up to 40% of adults with defined pollen allergy due to crossreacting allergens. Therapy involves allergen avoidance.

The eosinophilic gastroenteritides (eosinophilic esophagitis, gastritis, gastroenteritis), though not thought in general to be due to food allergy, deserve brief mention here. These syndromes are characterized pathologically by eosinophil infiltration and clinically by a variety of nonspecific symptoms, including abdominal pain, nausea, vomiting, and diarrhea, although the etiology and pathogenesis remain unclear in most cases. Some cases are due to hypersensitivity reactions to antigens derived from *Anycylostoma caninum*, a common dog hookworm that is poorly adapted to and causes nonpatent infection of humans.[496] Some cases may indeed be due to food allergy. Eosinophilic esophagitis is an inflammatory

disease of the esophagus characterized by the presence of a dense infiltrate of eosinophils restricted to the mucosa of this organ. It is manifest by chronic and/or recurrent dysphagia and episodes of esophageal alimentary impaction, with great variation in terms of intensity, frequency, and duration of the attacks. Eosinophilic esophagitis appears to be an antigendriven hypersensitivity reaction characterized by a mixed IgE-dependent/delayed-type reaction and a type 2 cytokine profile. Treatment options include nonpharmacologic approaches including elimination or elemental diets and/ or treatment with corticosteroids. The topical administration of fluticasone propionate has been demonstrated to improve symptoms and mobilize the pathologic infiltrate of eosinophils. The anti-IL-5 monoclonal anitbody (mepolizumab) has been shown to be effective in inhibiting both blood and tissue eosinophils in patients with eosinophilic esphagitis[453]; however, further studies are necessary to determine whether this treatment will eliminate the symptoms of disease. Acid suppression by a proton pump inhibitor may also be considered in view of the overlap between eosinophilic esophagitis and gastroesophageal reflux disease.

Atopic Dermatitis

AD is a common, chronic, relapsing, inflammatory skin disease characterized by dry skin, severe pruritis, secondary excoriation that can lead to lichenification, and a heightened susceptibility to cutaneous infections. AD can lead to significant suffering, both physical and psychologic. The prevalence of AD has increased in the last few decades; currently, 10% are affected at some point during childhood in the United States. Both environmental and genetic factors seem to play a role in susceptibility. Given the fact that the hereditability of atopy appears to be largely disease type-independent, it is notable that parental AD confers a higher risk for the development of AD than for either allergic asthma or allergic rhinitis in offspring, suggesting the likelihood of atopic dermatitis-specific genes.[497] Disease generally presents early in childhood and is associated with sensitivity to food and/or inhalant allergens. In adults, disease is primarily associated with sensitivity to inhalant allergens. Apart from exposure to specific food and inhalant allergens, environmental triggers such as irritating substances, emotional stress, climactic factors, hormones, and local infections are all known to be important in the expression of atopic dermatitis. Skin lesions generally display evidence of mast cell, eosinophil, and T-cell infiltration and activation. In contrast to other atopic disorders, a biphasic pattern of T-cell polarization or reactivity is present in atopic dermatitis: Th2 cytokines predominate in acute lesions, whereas chronic lesions express a mix of Th1

and Th2 cytokines.

Current treatment of AD is directed at symptomatic relief, skin hydration, reduction of cutaneous inflammation, and avoidance of inciting antigens. Therapies include antihistamines, topical immunosuppressive agents (glucocorticoids, FK506), moisturizers, environmental control measures for inhalant allergens, and food elimination diets for food allergens. However, in a subgroup of patients with moderate to severe AD, the disease is recalcitrant to topical therapy and systemic treatments become necessary. Shortterm treatment with a number of immunosuppressive and immunomodulating substances such as cyclosporine, azathioprine, mycophenolate mofetil, and methotrexate has proven effective in patients with moderate to severe AD.[498] Intravenous Igs and IFNγ exert immunomodulatory effects and thus may improve severe AD. In patients for whom cyclosporin is not suitable, or when there is a lack of response, alternative drugs should be considered, such as azathioprine or IFNγ. Intravenous Igs and the monoclonal antibody infliximab only have a place in the systemic therapy of AD when other drugs have failed. Mycophenolate mofetil has recently been introduced in the treatment of recalcitrant AD. Omalizumab is a monoclonal antibody with a possible future role in the treatment of AD, but further studies are needed.[499]

ASTHMA

The term asthma was coined by Hippocrates to refer to attacks of breathlessness and wheezing. Asthma is a complex inflammatory disease of the lung in which the prevalence, morbidity, and mortality have been increasing markedly over the last few decades. Asthma is a heterogeneous disorder with variations in the age of onset, severity of disease, and underlying pathogenesis. Although asthma is multifactorial in origin with both environmental and genetic influences, atopy is the strongest identifiable predisposing factor for the development of asthma. Most childhood asthma is allergic in nature and referred to as extrinsic or atopic asthma; however, asthma that develops in adulthood and some forms of childhood asthma are not associated with elevated IgE; this form is referred to as intrinsic or nonatopic asthma. In the most common form of the disease, extrinsic asthma, the inflammatory process is thought to arise as a result of inappropriate immune responses to commonly inhaled antigens. The inflammatory response in the asthmatic lung is characterized by infiltration of the airway wall with lymphocytes, predominantly CD4+ T cells, eosinophils, and degranulated mast cells. Structurally, the airways of asthmatics are characterized by mucus cell hyperplasia, subepithelial membrane thickening, and loss of epithelial cell integrity. These cellular findings have consistently been associated with the main physiologic abnormalities of the disease, including variable airflow obstruction and AHR. As discussed extensively in this chapter, the pathologic consequences of this disease are thought to arise as a result of skewed T-cell responses to inhaled antigens, which, in turn, lead to activation and recruitment of the primary effector cells, mast cells, eosinophils, and T cells. Activation of these cells results in the release of a plethora of mediators that individually or in concert induce changes in airway geometry and produce the symptoms of the disease.

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REFERENCES

1. von Pirquet C. Allergie. Munch Med Wochenschr (translated from the German by Prausnitz C). In: Gell PGH, Cooms RRA, editors. *Clinical Aspects of Immunology* . Vol 30. Oxford, United Kingdom: Blackwell Scientific Publications; 1963:1457.

2. Prausnitz C, Kustner H. Studies concerning sensitivity. *Zent bl Bakteriol I Orig* . 1921;86:160-169.

3. Ishizaka K, Ishizaka T, Lee EH. Physiochemical properties of reaginic antibody. II. Characteristic properties of reaginic antibody different from human gamma-A-isohemagglutinin and gamma-D-globulin. *J Allergy* . 1966;37:336-349.

4. Ishizaka K, Ishizaka T, Hornbrook MM. Physico-chemical properties of human reaginic antibody. IV. Presence of a unique immunoglobulin as a carrier of reaginic activity. *J Immunol* . 1966;97:75-85.

5. Ishizaka K, Ishizaka T, Hornbrook MM. Physicochemical properties of reaginic antibody. V. Correlation of reaginic activity wth gamma-Eglobulin antibody. *J Immunol* . 1966;97:840-853.

6. Holford-Strevens V, Warren P, Wong C, et al. Serum total immunoglobulin E levels in Canadian adults. *J Allergy Clin Immunol* . 1984;73: 516-522.

7. Peat JK, Britton WJ, Salome CM, et al. Bronchial hyperresponsiveness in two populations of Australian schoolchildren. III. Effect of exposure to environmental allergens. *Clin Allergy* . 1987;17:291-300.

8. Cline MG, Burrows B. Distribution of allergy in a population sample residing in Tucson, Arizona. *Thorax* . 1989;44:425-431.

9. Organization WH. Allergen nomenclature IUIS/WHO Allergen Nomenclature Subcommittee. *Bull World Health Org* . 1994;72:797-806.

10. Slater JE. Latex allergy. *J Allergy Clin Immunol* . 1994;94:139-149.

11. Turjanmaa K, Alenius H, Makinen-Kiljunen S, et al. Natural rubber latex allergy. *Allergy* . 1996;51:593-602.

12. Breiteneder H, Scheiner O. Molecular and immunological characteristics of latex allergens. *Int Arch Allergy Immunol* . 1998;116:83-92.

13. Theissen U, Theissen JL, Mertes N, et al. IgE-mediated hypersensitivity to latex in childhood. *Allergy* . 1997;52:665-669.

14. King C, Brennan S, Thompson PJ, Stewart GA. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J Immunol*. 1998;161:3645-3651.

15. Sun G, Stacey MA, Schmidt M, Mori L, Mattoli S. Interaction of mite allergens Der p 3 and Der p 9 with protease-activated receptor-2 expressed by lung epithelial cells. *J Immunol*. 2001;167:1014-1021.

16. Bhat RK, Page K, Tan A, Hershenson MB. German cockroach extract increases bronchial epithelial cell interleukin-8 expression. *Clin Exp Allergy*. 2003;33:33-42.

17. Kouzaki H, O'Grady SM, Lawrence CB, Kita H. Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. *J Immunol*. 2009;183:1427-1434.

18. Ring PC, Wan H, Schou C, et al. The 18 kDa form of cat allergen Felis domesticus 1 (Fel d1) is associated with gelatin- and fibronectin-degrading activity. *Clin Exp Allergy*. 2000;30:1085-1096.

19. Widmer F, Hayes PJ, Whittaker RG, Kumar RK. Substrate preference profiles of proteases released by allergenic pollens. *Clin Exp Allergy*. 2000;30:571-576.

20. Saeki K, Ozaki K, Kobayashi T, Ito S. Detergent alkaline proteases: enzymatic properties, genes, and crystal structures. *J Biosci Bioeng*. 2007;103:501-508.

21. Novey HS, Marchioli LE, Sokol WN, Wells ID. Papain-induced asthma-physiological and immunological features. *J Allergy Clin Immunol*. 1979;63:98-103.

22. Maleki SJ, Viquez O, Jacks T, et al. The major peanut allergen, Ara h 2, functions as a trypsin inhibitor, and roasting enhances this function. *J Allergy Clin Immunol*. 2003;112:190-195.

23. Traidl-Hoffmann C, Mariani V, Hochrein H, et al. Pollen-associated phytoprostanes inhibit dendritic cell interleukin-12 production and augment T helper type 2 cell polarization. *J Exp Med*. 2005;201:627-636.

24. Boldogh I, Bacsi A, Choudhury BK, et al. ROS generated by pollen NADPH oxidase provide a signal that augments antigen-induced allergic airway inflammation. *J Clin Invest*. 2005;115:2169-2179.

25. Trompette A, Divanovic S, Visintin A, et al. Allergenicity resulting from functional mimicry of a toll-like receptor complex protein. *Nature*. 2009;457:585-588.

26. Shreffler WG, Castro RR, Kucuk ZY, et al. The major glycoprotein allergen from Arachis hypogaea, Ara h 1, is a ligand of dendritic cellspecific ICAM-grabbing nonintegrin and acts as a Th2 adjuvant in vitro. *J Immunol*. 2006;177(6):3677-3685.

27. Shakib F, Schulz O, Sewell H. A mite subversive: cleavage of CD23 and CD25 by Der p 1 enhances allergenicity. *Immunol Today*. 1998;19: 313-316.

28. Karlsson MG, Davidsson A, Hellquist HB. Increase in CD4+ and CD45RO+ memory T cells in the nasal mucosa of allergic patients. *APMIS*. 1994;102:753-758.

29. Azzawi M, Bradley B, Jeffery PK, et al. Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. *Am Rev Respir Dis* . 1990;142:1407-1413.

30. Schauer U, Jung T, Heymanns J, Rieger CH. Imbalance of CD4+CD45R+ and CD4+CD29+ T helper cell subsets in patients with atopic diseases. *Clin Exp Immunol*. 1991;83:25-29.

31. Mosmann TR, Cherwinski H, Bond MW, et al. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*. 1986;136:2348-2357.

32. Street NE, Mosmann TR. Functional diversity of T lymphocytes due to secretion of different cytokine patterns. *FASEB J*. 1991;5:171-177.

33. Kapsenberg ML, Wierenga EA, Bos JD, et al. Functional subsets of allergen-reactive human CD4+ T cells. *Immunol Today*. 1991;12:392-395.

34. Del Prete GF, De Carli M, Mastromauro C, et al. Purified protein derivative of Mycobacterium tuberculosis and excretory-secretory antigen(s) of Toxocara canis expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. *J Clin Invest*. 1991;88:346-350.

35. Read S, Powrie F. CD4(+) regulatory T cells. *Curr Opin Immunol* . 2001; 13:644-649.

36. Bettelli E, T Korn, VK Kuchroo. Th17: the third member of the effector T cell trilogy. *Curr Opin Immunol* . 2007;19(6):652-657.

37. Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu Rev Immunol*. 1999;17:255-281.

38. Grewe M, Bruijnzeel-Koomen CA, Schopf E, et al. A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunol Today*. 1998;19:359-361.

39. Werfel T, Morita A, Grewe M, et al. Allergen specificity of skin-infiltrating T cells is not restricted to a type-2 cytokine pattern in chronic skin lesions of atopic dermatitis. *J Invest Dermatol*. 1996;107:871-876.

40. Fokkens WJ, Godthelp T, Holm AF, et al. Allergic rhinitis and inflammation: the effect of nasal corticosteroid therapy. *Allergy*. 1997;52:29-32.

41. Secrist H, Chelen CJ, Wen Y, et al. Allergen immunotherapy decreases interleukin 4 production in CD4+ T cells from allergic individuals. *J Exp Med*. 1993;178:2123-2130.

42. Gavett SH, Chen X, Finkelman F, et al. Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. *Am J Respir Cell Mol Biol*. 1994;10:587-593.

43. Corry DB, Grunig G, Hadeiba H, et al. Requirements for allergen-induced airway hyperreactivity in T and B cell-deficient mice. *Mol Med*. 1998;4:344-355.

44. Cohn L, Tepper JS, Bottomly K. IL-4-independent induction of airway hyperresponsiveness by Th2, but not Th1, cells. *J Immunol*. 1998;161:3813-3816.

45. Gavett SH, O'Hearn DJ, Li X, et al. Interleukin 12 inhibits antigeninduced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J Exp Med*. 1995;182:1527-1536.

46. Lack G, Bradley KL, Hamelmann E, et al. Nebulized IFN-gamma inhibits the development of secondary allergic responses in mice. *J Immunol*. 1996;157:1432-1439.

47. Li XM, Chopra RK, Chou TY, et al. Mucosal IFN-gamma gene transfer inhibits pulmonary allergic responses in mice. *J Immunol*. 1996;157:3216-3219.

48. Yin Z, Chen C, Szabo SJ, et al. T-Bet expression and failure of GATA-3 cross-regulation lead to default production of IFN-γ by γδ T cells. *J Immunol*. 2002;168:1566-1571.

49. Raby BA, Hwang ES, Van Steen K, et al. T-bet polymorphisms are associated with asthma and airway hyperresponsiveness. *Am J Respir Crit Care Med*. 2006;173:64-70.

50. Gavett SH, O'Hearn DJ, Karp CL, et al. Interleukin-4 receptor blockade prevents airway responses induced by antigen challenge in mice. *Am J Physiol*. 1997;272:L253-L261.

51. Kung TT, Stelts DM, Zurcher JA, et al. Involvement of IL-5 in a murine model of allergic pulmonary inflammation: prophylactic and therapeutic effect of an anti-IL-5 antibody. *Am J Respir Cell Mol Biol*. 1995;13:360-365.

52. Brusselle G, Kips J, Joos G, et al. Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am J Respir Cell Mol Biol*. 1995;12:254-259.

53. Foster PS, Hogan SP, Ramsay AJ, et al. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med*. 1996;183:195-201.

54. Lukacs NW, Strieter RM, Chensue SW, et al. Interleukin-4-dependent pulmonary eosinophil infiltration in a murine model of asthma. *Am J Respir Cell Mol Biol* . 1994;10:526-532.

55. Saito H, Howie K, Wattie J, et al. Allergen-induced murine upper airway inflammation: local and systemic changes in murine experimental allergic rhinitis. *Immunology*. 2001;104:226-234.

56. Okano M, Azuma M, Yoshino T, et al. Differential role of CD80 and CD86 molecules in the induction and the effector phases of allergic rhinitis in mice. *Am J Respir Crit Care Med*. 2001;164:1501-1507.

57. Lee SY, Huang CK, Zhang TF, et al. Oral administration of IL-12 suppresses anaphylactic reactions in a murine model of peanut hypersensitivity. *Clin Immunol*. 2001;101:220-228.

58. Spergel JM, Mizoguchi E, Oettgen H, et al. Roles of TH1 and TH2 cytokines in a murine model of allergic dermatitis. *J Clin Invest*. 1999;103:1103-1111.

59. Brusselle GG, Kips JC, Tavernier JH, et al. Attenuation of allergic airway inflammation in IL-4 deficient mice. *Clin Exp Allergy*. 1994;24:73-80.

60. Coyle AJ, Le Gros G, Bertrand C, et al. Interleukin-4 is required for the induction of lung Th2 mucosal immunity. *Am J Respir Cell Mol Biol* . 1995;13:54-59.

61. Rothenberg ME, Hogan SP. The eosinophil. *Annu Rev Immunol*. 2006; 24:147-174.

62. Hultner L, Druez C, Moeller J, et al. Mast cell growth-enhancing activity (MEA) is structurally related and functionally identical to the novel mouse T cell growth factor P40/TCGFIII (interleukin 9). *Eur J Immunol* . 1990;20:1413-1416.

63. Wills-Karp M, Luyimbazi J, Xu X, et al. Interleukin-13: central mediator of allergic asthma. *Science*. 1998;282:2258-2261.

64. Grunig G, Warnock M, Wakil AE, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science*. 1998;282:2261-2263.

65. Akbari O, Stock P, Meyer E, et al. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat Med*. 2003;9:582-588.

66. Lisbonne M, Diem S, de Castro Keller A, et al. Cutting edge: invariant V alpha 14 NKT

cells are required for allergen-induced airway inflammation and hyperreactivity in an experimental asthma model. *J Immunol*. 2003;171:1637-1641.

67. Meyer EH, Goya S, Akbari O, et al. Glycolipid activation of invariant T cell receptor+ NK T cells is sufficient to induce airway hyperreactivity independent of conventional CD4+ T cells. *Proc Natl Acad Sci U S A* . 2006;103:2782-2787.

68. Morishima Y, Ishii Y, Kimura T, et al. Suppression of eosinophilic airway inflammation by treatment with alpha-galactosylceramide. *Eur J Immunol* . 2005;35:2803-2814.

69. Hachem P, Lisbonne M, Michel ML, et al. Alpha-galactosylceramideinduced iNKT cells suppress experimental allergic asthma in sensitized mice: role of IFN-gamma. *Eur J Immunol*. 2005;35:2793-2802.

70. Akbari O, Faul JL, Hoyte EG, et al. CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma. *N Engl J Med* . 2006;354:1117-1129.

71. Sen Y, Yongyi B, Yuling H, et al. V alpha 24-invariant NKT cells from patients with allergic asthma express CCR9 at high frequency and induce Th2 bias of CD3+ T cells upon CD226 engagement. *J Immunol*. 2005;175:4914-4926.

72. Wingender G, Rogers P, Batzer G, et al. Invariant NKT cells are required for airway inflammation induced by environmenal antigens. *J Exp Med*. 2011;208(6):1151-1162.

73. Agea E, Russano A, Bistoni O, et al. Human CD1-restricted T cell recognition of lipids from pollens. *J Exp Med*. 2005;202:295-308.

74. Elkhal A, Pichavant M, He R, et al. CD1d restricted natural killer T cells are not required for allergic skin inflammation. *J Allergy Clin Immunol* . 2006;118:1363-1368.

75. Al-Ramli W, Prefontaine D, Chouiali F, et al. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J Allergy Clin Immunol* . 2009;123:1185-1187.

76. Lajoie S, Lewkowich IP, Suzuki Y, et al. Complement-mediated regulation of the IL-17A axis is a central genetic determinant of the severity of experimental allergic asthma. *Nat Immunol*. 2010;11:928-935.

77. Agache I, Ciobanu C, Agache C, Anghel M. Increased serum IL-17 is an independent risk factor for severe asthma. *Respir Med* . 2010;104:1131-1137.

78. Bullens DM, Truyen E, Coteur L, et al. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir Res*. 2006;7:135.

79. Wang JY, Shyur SD, Wang WH, et al. The polymorphisms of interleukin 17A (IL17A) gene and its association with pediatric asthma in Taiwanese population. *Allergy*.

2009;64:1056-1060.

80. Jin EH, Choi EY, Yang JY, Chung HT, Yang YS. Significant association between IL-17F promoter region polymorphism and susceptibility to asthma in a Korean population. *Int Arch Allergy Immunol* . 2010;155:106-110.

81. Randolph AG, Lange C, Silverman EK, et al. The IL12B gene is associated with asthma. *Am J Hum Genet*. 2004;75:709-715.

82. Hirota T, Suzuki Y, Hasegawa K, et al. Functional haplotypes of IL-12B are associated with childhood atopic asthma. *J Allergy Clin Immunol* . 2005;116:789-795.

83. Ivanov S, Palmberg L, Venge P, Larsson K, Linden A. Interleukin-17A mRNA and protein expression within cells from the human bronchoalveolar space after exposure to organic dust. *Respir Res*. 2005;6:44.

84. Pichavant M, Goya S, Meyer EH, et al. Ozone exposure in a mouse model induces airway hyperreactivity that requires the presence of natural killer T cells and IL-17. *J Exp Med*. 2008;205:385-393.

85. Saunders V, Breysse P, Clark J, Sproles A, Davila M, Wills-Karp M. Particulate matter-induced airway hyperresponsiveness is lymphocyte dependent. *Environ Health Perspect*. 2010;118:640-646.

86. Nakae S, Komiyama Y, Nambu A, et al. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity*. 2002;17:375-387.

87. Park SJ, Lee KS, Kim SR, et al. Peroxisome proliferator-activated receptor gamma agonist down-regulates IL-17 expression in a murine model of allergic airway inflammation. *J Immunol* . 2009;183:3259-3267.

88. Song C, Luo L, Lei Z. IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. *J Immunol* . 2008;181: 6117-6124.

89. Yang XO, Chang SH, Park H. Regulation of inflammatory responses by IL-17F. *J Exp Med*. 2008;205:1063-1075.

90. Peng J, Yang XO, Chang SH, Yang J, Dong C. IL-23 signaling enhances Th2 polarization and regulates allergic airway inflammation. *Cell Res*. 2010;20:62-71.

91. Lewkowich IP, Lajoie S, Clark JR, Herman NS, Sproles AA, Wills-Karp M. Allergen uptake, activation, and IL-23 production by pulmonary myeloid DCs drives airway hyperresponsiveness in asthma-susceptible mice. *PLoS One*. 2008;3:e3879.

92. McKinley L, Alcorn JF, Peterson A. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J Immunol* . 2008;181:4089-4097.

93. Wakashin H, Hirose K, Maezawa Y, et al. IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. *Am J Respir Crit Care Med* . 2008;178:1023-1032.

94. Wang YH, Voo KS, Liu B. A novel subset of CD4(+) T(H)2 memory/effector cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic allergic asthma. *J Exp Med*. 2010;207:2479-2491.

95. Hellings PW, Kasran A, Liu Z. Interleukin-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. *Am J Respir Cell Mol Biol*. 2003;28:42-50.

96. Schnyder-Candrian S, Togbe D, Couillin I, et al. Interleukin-17 is a negative regulator of established allergic asthma. *J Exp Med*. 2006;203: 2715-2725.

97. Besnard AG, Sabat R, Dumoutier L, et al. Dual role of IL-22 in allergic airway inflammation and its cross-talk with IL-17A. *Am J Respir Crit Care Med* . 2011;183:1153-1163.

98. Ghoreschi K, Laurence A, Yang XP, et al. Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. *Nature*. 2010;467:967-971.

99. Tanaka S, Yoshimoto T, Naka T, et al. Natural occurring IL-17 producing T cells regulate the initial phase of neutrophil mediated airway responses. *J Immunol* . 2009;183:7523-7530.

100. Saitoh T, Kusunoki T, Yao T, et al. Role of interleukin-17A in the eosinophil accumulation and mucosal remodeling in chronic rhinosinusitis with nasal polyps associated with asthma. *Int Arch Allergy Immunol* . 2010;151:8-16.

101. Liu T, Song CH, Liu AM, et al. Forkhead box P3+ T cells express interleukin-17 in nasal mucosa of patients with both allergic rhinitis and polyposis. *Clin Exp Immunol*. 2011;163:59-64.

102. Toda M, Leung DY, Molet S, et al. Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. *J Allergy Clin Immunol* . 2003;111:875-881.

103. Koga C, Kabashima K, Shiraishi N, Kobayashi M, Tokura Y. Possible pathogenic role of Th17 cells for atopic dermatitis. *J Invest Dermatol* . 2008;128:2625-2630.

104. Oyoshi MK, Murphy GF, Geha RS. Filaggrin-deficient mice exhibit TH17-dominated skin inflammation and permissiveness to epicutaneous sensitization with protein antigen.

J Allergy Clin Immunol . 2009;124:485-493.

105. Guttman-Yassky E, Lowes MA, Fuentes-Duculan J, et al. Low expression of the IL-23/Th17 pathway in atopic dermatitis compared to psoriasis. *J Immunol* . 2008;181:7420-7427.

106. Nograles KE, Zaba LC, Shemer A, et al. IL-22-producing “T22 T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing TH17 T cells. *J Allergy Clin Immunol* . 2009;123:1244-1252.

107. Eyerich S, Eyerich K, Pennino D, et al. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J Clin Invest* . 2009;119:3573-3585.

108. Wenzel SE, Schwartz LB, Langmack EL, et al. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am J Respir Crit Care Med* . 1999;160:1001-1008.

109. Zhang N, Van Zele T, Perez-Novo C, et al. Different types of T-effector cells orchestrate mucosal inflammation in chronic sinus disease. *J Allergy Clin Immunol* . 2008;122:961-968.

110. Wilson RH, Whitehead GS, Nakano H, Free ME, Kolls JK, Cook DN. Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. *Am J Respir Crit Care Med* . 2009;180:720-730.

111. Chen Y, Thai P, Zhao YH, Ho YS, DeSouza MM, Wu R. Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. *J Biol Chem*. 2003;278:17036-17043.

112. Park H, Li Z, Yang XO, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* . 2005;6:1133-1141.

113. Huang, F, Wachi S, Thai P, et al. Potentiation of IL-19 expression in airway epithelia by IL-17A and IL-4/IL-13: important implications in asthma. *J Allergy Clin Immunol* . 2008;121:1415-1422.

114. Vijlstra GJ, Ten Hacken NH, Hoffmann RF, van Oosterhout AJ, Heijink IH. Interleukin-17A induces glucocorticoid insensitivity in human bronchial epithelial cells. *Eur Respir J* . 2012;39:439-445.

115. Scherf W, Burdach S, Hansen G. Reduced expression of transforming growth factor beta 1 exacerbates pathology in an experimental asthma model. *Eur J Immunol* . 2005;35:198-206.

116. Wang Y, McCusker C. Neonatal exposure with LPS and/or allergen prevents experimental allergic airways disease: development of tolerance using environmental antigens. *J Allergy Clin Immunol* . 2006;118:143-151.

117. Zuany-Amorim C, Sawicka E, Manlius C, et al. Suppression of airway eosinophilia by killed Mycobacterium vaccae-induced allergen-specific regulatory T-cells. *Nat Med*. 2002;8:625-629.

118. Frick OL, Teuber SS, Buchanan BB, et al. Allergen immunotherapy with heat-killed Listeria monocytogenes alleviates peanut and foodinduced anaphylaxis in dogs. *Allergy*. 2005;60:243-250.

119. Matsumoto K, Inoue H, Fukuyama S, et al. Decrease of interleukin-10-producing T cells in the peripheral blood of severe unstable atopic asthmatics. *Int Arch Allergy Immunol*. 2004;134:295-302.

120. Ling EM, Smith T, Nguyen XD, et al. Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet*. 2004;363:608-615.

121. Karlsson MR, Rugtveit J, Brandtzaeg P. Allergen-responsive CD4+ CD25+ regulatory T cells in children who have outgrown cow's milk allergy. *J Exp Med*. 2004;199:1679-1688.

122. Karagiannidis C, Akdis M, Holopainen P, et al. Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma. *J Allergy Clin Immunol* . 2004;114:1425-1433.

123. Fainaru O, Woolf E, Lotem J, et al. Runx3 regulates mouse TGF-betamediated dendritic cell function and its absence results in airway inflammation. *EMBO J*. 2004;23:969-979.

124. Lewkowich IP, Herman NS, Schleifer KW, et al. CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J Exp Med*. 2005;202:1549-1561.

125. Ober C. Susceptibility genes in asthma and allergy. *Curr Allergy Asthma Rep* . 2001;1:174-179.

126. Thomsen SF, Duffy DL, Kyvik KO, Backer V. Genetic influence on the age of onset of asthma: a twin study. *J Allergy Clin Immunol* . 2010;626-630.

127. Ober C, Hoffjan S. Asthma genetics 2006: the long and winding road to gene discovery. *Genes Immun*. 2006;7:95-100.

128. Moffatt MF, Gut IG, Demenais D, et al. A large scale consortium-based genome-wide association study of asthma. *N Eng J Med* . 2010;363: 1211-1221.

129. Palmer CN, Irvine AD, Terron-Kiviatkowki A, Zhoa Y. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet*. 2006;38:441-446.

130. Sherrill JD, Gao PS, Stucke EM, Blanchard C. Variants of thymic stromal lymphopoietin and its receptor associate with eosinophilic eosophagitis. *J Allergy Clin Immunol*. 2010;126:160-165.

131. Bjorksten F, Suoniemi I, Koski V. Neonatal birch-pollen contact and subsequent allergy to birch pollen. *Clin Allergy*. 1980;10:585-591.

132. Carosso A, Ruffino C, Bugiani M, et al. The effect of birth season on pollenosis. *Ann Allergy*. 1986;56:300-303.

133. Wahn U, Lau S, Bergmann R, et al. Indoor allergen exposure is a risk factor for sensitization during the first three years of life. *J Allergy Clin Immunol* . 1997;99:763-769.

134. Prescott SL, Macaubas C, Holt BJ, et al. Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. *J Immunol*. 1998;160:4730-4737.

135. Martinez FD, Stern DA, Wright AL, et al. Association of interleukin-2 and interferon-gamma production by blood mononuclear cells in infancy with parental allergy skin tests and with subsequent development of atopy. *J Allergy Clin Immunol* . 1995;96:652-660.

136. Warner JA, Miles EA, Jones AC, et al. Is deficiency of interferon gamma production by allergen triggered cord blood cells a predictor of atopic eczema? *Clin Exp Allergy*. 1994;24:423-430.

137. Prescott SL, Macaubas C, Smallacombe T, et al. Reciprocal agerelated patterns of allergen-specific T-cell immunity in normal vs. atopic infants. *Clin Exp Allergy*. 1998;28(suppl 5):39-44; discussion 50-51.

138. Holt PG, Rowe J, Kusel M, et al. Toward improved predictors of risk for atopy and asthma among preschoolers: a prospective cohort study. *J Allergy Clin Immunol* . 2010;125:653-659.

139. Strachan DP. Hay fever, hygiene, and household size. *BMJ*. 1989;299: 1259-1260.

140. Wills-Karp M, Ewart SL. Time to draw breath: asthma-susceptibility genes are identified. *Nat Rev Genet*. 2004;5:376-387.

141. Von Mutius E, Vercelli D. Farm living: effects on childhood asthma and allergy. *Nat Rev Immunol*. 2010;10:861-868.

142. Remes ST, Castro-Rodriguez JA, Holberg CJ, et al. Dog exposure in infancy decreases the subsequent risk of frequent wheeze but not of atopy. *J Allergy Clin Immunol*. 2001;108:509-515.

143. Loss G, Apprich S, Waser M. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. *J Allergy Clin Immunol* . 2011;128:766-773.

144. Adlerberth I, Carlsson B, de Man P, et al. Intestinal colonization with Enterobacteriaceae in Pakistani and Swedish hospital-delivered infants. *Acta Paediatr Scand*. 1991;80:602-610.

145. Bottcher MF, Nordin EK, Sandin A, et al. Microflora-associated characteristics in faeces from allergic and nonallergic infants. *Clin Exp Allergy*. 2000;30:1590-1596.

146. Kalliomaki M, Kirjavainen P, Eerola E, et al. Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. *J Allergy Clin Immunol* . 2001;107:129-134.

147. Sudo N, Sawamura S, Tanaka K, et al. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol* . 1997;159: 1739-1745.

148. Brandtzaeg PE. Current understanding of gastrointestinal immunoregulation and its relation to food allergy. *Ann N Y Acad Sci* . 2002;964: 13-45.

149. Pabst R. The anatomical basis for the immune function of the gut. *Anat Embryol (Berl)*. 1987;176:135-144.

150. Hilty M, Burke C, Pedro H, et al. Disordered microbial communities in asthmatic airways. *PLOS One*. 2010;5:1-9.

151. Ivanov II, Atarashi K, Mane N, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell*. 2009;139(3):485-488.

152. Gao PS, Mao XQ, Baldini M, et al. Serum total IgE levels and CD14 on chromosome 5q31 [letter]. *Clin Genet*. 1999;56:164-165.

153. Fageras Bottcher M, Hmani-Aifa M, Lindstrom A, et al. A TLR4 polymorphism is associated with asthma and reduced lipopolysaccharideinduced interleukin-12(p70) responses in Swedish children. *J Allergy Clin Immunol* . 2004;114:561-567.

154. Raby BA, Klimecki WT, Laprise C, et al. Polymorphisms in toll-like receptor 4 are not associated with asthma or atopy-related phenotypes. *Am J Respir Crit Care Med* . 2002;166:1449-1456.

155. Adjers K, Karjalainen J, Pessi T, et al. Epistatic effect of TLR4 and IL4 genes on the risk of asthma in females. *Int Arch Allergy Immunol* . 2005;138:251-256.

156. Eder W, Klimecki W, Yu L, et al. Toll-like receptor 2 as a major gene for asthma in children of European farmers. *J Allergy Clin Immunol* . 2004;113:482-488.

157. Diaz-Sanchez D, Garcia MP, Wang M, et al. Nasal challenge with diesel exhaust particles can induce sensitization to a neoallergen in the human mucosa. *J Allergy Clin Immunol*. 1999;104:1183-1188.

158. D'Amato G. Outdoor air pollution, climate and allergic respiratory diseases: evidence of a link. *Clin Exp Allergy*. 2002;32:1391-1393.

159. Kulig M, Luck W, Wahn U. The association between pre- and postnatal tobacco smoke exposure and allergic sensitization during early childhood. Multicentre Allergy Study Group, Germany. *Hum Exp Toxicol*. 1999;18:241-244.

160. Ronchetti R, Macri F, Ciofetta G, et al. Increased serum IgE and increased prevalence of eosinophilia in 9-year-old children of smoking parents. *J Allergy Clin Immunol*. 1990;86:400-407.

161. Diaz-Sanchez D, Rumold R, Gong H Jr. Challenge with environmental tobacco smoke exacerbates allergic airway disease in human beings. *J Allergy Clin Immunol* . 2006;118:441-446.

162. Lee KK, Hegele RG, Manfreda J, et al. Relationship of early childhood viral exposures to respiratory symptoms, onset of possible asthma and atopy in high risk children: the Canadian Asthma Primary Prevention Study. *Pediatr Pulmonol* . 2007;42:290-297.

163. Singh AM, Moore PE, Gern JE, et al. Bronchiolitis to asthma: a review and call for studies of gene-virus interactions in asthma causation. *Am J Respir Crit Care Med* . 2007;175:108-119.

164. Miller RL, Ho S. Environmental epigenetics and asthma. Current concepts and call for studies. *Am J Respir Crit Care Med* . 2008;177:567-573.

165. Nicolae D, Cox NJ, Lester LA, et al. Fine mapping and positional candidate studies identify HLA-G as an asthma susceptibility gene on chromosome 6p21. *Am J Hum Genet*. 2005;76:349-357.

166. Tan Z, Randall G, Fan J, et al. Allele-specific targeting of microRNAs to HLA-G and risk of asthma. *Am J Hum Genet*. 2007;81:829-834.

167. Fedulov AV, Kobzik L. Allergy risk is mediated by dendritic cells with congenital epigenetic changes. *Am J Respir Cell Mol Biol* . 2011;44(3):285-292.

168. Breton CV, Byun HM, Wenten M, et al. Prenatal tobacco smoke exposure affects global and gene-specific DNA methylation. *Am J Respir Crit Care Med* . 2009;180(5):462-467.

169. Liu J, Ballaney M, Al-alem U, et al. Combined inhaled diesel exhaust particles and allergen exposure alter methylation of T helper genes and IgE production in vivo. *Toxicol Sci*. 2008;102(1):76-81.

170. Hollingsworth JW, Maruoka S, Boon K, et al. In utero supplementation with methyl donors enhances allergic airway disease in mice. *J Clin Invest*. 2008;118(10):3462-3469.

171. Matsui EC, Matsui W. Higher serum folate levels are associated with a lower risk of atopy and wheeze. *J Allergy Clin Immunol* . 2009;123(6): 1253-1259.

172. Whitrow MJ, Moore VM, Rumbold AR, Davies MJ. Effect of supplemental folic acid in pregnancy on childhood asthma: a prospective birth cohort study. *Am J Epidemiol*. 2009;170(12):1486-1493.

173. Brand D, Teich T, Dicke T, et al. Epigenetic regulation in murine offspring as a novel mechanism for transmaternal asthma protection induced by microbes. *J Allergy Clin Immunol*. 2011;128(3):618-625.

174. Riedler J, Braun-Fahrländer C, Eder W, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet*. 2001;358:1129-1133.

175. Michel O, Duchateau J, Sergyseis R, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet*. 2001;358:1129-1133.

176. Wills-Karp M, Santeliz J, Karp CL. The germless theory of allergic disease: revisiting the hygiene hypothesis. *Nature Rev Immunol*. 2001;1:69-75.

177. Michel O, Duchateau J, Sergyseis R. Effect of inhaled endotoxin on bronchial reactivity in asthmatic and normal subjects. *J Appl Physiol* . 1989;66:1059-1064.

178. Kline JN, Waldschmidt TJ, Businga TR. Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J Immunol*. 1998;160(6):2555-2559.

179. Redecke V, Hacker H, Datta SK, et al. Cutting edge: activation of tolllike receptor 2 induces a Th2 immune response and promotes experimental asthma. *J Immunol*. 2004;172:2739-2743.

180. Eisenbarth SC, Piggott DA, Huleatt JW, et al. Lipopolysaccharideenhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med*. 2002;196:1645-1651.

181. Patel M, Xu D, Kewin P, et al. TLR2 agonist ameliorates established allergic airway inflammation by promoting Th1 response and not via regulatory T cells. *J Immunol*. 2005;174:7558-7563.

182. Tan AM, Chen HC, Pochard P, Eisenbarth SC, Herrick CA, Bottomly HK. TLR4 signaling in stromal cells is critical for the initiation of allergic Th2 responses to inhaled antigen. *J Immunol*. 2010;184:3535-3544.

183. Liotta F, Frosali F, Querci V, et al. Human immature myeloid dendritic cells trigger a Th2-polarizing program via Jagged-1/Notch interaction. *J Allergy Clin Immunol*. 2008;121(4):1000-1005.

184. Hammad H, Chieppa M, Peiros F, et al. House dust mite allergen induces asthma via TLR4 triggered on airway structural cells. *Nat Med*. 2009;15:410-416.

185. Lavier S, Kunda YA, Apodaca TA, et al. Soluble MD2 increases TLR4 levels on the epithelial cell surface. *Cell Immunol*. 2009;255:8-16.

186. Mueller GA, Gosavi RA, Krahn JM, et al. Der p 5 crystal structure provides insight into the group 5 dust mite allergens. *J Biol Chem*. 2010;285(33):25394-25401.

187. Mueller GA, Edwards LL, Aloor JJ, et al. The structure of the dust mite allergen Der p 7 reveals similarities to innate immune proteins. *J Allergy Clin Immunol*. 2010;125(4):909-917.

188. Cambi A, Figdor CG. Levels of complexity in pattern recognition recoeptors by C-type lectins. *Curr Opin Immunol*. 2005;17:345-351.

189. Barrett N, Rahman OM, Fernandez JM. Dectin-2 mediates Th2 immunity through the generation of cysteinyl leukotrienes. *J Exp Med*. 2011;208(3):593-604.

190. Deslee G, Charbonnier AS, Hammad H, et al. Involvement of the mannose receptor in the uptake of Der p 1, a major mite allergen, by human dendritic cells. *J Allergy Clin Immunol*. 2002;110:763-770.

191. Nathan AT, Peterson EA, Chakir J, Wills-Karp M. Innate immune responses of airway epithelium to house dust mite are mediated through beta-glycan-dependent pathways. *J*

Allergy Clin Immunol. 2009;123:612-618.

192. Emara M, Royer PJ, Abbas Z, et al. Recognition of the major cat allergen Fed d 1 through the cysteine-rich domain of the mannose receptor determines its allergenicity. *J Biol Chem*. 2011;286(15):13033-13040.

193. Royer PJ, Emara M, Yang C, et al. The mannose receptor mediates the uptake of diverse native allergens by dendritic cells and determines allergen-induced T cell polarization through modulation of IDO activity. *J Immunol*. 2010;185(3):1522-1531.

194. Hattori T, Konno S, Hizawa N, et al. Genetic variants in the mannose receptor gene (MRC1) are associated with asthma in two independent populations. *Immunogenetics*. 2009;61(11-12):731-738.

195. Magalhaes JG, Rubino SJ, Travassos LH, et al. Nucleotide oligomerication domain-containing proteins instruct T cell helper type 2 immunity through stromal activation. *Proc Natl Acad Sci U S A*. 2011;108(36):14896-14901.

196. Weidinger S, Klopp N, Rummler L, et al. Association of NOD1 polymorphisms with atopic eczema and related phenotypes. *J Allergy Clin Immunol* . 2005;116:177-184.

197. Wan H, Winton HL, Soeller C, et al. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest*. 1999;104:123-133.

198. Schulz O, Sewell HF, Shakib F. Proteolytic cleavage of CD25, the alpha subunit of the human T cell interleukin 2 receptor, by Der p 1, a major mite allergen with cysteine protease activity. *J Exp Med*. 1998;187:271-275.

199. Takai T, Kato T, Ota M, et al. Recombinant Der p 1 and Der f 1 with in vitro enzymatic activity to cleave human CD23, CD25 and alpha1-antitrypsin, and in vivo IgE-eliciting activity in mice. *Int Arch Allergy Immunol* . 2005;137(3):194-200.

200. Asokananthan N, Graham PT, Stewart DJ, et al. House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *J Immunol*. 2002;169:4572-4578.

201. Schmidlin F, Amadesi S, Dabbagh K, et al. Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. *J Immunol*. 2002;169:5315-5321.

202. Kouzaki H, O'Grady SM, Lawrence CB, et al. Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. *J Immunol*. 2009;183(2):1427-1434.

203. Eisenbarth SC, Colegio OR, O'Connor W, et al. Crucial role for the Nalps inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature*. 2008;453:1122-1126.

204. Idzko M, Hammad H, van Nimwegen M, et al. Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nat Med*. 2007;13:913-919.

205. Muller T, Robaye B, Vieira RP, et al. The purinergic receptor P2Y2 receptor mediates chemotaxis of dendritic cells and eosinophils in allergic lung inflammation. *Allergy*. 2010;65(12):1545-1553.

206. Kool M, Wilart MA, van Nimwegen M, et al. An unexpected role for uric acid as an inducer of T helper 2 immunity to inhaled antigens and inflammatory mediator of allergic asthma. *Immunity*. 2011;34:527-540.

207. Wills-Karp M, Rani R, Dienger K. Trefoil factor 2 rapidly induces interleukin 33 to promote type 2 immunity during allergic asthma and hookworm infection. *J Exp Med*. 2012;209(3):607-622.

208. Ziegler A. The role of thymic stromal lymphopoietin (TSLP) in allergic disorders. *Curr Opin Immunol* . 2010;22:795-799.

209. Ying S, O'Connor B, Ratoff J, et al. Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. *J Immunol* . 2005;174:8183-8190.

210. Zhou B, Comeau MR, De Smedt T, et al. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nat Immunol* . 2005;6:1047-1053.

211. Al-Shami A, Spolski R, Kelly J, et al. A role for TSLP in the development of inflammation in an asthma model. *J Exp Med*. 2005;202:829-839.

212. Yoo J, Omori M, Gyarmati D, et al. Spontaneous atopic dermatitis in mice expressing an inducible thymic stromal lymphopoietin transgene specifically in the skin. *J Exp Med*. 2005;202:541-549.

213. Hunninghake GM, Soto-Quiros ME, Avila L, et al. TSLP polymorphisms are associated with asthma in a sex-specific fashion. *Allergy*. 2010;65(12):1566-1575.

214. Omori M, Ziegler S. Induction of IL-4 expression in CD4(+) T cells by thymic stromal lymphopoietin. *J Immunol* . 2007;178:1396-1404.

215. Ito T, Wang YH, Duramad O, et al. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med*.

2005;202:1213-1223.

216. Fort MM, Cheung J, Yen D, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity*. 2001;15:985-995.

217. Neill D, Wong SH, Bellosi A, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature*. 2010;464:1367-1370.

218. Moro K, Yamada T, Tanabe M, et al. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature*. 2010;463(7280):540-544.

219. Saenz SA, Siracusa MC, Perrigoue JG, et al. IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature*. 2010;464(7293):1362-1366.

221. Barlow JL, Hardman CS, Drynan LF, et al. Innnate IL-13-producing nuocytes arise during allergic inflammation and contribute to airways hyperreactivity. *J Allergy Clin Immunol*. 2012;129:191-198.

222. Corrigan CJ, Wang W, Meng Q, et al. Allergen-induced expression of IL-25 and IL-25 receptor in atopic asthmatic airways and late-phase cutaneous responses. *J Allergy Clin Immunol*. 2011;128(1):116-124.

223. Schmitz J, Owyang A, Oldham E, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity*. 2005;23:479-490.

224. Kim HY, Chang YJ, Subramanian S, et al. Innate lymphoid cells responding to IL-33 mediate airway hyperreactivity independently of adaptive immunity. *J Allergy Clin Immunol*. 2012;129(1):216-227.

225. Kearley J, Buckland KF, Mathie SA, Lloyd CM. Resolution of allergic inflammation and airway hyperreactivity is dependent upon disruption of the T1/ST2-IL-33 pathway. *Am J Respir Crit Care Med* . 2009;179(9):772-781.

226. Oshikawa K, Kuroiwa K, Tago K, et al. Elevated soluble ST2 protein levels in sera of patients with asthma with an acute exacerbation. *Am J Respir Crit Care Med* . 2001;164:277-281.

227. Prefontaine D, Jessica N, Fazila C, et al. Increased IL-33 expression by epithelial cells in bronchial asthma. *J Allergy Clin Immunol* . 2010;125:752-754.

228. Miller AM. Role of IL-33 in inflammation and disease. *J Inflamm (Lond)*. 2011;8(1):22.

229. Sakashita M, Yoshimoto T, Hirota T, et al. Association of serum interleukin-33 level and the interleukin-33 genetic variant with Japanese cedar pollinosis. *Clin Exp Allergy*. 2008;38:1875-1881.

230. Kamekura R, Kojima T, Takano K, et al. The role of IL-33 and its receptor ST2 in human nasal epithelium with allergic rhinitis. *Clin Exp Allergy*. 2012;42(2):218-228.

231. Chang YJ, Kim HY, Albacker LA, et al. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat Immunol*. 2011;12:631-638.

232. Bartemes KR, Iijima K, Kobayashi T, Kephart GM, McKenzie AN, Kita H. IL-33-responsive lineage- CD25+ CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. *J Immunol*. 2012;188:1503-1513.

233. Monticelli LA, Sonnenberg GF, Abt MC, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol*. 2011;12(11):1045-1054.

234. Mjosberg JM, Trifari S, Crellin NK, et al. Human IL-25-and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol*. 2011;12(11):1055-1062.

235. Lambrecht BN, De Veerman M, Coyle AJ, et al. Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J Clin Invest*. 2000;106:551-559.

236. Bellini A, Vittori E, Marini M, et al. Intraepithelial dendritic cells and selective activation of Th2-like lymphocytes in patients with atopic asthma. *Chest*. 1993;103:997-1005.

237. Fokkens WJ, Vroom TM, Rijntjes E, et al. Fluctuation of the number of CD-1(T6)-positive dendritic cells, presumably Langerhans cells, in the nasal mucosa of patients with an isolated grass-pollen allergy before, during, and after the grass- pollen season. *J Allergy Clin Immunol*. 1989;84:39-43.

238. Godthelp T, Fokkens WJ, Kleinjan A, et al. Antigen presenting cells in the nasal mucosa of patients with allergic rhinitis during allergen provocation. *Clin Exp Allergy*. 1996;26:677-688.

239. Cerio R, Griffiths CE, Cooper KD, et al. Characterization of factor XIIIa positive dermal dendritic cells in normal and inflamed skin. *Br J Dermatol* . 1989;121:421-431.

240. Moller GM, Overbeek SE, Van Helden-Meeuwsen CG, et al. Increased numbers of dendritic cells in the bronchial mucosa of atopic asthmatic patients: downregulation by

inhaled corticosteroids. *Clin Exp Allergy*. 1996;26:517-524.

241. Tschernig T, Debertin AS, Paulsen F, et al. Dendritic cells in the mucosa of the human trachea are not regularly found in the first year of life. *Thorax*. 2001;56:427-431.

242. Hamada K, Goldsmith CA, Goldman A, et al. Resistance of very young mice to inhaled allergen sensitization is overcome by coexposure to an air-pollutant aerosol. *Am J Respir Crit Care Med*. 2000;161: 1285-1293.

243. Jahnsen FL, Moloney ED, Hogan T, et al. Rapid dendritic cell recruitment to the bronchial mucosa of patients with atopic asthma in response to local allergen challenge. *Thorax*. 2001;56:823-826.

244. McWilliam AS, Napoli S, Marsh AM, et al. Dendritic cells are recruited into the airway epithelium during the inflammatory response to a broad spectrum of stimuli. *J Exp Med*. 1996;184:2429-2432.

245. Reibman J, Hsu Y, Chen LC, et al. Airway epithelial cells release MIP-3alpha/CCL20 in response to cytokines and ambient particulate matter. *Am J Respir Cell Mol Biol* . 2003;28:648-654.

246. Pichavant M, Charbonnier AS, Taront S, et al. Asthmatic bronchial epithelium activated by the proteolytic allergen Der p 1 increases selective dendritic cell recruitment. *J Allergy Clin Immunol* . 2005;115:771-778.

247. Keane-Myers A, Gause WC, Linsley PS, et al. B7-CD28/CTLA-4 costimulatory pathways are required for the development of T helper cell 2-mediated allergic airway responses to inhaled antigens. *J Immunol* . 1997;158:2042-2048.

248. Padrid PA, Mathur M, Li X, et al. CTLA4Ig inhibits airway eosinophilia and hyperresponsiveness by regulating the development of Th1/Th2 subsets in a murine model of asthma. *Am J Respir Cell Mol Biol* . 1998;18:453-462.

249. Akbari O, Freeman GJ, Meyer EH, et al. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat Med*. 2002;8:1024-1032.

250. Gajewska BU, Tafuri A, Swirski FK, et al. B7RP-1 is not required for the generation of Th2 responses in a model of allergic airway inflammation but is essential for the induction of inhalation tolerance. *J Immunol* . 2005;174:3000-3005.

251. Tesciuba AG, Subudhi S, Rother RP, et al. Inducible costimulatory regulates Th2-mediated inflammation, but not Th2 differentiation, in a model of allergic airway disease. *J Immunol*. 2001;167:1996-2003.

252. Hammad H, Lambrecht BN, Pochard P, et al. Monocyte-derived dendritic cells induce a house dust mite-specific Th2 allergic inflammation in the lung of humanized SCID mice: involvement of CCR7. *J Immunol*. 2002;169:1524-1534.

253. Hammad H, Charbonnier AS, Duez C, et al. Th2 polarization by Der p 1—pulsed monocyte-derived dendritic cells is due to the allergic status of the donors. *Blood*. 2001;98:1135-1141.

254. van den Heuvel MM, Vanhee DD, Postmus PE, et al. Functional and phenotypic differences of monocyte-derived dendritic cells from allergic and nonallergic patients. *J Allergy Clin Immunol*. 1998;101:90-95.

255. Akdis M, Verhagen J, Taylor A, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergenspecific T regulatory 1 and T helper 2 cells. *J Exp Med*. 2004;199:1567-1575.

256. Shilling RA, Pinto JM, Decker DC, et al. Cutting edge: polymorphisms in the ICOS promoter region are associated with allergic sensitization and Th2 cytokine production. *J Immunol*. 2005;175:2061-2065.

257. Jones G, Wu S, Jang N, et al. Polymorphisms within the CTLA4 gene are associated with infant atopic dermatitis. *Br J Dermatol* . 2006;154: 467-471.

258. Howard TD, Postma DS, Hawkins GA, et al. Fine mapping of an IgEcontrolling gene on chromosome 2q: Analysis of CTLA4 and CD28. *J Allergy Clin Immunol* . 2002;110:743-751.

259. Sohn MH, Kim SH, Song TW, et al. Cytotoxic T lymphocyte-associated antigen-4 gene polymorphisms confer susceptibility to atopic asthma in Korean children. *Pediatr Pulmonol*. 2007;42(6):542-547.

260. de Heer HJ, Hammad H, Soullie T, et al. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J Exp Med*. 2004;200:89-98.

261. Fallarino F, Asselin-Paturel C, Vacca C, et al. Murine plasmacytoid dendritic cells initiate the immunosuppressive pathway of tryptophan catabolism in response to CD200 receptor engagement. *J Immunol*. 2004;173:3748-3754.

262. Moseman EA, Liang X, Dawson AJ, et al. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. *J Immunol*. 2004;173:4433-4442.

263. Kohl J, Baelder R, Lewkowich IP, et al. A regulatory role for the C5a anaphylatoxin in type 2 immunity in asthma. *J Clin Invest*. 2006;116: 783-796.

264. Hagendorens MM, Ebo DG, Schuerwegh AJ, et al. Differences in circulating dendritic cell subtypes in cord blood and peripheral blood of healthy and allergic children. *Clin Exp Allergy*. 2003;33:633-639.

265. Matsuda H, Suda T, Hashizume H, et al. Alteration of balance between myeloid dendritic cells and plasmacytoid dendritic cells in peripheral blood of patients with asthma. *Am J Respir Crit Care Med* . 2002;166:1050-1054.

266. Hashizume H, Horibe T, Yagi H, et al. Compartmental imbalance and aberrant immune function of blood CD123+ (plasmacytoid) and CD11c+ (myeloid) dendritic cells in atopic dermatitis. *J Immunol* . 2005;174:2396-2403.

267. Charbonnier AS, Hammad H, Gosset P, et al. Der p 1-pulsed myeloid and plasmacytoid dendritic cells from house dust mite-sensitized allergic patients dysregulate the T cell response. *J Leukoc Biol* . 2003;73:91-99.

268. Sokol CL, Barton GM, Farr AG, Medzhitov R. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat Immunol* . 2008;9(3):310-318.

269. Sokol CL, Barton GM, Farr AG, Medzhitov R. Basophils function as antigen presenting cells for allergen-induced Th2 response. *Nat Immunol* . 2009;10:713-720.

270. Hammad H, Plantinga M, Deswarte K, et al. Inflammatory dendritic cellsnot basophils- are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J Exp Med*. 2010;207:2097-2111.

271. Tang H, Cao W, Kasturi SP, et al. The helper type 2 response to cysteine proteases required dendritic cell-basophil cooperation via ROSmediated signaling. *Nat Immunol* . 2010;11(7):608-617.

272. Paul WE, Zhu J. How are Th2-type immune responses initiated and amplified? *Nature Rev Immunol* . 2010;10:225-235.

273. Zhang DH, Cohn L, Ray P, et al. Transcription factor GATA-3 is differentially expressed in Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. *J Biol Chem*. 1997;272:21597-21603.

274. Yamane H, Zhu J, Paul WE. Independent roles for IL-2 and GATA-3 in stimulating naive CD4+ T cells to generate a Th2-inducing cytokine environment. *J Exp Med*. 2005;202:793-804.

275. Cote-Sierra J, Foucras G, Guo L, et al. Interleukin 2 plays a central role in Th2 differentiation. *Proc Natl Acad Sci U S A* . 2004;101:3880-3885.

276. Zhu J, Cote-Sierra J, Guo L, Paul WE. Stat5 activation plays a critical role in Th2 differentiation. *Immunity*. 2003;19:739-748.

277. Amsen D, Antov A, Jankovic D, et al. Direct regulation of Gata3 expression determines the T helper differentiation potential of Notch. *Immunity*. 2007;27:89-99.

278. Ho I-C, Hodge MR, Rooney JW, et al. The proto-oncogene c-maf is responsible for tissue-specific expression of interleukin-4. *Cell*. 1996;85: 973-983.

279. Yoshida H, Nishina H, Takimoto H, et al. The transcription factor NFATc1 regulates lymphocyte proliferation and Th2 cytokine production. *Immunity*. 1998;8:115-124.

280. Yang L, Cohn L, Zhang DH, et al. Essential role of nuclear factor kB in the induction eosinophilia in allergic airway inflammation. *J Exp Med*. 1998;188(9):1739-1750.

281. Taha R, Hamid Q, Cameron L, Olivenstein R. T helper type 2 cytokine receptors and associated transcription factors GATA-3, c-MAF, and signal transducer and activator of transcription factor-6 in induced sputum of atopic asthmatic patients. *Chest*. 2003;123:2074-2082.

282. Rosenwasser LJ, Klemm DJ, Dresback JK, et al. Promoter polymorphisms in the chromosome 5 gene cluster in asthma and atopy. *Clin Exp Allergy*. 1995;25(suppl 2):74-78; discussion 95-96.

283. Kawashima T, Noguchi E, Arinami T, et al. Linkage and association of an interleukin 4 gene polymorphism with atopic dermatitis in Japanese families. *J Med Genet*. 1998;35:502-504.

284. Tamura K, Arakawa H, Suzuki M, et al. Novel dinucleotide repeat polymorphism in the first exon of the STAT-6 gene is associated with allergic diseases. *Clin Exp Allergy*. 2001;31:1509-1514.

285. Robinson DS, Tsicopoulos A, Meng Q, et al. Increased interleukin-10 messenger RNA expression in atopic allergy and asthma. *Am J Respir Cell Mol Biol* . 1996;14:113-117.

286. Moverare R, Elfman L, Stalenheim G, et al. Study of the Th1/Th2 balance, including IL-10 production, in cultures of peripheral blood mononuclear cells from birch-pollen-allergic patients. *Allergy*. 2000;55:171-175.

287. Colavita AM, Hastie AT, Musani AI, et al. Kinetics of IL-10 production after segmental antigen challenge of atopic asthmatic subjects. *J Allergy Clin Immunol* . 2000;106:880-886.

288. Lamblin C, Desreumaux P, Colombel JF, et al. Overexpression of IL-10 mRNA in gut

mucosa of patients with allergic asthma. *J Allergy Clin Immunol* . 2001;107:739-741.

289. Takanashi S, Hasegawa Y, Kanehira Y, et al. Interleukin-10 level in sputum is reduced in bronchial asthma, COPD and in smokers. *Eur Respir J* . 1999;14:309-314.

290. Koning H, Neijens HJ, Baert MR, et al. T cells subsets and cytokines in allergic and non-allergic children. II. Analysis and IL-5 and IL-10 mRNA expression and protein production. *Cytokine*. 1997;9:427-436.

291. Yang X, Wang S, Fan Y, et al. IL-10 deficiency prevents IL-5 overproduction and eosinophilic inflammation in a murine model of asthmalike reaction. *Eur J Immunol* . 2000;30:382-391.

292. Grunig G, Corry DB, Leach MW, et al. Interleukin-10 is a natural suppressor of cytokine production and inflammation in a murine model of allergic bronchopulmonary aspergillosis. *J Exp Med*. 1997;185: 1089-1099.

293. Tournoy KG, Kips JC, Pauwels RA. Endogenous interleukin-10 suppresses allergen-induced airway inflammation and nonspecific airway responsiveness. *Clin Exp Allergy*. 2000;30:775-783.

294. Stampfli MR, Cwiartka M, Gajewska BU, et al. Interleukin-10 gene transfer to the airway regulates allergic mucosal sensitization in mice. *Am J Respir Cell Mol Biol* . 1999;21:586-596.

295. Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat Immunol*. 2001;2:725-731.

296. Read S, Powrie F. CD4(+) regulatory T cells. *Curr Opin Immunol* . 2001;13:644-649.

297. Jeannin P, Lecoanet S, Delneste Y, et al. IgE versus IgG4 production can be differentially regulated by IL-10. *J Immunol*. 1998;160:3555-3561.

298. Hodge S, Hodge G, Flower R, et al. Methyl-prednisolone up-regulates monocyte interleukin-10 production in stimulated whole blood. *Scand J Immunol* . 1999;49:548-553.

299. Pessi T, Sutas Y, Hurme M, et al. Interleukin-10 generation in atopic children following oral Lactobacillus rhamnosus GG. *Clin Exp Allergy*. 2000;30:1804-1808.

300. Bellinghausen I, Knop J, Saloga J. The role of interleukin 10 in the regulation of allergic immune responses. *Int Arch Allergy Immunol* . 2001;126:97-101.

301. Hobbs K, Negri J, Klinnert M, et al. Interleukin-10 and transforming growth factor-beta promoter polymorphisms in allergies and asthma. *Am J Respir Crit Care Med* .

1998;158:1958-1962.

302. Fattouh R, Midence NG, Arias K, et al. Transforming growth factor-beta regulates house dust mite-induced allergic airway inflammation but not airway remodeling. *Am J Respir Crit Care Med*. 2008;177(6):593-603.

303. Zheng SG, Wang JH, Stohl W, et al. TGF-beta requires CTLA-4 early after T cell activation to induce FoxP3 and generate adaptive CD4+CD25+ regulatory cells. *J Immunol*. 2006;176:3321-3329.

304. Li H, Romieu I, Wu H, et al. Genetic polymorphisms in transforming growth factor beta-1 (TGFB1) and childhood asthma and atopy. *Hum Genet*. 2007;121:529-538.

305. Heinzmann A, Bauer E, Ganter K, et al. Polymorphisms of the TGFbeta1 gene are not associated with bronchial asthma in Caucasian children. *Pediatr Allergy Immunol* . 2005;16:310-314.

306. Finkelman FD, Katona IM, Urban JF Jr, et al. IL-4 is required to generate and sustain in vivo IgE responses. *J Immunol*. 1988;141:2335-2341.

307. Kuperman D, Schofield B, Wills-Karp M, Grusby MJ. Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. *J Exp Med*. 1998;187(6):939-948.

308. Munitz A, Brandt EB, Mingler M, Finkelman FD, Rothenberg M. Distinct roles of IL-13 and IL-4 via IL-13 receptor alpha1 and the type II IL-4 receptor in asthma pathogenesis. *Proc Natl Acad Sci USA* . 2008;105:7240-7245.

309. Ishizaka K, Ishizaka T. Identification of γ E-antibodies as a carrier of reaginic activity. *J Immunol*. 1967;99:1187.

310. Vercelli D. Immunoglobulin E and its regulators. *Curr Opin Allergy Clin Immunol* . 2001;1:61-65.

311. Linehan LA, Warren WD, Thompson PA, et al. STAT6 is required for IL-4-induced germline Ig gene transcription and switch recombination. *J Immunol*. 1998;161:302-310.

312. Snapper CM, Zelazowski P, Rosas FR, et al. B cells from p50/NF-kappa B knockout mice have selective defects in proliferation, differentiation, germ-line CH transcription, and Ig class switching. *J Immunol*. 1996;156:183-191.

313. Harriman GR, Bradley A, Das S, et al. IgA class switch in I alpha exondeficient mice. Role of germline transcription in class switch recombination. *J Clin Invest*. 1996;97:477-485.

314. Oettgen HC, Geha RS. IgE in asthma and atopy: cellular and molecular connections. *J Clin Invest*. 1999;104:829-835.

315. Jabara HH, Fu SM, Geha RS, et al. CD40 and IgE: synergism between anti-CD40 monoclonal antibody and interleukin 4 in the induction of IgE synthesis by highly purified human B cells. *J Exp Med*. 1990;172:1861-1864.

316. Armitage RJ, Fanslow WC, Strockbine L, et al. Molecular and biological characterization of a murine ligand for CD40. *Nature*. 1992;357:80-82.

317. Bacharier LB, Jabara H, Geha RS. Molecular mechanisms of immunoglobulin E regulation. *Int Arch Allergy Immunol* . 1998;115:257-269.

318. Kawabe T, Naka T, Yoshida K, et al. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity*. 1994;1:167-178.

319. Renshaw BR, Fanslow WC III, Armitage RJ, et al. Humoral immune responses in CD40 ligand-deficient mice. *J Exp Med*. 1994;180:1889-1900.

320. Honjo T, Kinoshita K, Muramatsu M, et al. Molecular mechanisms of class switch recommendation: linkage with somatic hypermutation. *Annu Rev Immunol*. 2002;20:165-196.

321. Muramatsu M, Kinoshita K, Fagarasan S, et al. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell*. 2000;102:553-563.

322. Durandy A, Honjo T. Human genetic defects in class-switch recombination (hyper-IgM syndromes). *Curr Opin Immunol* . 2001;13:543-548.

323. Shapira SK, Vercelli D, Jabara HH, et al. Molecular analysis of the induction of immunoglobulin E synthesis in human B cells by interleukin 4 and engagement of CD40 antigen. *J Exp Med*. 1992;175:289-292.

324. Coffman RL, Carty J. A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-gamma. *J Immunol* . 1986;136: 949-954.

325. Pene J, Rousset F, Briere F, et al. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons gamma and alpha and prostaglandin E2. *Proc Natl Acad Sci U S A* . 1988;85:6880-6884.

326. de Boer BA, Kruize YC, Rotmans PJ, et al. Interleukin-12 suppresses immunoglobulin E production but enhances immunoglobulin G4 production by human peripheral blood mononuclear cells. *Infect Immun*. 1997;65:1122-1125.

327. Ozaki K, Spolski R, Feng CG, et al. A critical role for IL-21 in regulating immunoglobulin production. *Science*. 2002;298:1630-1634.

328. Suto A, Nakajima H, Hirose K, et al. Interleukin 21 prevents antigeninduced IgE production by inhibiting germ line C(epsilon) transcription of IL-4-stimulated B cells. *Blood*. 2002;100:4565-4573.

329. Avery DT, Deenick EK, Ma CS, et al. B cell-intrinsic signaling through IL-21 receptor and STAT3 is required for establishing long-lived antibody responses in humans. *J Exp Med*. 2010;207(1):155-171.

330. Wjst M, Lichtner P, Meitinger T, Grimbacher B. STAT3 single-nucleotide polymorphisms and STAT3 mutations associated with hyper-IgE syndrome are not responsible for increased serum IgE serum levels in asthma families. *Eur J Hum Genet* . 2009;17(3):352-356.

331. Wymann D, Akdis CA, Blesken T, et al. Enzymatic activity of soluble phospholipase A2 does not affect the specific IgE, IgG4 and cytokine responses in bee sting allergy. *Clin Exp Allergy*. 1998;28:839-849.

332. Jarvis D, Zock JP, Heinrich J, et al. Cat and dust mite allergen levels, specific IgG and IgG4, and respiratory symptoms in adults. *J Allergy Clin Immunol* . 2007;119:697-704.

333. Strait RT, Morris SC, Finkelman FD. IgG-blocking antibodies inhibit IgE-mediated anaphylaxis in vivo through both antigen interception and Fc gamma RIIb cross-linking. *J Clin Invest*. 2006;116:833-841.

334. Kinet JP. The high-affinity IgE receptor (Fc epsilon RI): from physiology to pathology. *Annu Rev Immunol* . 1999;17:931-972.

335. Reischl IG, Coward WR, Church MK. Molecular consequences of human mast cell activation following immunoglobulin E-high-affinity immunoglobulin E receptor (IgE-FcepsilonRI) interaction. *Biochem Pharmacol*. 1999;58:1841-1850.

336. Turner H, Kinet JP. Signalling through the high-affinity IgE receptor Fc epsilonRI. *Nature*. 1999;402:B24-30.

337. Costello PS, Turner M, Walters AE, et al. Critical role for the tyrosine kinase Syk in signalling through the high affinity IgE receptor of mast cells. *Oncogene*. 1996;13:2595-2605.

338. Malveaux FJ, Conroy MC, Adkinson NF Jr, et al. IgE receptors on human basophils. Relationship to serum IgE concentration. *J Clin Invest*. 1978;62:176-181.

339. Conroy MC, Adkinson NF Jr, Lichtenstein LM. Measurement of IgE on human basophils: relation to serum IgE and anti- IgE-induced histamine release. *J Immunol*. 1977;118:1317-1321.

340. MacGlashan DW Jr, Bochner BS, Adelman DC, et al. Down-regulation of Fc(epsilon)RI expression on human basophils during in vivo treatment of atopic patients with anti-IgE antibody. *J Immunol*. 1997;158:1438-1445.

341. Gosset P, Lamblin-Degros C, Tillie-Leblond I, et al. Modulation of highaffinity IgE receptor expression in blood monocytes: opposite effect of IL-4 and glucocorticoids. *J Allergy Clin Immunol*. 2001;107:114-122.

342. Daheshia M, Friend DS, Grusby MJ, et al. Increased severity of local and systemic anaphylactic reactions in gp49B1-deficient mice. *J Exp Med*. 2001;194:227-234.

343. Kepley CL, Cambier JC, Morel PA, et al. Negative regulation of FcepsilonRI signaling by FcgammaRII costimulation in human blood basophils. *J Allergy Clin Immunol*. 2000;106:337-348.

344. Katz HR, Vivier E, Castells MC, et al. Mouse mast cell gp49B1 contains two immunoreceptor tyrosine-based inhibition motifs and suppresses mast cell activation when coligated with the high-affinity Fc receptor for IgE. *Proc Natl Acad Sci U S A*. 1996;93:10809-10814.

345. Long EO. Regulation of immune responses through inhibitory receptors. *Annu Rev Immunol*. 1999;17:875-904.

346. Yokota A, Kikutani H, Tanaka T, et al. Two species of human Fc epsilon receptor II (Fc epsilon RII/CD23): tissue-specific and IL-4-specific regulation of gene expression. *Cell*. 1988;55:611-618.

347. Delespesse G, Suter U, Mossalayi D, et al. Expression, structure, and function of the CD23 antigen. *Adv Immunol*. 1991;49:149-191.

348. Abdelilah SG, Bouchaib L, Morita M, et al. Molecular characterization of the low-affinity IgE receptor Fc epsilonRII/CD23 expressed by human eosinophils. *Int Immunol*. 1998;10:395-404.

349. Yokota A, Yukawa K, Yamamoto A, et al. Two forms of the low-affinity Fc receptor for IgE differentially mediate endocytosis and phagocytosis: identification of the critical cytoplasmic domains. *Proc Natl Acad Sci U S A*. 1992;89:5030-5034.

350. Munoz O, Brignone C, Grenier-Brossette N, et al. Binding of anti-CD23 monoclonal antibody to the leucine zipper motif of FcepsilonRII/CD23 on B cell membrane promotes

its proteolytic cleavage. Evidence for an effect on the oligomer/monomer equilibrium. *J Biol Chem*. 1998;273:31795-31800.

351. Tsicopoulos A, Joseph M. The role of CD23 in allergic disease. *Clin Exp Allergy*. 2000;30:602-605.

352. Lemieux GA, Blumenkron F, Yeung N, et al. The low affinity IgE receptor (CD23) is cleaved by the metalloproteinase ADAM10. *J Biol Chem*. 2007;282(20):14836-14844.

353. Kisselgof AB, Oettgen HC. The expression of murine B cell CD23, in vivo, is regulated by its ligand, IgE. *Int Immunol* . 1998;10:1377-1384.

354. Bonnefoy JY, Lecoanet-Henchoz S, Gauchat JF, et al. Structure and functions of CD23. *Int Rev Immunol* . 1997;16:113-128.

355. Sherr E, Macy E, Kimata H, et al. Binding the low affinity Fc epsilon R on B cells suppresses ongoing human IgE synthesis. *J Immunol* . 1989;142:481-489.

356. Lee WT, Rao M, Conrad DH. The murine lymphocyte receptor for IgE. IV. The mechanism of ligand-specific receptor upregulation on B cells. *J Immunol* . 1987;139:1191-1198.

357. Lecoanet-Henchoz S, Gauchat JF, Aubry JP, et al. CD23 regulates monocyte activation through a novel interaction with the adhesion molecules CD11b-CD18 and CD11c-CD18. *Immunity*. 1995;3: 119-125.

358. Williams J, Johnson S, Mascali JJ, et al. Regulation of low affinity IgE receptor (CD23) expression on mononuclear phagocytes in normal and asthmatic subjects. *J Immunol*. 1992;149:2823-2829.

359. Jung CM, Prinz JC, Rieber EP, et al. A reduction in allergen-induced Fc epsilon R2/CD23 expression on peripheral B cells correlates with successful hyposensitization in grass pollinosis. *J Allergy Clin Immunol* . 1995;95:77-87.

360. Poole JA, Meng J, Reff M, et al. Anti-CD23 monoclonal antibody, lumiliximab, inhibited allergen-induced responses in antigen-presenting cells and T cells from atopic subjects. *J Allergy Clin Immunol* . 2005;116(4):780-788.

361. Ehrlich P. Contributions to the knowledge on granulated connective tissue cells and eosinophlic leukocytes. *Arch Anat Physiol Lpz*. 1879;3:166-169.

362. Weil SC, Hrisinko MA. A hybrid eosinophilic-basophilic granulocyte in chronic granulocytic leukemia. *Am J Clin Pathol* . 1987;87:66-70.

363. Denburg JA, Telizyn S, Messner H, et al. Heterogeneity of human peripheral blood

eosinophil-type colonies: evidence for a common basophil-eosinophil progenitor. *Blood*. 1985;66:312-318.

364. Leary AG, Ogawa M. Identification of pure and mixed basophil colonies in culture of human peripheral blood and marrow cells. *Blood*. 1984;64:78-83.

365. Valent P, Schmidt G, Besemer J, et al. Interleukin-3 is a differentiation factor for human basophils. *Blood*. 1989;73:1763-1769.

366. Denburg JA, Silver JE, Abrams JS. Interleukin-5 is a human basophilopoietin: induction of histamine content and basophilic differentiation of HL-60 cells and of peripheral blood basophil-eosinophil progenitors. *Blood*. 1991;77:1462-1468.

367. Yamaguchi M, Hirai K, Morita Y, et al. Hemopoietic growth factors regulate the survival of human basophils in vitro. *Int Arch Allergy Immunol* . 1992;97:322-329.

368. Tsuda T, Wong D, Dolovich J, et al. Synergistic effects of nerve growth factor and granulocyte-macrophage colony-stimulating factor on human basophilic cell differentiation. *Blood*. 1991;77:971-979.

369. Charlesworth EN, Hood AF, Soter NA, et al. Cutaneous late-phase response to allergen. Mediator release and inflammatory cell infiltration. *J Clin Invest*. 1989;83:1519-1526.

370. Liu MC, Hubbard WC, Proud D, et al. Immediate and late inflammatory responses to ragweed antigen challenge of the peripheral airways in allergic asthmatics. Cellular, mediator, and permeability changes. *Am Rev Respir Dis* . 1991;144:51-58.

371. Bascom R, Wachs M, Naclerio RM, et al. Basophil influx occurs after nasal antigen challenge: effects of topical corticosteroid pretreatment. *J Allergy Clin Immunol* . 1988;81:580-589.

372. Wershil BK, Galli SJ. The analysis of mast cell function in vivo using mast cell-deficient mice. *Adv Exp Med Biol* . 1994;347:39-54.

373. Austen KF, Boyce JA. Mast cell lineage development and phenotypic regulation. *Leuk Res*. 2001;25:511-518.

374. Irani AM, Craig SS, DeBlois G, et al. Deficiency of the tryptase-positive, chymase-negative mast cell type in gastrointestinal mucosa of patients with defective T lymphocyte function. *J Immunol* . 1987;138:4381-4386.

375. Ruitenberg EJ, Elgersma A. Absence of intestinal mast cell response in congenitally athymic mice during Trichinella spiralis infection. *Nature*. 1976;264:258-260.

376. Lantz CS, Boesiger J, Song CH, et al. Role for interleukin-3 in mastcell and basophil development and in immunity to parasites. *Nature*. 1998;392:90-93.

377. Godfraind C, Louahed J, Faulkner H, et al. Intraepithelial infiltration by mast cells with both connective tissue- type and mucosal-type characteristics in gut, trachea, and kidneys of IL-9 transgenic mice. *J Immunol* . 1998;160:3989-3996.

378. Stevens RL, Austen KF. Recent advances in the cellular and molecular biology of mast cells. *Immunol Today*. 1989;10:381-386.

379. Huang C, Sali A, Stevens RL. Regulation and function of mast cell proteases in inflammation. *J Clin Immunol* . 1998;18:169-183.

380. Church MK, Levi-Schaffer F. The human mast cell. *J Allergy Clin Immunol* . 1997;99:155-160.

381. Casale TB, Wood D, Richerson HB, et al. Elevated bronchoalveolar lavage fluid histamine levels in allergic asthmatics are associated with methacholine bronchial hyperresponsiveness. *J Clin Invest*. 1987;79:1197-1203.

382. Schwartz LB. Tryptase, a mediator of human mast cells. *J Allergy Clin Immunol* . 1990;86:594-598.

383. Schwartz LB. Tryptase: a clinical indicator of mast cell-dependent events. *Allergy Proc*. 1994;15:119-123.

384. Fisher MM, Baldo BA. The diagnosis of fatal anaphylactic reactions during anaesthesia: employment of immunoassays for mast cell tryptase and drug-reactive IgE antibodies. *Anaesth Intensive Care*. 1993;21:353-357.

385. Schwartz LB, Bradford TR, Rouse C, et al. Development of a new, more sensitive immunoassay for human tryptase: use in systemic anaphylaxis. *J Clin Immunol* . 1994;14:190-194.

386. Schwartz LB, Yunginger JW, Miller J, et al. Time course of appearance and disappearance of human mast cell tryptase in the circulation after anaphylaxis. *J Clin Invest*. 1989;83:1551-1555.

387. Murphy RC, Hammarstrom B, Samuelson B. Leukotriene C: a slow reacting substance from murine mastocytoma cells. *Proc Nat Acad Sci USA* . 1979;76:4275-4279.

388. Coles SJ, Neill KH, Reid LM, et al. Effects of leukotrienes C4 and D4 on glycoprotein and lysozyme by human bronchial mucosa. *Prostaglandins*. 1983;25:155-170.

389. Weiss JW, Drazen JM, McFadden ER Jr, et al. Airway constriction in normal humans produced by inhalation of leukotriene D. Potency, time course, and effect of aspirin therapy. *JAMA*. 1983;249:2814-2817.

390. Sampson A, Holgate S. Leukotrience modifers in the treatment of asthma: Look promising across the board of asthma severity. *BMJ*. 1998;1257-1258.

391. Rasmussen JB, Margolskee DJ, Eriksson LO, et al. Leukotriene (LT) D4 is involved in antigen-induced asthma: a study with the LTD4 receptor antagonist, MK-571. *Ann NY Acad Sci*. 1991;629:436.

392. Findlay SR, Barden JM, Easley CB. Effect of the oral leukotriene antagonist, ICI 204,219 on antigen-induced bronchoconstriction in subjects with asthma. *J Allergy Clin Immunol*. 1992;89:1040-1045.

393. Diamant Z, Timmers MC, van-der-Veen H, et al. The effect of MK-0591, a novel 5-lipoxygenase activating protein inhibitor, on leukotriene biosynthesis and allergen-induced airway responses in asthmatic subjects. *J Allergy Clin Immunol* . 1995;95:42-51.

394. Middleton E, Reed CE, Ellis EF, et al. *Allergy*. St. Louis, MI: Mosby; 1998.

395. Mencia-Huerta JM, Lewis RA, Razin E, et al. Antigen-initiated release of platelet-activating factor (PAF-acether) from mouse bone marrow-derived mast cells sensitized with monoclonal IgE. *J Immunol*. 1983;131:2958-2964.

396. Triggiani M, Hubbard WC, Chilton FH. Synthesis of 1-acyl-2-acetylsn-slycero-3-phosphocholine by an enriched preparation of the human lung mast cell. *J Immunol* . 1990;144:4773-4780.

397. Agius RM, Robinson C, Holgate ST. Release of histamine and newly generated mediators from human bronchoalveolar lavage cells. *Thorax*. 1985;40:220.

398. Hardy CC, Robinson C, Tattersfield AE, et al. The bronchoconstrictor effect of inhaled prostaglandin D2 in normal and asthmatic men. *N Engl J Med* . 1984;311:209-213.

399. Featherstone RL, Robinson C, Holgate ST, et al. Evidence for thromboxane receptor mediated contraction of guinea-pig and human airways in vitro by prostaglandin (PG) D2, 9 alpha, 11 beta-PGF2 and PGF2 alpha. *Naunyn Schmiedebergs Arch Pharmacol* . 1990;341:439-443.

400. Wensel SE, Fowler AA, Schwartz LB. Activation of pulmonary mast cells by bronchoalveolar allergen challenge. In vivo release of histamine and tryptase in atopic subjects with and without asthma. *Am Rev Respir Dis* . 1988;137:1002-1008.

401. O'Sullivan S, Dahlen B, Dahlen S-E, et al. Increased urinary excretion of the prostaglandin D2 metabolite 9a, 11b-prostaglandin F2 after aspirin challenge supports mast cell activation in aspirin-induced airway obstruction. *J Allergy Clin Immunol* . 1996;98:421-432.

402. Allegra J, Trautelein J, Demers L, et al. Peripheral plasma determinations of prostaglandin E in asthmatics. *J Allergy Clin Immunol* . 1976;58:546-550.

403. Szczeklik A. The cyclooxygenase theory of aspirin-induced asthma. *Eur Respir J* . 1990;3:588-593.

404. Kowalski ML. Aspirin sensitive rhinosinusitis and asthma. *Allergy Proc*. 1995;16:77-80.

405. Bradding P, Feather IH, Howarth PH, et al. Interleukin 4 is localized to and released by human mast cells. *J Exp Med*. 1992;176:1381-1386.

406. Church MK, Okayama Y, Bradding P. The role of the mast cell in acute and chronic allergic inflammation. *Ann N Y Acad Sci* . 1994;725:13-21.

407. Wershil BK, Mekori YA, Murakami T, et al. I-fibrin deposition in IgE-dependent immediate hypersensitivity reactions in mouse skin: demonstration of the role of mast cells using genetically mast cell-deficient mice locally reconstituted with cultured mast cells. *J Immunol* . 1987;139:2605-2614.

408. Oettgen HC, Martin TR, Wynshaw-Boris A, et al. Active anaphylaxis in IgE-deficient mice. *Nature*. 1994;370:367-370.

409. Konig P. The effects of cromolyn sodium and nedocromil sodium in early asthma prevention. *J Allergy Clin Immunol* . 2000;105:S575-S581.

410. Fahy JV, Fleming HE, Wong HH, et al. The effect of an anti-IgE monoclonal antibody on the early- and late-phase responses to allergen inhalation in asthmatic subjects. *Am J Respir Crit Care Med*. 1997;155:1828-1834.

411. Mehlhop PD, van de Rijn M, Goldberg AB, et al. Allergen-induced bronchial hyperreactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma. *Proc Natl Acad Sci U S A* . 1997;94:1344-1349.

412. Weller PF. Eosinophils: structure and functions. *Curr Opin Immunol* . 1994;6:85-90.

413. Gleich GJ. Mechanisms of eosinophil-associated inflammation. *J Allergy Clin Immunol*. 2000;105:651-663.

414. Weller PF, Lim K, Wan HC, et al. Role of the eosinophil in allergic reactions. *Eur*

Respir J Suppl. 1996;22:109s-115s.

415. Jacoby DB, Gleich GJ, Fryer AD. Human eosinophil major basic protein is an endogenous allosteric antagonist at the inhibitory muscarinic M2 receptor. *J Clin Invest*. 1993;91:1314-1318.

416. Wu W, Samoszuk MK, Comhair SA, et al. Eosinophils generate brominating oxidants in allergen-induced asthma. *J Clin Invest*. 2000;105:1455-1463.

417. Leonidas DD, Elbert BL, Zhou Z, et al. Crystal structure of human Charcot-Leyden crystal protein, an eosinophil lysophospholipase, identifies it as a new member of the carbohydrate-binding family of galectins. *Structure*. 1995;3:1379-1393.

418. Guo L, Johnson RS, Schuh JC. Biochemical characterization of endogenously formed eosinophilic crystals in the lungs of mice. *J Biol Chem*. 2000;275:8032-8037.

419. Wardlaw AJ. Molecular basis for selective eosinophil trafficking in asthma: a multistep paradigm. *J Allergy Clin Immunol* . 1999;104:917-926.

420. Nerlov C, Graf T. PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. *Genes Dev*. 1998;12:2403-2412.

421. Nerlov C, McNagny KM, Doderlein G, et al. Distinct C/EBP functions are required for eosinophil lineage commitment and maturation. *Genes Dev*. 1998;12:2413-2423.

422. Lee JJ, McGarry MP, Farmer SC, et al. Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *J Exp Med*. 1997;185:2143-2156.

423. Lobb RR, Pepinsky B, Leone DR, et al. The role of alpha 4 integrins in lung pathophysiology. *Eur Respir J Suppl* . 1996;22:104s-108s.

424. Ponath PD, Qin S, Post TW, et al. Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils. *J Exp Med*. 1996;183:2437-2448.

425. Uguccioni M, Mackay CR, Ochensberger B, et al. High expression of the chemokine receptor CCR3 in human blood basophils. Role in activation by eotaxin, MCP-4, and other chemokines. *J Clin Invest*. 1997;100:1137-1143.

426. Rothenberg ME, MacLean JA, Pearlman E, et al. Targeted disruption of the chemokine eotaxin partially reduces antigen- induced tissue eosinophilia. *J Exp Med*. 1997;185:785-790.

427. Yang Y, Loy J, Ryseck RP, et al. Antigen-induced eosinophilic lung inflammation

develops in mice deficient in chemokine eotaxin. *Blood*. 1998;92:3912-3923.

428. Collins PD, Marleau S, Griffiths-Johnson DA, et al. Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. *J Exp Med*. 1995;182:1169-1174.

429. Gounni AS, Lamkhioued B, Ochiai K, et al. High-affinity IgE receptor on eosinophils is involved in defence against parasites. *Nature*. 1994;367:183-186.

430. Tomassini M, Tsicopoulos A, Tai PC, et al. Release of granule proteins by eosinophils from allergic and nonallergic patients with eosinophilia on immunoglobulin-dependent activation. *J Allergy Clin Immunol* . 1991;88:365-375.

431. Rajakulasingam K, Till S, Ying S, et al. Increased expression of high affinity IgE (FcepsilonRI) receptor-alpha chain mRNA and proteinbearing eosinophils in human allergen-induced atopic asthma. *Am J Respir Crit Care Med* . 1998;158:233-240.

432. Barata LT, Ying S, Grant JA, et al. Allergen-induced recruitment of Fc epsilon RI+ eosinophils in human atopic skin. *Eur J Immunol* . 1997;27:1236-1241.

433. Ying S, Barata LT, Meng Q, et al. High-affinity immunoglobulin E receptor (Fc epsilon RI)-bearing eosinophils, mast cells, macrophages and Langerhans' cells in allergen-induced late-phase cutaneous reactions in atopic subjects. *Immunology*. 1998;93:281-288.

434. Kaneko M, Horie S, Kato M, et al. A crucial role for beta 2 integrin in the activation of eosinophils stimulated by IgG. *J Immunol* . 1995;155: 2631-2641.

435. Kita H, Kaneko M, Bartemes KR, et al. Does IgE bind to and activate eosinophils from patients with allergy? *J Immunol* . 1999;162: 6901-6911.

436. Kita H, Abu-Ghazaleh RI, Sur S, et al. Eosinophil major basic protein induces degranulation and IL-8 production by human eosinophils. *J Immunol* . 1995;154:4749-4758.

437. Martin LB, Kita H, Leiferman KM, et al. Eosinophils in allergy: role in disease, degranulation, and cytokines. *Int Arch Allergy Immunol* . 1996;109:207-215.

438. Shi HZ, Humbles A, Gerard C, et al. Lymph node trafficking and antigen presentation by endobronchial eosinophils. *J Clin Invest*. 2000;105:945-953.

439. Odemuyiwa SO, Ghahary A, Li Y, et al. Cutting edge: human eosinophils regulate T cell subset selection through indoleamine 2,3-dioxygenase. *J Immunol* . 2004;173:5909-5913.

440. Seminario MC, Gleich GJ. The role of eosinophils in the pathogenesis of asthma. *Curr Opin Immunol* . 1994;6:860-864.

441. Terada N, Konno A, Fukuda S, et al. Interleukin-5 gene expression in nasal mucosa and changes in amount of interleukin-5 in nasal lavage fluid after antigen challenge. *Acta Otolaryngol*. 1994;114:203-208.

442. Kiehl P, Falkenberg K, Vogelbruch M, et al. Tissue eosinophilia in acute and chronic atopic dermatitis: a morphometric approach using quantitative image analysis of immunostaining. *Br J Dermatol* . 2001;145: 720-729.

443. Andersson M, Andersson P, Venge P, et al. Eosinophils and eosinophil cationic protein in nasal lavages in allergen-induced hyperresponsiveness: effects of topical glucocorticosteroid treatment. *Allergy*. 1989;44:342-348.

444. Matsukura M, Yamada H, Yudate T, et al. Steroid-induced changes of eosinophils in atopic dermatitis. *Int Arch Allergy Immunol* . 1997;114 (Suppl 1):51-54.

445. Corry DB, Folkesson HG, Warnock ML, et al. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *J Exp Med*. 1996;183:109-117. Erratum *J Exp Med*. 1997;185(9):1715.

446. Lee JJ, Dimina D, Macias MP, et al. Defining a link with asthma in mice congenitally deficient in eosinophils. *Science*. 2004;305:1773-1776.

447. Humbles AA, Lloyd CM, McMillan SJ, et al. A critical role for eosinophils in allergic airways remodeling. *Science*. 2004;305(5691):1776-1779.

448. Walsh ER, Sahu N, Kearley J, et al. Strain-specific requirement for eosinophils in the recruitment of T cells to the lung during the development of allergic asthma. *J Exp Med*. 2008;205(6):1285-1292.

449. Leckie MJ, ten Brinke A, Khan J, et al. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet*. 2000;356:2144-2148.

450. Flood-Page PT, Menzies-Gow AN, Kay AB, Robinson DS. Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. *Am J Respir Crit Care Med*. 2003;167(2): 199-204.

451. Nair P, Pizzichini MM, Kjarsgaard M, et al. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N Engl J Med* . 2009;360:985-993.

452. Haldar P, Brightling CE, Hargadon B, et al. Mepolizumab and exacerbations of refractory eosinophilic asthma. *N Engl J Med* . 2009;360:973-984.

453. Rothenberg ME, Klion AD, Roufosse FE, et al. Treatment of patients with the hypereosinophilic syndrome with mepolizumab. *N Engl J Med* . 2008;358(12):1215-1228.

454. Yang M, Hogan SP, Henry PJ, et al. Interleukin-13 mediates airways hyperreactivity through the IL-4 receptor-alpha chain and STAT-6 independently of IL-5 and eotaxin. *Am J Respir Cell Mol Biol*. 2001;25: 522-530.

455. Zhu Z, Homer RJ, Wang Z, et al. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest*. 1999;103(6):779-788.

456. Rankin JA, Picarella DE, Geba GP, et al. Phenotypic and physiologic characterization of transgenic mice expressing interleukin 4 in the lung: lymphocytic and eosinophilic inflammation without airway hyperreactivity. *Proc Natl Acad Sci U S A* . 1996;93:7821-7825.

457. Kroegel C, Julius P, Matthys H, et al. Endobronchial secretion of interleukin-13 following local allergen challenge in atopic asthma: relationship to interleukin-4 and eosinophil counts. *Eur Respir J* . 1996;9:899-904.

458. Mattes J, Yang M, Siqueira A, et al. IL-13 induces airways hyperreactivity independently of the IL-4R alpha chain in the allergic lung. *J Immunol*. 2001;167:1683-1692.

459. Finkelman FD, Yang M, Perkins C, et al. Suppressive effect of IL-4 on IL-13-induced genes in mouse lung. *J Immunol*. 2005;174:4630-4638.

460. Webb DC, McKenzie AN, Koskinen AM, et al. Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperreactivity. *J Immunol*. 2000;165:108-113.

461. Danahay H, Atherton H, Jones G, et al. Interleukin-13 induces a hypersecretory ion transport phenotype in human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol*. 2002;282:L226-L236.

462. Chiaramonte MG, Donaldson DD, Cheever AW, et al. An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. *J Clin Invest*. 1999;104:777-785.

463. Zheng T, Zhu Z, Wang Z, et al. Inducible targeting of IL-13 to the adult lung causes matrix metalloproteinase- and cathepsin-dependent emphysema. *J Clin Invest*. 2000;106:1081-1093.

464. Hesse M, Modolell M, La Flamme AC, et al. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is

shaped by the pattern of L-arginine metabolism. *J Immunol*. 2001;167:6533-6544.

465. Zimmermann N, King NE, Laporte J, et al. Dissection of experimental asthma with DNA microarray analysis identifies arginase in asthma pathogenesis. *J Clin Invest*. 2003;111:1863-1874.

466. Lee CG, Homer RJ, Zhu Z, et al. Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1). *J Exp Med*. 2001;194:809-821.

467. Wen FQ, Kohyama T, Liu X, et al. Interleukin-4- and interleukin-13-enhanced transforming growth factor-beta2 production in cultured human bronchial epithelial cells is attenuated by interferon-gamma. *Am J Respir Cell Mol Biol* . 2002;26:484-490.

468. Ma B, Zhu Z, Homer RJ, et al. The C10/CCL6 chemokine and CCR1 play critical roles in the pathogenesis of IL-13-induced inflammation and remodeling. *J Immunol*. 2004;172:1872-1881.

469. Hogaboam CM, Bone-Larson CL, Lipinski S, et al. Differential monocyte chemoattractant protein-1 and chemokine receptor 2 expression by murine lung fibroblasts derived from Th1- and Th2-type pulmonary granuloma models. *J Immunol*. 1999;163:2193-2201.

470. Ingram JL, Rice A, Geisenhoffer K, et al. Interleukin-13 stimulates the proliferation of lung myofibroblasts via a signal transducer and activator of transcription-6-dependent mechanism: a possible mechanism for the development of airway fibrosis in asthma. *Chest*. 2003;123:422S-424S.

471. Blackburn MR, Lee CG, Young HW, et al. Adenosine mediates IL-13-induced inflammation and remodeling in the lung and interacts in an IL-13-adenosine amplification pathway. *J Clin Invest*. 2003;112:332-344.

472. Vargaftig BB, Singer M. Leukotrienes mediate murine bronchopulmonary hyperreactivity, inflammation, and part of mucosal metaplasia and tissue injury induced by recombinant murine interleukin-13. *Am J Respir Cell Mol Biol* . 2003;28:410-419.

473. Venkayya R, Lam M, Willkom M, et al. The Th2 lymphocyte products IL-4 and IL-13 rapidly induce airway hyperresponsiveness through direct effects on resident airway cells. *Am J Respir Cell Mol Biol* . 2002;26:202-208.

474. Kuperman DA, Huang X, Koth LL, et al. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med*. 2002;8(8):885-889.

475. Kirstein F, Horsnell WG, Kuperman DA, et al. Expression of IL-4 receptor alpha on

smooth muscle cells is not necessary for development of experimental allergic asthma. *J Allergy Clin Immunol*. 2010;126:347-354.

476. Perkins C, Yanase N, Smulian G, et al. Selective stimulation of IL-4 receptor on smooth muscle induces airway hyperresponsiveness in mice. *J Exp Med*. 2011;208:853-867.

477. Humbert M, Durham SR, Kimmitt P, et al. Elevated expression of messenger ribonucleic acid encoding IL-13 in the bronchial mucosa of atopic and nonatopic subjects with asthma. *J Allergy Clin Immunol* . 1997;99:657-665.

478. Till S, Durham S, Dickason R, et al. IL-13 production by allergen-stimulated T cells is increased in allergic disease and associated with IL-5, but not IFN-gamma expression. *Immunology*. 1997;91:53-57.

479. Ohshima Y, Yasutomi M, Omata N, et al. Dysregulation of IL-13 production by cord blood CD4+ T cells is associated with the subsequent development of atopic disease in infants. *Pediatr Res*. 2002;51:195-200.

480. Graves PE, Kabesch M, Halonen M, et al. A cluster of seven tightly linked polymorphisms in the IL-13 gene is associated with total serum IgE levels in three populations of white children. *J Allergy Clin Immunol* . 2000;105:506-513.

481. Heinzmann A, Mao XQ, Akaiwa M, et al. Genetic variants of IL-13 signalling and human asthma and atopy. *Hum Mol Genet*. 2000;9: 549-559.

482. Arima K, Umeshita-Suyama R, Sakata Y, et al. Upregulation of IL-13 concentration in vivo by the IL13 variant associated with bronchial asthma. *J Allergy Clin Immunol* . 2002;109:980-987.

483. Cameron L, Webster RB, Strempel JM, et al. Th2 cell-selective enhancement of human IL13 transcription by IL13-1112C>T, a polymorphism associated with allergic inflammation. *J Immunol*. 2006;177:8633-8642.

484. Portier P, Richet C. De'action anaphylactique de certains venins. *C Roy Soc Biol*. 1902;54:170.

485. Husain Z, Schwartz RA. Peanut allergy: an increasingly common lifethreatening disorder. *J Am Acad Dermatol* . 2012;66(1):136-143.

486. Khodoun M, Strait R, Orekov T, et al. Peanuts can contribute to anaphylactic shock by activating complement. *J Allergy Clin Immunol* . 2009;123(2):342-351.

487. De Carolis C, Perricone R, De Sanctis G, et al. Complement activation by Hymenoptera venom allergenic extracts. *J Allergy Clin Immunol* . 1982;70(3):219-220.

488. von Zabern I, Przyklenk H, Nolte R, Vogt W. Effect of different penicillin derivatives on complement components in human serum. *Int Arch Allergy Appl Immunol* . 1984;75(2):164-172.

489. Waite KJ. Blackley and the development of hay fever as a disease of civilization in the nineteenth century. *Med Hist*. 1995;39:186-196.

490. American Academy of Allergy Asthma and Immunology. Overview of allergic diseases. *Allergy Rep*. 2000;1.

491. Naclerio RM. Allergic rhinitis. *N Engl J Med* . 1991;325:860-869.

492. Kaliner M, Eggleston PA, Mathews KP. Rhinitis and asthma. *JAMA*. 1987;258:2851-2873.

493. Platts-Mills TA, Mueller GA, Wheatley LM. Future directions for allergen immunotherapy. *J Allergy Clin Immunol* . 1998;102:335-343.

494. Sampson HA. Food allergy. Part 1: immunopathogenesis and clinical disorders. *J Allergy Clin Immunol*. 1999;103:717-728.

495. Sampson HA. Food allergy. Part 2: diagnosis and management. *J Allergy Clin Immunol*. 1999;103:981-989.

496. Croese J, Loukas A, Opdebeeck J, et al. Human enteric infection with canine hookworms. *Ann Intern Med*. 1994;120:369-374.

497. Lee YA, Wahn U, Kehrt R, et al. A major susceptibility locus for atopic dermatitis maps to chromosome 3q21. *Nat Genet*. 2000;26:470-473.

498. Schneider LC, Baz Z, Zarcone C, et al. Long-term therapy with recombinant interferon-gamma (rIFN-γ) for atopic dermatitis. *Ann Allergy Asthma Immunol* . 1998;80:263-268.

499. Spector S. Omalizumab: efficacy in allergic disease. *Panminerva Med*. 2004;46:141-148.

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Chapter 46

Transplantation Immunology

Megan Sykes

Kathryn Wood

David H. Sachs

INTRODUCTION

The transplantation of organs and cells between individuals saves or prolongs thousands of lives each year. The growing list of organs transplanted includes corneas, kidneys, livers, hearts, lungs, small intestines, pancreata, and even hands and faces. Currently, clinical cellular transplantation includes islets of Langerhans and hematopoietic cells, but the list is likely to expand in the future to include other cell types, such as hepatocytes and myoblasts, which are currently under investigation in experimental models. Success of all types of transplants depends on the ability to avoid rejection due to a host-versus-graft immune response. Hematopoietic cell transplantation (HCT) is, in addition, associated with some special considerations, as the administration of a donor graft that contains mature T cells to a conditioned, and consequently immunoincompetent recipient, is associated with the risk of rejection in the graft-versus-host direction (ie, graft-versus-host disease [GVHD]). These transplants, performed between different members of the same species, are referred to as allotransplants. However, the success of organ transplantation has presented a new limitation, namely the insufficient supply of human organs. This organ shortage has led to consideration of alternative sources of organs, such as artificial organs, tissue grafts engineered from stem cells, and other species. The latter, referred to as xenografts, are a promising alternative, but present even greater immunologic challenges than allografts. This chapter presents an overview of the immunology of organ and cellular allotransplantation and xenotransplantation.

Allogeneic and xenogeneic responses differ from other immunologic responses in at least two fundamental ways. First, they exhibit extraordinary strength that includes multiple, redundant pathways. Second, they involve two different sets of antigen-presenting cells (APCs): those of the donor and those of the recipient. In this chapter, we will emphasize the uniqueness of and challenges presented by the immune response to transplantation.

ORIGINS OF TRANSPLANTATION IMMUNOLOGY

Early History

Although there were sporadic reports of tissue transplants in ancient times, skin grafting did not become an accepted practice until the late 1800s. Even then, however, many workers did not distinguish between autografts (where the donor and recipient are the same individual) and allografts (where the donor and recipient are of the same species), or even xenografts (where donor and recipient are of different species). The last of these formed the basis for an

extensive practice known as zoografting in which patients were subjected to grafts from animals ranging from pigs to frogs. According to Billingham, no one apparently cared whether the grafted skin “took” or merely promoted healing of the wound.[1] The results of these efforts led to a period of confusion in transplantation. Without any clear understanding of the processes involved, surgeons embarked on all sorts of transplants, and a series of operations were reported that we know, from our present understanding of the laws of transplantation, could not possibly have been successful. The transplantation of internal organs awaited the development of techniques for vascular anastomosis. In 1908, Carrel, one of the pioneers of vascular surgery, reported the results of en bloc allotransplantation of both kidneys in a series of nine cats.[2] He was able to obtain up to 25 days of urine output in some cats, but ultimately all of them died. The first successful clinical renal transplant was not performed until 1952 in Boston, using the kidney of an identical twin.[3]

During this same period, the closely related field of tumor transplantation gained momentum. Although many of the experiments with tumor transplants provided information essential to an understanding of transplantation immunology, this was not clear at the time. Many workers were committed to the idea that they were studying an effect peculiar to tumor tissues. In his Harvey lecture, Medawar summed up the confusion neatly by the statement, “Nearly everyone who supposed that he was using transplantation to study tumors was in fact using tumors to study transplantation.” Medawar was largely responsible for establishing the immune basis of transplant reactions. Following a series of grafting experiments in rabbits and mice, he concluded in 1945 that rejection of skin “belongs to the general category of actively acquired immune reactions.”[4]

History, Principles, and Discoveries of Immunogenetics

Inbred Strains

Rodents have provided an invaluable model for the study of the genetic basis for graft rejection, largely due to the availability of a large number of inbred strains. Such strains consist of genetically identical animals that have been produced by sequential pedigreed brother-sister matings for at least 20 generations. They are, therefore, homozygous for all autosomal chromosomes and produce identical homozygous

progeny. The reason that sequential inbreeding leads to homozygosity is illustrated in Figure 46.1. In essence, the probability of fixation of a given autosomal locus at each broter-sister mating is 1 in 8, which is mathematically equivalent to stating that on average, 1 in 8 of all segregating loci are fixed at each generation. If the locus in question is not fixed during this random breeding, then the chances that it will be fixed at the next breeding are just a little less than 1 in 8 (ie, 1 in 8 of the remaining unfixed loci). Thus, as indicated in Figure 46.1, the probability of fixation is given by the following formula:

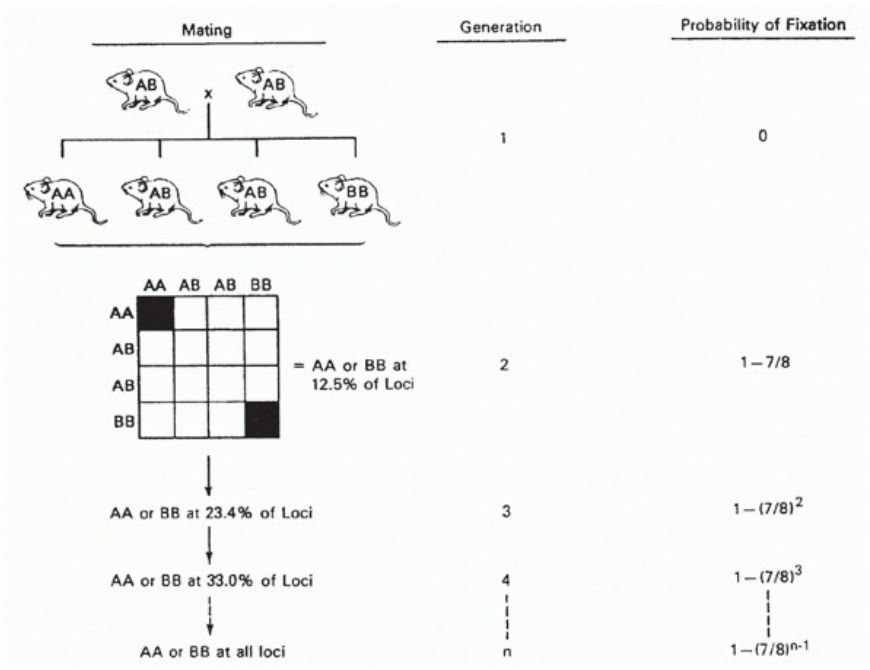

FIG. 46.1. Breeding Scheme for Inbred Strains.

Probability of fixation = 1- $(7/8)^{n-1}$ which describes a curve that asymptotes toward 100% fixation (Fig. 46.2). For practical purposes, one considers a strain inbred after 20 such brother-sister matings, as at this point there is a very small chance that any locus will not have reached homozygosity. Hundreds of such well-characterized inbred strains are now available.

Inbred strains have also been produced in several other species, including rats, guinea pigs, and rabbits, although in much more limited numbers. Both space requirements and other genetic features, such as gestation times, age of sexual maturity, and litter size, make production of inbred strains in larger species much more difficult. Over the past 40 years, studies in one of our laboratories (D.H.S.) have produced highly inbred miniature swine.[5,6] Swine were chosen for this purpose because they are one of the few large animal species in which breeding characteristics make genetic experiments possible. These characteristics include a large litter size, a short gestation time, early sexual maturity, and an estrous cycle every 3 weeks,[5] and have made it possible to develop major histocompatibility complex (MHC) homozygous lines

of miniature swine in a relatively short time, to isolate and characterize new MHC recombinants, and to carry out experiments involving the segregation of genetic characteristics. The Massachusetts General Hospital (MGH) miniature swine thus represent the only large animal model in which MHC genetics can be reproducibly controlled, and they have been particularly useful in studying the role of MHC matching on rejection and/or tolerance induction.[7]

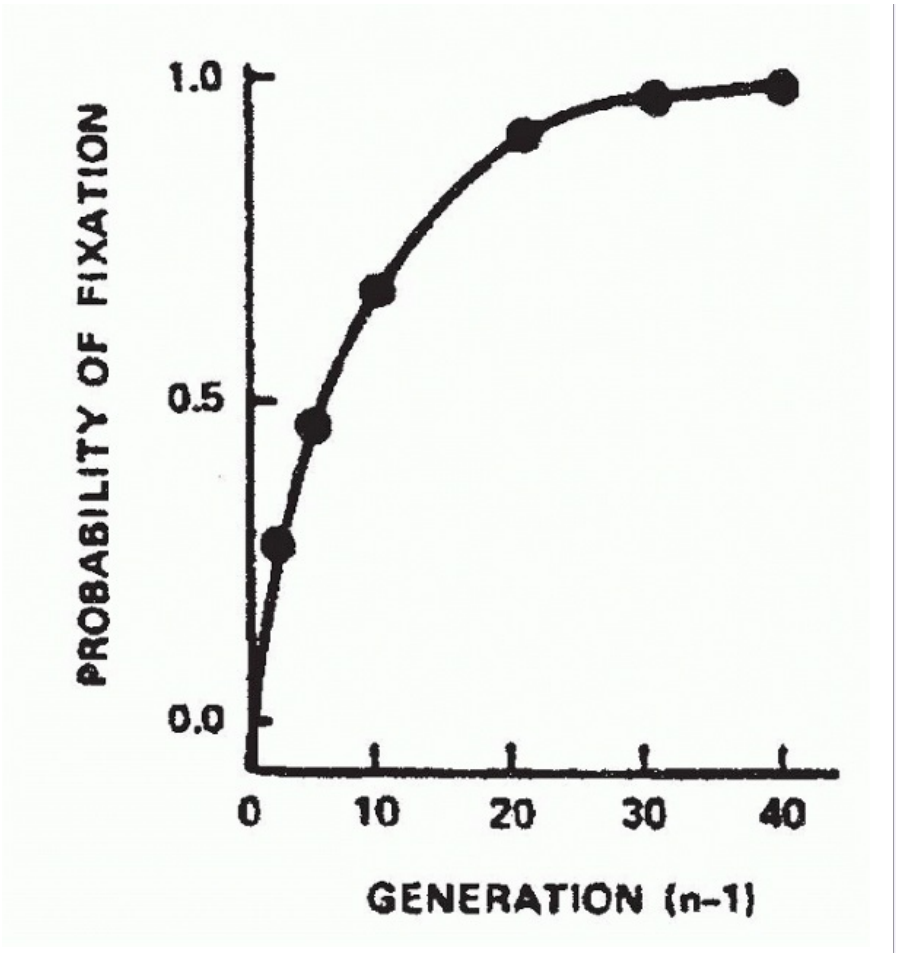

FIG. 46.2. Probablity of Fixation Curve.

At present, swine of three homozygous swine leukocyte antigen (SLA) haplotypes, SLA^a, SLA^c, SLA^d, and five lines bearing intra-SLA recombinant haplotypes are maintained, as illustrated in Figure 46.3. All of these lines differ by minor histocompatibility loci, thus providing a model in which most of the transplantation combinations relevant to human transplantation can be mimicked. In addition, one subline of SLA^{dd} animals was selected for further inbreeding in order to produce a fully inbred line of miniature swine. This subline has now reached a coefficient of inbreeding of > 94% and is now sufficiently inbred that reciprocal skin grafts among the offspring are not rejected.[6] This histocompatibility makes these animals particularly appropriate for adoptive transfer studies for the first time in a large animal model.[8]

Genetic Principles Governing Tissue Transplantation: The "Laws of Transplantation"

In the early 1900s, it was noted by tumor biologists that tumors arising in inbred animals could frequently be transplanted successfully to other animals of the same line, while this was usually impossible in outbred animals. Little studied this phenomenon systematically and in the process produced and characterized a large number of inbred strains of mice.[9] In summarizing the results of these studies of tumor grafting in mice, Little described what have since been called the five laws of transplantation (Table 46.1). Little's remarkable insight was

to reconcile these observations with the classical Mendelian principles by proposing that recipients would reject grafts if the donor expressed a product of any histocompatibility locus that was not expressed by the recipient. His explanation for the unusual inheritance pattern in Table 46.1 was to suggest, first, that there must be codominant expression of the histocompatibility genes, and second, that there must be a relatively large number of histocompatibility loci. Under these conditions, members of the F1 generation would express both parental alleles at all histocompatibility loci (and thus would fail to reject grafts from parental, F2, or subsequent generations) and members of the F2 generation would be unlikely to express all of the products of histocompatibility genes that are expressed by either parental generation (and thus would usually reject parental allografts).

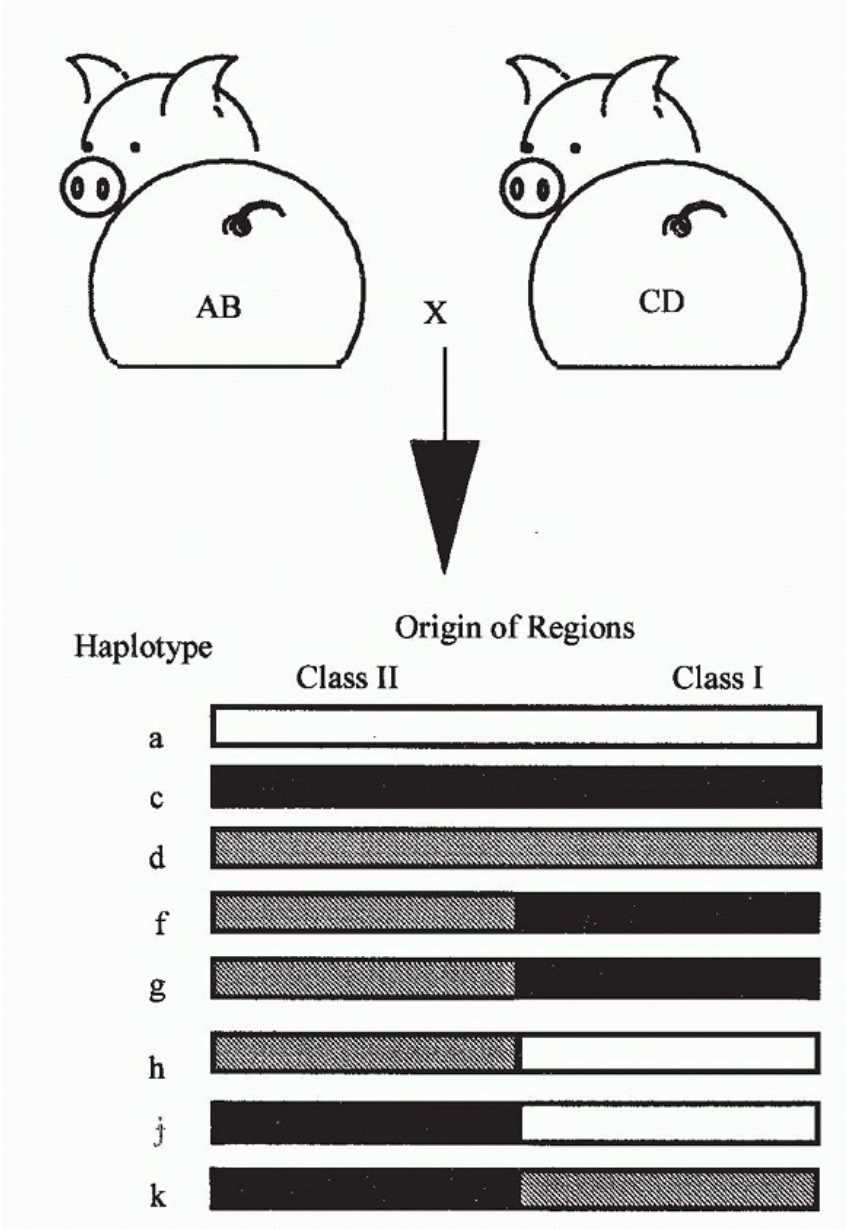

FIG. 46.3. Origin of Miniature Swine Haplotypes.

TABLE 46.1 The Laws of Transplantation

1. Transplants within inbred strains will succeed.

2. Transplants between inbred strains will fail.

3. Transplants from a member of an inbred parental strain to an F1 offspring will succeed but those in the reverse direction will fail.

4. Transplants from F_2 and all subsequent generations to F_1 animals will succeed.

5. Transplants from inbred parental strains to the F_2 generation will usually, but not always, fail.

Adapted from Little.9

Estimating the Number of Histocompatibility Genes

One can experimentally determine the number of histocompatibility loci by which any two inbred strains differ by breeding a large F2 population between these strains and then transplanting tissues from one of the parental strains to all of the F2 offspring, measuring the fraction of grafts that survive. As illustrated in Figure 46.4, if the two strains were to differ at only one histocompatibility locus,

one would predict that three-quarters of the grafts would survive. If, however, the two strains differed by two independently segregating histocompatibility loci, then one would predict that (three-quarters)^2 or nine-sixteenths of the grafts would survive. Similarly, if there were n loci by which these two strains differed, one would expect (threequarters)^n to be the fraction of surviving grafts. When this equation has been solved for “n” experimentally, using skin grafts as the challenging transplant, numbers as high as 30 to 50 have been reported.[10] Because there are only 20 chromosome pairs in the mouse genome, these larger numbers imply that many chromosomes carry more than one histocompatibility locus.

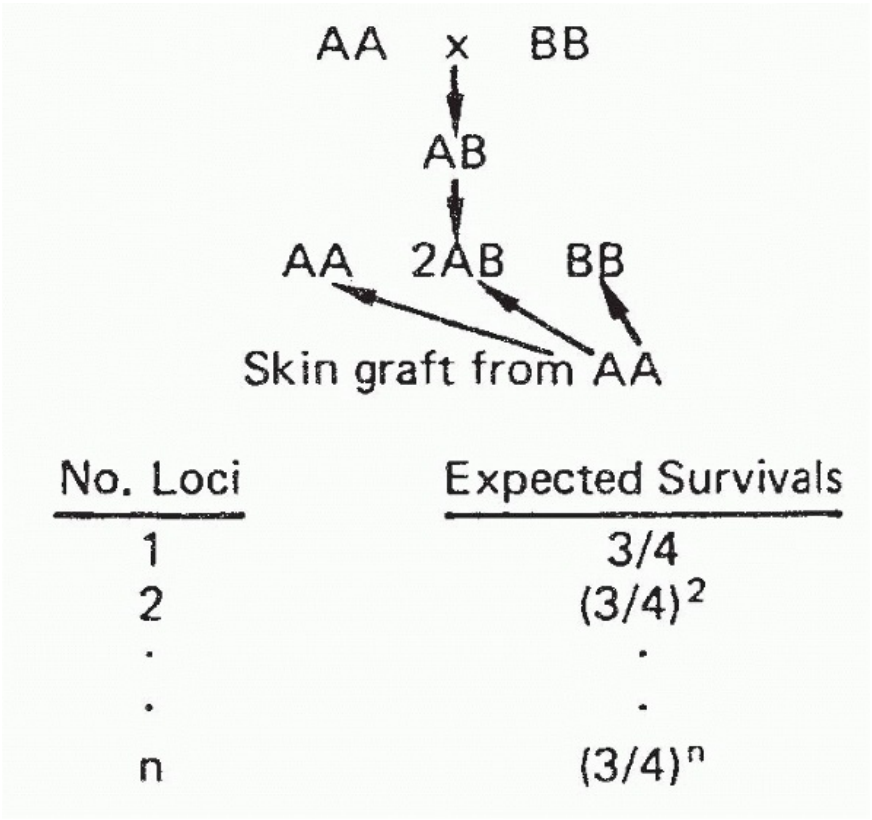

FIG. 46.4. Estimating the Number of Histocompatibility Loci.

Producing Congenic Strains: Identifying the Major Histocompatibility Complex

A process to generate strains differing from one another genetically at only a single one of these numerous histocompatibility loci was pursued by Snell at Jackson Laboratory and involved the production of congenic strains (inbred strains that differ from one another at only one independently segregating genetic locus) using the rejection of parental skin grafts as the trait used to select successive matings.[11] It became apparent during this process that one histocompatibility locus could be distinguished from all the others by the speed with which it caused skin graft rejection. This is now called the MHC. All of the other 30 to 50 histocompatibility loci have since been called minor histocompatibility loci. There are now a very large number of H-2 congenic strains of mice available (Table 46.2), as well as some that isolate minor histocompatibility loci and some rat congenic strains.

TABLE 46.2 List of H-2 Congenic Resistant Strains

Strain	H-2 Haplotype	Origin of Background	MHC
A	a	A	—
A./BY	b	A	Brackyury
A./CA	f	A	Caracal

A./SW	s	A	Swiss
BALB/c	d	BALB/c	BALB/c
BALB.B	b	BALB/c	C57BL/10
BALB.K	k	BALB/c	C3H
B6.AKR-H-2^k	k	C57BL/6	AKR
B6.SJL	s	C57BL/6	SJL
B10	b	C57BL/10	C57BL/10
B10.A	a	C57BL/10	A
B10.D2	d	C57BL/10	DBA/2
B10.M	f	C57BL/10	Outbred
B10.BR	k	C57BL/10	C57BR
B10.SM	v	C57BL/10	SM
B10.RIII	r	C57BL/10	RIII
B10.PL	u	C57BL/10	PL/J
C3H	k	C3H	C3H
C3H.SW	b	C3H	Swiss
C3H.JK	j	C3H	JK
C3H.NB	p	C3H	NB
D1.C	d	DBA/1	BALB/c
D1.LP	b	DBA/1	LP
LP.RIII	r	LP	RIII

MHC, major histocompatibility complex.

One of the most useful breeding schemes to produce congenic resistant (CR) lines is illustrated in Figure 46.5. Starting with two inbred strains, labeled Strain A and Strain B for simplicity, the objective is to obtain a strain that will share its entire genome with Strain A except for the major histocompatibility locus H-2, which will be derived from Strain B. The end product will be designated Strain A.B. Using the cross-intercross scheme illustrated in Figure 46.4, a skin graft or tumor graft from Strain A is placed onto all F2 offspring. Animals that reject the graft must be of genotype bb in at least one histocompatibility locus. Obviously, as there are many histocompatibility loci, most animals at this generation will reject the graft. However, if only animals rejecting vigorously are chosen, and if numerous such animals are selected, then one can be reasonably certain to have selected bb homozygotes at the H-2 locus by this procedure. Because mammalian genes are transferred as linked units in chromosomes, this process will always lead to the retention of a variable amount of bb genetic information at genes closely linked to the locus being selected. However, as described in the following, the occurrence of recombination during intercrossing generations leads also to fixation of the aa genotype at loci on the same chromosome as the MHC (chromosome 17 in mice) but at a variable distance from H-2. For practical purposes, animals that have been through at least nine cycles of such selected breeding are considered to be congenic.

As indicated in Table 46.2, H-2 congenic mouse strains are available on a variety of backgrounds. In general, the names of each of these strains follow the rule A.B, with Strain A being the background strain used in the production of the congenic and Strain B being the other parental strain from which the alternate allele at H-2 was selected. All of the early inbred mouse strains were assigned a small letter designation to represent the particular constellation of alleles that they possessed at genes in the MHC. This small letter designation is often called the haplotype designation, as indicated in Table 46.2. Thus, for example, Strain C57BL/10 (or B10) is assigned the haplotype designation H-2b and Strain DBA/2 the haplotype designation H-2d. Thus, the congenic strain B10.D2 represents a line in which the background is derived from the C57BL/10 and the MHC from the DBA/2. It thus resembles in almost every way the C57BL/10 congenic partner, except that it differs from this partner for all properties controlled by MHC-linked genes.

Intra-Major Histocompatibility Complex Recombinant Strains: Class I and II Antigens

As can be seen in Figure 46.5, every alternate generation in this mating scheme involves the crossing of animals heterozygous at H-2. Whenever heterozygotes are bred, there is always a possibility of recombination between autosomal chromosomes at meiosis. During the production of congenic lines, such recombination will tend to decrease the amount of linked genetic information carried into the congenic from the H-2 source. Therefore, the more backcrosses a particular congenic line has been subjected to, the closer

will be the boundaries on either side of H-2 at which the chromosome reverts to the background strain. Because it soon became apparent that the MHC was in fact made up of multiple loci, there was also the possibility for recombination within H-2 to occur during such crosses. Fortunately, mouse geneticists were aware of this possibility and saved numerous recombinants during the production of H-2 congenic lines. Indeed, it was through the detection and characterization of such recombinants that the linkage map of H-2 was constructed. Thus, for example, there are now a series of recombinants between strain C57BL/10(H-2b) and A/WySn(H-2a) that were isolated by Stimpfling during production of the

B10.A CR line and which have provided a great deal of information on the genetic fine structure of the H-2 complex. Strains B10.A(2R) and B10.A(4R), for example, have been used to map a variety of immune response genes within the MHC. Table 46.3 presents a listing of many of the most useful congenic recombinant strains now available and their known or presumed points of recombination. Among the most important contributions that came from the study of intra-MHC recombinant strains was the progressive understanding that the loci within the MHC encoded two general types of MHC antigens, now referred to as class I and class II MHC antigens.

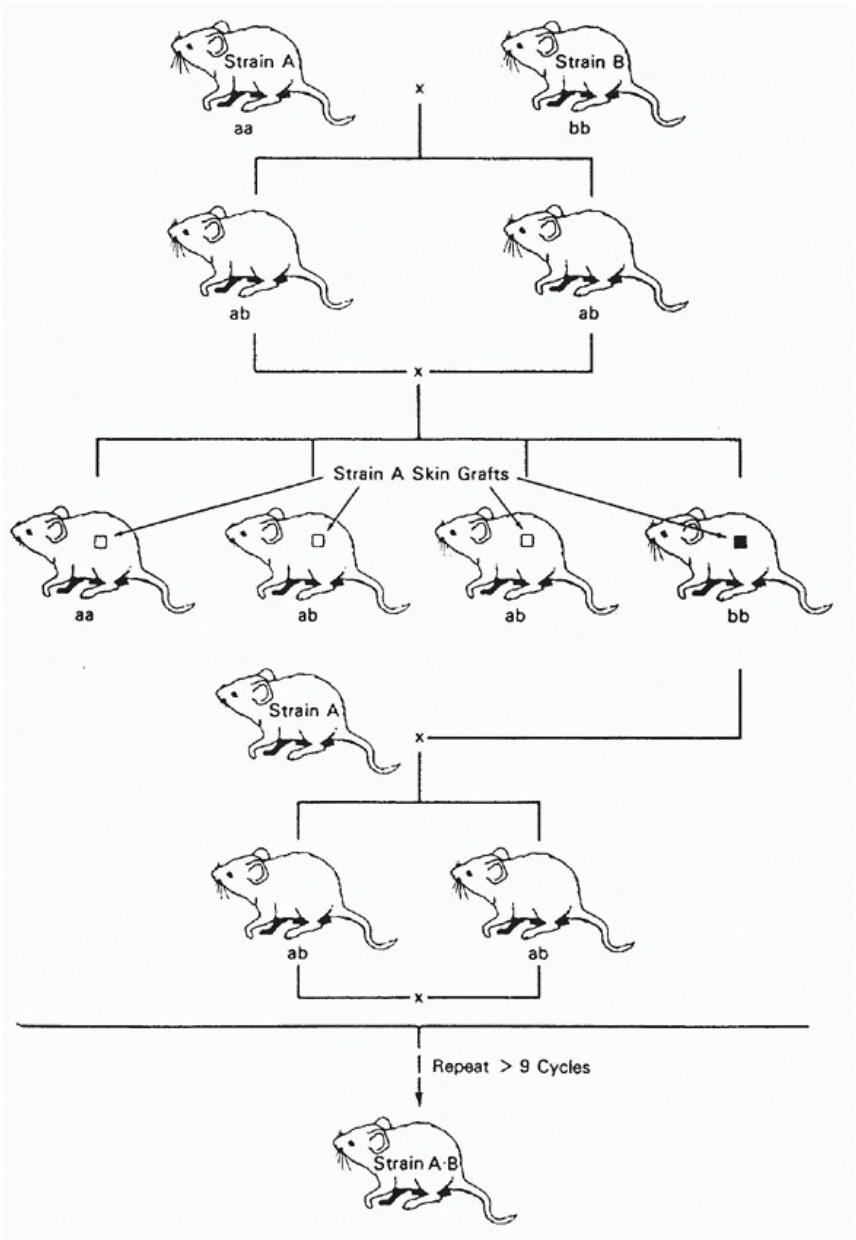

FIG. 46.5. Schematic Representation for Production of a Congenic Line.

TABLE 46.3 List of H-2 Recombinant Strains

Recombinant Interval Haplotypes	Parental Haplotypes	Haplotypes Designation	KAESD	Presence of Additional Recombinant Site	Strain-Bearing Recombinant
K-A	b/m	bq1	b/k k k q	Yes	B10.MBR
	s/a1	t1	slk k k d	Yes	A.TL
A-E	a/b	h4	k klb b b	No	B10.A(4R)
	b/a	i5	b blk d d	Yes	B10.A(5R)
	b/a	i3	b blk d d	Yes	B10.A(3R)
E-S	k/d	a	k k kld d	No	A, B10.A
S-D	d/b	g	d d d dlb	No	HTG, B10.HTG
	d/k	o2	d d d dlk	No	C3H.OH
	a/b	h1,h2	k k k dlb	Yes	B10.A(2R)
	k/q	m	k k k klq	No	AKR.M, B10.AKM
	q/a	y^2	q q q qld	No	B10.T(6R)
	s/A	t2	s s s sld	No	A.TH

Congenic recombinant haplotypes available from The Jackson Laboratory.

Note that many of the recombinants involve at least one haplotype already containing a point of recombination. These are indicated by “Yes” and are listed only under the recombinant interval representing the most recent recombination in the haplotype's history.

DONOR ANTIGENS RESPONSIBLE FOR GRAFT REJECTION

Major Histocompatibility Antigens

As discussed previously, the genetic analysis of graft rejection indicated that the antigens encoded within the MHC are of particular importance in graft rejection. Table 46.4 summarizes important aspects of the MHC antigens that are especially relevant for transplantation, while a much more detailed description of their structure and function can be found in Chapter 21.

Basic Features of Major Histocompatibility Complex Antigens

Polymorphism. The MHC antigens exhibit extraordinary polymorphism, providing the species with a broad capacity to present the peptides of, and thus respond to, a large number of pathogens. In the human leukocyte antigen (HLA) complex, for example, there are currently >600 named alleles at each of the HLA-A and B (class I) and >300 at the DRB1 (class II) locus.[12] The high degree of polymorphism has important consequences for transplantation. Given that there are three class I loci (A, B, and C) and three to four class II loci (DQ, DP, DRB1, ± an expressed DRB 3, 4, or 5 locus present in some haplotypes) on each haplotype, the likelihood of achieving identity for MHC antigens in two unrelated humans is extremely small, though for individuals with two major conserved HLA haplotypes,[13,14] the likelihood is increased.

TABLE 46.4 Summary of Features of Major Histocompatibility Complex

Class I antigens	Single polymorphic chain Three domains: alpha 1, 2, and 3 MW: 45,000 Associated with beta 2 microglobulin A, B, and C loci in humans Expressed on all tissues and cells
Class II antigens	Two polymorphic chains: alpha and beta Each with two domains: alpha 1 and 2, beta 1 and 2 MW: 33,000 and 28,000 DP, DQ, and DR loci in humans Expressed on macrophages, dendritic cells, and B cells; vascular endothelium; activated human T cells

Tissue Distribution. The tissue distribution of the two types of MHC antigens differs. Class I antigens are constitutively expressed on all nucleated cells, but at low levels on some types of cells.[15] Class II MHC antigens are more selective in their distribution.[16] They are especially frequent on macrophages, dendritic cells (DCs), and B-lymphocytes. They are present on other lymphoid cells under some circumstances and on vascular endothelium. Their expression on some tissues of the body is regulated (eg, by interferons [IFNs]).[17] One of the important distinctions between rodents and many larger species is the lack of constitutive expression of class II antigens on the vascular endothelium and other cell populations in rodents. In contrast, pigs, monkeys, and humans express class II antigens on

these tissues.[18,19,20]

Physiologic Function of Major Histocompatibility Complex Antigens . MHC antigens are called “histocompatibility” antigens because of their powerful role in causing graft rejection; however, they did not evolve in nature to prevent tissue grafting. While the name serves to emphasize the historical importance of transplantation in the discovery of the MHC, the essential role of MHC antigens is now understood to involve the presentation of peptides of foreign antigens to responding T cells (see Chapter 22).

The Importance of Major Histocompatibility Complex Antigens in Alloreactivity . MHC antigens are exceptionally important in stimulating T- and B-cell alloresponses. This section will

focus mainly on T-cell responses to MHC, as alloantibody responses are discussed later in the chapter.

VIGOROUS GRAFT REJECTION. Mouse skin grafts differing only in their MHC antigens are typically rejected in 8 to 10 days, whereas MHC-matched grafts may be rejected more slowly, depending on the number of minor histocompatibility antigen (MiHA) differences. In pigs, primarily vascularized organs, such as the kidney, may survive indefinitely in some cases, even without immunosuppression, if all of their MHC antigens are matched, whereas MHC-mismatched kidneys are always rejected within 2 weeks.[18]

STRONG IN VITRO ALLORESPONSES. Allogeneic MHC antigens stimulate an extraordinarily strong T-cell response in vitro, whereas responses to non-MHC antigens generally require in vivo priming. For the most part, cluster of differentiation (CD)4 cells recognize class II alloantigens and CD8 cells recognize class I alloantigens. However, this strong bias is more stringent for CD4 cells than CD8 cells. The standard in vitro assay of T helper function is the mixed lymphocyte response, which measures proliferation of T cells after allogeneic stimulation. Limiting dilution assays can be used to quantify alloreactive proliferating or cytokine-producing cells. Such analyses have led to frequency estimates of approximately 1% to 7% of T cells responding to a particular allogeneic donor,[21,22,23,24] whereas naïve T cells reactive with an exogenous peptide generally represent only approximately one in tens to hundreds of thousands of the same T-cell pool.[25,26,27]

Strong primary direct alloresponses of CD8+ T cells can be measured in vitro, either in standard cell-mediated lympholysis assays or in limiting dilution assays measuring cytotoxic T-lymphocyte (CTL) precursor frequencies. CTLs against MHC alloantigens can easily be generated from naive T cells following stimulation in vitro, whereas generation of CTLs to MiHAs generally requires that the T cells first be primed in vivo. Direct (without in vitro stimulation) cytotoxic activity,[28] increased alloreactive precursor frequencies, and modified CTL assays[29] have been used to demonstrate in vivo activation by alloantigens. ELISpot and flow cytometric carboxyfluorescein diacetate succinimidyl ester dye dilution assays and intracellular cytokine staining have enhanced the ability to detect alloreactive T cells.[24,30]

Direct Recognition of Allogeneic Major Histocompatibility Complex Antigens . The extraordinary strength of alloreactivity largely reflects the ability of T cells to recognize allogeneic MHC antigens presented on donor APC, referred to as “direct” allorecognition. Three different but not mutually exclusive hypotheses have been proposed to explain the high frequency of alloreactive T cells.

GENETIC BIAS. Because the thymus only positively selects T cells with some MHC reactivity,

a T-cell receptor (TCR) gene pool with intrinsic affinity for MHC molecules would allow for more efficient thymic selection. TCRs indeed have intrinsic affinity for MHC molecules.[31,32,33,34,35,36,37,38,39,40] Intrinsic allogeneic MHC reactivity is thereby prominent within a T-cell repertoire that has been negatively selected only by "self" MHC-peptide complexes.

THE "DETERMINANT DENSITY" HYPOTHESIS. As illustrated in Figure 46.6A, the density of specific peptide determinants presented by an APC would be quite low (as most MHC antigens present other peptides), whereas the density of a peptide-independent allogeneic MHC determinant on allogeneic APCs would be very high (as every MHC antigen would include the foreign determinant). These abundant allogeneic MHC determinants would activate many crossreactive T cells with relatively low affinities. This hypothesis requires that allogeneic MHC molecules can be recognized at least partly independently of the peptides they present. While peptide-independent and peptide-"promiscuous" alloreactive T cell clones have been described,[41,42,43,44,45,46,47,48,49,50] potential artefacts of in vitro culture and assay systems might have biased these results. Notably, human alloreactive T cells expanded in vivo in a graft-versus-host response were strongly dominated by peptide-specific clones,[51] and recent studies demonstrated that the requirement for peptide recognition limits TCR alloreactivity from being extended more broadly by their inherent MHC-binding capacity.[52]

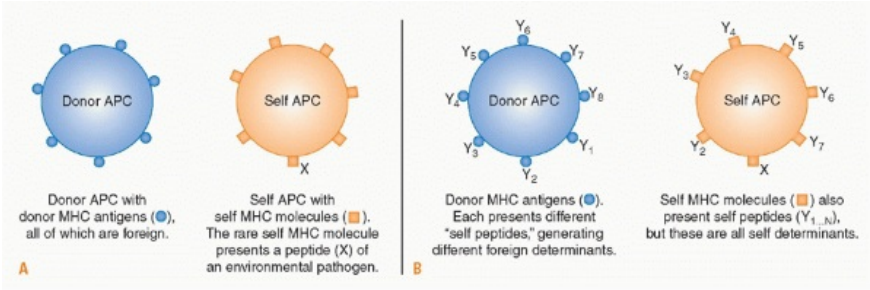

FIG. 46.6. A: Determinant density hypothesis. **B:** Determinant frequency hypothesis. APC, antigen-pressenting cell; MHC, major histocompatibility complex.

THE "DETERMINANT FREQUENCY" HYPOTHESIS. The third explanation for alloreactivity is based on the idea that allospecificities include the specific peptides presented by allogeneic MHC molecules,[53] for which there is strong evidence. Positive selection, which requires lower TCR affinity for self-MHC/peptide complexes than that involved in negative selection,[38] enriches for TCRs capable of seeing modified self-MHC antigens, which may cross-react on allogeneic MHC molecules. The repertoire of T cells positively selected with low affinity for self-MHC molecules plus peptides of self-proteins (say $X_{1,2...n}$), may cross-react with allogeneic MHC antigens presenting peptides of polymorphic or nonpolymorphic allogeneic proteins (eg, "Allo + X_1, Allo + X_2, …Allo + X_n") (see Fig. 46.6B). Nonpolymporphic peptides presented by allogeneic MHC molecules would be seen by different TCRs than those recognizing the same peptide with self-MHC, as both peptide and

MHC alpha helix residues contribute to the surface that is recognized by a TCR.[54,55,56,57] Thus, the set of determinants represented by "Self + $X_{1...n}$" would differ from that represented by "Allo + $X_{1...n}$." T cells strongly responsive to self-peptides on self-APCs (Self + X_1, Self + X_2, etc.) are eliminated by negative selection, which would not affect the response to the many peptides on allogeneic APCs (Allo + X_1, Allo + X_2, etc.). Consistent with this hypothesis, many alloreactive T cells have been shown to be peptide-specific or at least partially peptide-selective.[41,42,48,58,59,60,61,62,63] Cardiac allografting studies using DM-/-mice, which lack the capacity to replace invariant chain-derived CLIP peptide with a more diverse array of peptides, provide strong in vivo evidence for the importance of peptides in direct allorecognition.[64]

Overall, the available information supports the inherent MHC binding capacity of TCRs, combined with the determinant frequency notion, to explain the high frequency of T cells recognizing alloantigens directly.

Minor Histocompatibility Antigens

While initially defined by their ability to cause rapid graft rejection, MHC antigens are defined in part by the location of the genes encoding them and in part by the well-characterized structure of both class I and class II antigens (see Chapter 21). MiHAs, on the other hand, are those capable of eliciting a T-cell immune response, but which lack the structural characteristics of MHC products.[65] Rather than being allelic cell surface proteins, MiHAs are donor-specific peptides presented by MHC molecules that are shared by donor and recipient.[66,67,68,69,70,71,72,73] As individuals are tolerant to the peptides derived from their own proteins, they only respond to the peptides of another individual's proteins that have allelic variation. Unlike MHC antigens, MiHAs do not readily stimulate primary in vitro cell-mediated responses in mixed lymphocyte response and cell-mediated lympholysis assays, reflecting the low frequency of T cells recognizing them in the unprimed T-cell repertoire.

It has been estimated that there may be as many as 720 minor histocompatibility loci in mice,[74] some of which are autosomal and others of which are encoded on the Y chromosome. MiHAs can be expressed ubiquitously or in a tissue-selective manner.[75] Many proteins producing MiHAs have been identified,[71,72,73,76,77,78,79,80,81,82,83,84,85,86,87,88,89] some of which are intracellular proteins such as nuclear transcription factors and myosin, while others, like CD31 and CD19, are polymorphic cell surface glycoproteins.[90,91,92]

Some MiHAs are diallelic peptides, both of which can be represented by a particular MHC molecule,[76,78] resulting in bidirectional recognition (eg, the murine H13 locus[78]). Alternatively, allelic variation in MHC-binding capacity of a peptide can result in one allele being presented and the other not (eg, the human HA-1 minor antigen, in which only one of two allelic peptides binds effectively to HLA-A2[76]). Minor antigenic determinants can also result from the failure of one allele to be processed to a peptide. An example is HA-8, for which only one allele is effectively transported by the TAP complex, resulting in a null allele despite the presence of the MHC-binding peptide sequence in the molecule.[79]

Both helper determinants, recognized by CD4+ cells, and cytotoxic determinants, recognized by CD8+ cells, are required for effective cytotoxic T-cell responses to MiHAs.[93,94] When

multiple MiHA disparities exist, a phenomenon known as “immunodominance” may occur.[95,96,97,98,99,100] Removal of the immunodominant recognition can reveal strong responses to antigens that evoked weak or no responses before. This phenomenon may be due to competition between peptides for presentation by MHC molecules,[74] as well as differing durations of antigen presentation and TCR avidities.[101] An exceptional peptide, H60, is derived from an NKG2D-binding protein and produces responses that are comparable in potency to those elicited by MHC alloantigens, apparently due to the existence of a very high frequency of TCR in the naïve repertoire recognizing this peptide,[102] which may be a useful target for graft-versus-leukemia (GVL) effects in HCT.[103] However, immunodominance of CTL responses measured in vitro does not necessarily reflect the immunodominance of the same antigens in vivo.[74,104] possibly reflecting the importance of tissue distribution of minor antigen expression or of helper T-cell responses.[84]

The H-Y antigens are encoded on the Y chromosome and therefore expressed only by males.[81,82,105,106,107,108] They are of biologic significance, as they cause rejection of male skin grafts in syngeneic female mice and, in humans, female-to-male HCT is associated with increased GVHD rates compared to other combinations in the HLA-identical-related donor setting.

Human MiHAs have been identified as determinants recognized by CTLs, mainly in the setting of HLA-identical sibling donor HCT, in association with GVHD and marrow graft rejection.[77,79,84,85,109,110] Certain MiHA incompatibilities (eg, HA-1) may predispose to GVHD.[77,111] Immunodominance of CTL responses to particular H-Y and HA determinants as well as expansion of minor antigen-specific CTLs detected with tetrameric complexes of HLA molecules and minor antigenic peptides[85,110,112] have been associated with GVHD and marrow graft rejection.[113]

Most MiHAs identified to date are determinants recognized by CTL, reflecting the relative ease with which CTL assays can be used to measure peptide-specific responses. Newer techniques have allowed the recent identification of minor antigenic peptide epitopes recognized by CD4+ T cells.[114,115,116]

It is difficult to detect humoral responses to MiHAs, presumably because individual peptide/MHC complexes are too low in abundance on the cell surface to either stimulate an antibody response or be detected on the cell surface with antibodies. An exception is the recent discovery of antibodies against H-Y (Y chromosome-encoded) antigens in association with chronic GVHD (cGVHD).

Other Antigens of Potential Importance in Transplantation

Superantigens

Superantigens include products of endogenous retroviruses in mice, such as mammary tumor viruses, and bacterial products such as staphylococcal enterotoxin B. Like MHC antigens, superantigens can stimulate primary in vitro T-cell proliferative responses and activate a high proportion of the T-cell repertoire. However, these antigens are not presented as peptides in the binding groove of MHC molecules, but instead bind to distinct regions of class II MHC molecules, and engage nonvariable portions of Vβ components of the TCR, rather than the

hypervariable regions that recognize peptides. Endogenous superantigens are not classical transplantation antigens, perhaps because of their restricted tissue expression patterns.[117,118,119,120,121] However, they may contribute to GVHD in mice.[122]

Tissue-Specific Antigens

Some peptides are derived from proteins with limited tissue distribution,[123,124,125] which may not include hematopoietic cells used in traditional assays of alloreactivity. One implication is that transplantation tolerance induced by one set of donor cells might not always induce complete tolerance to donor cells of a different sort. For example, skin-specific antigens may be targets of skin graft rejection despite the presence of stable hematopoietic chimerism induced by HCT.[126] Tissue-specific proteins lacking allelic variation may still serve as alloantigens because the determinant formed by a given peptide with an allogeneic MHC molecule would be different from that formed by the same peptide with a recipient MHC molecule.

Several human MiHAs may be expressed only on hematopoietic cells,[75,127] and these may provide an opportunity to use graft-versus-host-alloreactive donor T cells (that recognize such antigens) to achieve GVL effects without GVHD (largely a disease affecting epithelial tissues). However, disparities for some of these minor antigens (eg, HA-1) have in fact been associated with an increased incidence of GVHD.[77,111]

Endothelial Glycoproteins

Blood Group Antigens. Blood group antigens are the products of glycosylation enzymes that are not the same in all individuals. They are expressed on erythrocytes and other cells and, importantly, on vascular endothelium where they may serve as the targets for “natural” antibody-mediated attack on blood vessels of organ grafts. Blood group A and B individuals each express their respective antigen, but O individuals have neither. “Natural” antibodies against blood group antigens an individual lacks probably arise due to cross-reactions with common carbohydrate determinants of environmental microorganisms. Type O individuals have antibodies to the antigens of A and B donors, whereas A and B individuals only have antibodies reactive with antigens from each other, and AB individuals have antibodies to neither. Therefore, O recipients can only receive transfusions from O donors, A and B recipients can receive transfusions from O donors or from individuals sharing their blood type, and AB recipients can receive blood from donors of any blood type. The same rules apply to the transplantation of most organs.[128] Recently, advances have been made in the ability to successfully transplant kidneys across ABO barriers by adsorbing antibody from the plasma and depleting B cells.[129,130,131] Other non-ABO blood group antigens on erythrocytes are irrelevant to organ transplantation because they are not expressed on vascular endothelium.

Blood group antigens are of lesser importance, but nevertheless significant, in HCT. ABO incompatibility in the hostversus-graft direction (“major” ABO mismatch) can lead to prolonged red cell aplasia following HCT; incompatibility in the graft-versus-host direction (“minor” mismatch) can result in initial hemolytic anemia, but this complication can be avoided by washing the donor HCT preparation to rid it of plasma.[132,133]

***Species-Specific Carbohydrate Determinants*.** Closely analogous to the blood group antigens are the carbohydrate determinants expressed on vascular endothelium that show species selectivity. For example, pigs, which are of interest as an organ source for xenotransplantation, have a glycosyl-transferase enzyme not expressed by humans that

glycosylates β-galactosyl N-acetyl glucosamine to form a Galα1-3Galβ1-4GlcNAc (αGal) determinant. This enzyme is present in most species but underwent a loss of function mutation in our nonhuman primate ancestors. In humans, a fucosyltransferase generates instead the H-substance from the same substrate, leading to blood group O. Preformed or "natural" antibodies are present in human serum that react to the nonself-pig determinant. Like the blood group antibodies, these natural antibodies probably arise from cross-reactions with environmental microorganisms,[134,135] and they also cause hyperacute rejection (HAR) of most primarily vascularized xenogeneic transplants.

"Missing Self" and Natural Killer Cell Recognition

In apparent violation of the laws of transplantation described previously, (AxB) F1 mice are capable of rejecting bone marrow from parental donors, a phenomenon termed hybrid resistance. This phenomenon, as well as rapid rejection of fully allogeneic marrow, is mediated by natural killer (NK) cells.[136] NK cells are large granular lymphocytes that lack TCRs and that have the ability to mediate cytolysis against certain tumor targets and hematopoietic cells. NK cells also produce a number of proinflammatory, hematopoietic, and even anti-inflammatory cytokines, and may be divided into subsets on the basis of their cytokine production pattern.[137]

The originally puzzling specificity of NK cell-mediated marrow rejection is due to the expression by NK cells of inhibitory receptors that recognize specific groups of class I

MHC alleles on target cells and prevent cytolysis by the NK cell. These class I receptors are type II C lectins (Ly49 family) or dimers of CD94 with NKG2 lectins in the mouse, and are either immunoglobulin family members (KIR) or CD94/NKG2 in humans. Recognition by an inhibitory receptor of a class I ligand results in intracellular transmission of an inhibitory signal via an immune receptor tyrosine-based inhibitory motif that interacts with a tyrosine phosphatase and counteracts activating signals transmitted from other cell surface molecules. Recognition of "self" class I inhibitory ligands prevents NK cells from killing normal autologous cells.[138,139] Other molecules expressed by infected or stressed cells activate NK cells, counterbalancing these inhibitory signals (see Chapter 17).

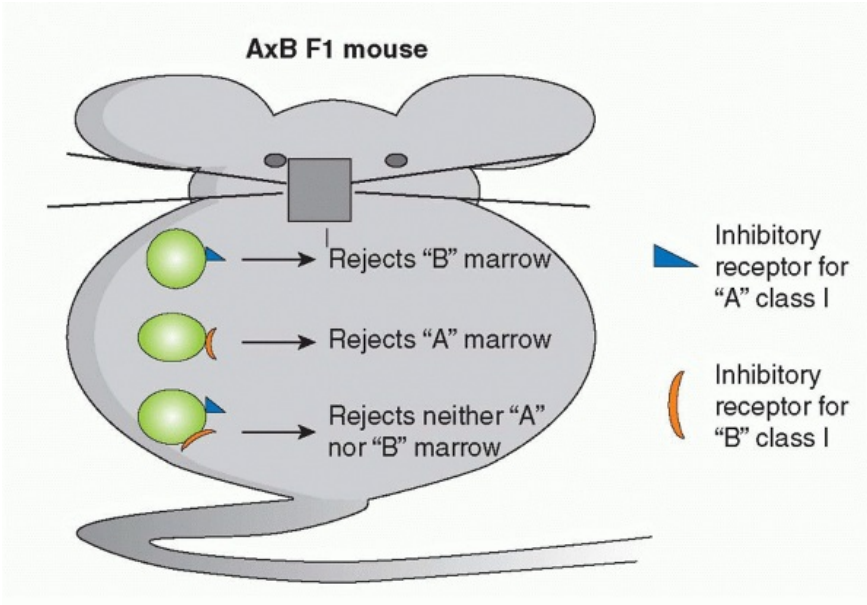

FIG. 46.7. An Explanation for Hybrid Resistance. Each *filled circle* represents a subset of natural killer cells.

Inhibitory receptors are clonally distributed on NK cells, each of which may express one or more different inhibitory receptor. For NK cells to be functional yet tolerant of "self," they must express at least one inhibitory receptor for a "self" class I MHC molecule.[140,141] Thus, as is illustrated in Figure 46.7, an AxB F1 recipient will have subsets of NK cells with inhibitory receptors that recognize MHC of either the A parent, the B parent, or both. The absence of "B" class I molecules on, for example, AA parental hematopoietic cells, permits subsets of (AxB)F1 NK cells that have inhibitory receptors only for class I molecules from the B parent to destroy AA cells. Thus, hybrid resistance and NK cell-mediated resistance to fully allogeneic marrow grafts can be explained on the basis of "missing self."[142] The roles of NK cells in transplantation are discussed in later sections.

MECHANISMS OF REJECTION

At least four distinct mechanisms that can cause graft rejection have been defined. According to the timeframe in which they tend to occur in clinical practice, they are, namely, HAR, accelerated rejection, acute rejection, and chronic rejection.

Rejection Caused by Preformed Antibodies (Hyperacute Rejection)

HAR occurs within minutes to hours after blood flow is established to a transplanted vascularized organ.[143,144,145] The phenomenon is visible and dramatic: the organ turns blue and its function ceases. Microscopically, there is extensive evidence of vascular thrombosis and hemorrhage. The important components involved in the mechanism of HAR include 1) donor endothelial antigens, 2) preformed antibodies that can bind these antigens, 3) complement, and 4) coagulation cascades, which are activated by the binding of preformed antibodies to the vascular endothelium. The interaction of these components leading to hyperacute rejection is diagrammed in Figure 46.8.

The role of complement in HAR is inferred both from the accumulation of various complement components in the grafts and from the fact that complement depletion leads to prolonged survival of xenografts.[146] Complement activation leads to production of active protein fragments and complexes of complement components, which cause tissue injury either directly or by recruiting effector cells that mediate destruction of the graft. In allogeneic combinations, this is initiated by antibody-mediated activation of complement through the classical pathway, whereas in xenogeneic combinations, the alternative pathway may also be involved.[147] In both cases, the membrane attack complex, produced by the ordered interaction of several complement components, initiates the destructive pathway.

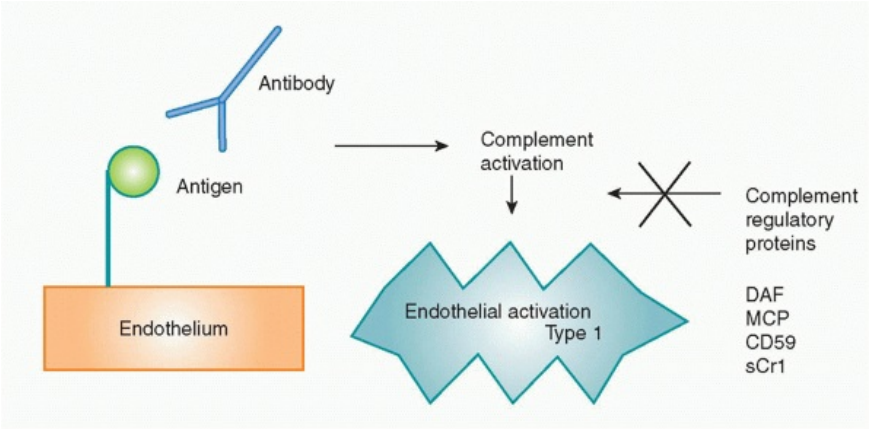

FIG. 46.8. Schematic Representation of Hyperacute Rejection.

Complement activation is controlled by several regulatory molecules, including complement receptor 1, decay accelerating factor (DAF; CD55), membrane cofactor protein (CD46), and CD59, which act at different stages along the cascade (see Chapter 36). Many of these molecules are produced by the vascular endothelial cells. Because these regulatory proteins prevent unwanted complement activation in the face of low levels of perturbation to the system, the titer and avidity of the preformed antibodies must be high enough to activate despite these downregulating molecules. Thus, preformed antibodies directed at MHC antigens almost always accomplish this activation, whereas the lower-affinity blood group antibodies lead to hyperacute rejection in only about 25% of kidneys. One of the reasons that hyperacute rejection is such an important feature in xenogeneic transplantation is that the complement regulatory proteins produced by the donor vascular endothelium of one species often do not function effectively with complement molecules derived from a different species. Because of this incompatibility, lower levels of an initial triggering signal lead to explosive complement activation.

Although the membrane attack complex is often thought of as a lytic molecule, its effect on the donor vascular endothelium, even before cell lysis, is to cause endothelial activation. This occurs rapidly, before there is time for new gene transcription or protein synthesis, and has been referred to as type I endothelial activation. The two principal manifestations of this activation are cell retraction, leading to gaps between endothelial cells, and initiation of coagulation pathways due to the loss of antithrombotic molecules from the endothelium.[148] Thus, type I endothelial activation is responsible for the two principal pathologic findings in hyperacute rejection: extravascular hemorrhage and edema, and intravascular thrombosis.

There are no known treatments that can stop the process of hyperacute rejection once it has started and, thus, it is essential to avoid the circumstances that initiate it. Experimentally, this can be accomplished for relatively short periods of time by administration of complement inhibitors, such as cobra venom factor, which depletes complement. In clinical practice, this is accomplished by avoiding transplantation in the face of preformed antibodies, both by avoiding blood-group antigen disparities and by testing recipients before transplantation (ie, a “cross-match”) to determine whether they have performed antidonor antibodies.

Not all organs and tissues are equally susceptible to hyperacute rejection. Most primarily vascularized organs, such as kidneys and hearts, are very susceptible, but the liver can often

survive without hyperacute rejection despite preexisting antidonor antibodies.[149] It is not clear whether this unusual feature of the liver reflects the large surface area of its vascular endothelium or an intrinsic property of liver endothelial cells. Nonetheless, hyperacute rejection of the liver has occurred in some cases, especially involving xenogeneic transplantation, indicating that its resistance to hyperacute rejection is not absolute. Skin grafts are relatively resistant to hyperacute rejection but high levels of antibody can cause a "white graft" (ie, a failure of blood vessels to communicate with those of the recipient)[150] Pancreatic islets are likewise resistant to this form of rejection.[151] Free cellular transplants, such as bone marrow cells or hepatocytes, that express some of the antigens recognized by preformed antibodies, are cleared quickly from the circulation by the reticular endothelial system, leading to resistance to engraftment.[152] In the case of HCT, this resistance can be overcome by transplanting larger numbers of cells.[152,153,154] Additionally, antibody-independent complement activation has been shown to be a significant factor diminishing the engraftment of porcine bone marrow in mice.[155]

Acute Humoral Rejection (Accelerated Rejection)

A second mechanism of rejection, also caused by antibodies, occurs as a result of antibodies that are induced very rapidly after a transplant is performed. This type of rejection has been called "acute humoral" or "accelerated" rejection because it typically occurs within the first 5 days after transplant. The process is characterized by fibrinoid necrosis of donor arterioles with intravascular thrombosis.[156]

Accelerated rejection is rare in allogeneic combinations because it requires that an antibody response occur before the T-cell response that is typically responsible for acute rejection episodes (see following discussion). Indeed, most allogeneic B-cell responses are T-cell-dependent. The best examples of accelerated rejection are probably those observed in vascularized organ transplants between closely related, concordant xenogeneic species and between discordant species following adsorption of anti-Gal antibodies. In these cases, the levels of preformed antibodies are not sufficient to cause hyperacute rejection, but antidonor antibodies appear rapidly (within 3 to 4 days) in association with the onset of rejection. The pathology of acute humoral rejection reveals a paucity of lymphocytes infiltrating the donor graft, antibody binding to donor vascular endothelium, and fibrinoid necrosis of the donor vessels. Vigorous anti-T-cell immunosuppression has little effect on acute humoral rejection, whereas immunosuppression with reagents that affect B-cell responses, such as cyclophosphamide, delays its onset until more typical T-cell-mediated rejection occurs.[157]

As in hyperacute rejection, the process of acute humoral rejection is usually initiated by antibody binding to antigens on the donor vascular endothelium. In this case, however, the subsequent endothelial changes occur more slowly, allowing time for gene transcription and new protein synthesis. This later form of activation has been called type II endothelial activation.[147,158] Many of its features appear to be mediated by the transcription factor NF-κB, which generates many of the responses associated with inflammation, including the secretion of inflammatory cytokines such as interleukin (IL)-1 and IL-8, and the expression of adhesion molecules such as E-selectin and intercellular adhesion molecule (ICAM)-1.[159] In addition, type II endothelial activation causes the loss of thrombomodulin and other prothrombotic changes.[160] Thus, the events following type II endothelial activation are associated with the pathologic changes that occur with "accelerated" rejection, including the tendency toward intravascular thrombosis and the inflammatory destruction of donor vessels

that occurs in the absence of infiltrating lymphocytes.

Just as there are regulatory processes for complement activation, there are regulatory molecules that counter the tendency toward intravascular coagulation and the process of type II endothelial activation (eg, tissue factor protein inhibitor [expressed by vascular endothelium, which inhibits factor Xa of the clotting cascade] and a number of other protective molecules, including Bcl-x_L, Bcl-2, and A20).[147,161] Although these are often thought of as antiapoptotic molecules, they also tend to inhibit activation mediated by NF-κB. Like the regulatory molecules of complement, some of these regulators may not function across species differences, leading to disorder regulation of the coagulation system.[162]

Although vigorous early antibody responses generate type II endothelial activation and accelerated rejection, later antibody responses often fail to do so. The process that enables transplanted organs to survive in the face of circulating antibodies that can bind endothelial antigens has been called “accommodation.”[147,163] This phenomenon has been observed in some allogeneic and xenogeneic combinations with preformed antibodies, but has so far been disappointingly ineffective in discordant xenotransplants. Resistance to type II endothelial activation has been achieved in vitro by pretreatment with low levels of antiendothelial antibodies that are insufficient to trigger activation.[164] The achievement of accommodation is associated with increased expression of the antiapoptotic genes described previously and with changes in the isotype of the recipient's antibody responses.[158,165]

An important difference between HAR and acute humoral rejection is that there is no known therapy to stop graft destruction by HAR, whereas acute humoral rejection can sometimes be reversed by desensitization.

Rejection Caused by T Cells (Acute Rejection and Graft-versus-Host Disease)

“Acute cellular rejection,” which is characterized by a mononuclear cell infiltrate in the graft, is the most common type of organ allograft rejection. Acute rejection is most common during the first 3 months after transplant, but may occur at any time, especially if immunosuppressive medication is withdrawn. Acute rejection is T cell-dependent, and its treatment, which is usually successful, includes increased doses of standard immunosuppressive drugs or antilymphocyte antibodies.

The use of newer immunosuppressive drugs and anti-T-cell antibodies has markedly reduced acute rejection rates. For example, the vast majority of kidney transplant recipients never experience an episode of acute rejection. It is now quite rare to lose a transplanted organ to cellmediated rejection during the first year after transplantation. However, the use of these highly effective immunosuppressive treatments is associated with significant morbidity.

Experimental models for acute rejection include nonprimarily vascularized skin grafts, heart graft fragments, artificial “sponge” allografts, or islet transplants in rodents, which may not accurately reflect the processes of rejection for primarily vascularized organs. While there are models of heart, kidney, liver, and other types of primarily vascularized organ transplants in rodents, these types of transplants are more tolerogenic and hence more easily accepted than similar transplants in large animals and humans. Studies of primarily vascularized organ transplants in large animals, such as monkeys or pigs, have obvious clinical relevance, but are expensive and require many special resources.

Acute GVHD is the counterpart of cellular rejection that involves graft-versus-host

alloreactivity, usually in the context of HCT, but also sometimes with organ transplants that carry significant amounts of donor lymphoid tissue (eg, liver). Like acute rejection, acute GVHD is T cell-dependent. T-cell depletion of the donor hematopoietic cell graft prevents GVHD but is associated with increased rates of graft rejection and relapse of malignant diseases.

The concepts of “direct” and “indirect” allorecognition introduced previously must be considered at both the sensitization and effector phases of an immune response. The definition of “indirect” recognition used in this chapter is based on which set of APCs (donor versus recipient) is presenting donor antigen. “Cross-priming” is a term specifically denoting sensitization of CD8 T cells through the indirect pathway. As shown in Figure 46.9, there are three major T-cell pathways to consider in relation to graft rejection. These include 1) direct recognition of donor alloantigens by CD4+ T cells, which generate effector CD4 cells and provide help for the generation of effector CD8 cells; 2) direct activation of CD8 T cells by donor APCs; and 3) CD4+ T-cell activation by recipient APCs presenting re-processed donor antigens (the indirect pathway of sensitization). This pathway is important in providing help for immunoglobulin production by B cells. A role in rejection for cross-primed CD8+ T cells is not included in Figure 46.9, because this pathway has not been shown to play a role in allograft rejection except under certain circumstances, as described in the following. The multiplicity of T-cell sensitization and effector pathways involved in graft rejection and GVHD is demonstrated by the frequent observation of rejection or GVHD when only CD4+ or CD8+ T cells are depleted.[166,167,168] As a result of the high precursor frequency of T cells that respond to allogeneic MHC antigens directly, populations of T cells that ordinarily have minimal significance become functionally important.

Sensitization and Cell Trafficking during Rejection and Graft versus Host Disease

Transplanted tissue contains passenger leukocytes of donor origin that have the characteristics of immature DCs.[169] In response to the inflammatory signals that are triggered by retrieval and transplantation, both within the tissue itself as well as in the recipient,[170] the donor-derived passenger leukocytes rapidly leave the graft and migrate to the secondary lymphoid tissues of the recipient.[171] Secondary lymphoid tissues comprise the spleen, lymph nodes, and gut- or mucosal-associated lymphoid tissue, and depending on the location of the graft, the passenger leukocytes will migrate to the tissue that drains the graft site where they encounter naïve T cells. After transplantation, both in situ within the tissue and during migration, the passenger leukocytes

acquire the phenotypic and functional characteristics of mature DCs, expressing high levels of MHC class I and II molecules as well as other cell surface costimulatory molecules necessary to fully activate naïve CD4+ and CD8+ T cells.[172] Once in the secondary lymphoid tissues they act as professional APCs, presenting antigens expressed in the transplanted tissue to recipient T cells via the direct pathway of allorecognition.[173]

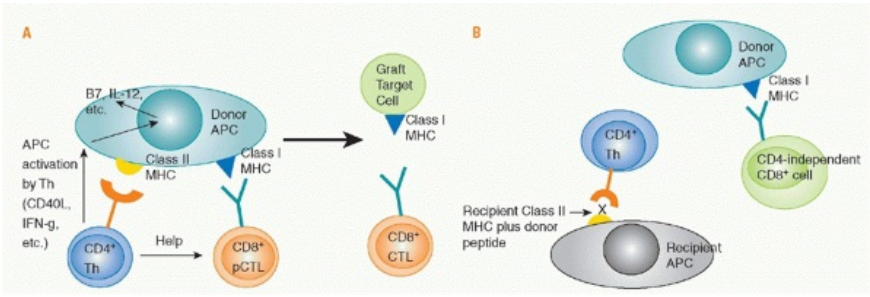

FIG. 46.9. Model of T-Cell-Mediated Rejection. A: Interactions between cluster of differentiation (CD)4+ Th, donor antigen-presenting complex, and CD8+ cytotoxic T lymphocyte. **B:** Additional pathways of T-cell sensitization that can lead to rejection. MHC, major histocompatibility complex.

Naïve T cells recirculate around the body and are constantly moving through the secondary lymphoid tissues sampling the APC, both host- and (after transplantation) donor-derived, for antigen.[174] If a naïve T cell with a TCR that can recognize a donor MHC molecule encounters the donor-derived passenger leukocyte in the draining lymphoid tissue as it recirculates, it will stop, interact, and differentiate into an antigen-experienced effector T cell. In support of the secondary lymphoid tissue being the primary site for sensitization of naive T cells and initiation of rejection after solid organ transplantation, Lakkis and colleagues[175] showed that cardiac allografts were not rejected in splenectomised aly/aly mice that lack secondary lymphoid tissue as a result of a mutation in the gene encoding NF-kB-inducing kinase[176] and suggested that in this situation permanent graft acceptance was due to immunologic ignorance. Other studies supporting the concept that secondary lymphoid tissues draining the graft are the key site for initiation of the immune response have followed the fate of T cells of a known specificity for donor antigen as they respond.[177]

Similarly, after bone marrow transplantation (BMT) the initiation of GVHD also takes place in the secondary lymphoid tissue, with evidence for the initial proliferation of donor CD4+ T cells followed by CD8+ T cells in secondary lymphoid organs with subsequent homing to the intestines, liver, and skin.[178,179,180] Visualization of T cells responding during the initiation of GVHD showed that while Peyer patches are involved, other secondary lymphoid tissues contribute to the activation of T cells that can home to the gut; mesenteric lymph nodes and spleen are also sites where gut homing T cells were activated.[179]

In solid organ transplantation, exclusive initiation of rejection in the secondary lymphoid tissues conflicts with the earlier hypothesis that rejection was initiated within the graft itself by donor endothelial cells lining the vessels that could activate T cells directly as they passed through the graft.[181,182] Since these early papers, there have been a number of studies that support this hypothesis. For example, human endothelial cells have been shown to activate naïve T cells in vitro.[183] In the mouse, APCs that are not of hematopoietic origin have been shown to activate CD8+ T cells in vitro and in vivo,[184] thus supporting the concept that T cells may be activated in the graft rather than in the secondary lymphoid tissue. Moreover, splenectomized lymphotoxin α and lymphotoxin β knockout mice that also lack secondary lymphoid tissues were found to reject cardiac allografts, albeit at a slower

than normal tempo.[185] Each of these models is subtly different immunologically, and therefore different components of the immune response to an allograft may be differentially affected by the presence or absence of secondary lymphoid tissues. Clearly, in the absence of secondary lymphoid tissue, the initiation of the rejection response by naïve T cells is less aggressive.

While antigen presentation via the direct pathway plays a dominant role in initiating the response to a transplant, a finite number of donor-derived passenger leukocytes is transferred within a transplanted organ. Thus the role of the direct pathway initiated by passenger leukocytes may diminish with time as eventually only "nonprofessional" APCs, including endothelial cells, remain to stimulate direct pathway T cells. Thus the role of endothelial cells within the graft may assume a greater significance with time after transplantation both for the initiation of the response and as a target for direct pathway effector cells. While activation of naïve T cells may occur predominantly in the secondary lymphoid tissues after transplantation, activation of memory T cells in presensitized recipients is quite different. Unlike naïve T cells, memory T cells can migrate to nonlymphoid tissues in the periphery[186] and can trigger rejection through pathways that are independent of secondary lymphoid tissues.[187]

Thus, in humans, where there are likely to be both naïve and memory T cells that can recognize or cross-react with donor MHC molecules, rejection may be initiated both within the secondary lymphoid tissue as well as within the allograft by naïve and memory T cells, respectively.

At the same time that donor-derived passenger leukocytes are leaving the graft, recipient leukocytes including APCs are attracted to the graft by the inflammatory mediators and chemokines released in the vicinity of the transplanted tissue. As these cells traffic through the graft, they phagocytose debris arising from tissue damage at the time of transplantation before migrating to the draining lymphoid tissue. The ingested antigens are processed and presented on recipient MHC molecules to T cells in the recipient lymphoid tissue.[188] In addition, soluble antigens released from the graft will also be transported in the blood to the draining lymphoid tissue, where they will be taken up and presented by resident APCs. Common antigenic peptides presented by the indirect pathway are the hypervariable peptide binding regions of MHC molecules.[189] Indirect pathway responses undoubtedly contribute to acute rejection, although the tempo of rejection may be slower due to the lower frequency of T cells that can respond. However, unlike direct pathway allorecognition, the indirect pathway is available for antigen presentation for as long as the graft remains in situ, and therefore becomes the dominant mode of allorecognition long term.

A third pathway of allorecognition has been described more recently, the so-called semidirect pathway that involves the capture of donor MHC-peptide complexes by host APCs. The exchange of fragments of cell membrane between cells that interact with one another is a well described phenomenon in cell biology. In the context of the immune response to an allograft, the transfer of membrane fragments from allogeneic cells expressing donor MHC molecules can result in the presentation of intact donor MHC molecules by recipient APCs to T cells. The significance of the semidirect pathway is still under investigation.[190]

Traffic of naïve lymphocytes is usually restricted to recirculation between the blood and lymphatic systems. However, once they have been primed in the secondary lymphoid tissues, activated lymphocytes as well as other activated leukocytes must be able to migrate into the

graft in order to destroy the transplanted tissue, a process known as leukocyte recruitment.

The inflammatory processes at the site of transplantation generate chemotactic cytokines called chemokines, and upregulation of chemokine receptor expression by activated leukocytes enables them to migrate along the chemoattractant gradient to reach the graft.[191]

Inflammatory signals also affect blood vessels in the vicinity of the transplant, causing vasodilation and endothelial activation. Activated endothelial cells rapidly externalize preformed granules called Weibel-Palade bodies that contain the adhesion molecule P-selectin[192] and rapidly upregulate expression of vascular cell adhesion molecule and CD62E (E-selectin). At the same time, chemokines released from the graft become tethered to the endothelium, and these alterations in endothelial surface markers advertise to passing leukocytes that an inflammatory process is occurring in the neighboring tissue.

Leukocytes are usually conveyed within the fast laminar flow at the center of blood vessels, but once activated leukocytes reach postcapillary venules in proximity to the graft, they are able to leave this rapid flow and move toward the edge of the vessel. This occurs in response to the local chemokine gradient and is assisted by the slower blood flow in the vasodilated blood vessels near the graft. Leukocyte extravasation is a multistep process. Initially, low-affinity interactions develop between endothelial P-selectin and sialyl-Lewis^X moieties that are present on the surface of activated leukocytes. These interactions continually form and break down, and the leukocyte "rolls" along the endothelial surface. If chemokines are present on the endothelial surface, conformational changes in leukocyte integrin molecules occur that allow them to bind other endothelial adhesion molecules such as ICAM-1. These higher-affinity interactions cause arrest of the leukocyte on the endothelial surface, allowing it to commence extravasation. Having entered the tissues, the activated leukocytes continue to migrate along chemokine gradients in order to invade the graft.

Antigen Recognition and T-Cell Help in Graft Rejection and Graft-versus-Host Disease

Role of Direct Cluster of Differentiation 4 Allorecognition . Priming of naïve, directly alloreactive T cells requires professional APCs that leave the graft and enter the recipient's lymphoid tissues. Direct CD4 T-cell sensitization by donor class II MHC antigens may both generate CD4+ effector cells and provide help for the activation, differentiation, and proliferation of cytotoxic CD8+ cells that directly recognize donor class I MHC antigens and destroy the graft (see Fig. 46.9). Depletion of donor APCs can markedly prolong graft survival,[193,194,195,196,197,198] illustrating the importance of direct allorecognition in inducing rejection. The CD4 help for CD8 cells consists of both cytokine (eg, IL-2) production and "conditioning" of the APC, for example by interactions of CD40 on the APC with CD40L on the activated CD4 cell. These interactions upregulate APC expression of CD80 and CD86 costimulatory molecules and cytokines such as IL-12 and MHC, making the cell a more effective APC. Studies of antiviral immunity indicate that CD4 help is needed for development of full effector function,[199] and for CD8 memory cell survival[200] and function.[201]

Studies involving very limited (not clinically relevant) antigenic disparities between donors and recipients suggested that a "three-cell cluster" model involving interactions between helper T cells, effector T cells, and APCs was essential for rejection.[202,203,204,205] However, studies involving more extensive, clinically relevant histoincompatibilities[206,207] suggest that CD4 helper cells sensitized by antigen presented on recipient APCs can provide help for directly alloreactive CD8+ effector cells. It remains possible that a "three-cell cluster"

is still essential for CD4 cells to provide help to CD8 cells mediating rejection, and that donor class I MHC/peptide complexes are transferred and picked up by recipient APCs. Recipient APCs with directly alloreactive CD8 T cells would thereby encounter their ligands on the same recipient APC that an indirectly alloreactive CD4 cell recognizes. Such transfer of class I/peptide antigens, resulting in this type of

"semidirect" antigen presentation, requires consideration in transplant models.[208,209,210,211]

CD4+ T cells alone can cause graft rejection (without CD8 cells) in the setting of class II or multiple minor histoincompatibilities,[198,212,213,214,215] indicating that CD4 T cells can mediate rejection effector functions. In BMT recipients, they can induce GVHD in the absence of CD8 cells in the setting of class II, full MHC, or multiple minor histoincompatibilities,[216,217,218,219] and can reject class II and minor antigen-mismatched bone marrow.[220,221]

Role of Indirect Cluster of Differentiation 4 Cell-Mediated Allorecognition. Indirectly alloreactive CD4 T cells have roles in skin and solid organ graft rejection,[210,222,223,224,225,226,227,228,229,230,231,232] including the provision of help for class-switched alloantibody responses.[233] This help requires cognate interactions between recipient class II-restricted indirectly alloreactive CD4 cells and host B cells that recognize donor MHC molecules through their immunoglobulin receptors, process them, and present donor MHC peptides with their class II molecules. CD4 cells also contribute to rejection of bone marrow grafts differing only at class I MHC loci, possibly implicating indirect allorecognition.[220,221]

Rejection by CD4 cells of skin grafts lacking class II MHC shows the strength of the indirect pathway of rejection.[206,222] Rejection of islet xenografts in mice may depend on indirectly xenoreactive CD4+ T cells.[234] Sensitization of indirect CD4 responses to donor MHC-derived peptides has been demonstrated in patients undergoing graft rejection, and these may be correlated with poor outcomes.[235,236,237,238,239,240,241,242] A major role for indirect allorecognition has been suggested in the setting of chronic rejection,[230,243,244,245,246] in part because of its role in inducing antibody responses, which are implicated in chronic rejection.[247,248,249,250,251] Moreover, the eventual replacement of donor APCs by recipient APCs implicates the latter in long-term graft recipients.[196,241,252,253] Consistently, direct alloresponses tend to subside over time in transplant patients.[254,255,256]

Nevertheless, donor APC depletion or the lack of donor class II MHC can prevent rejection in some situations.[193,194,195,196,197,198] An essential role for indirect allorecognition has not been demonstrated for acute rejection.[21,253,257,258] Indirectly alloreactive CD4+ cells alone fail to reject skin grafts with minimal class I or minor histoincompatibilities,[202,259,260] or to induce GVHD against class I MHC or minor histocompatibility barriers alone.[216,217] With rodent primarily vascularized allografts, donor APC depletion may, by preventing the strong direct alloresponse, allow the inherent tolerogenicity of the organ to prevail.

Role of Helper-Independent Cluster of Differentiation 8+ T Cells . CD8 T cells can readily reject skin and bone marrow allografts in the absence of CD4

cells,[166,202,220,221,261,262] and alloreactive CD8 T-cell memory can be generated and maintained without CD4 cells.[263] CD8 cells can also induce GVHD without CD4 T cells in the setting of full MHC, class I only, and minor antigen histoincompatibilities.[264,265] Direct recognition of recipient MiHAs on recipient APCs is essential for the induction of CD8-dependent, CD4-independent GVHD in MHC-identical, lethally irradiated mice,[266] but indirect[267] or "semidirect"[211] CD8 recognition of recipient antigens presented by donor APCs amplifies the process.

Together, these studies show that CD4 help is not critical for CD8 cell-mediated rejection or GVHD. However, the requirement for CD4 help may increase in the absence of inflammatory stimuli, as indicated by marked differences in the need for CD4 help for CD8-cell activation and GVL effects in the presence and absence of inflammation.[268,269] Grafts expressing only class I antigen disparities are usually rejected quite slowly, and CD4-independent rejection is relatively easily suppressed by cyclosporine.[270,271,272] Many primarily vascularized grafts that express only a class I antigen disparity require CD4+ cells to initiate rejection, and, when it occurs, CD4-independent rejection by CD8+ cells is dependent on the number of donor APCs in a graft.[166,222] CD4-indepenent CD8+ cells do not reject grafts expressing only a small number of minor antigen disparities and generate only weak helper responses even in the presence of multiple MiHA disparities. CD8+ helper cells also differ from CD4+ helper cells in being unable to provide help for other cell populations.[273] CD8+ cells alone cannot reject skin grafts with only limited class II antigen disparities.[202,218,259,274]

Cross-Primed Cluster of Differentiation 8 Cells . Peptides of exogenous antigens were originally thought to be presented by MHC class II antigens, whereas those of endogenous cellular antigens are presented by MHC class I molecules.[275,276] However, it is now clear that class I presentation of exogenous peptides (cross-presentation) is essential for many immune responses, including those against microbial and tumor antigens.[277,278,279,280,281] Several pathways have now been delineated for cross-presentation by class I molecules.[282,283,284] CD8 cell crosspriming was originally demonstrated in a transplantation model by Bevan[70] when minor antigen-disparate grafts with MHC antigens of type A were placed on MHC (A × B) F1 recipients and CD8+ cells became sensitized to the minor antigens presented by both A and B types of class I MHC molecules. Activation of cross-primed CD8 cells is strongly dependent on CD4 help and IL-2.[285] Cross-primed CD8 cells recognizing donor MiHAs and MHC-derived peptides are most likely to participate in rejection when there is sharing of class I alleles between the donor and recipient. Without such sharing, the self-class I/allogeneic peptide epitope cannot be presented by the parenchymal or endothelial cells of the graft.[286] However, even without class I sharing, indirect CD8+-cell sensitization can lead to skin allograft rejection, perhaps due to recognition of donor peptides presented by recipient endothelial cells on host-derived vessels that revascularize the graft.[287,288] Cross-primed CD8 cells might also contribute to graft rejection via indirect effector mechanisms upon antigen recognition on host APCs in the graft or by producing inflammatory cytokines.[289] Some of the rejection processes previously attributed to cross-primed CD8 cells may in fact be mediated by CD8 cells seeing intact donor MHC-peptide complexes on recipient APCs ("semidirect" presentation).

Effector Mechanisms of Rejection and Graft-versus-Host Disease

While cytotoxic T cells are important effectors of graft rejection and GVHD, additional mechanisms involve effector cells of the innate immune system and cytokines as final mediators of tissue destruction. The net result of this multiplicity of pathways is considerable redundancy of mechanisms of graft rejection and GVHD.

Cytotoxic Mechanisms of Graft Rejection and Graftversus-Host Disease . Rejecting organs contain proteins and messenger ribonucleic acid (RNA) encoding perforin, granzymes, and proteases associated with cell-mediated cytotoxicity.[290,291,292,293,294,295,296,297] The presence in urine of RNA encoding perforin and granzyme B has been associated with renal allograft rejection in humans.[298] Although the perforin/granzyme pathway is the major cytolytic pathway for CD8 T cells and CD4 cells tend to utilize the Fas/FasL pathway,[299] both subsets are capable of both types of cytolytic activity,[300,301] and the perforin pathway is available to both T-cell subsets mediating GVHD.[302] All of these cytotoxic proteins play contributory roles, and no single protein has been found to be critical for solid organ graft rejection,[303,304,305,306] GVHD,[307,308,309,310] or bone marrow graft rejection[311] in the presence of clinically relevant mismatches. Critical cytotoxic interactions have been identified in less relevant animal models involving Fas-dependent GVHD directed at isolated class II MHC disparities[310] and perforin-dependent rejection of K^b mutant class I-only mismatched heart allografts.[303] Fas ligand promotes lymphoid hypoplasia[312] and skin and liver GVHD,[312] and both Fas ligand and TRAIL are required for GVHD-related thymic destruction.[313] While the perforin-granzyme pathway contributes to GVHD,[310,312] the Fas pathway appears to be of greater overall importance. In contrast, the perforin/granzyme and TRAIL pathways predominate in antileukemic effects, especially of CD8 cells, and selective blockade of the Fas/FasL pathway may ameliorate CD8-mediated GVHD without eliminating GVL effects.[309,314,315,316,317,318]

Non-Cytotoxic T-Lymphocyte Effector Mechanisms in Graft Rejection and Graft-versus-Host Disease. T cells can effect rejection of grafts whose parenchymal cells do not express the TCR ligand, indicating the existence of “indirect” effector mechanisms. Entire skin grafts can be rejected when only the APCs are foreign,[319] indicating that nonselective destruction of grafted tissue can occur. Several studies have implicated indirect CD4 cell-mediated rejection of skin[320,321] and cardiac[322] allografts. Replacement of graft endothelium by the host was shown to be needed for rejection through this indirect effector mechanism.[322] GVHD of the liver and intestine can be induced by donor T cells in MHC-deficient hosts receiving wild-type host DCs, suggesting that indirect effector mechanisms may also mediate tissue injury of GVHD.[323,324] However, CD4-mediated GVHD against MiHAs is markedly attenuated when the target antigens are expressed only on hematopoietic cells.[325] Thus, “indirect” effector mechanisms can destroy transplanted tissue or recipient tissue in the case of GVHD, but less efficiently than direct cytotoxic mechanisms.

Another non-CTL graft rejection mechanism involves antibodies, which cause hyperacute rejection, acute humoral rejection, or chronic rejection through Fc receptor, complement-mediated, and other inflammatory pathways. B cells[326,327] and antibodies[327] contribute to

cGVHD in animal models and are implicated in human cGVHD.[328,329,330] B-cell depletion with rituximab has been reported to have efficacy against cGVHD.[330,331,332]

Cytokines as Mediators of Graft Rejection and Graftversus-Host Disease **.** Interactions between alloreactive CD4 helper cells producing cytokines of the "Th1" type and alloreactive cytotoxic CD8+ effector cells can mediate rejection and GVHD via direct cytotoxicity.[333] The "indirect" mechanisms of graft rejection and GVHD are likely to include cytokines.[298] "Th17" cells producing IL-17 and other proinflammatory cytokines promote rejection and GVHD.[334,335,336,337,338,339,340,341,342,343,344,345,346,347,348] The generation of Th-17 cells is antagonized by Th-1 cells and promoted by IL-23, transforming growth factor (TGF)-β, and IL-6. A great redundancy of rejection pathways is suggested by studies detecting both Th1 (IL-2, IFNγ) and Th2 (IL-4, IL-5, IL-10) cytokines in rejecting allografts.[296,349,350,351,352,353,354,355,356] Th2 can also mediate GVHD and rejection. There appears to be strain-dependent tissue specificity to the type of GVHD induced by the various Th subsets.[337,357,358] Thus, while the concept that Th2 cytokines are anti-inflammatory attracted interest in the transplantation field,[359,360,361,362,363,364,365,366,367] Th2 responses can clearly contribute to both graft rejection[359,368,369,370] and GVHD.[357,371,372,373] With a few special exceptions,[374,375,376,377] studies using various cytokine knockout mice as recipients have failed to reveal any single molecule that is essential for rejection[378,379,380,381,382,383,384] or GVHD.[371,385,386]

In GVHD, cytokines such as tumor necrosis factor (TNF)-α and IFNγ play a role. Macrophages are activated by lipopolysaccharides from the damaged gut epithelium and by IFNγ to release TNF-α, nitric oxide, and other mediators of tissue injury.[387,388,389,390,391] In certain models, TNF-α had been shown to be critical for wasting disease and intestinal GVHD.[392,393] While the relative contribution of cytokine-dependent mechanisms versus direct cell-mediated cytotoxicity to GVHD is still a matter of debate, GVHD is induced by T cells incapable of both perforin-mediated and Fas-mediated cytotoxicity, even in recipients lacking TNF receptor 1-mediated signaling,[310,394,395] demonstrating the redundancy of GVHD effector mechanisms.

Graft-Infiltrating Cells**.** Many types of cells infiltrate rejecting grafts, including CD4+ and CD8+ T cells, NK cells, and macrophages.[396,397,398,399,400,401,402,403,404] While B cells may be less prominent,[405] their presence has been associated with both acute and chronic rejection, and they are attracting increasing interest for their role not only as producers of antibody effectors of rejection, but also as APCs.[406] B cells may be located in tertiary lymphoid organs found in chronically rejecting allografts.[407]

The number of invading T cells in a graft is not necessarily correlated with the speed of rejection.[405] This finding has

suggested that certain critical elements of the graft, such as its blood vessels, are the actual site of graft destruction and, indeed, endothelialitis is an important hallmark of clinically significant rejection activity.[408]

Repertoire analysis of graft-infiltrating T cells in acutely rejecting grafts reveals marked

polyconality,[22,409,410,411,412] but only the donor-reactive CTLs show evidence of having been activated in vivo.[413] Oligoclonal dominance has been suggested in studies of tolerated rodent allografts[412] and in long-term rejected human kidneys.[414] T cells infiltrating xenografts included a broad TCR repertoire.[415,416,417] T cells mediating GVHD in the setting of multiple minor histoincompatibilities demonstrated a markedly skewed repertoire involving several different Vβ families.[418,419] Clinical studies suggest that the anti-MiHA TCR repertoire is most often polyclonal.[420]

Role of Natural Killer Cells . Although the role of NK cells in marrow rejection is well established in mice, the amount of resistance mediated by NK cells to allogeneic hematopoietic stem cells is limited and can be readily overcome by increasing the dose of donor stem cells administered.[421,422] Furthermore, a role for NK cells in resisting human allogeneic marrow engraftment has not been clearly demonstrated, although they might be expected to be important in recipients of reduced toxicity conditioning regimens. Indeed, patients with severe combined (T- and B-cell) immunodeficiency who have functional NK cells require cytotoxic conditioning to permit engraftment of haploidentical marrow, whereas those lacking NK cells do not.[423]

The ability of NK cells to be triggered by “missing self” may have utility in HCT.[424] Donor-derived NK cells with graft-versus-host reactivity due to the lack of donor class I MHC inhibitory ligands in the recipient can kill residual host leukemia cells and alloreactive cells that resist the marrow graft without causing GVHD. The alloreactive donor NK cells may also reduce susceptibility to GVHD by killing recipient APCs needed to activate donor T cells.[424,425] While striking antileukemic effects of KIR mismatching were detected in heavily conditioned patients receiving high doses of haploidentical CD34+ stem cells,[424] the effect of KIR incompatibility has been more variable in other clinical studies,[426,427,428,429,430,431] and the antitumor benefit is most evident for acute myelogenous leukemia.[432]

The possible role of NK cells in rejecting solid organ grafts is somewhat controversial. NK cells are prominent among cells infiltrating rejecting organ allografts and may be the earliest producers of inflammatory cytokines and chemokines and inducers of DC maturation.[433,434,435,436] If NK cells make an important contribution to solid organ allograft rejection under normal circumstances, they must be dependent on T cells, as mice lacking T cells are unable to reject nonhematopoietic allografts. Furthermore, whereas bone marrow allografts from class I deficient donors (β2m-/-) are subject to potent NK-mediated rejection (because these cells cannot trigger inhibitory receptors on host NK cells[437]), β2m-/- skin grafts are not rejected by β2m+ recipients.[438] NK cells have recently been reported to play a critical role in cardiac allograft rejection in CD28 knockout mice,[439,440] and NK cells can mediate a particular form of chronic allograft vasculopathy in a murine cardiac allograft model.[435] This lesion may be triggered by viral infection.[441]

Inhibitory receptors on NK cells are quite broad in their class I specificity,[442] and fully allogeneic class I MHC marrow is less susceptible to NK-mediated marrow destruction compared to class I-deficient marrow.[437,443] Because of the increased disparity of xenogeneic compared to allogeneic MHC molecules, NK cells may receive fewer inhibitory signals from xenogeneic than allogeneic cells. Indeed, transduction of HLA molecules into

porcine endothelial cells reduces NK cell-mediated xenogeneic cell adhesion and cytotoxicity.[444,445,446] However, some inhibitory receptors, such as killer cell lectin-like receptor G1, do recognize xenogeneic ligands such as e-cadherin.[447] NK cells may also be activated by interactions of activating receptors with ligands on xenogeneic cells,[448,449] of which several examples have been identified.[450,451] On balance, activating xenogeneic NK cell-target interactions are more effective than inhibitory interactions. Indeed, NK cells resist xenogeneic marrow engraftment to a greater extent than allogeneic marrow.[421,452,453,454] NK cells have also been implicated in the acute vascular rejection[455] that can destroy solid organ xenografts that have escaped hyperacute rejection (see following discussion) and in xenogeneic skin graft rejection.[456] As one mechanism by which NK cells mediate cytolysis is antibody-dependent cell-mediated cytotoxicity, it is possible that immunoglobulin G natural antibodies play a significant role in initiating NK cell-mediated rejection. NK cells also release cytokines, such as IFNγ, and TNF-α, which activate macrophages and endothelial cells, and induce inflammation.[455]

Role of Natural Killer T Cells . While NKT cells have apparent inhibitory effects on graft rejection[457,458,459,460] and GVHD,[461,462] they have also been reported to participate in rejection of tissues and bone marrow in mice.[463,464,465] The latter is due to the ability of NKT cells to activate NK cells.[465] NKT cells promote skin graft rejection by cross-primed CD8 cells via their ability to produce IFNγ.[466]

Role of Monocytes/Macrophages and Eosinophils as Effectors of Rejection . Classical delayed-type hypersensitivity (DTH) responses are thought to depend on the activation of macrophages by helper T cells through production of IFNγ. It is likely that proinflammatory cytokines and chemokines produced by activated monocytes and macrophages play a role in endothelial cell activation and lymphocyte recruitment. Additionally, activated macrophages may damage tissue through the production of toxic molecules such as nitric oxide.[467]

Macrophages play an especially important role in the rejection of cellular xenografts such as islets[468,469] in a T cell-dependent manner.[470] Macrophages cause almost immediate rejection of xenogeneic bone marrow, even in the absence of adaptive immunity.[471,472,473] Human macrophages can phagocytose porcine cells in an antibody- and complementindependent manner.[474] Additional studies have implicated macrophages in solid organ and skin xenograft rejection.[475,476,477,478,479,480] This prominent role for xenogeneic macrophages may reflect the combined ability of certain xenogeneic

receptors to activate macrophages,[481] whereas important inhibitory interactions, such as that between CD47 and its macrophage ligand SIRPα, are not effective.[482] Surprisingly, a system for monocyte-mediated recognition of allogeneic non-MHC nonself has been described.[483]

Eosinophils recruited to allografts by Th2 T-cell responses have been reported to be effectors of graft rejection in some experimental models, and eosinophils are often found clinically in rejecting allografts.[333] Th2-derived IL-4 and IL-5 recruit and activate eosinophils, which release highly cytotoxic substances from granules into the tissue.

Chronic Rejection and Chronic Graft-versus-Host Disease

Most experimental studies of rejection are performed without immunosuppression and, therefore, graft destruction usually occurs within the first several days or weeks by one of the mechanisms described previously. In clinical practice, however, the use of immunosuppression usually allows graft survival for much longer periods of time. Nonetheless, clinical survival statistics reveal that even when 1-year graft survival has been achieved, the loss of transplanted organs continues to occur at a rate of about 3% to 5% per year, and a significant proportion of this delayed or late graft failure appears to be due to immunologic mechanisms.

The term "chronic rejection" is commonly used to describe this later process of delayed graft destruction, although in kidney transplantation the Banff classification schema has proposed to replace this term with interstitial fibrosis and tubular atrophy.[484] As immunosuppressive reagents have become more effective at controlling acute rejection, chronic rejection has emerged as one of the most important problems in clinical practice. Indeed, while there has been ongoing improvement over the past 30 years in the 1-year graft survival rates for kidney transplants, the halflife for organs that have survived for 1 year has not changed significantly over that entire period of time; as a result of this ongoing loss, only about 50% of transplants are still functioning 10 years later.

Although almost every type of organ transplant suffers from deterioration in function over time, the pathologic manifestations are different in each case. Kidney biopsies tend to show interstitial fibrosis along with arterial narrowing from hyalinization of the vessels—hence the terminology interstitial fibrosis and tubular atrophy. In the heart, the process is manifested principally as a diffuse myointimal hyperplasia, proceeding to fibrosis of the coronary arteries that has often been referred to as "accelerated atherosclerosis" or "transplant arteriosclerosis." Chronic rejection in lung transplants primarily affects the bronchioles with progressive narrowing of these structures and is referred to as "bronchiolitis obliterans." The liver may be the one type of organ transplant that is relatively resistant to chronic rejection, but the progressive destruction of bile ducts referred to as the "vanishing bile duct syndrome" may be another manifestation of this process.

Some of the causes of chronic graft destruction may not be immunologic in origin.[485,486] Analysis of sequential kidney transplant biopsies suggests that chronic rejection represents cumulative and incremental damage to the graft from time-dependent nonimmunologic and immunologic causes.[487,488] Potential nonimmunologic factors that have been considered to contribute to the development of chronic rejection include the initial ischemic insult, the reduced mass of transplanted tissue (especially in the case of kidney transplants leading to hyperfiltration injury), the denervation of the transplanted organ, the hyperlipidemia and hypertension associated with immunosuppressive drugs, the immunosuppressive drugs themselves, and chronic viral injury, amongst others. Nonetheless, while these factors undoubtedly contribute to the process, there is a marked difference in survival between syngeneic and allogeneic transplants in experimental models. Thus, there is almost certainly an important immunologic component in most cases of chronic rejection.

Several important observations regarding the pathogenesis of chronic rejection have emerged from clinical practice, particularly the analysis of biopsy samples. In kidney transplants, two distinctive phases of injury of chronic allograft nephropathy have been described.[487] Previous studies have suggested that there is a high correlation between the onset of chronic rejection and a history of early acute and subclincial rejection

episodes.[489,490] Analysis of protocol biopsies has revealed that the onset of mild chronic rejection by 1 year after kidney transplantation is associated with an initial phase of early tubulointerstitial damage from ischemic injury that occurs before severe rejection is detected. Beyond 1 year, a later phase of chronic allograft nephropathy was characterized by microvascular and glomerular injury.[487] Importantly for long-term outcomes, the clinical data show that the process of chronic rejection is usually refractory to increases in immunosuppressive therapy, in contrast to acute rejection episodes that almost always respond to treatment. The development of chronic rejection has also frequently been associated with the presence of antidonor antibodies,[491,492] and the deposition of complement component C4d in the allograft.

Taken together, these clinical observations have suggested to some that chronic rejection is the result of donorspecific alloantibody production.[493] Moreover, there are also now data emerging to suggest that components of the innate immune system such as NK cells can also contribute,[494] in some cases in conjunction with alloantibody.[495] All of these suggestions may be correct, but neither the logic nor the evidence fully supports the exclusive involvement of one mechanism. The relationship between alloantibody, C4d deposition, and neointimal fibrosis is complex.[496] In the first place, alloantibody production often reflects activation of indirect pathway T cells (see previous discussion), and hence it might equally well be a marker for other rejection mechanisms as opposed to a cause of chronic rejection. Moreover, the presence of alloantibody and C4d may be transient, and there are clear examples of chronic changes in the graft in their absence.[496] In addition, early rejection episodes probably reflect primarily the degree of antidonor immunoreactivity, and there is no proven direct link with later deterioration of graft function. Therefore, even if sufficient

immunosuppression were given to prevent acute rejection, chronic rejection might still occur when the levels of immunosuppression are reduced over the long term, even if acute rejection had never occurred. Finally, experimental studies have suggested that the mechanisms of chronic rejection are not absolutely dependent on either antibody formation or on the occurrence of acute rejection episodes.

The uncertainties that arise from the interpretation of the clinical data make it important to develop experimental models for studying the mechanisms of chronic rejection. It is difficult in the laboratory, however, to mimic a process that may take 5 or 10 years to develop in patients treated with immunosuppressive drugs. Thus, the effort to study chronic rejection experimentally has depended on surrogate short-term pathologic markers that are thought to predict the long-term changes of chronic rejection. In particular, these studies have concentrated on the development of the myointimal proliferation that is thought to be the precursor of the chronic vascular changes typically observed in patients. In rodents, pigs, and primates, this has often been done with grafts after an initial period of immunosuppression that prevents acute rejection.[497,498,499] All of these experimental studies are subject to the caveat that the surrogate pathologic lesion occurs much earlier than the typical changes of chronic rejection in patients. Thus, the process being studied experimentally may not be the same as the clinical process.

Pathologic Manifestations of Experimental Chronic Rejection

The typical pathologic features of the experimental lesion associated with chronic rejection are shown in Figure 46.10.[500] The marked narrowing of the vascular lumen is caused by the

substantial proliferation of endothelial and then smooth muscle cells that can be host-derived.[501] Associated with this proliferation is progressive destruction of the media. In time, the cellular proliferation becomes less pronounced and is replaced by concentric fibrosis that narrows the vascular lumen. Immunohistologic staining indicates that there is increased expression of several adhesion molecules,[500] intracellular proteins such as vimentin,[502,503] and easily detectable levels of several molecules, including nitric oxide synthase,[504] acidic fibroblast growth factor, insulin-like growth factor, IFNγ,[505] and endothelin,[506] each of which may play a role. The abnormal expression of self-molecules in allografts undergoing chronic damage can lead to autoantibody formation.[507] Ultimately, the ischemia resulting from vascular occlusion results in fibrosis in the parenchyma of the organ and consequent organ dysfunction.[508] In the case of the lung or the liver, chronic injury may cause changes most prominently in the bronchioles or the bile ducts, but this is also associated with arterial lumen loss, which may be the primary lesion causing bronchiolitis obliterans or bile duct fibrosis, respectively.[508]

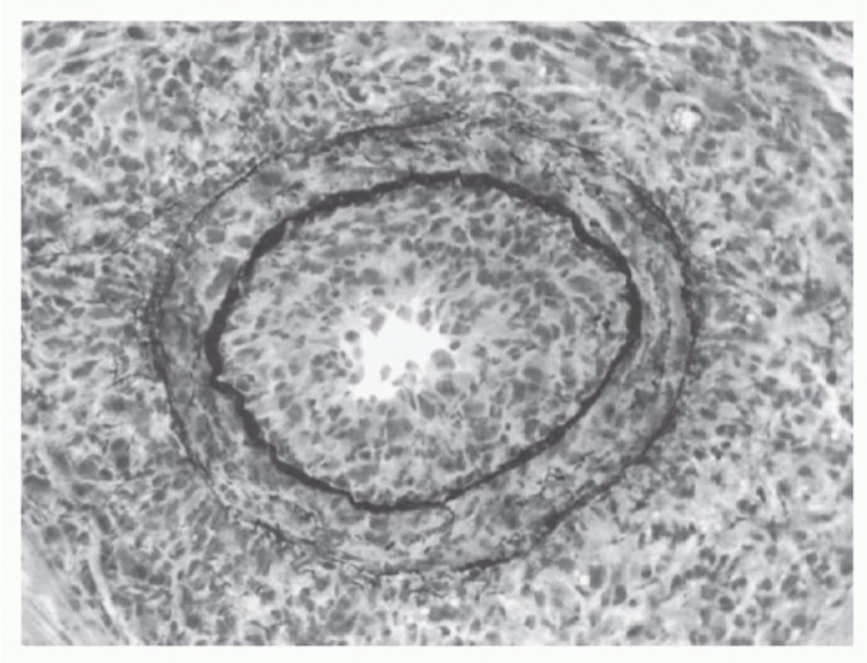

FIG. 46.10. Histology of Chronic Rejection.

Immunologic Mechanisms of Chronic Rejection

Rejection requires a dialogue between the innate and adaptive immune systems.[509] Innate immunity is most likely involved at the outset of the process that leads to the development of chronic rejection, and there is evidence to suggest that NK cells[494,510] and complement activation can be involved.[511] At the level of the adaptive response, because it is assumed that stimulation of direct pathway T cells is likely to diminish over time as donor APCs are replaced by recipient APCs (see previous discussion), it is commonly assumed that the predominant immune response that causes chronic rejection occurs through the indirect pathway.[512]

Studies in pigs have suggested that the vascular changes of chronic rejection are more apt to develop when there are class I antigenic disparities than when there are only class II disparities and that the development of the lesion depends especially on CD8+ T cells.[513] In mouse models, in contrast, there is evidence that either CD4+ or CD8+ T cells can produce the lesion and that either class I or class II antigenic disparities are sufficient to stimulate chronic rejection.[514] The finding that class II antigenic disparities are themselves sufficient to induce this pathology is consistent with the observation of class II MHC expression on the vascular endothelium and medial smooth muscle cells of mouse cardiac allografts with these vascular lesions.[515] Because class II MHC is not constitutively expressed by mouse vascular endothelial cells, indirect recognition of donor class II transferred from passenger leukocytes may be responsible for inducing an inflammatory response that leads to subsequent upregulation of class II on the donor vascular endothelium. In keeping with the prediction of many clinical studies, adoptive transfer experiments into mice with severe combined immunodeficiency have shown that alloantibodies in the absence of T cells can induce the typical pathologic vascular changes, and lesions can develop in T-cell-deficient mice.[516] However, T cells without B cells have also been shown to cause the lesion, although there may be somewhat less tendency to progress to end-stage fibrosis.[517]

Several studies have indicated that the induction of donor-specific tolerance can prevent the development of the vascular changes of chronic rejection, although not all of the short-term manipulations that have been effective in preventing acute rejection have necessarily prevented the later onset of chronic rejection. Remarkably, mice rendered

tolerant by neonatal injection of donor splenocytes, or by the induction of high levels of lasting, multilineage mixed chimerism with demonstrated central deletion of donorreactive T cells and permanent acceptance of donor-specific skin grafts, demonstrate graft vasculopathy in donor cardiac allografts.[510] Thus in the complete absence of antidonor T-cell reactivity, other cell types such as NK cells may induce these types of lesions in cardiac allografts. In addition, T-cell recognition of cardiac-specific antigens presented by donor MHC and not shared by donor hematopoietic cells could play a possible role in the development of these lesions in immunocompetent, tolerant mice.[494]

From these data, it seems likely that multiple immunologic mechanisms may be capable of creating the graft arteriosclerotic lesions that are characteristic of chronic rejection, and that T-cell alloreactivity is not essential for their induction. Whether there is a critical final common mediator involved in all of these pathways is not currently known. However, IFNγ has been shown to play an important role in the development of lesions in several models,[518,519] and signal transducer and activator of transcription (STAT)4-deficient mice, which do not respond to IL-12 and therefore cannot generate Th1 responses, show markedly reduced severity of graft vasculopathy compared to wild-type mice.[520] TGF-β has been shown to attenuate the lesions, but has also been detected within the lesions and implicated in the development of fibrosis.[521]

Chronic Graft-versus-Host Disease

cGVHD is the most common and severe complication among patients surviving for more than 100 days after allogeneic BMT. Clinically, acute and cGVHD can be distinguished on the basis of the time of onset, clinical manifestations, and distinct pathobiologic mechanisms. Acute GVHD usually occurs within 2 to 6 weeks following allogeneic BMT and primarily

affects the skin, liver, and the gastrointestinal tract with T-cell infiltration of the epithelia of the skin, mucous membranes, bile ducts, and gut. However, acute GVHD has been noted to occur later in recent protocols involving nonmyeloablative conditioning for HCT. In contrast, cGVHD involves a wider range of organs and clinical manifestations include scleroderma, liver failure, immune complex disease, glomerulonephritis, and autoantibody formation.

The pathogenesis of cGVHD, like chronic rejection, is poorly understood. The disease involves T-cell responses to alloantigens or autoantigens.[522] Because most BMT is performed between HLA-identical or closely HLA-matched pairs, alloreactivity may be directed against miHAs presented by shared MHC molecules, or against MHC alloantigens when present. T cells developing de novo in a recipient thymus that is damaged due to GVHD may result in the emergence of autoreactive T cells into the peripheral repertoire. The injury to target organs is poorly understood, but may involve inflammatory cytokines and fibrosis, as well as B-cell activation and production of autoantibodies.

The main risk factors for the onset of cGVHD are HLA disparity, donor and patient age and sex, source of progenitor cells, graft composition and previous acute GVHD. cGVHD can be treated providing it is identified sufficiently early after initiation. Even with treatment, extensive skin involvement, thrombocytopenia, and progression are poor prognostic factors.

PHYSIOLOGIC INTERACTIONS THAT MODULATE GRAFT REJECTION AND GRAFT-VERSUS-HOST DISEASE

Although graft rejection and GVHD often involve exceptionally strong immune responses, these responses are still accompanied by downregulatory components that can be manipulated to promote graft survival. While many of these manipulations are described in the section on tolerance, some of the regulatory components of the rejection response are briefly described here.

Downregulating Signals Following T-Cell Activation

Interactions between Fas and Fas ligand (FasL), which is upregulated during rejection responses, can mitigate GVHD and graft rejection by killing activated T cells and APCs.[306] FasL-deficient recipients are more susceptible than normal mice to the development of GVHD,[523] and FasL can promote resistance to rejection of tissues transplanted to some “privileged sites,” such as the testis or the anterior chamber of the eye.[524] However, forced overexpression of FasL causes a nonspecific inflammatory syndrome associated with prominent neutrophil infiltration[525] and can promote graft destruction.[526,527] Overexpression of FasL has, however, been reported to promote survival of heart allografts in recipients of FasL-expressing donor-specific transfusions (DSTs),[528] and of bone marrow[529] and islet[530] allografts.

CTL antigen (CTLA)4 helps maintain self-tolerance, as evidenced by the T-cell lymphoproliferative autoimmune syndrome that develops in CTLA4 knockout mice.[531,532] CTLA4 has been shown to play a role in T-cell tolerance in many systems.[384,533,534,535,536,537,538,539,540,541,542,543,544,545,546] Blockade of CTLA4 accelerates cardiac allograft rejection[547] and increases GVHD.[548] PD1, an additional downregulatory molecule expressed by activated T cells that recognizes the B7 family members PDL-1 and PDL-2, also mitigates graft rejection[549,550,551,552,553,554] and GVHD.[555,556,557] Interaction of one of the PD1 ligands, PD-L1, with its alternative

receptor B7.1, also mitigates graft rejection.[558] While PD1 plays an important modulatory role in the presence of extensive antigenic disparities between murine heart graft donors and recipients, the B- and T-lymphocyte attenuator (BTLA)-herpesvirus entry mediator (HVEM) inhibitory pathway predominates in the presence of more restricted antigenic disparities.[559] BTLA-HVEM interactions may also control GVHD,[560] but the opposing effects of BTLA and HVEM expressed by T cells on T-cell activation complicate interpretation of experiments, which have produced conflicting results with respect to GVHD[560,561] and islet allograft survival.[562,563]

Immunomodulatory Effects of Cytokines

There is considerable evidence of roles for IL-10 and TGF-β in downmodulating graft rejection[564,565,566] and GVHD,[372,567,568,569,570] and these cytokines may have therapeutic utility.[571,572,573,574,575] These

activities may reflect the important role of these cytokines in regulatory T (T_{reg})-cell and B-cell generation and function. On the other hand, IL-10 can enhance cytolytic mechanisms of islet graft rejection,[576] and high doses can accelerate GVHD.[372] Moreover, TGF-β is an important mediator of fibrotic pathologies in both chronic rejection and GVHD.[577,578] TGF-β, in concert with proinflammatory cytokines such as IL-1 and IL-6, also promotes the development of proinflammatory Th-17 cells.[579]

The inflammatory condition that develops in IL-2 knockout mice is clear evidence of the important immunomodulatory role of this cytokine, and studies in several graft rejection[580] and GVHD[581] models have confirmed such a role in transplantation. Much of this effect is due to the important role of IL-2 in survival and expansion of T_{reg} cells,[582,583] as is discussed elsewhere in this chapter. IL-2 can also promote activation-induced cell death of alloreactive CD8 cells.[582,584]

While IFNγ can also promote graft rejection, rejection is rapid or even accelerated in IFNγ knockout mice.[383,564,585,586,587] IFNγ has also been shown to play a downregulatory role in GVHD.[371,385,588] IFNγ has antiproliferative effects on T cells,[384,587] increases activation-induced cell death via the Fas/FasL pathway,[589,590,591] upregulates nitric oxide production,[592,593,594,595] and is necessary for T_{reg}-cell function in certain conditions.[596,597,598] Consistently, the major cytokines that induce IFNγ, IL-12 and IL-18, can inhibit graft rejection[382,384,599] and GVHD.[385,600,601] IL-12 and IL-18 act in an IFNγ and Fas-FasL-dependent manner,[385,601,602] which preserves or enhances GVL effects.[588,603,604,605,606] IFNγ promotes the GVL effect of CD8 T cells,[385,607,608] apparently by promoting lymphohematopoietic grat-versus-host responses while inhibiting tissue GVHD,[609] perhaps due in part to increased PDL1 expression by APCs,[610] promotion of donor T_{reg}-cell expansion,[610] and reduced Th17 differentiation.[337]

Despite interest in the notion that Th2 cytokines are antiinflammatory and may suppress rejection and GVHD,[359,360,361,362,363,364,365,366,367] IL-4 deficiency does not

accelerate graft rejection[564,611] and can actually downmodulate GVHD.[371] However, there clearly are situations in which an immunomodulatory effect is achieved by Th2 cytokines. For example, the use of total lymphoid irradiation can alter the balance between NKT cells and conventional T cells in BMT recipients, and IL-4 production by enriched recipient NKT cells downmodulates GVHD.[612] This approach, which also apparently involves T_{reg} cell enrichment when combined with antithymocyte serum,[613] has recently been extended to clinical trials of HLA-identical HCT for treatment of hematologic malignancies and renal allograft tolerance induction.[614,615,616,617]

The Presence of the Transplanted Organ

Vascularized organ allografts may be accepted spontaneously[618,619,620,621] or with a short course of immunosuppression in rodents or pigs.[621,622,623,624,625,626,627,628,629] The long survival of these transplanted organs can prevent rejection of other allografts from the same donor.[618,630,631] In clinical transplantation, long survival of a transplanted organ may diminish the rejection response, as much less immunosuppression is required late after transplantation than in the early period. Studies in transiently chimeric monkeys and patients achieving tolerance with HLA-mismatched combined kidney and BMT strongly implicate a role for the kidney itself in promoting tolerance.[632,633,634,635,636] T_{reg} cells have been implicated in many of these models (see following discussion). The capacity of transplanted organs to regulate their own survival is often confused with the capacity of a treatment to induce tolerance. For example, many transient immunosuppressive regimens achieve vascularized allograft survival in rodents, but the role of the immunosuppression is to allow a strong T_{reg} cell response induced by the graft to predominate under the controlled experimental conditions. These conditions do not usually apply to human transplantation, thus explaining the failure to translate the many tolerance regimens that succeed in rodents into clinical practice.

Role of Graft and Tissue Injury

Graft injury, such as that associated with ischemia-reperfusion and host tissue injury induced by conditioning therapy in the case of HCT, play an important role in promoting graft rejection and GVHD, respectively. Local inflammatory processes activate innate and consequently adaptive immunity and T-cell activation. Inflammation plays an important role in promoting APC migration from tissues to lymph nodes[637,638,639,640,641] and also promotes trafficking of activated T cells into tissues, as is illustrated dramatically in HCT models. Administration of large numbers of nontolerant donor lymphocytes to established mixed bone marrow chimeras (ie, animals not recently treated with conditioning therapy) leads to a graft-versus-host response that attacks only lymphohematopoietic tissues and does not cause GVHD, a disease of epithelial tissues such as skin, intestines, and liver.[642,643] In contrast, similar numbers of T cells cause rapidly lethal, severe GVHD in freshly irradiated hosts.[642,644] Conditioning rapidly induces production of chemokines in the GVHD target tissues, promoting immigration of T cells that then elicit a further cascade of chemokines that amplifies the response.[645] Upregulated adhesion molecules also promote leukocyte infiltration through the microvasculature of these tissues. Lethal total body irradiation (TBI) and cyclophosphamide, for example, upregulate the proinflammatory cytokines IL-1, IL-6 and TNF-α,[646,647,648] which can upregulate endothelial cell E-selectin, P-selectin, ICAM-1, and

vascular cell adhesion molecule-1.[649,650] In the absence of such host target tissue inflammation, mature, activated graft-versus-host-reactive effector T cells are unable to traffic into skin and induce injury.[644] Provision of a local tolllike receptor (TLR) stimulus promotes the entry of such cells into the skin and induces localized GVHD,[644] indicating that tissue inflammation provides a critical checkpoint for T-cell recruitment to GVHD target tissues. A systemic TLR stimulus in this setting promotes severe, multiorgan GVHD.[644] GVHD can also occur when very large numbers of nontolerant parental T cells are administered to genetically tolerant F1 hosts,[651] indicating that, in the presence of sufficiently powerful graft-versus-host responses, the need for tissue inflammation to induce GVHD can be bypassed, possibly due to inflammation induced by high systemic cytokine levels.

All forms of organ and tissue transplantation involve ischemic and traumatic injury to the donor tissue, which may be one of the reasons that rejection episodes occur most frequently early after transplantation. The surgical trauma associated with transplantation is associated with very early production of chemokines in the graft,[433,652] promoting infiltration of NK cells[433] and neutrophils,[653] which in turn perpetuate inflammation that promotes subsequent T-cell infiltration.[654] At least partly due to the influence of these cells of the innate immune system,[433] chemokines are produced before T-cell infiltration is seen. IFNγ, whose early production may require CD8 T-cell activation,[655] also activates macrophages to become effective APCs and release chemokines.[655,656,657] These phenomena, along with microbial exposures that drive innate immunity, contribute to “danger” signals that promote graft rejection.[658] Nevertheless, skin and cardiac allografts that are allowed to heal before being exposed to alloreactive T cells are rapidly rejected if there is sufficient antigenic disparity between donor and host.[659,660] Similarly, patients with long-standing allografts are rarely able to terminate immunosuppressive therapy without rejection. Therefore, “danger” signals are not a critical requirement for graft rejection, and it is better to picture the antigenic disparity and the recipient's immune responsiveness as the dominant features controlling graft rejection, while danger signals may influence the timing, intensity, or character of the rejection response.

Role of the Innate Immune System

The innate immune system comprises a group of cells and molecules (Table 46.5) that provide a first line of defense against pathogens and which also play an important role in allograft rejection. Primary adaptive immune system responses that rely on the activation and expansion of antigenspecific T and B cells take several days to reach maturity. In contrast, the innate immune system can be considered as a “preformed” defense mechanism that is immediately available to defend the host until either the dangerous stimulus is cleared or the adaptive immune system is able to mount an antigen-specific response.[661] Clearly, this is a somewhat simplistic view; while many components of the innate immune system can be recruited very quickly after transplantation, their activity can be amplified after activation.

TABLE 46.5 Components of the Innate Immune System

Cell	Primary function

Macrophage/neutrophil	Phagocytosis, opsonisation, antigen presentation Release of inflammatory mediators
Dendritic cell	Antigen uptake and presentation to lymphocytes
Natural killer cell	Release of cytokines Cytotoxic to virally infected or mutated cells
Eosinophil	Release of inflammatory mediators Killing of antibody-coated entities
Complement	Opsonisation, target cell lysis, and chemoattraction

The physical process of graft retrieval and implantation generates signals within the graft and the recipient that trigger rejection. The concept of “danger” triggering an immune response has evolved as an idea over many years.[170,661] Pattern recognition receptors exist to detect the unwanted presence of bacterial or viral pathogen-associated molecular patterns, but after transplantation the TLRs that form part of the pattern recognition receptor family can also be used to detect the molecules produced as a result of implantation of the graft, so called damage-associated molecular patterns (DAMPS). These signals include heat shock proteins, reactive oxygen species, high mobility group protein B1, complement breakdown products, nucleic acids (deoxyribonucleic acid and RNA), mitochondrial components, and molecules associated with tissue fibrosis that activate cells of the innate immune system via TLR ligation.

The immune system monitors the health of cells and responds to ones that have been injured and killed. Cell death is an inevitable consequence of the ischemia and reperfusion injury that is caused by organ and tissue retrieval. Dying cells expose intracellular DAMPs that can be recognized by components of the innate immune system.[662] The role of DAMPs may vary depending on the type of transplant performed and the degree of injury resulting from cell isolation or organ retrieval. Some DAMPs may be expressed in a tissue-restricted manner (eg, one class of DAMPs called alarmins include molecules such as β-defensins that are expressed primarily by leukocytes and therefore may be more relevant after hematopoietic stem cell transplantation or BMT). In addition, the location or type of injury may contribute to the outcome of dying cells triggering a response. Thus, the contribution of different DAMPs to triggering the innate immune system may vary with different types of transplant.

Macrophages and other phagocytic cells can ingest dying cells and necrotic tissue; when activated, they release cytokines such as TNF-α, IL-1, and IL-6, which all contribute to the local inflammatory environment. Production of active IL-1β requires proteolytic cleavage of the inactive form, pro-IL-1, a process linked to a multiprotein complex called an inflammasome that acts as a platform for caspase 1, the cysteine protease involved in the proteolytic maturation and secretion of IL-1β.[663] A number of different inflammasomes exist, but the nucleotide oligomerization domain-like receptor protein 3 inflammasome is the best studied to date. IL-1β can be highly deleterious to the tissue in which is produced; therefore, inflammasome activity is tightly controlled. As a consequence of these events, the early infiltration of macrophages into a graft at the onset of rejection has been suggested to be a

poor prognostic sign for transplant survival. Macrophage colony-stimulating factor, produced by tubular and mesangial cells, promotes macrophage infiltration and proliferation, and may play a pathogenic role in acute rejection.

Damaged tissue can also trigger complement activation in the absence as well as the presence of antibody; complement has been demonstrated to contribute to ischemia reperfusion

injury.[664] Activated complement components constitute a proteolytic cascade that generates a range of effector molecules.[170] The anaphylatoxins C5a and C3a are chemoattractant molecules that assist leukocytes to home to the graft while other soluble mediators are able to opsonise cells, targeting them for destruction by phagocytes.[665] Recognition of C3b, C4b, or their fragments covalently bound to target cells by complement receptors on the surface of leucocytes facilitates antigen presentation and T-and B-cell activation.[666,667] Generation of the terminal components of the complement cascade (C5b-9) results in formation of the membrane attack complex within the target cell membrane and initiation of target cell lysis. This has been demonstrated to play an important role in ischemia reperfusion injury.[664] In addition to the potential of the damaged tissue itself to activate complement, there is also evidence that natural immunoglobulin M antibody can trigger complement activation via both the classical and mannose-binding lectin pathway.[668] Studies in muscle reperfusion models initially identified natural immunoglobulin M as a major initiator of pathology through the activation of the complement system and recruitment of inflammatory cells. When the repertoire of natural immunoglobulin M antibodies was altered, significant protection of the myocardial tissue was observed with only limited apoptosis of cardiomyocytes and decreased neutrophil infiltration compared to when natural antibody was present.[669] As mentioned previously, there is also increasing evidence that complement can influence graft outcomes, contributing to the development of acute and/or chronic rejection, either directly or through antibodydependent mechanisms.[511,670]

NK cells are innate immune mediators that express cell surface receptors, including activating receptors that bind to widely expressed carbohydrate residues on self-cells and inhibitory receptors that bind self-MHC class I molecules. Activating NK cell receptors including NKG2D recognize natural stress signal ligands, whereas the inhibitory receptors include the CD94-NKG2A complex, KIR family in humans, and Ly49 family in mice. The possible role of NK cells in graft rejection is discussed previously. Absence of an appropriate MHC class I ligand on an allogeneic cell informs the NK cell that the allogeneic cell should be killed. Some malignant or virally infected cells downregulate MHC class I expression or express altered class I molecules as a strategy to evade CD8+ T-cell cytotoxicity. As a result, they are unable to stimulate inhibitory receptors and are vulnerable to NK cell killing. Thus, NK cells could contribute to tissue damage following cell and solid organ transplantation. While the role of NK cells in rejecting bone marrow (at least in mice) is clearer than for solid organ transplantation,[671] NK cells can have a marked and lasting impact in this setting,[672] particularly with a form of chronic cardiac graft vasculopathy in mice.[494] NK cells likely contribute to acute rejection in certain donor-recipient combinations where, even if they are not the major drivers of the responses, they have a significant impact by secreting IFNγ. Nevertheless the precise role of NK cells requires further elucidation as NK cells have also been shown to promote tolerance induction (see the following discussion) by killing donor APCs.[673]

Increasing evidence demonstrates the important role that components of the innate immune system play in activating the adaptive immune system. In particular, ligation of TLRs on DCs induces maturation, as defined by upregulation of costimulatory molecules and MHC class II, enhancing their ability to act as a bridge between the innate and adaptive immune systems.[674] TLRs are critical sentinels of the innate immune system and contribute to the early response after transplantation. Using HY grafts, Goldstein and colleagues showed that rejection was not triggered in the absence of MyD88 TLR signaling pathway due to the migration of a reduced number of mature DCs to the draining lymph nodes that resulted in the impaired generation of anti-graft-reactive T cells and Th1 immunity.[675] Further studies using fully allogeneic grafts have shown that multiple TLR signaling pathways contribute to the initiation of rejection.[676,677] There are also data indicating that TLR2, TLR4, and/or TLR9 can play a role in the ischemia-reperfusion injury in heart, brain, liver, intestine, and kidney.[678,679] Haptoglobin, a serum acute-phase reactant protein released by necrotic donor skin grafts, has recently been shown to promote graft rejection via a MyD88-dependent pathway.[680] As well as stimulating rejection, TLR signaling and activation of the innate immune system may also prevent or increase the difficulty of induction of tolerance to alloantigens[681] (see following discussion).

MANIPULATIONS TO PREVENT GRAFT REJECTION

Methods of preventing graft rejection can be divided into nonspecific immunosuppression, which reduces the overall immunocompetence of the recipient, and specific suppression of the response to the graft, leaving the immune system otherwise intact. The latter, known as tolerance, is a major goal of transplantation research.

Nonspecific Techniques

Immunosuppressive Medications

Reviewing the pharmacology of the nonspecific immunosuppressive drugs commonly used in clinical transplantation is beyond the scope of this chapter. It is important to note, however, that most of the major advances in clinical transplantation that have occurred over the past four decades have been made possible largely because of these agents. Most recipients of allogeneic organs today receive exogenous immunosuppression in the form of combination therapy with several drugs (Table 46.6), including steroids, a calcineurin inhibitor (cyclosporine or tacrolimus), and an antimetabolite (eg, azathioprine or mycophenolate mofetil). Newer drugs, including sirolimus and several other agents tested as subsitutes for the calcineurin inhibitors in some regimens and both steroid-free and single-drug regimens, are being tested,[682,683,684,685] as are strategies incorporating immunosuppressive antibodies.[686] In general terms, both standard and experimental immunosuppressive drugs suppress immune responses either by depleting immune cells, by blocking costimulation, or by inhibiting lymphocyte gene transcription (eg, cyclosporine, tacrolimus), cytokine

signal transduction (eg, rapamycin), or nucleotide synthesis (eg, azathioprine, mycophenolate mofetil).

TABLE 46.6 Immunosuppressive Medications

Type of Drug	Examples	Mode of Action
Antirejection medication		
First generation	Prednisone	Anti-inflammatory; inhibition of cytokines
	Azathioprine	Antimetabolite (purine analog)
Second generation	Cyclosporin	Calcineurin inhibition (downregulates IL-2)
	FK-506	Calcineurin inhibition (downregulates IL-2)
	MMF	Inhibitor of nucleotide synthesis
	Rapamycin	Inhibitor of signal transduction
Polyclonal antibodies	ATG	T-cell depletion
	Thymoglobulin	T-cell depletion
Monoclonal antibodies	Anti-CD3	T-cell sequestration
	Anti-CD25	Activated T cells
	Anti-CD2	T-cell depletion and costimulation blockade
	Anti-CD154	Costimulation blockade
		Costimulation

	CTLA4-Ig	blockade
	Anti-CD40	Costimulation blockade

ATG, anti-thymocyte globulin; CD, cluster of differentiation; CTLA, cytotoxic T-lymphocyte antigen; MMF, mycophenolate mofetil; IL, interleukin.

Anti-T-Cell Antibodies

The first therapeutic anti-T-cell antibodies used clinically were heterologous antisera prepared against human lymphocytes or thymocytes. These powerful immunosuppressants are still used today, both as induction agents and for the treatment of rejection episodes. Their major side effects include serum sickness and infectious complications. A variety of monoclonal antibodies (mAbs) are also being used actively in clinical transplantation or are being tested in clinical trials[687] (see Table 46.6). These include OKT3, Campath-1, and numerous other, newer mAbs. In general, these antibodies are now either "humanized" from the mouse versions by genetic engineering or prepared in mice carrying human immunoglobulin genes[688] to avoid immunization of the recipients.

mAbs to the α chain of the IL-2 receptor (CD25) have been used in an effort to achieve greater antigen specificity with anti-T-cell antibodies, based on the hypothesis that these mAbs might selectively eliminate only those T cells activated by the transplant. Clinical results using humanized anti-CD25 mAbs have so far demonstrated immunosuppression, but not tolerance induction. Some mAbs have also been used in attempts to block the effector mechanism of graft rejection, including anti-ICAM antibodies and anti-TNF antibodies. Agents that suppress T-cell costimulation, including anti-CD154, anti-CD40, and CTLA4-immunoglobulin, have shown promise,[687] and will be discussed further with regard to their potential value in tolerance induction protocols (see following discussion). CTLA4-immunoglobulin (belatacept) in particular, has recently been reported to have better cardiovascular and metabolic risk profiles than a calcineurin inhibitor in the control arm of a recent clinical trial.[689]

While mAb therapy has been extremely effective, several problems still exist, including toxicities related to the release of cytokines and immunogenicity, leading to antibody production against both constant region and idiotypic determinants of the antibody molecules.

Donor-Specific Tolerance Induction

The Need for Tolerance-Inducing Regimens

Immune tolerance denotes a state in which donor-specific nonresponsiveness is maintained without immunosuppressive agents. For the purpose of this discussion, we will define tolerance as acceptance of a donor organ by an otherwise intact immune system, and will qualify the term as "systemic" when it applies to the entire immune system so that the immune system fails to respond to cells or other grafts from the same donor. Transplantation tolerance is a desirable goal for three major reasons. First, while improvements in immunosuppressive therapy have dramatically increased the success of clinical organ transplantation, these drugs are associated with increased risks of infection and malignancy as well as metabolic

and organ toxicities that can result in additional organ failure. Second, despite improved immunosuppression, chronic rejection still contributes to constantly down-sloping long-term survival curves for organ allografts. Third, a critical shortage of allogeneic organs has increased interest in the use of other species as organ and tissue sources. However, immune barriers to xenografts are greater than those to allografts, and the induction of both B-cell and T-cell tolerance may be essential to the ultimate success of xenotransplantation.

Mechanisms of Transplantation Tolerance

Other chapters in this book describe the mechanisms by which tolerance to self-antigens is achieved (see Chapters 13 and 32). Rather than reiterate these in detail here, we will mention each and provide examples in which they have been implicated in transplantation. Mechanisms for inducing T- and B-cell tolerance include deletion, anergy, and suppression. In addition, a graft may simply be ignored by recipient lymphocytes but in most transplant settings this is unlikely

to be a reliable mechanism for sustaining tolerance. The thymus is the central organ for T-cell development. Induction of tolerance among developing thymocytes is referred to as “central,” as distinguished from the “peripheral” tolerance that may develop among already mature T cells when they encounter antigen in the peripheral tissues. The marrow is the central organ for B-cell development and, as for T cells, B-cell tolerance may also be thought of as central or peripheral.

Clonal Deletion

B Cells. When a developing B cell with autoreactivity encounters self-antigen, it undergoes developmental arrest. Recombination activating gene-dependent light chain receptor editing then takes place. If this results in the formation of a nonautoreactive immunoglobulin receptor, the B-cell survives; if not, the B cell undergoes apoptosis.[690] B cells are susceptible to tolerance at particular stages of development upon recognition of membrane-bound antigen.[691] Several checkpoints for elimination of autoreactive B cells during development in the marrow and after migration to the periphery have been identified.[692] Deletion, rather than receptor editing, eliminates autoreactive peripheral B cells,[693] which may also become anergized, depending on the nature of the antigen.[694,695]

In the context of transplantation, B-cell responses to allogeneic MHC molecules are largely T cell-dependent, so an absence of alloantibodies may not necessarily reflect intrinsic B-cell tolerance; an absence of T-cell help would produce a similar outcome. However, MHC-alloreactive B cells may be intrinsically tolerized by encounter with antigens in the absence of T-cell help, apparently by a deletional mechanism.[696,697]

Elimination of natural antibody (eg, xenoantibody) responses must target B cells in addition to T cells, as these antibodies are produced even in the absence of T cells.[698] Nevertheless, T-cell responses can clearly augment these antibody responses.[699,700,701] Cells of the B-1 subset, which produce natural antibodies responsible for xenograft hyperacute rejection,[702] can be deleted when their receptors are crosslinked by cell-bound antigen.[703] Initial anergy followed by deletion of preexisiting B cells and deletion/receptor editing is responsible for the long-term tolerance of natural antibody-producing B-1 cells in mice given hematopoietic cells expressing an antigen for which natural antibody-producing cells preexist in the

recipient.[153,704,705]

T Cells. Central T-cell tolerance involves deletion of developing thymocytes that recognize self-antigens presented by hematopoietic cells and thymic epithelial cells. In addition, antigens presented only by nonhematopoietic thymic stromal cells can induce T-cell anergy[706] or drive T_{reg}-cell differentiation.[707,708,709] Several hematopoietic cell types, including DCs,[710] B cells,[711] and thymocytes,[712,713] as well as thymic stromal cells,[714,715] can induce intrathymic deletion. Medullary epithelial cells producing tissue-specific antigens can mediate thymocyte deletion by presenting antigens directly or transferring them to DCs.[716] Transplantation tolerance induced by intrathymic deletion should be very reliable, as donor-reactive T cells would be largely absent. Durable mixed hematopoietic chimerism achieved with regimens that completely deplete preexisting T cells in the thymus and periphery results in donor- and recipient-specific tolerance through this central deletion mechanism.[717,718]

Mature peripheral T cells can also be deleted.[719,720,721,722,723,724] Peripheral CD8 cells may undergo "exhaustion" in the presence of persistent antigen.[725,726] Persistent antigen encountered on recipient nonhematopoietic cells leads to deletion and exhaustion of graft-versus-host-reactive CTLs following HCT.[556,727,728] Peripheral deletion of donor-reactive T cells has been demonstrated in mice receiving DSTs and/or BMT with costimulatory blockade,[533,729,730,731,732,733,734] and has been implicated in a model involving tolerance induction with anti-CD154 mAb and rapamycin.[735] While peripheral deletion in the BMT/anti-CD154 model is not simply explained by exhaustion or "activation-induced cell death,"[551,733,736] activation-induced cell death was implicated in tolerance induction with anti-CD154 mAb plus rapamycin.[737] In addition, veto cells (see the following) can delete alloreactive CTLs. A non-veto mechanism mediated by CD4-CD8- cytotoxic regulatory cells has been reported to delete alloreactive CD8+ T cells with the same specificity as the regulatory cells.[738]

Anergy. Anergy is a state that may result when lymphocytes recognize antigen without adequate accessory or costimulatory signals[706,739,740,741,742] or when they encounter ligands for which they have low affinity.[743,744]

Anergy is an important tolerance mechanism for the many self-reactive B cells that escape deletion in the bone marrow,[745,746] particularly if they recognize abundant but low-avidity antigens.[691,747] Anergy requires persistent antigen and is characterized by immunoglobulin receptor downregulation,[691] altered signaling patterns, and increased apoptosis upon antigen encounter.[747] T-cell tolerance and the resulting absence of help is important for the maintenance of B-cell anergy, as anergic B cells can be activated in the presence of high avidity antigen and T-cell help.[747] Anergy is responsible for the early tolerance of natural antibody-producing B-1 cells in mice rendered mixed chimeric with bone marrow cells expressing an antigen recognized by natural antibodyproducing cells in the recipient.[153,704,705]

A T cell is considered to be "anergic" if it cannot proliferate or produce IL-2 in response to the antigen for which it is specific. T-cell anergy has been associated with altered signaling and

tyrosine phosphorylation patterns.[748,749,750,751] T-cell anergy can often,[752] but not always,[753,754] be overcome by providing exogenous IL-2. Thymocytes are also susceptible to anergy induction by antigens presented on hematopoietic[711] or nonhematopoietic stromal[755,756,757] cells. Anergy is generally reversible in vivo and can be overcome by infection[758] or by removal of antigen,[759,760,761,762] and therefore may not be reliable in transplant recipients, in whom infections may perturb a nondeletional state of tolerance by activating innate immunity.[763,764] While deletion may follow the induction of anergy of T cells in the continued presence of antigen,[765,766] anergic T cells may also persist.[767,768] In a transplantation model involving BMT under cover of costimulatory blockade, peripheral donor-reactive CD4 and CD8 T cells are rendered

antigen-unresponsive prior to their deletion.[550,734,769] Anergic T cells may also have a suppressive effect on other T cells,[770,771] which may involve conditioning of suppressive APCs.[772]

Immunoregulation

Regulatory T Cells. The concept of antigen-specific suppression and the existence of a specialized population of T cells, known initially as suppressor cells and more recently as T_{reg} cells, that could control immune responsiveness, originated in the 1970s from the studies of Gershon and Kondo,[773] among others, who demonstrated the existence of complex regulatory networks involving several levels of T-cell-mediated suppression. In the specific setting of transplantation, a role for suppressor cells in tolerance induction was also identified.[774] However, despite the efforts of many groups through the 1980s, the cellular and molecular characteristics of these suppressor cells were never defined with any precision, even though the phenomenon of suppression was highly reproducible in vivo, particularly in transplant models.

In 1990, Hall and colleagues reexamined the function of T-cell subpopulations present in rats with long-term surviving cardiac allografts after cyclosporine treatment. Interestingly, they reported that tolerance could be adoptively transferred by CD25+CD4+ cells.[775] However, interest in characterizing suppressor/T_{reg} cells was not fully revived until Sakaguchi and colleagues followed up on data showing that susceptible strains of neonatally thymectomized mice developed multiorgan autoimmune disease. These workers found that neonatal thymectomy resulted in a loss of T cells with suppressor or regulatory properties that could be enriched amongst T cells expressing CD25, the alpha chain of the IL-2 receptor.[776]

Naturally occurring or thymus-derived CD25+CD4+ T cells with regulatory properties are generated in the thymus through interaction between the developing thymocytes with an intermediate affinity ligand, too low for negative selection, expressed on nonhematopoietic cells of the thymus for their positive selection.[777,778] Cortical thymic epithelium has been found to be the site of MHC class II expression required for the development of functional T_{reg} cells.[779] In addition to the naturally occurring populations of CD25+CD4+ T cells with regulatory activity, populations of T cells with regulatory properties that also express CD25 can be induced in the periphery following exposure to antigen under certain conditions.[780] These populations are known as antigen-induced or adaptive T_{reg} cells. It is likely that

following transplantation or exposure to alloantigen, T_{reg} cells that can control the immune response and prevent rejection will be derived from both the naturally occurring and induced populations (see the following). In addition to the wealth of data in mice, CD4+ T cells expressing high levels of CD25 in the peripheral blood and thymus have also been shown to have suppressive activity in humans[781,782,783,784,785,786] and are present in transplant recipients with long-term surviving liver allografts.[787,788]

CD25 is by no means an exclusive marker for T cells with regulatory activity. Although the kinetics of expression are different, CD25 is also expressed by activated T cells. Indeed in transplantation, mAbs targeting CD25 are licensed for use as antirejection therapies. Thus, there is a need to define other markers for T_{reg} cells. The list of potential cell surface markers has been growing steadily, and includes CD152 (CTLA4), CD103, CD127, LAP, and glucocorticoid-induced TNF receptor (GITR) related proteinl amongst many others. However, as yet, a T_{reg} cell-specific “perfect cell surface marker” has not been identified.

Intracellular proteins and transcription factors can also potentially provide a tool for identifying committed cell lineages. In both humans and mice, forkhead box protein 3 (foxp3), a forkhead/winged-helix transcription factor, has been shown to be a master regulator for the development and function of T_{reg} cells. In humans, mutations in the Foxp3 gene in patients with the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome results in uncontrolled activation/expansion of CD4+ T cells, resulting in severe immune deficiency.[789,790] Scurfy mice also have mutations in foxp3 and a related immune profile.[791] A direct link between foxp3 expression and regulatory activity came from retroviral gene transfer studies where transduction of naïve mouse T cells with foxp3 resulted in acquisition of regulatory function.[792] This correlation between foxp3 and regulation has been strengthened by the generation of foxp3 transgenic mice where cells expressing FOXP3 protein express either green[793] or red fluorescent protein,[794] allowing such cells to be identified and their function explored. While in mice the relationship between foxp3 expression and regulation may be tightly linked, the situation may not be so straightforward in humans, as FOXP3 protein has also been found to be expressed transiently in activated T cells.[795] The methylation status of the Foxp3 gene turns out to be a marker of the stability of FOXP3 expression.[796,797]

Recipient-derived CD25+CD4+ T cells were shown to have potent regulatory properties in both the induction and maintenance phases of tolerance to alloantigens in vivo in mice.[798,799,800,801] In BMT, donor CD25+CD4+ T cells present in the bone marrow inoculum were found to protect from GVHD.[802,803,804] In humans, $CD25^{hi}$CD4+ T cells have been found in the peripheral blood of immunosuppression-free allograft recipients,[787] and their presence may contribute to long-term graft survival.[805] As mentioned previously, T_{reg} cells with capacity to control immune responses to a transplant most likely comprise naturally-occurring and alloantigeninduced T_{reg} cell populations.[806] Naturally occurring T_{reg} cells present in naïve, unmanipulated adult mice can prevent rejection of allografts mismatched for a single minor histocompatibility antigen (eg, H-Y). However, they appear to be much less potent than T_{reg} cells induced following exposure to alloantigen, as studies comparing the activity of naturally occurring and induced T_{reg} cells directly suggest that 10 times fewer induced T_{reg} cells are needed to prevent allograft rejection. These findings may

explain why in situations where grafts are mismatched for multiple major and MiHAs it has sometimes been difficult to demonstrate that naturally occurring T_{reg} cells can control the rejection response.

Another situation where T_{reg} cells are less effective at controlling allograft rejection arises when donor alloantigen-reactive memory T cells preexist in the recipient. In this case, the kinetics of activation of the memory cells is very rapid and, unless very high numbers of T_{reg}s are present, the

balance between rejection and regulation is overwhelmingly in favor of rejection.[807] Importantly, this critical balance can be shifted in a number of ways, notably by employing strategies that increase the relative frequency and/or the activation status and consequently the functional activity of induced T_{reg}s that can then respond to donor alloantigens before or in the early period after transplantation[801,808] or by inhibiting the activity of the effector cells.

After transplantation, the ability to generate T_{reg}s in vivo may be influenced by the microenvironment. The balance between the presence of IL-6 and TGF-β in vivo can have a dramatic impact on which T-cell population develops. Importantly, T_{reg} generation can be prevented by inflammation,[809,810] which is commonly present in vivo after cell or organ transplantation, leading instead to the generation of cells capable of mediating tissue damage, including T_H17 cells, which produce IL-17.[809,810,811] T_{reg} stability in the face of an inflammatory response remains a controversial issue.[812,813]

The mechanisms of regulation effected by T_{reg}s to prevent allograft rejection involve a variety of different pathways. When T_{reg}s are reactivated by their cognate antigen through the TCR, they can rapidly and transiently produce IFNγ, which is needed to mediate their regulatory activity and prevent graft rejection.[814] IFNγ triggers the STAT-1 signaling pathway that also influences the functional activity of T_{reg}s.[815] Interestingly, IFNγ has been shown previously to be an essential mediator for the induction of tolerance in mouse models where costimulation blockade was used to prevent graft rejection.[816] The transient production of IFNγ by T_{reg}s can have multiple effects on cells with which they interact,[817] including other T cells, mediated either directly or indirectly through effects on APCs.[818] T_{reg} activity can result in inhibition of cytokine production and secretion, downregulation of costimulatory and/or adhesion molecule expression, inhibition of proliferation, induction of anergy, elimination of the effector population by promoting cell death, or even conversion of naïve and/or effector T cells to a regulatory phenotype, a process known as “infectious tolerance. „[783,819,820,821] IL-2, IL-10, and TGF-β may have a role in this phenomenon.[822] If this process is effective, any new T cells entering the repertoire after transplantation where immunoregulation is the dominant mechanism of tolerance will be converted to T_{reg}s, thereby propagating and reinforcing the tolerant state throughout the posttransplant course.

Indoleamine 2,3 dioxygenease (IDO), an enzyme that catalyses the initial and rate limiting step of the kyneurinine pathway of tryptophan catabolism, is known to be subject to transcriptional regulation by IFNγ[823,824] in a manner that is dependent on STAT-1 signaling.[825] IFNγ released by T_{reg}s can lead to the development of IDO-competent DCs

that may acquire the capacity to control T cells locally through tryptophan depletion, inhibiting alloimmune responses.[826,827,828] The molecular mechanism responsible for the inducing DCs to become IDO producers requires further investigation, but roles for TGF-β and IL-10 have been suggested.[820,829]

In addition, the activity of T_{reg}s in transplant models has been shown to be dependent on IL-10,[541,798,799,830,831,832,833,834] TGF-β, and CTLA-4.[799] CTLA-4 (CD152) is constitutively expressed by T_{reg}s, and engagement of CD80/86 on DCs can also induce IDO.[835] In addition, CTLA-4 has been found to modulate T-cell motility by activating leukocyte function-associated antigen (LFA)-1 clustering and adhesion,[836] thereby altering the threshold for T-cell activation.[836] While the mechanistic links between each of these mediators still requires clarification, it is important to note that many of the same molecules have also been found to be required for T_{reg} activity in other settings, including autoimmune diseases, suggesting that T_{reg}s capable of controlling disease states have many properties in common.

One property of T_{reg}s that can potentially be exploited in the context of transplantation is their ability to mediate bystander regulation in a defined microenvironment in vivo. To elaborate their functional activity, T_{reg}s need to be activated through their TCRs. Data obtained by Thornton and Shevach in vitro showed that reactivation of T_{reg}s with a defined antigen specificity through their TCR enabled the cells to suppress the response of other cells present in the same cultures.[837] Bushell and colleagues have shown that it is possible to exploit this observation in vivo to enable T_{reg}s that are generated and respond to an unrelated protein antigen, such as human gamma globulin, to control rejection when the cells are restimulated through TCR immediately before they are asked to function in vivo.[838]

There is evidence that the location in which T_{reg}s function in vivo may change with time after transplantation. Jones and colleagues have shown that while T_{reg}s are active in the draining lymph nodes in the first few days following skin transplantation, later in the posttransplant course T_{reg}s are found within the allograft itself.[839] Indeed, there is increasing evidence that an important site of immune regulation is within the allograft itself, where T_{reg}s function to create an environment that is permissive of control.[801,840] Moreover, reexposing T_{reg}s to antigen in a tissue may enable them to become more potent suppressors.[841]

Despite the current interest in the role of CD25+CD4+ T_{reg}s, it is important to remember that regulatory activity is not exclusive to CD4+ T cells, and CD8+,[842,843,844] CD8+CD28-,[845] TCR+CD4-CD8- ("double negative"),[846] and NKT cells[847,848] have also been shown to have regulatory activities in different situations after transplantation. In fact, regulatory mechanisms in both the innate and adaptive immune systems will most likely contribute to the overall outcome after transplantation. This is highlighted by the finding that both NKT cells and CD25+CD4+ T_{reg}s play a role in preventing acute GVHD after allogeneic BMT,[802,803,804,847] and by a recent study showing that inhibitory NK cell receptors can play a regulatory role in T-cell homeostasis.[849]

It may be possible to exploit T_{reg}s to control the immune response after transplantation either by developing strategies using immunosuppressive agents that promote T_{reg} generation

and/or do not inhibit their function or by generating T_{reg}s ex vivo and using them as a cellular therapy at different stages in the posttransplant course to prevent rejection and reestablish control. Understanding the impact of immunosuppressive drugs on T_{reg} generation and function is an important part of using the potential of T_{reg}s in vivo. Data from mouse models suggest that calcineurin inhibitors have the capacity to inhibit T-cell apoptosis after activation, thereby

preventing a reduction in the frequency of T cells that mediate rejection that would allow T_{reg}s to assume a more dominant functional role[850,851] (Bushell and Wood, personal communication). Similarly, administration of anti-CD25 monocloncal antibodies after the induction of T_{reg}s can prevent long-term graft survival (Fig. 46.11). On the other hand, other classes of immunosuppressive drugs, such as the mammalian target of rampamycin inhibitors sirolimus and everolimus, may enhance the generation of T_{reg}s.

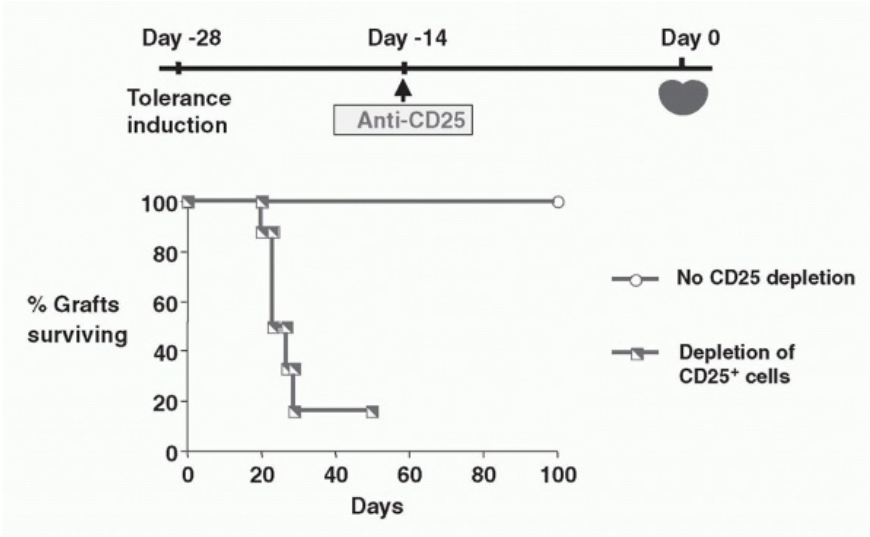

FIG. 46.11. Administration of Anti-Cluster of Differentiation 25 Monocloncal Antibodies after the Induction of Regulatory T Cells can Prevent Long-Term Graft Survival.

Strategies to generate and/or expand T_{reg}s ex vivo for therapeutic purposes are being developed with potential application in cell and organ transplantation. A variety of different approaches have been shown to be successful for generating and/or expanding T cells with regulatory capacity in both mice and humans, including exposure to TGF-β, IFNγ, and stimulation with anti-CD3/CD28 in the presence of IL-2 to name just some.[852,853,854] The development, maintenance, and expansion of T_{reg}s is critically dependent on IL-2.[673,855] Small molecule inhibitors such as phosphodiesterase inhibitors can be used to generate populations of T cells with regulatory activity. In each case, at the end of the culture period, the T cells that emerge have been shown either in vitro and in some studies in vivo to have regulatory activity that can prevent allograft rejection[854] or GVHD.[856,857]

Strategies for expanding human T cells with regulatory activity ex vivo usually rely on selection of a population of CD4+ T cells that contains regulatory cells, either by enriching for cells that express high levels of CD25 alone or low levels of CD127 in addition.[854,858,859]

In proof-of-concept studies using a humanized mouse transplant model, such ex vivo expanded T_{reg}s have been shown to be capable of controlling rejection.[854,860,861]

Antibody-Mediated Suppression. Idiotypes are unique antigenic determinants that characterize the binding sites of antibody or TCRs. These determinants can be antigenic and induce the production of anti-idiotypic antibodies.[862] Antibody-mediated suppression could theoretically occur through the recognition of idiotypes of antidonor immunoglobulin receptors. Anti-idiotypic antibodies can suppress antibody reactivity by directly binding to the antigen-binding site of the antibody, and the development of such antibodies has been suggested as one of the possible benefits of pre-kidney transplant blood transfusions. Such antibodies have also been suggested to contribute to the apparent hyporesponsiveness to noninherited maternal antigens in renal allograft recipients. In the past, it was also considered possible that anti-idiotypic antibodies might inhibit T-cell recognition of antigen, but this now seems unlikely, given that T cells recognize peptide/MHC complexes, whereas antibodies recognize epitopes of intact molecules. It continues to be an intriguing question whether normal regulatory mechanisms for B-cell responses might include anti-idiotypes, as suggested by Jerne.[862a] However, efforts to control transplantation using exogenous anti-idiotypic antibodies to either TCRs or B-cell receptors have been disappointing.[863]

Antibodies can also induce tolerance through a process known as enhancement. Enhancement is defined as prolongation of graft survival achieved by the presence of antigraft antibodies.[864] This phenomenon was first described in experiments involving allogeneic tumor growth. Subsequently, enhancing regimens using anti-MHC antibodies and/or soluble antigen were shown to produce long-term tolerance for rodent allogeneic kidney transplants.[865] The simple interpretation was that anti-MHC antibodies bind to the antigen and thereby block the immune response, but this explanation has not turned out to be sufficient. For example, tolerance following enhancement can be transferred by cells and not serum from enhanced recipients. Apparently, the administered antibody sets up a host reaction that leads to specific immunosuppression. An idiotype/anti-idiotype network would be an attractive explanation for this phenomenon. Unfortunately, the spectacular success obtained using enhancement for kidney graft survival in rats has not been observed for grafts in other species, and this approach has largely been abandoned, at least for the present.

Ignorance and Immune Privilege. Peripheral antigens may be ignored by T cells[866,867,868] or B cells[691] that recognize them. This may be due to the presentation of these antigens by

"non-professional APCs," or it may reflect a failure of recipient T cells to migrate to the antigen-bearing tissue, as in murine solid tumor models.[869] The level of peripheral antigen expression, how recently the responding T cell has emerged from the thymus,[866,870] and the presence of proin-flammatory cytokines[871,872] and costimulatory molecules[873] may all influence the decision of a T cell to ignore or respond to peripheral antigens. However, "ignorance" may be a precarious state, which can be upset by additional immunologic stimuli provoked by inflammation that may be induced, for example, by infections[868] or by presentation of antigen on professional APCs, as observed for endocrine allografts that are depleted of APCs prior to transplantation.[874]

Immunoprotection in specific niches may protect allografts from immune attack, either by

making antigen invisible to the immune system or by providing cell types in the microenvironment that protect the allogeneic cells. An example of the former is corneal transplantation, which is done without systemic immunosuppression due in part to the sequestered status of the anterior chamber of the eye.[875,876,877,878] However, a number of active mechanisms also contribute to this immune privilege.[876,879] An example of a cell that may be protected largely by other cells in its niche is the hematopoietic stem cell, which can survive in an allogeneic stem cell niche without immunosuppression.[880]

Strategies for Inducing Transplantation Tolerance

Strategies to Achieve Central Tolerance

Hematopoietic Chimerism. It has been known for 60 years that hematopoietic chimerism induced in utero is associated with transplantation tolerance.[881,882] The capacity of hematopoietic cells to induce intrathymic clonal deletion achieves tolerance to the most immunogenic allografts, such as fully MHC-mismatched skin and small bowel grafts.[452,883,884] However, HCT has not yet been routinely applied to the induction of tolerance in man. HCT for tolerance induction in rodents originally involved recipient treatment with lethal whole-body irradiation. Removal of mature donor T cells before transplantation prevented GVHD.[885,886,887,888] However, MHC-mismatched allogeneic HCT in humans has proved to be less successful and more dangerous than in rodents because of the toxicity associated with myeloablative conditioning, and the high risk of GVHD, and of engraftment failure when donor T-cell depletion is used to prevent GVHD.[889] Thus, more specific and effective methods of overcoming the barriers to marrow engraftment with minimal GVHD risk are needed for the application of HCT for organ allograft tolerance.

Achievement of a state of mixed rather than full allogeneic hematopoietic chimerism has several advangtages for allograft tolerance induction: 1) Mixed chimerism can be achieved with less toxic (nonmyeloablative) conditioning regimens than those that lead to full donor chimerism. Nonmyeloablative regimens preserve some host hematopoiesis so that life-threatening marrow failure is not a risk if donor marrow is rejected. 2) Mixed chimeras, unlike full chimeras, contain abundant recipient APCs in the periphery, allowing optimal antigen presentation to T cells that have developed in the host thymus, and which therefore preferentially recognize peptide antigens presented by host-type MHC molecules.[126,890] Antiviral CTL responses in mixed chimeras demonstrate exquisite specificity for recipient-derived MHC-restricting elements.[891,892] 3) Mixed chimeras contain hematopoietic cells from both the recipient and the donor in the thymus and hence delete both hostreactive and donor-reactive T cells.[717,893] Thymic stromal cells are less effective at inducing deletion, so intrathymic deletion of host-reactive cells is more complete in mixed compared to full chimeras.[717,894] Tolerance achieved with durable mixed chimerism is “systemic,” as evidenced by specific unresponsiveness to the donor and recipient in mixed lymphocyte response and cell-mediated lympholysis assays, and the acceptance of donor skin grafted at any time post-BMT.[452,895,896,897,898] In contrast, tolerance approaches that do not lead to durable chimerism generally do not induce systemic tolerance, suggesting that tolerance might be less robust.

Several nonmyeloablative approaches have been recently developed to permit the use of BMT to achieve mixed chimerism and specific tolerance, bringing the approach toward clinical application. In mixed chimeras prepared with a nonmyeloablative regimen consisting

of low dose (3 Gy) TBI, T-cell depleting mAbs, and thymic irradiation,[452] intrathymic deletion was shown to be the major mechanism maintaining long-term donor-specific tolerance.[717] Administration of donor MHC-specific antibody to eliminate donor chimerism from established mixed chimeras led to loss of tolerance and to the de novo appearance in the periphery of T cells with receptors that recognize donor antigens. However, if the recipient thymus was removed prior to elimination of chimerism, specific skin graft tolerance was preserved and donor-reactive TCR did not appear.[718] Thus, chimerism is needed only in the thymus and not in the periphery in order to ensure persistent tolerance of a peripheral repertoire that is deleted of donor-reactive cells. In contrast, peripheral anergy and suppression generally require persistent antigen to maintain tolerance. Because thymic APCs are continually turning over, this observation emphasizes the need for hematopoietic stem cell engraftment at sufficient levels to ensure an uninterrupted supply of donor APCs in the recipient thymus for the life of the mixed chimera. The absence of suppressive tolerance mechanisms makes these animals particularly vulnerable to breaking of tolerance when non-tolerant T cells emerge from the thymus after intentional depletion of donor antigen or after exogenous administration of nontolerant host-type T cells.[718,734]

The universal requirements for achievement of lasting chimerism are summarized in Figure 46.12. T-cell alloreactivity in both the thymus and periphery must be overcome in order to permit allogeneic stem cell engraftment and early seeding of the thymus with allogeneic APCs. Intrathymic alloreactivity can be overcome with irradiation,[452] high doses of T cell-depleting antibodies,[899] or costimulatory blockers.[731,896] Peripheral T cell-mediated alloreactivity can be overcome with T cell-depleting mAbs[452,900] or with costimulatory blockers.[731] The need for low-dose TBI or busulfan to facilitate engraftment of donor hematopoietic stem cells[899,901] can be eliminated by administering very high marrow doses[897,900,902] or by adding expanded recipient T_{reg} cells to donor marrow.[903]

The mechanisms of peripheral tolerance achieved with the combination of costimulatory blockade and allogeneic BMT are discussed in the section on peripheral tolerance.

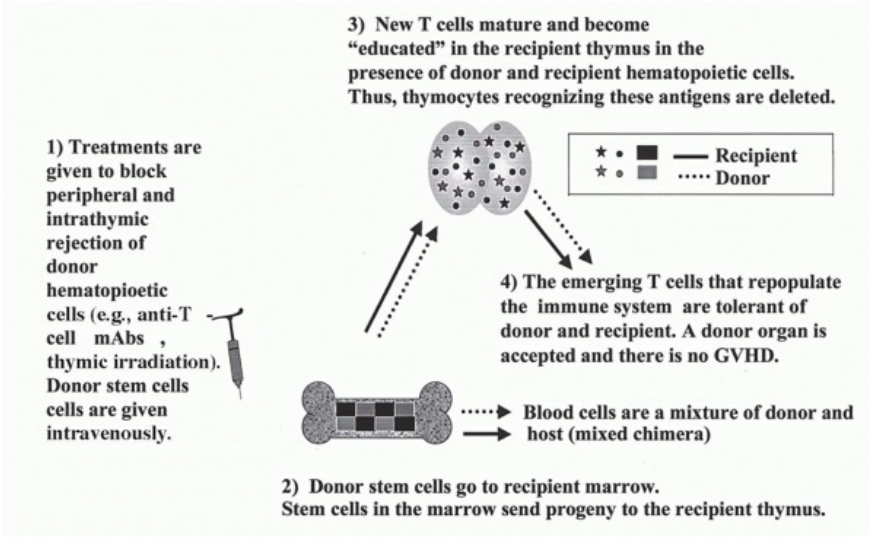

FIG. 46.12. Requirements for Induction of Long-Term Mixed Hematopoietic

Chimerism. GVHD, graft-versus-host disease.

Additional methods for achieving durable mixed chimerism for tolerance induction include the use of total lymphoid irradiation plus BMT[904,905] and various combinations of anti-T cell antibodies, irradiation, and immunosuppressive drugs in both large[906,907,908] and small[909,910,911,912] animals.

It has been difficult to achieve T-cell depletion with antibodies in large animals and humans that is as exhaustive as that achieved in the previously mentioned rodent models, perhaps due to the use of inadequate doses or suboptimal reagents. A second concern is that, if sufficiently exhaustive T-cell depletion could be achieved in humans, T-cell recovery from the thymus might be dangerously slow, especially in older individuals.[913] The ability to replace some[898] or all[731,897,902] T cell-depleting antibodies with costimulatory blockade is therefore of considerable interest.

GVHD does not occur in the rodent models discussed previously, despite the inclusion of T cells in the donor bone marrow graft, probably because of the presence of the T cell-depleting or costimulatory blocking antibodies in the serum of the hosts at the time of BMT.[914]

Extension of the Mixed Chimerism Approach to Xenotransplantation . Host treatment with mAbs to T cells and NK cells along with a low dose (3 Gy) of TBI has also permitted rat marrow engraftment in mice, resulting in mixed xenogeneic chimerism and donor-specific tolerance.[453] Both γδ T cells and NK cells play an important role in resisting xenogeneic marrow engraftment.[454] Anti-CD154 mAbs can overcome the requirement for CD4 cell depletion.[915] Mixed chimerism in the rat-to-mouse species combination was associated with tolerance of both T cells and natural xenoantibody-producing B cells.[453,916,917,918,919] Mixed xenogeneic chimerism thereby prevents HAR, acute vascular rejection, and cell-mediated rejection of vascularized cardiac xenografts.[154]

Pigs are widely believed to be the most suitable xenogeneic donor species for transplantation to humans, but transplantation from this species is impeded by the presence in human sera of Natural Antibodies (Nab) that cause hyperacute rejection of porcine vascularized xenografts. The major specificity recognized by human Nab on porcine tissues is a ubiquitous carbohydrate epitope, Galα1-3Galβ1-4GlcNAc-R (αGal). Humans lack a functional α1-3Gal transferase (GalT) enzyme, as do GalT knockout mice, which also make anti-αGal Nab. Both preexisting and newly developing B cells recognizing anti-αGal are tolerized by the induction of mixed chimerism in GalT knockout mice receiving αGal-expressing allogeneic or xenogeneic marrow.[153,704,920,921] The major immunoglobulin M Nab-producing cells in mice are splenic B-1b cells,[702] and these are tolerized rapidly by an anergy mechanism that is followed later by clonal deletion and/or receptor editing of Gal-specific B cells.[154,704,705,918,922] Splenic B cells, possibly of a similar subset, are also, along with immature plasma cells, the major anti-Gal immunoglobulin M-producing population in nonhuman primates and baboons.[923]

Studies in mixed allogeneic chimeras show that recipient NK cells are tolerized to the donor,[924] and studies in the ratto-mouse combination also indicate a state of NK-cell

tolerance.[925] While this tolerance is specific for the donor in mixed allogeneic chimeras, whose NK cells retain the ability to reject class I-deficient bone marrow,[924] the NK-cell tolerance in mixed xenogeneic chimeras is associated with global NK-cell unresponsiveness.[925] The most plausible explanation for these divergent results is that the presence of a xenogeneic cell population lacking any inhibitory ligand for recipient NK cells results in chronic activation of all NK cells and hence global anergy. Such "disarming" of NK cells could also explain dominant hyporesponsiveness in mixed chimeras involving class I-deficient donors or mosaic class I MHC transgenic mice.[926,927] In general, inhibitory NK receptors are less functional between species than activating receptors.[444,445,446,449,450,928,929,930,931,932,933,934,935,936] In mixed xenogeneic chimeras,

therefore, the xenogeneic cells may lack ligand that inhibit recipient NK cells, which become chronically activated and hence anergic. In contrast, allogeneic cells in mixed allogeneic chimeras express MHC molecules that will inhibit some subsets of recipient NK cells expressing relevant inhibitory receptors. Thus, in mixed allogeneic chimeras, only the subset of recipient NK cells lacking an inhibitory ligand for donor cells will chronically receive unopposed stimulation and hence become anergic; the consequence is a functional NK-cell repertoire that is inhibited by recognition of the donor (in addition to recipient) cells. The dominant tolerance exerted by mixed allogeneic or xenogeneic chimerism favors the "disarming" concept[927] over the "licensing" model, which proposes that inhibitory interactions license NK cells to become functional,[937] as the mechanism for NK-cell tolerance. Consistent with the disarming model, NK cells have been shown to tune their responsiveness in direct proportion to the number of inhibitory interactions they experience in vivo.[938] Moreover, continuous engagement of an NK cell-activating receptor renders NK cells hyporesponsive.[939,940] The complete lack of inhibitory interactions with xenogeneic cells could thereby render all NK cells anergic.

Despite this T-, B-, and NK-cell tolerance in mixed xenogeneic chimeras, the levels of donor chimerism decline gradually over time, due to a competitive advantage of recipient marrow over xenogeneic rat marrow as the host recovers from low-dose TBI.[941] Species specificity or selectivity of cytokines,[942,943] adhesion molecules, and other interactions that regulate hematopoiesis[944] probably account for this advantage. Achievement of xenogeneic hematopoietic repopulation has proved to be an even more formidable challenge in highly disparate (discordant) species combinations. Using immunodeficient mice transgenically expressing porcine hematopoietic cytokines to promote chimerism,[945] proof of principle was obtained that human T cells can be centrally tolerized in a human thymus to porcine xenoantigens via induction of mixed xenogeneic chimerism.[946]

Macrophages pose a major obstacle to engraftment of xenogeneic marrow from highly disparate species,[471,472] probably due to the failure of inhibitory ligands on xenogeneic cells to interact with macrophage inhibitory receptors, as observed for the porcine inhibitory ligand CD47 and the mouse[482] and human macrophage receptor SIRPα.[482,947,948]

Xenogeneic Thymic Transplantation. An alternative approach to achieving xenogeneic T-cell tolerance involves replacement of the recipient thymus with a xenogeneic donor thymus after host T-cell depletion and thymectomy. Immunocompetent mice treated in this way

reconstitute CD4+ T cells in xenogeneic porcine thymic grafts.[949] These cells repopulate the periphery, are competent to resist infection,[950] and are tolerant of porcine donor antigens.[949,951] Tolerance to both donor and host develops, at least in part, by intrathymic deletional mechanisms in these animals, reflecting the presence of class II^{high} APCs from both species within the thymic graft.[951,952] Studies using TCR-transgenic mouse recipients showed that positive selection in such grafts is mediated only by porcine thymic MHC, with no influence of mouse MHC.[953,954] However, excellent immune function is achieved in these mice. Importantly, human T cells can also develop normally and be rendered specifically tolerant of the porcine donor by developing in xenogeneic porcine thymus grafts.[955,956,957,958] This approach has demonstrated promise in pig-to-primate xenograft models.[959,960,961]

Transplantation of allogeneic[962] and concordant xenogeneic[963] thymic epithelial tissue obtained from fetuses before seeding with hematopoietic cells can also induce tolerance by generating donor-specific regulatory cells.[964,965] Transplantation of xenogeneic thymic tissue into congenitally athymic recipients has frequently resulted in the development of a multiorgan autoimmune syndrome, possibly due to the lack of recipient-type thymic epithelium needed for the development of regulatory cells with specificity for certain recipient antigens.[779,966,967,968,969,970] This complication occurs much less frequently in thymectomized, T cell-depleted mice than in congenitally athymic mice receiving porcine thymic transplants,[971] probably due to the persistence of regulatory cells derived from the host thymus prior to thymectomy and T-cell depletion. This autoimmunity can be avoided by adding recipient thymic epithelial cells to the thymic xenograft, resulting in improved deletion of recipient-reactive T cells and generation of T_{reg} cells that suppress autoimmune disease.[972]

Development of Chimerism without Host Conditioning

Developmentally Immunoincompetent Recipients. The first demonstration of allogeneic tolerance was obtained in Freemartin cattle, which are fraternal twins that share a placental circulation and develop mixed chimerism spontaneously in utero.[881,882,973] Injection of allogeneic hematopoietic cells to preimmune human fetuses has been used successfully to correct immunodeficiency diseases diagnosed in utero.[974,975,976] Low levels of chimerism have also been achieved in preimmune normal mouse and sheep fetuses.[977,978,979,980] While the ability of in utero HCT to induce transplantation tolerance has been somewhat unpredictable,[981,982] durable chimerism and renal allograft tolerance were achieved in pigs receiving in utero transplantation of T cell-depleted adult bone marrow.[983]

Acquired immune tolerance was first demonstrated by Medawar and colleagues, who injected allogeneic cells and tissue lysates into neonatal mice.[984] Untreated neonatal rodents can achieve skin graft tolerance if they are given allogeneic hematopoietic cells shortly after birth. Lasting microchimerism and sometimes sufficient levels of chimerism for flow cytometric detection (macrochimerism) have been observed.[985,986,987] Both intrathymic and extrathymic deletional mechanisms of tolerance have been implicated,[985,986] and regulatory cells probably play a role, as tolerance cannot be easily broken by the infusion of nontolerant host-type lymphocytes.[985,988,989] Neonatal mice tend to produce Th2 responses, which

have been implicated in tolerance.[362,363] The ability of allogeneic spleen cell infusions to induce tolerance may also reflect the high ratio of non-costimulatory APCs (T and B cells) in donor inocula to recipient T cells in the neonate.[990]

Adult Recipients. Very low levels of chimerism ("microchimerism") can exist for many years in the tissues of human recipients of solid organ allografts who did not receive HCT.[991] This observation led to the hypothesis that microchimerism, resulting from emigration of passenger leukocytes from the graft to recipient tissues, leads to donor-specific tolerance.[992] Microchimerism, which requires highly sensitive techniques for its detection, should be distinguished from the mixed chimerism discussed previously, in which multilineage chimerism is readily measurable by flow cytometry. Mechanisms by which microchimerism might promote peripheral T-cell tolerance include nonprofessional APC function of donor-derived B or T cells[993,994,995] and "veto" activity of T cells and NK cells.[996] However, microchimerism has not been shown to be required for or even necessarily associated with tolerance in a variety of models.[997,998,999,1000,1001]

Donor bone marrow cell infusions without specific myelosuppressive conditioning have been evaluated in efforts to achieve tolerance in patients receiving organ transplantation with standard chronic immunosuppressive therapy.[1002,1003] Such transplants were initially associated with significant risks[1004,1005] without having a measurable impact on acute rejection episodes or immunosuppressive medication doses in kidney[1006,1007] or liver[1008] transplant recipients, despite the persistence of and gradual increase in low levels of chimerism.[1007] A similar approach failed to achieve islet allograft tolerance in patients.[1009] However, long-term kidney allograft survival was favorable in donor marrow-infused compared to noninfused kidney graft recipients,[1010,1011] and immunologic assays suggested that bone marrow promoted donor-specific unresponsiveness.[1012] Immunosuppression withdrawal was not achieved.

Macroscopically detectable, though transient, chimerism has been observed in a nonhuman primate model that includes sublethal TBI.[1013] Myelosuppressive host conditioning may promote marrow engraftment by creating physical niches due to the destruction of host hematopoietic cells and by upregulating cytokines that promote hematopoiesis. Upregulation of chemokine SDF-1, which binds to stem cell CXCR4, and complement (C3) cleavage fragments are implicated in radiation-induced marrow injury and promotion of stem cell homing to that microenvironment.[1014] In mice, myelosuppression is required to promote engraftment of syngeneic marrow cells given in conventional doses,[899] but this requirement can be avoided using very high doses of marrow.[1015,1016] Engraftment of high doses of allogeneic marrow can be achieved without myelosuppressive treatment in mice and pigs receiving T cell-depleting mAbs,[900,1017] or in mice receiving costimulatory blockade.[897,902] The ability of expanded recipient T_{reg} cells to promote chimerism in nonmyelosuppressed recipients of conventional marrow doses[903] and advances in the ability to expand hematopoietic stem cells ex vivo[1018,1019] hold promise for the achievement of mixed chimerism without host myelosuppression in the future.

Strategies to Achieve Peripheral Tolerance

ANTIGEN INFUSION. DST/gene therapy with autologous cells: In rodents, DSTs have the ability to prolong allograft survival,[1020] and in certain donor/recipient combinations have been shown to induce operational tolerance.[1021] In most other species, DST alone has a less dramatic impact on graft outcome, but there is no doubt that it can influence graft survival in a positive manner and even in humans has been shown to be beneficial to graft outcome.[1022,1023] However, alongside these potential positive effects, infusion of cells expressing alloantigens from the organ donor can also have negative effects, including sensitizing the recipient to donor alloantigens, thereby increasing the risk of HAR. Moreover, with the introduction of erythropoietin into clinical practice, there was no longer a medical need to use transfusions to treat dialysis patients on transplant waiting lists; thus, the practice has largely stopped.

Investigations into the mechanisms by which donor alloantigen, either following infusion or as a result of donor antigens released from the graft at the time of transplantation, modifies the immune response to a subsequent transplant have provided important insights into how the adult immune system can be manipulated in vivo. Studies in rodents have shown that the level of induced unresponsiveness varies considerably depending on the quantity and source of alloantigen infused, as well as the immune status of the recipient.[1024] High doses of donor alloantigens expressed by cells of haematopoeitic origin can result in deletion of donor-reactive leukocytes, even in a host that is otherwise naïve. Lower doses of antigen resulted in the development of T cells with suppressive properties (see the following discussion). Interestingly, it was found not to be necessary to pretreat recipients with every donor major and MiHA they would subsequently encounter on the allograft. Exposure to a single donor alloantigen was sufficient to induce some graft prolongation as long as the allograft also expressed the same alloantigen.[1025,1026] This phenomenon was demonstrated in a number of experimental systems involving both transplantation and autoimmune disease, and is referred to as linked unresponsiveness or suppression.[1027,1028,1029] The mechanisms underpinning this effect have subsequently been elucidated and involve T_{reg} cells[1030] that once activated can mediate so-called bystander suppression, a mechanism identified initially in vitro[837] that also operates in vivo, thereby influencing the functional activity of other leukocytes present in the same microenvironment.[838]

An alternative strategy to using allogeneic cells for inducing specific unresponsiveness is to manipulate recipient cells to express defined MHC molecules that are subsequently expressed by the graft. The first proof-of-concept study using this approach transfected a fibroblast cell line of recipient origin with a mouse MHC class I gene, $H2K^b$.[1026] When the transfectants were used to pretreat recipient mice before transplantation, graft survival was prolonged. Obviously, cell lines are not applicable to the clinical situation, and therefore recipient or autologous bone marrow cells and hematopoietic stem cells were investigated as alternatives. This approach, using either replication defective retroviral or adenovirus constructs, has permitted markedly prolonged survival of class I-disparate skin grafts bearing the class I gene that was introduced into the autologous marrow[1031] and fully allogeneic heart allografts.[1032] In some settings, tolerance to the transduced antigens can be induced either by infusing

the transduced cells alone[1033] or by combining the transduced cells with other immune-modulating agents.[1034,1035] As with other approaches to tolerance induction, the persistence of the antigen, either in the form of the transduced cells, or after transplantation, from the graft, is critical to the maintenance of the unresponsive state in vivo.[1036]

TOLEROGENIC ANTIGEN-PRESENTING CELLS. DCs are central to the activation/priming of an immune response, but paradoxically they can also promote the development of tolerance.[173,1037,1038] One of the keys to both effects is the state of maturation of the DC when it functions in vivo and/or its lineage. Initially, immature myeloid DCs that express low levels of MHC class II and costimulatory molecules at the cell surface were identified as the dominant form of DCs that had the capacity to induce T-cell tolerance. In contrast, mature myeloid DCs expressing much higher levels of both MHC and costimulatory molecules were required for priming T-cell responses. However, mature DCs have subsequently also been shown to have the capacity to induce tolerance; therefore, the relationship between the state of maturity of a DC and its tolerogenic potential is now less clear. In addition, plasmacytoid DCs (pDCs) have also been found to have a role in tolerance induction. pDCs were originally defined by their capacity to secrete large amounts of type I IFNs in response to viruses and to play an essential role in protection against inflammatory responses to harmless antigens, but they have now also been shown to be able to induce human T_{reg} cells in vitro that produce significant amounts of interleukin IL-10, low IFNγ, and no IL-4, IL-5, or TGF-beta.[843] pDCs, unlike myeloid DCs, can rapidly express inducible T-cell costimulator-L upon maturation, which has been found to play a key role in the generation of IL-10-producing T_{reg} cells.[1039] In mice, pre-pDCs appear to be capable of facilitating hematopoietic stem cell engraftment, thereby promoting donor-specific skin graft tolerance in allogeneic recipients.[1040] However, the exact role and practicality of using pre-pDCs in this setting requires clarification as T cells are more abundant and hence more powerful in promoting engraftment. Significantly, donor-derived pre-pDCs infused 7 days before transplant were found to prolong subsequent heart allograft survival (from 9 to 22 days) in the absence of immunosuppressive therapy,[1041] but this effect was markedly enhanced by anti-CD154 mAb administration.[1042]

Immature DCs have been shown to promote tolerance to solid organ allografts and bone marrow grafts. For example, a single injection of immature donor-derived DCs 7 days before transplantation of an MHC-mismatched heart allograft extends[1043] or prolongs survival indefinitely[1044] in a donor-specific manner. The potential tolerogenic effects of immature DCs can be potentiated by the coadministration of immune-modulating agents such as costimulation blockade.[1045] Among several distinct approaches to generate stably immature DCs, pharmacologic manipulation may offer a promising and clinically applicable option. For example, sirolimus has been found to inhibit DC maturation and their effector functions.[1046] “Alternatively activated” or “regulatory” DCs, which have low costimulatory ability, were also found to protect MHC-mismatched skin grafts from rejection and to protect mice from lethal acute GVHD when administered 7 days before transplantation.[1047]

Reports showing that mature DCs can induce tolerance despite expressing high levels of MHC and costimulatory molecules include in vitro data with human cells demonstrating that maturation of human monocyte-derived DC with TNF-α and prostaglandin E2 triggered cross-priming and proliferation of CD8+ T cells with tolerogenic properties[1048] and that mature but not immature DCs can prime CD4+ T cells that inhibit allogeneic mixed leukocyte

reactions.[1049] In vivo, bone marrow-derived DCs matured with TNF-α, but not lipopolysaccharide or antibody to CD40, protected mice from CD4+ T cell-mediated experimental autoimmune encephalomyelitis, despite the expression of high levels of MHC class II and costimulatory molecules.[1041,1042,1050]

The molecular mechanisms used by pDCs to promote tolerance are complex,[1051] including evidence that they can promote the differentiation of Foxp3+ T_{reg}s. In transplantation models, tolerizing pDCs can acquire alloantigen in the allograft and then migrate through the blood to home to peripheral lymph nodes where they induce the generation of CCR4+CD4+CD25+Foxp3+ T_{reg}s.[1052] Infusion of donor-derived pDCs 7 days before transplant were significantly found to be capable of prolonging subsequent heart allograft survival (from 9 to 22 days) in the absence of immunosuppressive therapy,[1041] but this effect was markedly enhanced by anti-CD154 mAb administration.[1042] In mice, pre-pDCs appear to be the principal cell type that facilitates hematopoietic stem cell engraftment and induction of donor-specific skin graft tolerance in allogeneic recipients.[843] Although the concept that pDCs have the potential to promote transplantation tolerance has been suggested, the role of pDCs in experimental transplant tolerance remains poorly characterized and may be context dependent.

Taking the data using different populations of DCs together, it seems that both myeloid and pDCs can promote tolerance, and that maturation by itself is not the distinguishing feature that separates their immunogenic from their tolerogenic function. Indeed, maturation is more of a continuum than an “on-off” switch, and a “semimature” state, in which DCs are phenotypically mature but remain poor producers of proinflammatory cytokines, appears to be linked to tolerogenic function.[1053] The combination of DCs administered with costimulatory blockade may be the most promising approach identified thus far.

T-CELL-DEPLETING ANTIBODIES. Many tolerance induction strategies that have been investigated in small and large animals have used depletion of leukocytes (antithymocyte globulin, anti-CD52) or T cells (anti-CD3 with or without immunotoxin, anti-CD2, CD4, and CD8) to create an environment that allows reprogramming of the immune system.[1054] In small animals, the short-term depletion of T cells appears to be sufficient in some situations for tolerance to develop and be maintained in the long-term. The success rate can be enhanced by removing the thymus before transplantation to prevent repopulation of the periphery with T cells after transplantation.[1055] Initial data from primates

using anti-CD3 immunotoxin conjugated alone before transplantation or in combination with deoxyspergualin, a drug that inhibits NF-κB and therefore monocytes and macrophages, at the time of transplantation suggested that T-cell depletion can be used to induce tolerance to donor alloantigens.[1056,1057,1058] However, follow-up trials in humans undergoing renal transplantation and T-cell depletion with the anti-CD52 mAb alemtuzemab revealed that neither profound T-cell depletion alone or in combination with deoxyspergualin induced tolerance in humans.[1059,1060] Nevertheless, clinical results, across organ systems, reveal that acute rejection is lower[1061] and that steroid-free regimens with reduced maintenance doses of immunosuppression may be used after alemtuzemab therapy.[1062,1063,1064]

Depletion of leukocytes at the time of transplantation creates a transient immunodeficiency in the recipient, compromising the recipient's ability to reject the transplant. The degree and

duration of leukocyte depletion achieved in adults and pediatric transplant recipients determine how effective and for how long the graft is protected from immune attack.[1065,1066,1067] However, memory lymphocytes, including those capable of cross-reaction with allogeneic tissues, are not as readily depleted. Thus, while the intense immunosuppression provided by leukocyte-depleting antibodies prevents rejection in the rejection-prone early transplant period,[1061] active treatment can promote the homeostatic proliferation expansion of memory cells.

The downstream events that occur once leukocytes begin to reappear in the recipient's circulation are not well understood. An increased proportion of T_{reg} cells after alemtuzumab induction has been demonstrated and found to be most pronounced in calcineurin inhibitor-sparing protocols with early introduction of sirolimus.[1068] Interestingly, T_{reg} cells present in the peripheral blood of kidney transplant recipients who had received alemtuzumab induction were able to control the functional activity of Th17 cells that were also present.[1069] Marked enrichment of regulatory cells is observed in blood of patients receiving humanized anti-CD2 T cell-depleting therapy in conditioning regimens for BMT.[1070,1071] Enrichment for T_{reg} cells has also been demonstrated in mice after treatment with antithymocyte globulin.[1072,1073]

Studies using TCR-transgenic recipients have shown that when leukocytes are depleted, the maintenance of tolerance depends on transplantation of the graft within a time window of depletion of donor-reactive cells in the thymus and periphery.[1074] If the organ graft is transplanted at the appropriate time, donor-specific cells fail to repopulate from the thymus, whereas cells with reactivity to other antigens repopulate the periphery of recipients with long-term surviving organ grafts. These data can be used to suggest a mechanism for the long-term survival observed in primates treated with anti-CD3 immunotoxin complex. In this case, one can argue that the CD3+ T cells are depleted by the immunotoxin before transplantation. A window of opportunity is created such that when a renal allograft is transplanted, donor-reactive cells are absent from the periphery. As cells repopulate the periphery with time after the transplantation, donor-reactive cells are deleted or eliminated as a result of the presence of the surviving graft.

Although clinical trials utilizing T-cell depletion strategies alone have yet to demonstrate true immunologic tolerance, they have allowed for further larger-scale trials and have fueled combination of this strategy with administration of donor antigen in an attempt to achieve some level of mixed chimerism.[1075]

Costimulatory Blockade and Other Biologic Proteins

WITH SOLID ORGANS. T-cell activation, and hence rejection, is dependent upon multiple signals. Cell surface costimulatory molecules provide “signal 2” which, when combined with “signal 1” through TCR, triggers the activation of naïve T cells.[1076,1077] When signal 1 is forced to act on its own, T cells have been shown to undergo anergy or apoptosis.[1078] The original concept of a single costimulatory pathway has long been superseded, and it is now clear that there are multiple levels at which costimulation can participate both in initiating and determining the direction that an immune response takes.[1079,1080,1081] Nevertheless, targeting costimulatory pathways with either mAbs or recombinant fusion proteins specific for the costimulatory molecule itself or the ligand with which it interacts can be very effective at suppressing immune responses and in some cases may have the capacity to promote

tolerance to donor antigens in vivo.

Members of the immunoglobulin and TNF/TNF receptor superfamilies make up many of the costimulatory molecules that are integral to positive costimulation in the pathway of T-cell activation.[1082] Two pairs of ligandreceptor interactions that seem to play key roles in positive costimulation are CD40/CD40-ligand (CD154), which are members of the TNF:TNF receptor superfamily and CD80/CD86 and CD28, which belong to the immunoglobulin superfamily.[1083] Although the precise mechanisms that each of these costimulatory pathways plays during rejection and tolerance is still not completely understood, the complete abrogation or attenuation of either of these pathways can modulate the immune response to an allograft in vivo. Data from clinical trials using CD28 blockade as part of an immunosuppressive drug strategy has led to the licensing of CTLA-4-immunoglobulin (abatacept) for clinical use in patients with rheumatoid arthritis and of the mutated version of CTLA-4 immunoglobulin LEA29Y (belatacept) for use in kidney transplantation.[1084] Interestingly, anti-CTLA4 therapy (ipilimumab) has also been developed for clinical use, but in this case the approach is to augment immune responsiveness to tumour antigens.[1085]

The B7:Cluster of Differentiation 28/Cytotoxic T-Lymphocyte Antigen-4 Pathway . CD80 (B7-1) and CD86 (B7-2) are expressed as cell surface molecules by APCs and are responsible for delivering additional signals to T cells when they interact with CD28. CD86 and CD80 can also interact with a second molecule, CD152 (CTLA-4), which is expressed by T cells later in the activation process and is expressed constitutively by T_{reg} cells. CD86 and CD80 can exhibit preferential binding to CD28 and CD152, respectively.[1086] In contrast to CD28, CTLA-4 negatively regulates T-cell activation when it engages its ligand on the APC and, as described previously, is implicated in the control of clone size to maintain normal

homeostasis in the immune system.[1087] CTLA-4 is pivotal in regulating the threshold of signals during T-cell activation, and recent findings suggest that upregulation of CTLA-4 expression increases T-cell motility and overrides the TCR-induced stop signal required for stable conjugate formation between T cells and APCs. This results in reduced contact periods between T cells and APCs, leading to decreased cytokine production and proliferation.[1088]

Utilizing the B7:Cluster of Differentiation 28/Cytotoxic T-Lymphocyte Antigen-4 Pathway for Therapeutics. When CTLA-4-immunoglobulin, an immunoglobulin fusion protein of CTLA-4, was produced, it was shown to inhibit graft rejection in xenogeneic and allogeneic systems[1089,1090] and to promote engraftment of allogeneic stem cells.[731] In rodent models, CTLA-4-immunoglobulin therapy alone promoted tolerance to human islet xenografts in mice and, in combination with anti-CD154 (see the following), to vascularized allografts,[1089,1091] an effect that was enhanced when donor antigen was included in the treatment protocol.[1091,1092,1093]

The mechanism by which CTLA-4-immunoglobulin promotes long-term graft survival has been investigated in a mouse model. Blockade of CD80 and CD86 at the time of alloantigen recognition has been suggested to trigger T-cell apoptosis in the early phase after transplantation.[850,851] However, it should be noted that specific markers for alloantigen-specific T cells were not incorporated into this analysis, and therefore this mechanism has not been definitively demonstrated. When an antiapoptotic gene, bcl-x, was expressed in the responding lymphocytes, deletion did not occur, and graft prolongation was prevented. This

finding suggests that CTLA-4-immunoglobulin facilitates graft survival by reducing the number of donor-reactive cells that have to be controlled after transplant.

Although primate studies failed to achieve tolerance with CTLA-4-immunoglobulin,[1094] the theoretical foundation of blocking this pathway to promote graft survival continues to intrigue researchers.[1095] Additionally, it was known that the binding properties of CTLA-4 could be manipulated to optimize the ligation of both CD80 and CD86, a crucial component to experimental efforts at tolerance induction.[1083]

Belatacept. Experiments using CTLA-4-immunoglobulin laid the groundwork for further development of therapeutic agents targeting the B7:CD28/CTLA-4 pathway. Belatacept, LEA29Y, was originally derived from the fusion protein CTLA-4-immunoglobulin, or abatacept.[1096,1097] It differs from CTLA-4-immunoglobulin by two amino acids, conferring an approximately twofold increased binding capacity to CD80 and CD86. This increase in avidity allows for a 10-fold increase in the in vitro suppression of T-cell activation when compared to CTLA-4-immunoglobulin; in nonhuman primate studies, belatacept was found to prolong renal allograft survival and inhibit donor-specific alloantibody production both alone and in combination with other traditional immunosuppressive regimens.[1096] These and other findings allowed for the translation of LEA29Y to renal transplant patients. Results from clinical trials comparing belatacept to cyclosporine in partially randomized studies across 22 centers in North America and Europe of over 200 patients demonstrated that patients treated with belatacept-based therapy had improved renal function, reduction in chronic allograft nephropathy, and decreased calcineurin-related toxicity.[1098] Additionally, recent experiments in nonhuman primates using neonatal porcine islet grafts have revealed long-term xenograft survival under the cover of CD28-CD154 blockade with maintenance immunosuppression of sirolimus and belatacept.[1099] The use of belatacept in clinical transplantation has revealed some safety concerns, particularly when high doses were used.[1100]

Cluster of Differentiation 40-Cluster of Differentiation 154 Pathway . The CD40-CD154 pathway has been targeted to inhibit graft rejection using mAb therapy either alone[1101,1102,1103] or in combination with alloantigen infusion.[1104] CD154, or CD40-ligand, is a type 2 membrane protein of the TNF family and is expressed predominantly by activated CD4+ T cells and by a small proportion of CD8+ T cells, NK cells and eosinophils,[1105] and, significantly, on platelets as well.[1106] Structural models predict that CD154 forms a homotrimer that binds to CD40 on the surface of APCs. CD40 is expressed by B cells, macrophages, DCs, and thymic epithelium, and is inducible on the surface of endothelial cells and fibroblasts.

The CD40-CD154 pathway interaction is pivotal for the induction of humoral and cellular responses to nominal antigens as well as alloantigens. A CD40-immunoglobulin fusion protein and a blocking mAb to CD154 were shown to inhibit B-cell cycling, proliferation, and differentiation into plasma cells in response to T cell-dependent antigens.[1107] In vivo studies using CD154 mAb or CD40 or CD154 knockout mice[1108,1109] demonstrated a crucial role for this interaction in the generation of primary and secondary humoral responses to T cell-dependent antigens, class switching, and development of germinal centers. The lack of a humoral response in the absence of CD40-CD40L interaction is due not only to a lack of signaling through CD40 on the B-cell surface, but also to inhibition of priming of CD4+ T cells through CD40L.[1110]

The CD40-CD154 pathway is bidirectional. Not only does CD154 engagement on T cells augment T-cell activation, but also CD40 triggering on the APC primes the APC for stimulation. Signals through CD40 have been shown to upregulate expression of CD80 and CD86, as well as induce IL-12.[1111] Activation of DCs through CD40 promotes their ability to present antigen to T cells; this may explain why targeting CD154 and blocking its ability to interact with CD40 has a profound effect on T cell-dependent immune responses in vivo. Blocking CD154-CD40 interactions may promote tolerance induction by altering both this interaction and signals between APCs and immunoregulatory and suppressor T cells.[1112]

Utilizing the CD40-CD154 Pathway for Therapeutic Intervention in Cell and Organ Transplantation. Long-term acceptance of cardiac, renal, and islet allografts in several mouse and nonhuman primate models has been achieved with CD40 blockade using anti-CD154 mAb as monotherapy or in conjunction with anti-CD28.[1101,1104,1113,1114] However, with

the exception of BMT, in which durable chimerism and tolerance can be achieved with anti-CD154 mAb,[731,1115,1116] tolerance is not generated by anti-CD154 therapy alone in stringent models, in which therapy withdrawal leads to rejection.[1117,1118]

Further studies in the mouse revealed that only when rejection was dependent upon CD4+ T cells was CD154 blockade on its own effective at prolonging graft survival. In fact, several studies in donor-recipient combinations in which CD8+ T cells also play a role in rejection have shown that the CD8+ T-cell subset is unaffected by CD154 mAb therapy,[1119,1120] such that CD8+ T cells become activated, proliferate, and home to the graft in the presence of high-dose continued anti-CD154 mAb therapy. The addition of low dose TBI with donor hematopoietic cells can overcome this CD8+ T cell-mediated resistance.[1121]

Interest in this approach was also reflected in reports that a humanized mAb specific for CD154 (hu5c8) was capable of prolonging the survival of renal and islet allografts in rhesus monkeys.[1094,1102,1122] The initial data from these primate studies appeared encouraging, with rejection-free survival of the kidney grafts, provided that antibody therapy at a relatively high dose (25 mg/kg) was continued in the first 6 months after transplant. When anti-CD154 therapy was discontinued after the first month after transplant, rejection episodes did occur. Analysis of the status of recipients with long-term surviving grafts showed that peripheral lymphocytes from the monkeys failed to respond in vitro to donor antigens, but the recipients developed antidonor antibodies and biopsy samples from some of the long-term surviving grafts revealed T-cell infiltrates.

Together, these observations were sufficiently encouraging to initiate a pilot clinical study using Hu5c8 in renal transplantation. In this study, Hu5c8 was administered to seven patients with low-dose steroid alone, and five patients went on to experience episodes of rejection.[1123] Moreover, the unexpected complication of thrombogenesis in some patients treated with anti-CD154 highlighted that CD154 plays a key role in coagulation and clotting, with some reports suggesting that CD154 acts to stabilize thrombi while others implicate CD154 in platelet activation.[1106]

Other variants of costimulatory blockade that target different epitopes of CD154 have been developed with hopes of improved efficacy in transplantation without promotion of thrombogenesis. Experimental results in cardiac allografts of cynomolgus monkeys treated with an inhibitor of CD154, IDEC-131, either alone or in combination with leukocyte depletion in the form of antithymocyte globulin prolonged allograft survival; however, tolerance was not

induced, as alloantibody production and transplant vasculopathy, while delayed, still occurred.[1124] Still other anti-CD154 antibodies, such as ABI793, have been developed, but have been plagued with continued thromboembolic complications.[1125] Whatever the role that CD154 may play in prolonging allograft survival, it is clear that this molecule acts via independent pathways in a variety of cascades unrelated to tolerance induction.[1083]

Further studies have been undertaken to evaluate antibodies targeting CD40 in order to bypass the potential ramifications of CD154 blockade. Initial animal knockout models revealed a propensity of CD154 knockout mice to develop unstable thrombi, a phenomenon not seen in CD40 knockout mice.[1126] Studies in primate renal allograft models reveal promising results with a short course of low-dose calcineurin inhibitor administered concomitantly with anti-CD40/anti-CD86 costimulation blockade[1127] or with anti-CD40 therapy alone.[1128] Trials using a fully humanized anti-CD40 antiagonist are currently in phase II clinical development.

Targeting Cluster of Differentiation 3 and Accessory Molecules . Initially, administration of depleting anti-CD4 and anti-CD8 mAbs was shown to result in prolonged graft survival.[1129,1130,1131] That this treatment strategy resulted in antigen-specific tolerance was first shown most clearly when a protein antigen was administered in conjunction with a depleting anti-CD4 mAb.[1132,1133,1134] Refinements of these types of protocols have resulted in the ability to achieve long-term T-cell unresponsiveness to protein and alloantigens in the absence of T-cell depletion in experimental models.[1135,1136,1137] In fact, many other accessory molecules, other than anti-CD4 and anti-CD8, have been targeted in an attempt to induce tolerance in models of bone marrow,[1138,1139] islet,[1140] renal,[1141,1142] and cardiac allografts,[1139,1143] to name a few.[1144]

OKT3, a murine antihuman CD3 mAb, received approval for human use in 1986 in kidney transplant patients undergoing rejection and eventually for liver and cardiac transplant recipients as well.[1145] Although widely used, OKT3 brings with it the undesired complications of the human antimouse antibody response as well as a first dose reaction characterized by fevers, chills, and gastrointestinal, respiratory, and cardiac complications.[1146,1147] These ramifications are thought to be the result of CD3 cross-linking activating the signaling pathways downstream of the TCR, leading to transient T-cell activation and subsequent cytokine release.[1145] Therefore, investigators have attempted to construct pharmacotherapeutics that mimic the efficacy of OKT3 with less immunogenicity.[1148] These designer anti-CD3 monoclonals retain T-cell stimulatory capacity, albeit greatly impaired, as a consequence of their failure to readily cross-link targeted CD3 molecules on the cell surface. Interestingly, these antibodies stimulate T cells to express TGF-β, a potent, immunosuppressive cytokine, an effect crucial to immune tolerizing effects achieved in autoimmune models.[1148] Early clinical studies using the humanized “nonstimulatory” anti-CD3 antibodies, teplizumab and otelixizumab, showed some benefit in patients with new onset type 1 diabetes,[1145,1149,1150,1151] though lasting insulin independence was not achieved. While anti-CD3 mAbs have the potential to tilt the balance of immunity toward tolerance, adjunctive agents may be required to enable use safe nontoxic doses in attempts to reinstate tolerance. As yet, neither has been introduced into clinical transplantation.

Along with anti-CD3, antibodies to CD11a (LFA-1) and its ligands, ICAM-1, -2, and -3, have been investigated and have led to prolonged graft survival in many of the aforementioned models. LFA-1 has been implicated as an essential molecule for cellular trafficking and motility as well

as T-cell activation.[1152,1153] Additionally, the interaction of LFA-1 and the ICAM molecules may serve as a costimulatory pairing for T-cell activation.[1154] Preclinical studies in transplant models have shown that combining anti-LFA-1 with costimulatory blockade may be efficacious[1155]; these findings are supported by results of a pilot clinical study using efalizumab in islet transplantation.[1156]

Operational tolerance induced in animals by these strategies has been shown to develop over several weeks after the initial antigen encounter.[1157,1158] When a combination of donor antigen and mAb therapy targeting accessory molecules is used, the precise mechanism of tolerance induction in part depends on the amount of antigen infused.[1137] Deletion also may be one of the initial mechanisms of tolerance,[1024,1159] but in the absence of complete deletion of donor-reactive cells, immunoregulation develops. When antibodies targeting accessory molecules are used as therapeutic agents at the time of transplantation, immunoregulation is the dominant mechanism that comes into play to maintain tolerance in the longer term.

In these systems, tolerance to donor antigens is induced and/or maintained as a result of the development of a population of T_{reg} and suppressor T cells that can mediate unresponsiveness to the initiating donor antigen as well as other antigens present on the graft (linked unresponsiveness).[1160] In mice and rats, this type of tolerance has been shown to be infectious[1161]; it can be transferred from one generation of cells to another, provided that there is sufficient period of contact between the two populations.

The maintenance of tolerance in these systems requires the persistent presence of antigen in the form of the organ when the thymus is still functional.[1162] In the absence of donor antigen, tolerance is eventually lost, presumably as a result of the export of naïve T cells from the thymus into the periphery. Quantitatively, if these cells fail to encounter antigen, they eventually outnumber the unresponsive T cells induced by the monoclonal antibody therapy.

Costimulatory Blockade with Infusion of Donor Cells

While long-term tolerance is maintained by intrathymic deletion in mixed chimeras prepared with costimulatory blockade,[731,897,898] initial tolerance of peripheral T cells involves specific deletion of donor-reactive CD4[734] and CD8[533] T cells. The deletion of CD4 cells achieved following BMT with anti-CD154 is not due to binding of anti-CD154 mAb to activated T cells, and only requires absence of the CD154-CD40 interaction.[1163] Peripheral deletion of donorspecific CD8 cells occurs within 1 to 2 weeks. CD25-negative CD4 cells are required for CD8 tolerance only during this initial 2-week period.[533] Deletion of peripheral donor-specific CD4 and CD8 cells is preceded by specific unresponsiveness toward the donor.[550,734,1163] Regulatory cells do not appear to play a major role in maintaining the long-term tolerance in this model, as tolerance and chimerism are obliterated by the infusion of relatively small numbers of nontolerant recipient-type lymphocytes and linked suppression is not observed.[734] Because hematopoietic stem cell engraftment ensures complete central

deletional tolerance in these longterm chimeras,[731,732,897,898] and specific peripheral deletion is quite complete, the absence of donor-reactive T cells may preclude the expansion and maintainence of specific regulatory mechanisms.

Administration of DST with anti-CD40L mAb prevents islet allograft rejection,[1164] but in contrast to BMT, does not achieve central deletion[896,897] and is most effective in thymectomized mice.[540,729,1165] The inability to resist breaking of tolerance by new thymic emigrants in this model argues against powerful peripheral regulatory mechanisms, but CD4 cells are needed to promote skin graft acceptance.[540] DST promotes peripheral deletion of donor-reactive CD8 T cells[729] and can be replaced by CD8 depletion with mAbs.[1166] The combination of anti-CD154, BMT, and DST seems to be particularly effective in achieving mixed chimerism, largely because of the capacity of DST to overcome residual CD8 T cell-mediated alloresistance.[730,1167]

Costimulatory blockers can also be used to prevent GVHD, using B7/CD28 blockade or anti-CD154 mAb,[1168,1169,1170] or pre-BMT exposure of donor T cells to recipient alloantigens in the presence of anti-CD40L.[1171] Clinically, HLA-mismatched BMT has been attempted with T cells anergized by exposures to recipient alloantigens in the presence of CD28/B7 blockade, with apparently reduced GVHD.[1172,1173]

A Large Animal Model

MHC-defined, inbred miniature swine have provided an instructive model for delineation of the role of various histoincompatibilities in tolerance and rejection in large animals. Studies of pig renal transplantation have demonstrated that spontaneous tolerance can be induced by organ grafts when MHC antigens are matched. The ability to achieve such tolerance is dependent on one or possibly two non-MHC-linked genetic loci in the recipient animals. The presence of the “acceptor” phenotype also permits the spontaneous acceptance of single haplotype class I-mismatched kidney grafts.[7] Graft acceptance is associated with donor-specific CTL unresponsiveness, apparently due to a deficiency in help for these CTL, and not due to a deletional mechanism. Thus, in class II-matched, class I-mismatched porcine donor-recipient pairs, a 12-day course of high-dose (10 mg/kg/day) cyclosporine (CsA) permits long-term renal allograft acceptance in 100% of cases.[7]

The requirement that class II antigens be matched between donor and recipient in order for this tolerance to be achieved may reflect the influence of a major difference in class II antigen expression that exists between large and small animals. Unlike large animals and man, in which class II antigens are expressed constitutively on vascular endothelial cells, the corresponding endothelial cells of rodent species do not express MHC class II molecules.[18,19] Consistent with this interpretation, the use of a short course of CsA can facilitate the ability of renal allografts to induce tolerance in rodents across fully MHC-mismatched barriers, but tolerance induction in swine requires class II matching between donor and recipient for uniform success.

Animals accepting class II-matched allografts are systemically tolerant to the donor's class I and minor antigens, as indicated by the fact that the accepted graft can be removed and replaced by a second donor-matched graft,

which is accepted without immunosuppressive therapy. This ability of CsA to facilitate

tolerance induction, and the ability of exogenous IL-2 to prevent the induction of tolerance in this model,[7] is consistent with the interpretation that induction of tolerance of donor class I-reactive CTLs is due, at least in part, to the absence of adequate T-cell "help" during the time of initial exposure to antigen. A selective decrease of expression of the Th1-associated cytokine IFNγ relative to the Th2-associated cytokine IL-10 has been observed in these accepted grafts.[1174] The thymus appears to play a role in the induction of tolerance among preexisting peripheral T cells in this model, as removal of the host thymus prior to kidney allotransplantation leads to rejection.[1175,1176,1177] The possible mechanisms responsible for this role of the thymus in inducing peripheral tolerance phenomena are discussed elsewhere in this chapter. The kidney allograft itself clearly plays an important role in the tolerance induced in this model. Class II-matched cardiac allografts are not accepted after a similar short course of CsA, but they are accepted if grafted to animals that are tolerized in this manner to kidney allografts bearing the same mismatched class I alleles as the donor heart.[631] The mechanisms of this tolerance has been demonstrated to involve T_{reg} cells that can specifically suppress antidonor CTL responses.[1178,1179,1180]

The Relationship between Peripheral T-Cell Tolerance and Central Tolerance

The thymus can play an active role in tolerizing peripheral T cells. For example, in the pig model involving a short course of CsA with kidney transplantation, the recipient thymus is required to achieve tolerance among preexisting peripheral T cells.[1176] In rats, peripheral tolerance can be achieved by intrathymic injection of donor antigens combined with peripheral organ transplantation.[1181,1182,1183] The capacity of T cells that are activated in the periphery to migrate to the thymus,[1184,1185,1186] migration of donor graft APCs to the thymus where they induce thymic T-cell deletion,[1187,1188] or the development of T_{reg} cells that migrate to the periphery and promote tolerance[1189,1190] may contribute to this role for the thymus in peripheral tolerance induction. T_{reg}s have indeed been implicated in both the pig model and the rat intrathymic injection model.[1179,1191,1192] The intrathymic injection approach has not been successful in "high responder" rat strain combinations[1193,1194] and has not successfully prevented xenograft rejection or chronic rejection of cardiac allografts.[1195] While one attempt to use this approach in nonhuman primates was discouraging,[1196] donor-specific skin graft prolongation was reported in three animals receiving allogeneic or xenogeneic (human) CD34+ cells intrathymically.[1197]

From Animal Models to Clinical Transplantation Tolerance

Almost every transplant clinician has treated patients who have chosen to withdraw immunosuppressive therapy for a variety of reasons. While the majority of such patients reject their allograft, rare cases achieve "spontaneous" tolerance in this manner, demonstrating that tolerance can be achieved in humans. Blood biomarkers have been described that identify tolerant patients who have been successfully weaned from immunosuppression,[1198,1199,1200] but so far none have been used to identify such patients before immunosuppression withdrawal and thereby shown to have predictive value. In general, short-term results of most organ allograft transplants are excellent, making it essential to have reliable methods of inducing tolerance in order to ethically justify their use in place of conventional chronic immunosuppressive therapies. Because the risk of rejection

due to immunosuppression withdrawal is difficult to accept in patients, it is our opinion that extension of results from animal models to humans should only be attempted after 1) rodent studies have demonstrated robust tolerance in multiple strain combinations using extensively histoincompatible, highly immunogenic grafts such as skin; 2) efficacy has been demonstrated in large animal models; and 3) acceptable toxicity has been demonstrated in large animal models. When complete removal of immunosuppression is likely to be achieved, the level of toxicity accepted in the short term may be somewhat higher than that which could be acceptable on a longterm basis. Most efforts to achieve peripheral tolerance in larger animals have not been as effective as in rodent models.

The extensive animal data that have been accumulated on the ability of mixed chimerism induced with reduced intensity conditioning to achieve robust transplantation tolerance, including its demonstration in large animal models,[1013,1201] combined with clinical data obtained in patients with a more conventional indication for HCT, have allowed clinical evaluation of this approach for tolerance induction. Mixed chimerism can be achieved with reduced toxicity using nonmyeloablative conditioning in patients with hematologic malignancies,[1202] and lymphohematopoietic graft-versus-host reactions induced by donor lymphocyte infusion (DLI) can be used to achieve graft-versus-tumor effects.[1203] These observations provided an opportunity to evaluate the potential of this approach to induce transplantation tolerance in patients with a hematologic malignancy, multiple myeloma, and consequent renal failure. Recipients of a simultaneous nonmyeloablative BMT and renal allograft from HLA-identical siblings achieved either transient or durable mixed chimerism or, in a few cases, full allogeneic chimerism (some after DLIs). All three subsets of patients accepted their kidney grafts without any immunosuppression for follow-up periods of up to 14 years, with very good tumor responses.[1204,1205] In vitro studies suggested that, in patients who lost their chimerism, tolerance may be specific for donor antigens expressed by the kidney, while responses to antigens expressed on hematopoietic cells but not the kidney may even be sensitized.[1204] T_{reg} cells may also be involved.[634,1204] These promising efficacy results, combined with safety data in patients receiving HLA-mismatched transplants for hematologic malignancies,[1206] cleared the way for trials of HLA-mismatched haploidentical bone marrow and kidney grafts in patients with no malignant disease (ie, BMT was performed solely for allograft tolerance induction), using a regimen that had led to transient mixed chimerism without GVHD in an initial cohort of patients with hematologic malignancies. Donor kidneys were accepted off immunosuppression in 7 of 10 patients, with

follow-up for as long as 10 years in the first patient at the time of this writing. These studies were the first intentional protocol to achieve transplantation tolerance across HLA barriers in humans. Mechanistic studies implicated regulatory cells and the kidney graft itself as possible contributors to long-term, systemic, donor-specific tolerance that may involve eventual peripheral deletion of donor-reactive T cells.[635,636,1207,1208] Several additional protocols have recently used different nonmyeloablative HCT protocols to achieve allograft tolerance across minor histocompatibility[615,617] or HLA[1209] barriers. The different mechanisms involved with these various approaches have recently been reviewed.[1210,1211] Both T_{reg}s and inducible NKT cells have been implicated in the HLA-identical combined kidney-BMT protocol developed at Stanford.[615,617,1211] A poorly understood “facilitating cell” population was suggested to be involved in the full chimerism achieved across HLA barriers, reportedly without GVHD, in five patients at Northwestern University. However, the safety of this latter

protocol remains to be established, and a life-threatening complication led to infusion of recipient lymphocytes for rescue, resulting in graft rejection in one of the patients.[1209] Animal studies would predict that these full chimeras with extensive (up to five of six) HLA mismatches might not regain full immunocompetence.[891,892]

TRANSPLANTATION OF SPECIFIC ORGANS AND TISSUES

Kidney Transplantation

Kidneys are the most frequently transplanted human organs, with approximately 10,000 kidney transplants being performed annually in the United States. Renal allograft survival has increased steadily since its inception, and at present there is over 95% patient survival and over 91% organ survival at 1 year.[1212] However, even well-matched recipients of renal transplants must continue to take immunosuppressive medications for the rest of their lives, with complications including an increased risk of infection, cancer, hypertension, and metabolic bone disease. In addition, even when immunosuppression is well managed, there is an inexorable loss of kidneys to chronic rejection at a rate of approximately 5% to 7% per year. Both of these problems might be eliminated by the induction of tolerance, for which clinical trials have recently begun in earnest[615,635,1204,1208,1213,1214] (see previous discussion).

As for other organs, a major obstacle in kidney transplantation is the shortage of organs, which has been a paradoxical consequence of the success of this field. Unlike hearts and livers, where the inadequate supply of cadaveric organs spells death for many potential recipients, candidates for renal transplants are instead faced with long periods on dialysis while they wait for a kidney. This waiting time can be 4 or more years even for unsensitized candidates and even greater for those with high levels of sensitization. To avoid this delay, many patients are now being offered kidneys from living donors, and in many transplant centers the number of living donor renal transplants performed per year now exceeds the number of transplants from cadaver donors.

Sensitization of renal transplant candidates usually results from prior antigen exposure, either by blood transfusions or previous transplants. Such highly sensitized patients are said to have high “panel reactive antibody”; they may wait many years to obtain a kidney that is cross-match negative, and some never receive a transplant at all, despite the recent development of antibody desensitization procedures.[1215] Indeed, as discussed in the following, these patients may be among the first to benefit if and when xenotransplantation becomes a clinical reality.[1216]

Finally, late failure of kidney transplants, or “chronic rejection,” is a continuing problem to the field. This form of graft loss may occur many years after transplantation and it probably involves both immunologic reactivity and other factors, including the effects of the early ischemic injury and the ongoing effects of drugs and metabolic abnormalities in the recipient.[1217] This process leads to an inexorable loss of organs. Thus, although the 1-year survival rate for kidney transplants has improved from roughly 40% to over 90% over the past 40 years, at 5 years there is only 71% graft survival. Indeed, the half-life for kidney transplants has barely increased during the same period and remains less than 10 years.

Liver Transplantation

Transplantation of the liver represents a major technical challenge. For this reason, the organ

and patient survival rates have not been as high as those for renal transplantation, but have nevertheless increased yearly, with a current 1-year organ survival rate of about 80% and patient survival rate of about 86%.[1212] There are currently about 6,000 liver transplants carried out in the United States per year. The severe shortage of available organs has recently led to use of living donors, although the number is still small, accounting for only about 5% of liver transplants, or about 300 cases per year. Immunologically, the liver is relatively resistant to early antibody-mediated rejection, allowing successful transplantation even across blood group barriers and in the face of a positive cross-match, although a negative cross-match is generally considered preferable.[1218,1219,1220] In addition, longterm survival of liver transplants does not appear to be improved by HLA matching between donor and recipient,[1221] and many centers do not even type recipients and donors. Both of these immunologic features may be responsible for the fact that the long-term results of liver transplantation are almost as good as those for kidney, with 65% graft survival and 71% patient survival at 5 years. Finally, transplantation of the liver carries with it large numbers of donor lymphoid cells, thus creating the potential for inducing GVHD.[1222] The symptoms can range from antibody-dependent hemolysis of recipient red blood cells across a blood-group incompatibility to severe or even fatal, full-blown GVHD.

Heart and Lung Transplantation

There are approximately 2,000 heart transplants performed annually in the United States. As might be expected for this vital organ, the 1-year statistics for heart transplantation are approximately the same for patient (87%) and graft (86%) survival.[1212] One of the immunologic issues of particular importance in heart transplantation is the high rate of

atherosclerotic disease in the coronary arteries of the transplanted organ. This atherosclerotic disease is generally considered to be a manifestation of chronic rejection, although the causes appear to include more than just the immune response. Nevertheless, patient and graft survivals are about 70% for heart transplants at 5 years.

The number of lung transplants performed annually in the United States is approximately 1,200, while only about 30 combined heart and lung transplants are performed. The statistics for lung transplant survival are about the same as for liver (82% graft survival and 83% patient survival) over the first year, but then decrease much faster, with 5-year survivals of only about 45% for grafts and 46% for patients.[1212] A major cause of graft loss is a process called bronchiolitis obliterans, thought to be the pulmonary manifestation of chronic rejection.[1223] However, these patients are also highly susceptible to pulmonary infections, which undoubtedly contribute to the poor long-term statistics.

Pancreas and Islet Transplantation

Transplantation of the whole pancreas was almost without success until about 1980, largely for technical reasons. More recently, successful pancreas transplantation to treat diabetes mellitus has been achieved using new technical approaches, with success rates of about 92% at 1 year, approaching those for kidney transplantation, as long as the two organs are transplanted together. There are approximately 850 simultaneous kidney/pancreas transplants per year in the United States. The lower survival rates (77% graft survival at 1 year) achieved when pancreas transplantation is performed alone (about 500 cases annually) probably reflect the difficulty in diagnosing rejection episodes involving this organ. By the time blood sugar levels begin to rise, destruction of the pancreas is generally so far advanced that

it cannot be reversed by immunosuppressive therapy. Measurement of the serum creatinine, reflecting early dysfunction of a simultaneous kidney transplant, allows much earlier detection of rejection and, thus, better outcomes.

Simultaneous transplantation of both a kidney and a pancreas from a single donor has demonstrated, surprisingly, that rejection activity in one organ is not always associated with rejection activity in the other. It is not known whether this occasional dichotomy reflects tissue-specific antigens or localized inflammatory (or regulatory) events in one but not the other organ.

Most pancreas transplantation is carried out as a curative treatment for diabetes mellitus. For this purpose, the potential also exists of transplanting only the insulin-producing islet cells rather than the whole pancreas. In rodent models, both allogeneic and xenogeneic islet transplants have successfully achieved normoglycemia in diabetic animals.[1224,1225] Attempts to extend these results to patients, however, have met with limited success,[1226,1227] and until recently, the results of whole organ pancreas transplantation in correcting the hyperglycemia of diabetes have been far superior to those of islet transplantation.[1228,1229,1230] In 2000, the “Edmonton Protocol”[1231] showed much improved survival of islet transplants at 1 year in 4 of 12 patients, using a new combination of immunosuppressive drugs that appeared to cause less damage to transplanted islets. However, this protocol required use of two or sometimes three pancreases for a single recipient, and by 5 years, in a much larger series of patients, only 10% of patients have remained insulin independent.[1232,1233,1234] Thus, these results, while representing an improvement, remain much inferior to the results of whole organ pancreas transplantation.

Hematopoietic Cell Transplantation

BMTs, and more recently, transplants of hematopoietic stem cells and progenitors mobilized from the marrow into peripheral blood by treatment with granulocyte-colony stimulating factor, are used most commonly for the treatment of otherwise incurable leukemias and lymphomas, aplastic anemia, and congenital immunodeficiency states. Additional applications include hemoglobinopathies and inborn errors of metabolism. Autologous HCT is used for hematologic rescue following high-dose chemo-/radiotherapy for the treatment of malignancies and is currently being explored as a treatment for autoimmune diseases. However, autologous HCT will not be considered further here as it does not involve the broaching of any immunologic barriers.

One fundamental difference between HCT and the transplantation of all other organs is that the recipient's treatment for his or her malignancy usually results in ablation of the immune and hematopoietic systems prior to transplantation (ie, the “conditioning” for transplantation is myeloablative). Originally, hematopoietic cell allografts were administered only as a means of replacing ablated host hematopoiesis. However, clinical experience revealed that one of the main therapeutic benefits of allogeneic HCT is due to the GVL effect of donor lymphocytes.[1235] With the recognition of this immunotherapeutic benefit of allogeneic HCT, clinicians began to evaluate less toxic, nonmyeloablative conditioning as a means of allowing allogeneic marrow to engraft so that donor lymphocytes can mediate GVL effects.[1202,1203,1236,1237] In contrast to HCT for malignancies and other indications in immunocompetent recipients, transplantation for immunodeficiency states does not require myeloablation or immunoablation in order to achieve alloengraftment.

Another major distinguishing feature of HCT is that the recovering immune system in durable

chimeras is tolerant of the donor alloantigens, so there is no requirement for immunosuppressive therapy to prevent allograft rejection once the initial immune resistance to the allograft has been overcome. However, initial rejection must be prevented in order to achieve this state. Mechanisms of hematopoietic cell rejection have been discussed previously. In humans, while the importance of class I mismatching, and particularly that of HLA-C,[1238] might suggest a role for NK cell-mediated rejection, clear evidence for NK cell-mediated rejection has not yet been obtained.[1239] In contrast, a role for classical CTLs is well established in the rejection of even HLA-identical donor marrow following myeloablative conditioning,[1240] particularly when the donor product is T cell depleted[1241,1242] or lowintensity conditioning is used.[1243] Expanded CTLs and or Th against donor MiHAs have been detected in association

with expanded recipient T cells in patients losing chimerism after nonmyeloablative HLA-identical HCT.[1204,1244] Rejection occurs more frequently in the setting of HLA-mismatched related or unrelated donor transplants[1238,1245] and increases even further with T-cell depletion.[1246,1247] Antidonor CTLs specific for the mismatched donor class I[1248] or class II[28,634] allele and class II-specific helper cells[634] have been associated with HLA-mismatched marrow rejection. Donor-specific alloantibodies in presensitized patients are predictive of rejection of mismatched transplants.[1249]

A third unique feature of HCT (as well as transplants of other organs that are rich in lymphoid tissue, such as small intestinal grafts and, to a lesser extent, liver grafts) is the ability of T cells in the allograft to mount an immunologic attack on the recipient's tissues, resulting in GVHD. While GVHD rates and severity are reduced using prophylaxis with nonspecific immunosuppressive drugs, GVHD still afflicts 30% to 50% of recipients of HLA-matched sibling HCT, though this may be reduced by substituting a mammalian target of rampamycin inhibitor for calcineurin inhibitors.[1250] HCT can be extended to additional patients by including umbilical cord blood registries in the donor search, as HLA mismatching is better tolerated in cord blood, possibly due to the naivety/immaturity of the immune cells.[1251,1252] The frequency and severity of the GVHD that develops when extensive HLA barriers are traversed has essentially precluded the routine performance of such transplants from adult donors, though some recent clinical trials using nonmyeloablative conditioning have achieved acceptable GVHD rates.[1206,1253] The establishment of large marrow donor registries and the recognition of common HLA haplotypes has permitted the performance of closely matched transplants from unrelated donors, but these transplants are also associated with a high incidence of severe GVHD, due to the existence of subtle HLA mismatches and more extensive minor histoincompatibilities[13,1251,1254,1255,1256] (see section on Donor Antigens Responsible for Graft Rejection).

The GVL effect of allogeneic lymphocytes, especially T cells, transferred with the donor graft, is due largely to recognition by donor T cells of host alloantigens, which are also expressed on malignant cells. Therefore, while T-cell depletion of the donor graft decreases the incidence of GVHD, this benefit is offset by increased relapse rates[1256] and increased risk of engraftment failure due to rejection.[1246] Graft rejection can be offset by more intensive host conditioning and high doses of donor hematopoietic stem cells, even with extensively (one haplotype) HLA-mismatched stem cell grafts,[1257] but at the expense of delayed

immune reconstitution.[1257,1258] While T cell-mediated GVL effects due to graftversus-host reactivity are greatly diminished with such an approach, this may be compensated for by the ability of NK cells to mediate GVL when the host lacks inhibitory MHC ligands recognized by the donor, at least for acute myelogenous leukemia.[424] Such alloreactive NK cells may also help to promote donor engraftment by eliminating alloresistant host T cells.[424]

A major and elusive goal in the HCT field has been to separate the GVL effect of donor T cells from their potential to cause GVHD. Recently, several new approaches for inhibiting GVHD have been attempted.[1259] The use of costimulatory blockade has been discussed previously. Blockade of proinflammatory cytokines such as TNF-α and IL-1 has shown some efficacy in animal models but were less effective in clinical trials.[386] Many other strategies, such as immune deviation and the use of NKT cells, have been discussed elsewhere in the chapter.

Another approach to separating GVL from the GVHD-inducing capacity of MHC-directed alloreactivity is to separate the HCT and the administration of donor T cells in time, so that the T cells are given after some host recovery from the initial conditioning regimen has occurred. Established mixed hematopoietic chimeras are immunologically tolerant of their original marrow donor's antigens. As expected, a graft-versus-host reaction occurs after administration of nontolerant DLIs, resulting in conversion of mixed hematopoietic chimerism to full donor chimerism. Remarkably, this powerful graft-versus-host alloreaction against lymphohematopoietic cells is not associated with GVHD in mice, even though donor T cells are given in numbers that would cause rapidly lethal GVHD in freshly conditioned recipients.[642,643] Although antihost MHC alloreactivity mediates the most potent GVL effects[268,1260] and GVH-reactive donor T cells can clearly be shown to be activated and proliferating in mixed chimeric recipients of DLI,[268,644] these cells do not migrate to GVHD target tissues (epithelial tissues such as skin, intestines, and liver) unless there are inflammatory signals in those tissues.[644] Such inflammatory signals can be induced by conditioning therapy[645] or by TLR activation,[644] which may be induced by infection or gut translocation of bacteria. The observation that graft-versus-host reactions can be confined to the lymphohematopoietic system suggests an approach to separating GVHD from GVL reactions, as hematologic malignancies reside largely in the lymphohematopoietic system. Proof of principle has been obtained that similar results can be achieved in patients receiving nonmyeloablative BMT with in vivo T-cell depletion of the donor and recipient, followed by delayed DLI for the treatment of lymphomas.[1203,1206] In addition to recovery of epithelial GVHD target tissues from conditioning-induced injury, increasing resistance to GVHD with time may be conferred by recovering T_{reg}-cell populations that downregulate graftversus-host reactions.[1261,1262,1263] Delayed DLI is somewhat variably associated with GVHD in patients, but generally to a lesser degree than would be expected in freshly conditioned recipients of similar cell numbers.[1235,1264]

Graft-versus-host reactions can also be confined to lymphohematopoietic tissues to prevent GVHD by preventing their egress from lymphoid tissues with FTY-720,[1265] preventing attraction to epithelial target tissues by blocking chemokines,[1266,1267,1268] or by reducing tissue inflammation with keratinocyte growth factor,[1269,1270] TLR or MyD88 inhibition, adenosine triphosphate inhibitors, or the nucleotide oligomerization domain/CARD15 axis.[1259]

The surprising protective effect of IL-2 against GVHD[581] may be due in part to its ability to promte T_{reg} expansion, an effect that can be enhanced by administering rapamycin.[1271] Additional approaches to expanding T_{reg}s in vivo include manipulation of DCs with histone deactylase inhibitors,

which results in numerous immunomodulatory effects in addition to T_{reg} expansion.[1259] A clinical trial has been initiated using expanded third-party T_{reg}s in patients receiving cord blood transplantation, but efficacy was not determined in the phase I trial.[1272]

A variety of promising inhibitors of T cells are being explored clinically, such as IL-21 blockade,[1273] protein kinase C theta blockade, and proteasome inhibitors.[1259] Imatinib, a tyrosine kinase inhibitor with strong activity against Philadelphia chromosome-positive leukemias, also inhibits platelet-derived growth factor receptor (PDGF-R) signaling and has promising activity against the fibrosis associated with cGVHD.[1259]

Additional strategies for separating GVHD from allogeneic graft-versus-tumor effects include the transduction of donor T cells with suicide genes so that the alloresponse can be turned off at will, hopefully after residual tumor has been eradicated.[1210,1274,1275] Another approach is to avoid the graftversus-host alloresponse and try to target the donor immune response to tumor-specific antigens.[1276] Minor histocompatibility alloantigens expressed by lymphohematopoietic cells (including leukemias and lymphomas) may also be targeted using in vitro expanded CTLs.[75,1277] Immunization of HCT donors with tumor antigens could help to overcome the very limited frequency of T cells with these specificities,[103] as memory CD4 cells may have reduced GVHD-inducing capacity compared to naïve cells.[1278,1279]

Xenotransplantation

Over the past two decades, the increasing shortage of allogeneic donor organs has evoked a worldwide resurgence of interest in xenotransplantation, that is, the replacement of human organs or tissues with those from a donor of a different species. Routine clinical application of this therapeutic modality is still in the future. However, recent progress, which is reviewed briefly here, offers cause for optimism.

Concordant versus Discordant Xenotransplantation

Xenotransplants have been classified into two groups— “concordant” and “discordant”—on the basis of phylogenetic distance between the species combination, speed of the rejection, and levels of detectable preformed antibodies.[1280] Animals that are evolutionarily closely related and that have minimal or no preformed natural antibodies specific for each other are called “concordant,” whereas animals that belong to evolutionarily distant species and reject organs in a hyperacute manner are termed “discordant.”

Choice of Donor Species for Clinical Xenotransplantation

From a phylogenetic viewpoint, nonhuman primate organs would undoubtedly be the most similar to allotransplants immunologically. However, due to considerations of size, availability, and likelihood of transmission of infectious disease, most investigators have decided against the use of primates as a future source of xenogeneic organs. Instead, the discordant species, swine, has been chosen by many as the most suitable xenograft donor. The pig has

essentially unlimited availability, as well as favorable breeding characteristics, and many of its organ systems are similar to those of humans. Partially inbred miniature swine are a particularly attractive choice, because of their size (adult weights of approximately 120 kg), their physiology (also similar to humans for many organ systems), and their breeding characteristics, which have permitted inbreeding and genetic manipulation.[1281]

Mechanisms of Xenograft Rejection

Xenografts are subject to all four of the rejection mechanisms described previously in this chapter and give rise to more powerful immune responses than allografts, probably for each type of rejection. Primates have large amounts of "natural antibodies," so-called because they are present even though a primate has never been exposed to tissues from pigs. The reason for the presence of these antibodies is that during evolution, at the level of Old World primates, the gene for a particular enzyme was apparently lost due to mutation. This enzyme, α-1,3-galactosyltransferase, puts the sugar αgalactose (αGal) onto cell surface proteins in all species except for Old World primates and humans.[1282] Because the αGal antigen is found on bacteria and other environmental antigens, humans and Old World primates make a large amount of antibody against αGal. It is these anti-Gal antibodies that then react with pig tissues after xenotransplantation, causing vigorous rejection.

In addition, the hyperacute rejection that occurs with pig-to-primate transplantation is more vigorous than in the case of allogeneic blood group disparities. This is true, at least in part, because the complement regulatory proteins expressed by pig endothelium are less efficient in controlling human complement activation than are the human regulatory proteins expressed by human organs. Thus, these molecular incompatibilities also contribute to the increased intensity of the hyperacute rejection mechanism.

Similarly, the factors responsible for accelerated graft rejection are more prominent in xenogeneic than in allogeneic transplantation, probably also because the process is magnified considerably by the failure of regulatory molecules to function effectively with human coagulation factors, thus increasing the tendency toward intravascular thrombosis.[161] The available evidence also suggests that cellmediated immune responses to xenografts are more powerful than those directed to allografts.[1283] Initially, there was some uncertainty about this point as cell-mediated immune responses to xenogeneic stimulating cells were first studied using mouse T cells, for which molecular incompatibilities with human cells lead to weaker direct recognition of xenogeneic than allogeneic stimulators in vitro. In this case, the incompatibilities turned out to involve the accessory molecules that are required for T-cell activation rather than a lack of antigens that stimulate TCRs. Thus, it seemed that cellmediated rejection in vivo might also be weak. However, cell-mediated xenograft rejection, even by mice, has consistently been found to be extremely powerful in vivo, apparently initiated by CD4+ T cells responding to the many additional antigenic peptides through the indirect pathway.

For reasons of potential clinical applicability, greatest attention has been directed at investigation of the

human-antipig cellular response. In contrast to the murine studies, direct responses by human CD4 and CD8 T cells to pig stimulators can be readily measured in vitro.[1284] In addition, the cell-mediated reaction in vitro has been found to include a significant contribution by NK cells that can lyse pig targets. Most of the other molecular interactions involved in cellular immunity that have been examined appear to be at least partially

functional in primate antipig responses. Therefore, human-antipig T-cell responses are likely to be as great or greater than those in allogeneic combinations.

Therapeutic Strategies for Xenotransplantation

There are three main strategies that have been pursued to achieve long-term survival of xenogeneic transplants. The first has been to seek nonspecific immunosuppressive drugs that might prove especially effective for xenotransplantation. Unfortunately, none of the new drugs that have contributed to improved outcomes for allografts have proven sufficient alone to make xenografting possible, and considering the immunologic barriers to xenotransplantation discussed previously, it is unlikely that any such drug exists. Furthermore, the heightened immune response to xenografts compared to allografts suggests that larger amounts of exogenous immunosuppression will be required to achieve xenograft survival comparable to that of allografts. Given the narrow therapeutic window that already exists in allogeneic transplantation, most investigators believe that more than just immunosuppressive drugs will be needed to accomplish widespread clinical application of xenogeneic transplantation. Data so far for long-term survival of functioning pig-to-primate transplants of organs[960] support this impression. However, for islets, recent results with immunosuppression have become sufficiently promising that clinical trials are planned in the United States[1285] and are apparently already underway in New Zealand, although precise details are so far unpublished.

The second therapeutic approach has been to use genetic engineering of donor animals to lessen the immunologic barriers to xenografts. Because the two features that distinguish xenografts from allografts are the larger number of antigens and the molecular incompatibilities between species, these genetic modifications have been aimed primarily at correcting these two disadvantages of xenografts (Fig. 46.13). The first transgenic pigs produced by genetic engineering for xenotransplantation attempted to overcome the species selectivity of complement regulatory proteins. Transgenic pigs were produced that expressed human genes for several of these proteins. Organs from animals expressing one of these molecules (hDAF) have been studied extensively and appear to be more resistant to hyperacute rejection than are those from wild-type pigs,[366] but are nevertheless susceptible to acute humoral rejection.[1286] Numerous other transgenes (see Fig. 46.13) have been tested in attempts to further alter the primate host's humoral response to pig xenografts, but none to date has prolonged the survival of organ transplants beyond the survivals achieved with hDAF organs.[161]

The alternative genetic engineering strategy to the addition of transgenes is the removal or inactivation of genes through knockout technology.[1287] The advent of cloning through nuclear transfer technology, first demonstrated in the famous sheep “Dolly,”[1288] has made knockout technology possible in several large animal species, including pigs. As, as described previously, the major stumbling block in pig-to-primate xenotransplantation has been the large amount of natural antibody in primates directed toward the Gal determinant, investigators have utilized this technology to eliminate this same enzyme from pigs.[1289,1290,1291,1292] These new knockout pigs (called GalT-KO), like humans and Old World primates, do not put αGal on to the surface of their

cells. As a result, xenotransplants can now be performed without the powerful rejection previously caused by natural anti-Gal antibodies. The results have been remarkable, with

increased survivals of both heart and kidney transplants from pigs to baboons.[961,1293,1294] Using immunosuppressive drugs, organ survivals were prolonged using these new Gal knockout (GalT-KO) pigs, but new antibodies soon appeared, causing rejection.[961,1293,1294] However, using a regimen directed toward induction of tolerance (see the following), organ survivals were prolonged markedly, and no rejection was seen.[960] There are still problems to be resolved before such transplants will be attempted in patients, but survivals are now being measured in months rather than in days, as they were only a few years ago.

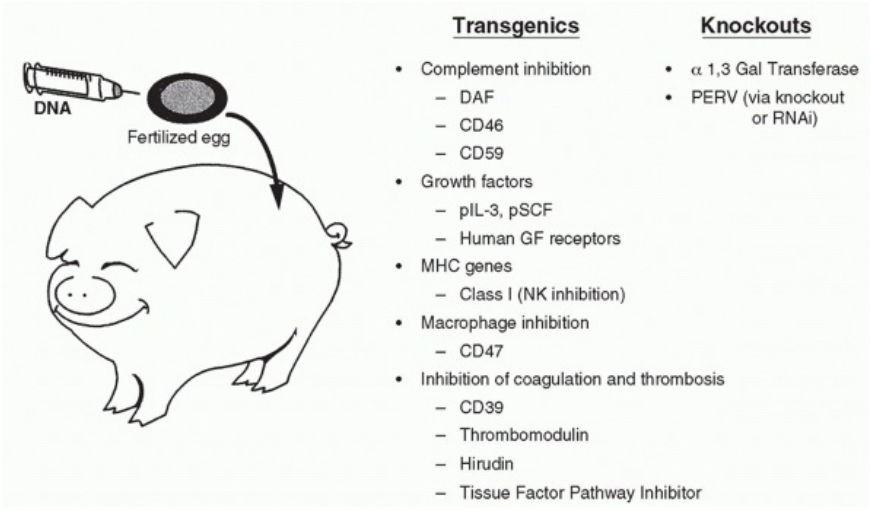

FIG. 46.13. Genetically Modified Pigs as Xenograft Source Animals. GF, growith factors; NK, natural killer; PERV, pig endogenous retroviruses.

The third strategy to achieve successful xenotransplantation is the induction of tolerance of donor antigens. Potential applications of this strategy have been described previously in this chapter, with reference mainly to transplantation in rodent models. There have been attempts to utilize either mixed chimerism or thymic transplantation to induce tolerance across xenogeneic barriers in primates. So far, long-term success by the mixed chimerism approach has been attained only for concordant cynomolgus monkey to baboon renal transplants.[1295] Both mixed chimerism and thymic transplantation approaches had been attempted for the discordant pig to baboon combination before the availability of GalT-KO swine, but with limited success.[1296,1297,1298] However, combining the thymic transplantation approach with the use of the GalT-KO as a source of donor kidneys has extended survivals to over 3 months.[960] Thus, it seems possible that elimination of the natural antibody problem along with tolerance induction could make discordant xenotransplantation as successful as allogeneic transplantation in providing a long-term solution for patients waiting for transplants. Of course, it is possible that when these barriers are overcome, other obstacles, not yet apparent, will still limit the survival of xenogeneic transplants, and additional measures will be required to achieve success.

Nonimmunologic Barriers to Xenotransplantation

In addition to the immunologic mechanisms that prevent successful xenografting, there are two other potentially important obstacles to clinical application. First, molecular

incompatibilities between species may cause physiologic dysfunction of xenogeneic organs. This kind of incompatibility is least likely for the heart, for which the function, albeit vital, is relatively simple. It could certainly become an obstacle, however, for the liver, as the liver is known to produce so many different products, including serum proteins and enzymes, that it is likely that at least some of these products may not function properly in a primate host. On the other hand, if the number of incompatibilities leading to physiologic dysfunction is limited, they could be correctable through knockout and transgenic technology. Thus, the physiologic dysfunction of xenogeneic organs is unlikely to be an insurmountable barrier to xenotransplantation.

The other nonimmunologic barrier to xenotransplantation is the risk of cross-species transfer of infectious agents, potentially creating a health hazard, not only for the recipient, but also for society as a whole. This possibility has gained significant attention, both in the scientific literature and in the lay press, and the issue has become confused by enormous uncertainties about the true risks that are involved. “Zoonosis” is a term that has been used for some time to describe the general process of cross-species infection, and the term “xenozoonosis” has been developed to describe infection transmission that might occur as a result of xenotransplantation.[1299] It is important to realize that from the point of view of the individual recipient, the risk of transmitting infection by xenotransplantation is likely to be less than by current clinical allotransplantation, both because of the natural resistance to cross-species transmission of infectious diseases and because it should be possible to screen for and eliminate the presence of known pathogens from the herd of donor pigs.

The major infectious concern, therefore, is that endogenous retroviral sequences from donor cells might infect the recipient's cells, giving rise to previously unrecognized pathogenic viruses.[1300] The concern has been raised that such new viruses might prove hazardous to other human beings in addition to the xenograft recipient.[1301] However, to date there is no evidence that such cross-species transfer after a pig-to-human transplant would generate a virus that would be infectious or pathogenic. Indeed, studies of humans exposed to pig tissues have not revealed any cases of detectable pig endogenous retroviruses.[1302,1303] Nevertheless, the concern about infections from xenotransplantation involves fear of the unknown, for which it is impossible to assign an accurate level of risk. At this time, therefore, public health agencies and members of the transplant community are attempting to design rational approaches for identifying the true risks of xenotransplantation and detecting untoward events rapidly, while at the same time allowing further progress in this potentially enormously important field of transplantation.[1304]

Clinical Progress in Xenotransplantation

Early clinical efforts in xenotransplantation took place in the 1960s and involved organ transplants from nonhuman primates. One of the patients survived for 9 months with normal renal function provided by the kidney of a chimpanzee.[1305] Additional clinical trials thereafter, using baboon hearts and livers, were considerably less successful. More recent clinical trials have involved fetal pig cells transplanted into the brains of patients with Parkinson or Huntington diseases. Survival of pig tissue 8 months after the transplant was documented in a patient taking only moderate doses of immunosuppression.[1306] These studies suggest that cellular xenotransplantation may be achieved more easily, and thus may be performed sooner, than solid organ transplants, especially because free cellular transplants lack the vascular endothelium that is the target for both hyperacute and accelerated rejection. Indeed, xenogeneic islet transplants have already been performed in

New Zealand (see www.sciencemediacentre.co.nz/2011/03/22/nzbio-the-potential-of-livingcell-therapies/), and the recent long-term successes reported for porcine islet transplants into nonhuman primates[1307] has made it likely that clinical trials will soon be initiated in the

United States. For organ xenotransplantation, most investigators believe that further clinical trials should await a reasonable expectation of success on the basis of pig-to-nonhuman primate experimental studies, a goal that is becoming closer but has not yet been realized.

SOME IMMUNOLOGIC ISSUES IN CLINICAL TRANSPLANTATION

The Effect of Antigen Matching on Organ Graft Survival: Clinical Data

In the absence of transplantation antigen disparities, graft rejection does not occur. However, the importance of antigen matching is one of the controversial issues in clinical transplantation. The evidence from transplantation of kidneys using living related donors provides a clinical demonstration of the importance of HLA antigen matching in subsequent graft survival. Data from a large international database on kidney allograft survival from 1985 to 1999 showed a survival half-life of HLA-identical sibling allografts of 23.4 years, as compared to 12.8 years for haploidentical-related allografts. These data support the basic concept that antigen matching matters and that the use of HLA-matched sibling donors is advantageous. The advantage of complete HLA matching for unrelated donors is also clear, although less compelling than for siblings, and has led to the United Network for Organ Sharing (UNOS)-mandated shipping of kidneys to distant centers for fully matched transplants from deceased donors. Whether or not there are advantages to partial HLA matches over full mismatches remains a subject of controversy, and most surgeons consider the length of the ischemic time necessitated by shipping of deceased donor organs as more important than the degree of HLA mismatch, as long as there is a negative crossmatch (see the following).

Human Leukocyte Antigen in Hematopoietic Cell Transplantation

In contrast to the results described previously for solid organ transplants, the importance of HLA matching for unrelated HCT is unquestioned. Although restriction to HLA-matched donors has severely limited the availability of HCT (only 25% of individuals have an HLA-identical sibling; another 5% has a single antigen-mismatched-related donor), severe GVHD and rejection have been prohibitive limitations in the presence of extensive HLA disparity, as is discussed previously in this chapter. High-resolution, polymerase chain reaction-based HLA typing methods have improved the ability to identify mismatches in unrelated donors, and matching at this level has improved outcomes.[1308,1309] While genotypic matching with unrelated donors is possible for patients with major conserved HLA haplotypes,[13,14] this level of typing reduces the chance of finding a fully matched unrelated donor for patients with uncommon HLA types, even with the availability of large registries such as the National Marrow Donor Program in the United States.[1255] Already, many patients do not succeed in finding an unrelated donor, and thus the ongoing effort to identify “acceptable” mismatches in the unrelated donor setting is important.[1310,1311]

Crossmatch

There are several tests designed to detect preexisting antibodies with specificity for donor antigens in the serum of potential recipients. First, recipients and donors must be matched for

ABO blood type, because, with the exception of organs from donors of the A_2 blood group, transplantation across blood group barriers may cause hyperacute rejection (see previous discussion). Second, immediate pretransplant sera from prospective recipients are tested for reactivity against lymphocytes of potential donors. This "crossmatch" is generally performed by a two-step, antibody-mediated, complement-dependent cytotoxicity assay, although methods utilizing flow cytometry and luminex beads are now becoming more popular in many centers. However, whether a positive crossmatch detected only by the highly sensitive luminex bead assay should be considered in allocation of deceased donor kidneys is controversial.[1312]

"Sensitized" Candidates for Organ Transplantation

Because kidneys and many other vascularized organs cannot be transplanted safely into recipients with preexisting antibodies, the clinical goal is to avoid transplantation of organs that express HLA antigens against which the recipient has been sensitized. Except for blood group antibodies, recipient sensitization to transplantation antigens generally occurs by prior exposure to alloantigens as a result of blood transfusion, previous organ transplantation, or, in women, by exposure to paternal antigens on fetal cells during pregnancy and parturition. The degree of sensitization against a potential kidney donor is measured by testing recipient serum on a panel of lymphocytes expressing a broad representation of HLA antigens, thereby assessing panel reactive antibody, as defined previously. Highly sensitized candidates may wait many years to receive a kidney transplant.

The level of sensitization may decrease over time, leading to a negative crossmatch with recently obtained serum, but a positive crossmatch using previously collected sera. Transplantation in the face of this "historical positive crossmatch" has been performed successfully. Until recently, obtaining crossmatch-negative donors by searching for well-matched organs or waiting for a decline in the level of sensitization represented the primary solutions available for sensitized patients. Recently, however, several centers have begun to transplant kidneys in the face of antidonor antibodies by a variety of "desensitization" procedures, including plasmapheresis,[1313] treatment with anti-CD20 mAb (rituximab),[1314,1315,1316] intravenous immunoglobulin,[1317] and/or bortezamib (Velcade, Millenium Pharmaceuticals, Cambridge, MA),[1318] followed by immunosuppression geared toward preventing antibody responses.[130,158,1319]

The Diagnosis of Rejection

In clinical organ transplantation, the most obvious manifestation of the rejection process is usually diminished function of the transplanted organ, but it is important to confirm the immunologic origin of the event before increasing immunosuppression. The measurement of urinary perforin and granzyme B levels may be useful in this diagnosis.[1320,1321]

Nevertheless, the "gold standard" remains histologic examination of the biopsy of the transplanted organ itself. Pathologists have been able to identify the abnormal lymphocytic infiltrate within grafts, to grade the intensity of the infiltrate, and, for some organs, to describe histologic findings characterizing the effects of immunologic injury.[165] Some pathologic changes, including a lymphocytic infiltrate of the vascular wall, seem to be well correlated with rejection activity. In addition, pathologic changes suggesting nonimmunologic causes of renal dysfunction may be helpful in patient management. The immunohistologic finding of

deposition of the complement component C4d in the peritubular capillaries is generally considered to be indicative of antibody-mediated rejection, especially in the kidney.[1322]

Despite the widespread reliance on the biopsy to define episodes of rejection, however, rejection is sometimes difficult to differentiate from drug toxicity and/or viral infection. Furthermore, when routine "protocol" biopsies of well-functioning transplanted organs have been performed, histologic findings have often revealed cellular infiltrates similar to those of rejection. These results are consistent with several experimental models of tolerance induction that have shown intense lymphocytic infiltrates in organs that go on to survive indefinitely and/or in recipients who develop tolerance to the donor antigens.[1323,1324] These studies suggest that the amount of lymphocytic infiltrate detected pathologically may not be helpful in diagnosing rejection episodes and determining the need for treatment.

How Much Immunosuppression is Enough?

While the majority of transplant recipients respond immunologically to their new organ despite immunosuppression, some patients seem never to generate any rejection activity and maintain their transplanted organ with very small doses of immunosuppressive drugs. Indeed, a few patients have been known to stop all of their medications but have kept their transplant for years without rejection. On the other hand, some patients seem to require and tolerate very high doses of exogenous immunosuppression, while others seem to be severely immunocompromised by low doses of these drugs. These observations make it clear that the amount of immunosuppression that is required or that is safe is not the same for every individual or for all grafts. Unfortunately, there is no well-established assay to determine the amount of immunosuppression an individual requires and can safely tolerate for their particular transplant.

CONCLUSION

The great danger in any textbook chapter is that the need to summarize what we think is known will obscure the much greater amount still left to be learned. For example, we have recently gained important insight into the role of APCs in T-cell sensitization, but we still have not explored adequately the role that indirect presentation of alloantigens plays in graft rejection. During the past two decades, we have learned much about the generation and function of CTLs and about their likely role in some mechanisms of graft rejection and GVHD; however, our understanding of noncytolytic mechanisms of rejection and GVHD, and of the role of B cells and alloantibodies, is much more limited. Finally, this chapter has outlined several techniques for the generation of immunologic tolerance to alloantigens in experimental systems; however, the first human beings have only recently been transplanted with tolerance-inducing regimens that allow the early discontinuation of nonspecific immunosuppression and the mechanisms of tolerance, which involve the kidney graft itself, are incompletely understood. The encouraging initial results raise hopes that routine tolerance induction may soon become a broader clinical reality that can be extended to other organs besides the kidney. It is, of course, the great fascination of transplantation immunology that new insights into basic immunologic issues will likely have important consequences for clinical transplantation in the future.

REFERENCES

1. Billingham RE. Transplantation: past, present and future. *J Invest Dermatol* . 1963;41:165-180.

2. Carrel A. Transplantation in mass of kidneys. *J Exp Med*. 1908;10:98-140.

3. Murray JE. The 50th anniversary of the first successful human organ transplant. *Rev Invest Clin*. 2005;57:118-119.

4. Medawar PB. A second study of the behaviour and fate of skin homografts in rabbits; a report to the War Wounds Committee of the Medical Research Council. *J Anat*. 1945;79:157-176.

5. Sachs DH. MHC homozygous miniature swine. In: Swindle MM, Moody DC, Phillips LD, eds. *Swine as Models in Biomedical Research*. 1st ed. Ames, IA: Iowa State University Press; 1992:3-15.

6. Mezrich JD, Haller GW, Arn JS, Houser SL, Madsen JC, Sachs DH. Histocompatible miniature swine: an inbred large-animal model. *Transplantation*. 2003;75:904-907.

7. Gianello P, Fishbein JM, Sachs DH. Tolerance to primarily vascularized allografts in miniature swine. *Immunol Rev*. 1993;133:19-44.

8. Okumi M, Fishbein JM, Griesemer AD, et al. Role of persistence of antigen and indirect recognition in the maintenance of tolerance to renal allografts. *Transplantation*. 2008;85:270-280.

9. Little CC. The genetics of tissue transplantation in mammals. *Cancer Res*. 1924;8:75-95.

10. Bailey DW, Mobraaten LE. Estimates of the number of loci contributing to the histoincompatibility between C57BL-6 and BALB-c strains of mice. *Transplantation*. 1969;7:394-400.

11. Snell GD. Methods for the study of histocompatibility genes. *J Genet*. 1948;49:87-108.

12. Marsh SG, Albert ED, Bodmer WF, et al. Nomenclature for factors of the HLA system, 2010. *Tissue Antigens*. 2010;75:291-455.

13. Baschal EE, Aly TA, Jasinski JM, et al. Defining multiple common “completely” conserved major histocompatibility complex SNP haplotypes. *Clin Immunol*. 2009;132:203-214.

14. Morishima S, Ogawa S, Matsubara A, et al. Impact of highly conserved HLA haplotype on acute graft-versus-host disease. *Blood*. 2010;115:4664-4670.

15. Harris HW, Gill TJ. Expression of class I transplantation antigens. *Transplantation*. 1986;42:109.

16. Daar AS, Fuggle SV, Fabre JW, Ting A, Morris PJ. The detailed distribution of MHC class II antigens in normal human organs. *Transplantation*. 1984;38:293-298.

17. Glimcher LH, Kara CJ. Sequences and factors: a guide to MHC class-II transcription. *Ann Rev Immunol*. 1992;10:13-49.

18. Pescovitz MD, Sachs DH, Lunney JK, Hsu SM. Localization of class II MHC antigens on porcine renal vascular endothelium. *Transplantation*. 1984;37:627-630.

19. Choo JH, Seebach JD, Nickeleit V, et al. Species differences in the expression of MHC class II on coronary artery endothelium: implications for cell-mediated xenoreactivity. *Transplantation*. 1997;64:1315-1322.

20. Houser SL, Benjamin LC, Wain JC, Madsen JC, Allan JS. Constitutive expression of major histocompatiblity complex class II antigens in pulmonary epithelium and endothelium varies among different species. *Transplantation*. 2004;77:605-607.

21. Guillet M, Sebille F, Soulillou J. TCR usage in naive and committed alloreactive cells: implications for the understanding of TCR biases in transplantation. *Curr Opin Immunol*. 2001;13:566-571.

22. Sebille F, Gagne K, Guillet M, et al. Direct recognition of foreign MHC determinants by naive T cells mobilizes specific Vbeta families without skewing of the complementarity-determining region 3 length distribution. *J Immunol*. 2001;167:3082-3088.

23. Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. *J Immunol Methods*. 1994;171:131-137.

24. Noorchashm H, Lieu YK, Rostami SY, et al. A direct method for the calculation of alloreactive CD4+ T cell prescursor frequency. *Transplantation*. 1999;67:1281-1284.

25. Fischer Lindahl K, Wilson DB. Histocompatibility antigen-activated cytotoxic T lymphocytes II. Estimates of frequency and specificity of precursors. *J Exp Med*. 1977;145:508-522.

26. Teh HS, Harley E, Phillips RA, Miller RG. Quantitative studies on the precursors of cytotoxic lymphocytes I. Characterization of a clonal assay and determination of the size of clones derived from single precursors. *J Immunol*. 1977;118:1049-1056.

27. Suchin EJ, Langmuir PB, Palmer E, Sayegh MH, Wells AD, Turka LA. Quantifying the frequency of alloreactive T cells in vivo: new answers to an old question. *J Immunol*. 2001;166:973-981.

28. Fleischhauer K, Zino E, Mazzi B, et al. Peripheral blood stem cell allograft rejection mediated by CD4(+) T lymphocytes recognizing a single mismatch at HLA-DPbeta1*0901. *Blood*. 2001;98:1122-1126.

29. Orosz CG, Bishop DK. Limiting dilution analysis of alloreactive T-cell status and distribution during allograft rejection. *Hum Immunol*. 1990;28:72-81.

30. Volk H-D, Kern F. Insights into the specificity and function of (allo) antigen-reactive T cells. *Am J Transplant*. 2001;1:109-114.

31. Blackman M, Yague J, Kubo R, et al. The T cell repertoire may be biased in favor of MHC recognition. *Cell*. 1986;47:349-357.

32. Kappler JW, Wade T, White J, et al. A T cell receptor V beta segment that imparts reactivity to a class II major histocompatibility complex product. *Cell*. 1987;49:263-271.

33. Zerrahn J, Held W, Raulet DH. The MHC reactivity of the T cell repertoire prior to positive and negative selection. *Cell*. 1997;88:627-636.

34. Surh CD, Lee DS, Fung-Leung W, Karlsson L, Sprent J. Thymic selection by a single MHC/peptide ligand produces a semidiverse repertoire of CD4+ T cells. *Immunity*. 1997;7:209-219.

35. Bevan MJ. In thymic selection, peptide diversity gives and takes away. *Immunity*. 1997;7:175-178.

36. Tourne S, Miyazaki T, Oxenius A, et al. Selection of a broad repertoire of CD4+ T cells in H-2Ma0/0 mice. *Immunity*. 1997;7:187-195.

37. Grubin CE, Kovats S, deRoos P, Rudensky AY. Deficient positive selection of CD4 T cells in mice displaying altered repertoires of MHC class II-bound self-peptides. *Immunity*. 1997;7:197-208.

38. Huseby ES, White J, Crawford F, et al. How the T cell repertoire becomes peptide and MHC specific. *Cell*. 2005;122:247-260.

39. Huseby E, Kappler J, Marrack P. TCR-MHC/peptide interactions: kissing-cousins or a shotgun wedding? *Eur J Immunol* . 2004;34:1243-1250.

40. Scott-Browne JP, Crawford F, Young MH, et al. Evolutionarily conserved features contribute to αβ T cell receptor specificity. *Immunity*. 2011;35:526-535.

41. Rotzschke O, Falk K, Faath S, Rammensee HG. On the nature of peptides involved in T cell alloreactivity. *J Exp Med*. 1991;174:1059-1071.

42. Obst R, Netuschil N, Klopfer K, Stevanovic S, Rammensee HG. The role of peptides in T cell alloreactivity is determined by self-major histocompatibility complex molecules. *J Exp Med*. 2000;191:805-812.

43. Crawford F, Huseby E, White J, Marrack P, Kappler JW. Mimotopes for alloreactive and conventional T cells in a peptide-MHC display library. *PLoS Biol*. 2004;2:E90.

44. Berkowitz N, Braunstein NS. T cell responses specific for subregions of allogeneic MHC molecules. *J Immunol*. 1992;148:309-317.

45. Müellbacher A, Hill AB, Blanden RV, Cowden WB, King NJ, Hla RT. Alloreactive cytotoxic T cells recognize MHC class I antigen without peptide specificity. *J Immunol*. 1991;147:1765-1772.

46. Smith PA, Brunmark A, Jackson MR, Potter TA. Peptide-independent recognition by alloreactive cytotoxic T lymphocytes (CTL). *J Exp Med*. 1997;185:1023-1033.

47. Crumpacker DB, Alexander J, Cresswell P, Engelhard VH. Role of endogenous peptides in murine allogeneic cytotoxic T cell responses assessed using transfectants of the antigen-processing mutant 174xCEM. T2. *J Immunol*. 1992;148:3004-3011.

48. Heath WR, Kane KP, Mescher MF, Sherman LA. Alloreactive T cells discriminate among a diverse set of endogenous peptides. *Proc Natl Acad Sci U S A* . 1991;88:5101-5105.

49. Aosai F, Ohlen C, Ljunggren HG, et al. Different types of allospecific CTL clones identified by their ability to recognize peptide loading-defective target cells. *Eur J Immunol*. 1991;21:2767-2774.

50. Felix NJ, Donermeyer DL, Horvath S, et al. Alloreactive T cells respond specifically to multiple distinct peptide-MHC complexes. *Nat Immunol*. 2007;8:388-397.

51. Amir AL, van der Steen DM, Hagedoorn RS, et al. Allo-HLA-reactive T cells inducing graft-versus-host disease are single peptide specific. *Blood*. 2011;118:6733-6742.

52. Morris GP, Ni PP, Allen PM. Alloreactivity is limited by the endogenous peptide repertoire. *Proc Natl Acad Sci U S A* . 2011;108:3695-3700.

53. Matzinger P, Bevan MJ. Why do so many lymphocytes respond to major histocompatibility complex antigens? *Cell Immunol*. 1977;29:1.

54. Garboczi D, Ghosh P, Utz U, et al. Structure ofthe complex between human T-cell receptor, viral peptide and HLA-A2. *Nature*. 1996;384:134-141.

55. Reinherz EL, Tan K, Tang L, et al. The crystal structure of a T cell receptor in complex with peptide and MHC class II. *Science*. 1999;286: 1913-1921.

56. Speir JA, Garcia KC, Brunmark A, et al. Structural basis of 2C TCR allorecognition of H-2Ld peptide complexes. *Immunity*. 1998;8:553-562.

57. Rudolph MG, Speir J, Brunmark A, et al. The crystal structures of K(bm1) and K(bm8) reveal that subtle changes in the peptide environment impact thermostability and alloreactivity. *Immunity*. 2001;14:231-242.

58. Demotz S, Sette A, Sakaguchi K, Buchner R, Appella E, Grey HM. Self peptide requirement for class II major histocompatibility complex allorecognition. *Proc Nat Acad Sci U S A*. 1991;88:8730-8734.

59. Weber DA, Terrell NK, Zhang Y, et al. Requirement for peptide in alloreactive CD4+ T cell recognition of class II MHC molecules. *J Immunol*. 1995;154:5153-5164.

60. Alexander-Miller MA, Burke K, Koszinowski UH, Hansen TH, Connolly JM. Alloreactive cytotoxic T lymphocytes generated in the presence of viral-derived peptides show exquisite peptide and MHC specificity. *J Immunol*. 1993;151:1-10.

61. Smith KD, Huczko E, Engelhard VH, Li VY, Lutz CT. Alloreactive cytotoxic T lymphocytes focus on specific major histocompatibility complex-bound peptides. *Transplantation*. 1997;64:351-359.

62. Moris A, Teichgraber V, Gauthier L, Buhring HJ, Rammensee HG. Cutting edge: characterization of allorestricted and peptide-selective alloreactive T cells using HLA-tetramer selection. *J Immunol*. 2001;166: 4818-4821.

63. Moris A, Wernet D, Stevanovic S, Rammensee HG. The peptide-specific alloreactive human T cell repertoire varies largely between individuals and is not extended in HLA-A*0205—anti-HLA-A*0201 pairings. *Int Immunol*. 2001;13:863-870.

64. Felix NJ, Brickey WJ, Griffiths R, et al. H2-DMalpha(-/-) mice show the importance of major histocompatibility complex-bound peptide in cardiac allograft rejection. *J Exp Med*. 2000;192:31-40.

65. Simpson E. Non-H-2 histocompatibiltiy antigens: can they be retroviral products? *Immunol Today*. 1987;8:176-177.

66. Roopenian DC, Widmer MB, Orosz CG, Bach FH. Response against single minor histocompatibility antigens. I. Functional immunogenetic analysis of cloned cytolytic T cells. *J Immunol*. 1983;131:2135-2140.

67. Roopenian DC, Orosz CG, Bach FH. Responses against single histocompatibility antigens. II. Analysis of cloned helper T cells. *J Immunol*. 1984;132:1080-1084.

68. Tekolf WA, Shaw S. Primary in vitro generation of cytotoxic cells specific for human minor histocompatibility antigens between HLA-identical siblings. *J Immunol*. 1984;132:1756-1760.

69. Czitrom AA, Gascoigne NR, Edwards S, Waterfield DJ. Induction of minor alloantigen-specific T cell subsets in vivo: recognition of processed antigen by helper but not by cytotoxic T cell precursors. *J Immunol*. 1984;133:33-39.

70. Bevan MJ. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med*.

1976;143:1283.

71. Wallny HJ, Rammensee HG. Identification of classical minor histocompatibility antigen as cell-derived peptide. *Nature*. 1990;343:275-278.

72. Elliott T, Townsend A, Cerundolo V. Antigen presentation. Naturally processed peptides. *Nature*. 1990;348:195-197.

73. Falk K, Rotzschke O, Rammensee HG. Cellular peptide composition governed by major histocompatibility complex class I molecules. *Nature*. 1990;348:248-251.

74. Perreault C, Roy DC, Fortin C. Immunodominant minor histocompatibility antigens: the major ones. *Immunol Today*. 1998;19:69-74.

75. Mutis T, Verdijk R, Schrama E, Esendam B, Brand A, Goulmy E. Feasibility of immunotherapy of relapsed leukemia with ex vivo-generated cytotoxic T lymphocytes specific for hematopoietic systemrestricted minor histocompatibility antigens. *Blood*. 1999;93:2336-2341.

76. den Haan JMM, Meadows LM, Wang W, et al. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. *Science*. 1998;279:1054-1057.

77. den Haan JMM, Sherman NE, Blokland E, et al. Identification of a graft versus host disease-associated human minor histocompatibility antigen. *Science*. 1995;268:1476-1480.

78. Mendoza LM, Paz P, Zuberi A, Christianson G, Roopenian D, Shastri N. Minors held by major: the H13 minor histocompatibility locus defined as a peptide/MHC class I complex. *Immunity*. 1997;7:461-472.

79. Brickner AG, Warren EH, Caldwell JA, et al. The immunogenicity of a new human minor histocompatibility antigen results from differential antigen processing. *J Exp Med*. 2001;193:195-206.

80. Warren EH, Gavin MA, Simpson E, et al. The human UTY gene encodes a novel HLA-B8-restricted H-Y antigen. *J Immunol*. 2000; 164:2807-2814.

81. Perreault C, Roy DC, Fortin C. Immunodominant minor histocompatibility antigens: the major ones. *Immunol Today*. 1998;19:69-74.

82. Simpson E, Roopenian D, Goulmy E. Much ado about minor histocompatibility antigens. *Immunol Today*. 1998;19:108-111.

83. Wang W, Meadows LR, den Haan JMM, et al. Human H-Y: a malespecific histocompatibility antigen derived from the SMCY protein. *Science*.1995;269:1588-1590.

84. Goulmy E. Human minor histocompatibility antigens. *Curr Opin Immunol* . 1996;8:75-81.

85. Vogt MH, Goulmy E, Kloosterboer FM, et al. UTY gene codes for an HLA-B60-restricted human male-specific minor histocompatibility antigen involved in stem cell graft rejection: characterization of the critical polymorphic amino acid residues for T-cell recognition. *Blood*. 2000;96:3126-3132.

86. Pierce RA, Field ED, Mutis T, et al. The HA-2 minor histocompatibility antigen is derived from a diallelic gene encoding a novel human class I myosin protein. *J Immunol*. 2001;167:3223-3230.

87. Simmons WA, Summerfield SG, Roopenian DC, et al. Novel HY peptide antigens presented by HLA-B27. *J Immunol*. 1997;159:2750-2759.

88. Malarkannan S, Horng T, Eden P, et al. Differences that matter: major cytotoxic T cell-stimulating minor histocompatibility antigens. *Immunity*. 2000;13:333-344.

89. Dierselhuis M, Goulmy E. The relevance of minor histocompatibility antigens in solid organ transplantation. *Curr Opin Organ Transplant* . 2009;14:419-425.

90. Nichols WC, Antin JH, Lunetta KL, et al. Polymorphism of adhesion molecule CD31 is not a significant risk factor for graft-versus-host disease. *Blood*. 1996;88:4429-4434.

91. Grumet FC, Hiraki DD, Brown BWM, et al. CD31 mismatching affects marrow transplantation outcome. *Biol Blood Marrow Transplant* . 2001;7:503-512.

92. Sarcon AK, Desierto MJ, Zhou W, et al. Role of perforin-mediated cell apoptosis in murine models of infusion-induced bone marrow failure. *Exp Hematol*. 2009;37:477-486.

93. Roopenian DC, David AP, Christianson GJ, Mobraaten LE. The functional basis of minor histocompatibility loci. *J Immunol*. 1993; 151:4595-4605.

94. Ryu SJ, Jung KM, Yoo HS, et al. Cognate CD4 help is essential for the reactivation and expansion of CD8 memory T cells directed against the hematopoietic cell-specific dominant minor histocompatibility antigen, H60. *Blood*. 2009;113:4273-4280.

95. Elkins WL. Decreased immunogenicity of a transplantation antigen in hosts sensitized to other isoantigens of its cellular vehicle. *J Immunol*. 1964;92:275.

96. Johnson LL, Bailey DW, Mobraaten LE. Antigenic competition between minor (non-H-2) histocompatibility antigens. *Immunogenetics*. 1981;13:451-455.

97. Wettstein PJ, Bailey DW. Immunodominance in the immune response to “multiple” histocompatibility antigens. *Immunogenetics*. 1982;16: 47-58.

98. Wettstein PJ. Immunodominance in the T cell response to multiple non-H-2

histocompatibility antigens III. Single histocompatibility antigens dominate the male antigen. *J Immunol*. 1986;137:2073.

99. Lai PK. Antigen competition in cytotoxic T cell response to minor histocompatibility antigens. *Transplantation*. 1985;39:638-643.

100. Nevala WK, Wettstein PJ. The preferential cytolytic T lymphocyte resonse to immunodominant minor histocompatibility antigen peptides. *Transplantation*. 1996;62:283-291.

101. Yoshimura Y, Yadav R, Christianson GJ, Ajayi WU, Roopenian DC, Joyce S. Duration of alloantigen presentation and avidity of T cell antigen recognition correlate with immunodominance of CTL response to minor histocompatibility antigens. *J Immunol*. 2004;172:6666-6674.

102. Choi EY, Christianson GJ, Yoshimura Y, et al. Immunodominance of H60 is caused by an abnormally high precursor T cell pool directed against its unique minor histocompatibility antigen peptide. *Immunity*. 2002;17:593-603.

103. Li N, Matte-Martone C, Zheng H, et al. Memory T cells from minor histocompatibility antigen-vaccinated and virus-immune donors improve GVL and immune reconstitution. *Blood*. 2011;118:5965-5976.

104. Korngold R, Wettstein PJ. Immunodominance in the graft-vs-host disease T cell response to minor histocompatibility antigens. *J Immunol*. 1990;145:4079-4088.

105. Gordon RD, Simpson E, Samelson LE. In vitro cell-mediated immune responses to the male specific (H-Y) antigen in mice. *J Exp Med*. 1975; 142:1108.

106. Simpson E, Gordon RD. Responsiveness to H-Y antigen, Ir gene complementation, and target cell specificity. *Immunol Rev*. 1997;35:59.

107. Simpson E. The role of H-Y as a minor transplantation antigen. *Immunol Today*. 1982;3:97-106.

108. Pierce RA, Field ED, den Haan JM, et al. Cutting edge: the HLAA* 0101-restricted HY minor histocompatibility antigen originates from DFFRY and contains a cysteinylated cysteine residue as identified by a novel mass spectrometric technique. *J Immunol*. 1999;163:6360-6364.

109. Voogt PJ, Fibbe WE, Marijt WAF, et al. Rejection of bone-marrow graft by recipient-derived cytotoxic T lymphocytes against minor histocompatibility antigens. *Lancet*. 1990;335:131-134.

110. Vogt MH, de Paus RA, Voogt PJ, Willemze R, Falkenburg JH. DFFRY codes for a new human male-specific minor transplantation antigen involved in bone marrow graft rejection. *Blood*. 2000;95:1100-1105.

111. Goulmy E, Schipper R, Pool J, et al. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. *New Engl J Med* . 1996;334:281-285.

112. Mutis T, Gillespie G, Schrama E, Falkenburg JH, Moss P, Goulmy E. Tetrameric HLA class I-minor histocompatibility antigen peptide complexes demonstrate minor histocompatibility antigen-specific cytotoxic T lymphocytes in patients with graft-versus-host disease. *Nat Med*. 1999;5:839-842.

113. Rufer N, Wolpert E, Helg C, et al. HA-1 and the SMCY-derived peptide FIDSYICQV (H-Y) are immunodominant minor histocompatibility antigens after bone marrow transplantation. *Transplantation*. 1998;66:910-916.

114. Scott D, Addey C, Ellis P, et al. Dendritic cells permit identification of genes encoding MHC class II- restricted epitopes of transplantation antigens. *Immunity*. 2000;12:711-720.

115. Milosevic S, Bachnick B, Karim K, et al. Identification of MHC II-restricted minor histocompatibility antigens after HLA-identical stem-cell transplantation. *Transplantation*. 2010;90:1030-1035.

116. Spaapen RM, de Kort RA, van den Oudenalder K, et al. Rapid identification of clinical relevant minor histocompatibility antigens via genome-wide zygosity-genotype correlation analysis. *Clin Cancer Res* . 2009;15:7137-7143.

117. Schorle H, Holtschke T, Hunig T, Schimpl A, Horak I. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature*. 1991;352:621-624.

118. Arase-Fukushi N, Arase H, Ogasawara K, Good RA, Onoe K. Production of minor lymphocyte stimulatory-1a antigen from activated CD4+ or CD8+ T cells. *J Immunol* . 1993;151:4445-4454.

119. Jarvis CD, Germain RN, Hager GL, Damschroder M, Matis LA. Tissue-specific expression of messenger RNAs encoding endogenous viral superantigens. *J Immunol* . 1994;152:1032-1038.

120. Moore NC, Anderson G, McLoughlin DE, Owen JJ, Jenkinson EJ. Differential expression of Mtv loci in MHC class II-positive thymic stromal cells. *J Immunol* . 1994;152:4826-4831.

121. Ardavin C, Waanders G, Ferrero I, et al. Expression and presentation of endogenous mouse mammary tumor virus superantigens by thymic and splenic dendritic cells and B cells. *J Immunol* . 1996;157:2789-2794.

122. Jones MS, Riley R, Hamilton BL, Paupe J, Perez D, Levy RB. Endogenous superantigens in allogeneic bone marrow transplant recipients rapidly and selectively expand donor T cells which can produce IFN-gamma. *Bone Marrow Transplant* .

1994;14:725-735.

123. Steinmuller D, Wachtel SS. Transplantation biology and immuno genetics of murine skin-specific antigens. *Transplant Proc*. 1990;12(suppl 1): 100-106.

124. Rosengard BR, Kortz EO, Ojikutu CA, et al. The failure of skin grafting to break tolerance to class I disparate renal allografts in miniature swine despite inducing marked anti-donor cellular immunity. *Transplantation*. 1991;52:1044-1052.

125. Lorenz R, Allen PM. Processing and presentation of self proteins. *Immunol Rev*. 1998;106:115-127.

126. Ildstad ST, Wren SM, Bluestone JA, Barbieri SA, Sachs DH. Characterization of mixed allogeneic chimeras. Immunocompetence, in vitro reactivity, and genetic specificity of tolerance. *J Exp Med*. 1985;162:231-244.

127. Warren EH, Greenberg PD, Riddell SR. Cytotoxic T-lymphocytedefined human minor histocompatibility antigens with a restricted tissue distribution. *Blood*. 1998;91:2197-2207.

128. Szulman AE. The histological distribution of the blood group substances in man as disclosed by immunofluorescence. II. The H antigen and its relation to A and B antigens. *J Exp Med*. 1962;115:977-996.

129. Montgomery RA, Locke JE, King KE, et al. ABO incompatible renal transplantation: a paradigm ready for broad implementation. *Transplantation*. 2009;87:1246-1255.

130. Jordan SC, Peng A, Vo AA. Therapeutic strategies in management of the highly HLA-sensitized and ABO-incompatible transplant recipients. *Contrib Nephrol*. 2009;162:13-26.

131. Jordan SC, Peng A, Vo AA. Therapeutic strategies in management of the highly HLA-sensitized and ABO-incompatible transplant recipients. *Contrib Nephrol*. 2009;162:13-26.

132. Seebach JD, Stussi G, Passweg JR, et al. ABO blood group barrier in allogeneic bone marrow transplantation revisited. *Biol Blood Marrow Transplant* . 2009;11:1006-1013.

133. Blin N, Traineau R, Houssin S, et al. Impact of donor-recipient major ABO mismatch on allogeneic transplantation outcome according to stem cell source. *Biol Blood Marrow Transplant*. 2010;16:1315-1323.

134. Platt JL, Bach FH. The barrier to xenotransplantation. *Transplantation*. 1991;52:937-947.

135. Fischel RJ, Bolman RM III, Platt JL, Najarian JS, Bach FH, Matas AJ. Removal of IgM anti-endothelial antibodies results in prolonged cardiac xenograft survival. *Transplant Proc*. 1990;22:1077-1078.

136. Kiessling R, Hochman PS, Haller O, Shearer GM, Wigzell H, Cudkowicz G. Evidence for a similar or common mechanism for natural killer activity and resistance to hemopoietic grafts. *Eur J Immunol* . 1997;7:655-663.

137. Peritt D, Robertson S, Gri G, Showe L, Aste-Amezaga M, Trinchieri G. Differentiation of human NK cells into NK1 and NK2 subsets. *J Immunol* . 1998;161:5821-5824.

138. Karlhofer FM, Ribaudo RK, Yokoyama WM. MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. *Nature*. 1992;358:66-70.

139. Moretta L, Ciccone E, Moretta A, Hoglund P, Ohlen C, Karre K. Allorecognition by NK cells: nonself or no self? *Immunol Today*. 1992;13:300-306.

140. Anfossi N, Andre P, Guia S, et al. Human NK cell education by inhibitory receptors for MHC class I. *Immunity*. 2006;25:331-342.

141. Raulet DH, Vance RE. Self-tolerance of natural killer cells. *Nat Rev Immunol* . 2006;6:520-531.

142. Moretta L, Ciccone E, Moretta A, Hoglund P, Ohlen C, Karre K. Allorecognition by NK cells: nonself or no self? *Immunol Today*. 1992;13:300-306.

143. Alwayn IP, Basker M, Buhler L, Cooper DK. The problem of anti-pig antibodies in pig-to-primate xenografting: current and novel methods of depletion and/or suppression of production of anti-pig antibodies. *Xenotransplantation*. 1999;6:157-168.

144. Hammer C, Land W, Brendel W. Experimental xenotransplantation in distantly related species systems. Mechanism of the xenogeneic hyperacute rejection reaction and its modification due to actively and passively produced enhancement [in German]. *Res Exp Med (Berl)*. 1973;159:239-265.

145. Rosenberg JC, Broersma RJ, Bullemer G, Mammen EF, Lenaghan R, Rosenberg BF. Relationship of platelets, blood coagulation, and fibrinolysis to hyperacute rejection of renal xenografts. *Transplantation*. 1969;8:152-161.

146. Dalmasso AP, Platt JL, Bach FH. Reaction of complement with endothelial cells in a model of xenotransplantation. *Clin Exp Immunol*. 1999;86(suppl 1):31-35.

147. Robson SC, Schulte AE, Bach FH. Factors in xenograft rejection. *Ann N Y Acad Sci*. 1999;875:261-276.

148. Platt JL, Vercellotti GM, Lindman BJ, Oegema TR Jr, Bach FH, Dalmasso AP. Release of heparan sulfate from endothelial cells. Implications for pathogenesis of hyperacute rejection. *J Exp Med*. 1990;171:1363-1368.

149. Doyle HR, Marino IR, Morelli F, et al. Assessing risk in liver transplantation. Special reference to the significance of a positive cytotoxic crossmatch. *Ann Surg*. 1996;224:168-

177.

150. Marshall DC, Friedman EA, Goldstein DP, Henry L, Merrill JP. Clinical criteria for evaluating first set, accelerated, and white graft rejection in human skin homografts. *Surg Forum*. 1969;12:469-470.

151. Mirenda V, Le Mauff B, Cassard A, et al. Intact pig pancreatic islet function in the presence of human xenoreactive natural antibody binding and complement activation. *Transplantation*. 1997;63:1452-1462.

152. Aksentijevich I, Sachs DH, Sykes M. Humoral tolerance in xenogeneic BMT recipients conditioned with a non-myeloablative regimen. *Transplantation*. 1992;53:1108-1114.

153. Ohdan H, Swenson KG, Kitamura H, Yang YG, Sykes M. Tolerization of Galα1,3Gal-reactive B cells in presensitized α1,3-galactosyltransferasedeficient mice by nonmyeloablative induction of mixed chimerism. *Xenotransplantation*. 2002;8:227-238.

154. Ohdan H, Yang YG, Swenson KG, Kitamura H, Sykes M. T cell and B cell tolerance to galα1,3gal-expressing heart xenografts is achieved in α1,3-galactosyltransferase-deficient mice by non-myeloablative induction of mixed chimerism. *Transplantation*. 2001;71:1532-1542.

155. Yang Y-G, Chen AM, Sergio JJ, Zhou Y, Sykes M. Role of antibodyindependent complement activation in rejection of porcine bone marrow cells in mice. *Transplantation*. 2000;69:163-165.

156. Trpkov K, Campbell P, Pazderka F, Cockfield S, Solez K, Halloran PF. Pathologic features of acute renal allograft rejection associated with donor-specific antibody, analysis using the Banff grading schema. *Transplantation*. 1996;61:1586-1592.

157. Van den Bogaerde J, Aspinall R, Wang MW, et al. Induction of longterm survival of hamster heart xenografts in rats. *Transplantation*. 1991;52:15-20.

158. Bach FH, Ferran C, Hechenleitner P, et al. Accomodation of vascularized xenografts: Expression of “protective genes” by donor endothelial cells in a host Th2 cytokine environment. *Nat Med*. 1997;3:196-204.

159. Millan MT, Geczy C, Stuhlmeier KM, Goodman DJ, Ferran C, Bach FH. Human monocytes activate porcine endothelial cells, resulting in increased E-selectin, interleukin-8, monocyte chemotactic protein-1, and plasminogen activator inhibitor-type-1 expression. Transplantation 63: 421-429.

160. Siegel JB, Grey ST, Lesnikoski BA, et al. Xenogeneic endothelial cells activate human prothrombin. *Transplantation*. 1997;64:888-896.

161. Sachs DH, Sykes M, Robson SC, Cooper DK. Xenotransplantation. *Adv Immunol*.

2001;79:129-223.

162. Robson SC, Cooper DK, d'Apice AJ. Disordered regulation of coagulation and platelet activation in xenotransplantation. *Xenotransplantation*. 2000;7:166-176.

163. Galili U. Xenotransplantation and ABO incompatible transplantation: the similarities they share. *Transfus Apher Sci* . 2006;35:45-58.

164. Dorling A, Stocker C, Tsao T, Haskard DO, Lechler RI. In vitro accommodation of immortalized porcine endothelial cells: resistance to complement mediated lysis and down-regulation of VCAM expression induced by low concentrations of polyclonal human IgG antipig antibodies. *Transplantation*. 1996;62:1127-1136.

165. Dorling A, Jordan W, Brookes P, Delikouras A, Lechler RI. "Accommodated" pig endothelial cells promote nitric oxide-dependent Th-2 cytokine responses from human T cells. *Transplantation*. 2001; 72:1597-1602.

166. Auchincloss H Jr, Ghobrial RR, Russell PS, Winn HJ. Anti-L3T4 in vivo prevents alloantibody formation after skin grafting without prolonging graft survival. *Transplantation*. 1988;45:1118-1123.

167. Auchincloss H Jr, Mayer T, Ghobrial R, Winn HJ. T-cell subsets, bm mutants, and the mechanisms of allogeneic skin graft rejection. *Immunol Res*. 1989;8:149-164.

168. Martin PJ, Rowley SD, Anasetti C, et al. A phase I-II clinical trial to evaluate removal of CD4 cells and partial depletion of CD8 cells from donor marrow for HLA-mismatched unrelated recipients. *Blood*. 1999;94:2192-2199.

169. Hart DN, Fabre JW. Demonstration and characterization of Ia-positive dendritic cells in the interstitial connective tissues of rat heart and other tissues, but not brain. *J Exp Med*. 1981;154:347-361.

170. Wood KJ, Goto R. Mechanisms of rejection: current perspectives. *Transplantation*. 2012;93:1-10.

171. Larsen CP, Morris PJ, Austyn JM. Migration of dendritic leukocytes from cardiac allografts into host spleens. *J Exp Med*. 1989;171: 307-314.

172. Pierre P, Turley SJ, Gatti E, et al. Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature*. 1997;388:787-792.

173. van Kooten C, Lombardi G, Gelderman KA, et al. Dendritic cells as a tool to induce transplantation tolerance: obstacles and opportunities. *Transplantation*. 2011;91:2-7.

174. Stoll S, Delon J, Brotz TM, Germain RN. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. *Science*. 2002;296: 1873-1876.

175. Lakkis FG, Arakelov A, Konieczny BT, Inoue Y. Immunologic “ignorance” of vascularized organ transplants in the absence of secondary lymphoid tissue. *Nat Med*. 2000;6:686-688.

176. Ochsenbein AF, Pinschewer DD, Sierro S, Horvath E, Hengartner H, Zinkernagel RM. Protective long-term antibody memory by antigen-driven and T help-dependent differentiation of long-lived memory B cells to short-lived plasma cells independent of secondary lymphoid organs. *Proc Natl Acad Sci U S A* . 2000;97:13263-13268.

177. Jones ND, Carvalho-Gaspar M, Luo S, Brook MO, Martin L, Wood KJ. Effector and memory CD8+ T cells can be generated in response to alloantigen independently of CD4+ T cell help. *J Immunol* . 2006;176:2316-2323.

178. Nguyen VH, Zeiser R, Dasilva DL, et al. In vivo dynamics of regulatory T-cell trafficking and survival predict effective strategies to control graft-versus-host disease following allogeneic transplantation. *Blood*. 2007;109:2649-2656.

179. Beilhack A, Schulz S, Baker J, et al. In vivo analyses of early events in acute graft-versus-host disease reveal sequential infiltration of T-cell subsets. *Blood*. 2005;106:1113-1122.

180. Murai M, Yoneyama H, Ezaki T, et al. Peyer's patch is the essential site in initiating murine acute and lethal graft-versus-host reaction. *Nat Immunol* . 2003;4:154-160.

181. Vetto RM, Lawson R. The role of vascular endothelium in the afferent pathway as suggested by alymphatic homotransplantation. *Transplantation*. 1967;5:1537-1539.

182. Barker C, Bilingham R. The role of afferent lymphatics in the rejection of skin homografts. *J Exp Med*. 1968;128:197-221.

183. Hughes CC, Savage CO, Pober JS. Endothelial cells augment T cell interleukin 2 production by a contact-dependent mechanism involving CD2/LFA-3 interaction. *J Exp Med*. 1990;171:1453-1467.

184. Kreisel D, Krupnick AS, Gelman AE, et al. Non-hematopoietic allograft cells directly activate CD8+ T cells and trigger acute rejection: an alternative mechanism of allorecognition. *Nat Med*. 2002;8:233-239.

185. Zhou P, Hwang KW, Palucki D, et al. Secondary lymphoid organs are important but not absolutely required for allograft responses. *Am J Transplant*. 2003;3:259-266.

186. Reinhardt RL, Khoruts A, Merica R, Zell T, Jenkins MK. Visualizing the generation of memory CD4 T cells in the whole body. *Nature*. 2001;410:101-105.

187. Chalasani G, Dai Z, Konieczny BT, Baddoura FK, Lakkis FG. Recall and propagation of allospecific memory T cells independent of secondary lymphoid organs. *Proc Natl Acad Sci U S A* . 2002;99:6175-6180.

188. Lechler RI, Batchelor JR. Immunogenicity of retransplanted rat kidney allografts. Effect of inducing chimerism in the first recipient and quantitative studies on immunosuppression of the second recipient. *J Exp Med*. 1982;156:1835-1841.

189. Benichou G, Takizawa PA, Olson CA, McMillan M, Sercarz EE. Donor major histocompatibility complex (MHC) peptides are presented by recipient MHC molecules during graft rejection. *J Exp Med*. 1992;175:305-308.

190. Smyth LA, Afzali B, Tsang J, Lombardi G, Lechler RI. Intercellular transfer of MHC and immunological molecules: molecular mechanisms and biological significance. *Am J Transplant*. 2007;7:1442-1449.

191. Stein JV, Nombela-Arrieta C. Chemokine control of lymphocyte trafficking: a general overview. *Immunology*. 2005;116:1-12.

192. Weibel ER, Palade GE. New cytoplasmic components in arterial endothelia. *J Cell Biol*. 1964;23:101-112.

193. Lafferty KJ, Bootes A, Dart G, Talmage DW. Effect of organ culture on the survival of thyroid allografts in mice. *Transplantation*. 1976;22:138-149.

194. Lafferty KJ, Prowse SJ, Simeonovic CJ, Warren HS. Immunobiology of tissue transplantation: a return to the passenger leukocyte concept. *Ann Rev Immunol*. 1983;1:143-173.

195. Sollinger HW, Burkholder PM, Rasmus WR, Bach FH. Prolonged survival of xenografts after organ culture. *Surgery*. 1977;81:74.

196. Lechler RI, Batchelor JR. Restoration of immunogenicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells. *J Exp Med*. 1982;155:31-41.

197. Faustman D, Hauptfeld V, Lacy P, Davie J. Prolongation of murine islet allograft survival by pretreatment of islets with antibody directed to Ia determinants. *Proc Natl Acad Sci U S A*. 1981;78:5156-5159.

198. Pietra BA, Wiseman A, Bolwerk A, Rizeq M, Gill RG. CD4 T cell-mediated cardiac allograft rejection requires donor but not host MHC class II. *J Clin Invest*. 2000;106:1003-1010.

199. Barber DL, Wherry EJ, Masopust D, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*. 2005;439:682-687.

200. Janssen EM, Droin NM, Lemmens EE, et al. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature*. 2005;434:88-93.

201. Williams MA, Tyznik AJ, Bevan MJ. Interleukin-2 signals during priming are required

for secondary expansion of CD8+ memory T cells. *Nature*. 2006;441:890-893.

202. Rosenberg AS, Mizuochi T, Sharrow SO, Singer A. Phenotype, specificity, and function of T cell subsets and T cell interactions involved in skin allograft rejection. *J Exp Med*. 1987;165:1296-1315.

203. Cobbold SP, Jayasuriya A, Nash A, Prospero TD, Waldmann H. Therapy with monoclonal antibodies by elimination of T cell subsets in vivo. *Nature*. 1984;312:548-551.

204. Mitchison NA, O'Malley C. Three-cell type clusters of T cells with antigen presenting cells best explain the epitope linkage and noncognate requirements of the in vivo cytolytic response. *Eur J Immunol* . 1987;17:1579.

205. Bennett SR, Carbone FR, Karamalis F, Miller JF, Heath WR. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. *J Exp Med*. 1987;186:65-70.

206. Lee RS, Grusby MJ, Laufer TM, Colvin R, Glimcher LH, Auchincloss H Jr. CD8+ effector cells responding to residual class I antigens, with help from CD4+ cells stimulated indirectly, cause rejection of “major histocompatibility complex-deficient” skin grafts. *Transplantation*. 1987;63:1123-1133.

207. Lee RS, Grusby MJ, Glimcher LH, Winn HJ, Auchincloss H Jr. Indirect recognition by helper cells can induce donor-specific cytotoxic T lymphocytes in vivo. *J Exp Med*. 1994;179:865-872.

208. Dolan BP, Gibbs KD Jr, Ostrand-Rosenberg S. Dendritic cells crossdressed with peptide MHC class I complexes prime CD8+ T cells. *J Immunol* . 2006;177:6018-6024.

209. Smyth LA, Afzali B, Tsang J, Lombardi G, Lechler RI. Intercellular transfer of MHC and immunological molecules: molecular mechanisms and biological significance. *Am J Transplant*. 2007;7:1442-1449.

210. Afzali B, Lombardi G, Lechler RI. Pathways of major histocompatibility complex allorecognition. *Curr Opin Organ Transplant* . 2008; 13:438-444.

211. Wang X, Li H, Matte-Martone C, et al. Mechanisms of antigen presentation to T cells in murine graft-versus-host disease: cross-presentation and the appearance of cross-presentation. *Blood*. 2011;118:6426-6437.

212. Bishop DK, Chan S, Li W, Ensley RD, Xu S, Eichwald EJ. CD4-positive helper T lymphocytes mediate mouse cardiac allograft rejection independent of donor alloantigen specific cytotoxic T lymphocytes. *Transplantation*. 1993;56:892-897.

213. Benham AM, Sawyer GJ, Fabre JW. T and B cell responsiveness to donor class I MHC molecules and peptides in long survivors with kidney allografts. *Transplantation*. 1996;61:1455-1460.

214. Rosenberg AS, Singer A. Cellular basis of skin allograft rejection: an in vivo model of immune-mediated tissue destruction. *Ann Rev Immunol*. 1992;10:333-358.

215. Braun YM, McCormack A, Webb G, Batchelor RJ. Mediation of acute but not chronic rejection of MHC-incompatible rat kidney grafts by alloreactive CD4 T cells activated by the direct pathway of sensitization. *Transplantation*. 1993;55:177-182.

216. Korngold R, Sprent J. Surface markers of T cells causing lethal graft-vs-host disease to class I vs class II H-2 differences. *J Immunol*. 1985;135:3004-3010.

217. Korngold R, Sprent J. Variable capacity of L3T4+ T cells to cause lethal graft-versus-host disease across minor histocompatibility barriers in mice. *J Exp Med*. 1987;165:1552-1564.

218. Sprent J, Schaefer M, Lo D, Korngold R. Properties of purified T cell subsets. II. In vivo responses to class I vs. class II H-2 differences. *J Exp Med*. 1986;163:998-1011.

219. Sykes M, Harty MW, Pearson DA. Strain dependence of IL-2-induced GVHD protection: evidence that IL-2 inhibits selected CD4 functions. *J Immunother*. 1994;15:11-21.

220. Sharabi Y, Sachs DH, Sykes M. T cell subsets resisting induction of mixed chimerism across various histocompatibility barriers. In: Gergely J, Benczur M, Falus A, et al., eds. *Progress in Immunology VIII. Proceedings of the Eighth International Congress of Immunology*. Budapest, Hungary: Springer-Verlag, Heidelberg; 1992:801-805.

221. Vallera DA, Taylor PA, Sprent J, Blazar BR. The role of host T cell subsets in bone marrow rejection directed to isolated major histocompatibility complex class I versus class II differences of bm1 and bm12 mutant mice. *Transplantation*. 1994;57:249-256.

222. Auchincloss H Jr, Lee R, Shea S, Markowitz JS, Grusby MJ, Glimcher LH. The role of “indirect” recognition in initiating rejection of skin grafts from major histocompatibility complex class II-deficient mice. *Proc Natl Acad Sci U S A*. 1993;90:3373-3377.

223. Susskind B, Iannotti MR, Shornick MD, Steward NS, Gorka J, Mohanakumar T. Indirect allorecognition of HLA class I peptides by CD4+ cytolytic T lymphocytes. *Hum Immunol*. 1996;46:1-9.

224. Benham AM, Sawyer GJ, Fabre JW. Indirect T cell allorecognition of donor antigens contributes to the rejection of vascularized kidney allografts. *Transplantation*. 1995;59:1028-1032.

225. Sayegh MH, Watschinger B, Carpenter CB. Mechanisms of T cell recognition of alloantigen. The role of peptides. *Transplantation*. 1994;57:1295-1302.

226. Liu Z, Sun YK, Xi YP, et al. Contribution of direct and indirect recognition pathways to T cell alloreactivity. *J Exp Med*. 1993;177:1643-1650.

227. Rogers NJ, Lechler RI. Allorecognition. *Am J Transplant*. 2001;1: 97-102.

228. Priestley CA, Spencer SC, Sawyer GJ, Fabre JW. Suppression of kidney allograft rejection across full MHC barriers by recipient-specific antibodies to class II MHC antigens. *Transplantation*. 1992;53:1024-1032.

229. Fangmann J, Dalchau R, Fabre JW. Rejection of skin allografts by indirect allorecognition of donor class I major histocompatibility complex peptides. *J Exp Med*. 1992;175:1521-1529.

230. Gokmen MR, Lombardi G, Lechler RI. The importance of the indirect pathway of allorecognition in clinical transplantation. *Curr Opin Immunol* . 2008;20:568-574.

231. Kelishadi SS, Azimzadeh AM, Zhang T, et al. Preemptive CD20+ B cell depletion attenuates cardiac allograft vasculopathy in cyclosporinetreated monkeys. *J Clin Invest*. 2010;120:1275-1284.

232. Kwun J, Knechtle SJ. Overcoming chronic rejection-can it B? *Transplantation*. 2009;88:955-961.

233. Steele DJ, Laufer TM, Smiley ST, et al. Two levels of help for B cell alloantibody production. *J Exp Med*. 1996;183:699-703.

234. Gill RG, Rosenberg AS, Lafferty KJ, Singer A. Characterization of primary T cell subsets mediating rejection of pancreatic islet grafts. *J Immunol* . 1989;143:2176-2178.

235. Gallon L, Watschinger B, Murphy B, Akalin E, Sayegh MH, Carpenter CB. The indirect pathway of allorecognition: the occurrence of selfrestricted T cell recognition of allo-MHC peptides early in acute renal allograft rejection and its inhibition by conventional immunosuppression. *Transplantation*. 1995;59:612-616.

236. Shirwan H, Learner M, Wang HK, Makowka L, Cramer DV. Peptides derived from a-helices of allogeneic class I major histocompatibility complex antigens are potent inducers of CD4+ and CD8+ T cell and B cell responses after cardiac allograft rejection. *Transplantation*. 1995;59:401-410.

237. Benichou G, Fedoseyeva E, Lehmann PV, et al. Limited T cell response to donor MHC peptides during allograft rejection: implications for selective immune therapy in transplantation. *J Immunol* . 1994;153: 938-945.

238. Liu Z, Harris PE, Colovai AI, Reed EF, Maffei A, Suciu-Foca N. Indirect recognition of donor MHC class II antigens in human transplantation. *Clin Immunol Immunopathol* . 1996;78:228-235.

239. Molajoni ER, Cinti P, Orlandini A, et al. Mechanisms of liver allograft rejection: the indirect recognition pathway. *Hum Immunol* . 1997;53:57-63.

240. Liu Z, Colovai AI, Tugulea S, et al. Indirect recognition of donor HLA-DR peptides in organ allograft rejection. *J Clin Invest*. 1996; 98:1150-1157.

241. Vella JP, Spadafora-Ferreira M, Murphy B, et al. Indirect allorecognition of major histocompatibility complex allopeptides in human renal transplant recipients with chronic graft dysfunction. *Transplantation*. 1997;64:795-800.

242. Tugulea S, Ciubotariu R, Colovai AI, et al. New strategies for early diagnosis of heart allograft rejection. *Transplantation*. 1997;64:842-847.

243. Benichou G, Takizawa PA, Olson CA, McMillan M, Sercarz EE. Donor major histocompatibility complex (MHC) peptides are presented by recipient MHC molecules during graft rejection. *J Exp Med*. 1992;175:305-308.

244. Bradley JA, Mowat AM, Bolton EM. Processed MHC class I alloantigen as the stimulus for CD4+ T-cell dependent antibody-mediated graft rejection. *Immunol Today*. 1992;13:434-437.

245. Hornick PI, Mason PD, Baker RJ, et al. Significant frequencies of T cells with indirect anti-donor specificity in heart graft recipients with chronic rejection. *Circulation*. 2000;101:2405-2410.

246. Haynes LD, Jankowska-Gan E, Sheka A, et al. Donor-specific indirect pathway analysis reveals a B-cell-independent signature which reflects outcomes in kidney transplant recipients. *Am J Transplant*. 2012;12:640-648.

247. Rose EA, Smith CR, Petrossian GA, Barr ML, Reemtsma K. Humoral immune responses after cardiac transplantation: correlation with fatal rejection and graft atherosclerosis. *Surgery*. 1989;106:203-207.

248. Russell PS, Chase CM, Winn HJ, Colvin RB. Coronary atherosclerosis in transplanted mouse hearts. II. Importance of humoral immunity. *J Immunol*. 1994;152:5135-5141.

249. Russell PS, Chase CM, Colvin RB. Alloantibody- and T cell-mediated immunity in the pathogenesis of transplant arteriosclerosis: lack of progression to sclerotic lesions in B cell-deficient mice. *Transplantation*. 1997;64:1531-1536.

250. Shi C, Lee WS, He Q, et al. Immunologic basis of transplant-associated arteriosclerosis. *Proc Natl Acad Sci U S A* . 1996;93:4051-4056.

251. Ohishi K, Varnum-Finney B, Bernstein ID. Delta-1 enhances marrow and thymus repopulating ability of human CD34(+)CD38(-) cord blood cells. *J Clin Invest*. 2002;110:1165-1174.

252. Krasinskas AM, Eiref SD, McLean AD, et al. Replacement of graftresident donor-type antigen presenting cells alters the tempo and pathogenesis of murine cardiac

allograft rejection. *Transplantation*. 2000;70:514-521.

253. Ciubotariu R, Liu Z, Colovai AI, et al. Persistent allopeptide reactivity and epitope spreading in chronic rejection of organ allografts. *J Clin Invest*. 1998;101:398-405.

254. Mason PD, Robinson CM, Lechler RI. Detection of donor-specific hyporesponsiveness following late failure of human renal allografts. *Kidney Int*. 1996;50:1019-1025.

255. Hornick PI, Mason PD, Yacoub MH, Rose ML, Batchelor R, Lechler RI. Assessment of the contribution that direct allorecognition makes to the progression of chronic cardiac transplant rejection in humans. *Circulation*. 1998;97:1257-1263.

256. Baker RJ, Hernandez-Fuentes MP, Brookes PA, et al. Loss of direct and maintenance of indirect alloresponses in renal allograft recipients: implications for the pathogenesis of chronic allograft nephropathy. *J Immunol*. 2001;167:7199-7206.

257. Shirwan H. Chronic allograft rejection: do the Th2 cells preferentially induced by indirect alloantigen recognition play a predominant role? *Transplantation*. 1999;68:715-726.

258. Lee RS, Yamada K, Houser SL, et al. Indirect recognition of allopeptides promotes the development of cardiac allograft vasculopathy. *Proc Natl Acad Sci U S A*. 2001;98:3276-3281.

259. Rosenberg A, Mizuochi T, Singer A. Analysis of T-cell subsets in rejection of Kb mutant skin allografts differing at class I MHC. *Nature*. 1986;322:829-831.

260. Krieger NR, Yin DP, Fathman CG. CD4+ but not CD8+ cells are essential for allorejection. *J Exp Med*. 1986;184:2013-2018.

261. Ben-Nun A, Lando Z, Dorf MA, Burakoff SJ. Analysis of cross-reactive antigen-specific T cell clones: specific recognition of two major histocompatibility complex (MHC) and two non-MHC antigens by a single clone. *J Exp Med*. 1983;157:2147-2153.

262. Kitagawa S, Sato S, Hori S, Hamaoka T, Fujiwara H. Induction of anti-allo-class I H-2 tolerance by inactivation of CD8+ helper T cells, and reversal of tolerance through introduction of third-party helper T cells. *J Exp Med*. 1990;172:105-113.

263. Jones ND, Carvalho-Gaspar M, Luo S, Brook MO, Martin L, Wood KJ. Effector and memory CD8+ T cells can be generated in response to alloantigen independently of CD4+ T cell help. *J Immunol*. 2006;176:2316-2323.

264. Pietryga DW, Blazar BR, Soderling CC, Vallera DA. The effect of T subset depletion on the incidence of lethal graft-versus-host disease in a murine major-histocompatibility-mismatched transplantation system. *Transplantation*. 1987;43:442-445.

265. Korngold R, Sprent J. Purified T cell subsets and lethal graft-versushost disease in mice. In: Gale RP, Champlin R, eds. *Progress in Bone Marrow Transplantation*. New York, NY: Alan Liss Inc; 1987:213.

266. Shlomchik WD, Couzens MS, Tang CB, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science*. 1999;285:412-415.

267. Matte CC, Liu J, Cormier J, et al. Donor APCs are required for maximal GVHD but not for GVL. *Nat Med*. 2004;10:987-992.

268. Chakraverty R, Eom HS, Sachs J, et al. Host MHC Class II+ antigen-presenting cells and CD4 cells are required for CD8-mediated graft-versus-leukemia responses following delayed donor leukocyte infusions. *Blood*. 2006;108:2106-2113.

269. Chakraverty R, Flutter B, Fallah-Arani F, et al. The host environment regulates the function of CD8+ graft-versus-host-reactive effector cells. *J Immunol*. 2008;181:6820-6828.

270. Auchincloss H Jr, Winn HJ. Murine CD8+ T cell helper function is particularly sensitive to CsA suppression in vivo. *J Immunol*. 1989;143:3940-3943.

271. Fidelus RK, Ferguson RM, Widmer MB, Wee SL, Bach FH, Orosz CG. Effect of cyclosporin A on murine and human T helper cell clones. *Transplantation*. 1982;34:308-311.

272. Orosz CG, Roopenian DC, Widmer MB, Bach FM. Analysis of cloned T cell function. II. Differential blockade of various cloned T cell functions by cyclosporine. *Transplantation*. 1983;36:706-711.

273. Rosenberg AS, Mizuochi T, Singer A. Evidence for involvement of dual-function T cells in rejection of MHC class I disparate skin grafts. Assessment of MHC class I alloantigens as in vivo helper determinants. *J Exp Med*. 1988;168:33-45.

274. Ichikawa T, Nakayama E, Uenaka A, Monden M, Mori T. Effector cells in allelic H-2 class I-incompatible skin graft rejection. *J Exp Med*. 1987;166:982-990.

275. Monaco JJ.A molecular model of MHC class-I-restricted antigen processing. *Immunol Today*. 1992;13:173-178.

276. Neefjes JJ, Ploegh HL. Intracellular transport of MHC class II molecules. *Immunol Today*. 1992;13:179-183.

277. Grant EP, Rock KL. MHC class I-restricted presentation of exogenous antigen by thymic antigen-presenting cells in vitro and in vivo. *J Immunol*. 1992;148:13-18.

278. Michalek MT, Benacerraf B, Rock KL. The class II MHC-restricted presentation of endogenously synthesized ovalbumin displays clonal variation, requires

endosomal/lysosomal processing, and is upregulated by heat shock. *J Immunol*. 1992;148:1016-1024.

279. Kurts C, Heath WR, Carbone FR, et al. Constitutive class I-restricted exogenous presenetation of self antigens in vivo. *J Exp Med*. 1996; 184:923-930.

280. Sigal LJ, Rock KL. Bone marrow-derived antigen-presenting cells are required for the generation of cytotoxic T lymphocyte responses to viruses and use transporter associated with antigen presentation (TAP)-dependent and - independent pathways of antigen presentation [In Process Citation]. *J Exp Med*. 2000;192:1143-1150.

281. Kurts C, Robinson BW, Knolle PA. Cross-priming in health and disease. *Nat Rev Immunol*. 2010;10:403-414.

282. den Haan JM, Bevan MJ. Antigen presentation to CD8(+) T cells: crosspriming in infectious diseases. *Curr Opin Immunol* . 2001;13:437-441.

283. Rock KL, Farfan-Arribas DJ, Shen L. Proteases in MHC class I presentation and cross-presentation. *J Immunol*. 2010;184:9-15.

284. Shen L, Rock KL. Priming of T cells by exogenous antigen cross-presented on MHC class I molecules. *Curr Opin Immunol* . 2006;18:85-91.

285. Blachere NE, Morris HK, Braun D, et al. IL-2 is required for the activation of memory CD8+ T cells via antigen cross-presentation. *J Immunol*. 2006;176:7288-7300.

286. Valujskikh A, Zhang Q, Heeger PS. CD8 T cells specific for a donor-derived, self-restricted transplant antigen are nonpathogenic bystanders after vascularized heart transplantation in mice. *J Immunol*. 2006;176:2190-2196.

287. Valujskikh A, Lantz O, Celli S, Matzinger P, Heeger PS. Cross-primed CD8(+) T cells mediate graft rejection via a distinct effector pathway. *Nat Immunol*. 2002;3:844-851.

288. He C, Heeger PS. CD8 T cells can reject major histocompatibility complex class I-deficient skin allografts. *Am J Transplant*. 2004;4:698-704.

289. Williams NS, Engelhard VH. Perforin-dependent cytotoxic activity and lymphokine secretion by CD4+ T cells are regulated by CD8+ T cells. *J Immunol*. 1997;159:2091-2099.

290. Griffiths GM, Mueller C. Expression of perforin and granzymes in vivo: potential diagnostic markers for activated cytotoxic cells. *Immunol Today*. 1991;12:415-418.

291. Griffiths GM, Namikawa R, Mueller C, et al. Granzyme A and perforin as markers for rejection in cardiac transplantation. *Eur J Immunol* . 1991;21:687-692.

292. Linsley PS, Brady W, Urnes M, Grosmaire LS, Damle NK, Ledbetter JA. CTLA-4 is a

second receptor for the B cell activation antigen B7. *J Exp Med*. 1991;174:561-569.

293. Lipman ML, Stevens CA, Bleackley CR, et al. The strong correlation of cytotoxic T lymphocyte-specific serine protease gene transcripts with renal allograft rejection. *Transplantation*. 1992;53:73-79.

294. Thiele DL, Geissler GH, Williams FH, Lipsky PE. The role of leucylleucine methyl ester-sensitive cytotoxic cells in skin allograft rejection. *Transplantation*. 1992;53:1334-1340.

295. Chen RH, Ivens KW, Alpert S, et al. The use of granzyme A as a marker of heart transplant rejection in cyclosporine or anti-CD4 monoclonal antibody-treated rats. *Transplantation*. 1993;55:146-153.

296. Sharma VK, Bologa RM, Li B, et al. Molecular executors of cell death—differential intrarenal expression of Fas ligand, Fas, granzyme B, and perforin during acute and/or chronic rejection of human renal allografts. *Transplantation*. 1996;62:1860-1866.

297. Lipman ML, Stevens AC, Strom TB. Heightened intragraft CTL gene expression in acutely rejecting renal allografts. *J Immunol*. 1994;152: 5120-5127.

298. Li B, Hartono C, Ding R, et al. Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. *New Engl J Med*. 2001;344:947-954.

299. Graubert TA, Russell JH, Ley TJ. The role of granzyme B in murine models of acute graft-versus-host disease and graft rejection. *Blood*. 1996;87:1232-1237.

300. Williams NS, Engelhard VH. Identification of a population of CD4+ CTL that utilizes a perforin- rather than a Fas ligand-dependent cytotoxic mechanism. *J Immunol*. 1996;156:153-159.

301. Hahn S, Gehri R, Erb P. Mechanism and biological significance of CD4-mediated cytotoxicity. *Immunol Rev*. 1995;146:57-79.

302. Blazar BR, Taylor PA, Vallera DA. CD4+ and CD8+ T cells each can utilize a perforin-dependent pathway to mediate lethal graft-versushost disease in major histocompatibiity complex-disparate recipients. *Transplantation*. 1997;64:571-576.

303. Schulz M, Schuurman HJ, Joergensen J, et al. Acute rejection of vascular heart allografts by perforin-deficient mice. *Eur J Immunol*. 1995;25:474-480.

304. Selvaggi G, Ricordi C, Podack ER, Inverardi L. The role of the perforin and Fas pathways of cytotoxicity in skin graft rejection. *Transplantation*. 1996;62:1912-1915.

305. Ito A, Minagawa M, Tomiyama K, Ito M, Kawai K. Cytotoxic pathways in the skin allograft rejection by CD4+ cells. *Transplantation*. 1999;68:97-100.

306. Larsen CP, Alexander DZ, Hendrix R, Ritchie SC, Pearson TC. Fasmediated cytotoxicity. An immunoeffector or immunoregulatory pathway in T cell-mediated immune responses? *Transplantation*. 1995;60:221-224.

307. Braun MY, Lowin B, French L, Acha-Orbea H, Tschopp J. Cytotoxic T cells deficient in both functional Fas ligand and perforin show residual cytolytic activity yet lose their capacity to induce acute graft-versushost disease. *J Exp Med*. 1996;183:657-661.

308. Baker MB, Riley RL, Podack ER, Levy RB. Graft-versus-host diseaseassociated lymphiod hypoplasia and B cell dysfunction is dependent upon donor T cell-mediated Fas-ligand function, but not perforin function. *Proc Natl Acad Sci U S A* . 1997;94:1366-1371.

309. Tsukada N, Kobata T, Aizawa Y, Yagita H, Okumura K. Graft-versusleukemia effect and graft-versus-host disease can be differentiated by cytotoxic mechanisms in a murine model of allogeneic bone marrow transplantation. *Blood*. 1999;93:2738-2747.

310. Graubert TA, DiPersio JF, Russell JH, Ley TJ. Perforin/granzymedependent and independent mechanisms are both important for the development of graft-versus-host disease after murine bone marrow transplantation. *J Clin Invest*. 1997;100:904-911.

311. Baker MB, Podack ER, Levy RB. Perforin- and Fas-mediated cytotoxic pathways are not required for allogeneic resistance to bone marrow grafts in mice. *Biol Blood Marrow Transplant*. 1995;1:69-73.

312. Baker MB, Altman NH, Podack ER, Levy RB. The role of cell-mediated cytotxicity in acute GVHD after MHC-matched allogeneic bone marrow transplantation in mice. *J Exp Med*. 1996;183:2645-2656.

313. Na IK, Lu SX, Yim NL, et al. The cytolytic molecules Fas ligand and TRAIL are required for murine thymic graft-versus-host disease. *J Clin Invest*. 2010;120:343-356.

314. Schmaltz C, Alpdogan O, Horndasch KJ, et al. Differential use of Fas ligand and perforin cytotoxic pathways by donor T cells in graft-versushost disease and graft-versus-leukemia effect. *Blood*. 2001;97:2886-2895.

315. Pan L, Teshima T, Hill GR, et al. Granulocyte colony-stimulating factor-mobilized allogeneic stem cell transplantation maintains graftversus-leukemia effects through a perforin-dependent pathway while preventing graft-versus-host disease. *Blood*. 1999;93:4071-4078.

316. Hsieh MH, Korngold R. Differential use of FasL- and perforin-mediated cytolytic mechanisms by T-cell subsets involved in graftversus-myeloid leukemia responses. *Blood*. 2000;96:1047-1055.

317. Hsieh MH, Patterson AE, Korngold R. T-cell subsets mediate graftversus-myeloid leukemia responses via different cytotoxic mechanisms. *Biol Blood Marrow Transplant* .

2000;6:231-240.

318. Schmaltz C, Alpdogan O, Kappel BJ, et al. T cells require TRAIL for optimal graft-versus-tumor activity. *Nat Med*. 2002;8:1433-1437.

319. Doody DP, Stenger KS, Winn HJ. Immunologically nonspecific mechanisms of tissue destruction in the rejection of skin grafts. *J Exp Med*. 1994;179:1645-1652.

320. Wecker H, Grusby MJ, Auchincloss H Jr. Effector cells must recognize antigens expressed in the graft to cause efficient skin graft rejection in SCID mice. *Transplantation*. 1995;59:1223-1227.

321. Braun MY, Grandjean I, Feunou P, et al. Acute rejection in the absence of cognate recognition of allograft by T cells. *J Immunol*. 2001;166:4879-4883.

322. Kapessidou Y, Habran C, Buonocore S, et al. The replacement of graft endothelium by recipient-type cells conditions allograft rejection mediated by indirect pathway CD4(+) T cells. *Transplantation*. 2006;81: 726-735.

323. Teshima T, Ordemann R, Reddy P, et al. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. *Nat Med*. 2002;8:575-581.

324. Duffner UA, Maeda Y, Cooke KR, et al. Host dendritic cells alone are sufficient to initiate acute graft-versus-host disease. *J Immunol*. 2004;172:7393-7398.

325. Jones SC, Murphy GF, Friedman TM, Korngold R. Importance of minor histocompatibility antigen expression by nonhematopoietic tissues in a CD4+ T cell-mediated graft-versus-host disease model. *J Clin Invest*. 2003;112:1880-1886.

326. Zhang C, Todorov I, Zhang Z, et al. Donor CD4+ T and B cells in transplants induce chronic graft-versus-host disease with autoimmune manifestations. *Blood*. 2006;107:2993-3001.

327. Srinivasan M, Flynn R, Price A, et al. Donor B-cell alloantibody deposition and germinal center formation are required for the development of murine chronic GVHD and bronchiolitis obliterans. *Blood*. 2012;119:1570-1580.

328. Greinix HT, Pohlreich D, Kouba M, et al. Elevated numbers of immature/transitional CD21- B lymphocytes and deficiency of memory CD27_ B cells idenitfy patients with active chronic graft-versus-host disease. *Biol Blood Marrow Transplant*. 2008;14:208-219.

329. Sarantopoulos S, Stevenson KE, Kim HT, et al. Altered B-cell homeostasis and excess BAFF in human chronic graft-versus-host disease. *Blood*. 2009;113:3865-3874.

330. Sarantopoulos S, Stevenson KE, Kim HT, et al. Recovery of B-cell homeostasis after rituximab in chronic graft-versus-host disease. *Blood*. 2011;117:2275-2283.

331. van Dorp S, Resemann H, te Boome L, et al. The immunological phenotype of rituximab-sensitive chronic graft-versus-host disease: a phase II study. *Haematologica*. 2011;96:1380-1384.

332. Zaja F, Bacigalupo A, Patriarca F, et al. Treatment of refractory chronic GVHD with rituximab: a GITMO study. *Bone Marrow Transplant* . 2007;40:273-277.

333. Le Moine A, Goldman M, Abramowicz D. Multiple pathways to allograft rejection. *Transplantation*. 2002;73:1373-1381.

334. Steinman L. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat Med*. 2007;13:139-145.

335. Carlson MJ, West ML, Coghill JM, et al. In vitro differentiated TH17 cells mediate lethal acute graft-versus-host disease with severe cutaneous and pulmonary pathology. *Blood*. 2008;113:1365-1374.

336. Oliveira CC, van Veelen PA, Querido B, et al. The nonpolymorphic MHC Qa-1b mediates CD8+ T cell surveillance of antigen-processing defects. *J Exp Med*. 2010;207:207-212.

337. Yi T, Chen Y, Wang L, et al. Reciprocal differentiation and tissuespecific pathogenesis of Th1, Th2, and Th17 cells in graft-versus-host disease. *Blood*. 2009;114:3101-3112.

338. Dander E, Balduzzi A, Zappa G, et al. Interleukin-17-producing T-helper cells as new potential player mediating graft-versus-host disease in patients undergoing allogeneic stem-cell transplantation. *Transplantation*. 2009;88:1261-1272.

339. Iclozan C, Yu Y, Liu C, et al. T helper17 cells are sufficient but not necessary to induce acute graft-versus-host disease. *Biol Blood Marrow Transplant* . 2010;16:170-178.

340. Ratajczak P, Janin A, de Peffault LR, et al. Th17/Treg ratio in human graft-versus-host disease. *Blood*. 2010;116:1165-1171.

341. Hanash AM, Kappel LW, Yim NL, et al. Abrogation of donor T-cell IL-21 signaling leads to tissue-specific modulation of immunity and separation of GVHD from GVL. *Blood*. 2011;118:446-455.

342. Yu Y, Wang D, Liu C, et al. Prevention of GVHD while sparing GVL effect by targeting Th1 and Th17 transcription factor T-bet and RORgammat in mice. *Blood*. 2011;118:5011-5020.

343. Chen Y, Wood KJ. Interleukin-23 and TH17 cells in transplantation immunity: does 23+17 equal rejection? *Transplantation*. 2007;84:1071-1074.

344. Yuan X, Paez-Cortez J, Schmitt-Knosalla I, et al. A novel role of CD4 Th17 cells in mediating cardiac allograft rejection and vasculopathy. *J Exp Med*. 2008;205:3133-3144.

345. Chen L, Ahmed E, Wang T, et al. TLR signals promote IL-6/IL-17-dependent transplant rejection. *J Immunol*. 2009;182:6217-6225.

346. Hester J, Mills N, Shankar S, Carvalho-Gaspar M, Friend P, Wood KJ. Th17 cells in alemtuzumab-treated patients: the effect of longterm maintenance immunosuppressive therapy. *Transplantation*. 2011;91:744-750.

347. Vokaer B, Van RN, Lemaitre PH, et al. Critical role of regulatory T cells in Th17-mediated minor antigen-disparate rejection. *J Immunol*. 2010;185:3417-3425.

348. Chadha R, Heidt S, Jones ND, Wood KJ. Th17: contributors to allograft rejection and a barrier to the induction of transplantation tolerance? *Transplantation*. 2011;91:939-945.

349. Merville P, Lambert C, Durand I, et al. High frequency of IL-10-secreting CD4+ graft-infiltrating T lymphocytes in promptly rejected kidney allografts. *Transplantation*. 1995;59:1113-1119.

350. Pavlakis M, Strehlau J, Lipman M, Shapiro M, Maslinski W, Strom TB. Intragraft IL-15 transcripts are increased in human renal allograft rejection. *Transplantation*. 1996;62:543-545.

351. Truong LD, Shappell S, Barrios R, Gonzalez J, Suki WN, Solez K. Immunohistochemistry and molecular biology markers of renal transplant rejection: diagnostic applications. *Transplant Rev*. 1996;10:187-208.

352. Carlquist JF, Edelman LS, White W, Shelby J, Anderson JL. Cytokines and rejection of mouse cardiac allografts. *Transplantation*. 1996; 62:1160-1166.

353. O'Connell PJ, Pacheco-Silva A, Nickerson PW, et al. Unmodified pancreatic islet allograft rejection results in the preferential expression of certain T cell activation transcripts. *J Immunol*. 1993;150:1093-1104.

354. Strehlau J, Pavlakis M, Lipman M, et al. Quantitative detection of immune activation transcripts as a diagnostic tool in kidney transplantation. *Proc Nat Acad Sci U S A*. 1997;94:695-700.

355. Fairchild RL, VanBuskirk AM, Kondo T, Wakely ME, Orosz CG. Expression of chemokine genes during rejection and long-term acceptance of cardiac allografts. *Transplantation*. 1997;63:1807-1812.

356. Martinez OM, Lang T, Villanueva JC, Esquivel CO, So S, Krams SM. Allograft rejection is associated with a Th2-dominant cytokine profile. *Transplantation*. 1995;9:A497.

357. Nikolic B, Lee S, Bronson RT, Grusby MJ, Sykes M. Th1 and Th2 cells both contribute to acute GVHD, and each subset has distinct endorgan targets. *J Clin Invest*. 2000;105:1289-1293.

358. Broady R, Yu J, Chow V, et al. Cutaneous GVHD is associated with the expansion of tissue-localized Th1 and not Th17 cells. *Blood*. 2010;116:5748-5751.

359. Piccotti JR, Chan SY, VanBuskirk AM, Eichwald EJ, Bishop DK. Are Th2 helper T lymphocytes beneficial, deleterious, or irrelevant in promoting allograft survival? *Transplantation*. 1997;63:619-624.

360. Sayegh MH, Akalin E, Hancock WW, et al. CD28-B7 blockade after alloantigenic challenge in vivo inhibits Th1 cytokines but spares Th2. *J Exp Med*. 1995;181:1869-1874.

361. Waaga AM, Gasser M, Kist-van Holthe JE, et al. Regulatory functions of self-restricted MHC class II allopeptide- specific Th2 clones in vivo. *J Clin Invest*. 2001;107:909-916.

362. Chen N, Field EH. Enhanced type 2 and diminished type 1 cytokines in neonatal tolerance. *Transplantation*. 1995;59:933-941.

363. Donckier V, Wissing M, Bruyns C, et al. Critical role of interleukin 4 in the induction of neonatal transplantation tolerance. *Transplantation*. 1995;59:1571-1576.

364. Inoue Y, Konieczny BT, Wagener ME, McKenzie AN, Lakkis FG. Failure to induce neonatal tolerance in mice that lack both IL-4 and IL-13 but not in those that lack IL-4 alone. *J Immunol*. 2001;167:1125-1128.

365. Fowler DH, Kurasawa K, Smith R, Eckhaus MA, Gress RE. Donor CD4-enriched cells of Th2 cytokine phenotype regulate graft-versushost disease without impairing allogeneic engraftment in sublethally irradiated mice. *Blood*. 1994;84:3540-3549.

366. Antin JH, Ferrara JLM. Cytokine dysregulation in acute graft-versushost disease. *Blood*. 1992;80:2964-2968.

367. Krenger W, Cooke KR, Crawford JM, et al. Transplantation of polarized type 2 donor T cells reduces mortality caused by experimental graft-versus-host disease. *Transplantation*. 2006;62:1278-1285.

368. Chan SY, DeBruyne LA, Goodman RE, Eichwald EJ, Bishop DK. In vivo depletion of CD8+ T cells results in Th2 cytokine production and alternate mechanisms of allograft rejection. *Transplantation*. 1995; 59:1155-1161.

369. Qian S, Li W, Li Y, et al. Systemic administration of cellular interleukin-10 can exacerbate cardiac allograft rejection in mice. *Transplantation*. 1996;62:1709-1714.

370. Zhou P, Szot GL, Guo Z, et al. Role of STAT4 and STAT6 signaling in allograft rejection and CTLA4-Ig- mediated tolerance. *J Immunol*. 2001;165:5580-5587.

371. Murphy WJ, Welniak LA, Taub DD, et al. Differential effects of the absence of interferon-γ and IL-4 in acute graft-versus-host disease after allogeneic bone marrow transplantation in mice. *J Clin Invest*. 1998;102:1742-1748.

372. Blazar BR, Taylor PA, Panoskaltsis-Mortari A, et al. Interleukin-10 dose-dependent regulation of CD4+ and CD8+ T cell-mediated graftversus-host disease. *Transplantation*. 1998;66:1220-1229.

373. De Wit D, Van Melchelen M, Zanin C, et al. Preferential activation of Th2 cells in chronic graft-versus-host reaction. *J Immunol*. 1993;150: 361-366.

374. Diamond AS, Gill RG. An essential contribution by IFN-gamma to CD8+ T cell-mediated rejection of pancreatic islet allografts. *J Immunol*. 2000;165:247-255.

375. Ring GH, Saleem S, Dai Z, et al. Interferon-γ is necessary for initiating the acute rejection of major histocompatibility complex class II-disparate skin allografts. *Transplantation*. 1999;67:1362-1383.

376. Guo Z, Wang J, Meng L, et al. Cutting edge: membrane lymphotoxin regulates CD8(+) t cell-mediated intestinal allograft rejection. *J Immunol*. 2001;167:4796-4800.

377. Wiseman AC, Pietra BA, Kelly BP, Rayat GR, Rizeq M, Gill RG. Donor IFN-gamma receptors are critical for acute CD4(+) T cell-mediated cardiac allograft rejection. *J Immunol*. 2001;167:5457-5463.

378. Steiger J, Nickerson PW, Steurer W, Moscovitch-Lopatin M, Strom TB. IL-2 knockout recipient mice reject islet cell allografts. *J Immunol*. 1995;155:489-498.

379. Dalloul AH, Chmouzis E, Ngo K, Fung-Leung WP. Adoptively transferred CD4+ lymphocytes from CD8-/- mice are sufficient to mediate the rejection of MHC class II or class I disparate skin grafts. *J Immunol*. 1996;156:4114-4119.

380. Nickerson P, Zheng XX, Steiger J, et al. Prolonged islet allograft acceptance in the absence of interleukin 4 expression. *Transplant Immunol*. 1996;4:81-85.

381. Roy-Chaudhury P, Manfro RC, Steiger J, et al. IL-2 and IL-4 double knock-out mice reject islet allografts: a role for novel T-cell growth factors? *Transplant Proc*. 1997;29:1083-1084.

382. Piccotti JR, Li K, Chan SY, et al. Alloantigen-reactive Th1 development in IL-12-deficient mice. *J Immunol*. 1998;160:1132-1138.

383. Saleem S, Konieczny BT, Lowry RP, Baddoura FK, Lakkis FG. Acute rejection of vascularized heart allografts in the absence of IFN-gamma. *Transplantation*.

1996;62:1908-1911.

384. Lakkis FG, Dai Z. The role of cytokines, CTLA-4 and costimulation in transplant tolerance and rejection. *Curr Opin Immunol* . 1999;11: 504-508.

385. Yang YG, Dey B, Sergio JJ, Pearson DA, Sykes M. Donor-derived Interferon γ is required for inhibition of acute GVHD by interleukin 12. *J Clin Invest*. 1998;102:2126-2135.

386. Murphy WJ, Blazar BR. New strategies for preventing graft-versushost disease. *Curr Opin Immunol* . 1999;11:509-515.

387. Nestel FP, Price KS, Seemayer TA, Lapp WS. Macrophage priming and lipopolysaccharide-triggered release of tumor necrosis factor α during graft-versus-host disease. *J Exp Med*. 1992;175:405-413.

388. Hill GR, Crawford JM, Cooke KR, et al. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. *Blood*. 1997;90:3204-3213.

389. Bogdan C. Nitric oxide and the immune response. *Nat Immunol* . 2001;2:907-916.

390. Garside P, Hutton AK, Severn A, Liew FY, Mowat AM. Nitric oxide mediates intestinal pathology in graft-vs-host disease. *Eur J Immunol* . 1992;22:2141-2145.

391. Hoffman RA, Nussler NC, Gleixner SL, et al. Attenuation of lethal graft-versus-host disease by inhibition of nitric oxide synthase. *Transplantation*. 1997;63:94-100.

392. Hattori K, Hirano T, Miyajima H, et al. Differential effects of anti-Fas ligand and anti-tumor necrosis factor α antibodies on acute graftversus-host disease pathologies. *Blood*. 1998;91:4051-4055.

393. Lin T, Brunner T, Tietz B, et al. Fas ligand- mediated killing by intestinal intraepithelial lymphocytes. Participation in intestinal graftversus-host disease. *J Clin Invest*. 1998;101:570-577.

394. Jiang Z, Podack E, Levy RB. Major histocompatibility complex-mismatched allogeneic bone marrow transplantation using perforin and/or Fas ligand double-defective CD4(+) donor T cells: involvement of cytotoxic function by donor lymphocytes prior to graft-versus-host disease pathogenesis. *Blood*. 2001;98:390-397.

395. Jiang Z, Jones M, Levy RB. FasL and perforin cotyotoxically impaired donor T-cells induce GVHD in TNF receptor 1 (p55) knockout MHC I/II mismatched recipients. *Biol Blood Marrow Transplant*. 2001;7:76.

396. Roberts PJ, Hayry P. Sponge matrix allografts. A model for analysis of killer cells infiltrating mouse allografts. *Transplantation*. 1976;21:437.

397. Strom TB, Tilney NL, Paradyez J, Banceqicz J, Carpenter CB. Cellular components of allograft rejection: identity, specificity, and cytotoxic function of cells infiltrating acutely rejecting allografts. *J Immunol*. 1977;118:2020-2026.

398. Hall B, Dorsch S. Cells mediating allograft rejection. *Immunol Rev*. 1984;77:570.

399. Ascher NL, Hoffman RA, Hanto DW, Simmons RL. Cellular basis of allograft rejection. *Immunol Rev*. 1984;77:216-230.

400. Hall B, Bishop G, Farnsworth A, et al. Identification of the cellular subpopulations infiltrating rejecting cadaver renal allografts: preponderance of the T4 subset of T cells. *Transplantation*. 1984;37:564-570.

401. Hayry P, von Willebrand E, Parthenais E, et al. The inflammatory mechanisms of allograft rejection. *Immunol Rev*. 1984;77:85-142.

402. Bradley AJ, Bolton EM. The T-cell requirements for allograft rejection. *Transplant Rev*. 1992;6:115-129.

403. Preffer FI, Colvin RB, Leary CP, et al. Two color flow cytometry and functional analysis of lymphocytes cultured from human renal allografts: identification of a Leu 2+3+ subpopulation. *J Immunol*. 1986;137:2823-2830.

404. Tilney NL, Strom TB, MacPherson SG, Carpenter CB. Surface properties and functional characteristics of infiltrating cells harvested from acutely rejecting cardiac allografts in inbred rats. *Transplantation*. 1975;20:323-330.

405. Mayer TG, Bhan AK, Winn HJ. Immunohistochemical analysis of skin graft rejection in mice: kinetics of lymphocyte infiltration in grafts of limited immunogenetic disparity. *Transplantation*. 1988;46:890-899.

406. Clatworthy MR. Targeting B cells and antibody in transplantation. *Am J Transplant*. 2011;11:1359-1367.

407. Thaunat O, Patey N, Caligiuri G, et al. Chronic rejection triggers the development of an aggressive intragraft immune response through recapitulation of lymphoid organogenesis. *J Immunol*. 2010;185:717-728.

408. Colvin RB. The renal allograft biopsy. *Kidney Int*. 1996;50:1069-1082.

409. Douillard P, Cuturi MC, Brouard S, Josien R, Soullilou JP. T cell receptor repertoire usage in allotransplantation: an overview. *Transplantation*. 1999;68:913-921.

410. Moreau JF, Peyrat MA, Vie H, Bonneville M, Soulillou JP. T cell colony-forming frequency of mononucleated cells extracted from rejected human kidney transplants. *Transplantation*. 1985;39:649.

411. Bonneville M, Moreau JF, Blokland E, et al. T lymphocyte cloning from rejected human kidney allograft. Recognition repertoire of alloreactive T cell clones. *J Immunol*. 1998;141:4187-4195.

412. Guillet M, Brouard S, Gagne K, et al. Different qualitative and quantitative regulation of V beta TCR transcripts during early acute allograft rejection and tolerance induction. *J Immunol*. 2002;168:5088-5095.

413. Orosz CG, Horstemeyer B, Zinn NE, Bishop DK. Development and evaluation of a limiting dilution analysis technique that can discriminate in vivo alloactivated cytotoxic T lymphocytes from their naive CTL precursors. *Transplantation*. 1989;47:189-194.

414. Gagne K, Brouard S, Giral M, et al. Highly altered Vβ repertoire of T cells infiltrating long-term rejected kidney allografts. *J Immunol*. 2000;164:1553-1563.

415. Brouard S, Sebille F, Vanhove B, et al. T cell repertoire alterations in allograft and xenograft rejection processes. *Transplant Proc*. 2000;32: 924-925.

416. Brouard S, Vanhove B, Gagne K, et al. T cell repertoire alterations of vascularized xenografts. *J Immunol*. 1999;162:3367-3377.

417. Guillet M, Gagne K, Lair D, et al. Different patterns of TCR beta chain regulation following allo- and xeno-transplantation. *Xenotransplant*. 2004;11:315-322.

418. Friedman TM, Gilbert M, Briggs C, Korngold R. Repertoire analysis of CD8+ T cell responses to minor histocompatibility antigens involved in graft-versus-host disease. *J Immunol*. 1998;161:41-48.

419. Friedman TM, Statton D, Jones SC, Berger MA, Murphy GF, Korngold R. Vbeta spectratype analysis reveals heterogeneity of CD4+ T-cell responses to minor histocompatibility antigens involved in graft-versus-host disease: correlations with epithelial tissue infiltrate. *Biol Blood Marrow Transplant* . 2001;7:2-13.

420. Scheinberg P, Price DA, Ambrozak DR, Barrett AJ, Douek DC. Alloreactive T cell clonotype recruitment in a mixed lymphocyte reaction: implications for graft engineering. *Exp Hematol*. 2006;34: 788-795.

421. Lee LA, Sergio JJ, Sykes M. Natural killer cells weakly resist engraftment of allogeneic long-term multilineage-repopulating hematopoietic stem cells. *Transplantation*. 1996;61:125-132.

422. Aguila HL, Weissman IL. Hematopoietic stem cells are not direct cytotoxic targets of natural killer cells. *Blood*. 1996;87:1225-1231.

423. O'Reilly RJ, Brochstein J, Collins N, et al. Evaluation of HLA-haplotye disparate parental marrow grafts depleted of T lymphocytes by differential agglutination with a soybean lectin and E-rosette depletion for the treatment of severe combined

immunodeficiency. *Vox Sang*. 1986;51(suppl 2):81-86.

424. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. 2002;295:2097-2100.

425. Moretta L, Locatelli F, Pende D, Marcenaro E, Mingari MC, Moretta A. Killer Ig-like receptor-mediated control of natural killer cell alloreactivity in haploidentical hematopoietic stem cell transplantation. *Blood*. 2011;117:764-771.

426. Davies SM, Ruggieri L, DeFor T, et al. Evaluation of KIR ligand incompatibility in mismatched unrelated donor hematopoietic transplants. Killer immunoglobulin-like receptor. *Blood*. 2002;100:3825-3827.

427. Beelen DW, Ottinger HD, Ferencik S, et al. Genotypic inhibitory killer immunoglobulin-like receptor ligand incompatibility enhances the long-term antileukemic effect of unmodified allogeneic hematopoietic stem cell transplantation in patients with myeloid leukemias. *Blood*. 2005;105:2594-2600.

428. Elmaagacli AH, Ottinger H, Koldehoff M, et al. Reduced risk for molecular disease in patients with chronic myeloid leukemia after transplantation from a KIR-mismatched donor. *Transplantation*. 2005;79:1741-1747.

429. Brunstein CG, Wagner JE, Weisdorf DJ, et al. Negative effect of KIR alloreactivity in recipients of umbilical cord blood transplant depends on transplantation conditioning intensity. *Blood*. 2009;113:5628-5634.

430. Baron F, Petersdorf EW, Gooley T, et al. What is the role for donor natural killer cells after nonmyeloablative conditioning? *Biol Blood Marrow Transplant* . 2009;15:580-588.

431. Symons HJ, Leffell MS, Rossiter ND, Zahurak M, Jones RJ, Fuchs EJ. Improved survival with inhibitory killer immunoglobulin receptor (KIR) gene mismatches and KIR haplotype B donors after nonmyeloablative, HLA-haploidentical bone marrow transplantation. *Biol Blood Marrow Transplant* . 2010;16:533-542.

432. Cooley S, Weisdorf DJ, Guethlein LA, et al. Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. *Blood*. 2010; 116:2411-2419.

433. Kondo T, Morita K, Watarai Y, et al. Early increased chemokine expression and production in murine allogeneic skin grafts is mediated by natural killer cells. *Transplantation*. 2000;69:969-977.

434. Obara H, Nagasaki K, Hsieh CL, et al. IFN-gamma, produced by NK cells that infiltrate liver allografts early after transplantation, links the innate and adaptive immune responses. *Am J Transplant*. 2005;5:2094-2103.

435. Kitchens WH, Uehara S, Chase CM, et al. The changing role of natural killer cells in

solid organ rejection and tolerance. *Transplantation*. 2006;81:811-817.

436. Piccioli D, Sbrana S, Melandri E, Valiante NM. Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. *J Exp Med*. 2002;195:335-341.

437. Bix M, Liao NS, Zijlstr M, Loring J, Jaenisch E, Raulet D. Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice. *Nature*. 1991;349:329-331.

438. Zijlstra M, Auchincloss H Jr, Loring JM, Chase CM, Russell PS, Jaenisch R. Skin graft rejection by beta2-microglobulin-deficient mice. *J Exp Med*. 1992;175:885-893.

439. Maier S, Tertilt C, Chambron N, et al. Inhibition of natural killer cells results in acceptance of cardiac allografts in CD28-/-mice. *Nat Med*. 2001;7:557-562.

440. McNerney ME, Lee KM, Zhou P, et al. Role of natural killer cell subsets in cardiac allograft rejection. *Am J Transplant*. 2006;6:505-513.

441. Graham JA, Wilkinson RA, Hirohashi T, et al. Viral infection induces de novo lesions of coronary allograft vasculopathy through a natural killer cell-dependent pathway. *Am J Transplant*. 2009;9:2479-2484.

442. Renard V, Cambiaggi A, Vely F, et al. Transduction of cytotoxic signals in natural killer cells: a general model of fine tuning between activatory and inhibitory pathways in lymphocytes. *Immunol Rev*. 1997;155:205-221.

443. Hoglund P, Sundback J, Olsson-Alheim MY, et al. Host MHC class I gene control of NK cell specificity in the mouse. *Immunol Rev*. 1997;155:11-28.

444. Sasaki H, Xu XC, Smith SM, Howard T, Mohanakumar T. HLA-G expression protects porcine endothelial cells against natural killer cellmediated xenogeneic cytotoxicity. *Transplantation*. 1999;67:31-37.

445. Forte P, Pazmany L, Matter-Reissmann UB, Stussi G, Schneider MK, Seebach JD. HLA-G inhibits rolling adhesion of activated human NK cells on porcine endothelial cells. *J Immunol*. 2001;167:6002-6008.

446. Forte P, Baumann BC, Weiss EH, Seebach JD. HLA-E expression on porcine cells: protection from human NK cytotoxicity depends on peptide loading. *Am J Transplant*. 2005;5:2085-2093.

447. Ito M, Maruyama T, Saito N, Koganei S, Yamamoto K, Matsumoto N. Killer cell lectin-like receptor G1 binds three members of the classical cadherin family to inhibit NK cell cytotoxicity. *J Exp Med*. 2006;203:289-295.

448. Moretta A, Biassoni R, Bottino C, Mingari MC, Moretta L. Natural cytotoxicity receptors that trigger human NK cell-mediated cytolysis. *Immunol Today*. 2000;21:228-234.

449. Nakamura MC, Naper C, Niemi EC, et al. Natural killing of xenogeneic cells mediated by the mouse Ly-49D receptor. *J Immunol*. 1999;163:4694-4700.

450. Forte P, Lilienfeld BG, Baumann BC, Seebach JD. Human NK cytotoxicity against porcine cells is triggered by NKp44 and NKG2D. *J Immunol*. 2005;175:5463-5470.

451. Christiansen D, Mouhtouris E, Milland J, Zingoni A, Santoni A, Sandrin MS. Recognition of a carbohydrate xenoepitope by human NKRP1A (CD161). *Xenotransplant*. 2006;13:440-446.

452. Sharabi Y, Sachs DH. Mixed chimerism and permanent specific transplantation tolerance induced by a non-lethal preparative regimen. *J Exp Med*. 1989;169:493-502.

453. Sharabi Y, Aksentijevich I, Sundt TM III, Sachs DH, Sykes M. Specific tolerance induction across a xenogeneic barrier: production of mixed rat/mouse lymphohematopoietic chimeras using a nonlethal preparative regimen. *J Exp Med*. 1990;172:195-202.

454. Nikolic B, Cooke DT, Zhao G, Sykes M. Both γ/ δT cells and NK cells inhibit the engraftment of xenogeneic rat bone marrow cells in mice. *J Immunol*. 2001;166:1398-1404.

455. Goodman DJ, Millan MT, Ferran C, Bach FH. Mechanisms of delayed xenograft rejection. In: Cooper DKC, Kemp E, Platt JL, White DJG, eds. *Xenotransplantation: The Transplantation of Organs and Tissues between Species*. 2nd ed. Heidelberg, Germany: Springer; 1997:77-94.

456. Gourlay WA, Chambers WH, Monaco A, Maki T. Importance of natural killer cells in the rejection of hamster skin xenografts. *Transplantation*. 1998;65:727-734.

457. Seino KK, Fukao K, Muramoto K, et al. Requirement for natural killer T (NKT) cells in the induction of allograft tolerance. *Proc Natl Acad Sci U S A* . 2001;98:2577-2581.

458. Sonoda KH, Taniguchi M, Stein-Streilein J. Long-term survival of corneal allografts is dependent on intact CD1d- reactive NKT cells. *J Immunol*. 2002;168:2028-2034.

459. Jiang X, Shimaoka T, Kojo S, et al. Cutting edge: critical role of CXCL16/CXCR6 in NKT cell trafficking in allograft tolerance. *J Immunol*. 2005;175:2051-2055.

460. Oh K, Kim S, Park SH, et al. Direct regulatory role of NKT cells in allogeneic graft survival is dependent on the quantitative strength of antigenicity. *J Immunol*. 2005;174:2030-2036.

461. Zeng D, Lewis D, Dejbakhsh-Jones S, et al. Bone marrow NK1.1- and NK1.1+ T cells reciprocally regulate acute graft versus host disease. *J Exp Med*. 1999;189:1073-1081.

462. Haraguchi K, Takahashi T, Matsumoto A, et al. Host-residual invariant NK T cells attenuate graft-versus-host immunity. *J Immunol*. 2005;175:1320-1328.

463. Toyofuku A, Yasunami Y, Nabeyama K, et al. Natural killer T-cells participate in rejection of islet allografts in the liver of mice. *Diabetes*. 2006;55:34-39.

464. Jukes JP, Wood KJ, Jones ND. Natural killer T cells: a bridge to tolerance or a pathway to rejection? *Transplantation*. 2007;84:679-681.

465. Nierlich PN, Klaus C, Bigenzahn D, et al. The role of natural killer T cells in costimulation blockade-based mixed chimerism. *Transpl Int*. 2010;23:1179-1189.

466. Mattarollo SR, Yong M, Tan L, Frazer IH, Leggatt GR. Secretion of IFN-gamma but not IL-17 by CD1d-restricted NKT cells enhances rejection of skin grafts expressing epithelial cell-derived antigen. *J Immunol*. 2010;184:5663-5669.

467. Bogdan C. Nitric oxide and the immune response. *Nat Immunol*. 2001;2:907-916.

468. Wu G, Korsgren O, Zhang J, et al. Pig islet xenograft rejection is markedly delayed in macrophage-depleted mice: a study in streptozotocin diabetic animals. *Xenotransplant*. 2000;7:214-220.

469. Andres A, Toso C, Morel P, et al. Macrophage depletion prolongs discordant but not concordant islet xenograft survival. *Transplantation*. 2005;79:543-549.

470. Yi S, Hawthorne WJ, Lehnert AM, et al. T cell-activated macrophages are capable of both recognition and rejection of pancreatic islet xenografts. *J Immunol*. 2003;170:2750-2758.

471. Terpstra W, Leenen PJ, van den Bos C, et al. Facilitated engraftment of human hematopoietic cells in severe combined immunodeficient mice following a single injection of Cl2MDP liposomes. *Leukemia*. 1997;11:1049-1054.

472. Abe M, Cheng J, Qi J, et al. Elimination of porcine hematopoietic cells by macrophages in mice. *J Immunol*. 2002;168:621-628.

473. Basker M, Alwayn IP, Buhler L, et al. Clearance of mobilized porcine peripheral blood progenitor cells is delayed by depletion of the phagocytic reticuloendothelial system in baboons. *Transplantation*. 2001;72:1278-1285.

474. Ide K, Ohdan H, Kobayashi T, et al. Antibody- and complement-independent phagocytotic and cytolytic activities of human macrophages toward porcine cells. *Xenotransplant*. 2005;12:181-188.

475. Blakely ML, Van Der Werf W, Berndt MC, Dalmasso AP, Bach FH, Hancock WW. Activation of intragraft endothelial and mononuclear cells during discordant xenograft rejection. *Transplantation*. 1994; 58:1059-1066.

476. Candinas D, Belliveau S, Koyomada N, et al. T cell independence of macrophage and natural killer cell infiltration, cytokine production, and endothelial activation during delayed xenograft rejection. *Transplantation*. 1996;62:1920-1927.

477. Lin Y, Vandeputte M, Waer M. Natural killer cell- and macrophagemediated rejection of concordant xenografts in the absence of T and B cell responses. *J Immunol*. 1997;158:5658-5667.

478. Wu GS, Korsgren O, Can Rooijen N, Wennberg L, Tibell A. The effect of macrophage depletion on delayed xenograft rejection: studies in the guinea pig-to-C6-deficient rat heart transplantation model. *Xenotransplant*. 1999;6:262-270.

479. Xia G, Ji P, Rutgeerts O, Waer M. Natural killer cell- and macrophage mediated discordant guinea pig→rat xenograft rejection in the absence of complement, xenoantibody and T cell immunity. *Transplantation*. 2000;70:86-93.

480. Zhao Y, Xiong W, Yang T, Prall A, Baxter BT, Langnas AN. Xenogeneic skin graft rejection in M-CSF/macrophage deficient osteopetrotic mice. *Xenotransplant*. 2003;10:232-239.

481. Rees MA, Butler AJ, Brons IG, Negus MC, Skepper JN, Friend PJ. Evidence of macrophage receptors capable of direct recognition of xenogeneic epitopes without opsonization. *Xenotransplant*. 2005;12:13-19.

482. Wang H, Verhalen J, Madariaga ML, et al. Attenuation of phagocytosis of xenogeneic cells by manipulating CD47. *Blood*. 2007;109:836-842.

483. Zecher D, van Rooijen N, Rothstein DM, Shlomchik WD, Lakkis FG. An innate response to allogeneic nonself mediated by monocytes. *J Immunol*. 2009;183:7810-7816.

484. Solez K, Colvin RB, Racusen LC, et al. Banff'05 meeting report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). *Am J Transplant*. 2007;7:518-526.

485. Russell P, Chase C, Colvin R. Accelerated atheromatous lesions in mouse hearts transplanted into apolipoprotein E deficient recipients. *Am J Pathol*. 1996;149:91-99.

486. Schmid C, Heemann UW, Tilney NL. Factors contributing to the development of chronic rejection in heterotopic rat heart transplantation 1. *Transplantation*. 1997;64:222-228.

487. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR. The natural history of chronic allograft nephropathy. *N Engl J Med*. 2003;349:2326-2333.

488. Nankivell BJ, Kuypers DR. Diagnosis and prevention of chronic kidney allograft loss. *Lancet*. 2011;378:1428-1437.

489. Cosio FG, Pelletier RP, Falkenhain ME, et al. Impact of acute rejection and early allograft function on renal allograft survival. *Transplantation*. 1997;63:1611-1615.

490. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR. Natural history, risk factors, and impact of subclinical rejection in kidney transplantation. *Transplantation*. 2004;78:242-249.

491. Petersen VP, Olsen TS, Kissmeyer-Nielsen F, et al. Late failure of human renal transplants: an analysis of transplant disease and graft failure among 125 recipients surviving for one to eight years. *Medicine*. 1995;54:45-71.

492. Reed EF, Hong B, Ho E, et al. Monitoring of soluble HLA alloantigens and anti-HLA antibodies identifies heart allograft recipients at risk of transplant-associated coronary artery disease. *Transplantation*. 1996;61:566-572.

493. Terasaki PI, Cai J. Human leukocyte antigen antibodies and chronic rejection: from association to causation. *Transplantation*. 2008;86: 377-383.

494. Uehara S, Chase CM, Kitchens WH, et al. NK cells can trigger allograft vasculopathy: the role of hybrid resistance in solid organ allografts. *J Immunol*. 2005;175:3424-3430.

495. Hirohashi T, Chase CM, Della Pelle P, et al. A novel pathway of chronic allograft rejection mediated by NK cells and alloantibody. *Am J Transplant*. 2012;12:313-321.

496. Uehara S, Chase CM, Cornell LD, Madsen JC, Russell PS, Colvin RB. Chronic cardiac transplant arteriopathy in mice: relationship of alloantibody, C4d deposition and neointimal fibrosis. *Am J Transplant*. 2007;7:57-65.

497. Ensminger SM, Spriewald BM, Sorensen HV, et al. Critical role for il-4 in the development of transplant arteriosclerosis in the absence of CD40-CD154 costimulation. *J Immunol*. 2001;167:532-541.

498. Allan JS, Wain JC, Schwarze ML, et al. Modeling chronic lung allograft rejection in miniature swine. *Transplantation*. 2002;73:447-453.

499. Smith RN, Kawai T, Boskovic S, et al. Chronic antibody mediated rejection of renal allografts: pathological, serological and immunologic features in nonhuman primates. *Am J Transplant*. 2006;6:1790-1798.

500. Geraghty JG, Stoltenberg RL, Sollinger HW, Hullett DA. Vascular smooth muscle cells and neointimal hyperplasia in chronic transplant rejection. *Transplantation*. 1996;62:502-509.

501. Shimizu K, Sugiyama S, Aikawa M, et al. Host bone-marrow cells are a source of donor intimal smooth- muscle- like cells in murine aortic transplant arteriopathy. *Nat Med*.

2001;7:738-741.

502. Wheeler CH, Collins A, Dunn M, Crisp S, Yacoub MH, Rose ML. Characterization of endothelial antigens associated with transplantassociated coronary artery disease. *J Heart Lung Transplant*. 1995;14: S188-S197.

503. Dunn MJ, Crisp SJ, Rose ML, Taylor PM, Yacoub MH. Antiendothelial antibodies and coronary artery disease after cardiac transplantation. *Lancet*. 1992;339:1566-1570.

504. Russell ME, Wallace AF, Wyner LR, Newell JB, Karnovsky MJ. Upregulation and modulation of inducible nitric oxide synthase in rat cardiac allografts with chronic rejection and transplant arteriosclerosis. *Circulation*. 1995;92:457-464.

505. Tellides G, Tereb DA, Kirkiles-Smith NC, et al. Interferon-[gamma] elicits arteriosclerosis in the absence of leukocytes. *Nature*. 2000;403:207-211.

506. Watschinger B, Sayegh MH, Hancock WW, Russell M. Upregulatoin of endothelin-1 mRNA and peptide expression in rat cardiac allografts with rejection and arteriosclerosis. *Am J Pathol*. 1995;146:1065-1072.

507. Wilkes DS. Autoantibody formation in human and rat studies of chronic rejection and primary graft dysfunction. *Sem Immunol*. 2012;24: 131-135.

508. Libby P, Pober JS. Chronic rejection. *Immunity*. 2001;14:387-397.

509. Wood KJ, Goto R. Mechanisms of rejection: current perspectives. *Transplantation*. 2012;93:1-10.

510. Russell PS, Chase CM, Sykes M, Ito H, Shaffer J, Colvin RB. Tolerance, mixed chimerism and chronic transplant arteriopathy. *J Immunol*. 2001;167:5731-5740.

511. Pratt JR, Basheer SA, Sacks SH. Local synthesis of complement component C3 regulates acute renal transplant rejection. *Nat Med*. 2002;8:582-587.

512. Bolton EM, Bradley JA, Pettigrew GJ. Indirect allorecognition: not simple but effective. *Transplantation*. 2008;85:667-669.

513. Lee RS, Yamada K, Houser SL, et al. Indirect recognition of allopeptides promotes the development of cardiac allograft vasculopathy. *Proc Natl Acad Sci U S A*. 2001;98:3276-3281.

514. Madsen JC, Sachs D, Fallon J, Weissman N. Cardiac allograft vasculopathy in partially inbred miniature swine. I. Time course, pathology, and dependence on immune mechanisms. *J Thoracic Cardiovasc Surg*. 1996;111:1230-1239.

515. Russell PS, Chase CM, Winn HJ, Colvin RB. Coronary atherosclerosis in transplanted mouse hearts. III. Effects of recipient treatment with a monoclonal antibody

to interferon-gamma. *Transplantation*. 1994;57:1367-1371.

516. Shi C, Lee WS, He Q, et al. Immunologic basis of transplant-associated arteriosclerosis. *Proc Natl Acad Sci U S A* . 1996;93:4051-4056.

517. Russell PS, Chase CM, Colvin RB. Alloantibody- and T cell-mediated immunity in the pathogenesis of transplant arteriosclerosis: lack of progression to sclerotic lesions in B cell-deficient mice. *Transplantation*. 1997;64:1531-1536.

518. Tellides G, Tereb DA, Kirkiles-Smith NC, et al. Interferon-[gamma] elicits arteriosclerosis in the absence of leukocytes. *Nature*. 2000;403: 207-211.

519. Raisanen-Sokolowski A, Glysing-Jensen T, Koglin J, Russell ME. Reduced transplant arteriosclerosis in murine cardiac allografts placed in interferon-gamma knockout recipients. *Am J Pathol* . 1998;152: 359-365.

520. Koglin J, Glysing-Jensen T, Gadiraju S, Russell ME. Attenuated cardiac allograft vasculopathy in mice with targeted deletion of the transcription factor STAT4. *Circulation*. 2000;101:1034-1039.

521. Spriewald BM, Ensminger SM, Billing JS, Morris PJ, Wood KJ. Increased expression of transforming growth factor-[beta] and eosinophil infiltration is associated with the development of transplant arteriosclerosis in long-term surviving cardiac allografts. *Transplantation*. 2003;76:1105-1111.

522. Coghill JM, Sarantopoulos S, Moran TP, Murphy WJ, Blazar BR, Serody JS. Effector CD4+ T cells, the cytokines they generate, and GVHD: something old and something new. *Blood*. 2011;117:3268-3276.

523. Varas F, Grande T, Ramirez A, Bueren JA. Implantation of bone marrow beneath the kidney capsule results in transfer not only of functional stroma but also of hematopoietic repopulating cells. *Blood*. 2000;96:2307-2309.

524. Bellgrau D, Gold D, Selawry H, Moore J, Franzusoff A, Duke RC. A role for CD95 ligand in preventing graft rejection. *Nature*. 1995;377: 630-632.

525. Allison J, Georgiou HM, Strasser A, Vaux DL. Transgenic expression of CD95 ligand on islet beta cells induces a granulocytic infiltration but does not confer immune privilege upon islet allograft. *Proc Natl Acad Sci U S A* . 1997;94:3943-3947.

526. Kang SM, Schneider DB, Lin Z, et al. Fas ligand expression in islets of Langerhans does not confer immune privilege and instead targets them for rapid destruction. *Nat Med*. 1997;3:738-743.

527. Buonocore S, Paulart F, Le Moine A, et al. Dendritic cells overexpressing CD95 (Fas) ligand elicit vigorous allospecific T-cell responses in vivo. *Blood*. 2003;101:1469-1476.

528. Yolcu ES, Gu X, Lacelle C, et al. Induction of tolerance to cardiac allografts using donor splenocytes engineered to display on their surface an exogenous fas ligand protein. *J Immunol*. 2008;181:931-939.

529. Pearl-Yafe M, Yolcu ES, Stein J, et al. Fas ligand enhances hematopoietic cell engraftment through abrogation of alloimmune responses and nonimmunogenic interactions. *Stem Cells*. 2007;25:1448-1455.

530. Yolcu ES, Zhao H, Bandura-Morgan L, et al. Pancreatic islets engineered with SA-FasL protein establish robust localized tolerance by inducing regulatory T cells in mice. *J Immunol*. 2011;187:5901-5909.

531. Chambers CA, Sullivan TJ, Allison JP. Lymphoproliferation in CTLA-4-deficient mice is mediated by costimulation-dependent activation of CD4+ T cells. *Immunity*. 1997;7:885-895.

532. Bachmann MF, Kohler G, Ecabert B, Mak TW, Kopf M. Lymphoproliferative disease in the absence of CTLA-4 is not T cell autonomous. *J Immunol*. 1999;163:1128-1131.

533. Fehr T, Takeuchi Y, Kurtz J, Sykes M. Early regulation of CD8 T cell alloreactivity by CD4+CD25- T cells in recipients of anti-CD154 antibody and allogeneic BMT is followed by rapid peripheral deletion of donor-reactive CD8+ T cells, precluding a role for sustained regulation. *Eur J Immunol* . 2005;35:2679-2690.

534. Kurtz J, Raval F, Vallot C, Der J, Sykes M. CTLA4 on alloreactive CD4 T cells interacts with recipient CD80/86 to promote tolerance. *Blood*. 2009;113:3475-3484.

535. Frauwirth KA, Alegre ML, Thompson CB. Induction of T cell anergy in the absence of CTLA-4/B7 interaction. *J Immunol*. 2000;164: 2987-2993.

536. Greenwald RJ, Boussiotis VA, Lorsbach RB, Abbas AK, Sharpe AH. CTLA-4 regulates induction of anergy in vivo. *Immunity*. 2001;14: 145-155.

537. Gribben JG, Freeman GJ, Boussiotis VA, et al. CTLA4 mediates antigen-specific apoptosis of human T cells. *Proc Natl Acad Sci U S A* . 1995; 92:811-815.

538. Issazadeh S, Zhang M, Sayegh MH, Khoury SJ. Acquired thymic tolerance: role of CTLA4 in the initiation and maintenance of tolerance in a clinically relevant autoimmune disease model. *J Immunol*. 1999;162:765.

539. Judge TA, Wu Z, Zheng ZG, Sharpe AH, Sayegh MH, Turka LA. The role of CD80, CD86 and CTLA4 in alloimmune responses and the induction of long-term allograft survival. *J Immunol*. 1999;162:1947-1951.

540. Markees TG, Phillips NE, Gordon EJ, et al. Long-term survival of skin allografts induced by donor splenocytes and anti-CD154 antibody in thymectomized mice requires CD4+ T cells, Interferon-γ, and CTLA4. *J Clin Invest*. 1998;101:2446-2455.

541. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med*. 2000;192:295-302.

542. Samoilova EB, Horton JL, Zhang H, Khoury SJ, Weiner HL, Chen Y. CTLA-4 is required for the induction of high dose oral tolerance. *Int Immunol*. 1998;10:491-498.

543. Shrikant P, Khoruts A, Mescher MF. CTLA-4 blockade reverses CD8+ T cell tolerance to tumor by a CD4+ T cell- and IL-2-dependent mechanism. *Immunity*. 1999;11:483-493.

544. Walunas TL, Bluestone JA. CTLA-4 regulates tolerance induction and T cell differentiation in vivo. *J Immunol*. 1998;160:3855-3860.

545. Zheng XX, Markees TG, Hancock WW, et al. CTLA4 signals are required to optimally induce allograft tolerance with combined donorspecific transfusion and anti-CD154 monoclonal antibody treatment. *J Immunol*. 1999;162:4983-4990.

546. Chai JG, Vendetti S, Amofah E, Dyson J, Lechler R. CD152 ligation by CD80 on T cells is required for the induction of unresponsiveness by costimulation-deficient antigen presentation. *J Immunol*. 2000;165:3037-3042.

547. Lin H, Rathmell JC, Gray GS, Thompson CB, Leiden JM, Alegre ML. Cytotoxic T lymphocyte antigen 4 (CTLA4) blockade accelerates the acute rejection of cardiac allografts in CD28-deficient mice: CTLA4 can function independently of CD28. *J Exp Med*. 1998;188:199-204.

548. Blazar BR, Taylor PA, Panoskaltsis-Mortari A, Sharpe AH, Vallera DA. Opposing roles of CD28:B7 and CTLA-4:B7 pathways in regulating in vivo alloresponses in murine recipients of MHC disparate T cells. *J Immunol*. 1999;162:6368-6377.

549. Sandner SE, Clarkson MR, Salama AD, et al. Role of the programmed death-1 pathway in regulation of alloimmune responses in vivo. *J Immunol*. 2005;174:3408-3415.

550. Haspot F, Fehr T, Gibbons C, et al. Peripheral deletional tolerance of alloreactive CD8 but not CD4 cells is dependent on the PD-1/PD-L1 pathway. *Blood*. 2008;112:2149-2155.

551. Lucas CL, Workman CJ, Beyaz S, et al. LAG-3, TGF-β, and cellintrinsic PD-1 inhibitory pathways contribute to CD8 but not CD4 T-cell tolerance induced by allogeneic BMT with anti-CD40L. *Blood*. 2011;117:5532-5540.

552. Koehn BH, Ford ML, Ferrer IR, et al. PD-1-dependent mechanisms maintain peripheral tolerance of donor-reactive CD8+ T cells to transplanted tissue. *J Immunol*. 2008;181:5313-5322.

553. Schuchmann M, Meyer RG, Distler E, et al. The programmed death (PD)-1/PD-ligand 1 pathway regulates graft-versus-host-reactive CD8 T cells after liver transplantation. *Am J Transplant*. 2008;8:2434-2444.

554. Wang W, Carper K, Malone F, et al. PD-L1/PD-1 signal deficiency promotes allogeneic immune responses and accelerates heart allograft rejection. *Transplantation*. 2008;86:836-844.

555. Blazar BR, Carreno BM, Panoskaltsis-Mortari A, et al. Blockade of programmed death-1 engagement accelerates graft-versus-host disease lethality by an IFN-gamma-dependent mechanism. *J Immunol*. 2003;171:1272-1277.

556. Flutter B, Edwards N, Fallah-Arani F, et al. Nonhematopoietic antigen blocks memory programming of alloreactive CD8+ T cells and drives their eventual exhaustion in mouse models of bone marrow transplantation. *J Clin Invest*. 2010;120:3855-3868.

557. Asakura S, Hashimoto D, Takashima S, et al. Alloantigen expression on non-hematopoietic cells reduces graft-versus-leukemia effects in mice. *J Clin Invest*. 2010;120:2370-2378.

558. Yang J, Riella LV, Chock S, et al. The novel costimulatory programmed death ligand 1/B7.1 pathway is functional in inhibiting alloimmune responses in vivo. *J Immunol*. 2011;187:1113-1119.

559. Tao R, Wang L, Han R, et al. Differential effects of B and T lymphocyte attenuator and programmed death-1 on acceptance of partially versus fully MHC-mismatched cardiac allografts. *J Immunol*. 2005;175: 5774-5782.

560. Albring JC, Sandau MM, Rapaport AS, et al. Targeting of B and T lymphocyte associated (BTLA) prevents graft-versus-host disease without global immunosuppression. *J Exp Med*. 2010;207:2551-2559.

561. del Rio ML, Kurtz J, Perez-Martinez C, et al. B- and T-lymphocyte attenuator targeting protects against the acute phase of graft versus host reaction by inhibiting donor anti-host cytotoxicity. *Transplantation*. 2011;92:1085-1093.

562. Truong W, Plester JC, Hancock WW, et al. Negative and positive co-signaling with anti-BTLA (PJ196) and CTLA4Ig prolongs islet allograft survival. *Transplantation*. 2007;84:1368-1372.

563. Truong W, Plester JC, Hancock WW, et al. Combined coinhibitory and costimulatory modulation with anti-BTLA and CTLA4Ig facilitates tolerance in murine islet allografts. *Am J Transplant*. 2007;7:2663-2674.

564. Raisanen-Sokolowski A, Mottram PL, Glysing-Jensen T, Satoskar A, Russell ME. Heart transplants in interferon-gamma, interleukin 4, and interleukin 10 knockout mice. Recipient environment alters graft rejection. *J Clin Invest*. 1997;100:2449-2456.

565. Gorczynski RM, Hadidi S, Yu G, Clark DA. The same immunoregulatory molecules contribute to successful pregnancy and transplantation. *Am J Reprod Immunol* . 2002;48:18-26.

566. Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev*. 2006;212:28-50.

567. Lin MT, Storer B, Martin PJ, et al. Genetic variation in the IL-10 pathway modulates severity of acute graft-versus-host disease following hematopoietic cell transplantation: synergism between IL-10 genotype of patient and IL-10 receptor beta genotype of donor. *Blood*. 2005;106:3995-4001.

568. Banovic T, MacDonald KP, Morris ES, et al. TGF-beta in allogeneic stem cell transplantation: friend or foe? *Blood*. 2005;106:2206-2214.

569. Kim DH, Lee NY, Sohn SK, et al. IL-10 promoter gene polymorphism associated with the occurrence of chronic GVHD and its clinical course during systemic immunosuppressive treatment for chronic GVHD after allogeneic peripheral blood stem cell transplantation. *Transplantation*. 2005;79:1615-1622.

570. Coomes SM, Moore BB. Pleiotropic effects of transforming growth factor-beta in hematopoietic stem-cell transplantation. *Transplantation*. 2010;90:1139-1144.

571. Qin L, Chavin KD, Ding Y, et al. Gene transfer for transplantation: prolongation of allograft survival with transforming growth factor-b1. *Ann Surg*. 1994;220:508-519.

572. Qin L, Chavin KD, Ding Y, et al. Retrovirus-mediated transfer of viral IL-10 gene prolongs murine cardiac allograft survival. *J Immunol* . 1996;156:2316-2323.

573. Weimer R, Zipperle S, Daniel V, Carl S, Staehler G, Opelz G. Pretransplant CD4 helper function and interleukin 10 response predict risk of acute kidney graft rejection. *Transplantation*. 1996;62: 1606-1614.

574. Klebe S, Sykes PJ, Coster DJ, Krishnan R, Williams KA. Prolongation of sheep corneal allograft survival by ex vivo transfer of the gene encoding interleukin-10. *Transplantation*. 2001;71:1214-1220.

575. Zhang W, Zhang D, Shen M, et al. Combined administration of a mutant TGF-beta1/Fc and rapamycin promotes induction of regulatory T cells and islet allograft tolerance. *J Immunol* . 2010;185:4750-4759.

576. Zheng XX, Steele AW, Nickerson PW, Steurer W, Steiger J, Strom TB. Administration of noncytolytic IL-10/Fc in murine models of lipopolysaccharide-induced septic shock and allogeneic islet transplantation. *J Immunol* . 1995;154:5590-5600.

577. Booth AJ, Bishop DK. TGF-beta, IL-6, IL-17 and CTGF direct multiple pathologies of

chronic cardiac allograft rejection. *Immunotherapy*. 2010;2:511-520.

578. Coomes SM, Moore BB. Pleiotropic effects of transforming growth factor-beta in hematopoietic stem-cell transplantation. *Transplantation*. 2010;90:1139-1144.

579. Regateiro FS, Howie D, Cobbold SP, Waldmann H. TGF-beta in transplantation tolerance. *Curr Opin Immunol* . 2011;23:660-669.

580. Dai Z, Konieczny BT, Baddoura FK, Lakkis FG. Impaired alloantigenmediated T cell apoptosis and failure to induce long-term allograft survival in IL-2-deficient mice. *J Immunol*. 1998;161:1659-1663.

581. Sykes M, Romick ML, Hoyles KA, Sachs DH. In vivo administration of interleukin 2 plus T cell-depleted syngeneic marrow prevents graftversus-host disease mortality and permits alloengraftment. *J Exp Med*. 1990;171:645-658.

582. Malek TR, Bayer AL. Tolerance, not immunity, crucially depends on IL-2. *Nat Rev Immunol*. 2004;4:665-674.

583. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* . 2005;6:1142-1151.

584. Dai Z, Arakelov A, Wagener M, Konieczny BT, Lakkis FG. The role of the common cytokine receptor γ-chain in regulating IL-2-dependent, activation-induced CD8+ T cell death. *J Immunol* . 1999;163:3131-3137.

585. Goes N, Urmson J, Vincent D, Halloran PF. Acute renal injury in the interferon-gamma gene knockout mouse: effect on cytokine gene expression. *Transplantation*. 1995;60:1560-1564.

586. Konieczny BT, Dai Z, Elwood ET, et al. IFN-γ is critical for long-term allograft survival induced by blocking the CD28 and CD40 ligand T cell costimulation pathways. *J Immunol*. 1998;160:2059-2064.

587. Hassan AT, Dai Z, Konieczny BT, et al. Regulation of alloantigen-mediated T-cell proliferation by endogenous interferon-γ. *Transplantation*. 1999;68:124-129.

588. Yang YG, Sergio JJ, Pearson DA, Szot GL, Shimizu A, Sykes M. Interleukin-12 preserves the graft-versus-leukemia effect of allogeneic CD8 T cells while inhibiting CD4-dependent graft-vs-host disease in mice. *Blood*. 1997;90:4651-4660.

589. Oyaizu N, McCloskey TW, Than S, Hu R, Kalyanaraman VS, Pahwa S. Cross-linking of CD4 molecules upregulates Fas antigen expression in lymphocytes by inducing interferon-gamma and tumor necrosis factor-α secretion. *Blood*. 1994;84:2622-2631.

590. Novelli F, D'Elios MM, Bernabei P, et al. Expression and role in apoptosis of the α- and β-chains of the IFN-gamma receptor on human Th1 and Th2 clones. *J Immunol* .

1997;159:206-213.

591. Rafaeli Y, Van Parijs L, Alexander SI, Abbas AK. Interferon gamma is required for activation-induced death of T lymphocytes. *J Exp Med*. 2002;196:999-1005.

592. Willenborg DO, et al. IFN-gamma is critical to the control of murine autoimmune encephalomyelitis and regulates both in the periphery and in the target tissue: a possible role for nitric oxide. *J Immunol*. 1999;163:5278-5286.

593. Koblish HK, Hunter CA, Wysocka M, Trinchieri G, Lee WM. Immune suppression by recombinant interleukin (rIL)-12 involves interferon γ induction of nitric oxide synthase 2 (iNOS) activity: inhibitors of NO generation reveal the extent of rIL-12 vaccine adjuvant effect. *J Exp Med*. 1998;188:1603-1610.

594. Tarrant TK, Silver PB, Wahlsten JL, et al. Interleukin 12 protects from a T helper type 1-mediated autoimmune disease, experimental autoimmune uveitis, through a mechanism involving interferon-γ, nitric oxide, and apoptosis. *J Exp Med*. 1999;189:219-230.

595. Lasarte JJ, Corrales FJ, Casares N, et al. Different doses of adenoviral vector expressing IL-12 enhance or depress the immune response to a coadministered antigen: the role of nitric oxide. *J Immunol*. 1999;162: 5270-5277.

596. Sawitzki B, Kingsley CI, Oliveira V, Karim M, Herber M, Wood KJ. IFN-gamma production by alloantigen-reactive regulatory T cells is important for their regulatory function in vivo. *J Exp Med*. 2005;201: 1925-1935.

597. Wood KJ, Sawitzki B. Interferon gamma: a crucial role in the function of induced regulatory T cells in vivo. *Trends Immunol*. 2006;27: 183-187.

598. Wood KJ, Feng G, Wei B, Sawitzki B, Bushell AR. Interferon gamma: friend or foe? *Transplantation*. 2007;84:S4-S5.

599. Piccotti JR, Chan SY, Goodman RE, Magram J, Eichwald EJ, Bishop DK. IL-12 antagonism induces T helper 2 responses, yet exacerbates cardiac allograft rejection. Evidence against a dominant protective role for T helper 2 cytokines in alloimmunity. *J Immunol*. 1996;157:1951-1957.

600. Sykes M, Szot GL, Nguyen PL, Pearson DA. Interleukin-12 inhibits murine graft-vs-host disease. *Blood*. 1995;86:2429-2438.

601. Reddy P, Teshima T, Kukuruga M, et al. Interleukin-18 regulates acute graft-versus-host disease by enhancing Fas-mediated donor T cell apoptosis. *J Exp Med*. 2001;194:1433-1440.

602. Dey B, Yang YG, Szot GL, Pearson DA, Sykes M. IL-12 inhibits GVHD through a Fas-mediated mechanism associated with alterations in donor T cell activation and

expansion. *Blood*. 1998;91:3315-3322.

603. Sykes M, Romick ML, Sachs DH. Interleukin 2 prevents graft-vs-host disease while preserving the graft-vs-leukemia effect of allogeneic T cells. *Proc Natl Acad Sci U S A* . 1990;87:5633-5637.

604. Sykes M, Harty MW, Szot GL, Pearson DA. Interleukin-2 inhibits graft-versus-host disease-promoting activity of CD4+ cells while preserving CD4- and CD8-mediated graft-versus-leukemia effects. *Blood*. 1994;83:2560-2569.

605. Teshima T, Hill GR, Pan L, et al. IL-11 separates graft-versus-leukemia effects from graft-versus-host disease after bone marrow transplantation. *J Clin Invest*. 1999;104:317-325.

606. Reddy P, Teshima T, Hildebrandt G, et al. Pretreatment of donors with interleukin-18 attenuates acute graft-versus-host disease via STAT6 and preserves graft-versus-leukemia effects. *Blood*. 2003;101:2877-2885.

607. Yang YG, Qi J, Wang MG, Sykes M. Donor-derived interferon γ separates graft-versus-leukemia effects and graft-versus-host disease induced by donor CD8 T cells. *Blood*. 2002;99:4207-4215.

608. Yang Y, Wang H, Yu H, et al. IFN-gamma promotes graft-versus-leukemia effects without directly interacting with leukemia cells in mice after allogeneic hematopoietic cell transplantation. *Blood*. 2011;118: 3721-3724.

609. Wang H, Asavaroengchai W, Yeap BY, et al. Paradoxical effects of IFN-gamma in graft-versus-host disease reflect promotion of lymphohematopoietic graft-versus-host reactions and inhibition of epithelial tissue injury. *Blood*. 2009;113:3612-3619.

610. Yi T, Li X, Yao S, et al. Host APCs augment in vivo expansion of donor natural regulatory T cells via B7H1/B7.1 in allogeneic recipients. *J Immunol* . 2011;186:2739-2749.

611. Li XC, Roy-Chaudhury P, Hancock WW, et al. IL-2 and IL-4 double knockout mice reject islet allografts: a role for novel T cell growth factors in allograft rejection. *J Immunol*. 1998;161:890-896.

612. Lan F, Zeng D, Higuchi M, Huie P, Higgins JP, Strober S. Predominance of NK1.1(+)TCRalphabeta(+) or DX5(+)TCRalphabeta(+) T cells in mice conditioned with fractionated lymphoid irradiation protects against graft-versus-host disease: “natural suppressor” cells. *J Immunol* . 2001;167:20872096.

613. Nador RG, Hongo D, Yao Z, Strober S. The changed balance of regulatory and naive T cells promotes tolerance after TLI and anti-T-cell antibody conditioning. *Am J Transplant*. 2009;10:262-272.

614. Lowsky R, Takahashi T, Liu YP, et al. Protective conditioning for acute graft-versus-host disease. *N Engl J Med* . 2005;353:1321-1331.

615. Scandling JD, Busque S, Dejbakhsh-Jones S, et al. Tolerance and chimerism after renal and hematopoietic-cell transplantation. *N Engl J Med* . 2008;358:362-368.

616. Kohrt HE, Turnbull BB, Heydari K, et al. TLI and ATG conditioning with low risk of graft-versus-host disease retains antitumor reactions after allogeneic hematopoietic cell transplantation from related and unrelated donors. *Blood*. 2009;114:1099-1109.

617. Scandling JD, Busque S, Shizuru JA, Engleman EG, Strober S. Induced immune tolerance for kidney transplantation. *N Engl J Med* . 2011;365:1359-1360.

618. Russell P, Chase C, Colvin R, Plate J. An analysis of the immune status of mice bearing long-term H-2 incompatible transplants. *J Exp Med*. 1979;147:1449-1468.

619. Russell PS, Chase CM, Colvin RB, Plate JM. Kidney transplants in mice: an analysis of the immune status of mice bearing long-term H-2 incompatible transplants. *J Exp Med*. 1978;147:1449-1468.

620. Qian S, Thai NL, Lu L, Fung JJ, Thomson AW. Liver transplant tolerance: mechanistic insights from animal models, with particular reference to the mouse. *Transplant Rev*. 1997;11:151-164.

621. Calne RY, White HJ, Yoffa DE, et al. Prolonged survival of liver transplants in the pig. *Br Med J* . 1967;4:645-648.

622. Burdick RC, Russell PS. Antigenic requirement for induced rejection of long-surviving murine heart transplants. *J Immunol* . 1982;128: 1551-1554.

623. Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation*. 1973;16:343-350.

624. Salom RN, Maguire JA, Hancock WW. Mechanism of a clinically relevant protocol to induce tolerance of cardiac allografts. *Transplantation*. 1993;56:1309-1314.

625. Sayegh MH, Khoury SJ, Hancock WW, Weiner HL, Carpenter CB. Induction of immunity and oral tolerance with poymorphic class II major histocompatibility complex allopeptides in the rat. *Proc Natl Acad Sci U S A* . 1992;89:7762-7766.

626. Mottram PL, Han WR, Purcell LJ, McKenzie IF, Hancock WW. Increased expression of IL-4 and IL-10 and decreased expression of IL-2 and interferon-gamma in long-surviving mouse heart allografts after brief CD4-monoclonal antibody therapy. *Transplantation*. 1995;59: 559-565.

627. Lehmann M, Graser E, Risch K, et al. Anti-CD4 monoclonal antibody-induced

allograft tolerance in rats despite persistence of donor-reactive T cells. *Transplantation*. 1997;64:1181-1187.

628. Hancock WW, Lu B, Gao W, et al. Requirement of the chemokine receptor CXCR3 for acute allograft rejection. *J Exp Med*. 2000;192: 1515-1520.

629. Brynger H, Rydberg B, Samuelsson B, Sandberg L. Experience with 14 renal transplants with kidneys from blood group A (subgroup A2) to O recipients. *Trans Proc*. 1984;16:1175-1176.

630. Russell PS, Chase CM, Colvin RB, Plate JMD. Induced immune destruction of long-surviving H-2 incompatible kidney transplants in mice. *J Exp Med*. 1978;147:1469-1486.

631. Madsen JC, Yamada K, Allan JS, et al. Transplantation tolerance prevents cardiac graft vasculopathy in major histocompatiblity complex class I-disparate miniature swine. *Transplantation*. 1998;65:304-313.

632. Kawai T, Sogawa H, Koulmanda M, et al. Long-term islet allograft function in the absence of chronic immunosuppression: a case report of a nonhuman primate previously made tolerant to a renal allograft from the same donor. *Transplantation*. 2001;72:351-354.

633. Aoyama A, Ng CY, Millington TM, et al. Comparison of lung and kidney allografts in induction of tolerance by a mixed-chimerism approach in cynomolgus monkeys. *Transplant Proc*. 2009;41:429-430.

634. Shaffer J, Villard J, Means TK, et al. Regulatory T-cell recovery in recipients of haploidentical nonmyeloablative hematopoietic cell transplantation with a humanized anti-CD2 mAb, MEDI-507, with or without fludarabine. *Exp Hematol*. 2007;35:1140-1152.

635. Kawai T, Cosimi AB, Spitzer TR, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. *New Engl J Med* . 2008;358:353-361.

636. Andreola G, Chittenden M, Shaffer J, et al. Mechanisms of donor-specific tolerance in recipients of haploidentical combined bone marrow/kidney transplantation. *Am J Transplant*. 2011;11:1236-1247.

637. Moodycliffe AM, Shreedhar V, Ullrich SE, et al. CD40-CD40 ligand interactions in vivo regulate migration of antigen-bearing dendritic cells from the skin to draining lymph nodes. *J Exp Med*. 2000;191: 2011-2020.

638. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature*. 1998;392:245-252.

639. Kripke ML, Munn CG, Jeevan A, Tang JM, Bucana C. Evidence that cutaneous antigen-presenting cells migrate to regional lymph nodes during contact sensitization. *J Immunol*. 1990;145:2833-2838.

640. Cumberbatch M, Dearman RJ, Kimber I. Langerhans cells require signals from both tumour necrosis factor alpha and interleukin 1 beta for migration. *Adv Exp Med Biol* . 1997;417:125-128.

641. Cumberbatch M, Kimber I. Dermal tumour necrosis factor-alpha induces dendritic cell migration to draining lymph nodes, and possibly provides one stimulus for Langerhans' cell migration. *Immunology*. 1997;75:257-263.

642. Sykes M, Sheard MA, Sachs DH. Graft-versus-host-related immunosuppression is induced in mixed chimeras by alloresponses against either host or donor lymphohematopoietic cells. *J Exp Med*. 1988;168:2391-2396.

643. Pelot MR, Pearson DA, Swenson K, et al. Lymphohematopoietic graft-vs-host reactions can be induced without graft-vs-host disease in murine mixed chimeras established with a cyclophosphamidebased non-myeloablative conditioning regimen. *Biol Blood Marrow Transplant*. 1999;5:133-143.

644. Chakraverty R, Cote D, Buchli J, et al. An inflammatory checkpoint regulates recruitment of graft-versus-host-reactive T cells to peripheral tissues. *J Exp Med*. 2006;203:2021-2031.

645. Mapara MY, Leng C, Kim YM, et al. Expression of chemokines in GVHD target organs is influenced by conditioning and genetic factors and amplified by GVHR. *Biol Blood Marrow Transplant*. 2006;12:623-634.

646. Xun CQ, Thompson JS, Jennings CD, Brown SA, Widmer MB. Effect of total body irradiation, busulfan-cyclophosphamide, or cyclophosphamide conditioning on inflammatory cytokine release and development of acute and chronic graft-versus-host disease in H-2-incompatible transplanted SCID mice. *Blood*. 1994;83:2360-2367.

647. Krivenko SI, Drik SI, Komarovskaya ME, Karkanitsa LV. Ionizing radiation increases TNF/cachectin production by human peripheral blood mononuclear cells in vitro. *Int J Hematol*. 1992;55:127.

648. Schwaighofer H, Kernan NA, O'Reilly RJ, et al. Serum levels of cytokines and secondary messages after T-cell-depleted and non-T-celldepleted bone marrow transplantation. Influence of conditioning and hematopoietic reconstitution. *Transplantation*. 1996;62:947-953.

649. Carlos TM, Harlan JM. Leukocyte-endothelial adhesion molecules. *Blood*. 1994;84:2068-2101.

650. Pober JS, LaPierre LA, Stolpen AH, et al. Activation of cultured human endothelial cells by recombinant lymphotoxin: comparison with tumor necrosis factor and interleukin 1 species. *J Immunol* . 1987;138:3319.

651. Allen RD, Staley TA, Sidman CL. Differential cytokine expression in acute and chronic murine graft-versus-host disease. *Eur J Immunol* . 1993;23:333-337.

652. Morita K, Miura M, Paolone DR, et al. Early chemokine cascades in murine cardiac grafts regulate T cell recruitment and progression of acute allograft rejection. *J Immunol*. 2001;167:2979-2984.

653. Miura M, Fu X, Zhang QW, Remick DG, Fairchild RL. Neutralization of Gro alpha and macrophage inflammatory protein-2 attenuates renal ischemia/reperfusion injury. *Am J Pathol*. 2001;159:2137-2145.

654. El Sawy T, Belperio JA, Strieter RM, Remick DG, Fairchild RL. Inhibition of polymorphonuclear leukocyte-mediated graft damage synergizes with short-term costimulatory blockade to prevent cardiac allograft rejection. *Circulation*. 2005;112:320-331.

655. Kapoor A, Morita K, Engeman TM, et al. Early expression of interferon-gamma inducible protein 10 and monokine induced by interferon-gamma in cardiac allografts is mediated by CD8+ T cells. *Transplantation*. 2000;69:1147-1155.

656. Hancock WW, Gao W, Faia KL, Csizmadia V. Chemokines and their receptors in allograft rejection. *Curr Opin Immunol* . 2000;12:511-516.

657. Hancock WW, Gao W, Csizmadia V, Faia KL, Shemmeri N, Luster AD. Donor-derived IP-10 initiates development of acute allograft rejection. *J Exp Med*. 2001;193:975-980.

658. Matzinger P. Tolerance, danger, and the extended family. *Ann Rev Immunol*. 1994;12:991-1045.

659. Bingaman AW, Ha J, Waitze SY, et al. Vigorous allograft rejection in the absence of danger. *J Immunol*. 2000;164:3065-3071.

660. Anderson CC, Carroll JM, Gallucci S, Ridge JP, Cheever AW, Matzinger P. Testing time-, ignorance-, and danger-based models of tolerance. *J Immunol*. 2001;166:3663-3671.

661. Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol*. 2002;20:197-216.

662. Rock KL, Lai JJ, Kono H. Innate and adaptive immune responses to cell death. *Immunol Rev*. 2011;243:191-205.

663. Gross O, Thomas CJ, Guarda G, Tschopp J. The inflammasome: an integrated view. *Immunol Rev*. 2011;243:136-151.

664. Sacks SH, Chowdhury P, Zhou W. Role of the complement system in rejection. *Curr Opin Immunol*. 2003;15:487-492.

665. Sacks SH. Complement fragments C3a and C5a: the salt and pepper of the immune

response. *Eur J Immunol* . 2010;40:668-670.

666. Marsh JE, Farmer CK, Jurcevic S, Wang Y, Carroll MC, Sacks SH. The allogeneic T and B cell response is strongly dependent on complement components C3 and C4. *Transplantation*. 2001;72:1310-1318.

667. Carroll MC.The complement system in regulation of adaptive immunity. *Nat Immunol*. 2004;5:981-986.

668. Chan RK, Ibrahim SI, et al. The differing roles of the classical and mannose-binding lectin complement pathways in the events following skeletal muscle ischemia-reperfusion. *J Immunol* . 2006;177: 8080-8085.

669. Zhang M, Michael LH, Grosjean SA, Kelly RA, Carroll MC, Entman ML. The role of natural IgM in myocardial ischemia-reperfusion injury. *J Mol Cell Cardiol* . 2006;41:62-67.

670. Brown KM, Kondeatis E, Vaughan RW, et al. Influence of donor C3 allotype on late renal-transplantation outcome. *N Engl J Med* . 2006;354:2014-2023.

671. Vilches C, Parham P. Do NK-cell receptors and alloreactivity affect solid organ transplantation? *Transpl Immunol* . 2006;17:27-30.

672. Benichou G, Yamada Y, Aoyama A, Madsen JC. Natural killer cells in rejection and tolerance of solid organ allografts. *Curr Opin Organ Transplant* . 2010. Epub ahead of print.

673. Yu G, Xu X, Vu MD, Kilpatrick ED, Li XC. NK cells promote transplant tolerance by killing donor antigen-presenting cells. *J Exp Med*. 2006;203:1851-1858.

674. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* . 2004;5:987-995.

675. Goldstein DR, Tesar BM, Akira S, Lakkis FG. Critical role of the tolllike receptor signal adaptor protein MyD88 in acute allograft rejection. *J Clin Invest*. 2003;111:1571-1578.

676. Obhrai JS, Oberbarnscheidt MH, Hand TW, Diggs L, Chalasani G, Lakkis FG. Effector T cell differentiation and memory T cell maintenance outside secondary lymphoid organs. *J Immunol* . 2006;176: 4051-4058.

677. McKay D, Shigeoka A, Rubinstein M, Surh C, Sprent J. Simultaneous deletion of MyD88 and Trif delays major histocompatibility and minor antigen mismatch allograft rejection. *Eur J Immunol* . 2006;36: 1994-2002.

678. Arumugam TV, Okun E, Tang SC, Thundyil J, Taylor SM, Woodruff TM. Toll-like receptors in ischemia-reperfusion injury. *Shock*. 2009;32:4-16.

679. Arslan F, Smeets MB, O'Neill LA, et al. Myocardial ischemia/reperfusion injury is mediated by leukocytic toll-like receptor-2 and reduced by systemic administration of a novel anti-toll-like receptor-2 antibody. *Circulation*. 2010;121:80-90.

680. Shen H, Song Y, Colangelo CM, et al. Haptoglobin activates innate immunity to enhance acute transplant rejection in mice. *J Clin Invest*. 2012;122:383-387.

681. Chen L, Wang T, Zhou P, et al. TLR engagement prevents transplantation tolerance. *Am J Transplant*. 2006;6:2282-2291.

682. Burkhart C, Heusser C, Morris RE, et al. Pharmacodynamics in the development of new immunosuppressive drugs. *Ther Drug Monit* . 2004;26:588-592.

683. Gummert JF, Ikonen T, Morris RE. Newer immunosuppressive drugs: a review. *J Am Soc Nephrol*. 1999;10:1366-1380.

684. Raimondo ML, Burroughs AK. Single-agent immunosuppression after liver transplantation: what is possible? *Drugs*. 2002;62:1587-1597.

685. Regazzi MB, Alessiani M, Rinaldi M. New strategies in immunosuppression. *Transplant Proc*. 2005;37:2675-2678.

686. Krieger NR, Knechtle SJ. Novel immunosuppressive agents in tolerance induction. *Curr Drug Targets Cardiovasc Haematol Disord* . 2002;2:97-103.

687. Cruzado JM, Bestard O, Grinyo JM. New immunosuppressive protocols with the advent of novel biological drugs. *Transplantation*. 2009;88:S20-S23.

688. Lonberg N. Human antibodies from transgenic animals. *Nat Biotechnol*. 2005;23:1117-1125.

689. Vanrenterghem Y, Bresnahan B, Campistol J, et al. Belatacept-based regimens are associated with improved cardiovascular and metabolic risk factors compared with cyclosporine in kidney transplant recipients (BENEFIT and BENEFIT-EXT studies). *Transplantation*. 2011;91:976-983.

690. Nemazee D. Receptor editing in lymphocyte development and central tolerance. *Nat Rev Immunol*. 2006;6:728-740.

691. Goodnow CC. Transgenic mice and analysis of B-cell tolerance. *Ann Rev Immunol*. 1992;10:489-518.

692. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. *Science*. 2003;301:1374-1377.

693. Ota T, Ota M, Duong BH, Gavin AL, Nemazee D. Liver-expressed Igkappa

superantigen induces tolerance of polyclonal B cells by clonal deletion not kappa to lambda receptor editing. *J Exp Med*. 2011;208:617-629.

694. Hartley SB, Crosbie J, Brink RA, Kantor AB, Basten A, Goodnow CC. Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. *Nature*. 1991;353: 765-769.

695. Cambier JC, Gauld SB, Merrell KT, Vilen BJ. B-cell anergy: from transgenic models to naturally occurring anergic B cells? *Nat Rev Immunol* . 2007;7:633-643.

696. Li Y, Ma L, Shen J, Chong AS. Peripheral deletion of mature alloreactive B cells induced by costimulation blockade. *Proc Natl Acad Sci U S A* . 2007;104:12093-12098.

697. Li Y, Ma L, Yin D, Shen J, Chong AS. Long-term control of alloreactive B cell responses by the suppression of T cell help. *J Immunol* . 2008;180:6077-6084.

698. Ohdan H, Yang YG, Swenson KG, Thall AD, Sykes M. In vivo T-cell depletion enhances production of anti-galα1,3gal natural antibodies in α1,3-galactosyltransferase-deficient mice. *Transplantation*. 2000;69: 910-913.

699. Cretin N, Bracy J, Hanson K, Iacomini J. The role of T cell help in the production of antibodies specific for Gal alpha 1-3Gal. *J Immunol* . 2002;168:1479-1483.

700. Rodriguez-Barbosa JI, Zhao Y, Barth R, et al. Enhanced CD4 reconstitution by grafting neonatal porcine tissue in alternative locations is associated with donor-specific tolerance and suppression of pre-existing xenoreactive T cells. *Transplantation*. 2001;72:1223-1231.

701. Griesemer A, Liang F, Hirakata A, et al. Occurrence of specific humoral non-responsiveness to swine antigens following administration of GalT-KO bone marrow to baboons. *Xenotransplantation*. 2010;17:300-312.

702. Ohdan H, Swenson KG, Kruger-Gray HW, et al. Mac-1-negative B-1b phenotype of natural antibody-producing cells, including those responding to Galα1,3Gal epitopes in α1,3-galactosyltransferase deficient mice. *J Immunol* . 2000;165:5518-5529.

703. Murakami M, Tsubata T, Okamoto M, et al. Antigen-induced apoptotic death of Ly-1 B cells responsible for autoimmune disease in transgenic mice. *Nature*. 1992;357:77-80.

704. Ohdan H, Yang YG, Shimizu A, Swenson KG, Sykes M. Mixed bone marrow chimerism induced without lethal conditioning prevents T cell and anti-Galα1,3Gal-mediated graft rejection. *J Clin Invest*. 1999;104:281-290.

705. Kawahara T, Shimizu I, Ohdan H, Zhao G, Sykes M. Differing mechanisms of early and late B cell tolerance induced by mixed chimerism. *Am J Transplant*. 2005;5:2821-2829.

706. Ramsdell F, Fowlkes BJ. Clonal deletion versus clonal anergy: the role of the thymus in inducing self tolerance. *Science*. 1990;248:1342-1348.

707. Bensinger SJ, Bandeira A, Jordan MS, Caton AJ, Laufer TM. Major histocompatibility complex class II-positive cortical epithelium mediates the selection of CD4(+)25(+) immunoregulatory T cells. *J Exp Med*. 2001;194:427-438.

708. Jordan MS, Boesteanu A, Reed AJ, et al. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol*. 2001;2:301-306.

709. Baldwin TA, Hogquist KA, Jameson SC. The fourth way? Harnessing aggressive tendencies in the thymus. *J Immunol*. 2004;173:6515-6520.

710. Brocker T, Riedinger M, Karjalainen K. Targeted expression of major histocompatibiity complex (MHC) class II molecules demonstrates that dendritic cells can induce negative but not positive selection of thymocytes in vivo. *J Exp Med*. 1997;185:541-550.

711. Inaba M, Inaba K, Hosono M, et al. Distinct mechanisms of neonatal tolerance induced by dendritic cells and thymic B cells. *J Exp Med*. 1991;173:549-559.

712. Schonrich G, Strauss G, Muller KP, et al. Distinct requirements of positive and negative selection for selecting cell type and CD8 interaction. *J Immunol*. 1993;151:4098-4105.

713. Schulz R, Mellor AL. Self major histocompatibility complex class I antigens expressed solely in lymphoid cells do not induce tolerance in the CD4+ T cell compartment. *J Exp Med*. 1996;184:1573-1578.

714. Oukka M, Colucci-Guyon E, Tran PL, Cohen-Tannoudji M, Kosmatopoulos K. CD4 T cell tolerance to nuclear proteins induced by medullary thymic epithelium. *Immunity*. 1996;4:545-553.

715. Hoffman MW, Heath WR, Ruschmeyer D, Miller JF. Deletion of hiigh-avidity T cells by thymic epithelium. *Proc Natl Acad Sci U S A* . 1995;92:9851-9855.

716. Gallegos AM, Bevan MJ. Central tolerance: good but imperfect. *Immunol Rev*. 2006;209:290-296.

717. Tomita Y, Khan A, Sykes M. Role of intrathymic clonal deletion and peripheral anergy in transplantation tolerance induced by bone marrow transplantation in mice conditioned with a non-myeloablative regimen. *J Immunol*. 1994;153:1087-1098.

718. Khan A, Tomita Y, Sykes M. Thymic dependence of loss of tolerance in mixed allogeneic bone marrow chimeras after depletion of donor antigen. Peripheral mechanisms do not contribute to maintenance of tolerance. *Transplantation*. 1996;62:380-387.

719. Ferber I, Schonrich G, Schenkel J, Mellor AL, Hammerling GJ, Arnold B. Levels of peripheral T cell tolerance induced by different doses of tolerogen. *Science*. 1994;263:674-676.

720. Webb SR, Hutchinson J, Hayden K, Sprent J. Expansion/deletion of mature T cells exposed to endogenous superantigens in vivo. *J Immunol*. 1994;152:586-597.

721. Rocha B, Von Boehmer H. Peripheral selection of the T cell repertoire. *Science*. 1991;251:1225-1228.

722. Kurts C, Kosada H, Carbone FR, Miller JF, Heath WR. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8+ T cells. *J Exp Med*. 1997;186:239-245.

723. Heath WR, Kurts C, Miller JF, Carbone FR. Cross-tolerance: a pathway for inducing tolerance to peripheral tissue antigens. *J Exp Med*. 1998;187:1549-1553.

724. Carbone FR, Kurts C, Bennett SR, Miller JF, Heath WR. Crosspresentation: a general mechanism for CTL immunity and tolerance. *Immunol Today*. 1998;19:368-372.

725. Moskophidis D, Lechner F, Pircher H, Zinkernagel RM. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature*. 1993;362:758-761.

726. Redmond WL, Hernandez J, Sherman LA. Deletion of naive CD8 T cells requires persistent antigen and is not programmed by an initial signal from the tolerogenic APC. *J Immunol*. 2003;171:6349-6354.

727. Dey B, Yang YG, Preffer F, et al. The fate of donor TCR transgenic T cells with known host antigen specificity in a GVHD model. *Transplantation*. 1999;68:141-149.

728. Mapara MY, Kim YM, Marx J, Sykes M. DLI-mediated GVL effects in mixed chimeras established with a non-myeloablative conditioning regimen: extinction of GVL effects coincides with loss of alloreactive cells following conversion to full donor chimerism. *Transplantation*. 2003;76:297-305.

729. Iwakoshi NN, Markees TG, Turgeon N, et al. Skin allograft maintenance in a new synchimeric model system of tolerance. *J Immunol*. 2001;167:6623-6630.

730. Seung E, Mordes JP, Rossini AA, Greiner DL. Hematopoietic chimerism and central tolerance created by peripheral-tolerance induction without myeloablative conditioning. *J Clin Invest*. 2003;112:795-808.

731. Wekerle T, Sayegh MH, Hill J, et al. Extrathymic T cell deletion and allogeneic stem cell engraftment induced with costimulatory blockade is followed by central T cell tolerance. *J Exp Med*. 1998;187:2037-2044.

732. Wekerle T, Sayegh MH, Chandraker A, Swenson KG, Zhao Y, Sykes M. Role of peripheral clonal deletion in tolerance induction with bone marrow transplantation and costimulatory blockade. *Transplant Proc*. 1999;31:680.

733. Wekerle T, Kurtz J, Sayegh MH, et al. Peripheral deletion after bone marrow transplantation with costimulatory blockade has features of both activation-induced cell death and passive cell death. *J Immunol*. 2001;166:2311-2316.

734. Kurtz J, Shaffer J, Anosova N, Benichou G, Sykes M. Mechanisms of early peripheral CD4 T cell tolerance induction by anti-CD154 monoclonal antibody and allogeneic bone marrow transplantation: evidence for anergy and deletion, but not regulatory cells. *Blood*. 2004;103:4336-4343.

735. Wells AD, Li XC, Li Y, et al. Requirement for T cell apoptosis in the induction of peripheral transplantation tolerance. *Nat Med*. 1999;5:1303-1312.

736. Kurtz J, Lie A, Griffith M, et al. Lack of role for CsA-sensitive or Fas pathways in the tolerization of CD4+ T cells via BMT and anti-CD40L. *Am J Transplant*. 2003;3:804-816.

737. Li Y, Li XC, Zheng XX, Wells AD, Turka LA, Strom TB. Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. *Nat Med*. 1999;5:1298-1302.

738. Zhang ZX, Yang L, Young KJ, DuTemple B, Zhang L. Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. *Nat Med*. 2000;6:782-789.

739. Schwartz RH. A cell culture model for T lymphocyte clonal anergy. *Science*. 1990;248:1349-1356.

740. Nossal GJ, Pike BL. Clonal anergy: persistence in tolerant mice of antigen-binding B lymphocytes incapable of responding to antigen or mitogen. *Proc Natl Acad Sci U S A*. 1980;77:1602-1606.

741. Nossal GJV. Cellular mechanisms of immunologic tolerance. *Ann Rev Immunol*. 1983;1:33-62.

742. Appleman LJ, Boussiotis VA. T cell anergy and costimulation. *Immunol Rev*. 2003;192:161-180.

743. Allen PM. Peptides in positive and negative selection: a delicate balance. *Cell*. 1994;76:593-596.

744. Alam SM, Travers PJ, Wung JL, et al. T-cell-receptor affinity and thymocyte positive selection. *Nature*. 1996;381:616-620.

745. Fang W, Weintraub BC, Dunlap B, et al. Self-reactive B lymphocytes overexpressing

Bcl-xL escape negative selection and are tolerized by clonal anergy and receptor editing. *Immunity*. 1998;9:35-45.

746. Benschop RJ, Aviszus K, Zhang X, Manser T, Cambier JC, Wysocki LJ. Activation and anergy in bone marrow B cells of a novel immunoglobulin transgenic mouse that is both hapten specific and autoreactive. *Immunity*. 2001;14:33-43.

747. Ferry H, Leung JC, Lewis G, et al. B-cell tolerance. *Transplantation*. 2006;81:308-315.

748. Jenkins MK, Pardoll DM, Mizuguchi J, Chused TM, Schwartz RH. Molecular events in the induction of a nonresponsive state in interleukin 2-producing helper T-lymphocyte clones. *Proc Natl Acad Sci U S A* . 1987;84:5409-5413.

749. Migita K, Eguchi K, Kawabe Y, Tsukada T, Ichinose Y, Nagataki S. DefectiveTCR-mediatedsignalinginanergic T cells. *J Immunol*. 1995;155: 5083-5087.

750. Sloan-Lancaster J, Shaw AS, Rothbard JB, Allen PM. Partial T cell signalling: altered phospho-zeta and lack of zap70 recruitment in APL-induced T cell anergy. *Cell*. 1994;79:913-922.

751. McKay DB, Irie HY, Hollander G, et al. Antigen-induced unresponsiveness results in altered T cell signaling. *J Immunol*. 1999;163: 6455-6461.

752. Schwartz RH. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell*. 1992;71:1065-1068.

753. Bhandoola A, Cho EA, Yui K, et al. Reduced CD3-mediated protein tyrosine phosphorylation in anergic CD4+ and CD8+ T cells. *J Immunol*. 1993;151:2355-2367.

754. Wells AD, Walsh MC, Bluestone JA, Turka LA. Signaling through CD28 and CTLA-4 controls two distinct forms of T cell anergy. *J Clin Invest*. 2001;108:895-903.

755. Ramsdell F, Lantz T, Fowlkes BJ. A nondeletional mechanism of thymic self tolerance. *Science*. 1989;246:1038-1041.

756. van Meerwijk JP, Macdonald HR. In vivo T-lymphocyte tolerance in the absence of thymic clonal deletion mediated by hematopoietic cells. *Blood*. 1999;93:3856-3862.

757. Vandekerckhove BA, Namikawa R, Bacchetta R, Roncarolo MG. Human hematopoietic cells and thymic epithelial cells induce tolerance via different mechanisms in the the SCID-hu mouse thymus. *J Exp Med*. 1992;175:1033-1043.

758. Rocken M, Urban JF, Shevach EM. Infection breaks T cell tolerance. *Nature*. 1992;359:79-82.

759. Morecki S, Leshem B, Eid A, Slavin S. Alloantigen persistence in induction and

maintenance of transplantation tolerance. *J Exp Med*. 1987;165:1468-1480.

760. Rocha B, Tanchot C, Von Boehmer H. Clonal anergy blocks in vivo growth of mature T cells and can be reversed in the absence of antigen. *J Exp Med*. 1993;177:1517-1521.

761. Ramsdell F, Fowlkes BJ. Maintenance of in vivo tolerance by persistence of antigen. *Science*. 1992;257:1130-1134.

762. Pape KA, Merica R, Mondino A, Khoruts A, Jenkins MK. Direct evidence that functionally impaired CD4+ T cells persist in vivo following induction of peripheral tolerance. *J Immunol*. 1998;160:4719-4729.

763. Alegre ML, Chen L, Wang T, Ahmed E, Wang CR, Chong A. Antagonistic effect of toll-like receptor signaling and bacterial infections on transplantation tolerance. *Transplantation*. 2009;87:S77-S79.

764. Wang T, Ahmed EB, Chen L, et al. Infection with the intracellular bacterium, Listeria monocytogenes, overrides established tolerance in a mouse cardiac allograft model. *Am J Transplant*. 2010;10:1524-1533.

765. Fink PJ, Fang CA, Turk GL. The induction of peripheral tolerance by the chronic activation and deletion of CD4+Vβ5+ cells. *J Immunol*. 1994;152:4270-4281.

766. Chai JG, Bartok I, Scott D, Dyson J, Lechler R. T:T antigen presentation by activated murine CD8+ T cells induces anergy and apoptosis. *J Immunol*. 1998;160:3655-3665.

767. Rocha B, Grandien A, Freitas AA. Anergy and exhaustion are independent mechanisms of peripheral T cell tolerance. *J Exp Med*. 1995;181:993-1003.

768. Migita K, Ochi A. The fate of anergic T cells in vivo. *J Immunol*. 1993;150:763-770.

769. Fehr T, Wang S, Haspot F, et al. Rapid deletional peripheral CD8 T cell tolerance induced by allogeneic bone marrow: role of donor class II MHC and B cells. *J Immunol*. 2008;181:4371-4380.

770. Lombardi G, Sidhu S, Batchelor R, Lechler R. Anergic T cells as suppressor cells in vitro. *Science*. 1994;264:1587-1589.

771. Chai JG, Bartok I, Chandler P, et al. Anergic T cells act as suppressor cells in vitro and in vivo. *Eur J Immunol* . 1999;29:686-692.

772. Vendetti S, Chai JG, Dyson J, Simpson E, Lombardi G, Lechler R. Anergic T cells inhibit the antigen-presenting function of dendritic cells. *J Immunol*. 2000;165:1175-1181.

773. Gershon RK, Kondo K. Infectious immunological tolerance. *Immunology*. 1971;903-914.

774. Kilshaw PJ, Brent L, Pinto M. Suppressor T cells in mice made unresponsive to skin allografts. *Nature*. 1975;255:489-491.

775. Hall BM, Pearce NW, Gurley K, Dorsch SE. Specific unresponsiveness in rats with prolonged cardiac allograft survival after treatment with cyclosporine. *J Exp Med*. 1990;171:141-157.

776. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*. 1995;155:1151-1164.

777. Shevach EM. Regulatory T cells in autoimmmunity*. *Annu Rev Immunol*. 2000;18:423-449.

778. Seddon B, Mason D. The third function of the thymus. *Immunol Today*. 2000;21:95-99.

779. Bensinger SJ, Bandeira A, Jordan MS, Caton AJ, Laufer TM. Major histocompatibility complex class II-positive cortical epithelium mediates the selection of CD4(+)25(+) immunoregulatory T cells. *J Exp Med*. 2001;194:427-438.

780. Wood KJ, Sakaguchi S. Regulatory lymphocytes: regulatory T cells in transplantation tolerance. *Nat Rev Immunol*. 2003;3:199-210.

781. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. Cd4(+) cd25(high) regulatory cells in human peripheral blood. *J Immunol*. 2001;167:1245-1253.

782. Levings MK, Sangregorio R, Roncarolo MG. Human CD25(+)CD4(+) T regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med*. 2001;193:1295-1302.

783. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+) CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med*. 2001;193:1285-1294.

784. Ng WF, Duggan PJ, Ponchel F, et al. Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. *Blood*. 2001;98:2736-2744.

785. Stephens LA, Mottet C, Mason D, Powrie F. Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity in vitro. *Eur J Immunol*. 2001;31:1247-1254.

786. Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN. Human anergic/suppressive CD4+CD25+ T cells: a highly differentiated and apoptosis-prone population. *Eur J Immunol*. 2001;31: 1122-1131.

787. Li Y, Koshiba T, Yoshizawa A, et al. Analyses of peripheral blood mononuclear cells in operational tolerance after pediatric living donor liver transplantation. *Am J Transplant*. 2004;4:2118-2125.

788. Martínez-Llordella M, Puig-Pey I, Orlando G, et al. Multiparameter immune profiling of operational tolerance in liver transplantation. *Am J Transplant*. 2007;7:309-319.

789. Wildin RS, Smyk-Pearson S, Filipovich AH. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. *J Med Gen*. 2002;39:537-545.

790. Brunkow ME, Jeffery EW, Hjerrild KA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet*. 2001;27:68-73.

791. Ramsdell F. Foxp3 and natural regulatory T cells: key to a cell lineage? *Immunity*. 2003;19:165-168.

792. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor foxp3. *Science*. 2003;299:1057-1061.

793. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4(+)CD25(+) regulatory T cells. *Nat Immunol*. 2003;4:330-336.

794. Wan YY, Flavell RA. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proc Natl Acad Sci U S A* . 2005;102:5126-5131.

795. Morgan ME, van Bilsen JH, Bakker AM, et al. Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. *Hum Immunol*. 2005;66:13-20.

796. Josefowicz SZ, Rudensky A. Control of regulatory T cell lineage commitment and maintenance. *Immunity*. 2009;30:616-625.

797. Huehn J, Polansky JK, Hamann A. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? *Nat Rev Immunol* . 2009;9:83-89.

798. Hara M, Kingsley CI, Niimi M, et al. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J Immunol* . 2001;166:3789-3796.

799. Kingsley CI, Karim M, Bushell AR, Wood KJ. CD25+CD4+ regulatory T cells prevent graft rejection: CTLA-4- and IL-10- dependent immunoregulation of alloresponses. *J Immunol*. 2002;168:1080-1086.

800. Sanchez-Fueyo A, Weber M, Domenig C, Strom TB, Zheng XX. Tracking the immunoregulatory mechanisms active during allograft tolerance. *J Immunol* . 2002;168:2274-2281.

801. Graca L, Thompson S, Lin CY, Adams E, Cobbold SP, Waldmann H. Both CD4(+)CD25(+) and CD4(+)CD25(-) regulatory cells mediate dominant transplantation tolerance. *J Immunol*. 2002;168:5558-5565.

802. Taylor PA, Noelle RJ, Blazar BR. CD4(+)CD25(+) immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. *J Exp Med*. 2001;193:1311-1318.

803. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donortype CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med*. 2002;196:389-399.

804. Cohen JL, Trenado A, Vasey D, Klatzmann D, Salomon BL. CD4(+) CD25(+) immunoregulatory T cells: new therapeutics for graft-versus-host disease. *J Exp Med*. 2002;196:401-406.

805. Kawai T, Cosimi AB, Spitzer TR, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med*. 2008;358:353-361.

806. Wood KJ, Bushell A, Jones ND. Immunologic unresponsiveness to alloantigen in vivo: a role for regulatory T cells. *Immunol Rev*. 2011; 241:119-132.

807. Yang J, Brook MO, Carvalho-Gaspar M, et al. Allograft rejection mediated by memory T cells is resistant to regulation. *Proc Natl Acad Sci U S A* . 2007;104:19954-19959.

808. Francis RS, Feng G, Tha-In T, Lyons IS, Wood KJ, Bushell A. Induction of transplantation tolerance converts potential effector T cells into graft-protective regulatory T cells. *Eur J Immunol* . 2011;41:726-738.

809. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity*. 2006;24:179-189.

810. Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*. 2006;441:235-238.

811. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity*. 2006;24:677-688.

812. Hori S. Regulatory T cell plasticity: beyond the controversies. *Trends Immunol*. 2011;32:295-300.

813. Bailey-Bucktrout SL, Bluestone JA. Regulatory T cells: stability revisited. *Trends Immunol*. 2011;32:301-306.

814. Sawitzki B, Kingsley CI, Oliveira V, Karim M, Herber M, Wood KJ. IFN-gamma production by alloantigen-reactive regulatory T cells is important for their regulatory function in vivo. *J Exp Med*. 2005;201:1925-1935.

815. Wei B, Baker S, Wieckiewicz J, Wood KJ. IFN-gamma triggered STAT1-PKB/AKT signalling pathway influences the function of alloantigen reactive regulatory T cells. *Am J Transplant*. 2010;10:69-80.

816. Konieczny BT, Dai Z, Elwood ET, et al. IFN-γ is critical for long-term allograft survival induced by blocking the CD28 and CD40 ligand T cell costimulation pathways. *J Immunol*. 1998;160:2059-2064.

817. Wood KJ, Sawitzki B. Interferon gamma: a crucial role in the function of induced regulatory T cells in vivo. *Trends Immunol*. 2006;27: 183-187.

818. Cederbom L, Hall H, Ivars F. CD4+CD25+ regulatory T cells downregulate co-stimulatory molecules on antigen-presenting cells. *Eur J Immunol* . 2000;30:1538-1543.

819. Qin S, Cobbold S, Tighe H, Benjamin R, Waldmann H. CD4 monoclonal antibody pairs for immunosuppression and tolerance induction. *Eur J Immunol* . 1987;17:1159-1165.

820. Dieckmann D, Bruett CH, Ploettner H, Lutz MB, Schuler G. Human CD4(+)CD25(+) regulatory, contact-dependent T cells induce interleukin 1-producing, contact-independent type 10-like [correction of type 1-like] regulatory T cells. *J Exp Med*. 2002;196:247-253.

821. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med*. 1998;188:287-296.

822. Zheng SG, Wang JH, Gray JD, Soucier H, Horwitz DA. Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, adn IL-10. *J Immunol*. 2004;172:5213-5221.

823. Hassanain HH, Chon SY, Gupta SL. Differential regulation of human indoleamine 2,3-dioxygenase gene expression by interferonsgamma and -alpha. Analysis of the regulatory region of the gene and identification of an interferon-gamma-inducible DNA-binding factor. *J Biol Chem*. 1993;268:5077-5084.

824. Orabona C, Puccetti P, Vacca C, et al. Toward the identification of a tolerogenic signature in IDO-competent dendritic cells. *Blood*. 2006; 107:2846-2854.

825. Grohmann U, Orabona C, Fallarino F, et al. CTLA-4-Ig regulates tryptophan catabolism in vivo. *Nat Immunol*. 2002;3:1097-1101.

826. Mellor AL, Keskin DB, Johnson T, Chandler P, Munn DH. Cells expressing

indoleamine 2,3-dioxygenase inhibit T cell responses. *J Immunol*. 2002;168:3771-3776.

827. Terness P, Bauer TM, Rose L, et al. Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. *J Exp Med*. 2002;196:447-457.

828. Beutelspacher SC, Pillai R, Watson MP, et al. Function of indoleamine 2,3-dioxygenase in corneal allograft rejection and prolongation of allograft survival by over-expression. *Eur J Immunol* . 2006;36:690-700.

829. Jonuleit H, Schmitt E, Kakirman H, Stassen M, Knop J, Enk AH. Infectious tolerance: human CD25(+) regulatory T cells convey suppressor activity to conventional CD4(+) T helper cells. *J Exp Med*. 2002;196:255-260.

830. Josien R, Douillard P, Guillot C, et al. A critical role for transforming growth factor-beta in donor transfusion-induced allograft tolerance. *J Clin Invest*. 1998;102:1920-1926.

831. Read S, Greenwald R, Izcue A, et al. Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function in vivo. *J Immunol*. 2006;177:4376-4383.

832. Walunas TL, Lenschow DJ, Bakker CY, et al. CTLA-4 can function as a negative regulator of T cell activation. *Immunity*. 1994;1:405-413.

833. Waterhouse P, Penninger JM, Timms E, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science*. 1995;270:985-988.

834. Takahashi T, Tagami T, Yamazaki S, et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med*. 2000;192:303-310.

835. Fallarino F, Grohmann U, Hwang KW, et al. Modulation of tryptophan catabolism by regulatory T cells. *Nat Immunol*. 2003;4:1206-1212.

836. Schneider H, Valk E, da Rocha DS, Wei B, Rudd CE. CTLA-4 upregulation of lymphocyte function-associated antigen 1 adhesion and clustering as an alternate basis for coreceptor function. *Proc Natl Acad Sci U S A* . 2005;102:12861-12866.

837. Thornton AM, Shevach EM. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J Immunol*. 2000;164:183-190.

838. Karim M, Feng G, Wood KJ, Bushell AR. CD25+CD4+ regulatory T cells generated by exposure to a model protein antigen prevent allograft rejection: antigen-specific reactivation in vivo is critical for bystander regulation. *Blood*. 2005;105:4871-4877.

839. Carvalho-Gaspar M, Jones ND, Luo S, Martin L, Brook MO, Wood KJ. Location and time-dependent control of rejection by regulatory T cells culminates in a failure to generate memory T cells. *J Immunol*. 2008;180:6640-6648.

840. Kendal AR, Chen Y, Regateiro FS, et al. Sustained suppression by Foxp3+ regulatory T cells is vital for infectious transplantation tolerance. *J Exp Med*. 2011;208:2043-2053.

841. Rosenblum MD, Gratz IK, Paw JS, Lee K, Marshak-Rothstein A, Abbas AK. Response to self antigen imprints regulatory memory in tissues. *Nature*. 2011;480:538-542.

842. Hutchinson IV. Suppressor T cells in allogeneic models. *Transplantation*. 1986;41:547-555.

843. Gilliet M, Liu YJ. Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. *J Exp Med*. 2002;195:695-704.

844. Zhou J, Carr RI, Liwski RS, Stadnyk AW, Lee TD. Oral exposure to alloantigen generates intragraft CD8(+) regulatory cells. *J Immunol*. 2001;167:107-113.

845. Ciubotariu R, Colovai AI, Pennesi G, et al. Specific suppression of human CD4+ Th cell responses to pig MHC antigens by CD8+CD28-regulatory T cells. *J Immunol*. 1998;161:5193-5202.

846. Zhang ZX, Yang L, Young KJ, DuTemple C, Zhang L. Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. *Nat Med*. 2000;6:782-789.

847. Zeng D, Lewis D, Dejbakhsh-Jones S, et al. Bone marrow NK1.1- and NK1.1+ T cells reciprocally regulate acute graft versus host disease. *J Exp Med*. 1999;189:1073-1081.

848. Seino KK, Fukao K, Muramoto K, et al. Requirement for natural killer T (NKT) cells in the induction of allograft tolerance. *Proc Natl Acad Sci U S A*. 2001;98:2577-2581.

849. Chambers CA, Kang J, Wu Y, Held W, Raulet DH, Allison JP. The lymphoproliferative defect in CTLA-4-deficient mice is ameliorated by an inhibitory NK cell receptor. *Blood*. 2002;99:4509-4516.

850. Wells AD, Li XC, Li Y, et al. Requirement for T cell apoptosis in the induction of peripheral transplantation tolerance. *Nat Med*. 1999;5:1303-1312.

851. Li Y, Li XC, Zheng XX, Wells AD, Turka LA, Strom TB. Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. *Nat Med*. 1999;5:1298-1302.

852. Yamagiwa S, Gray JD, Hashimoto S, Horwitz DA. A role for TGF-beta in the generation and expansion of CD4+CD25+ regulatory T cells from human peripheral blood. *J Immunol*. 2001;166:7282-7289.

853. Zheng SG, Gray JD, Ohtsuka K, Yamagiwa S, Horwitz DA. Generation ex vivo of TGF-beta-producing regulatory T cells from CD4+CD25-precursors. *J Immunol*. 2002;169:4183-4189.

854. Nadig SN, Wieckiewicz J, Wu DC, et al. In vivo prevention of transplant arteriosclerosis by ex vivo-expanded human regulatory T cells. *Nat Med*. 2010;16:809-813.

855. Malek TR. The main function of IL-2 is to promote the development of T regulatory cells. *J Leukoc Biol*. 2003;74:961-965.

856. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graftversus-host disease lethality. *Blood*. 2002;99:3493-3499.

857. Koreth J, Matsuoka K, Kim HT, et al. Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med*. 2011;365:2055-2066.

858. Seddiki N, Santner-Nanan B, Martinson J, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med*. 2006;203:1693-1700.

859. Liu W, Putnam AL, Xu-Yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4(+) T reg cells. *J Exp Med*. 2006;203:1701-1711.

860. Issa F, Hester J, Goto R, Nadig SN, Goodacre TE, Wood K. Ex vivo-expanded human regulatory T cells prevent the rejection of skin allografts in a humanized mouse model. *Transplantation*. 2010;90:1321-1327.

861. Sagoo P, Ali N, Garg G, Nestle FO, Lechler RI, Lombardi G. Human regulatory T cells with alloantigen specificity are more potent inhibitors of alloimmune skin graft damage than polyclonal regulatory T cells. *Sci Transl Med*. 2011;3:83ra42.

862. Köhler H. The immune network revisited. In: Köhler H, Urbain J, Cazenave P, eds. *Idiotype in Biology and Medicine*. New York: Academic Press; 1984:3-14.

862a. Jerne NK. Idiotypic networks and other preconceived ideas. *Immunol Rev*. 1984;79:5-24.

863. Bluestone JA, Leo O, Epstein SL, Sachs DH. Idiotypic manipulation of the immune response to transplantation antigens. *Immunol Rev*. 1986;90:5-27.

864. Carpenter CB, D'Apice AJ, Abbas AK. The role of antibody in the rejection and enhancement of rat organ allografts: non-Ag-B (Ia) antigens. *Adv Immunol*. 1976;22:1.

865. Batchelor J, Welsh K. Mechanisms of enhancement of kidney allograft survival. A form of operational tolerance. *Br Med Bull*. 1976;32:113-117.

866. Arnold B, Schonrich G, Hammerling GJ. Multiple levels of peripheral tolerance. *Immunol Today*. 1993;14:12-14.

867. Bohme J, Haskins K, Stecha P, et al. Transgenic mice with I-A on islet cells are normoglycemic but immunologically intolerant. *Science*. 1989;244:1179-1183.

868. Ohashi PS, Oehen S, Buerki K, et al. Ablation of “tolerance” and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell*. 1991;65:305-317.

869. Ochsenbein AF, Sierro S, Odermatt B, et al. Roles of tumour localization, second signals and cross priming in cytotoxic T-cell induction. *Nature*. 2001;411:1058-1064.

870. Hammerling GJ, Schonrich G, Momburg F, et al. Non-deletional mechanisms of peripheral and central tolerance: studies with transgenic mice with tissue-specific expression of a foreign MHC class I antigen. *Immunol Rev*. 1991;122:47-67.

871. Picarella DE, Kratz A, Li C, Ruddle NH, Flavell RA. Transgenic tumor necrosis factor (TNF)-α production in pancreatic islets leads to insulinitis, not diabetes: distinct patterns of inflammation in TNF-α and TNF-β transgenic mice. *J Immunol*. 1993;150:4136-4150.

872. Heath WR, Allison J, Hoffman MW, et al. Autoimmune diabetes as a consequence of locally produced interleukin-2. *Nature*. 1992;359:547-549.

873. von Herrath MG, Guerder S, Lewicki H, Flavell RA, Oldstone MB. Coexpression of B7-1 and viral (“self”) transgenes in pancreatic β cells can break peripheral ignorance and lead to spontaneous autoimmune diabetes. *Immunity*. 1995;3:727-738.

874. Lafferty KJ, Babcock SK, Gill RG. Prevention of rejection by treatment of the graft: an overview. *Prog Clin Biol Res*. 1986;224:87-117.

875. Medawar PB. Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. *Br J Exp Pathol* . 1948;29:58-69.

876. George AJ, Larkin DF. Corneal transplantation: the forgotten graft. *Am J Transplant*. 2004;4:678-685.

877. Dietrich T, Bock F, Yuen D, et al. Cutting edge: lymphatic vessels, not blood vessels, primarily mediate immune rejections after transplantation. *J Immunol*. 2010;184:535-539.

878. Fu H, Khan A, Coe D, et al. Arginine depletion as a mechanism for the immune privilege of corneal allografts. *Eur J Immunol* . 2011;41:2997-3005.

879. Streilein JW. Regional immunity and ocular immune privilege. *Chem Immunol*. 1999;73:11-38.

880. Fujisaki J, Wu J, Carlson AL, et al. In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. *Nature*. 2011;474:216-219.

881. Owen RD. Immunogenetic consequences of vascular anastomoses between bovine twins. *Science*. 1945;102:400-401.

882. Billingham RE, Lampkin GH, Medawar PB, Williams HL. Tolerance to homografts, twin diagnosis, and the freemartin condition in cattle. *Heredity*. 1952;6:201-212.

883. Main JM, Prehn RT. Succesful skin homografts after the administration of high dosage X radiation and homologous bone marrow. *J Natl Cancer Inst* . 1955;15:1023.

884. Orloff MS, Fallon MA, DeMara E, et al. Induction of specific tolerance to small-bowel allografts. *Surgery*. 1994;116:222-228.

885. Auchincloss H Jr, Sachs DH. Mechanisms of tolerance in murine radiation bone marrow chimeras: I. Nonspecific suppression of alloreactivity by spleen cells from early but not late chimeras. *Transplantation*. 1983;36:436.

886. Auchincloss H Jr, Sachs DH. Mechanisms of tolerance in murine radiation bone marrow chimeras II. Absence of nonspecific suppression in mature chimeras. *Transplantation*. 1983;36:442.

887. Almaraz R, Ballinger W, Sachs DH, Rosenberg SA. The effect of peripheral lymphoid cells on the incidence of lethal graft versus host disease following allogeneic mouse bone marrow transplantation. *J Surg Res*. 1983;34:133-144.

888. Sharp TG, Sachs DH, Fauci AS, Messerschmidt GL, Rosenberg SA. T-cell depletion of human bone marrow using monoclonal antibody and complement mediated lysis. *Transplantation*. 1983;35:112-120.

889. Martin P. 1999. Overview of marrow transplantation immunology. In: Forman SJ, Blume KG, Thomas ED, eds. *Hematopoietic Cell Transplantation*. 2nd ed. Cambridge, United Kingdom: Blackwell Science Inc; 1999:19-27.

890. Singer A, Hathcock KS, Hodes RJ. Self recognition in allogeneic radiation chimeras. A radiation resistant host element dictates the self specificity and immune response gene phenotype of T-helper cells. *J Exp Med*. 1981;153:1286-1301.

891. Ruedi E, Sykes M, Ildstad ST, et al. Antiviral T cell competence and restriction specificty of mixed allogeneic (P1+P2→P1) irradiation chimeras. *Cell Immunol* . 1989;121:185-195.

892. Koehn BH, Williams MA, Borom K, et al. Fully MHC-disparate mixed hemopoietic chimeras show specific defects in the control of chronic viral infections. *J Immunol* . 2007;179:2616-2626.

893. Manilay JO, Pearson DA, Sergio JJ, Swenson KG, Sykes M. Intrathymic deletion of alloreactive T cells in mixed bone marrow chimeras prepared with a nonmyeloablative conditioning regimen. *Transplantation*. 1998;66:96-102.

894. Yoshikai Y, Ogimoto M, Matsuzaki G, Nomoto K. Bone marrow-derived cells are essential for intrathymic deletion of self-reactive T cells in both the host- and donor-derived thymocytes of fully allogeneic bone marrow chimeras. *J Immunol*. 1990;145:505-509.

895. Ildstad ST, Wren SM, Bluestone JA, Barbieri SA, Sachs DH. Characterization of mixed allogeneic chimeras. Immunocompetence, in vitro reactivity, and genetic specificity of tolerance. *J Exp Med*. 1985;162:231-244.

896. Wekerle T, Sayegh MH, Ito H, et al. Anti-CD154 or CTLA4Ig obviates the need for thymic irradiation in a non-myeloablative conditioning regimen for the induction of mixed hematopoietic chimerism and tolerance. *Transplantation*. 1989;68:1348-1355.

897. Wekerle T, Kurtz J, Ito H, et al. Allogeneic bone marrow transplantation with costimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment. *Nat Med*. 2000;6:464-469.

898. Ito H, Kurtz J, Shaffer J, Sykes M. CD4 T cell-mediated alloresistance to fully MHC-mismatched allogeneic bone marrow engraftment is dependent on CD40-CD40L interactions, and lasting T cell tolerance is induced by bone marrow transplantation with initial blockade of this pathway. *J Immunol*. 2001;166:2970-2981.

899. Tomita Y, Sachs DH, Sykes M. Myelosuppressive conditioning is required to achieve engraftment of pluripotent stem cells contained in moderate doses of syngeneic bone marrow. *Blood*. 1994;83:939-948.

900. Sykes M, Szot GL, Swenson K, Pearson DA. Induction of high levels of allogeneic hematopoietic reconstitution and donor-specific tolerance without myelosuppressive conditioning. *Nat Med*. 1997;3:783-787.

901. Ito H, Takeuchi Y, Shaffer J, Sykes M. Local irradiation enhances congenic donor pluripotent hematopoietic stem cell engraftment similarly in irradiated and non-irradiated sites. *Blood*. 2003;103:1949-1954.

902. Durham MM, Bingaman AW, Adams AB, et al. Cutting edge: administration of anti-CD40 ligand and donor bone marrow leads to hemopoietic chimerism and donor-specific tolerance without cytoreductive conditioning. *J Immunol*. 2000;165:1-4.

903. Pilat N, Baranyi U, Klaus C, et al. Treg-therapy allows mixed chimerism and transplantation tolerance without cytoreductive conditioning. *Am J Transplant*. 2010;10:751-762.

904. Slavin S, Fuks Z, Strober S, Kaplan H, Howard RJ, Sutherland DER. Transplantation tolerance across major histocompatibility barriers after total lymphoid

irradiation. *Transplantation*. 1979;28:359.

905. Slavin S, Strober S, Fuks Z, Kaplan HS. Induction of specific tissue transplantation tolerance using fractionated total lymphoid irradiation in adult mice: long-term survival of allogeneic bone marrow and skin grafts. *J Exp Med*. 1977;146:34.

906. Huang CA, Fuchimoto Y, Sheir-Dolberg R, Murphy MC, Neville DM Jr, Sachs DH. Stable mixed chimerism and tolerance using a non-myeloablative preparative regimen in a large animal model. *J Clin Invest*. 2000;105:173-181.

907. Fuchimoto Y, Huang CA, Yamada K, et al. Mixed chimerism and tolerance without whole body irradiation in a large animal model. *J Clin Invest*. 2000;105:1779-1789.

908. Hettiaratchy S, Melendy E, Randolph MA, et al. Tolerance to composite tissue allografts across a major histocompatibility barrier in miniature swine. *Transplantation*. 2004;77:514-521.

909. Mayumi H, Good RA. Long-lasting skin allograft tolerance in adult mice induced across fully allogeneic (multimajor H-2 plus multiminor histocompatibility) antigen barriers by a tolerance-inducing method using cyclophosphamide. *J Exp Med*. 1989;169:213.

910. De Vries-van der Zwan A, Besseling AC, De Waal LP, Boog CJ. Specific tolerance induction and transplantation: a single-day protocol. *Blood*. 1997;89:2596-2601.

911. Colson YL, Wren SM, Schuchert MJ, et al. A nonlethal conditioning approach to achieve durable multilineage mixed chimerism and tolerance across major, minor, and hematopoietic histocompatibility barriers. *J Immunol*. 1995;155:4179-4188.

912. Nomoto K, Yung-Yun K, Omoto K, Umesue M, Murakami Y, Matsuzaki G. Tolerance induction in a fully allogeneic combination using anti-T cell receptor-αβ monoclonal antibody, low dose irradiation, and donor bone marrow transfusion. *Transplantation*. 1995;59:395-401.

913. Haynes BF, Markert ML, Sempowski GD, Patel DD, Hale LP. The role of the thymus in immune reconstiutution in aging, bone marrow transplantion, and HIV-1 infection. *Ann Rev Immunol*. 2000;18:529-560.

914. Tomita Y, Khan A, Sykes M. Mechanism by which additional monoclonal antibody injections overcome the requirement for thymic irradiation to achieve mixed chimerism in mice receiving bone marrow transplantation after conditioning with anti-T cell mAbs and 3 Gy whole body irradiation. *Transplantation*. 1996;61:477-485.

915. Ito H, Takeuchi Y, Shaffer J, Sykes M. Anti-CD40L monoclonal antibodies can replace anti-CD4 monoclonal antibodies for the nonmyeloablative induction of mixed xenogeneic chimerism. *Transplantation*. 1996;82:251-257.

916. Aksentijevich I, Sachs DH, Sykes M. Humoral tolerance in xenogeneic BMT

recipients conditioned with a non-myeloablative regimen. *Transplantation*. 1992;53:1108-1114.

917. Lee LA, Sergio JJ, Sachs DH, Sykes M. Mechanism of tolerance in mixed xenogeneic chimeras prepared with a non-myeloablative conditioning regimen. *Transplant Proc*. 1994;26:1197-1198.

918. Ohdan H, Yang YG, Swenson KG, Kitamura H, Sykes M. T cell and B cell tolerance to galα1,3gal-expressing heart xenografts is achieved in α1,3-galactosyltransferase-deficient mice by non-myeloablative induction of mixed chimerism. *Transplantation*. 2001;71:1532-1542.

919. Nikolic B, Lei H, Pearson DA, Sergio JA, Swenson KA, Sykes M. Role of intrathymic rat class II cells in maintaining deletional tolerance in xenogeneic rat→mouse bone marrow chimeras. *Transplantation*. 1998;65:1216-1224.

920. Yang YG, deGoma E, Ohdan H, et al. Tolerization of anti-galα1-3gal natural antibody-forming B cells by induction of mixed chimerism. *J Exp Med*. 1998;187:1335-1342.

921. Ohdan H, Yang YG, Shimizu A, Swenson KG, Sykes M. Mixed bone marrow chimerism induced without lethal conditioning prevents T cell and anti-Galα1,3Gal-mediated graft rejection. *J Clin Invest*. 1999;104:281-290.

922. Kawahara T, Shimizu I, Ohdan H, Zhao G, Sykes M. Differing mechanisms of early and late B cell tolerance induced by mixed chimerism. *Am J Transplant*. 2005;5:2821-2829.

923. Xu Y, Yang YG, Ohdan H, et al. Characterization of anti-Gal antibody-producing cells of baboons and humans. *Transplantation*. 2006;81:940-948.

924. Zhao Y, Ohdan H, Manilay JO, Sykes M. NK cell tolerance in mixed allogeneic chimeras. *J Immunol*. 2003;170:5398-5405.

925. Kawahara T, Rodriguez-Barbosa JI, Zhao Y, Zhao G, Sykes M. Global unresponsiveness as a mechanism of natural killer cell tolerance in mixed xenogeneic chimeras. *Am J Transplant*. 2007;7:2090-2097.

926. Wu MF, Raulet DH. Class I-deficient hemopoietic cells and non-hemopoietic cells dominantly induce unresponsiveness of natural killer cells to class I-deficient bone marrow cells. *J Immunol*. 1997; 158:1628-1633.

927. Raulet DH, Vance RE. Self-tolerance of natural killer cells. *Nat Rev Immunol*. 2006;6:520-531.

928. Forte P, Baumann BC, Schneider MK, Seebach JD. HLA-Cw4 expression on porcine endothelial cells reduces cytotoxicity and adhesion mediated by CD158a+ human NK

cells. *Xenotransplant*. 2009;16:19-26.

929. Weiss EH, Lilienfeld BG, Muller S, et al. HLA-E/human beta2-microglobulin transgenic pigs: protection against xenogeneic human anti-pig natural killer cell cytotoxicity. *Transplantation*. 2009;87:35-43.

930. Torgersen KM, Salcedo M, Vaage JT, et al. Major histocompatibility complex class I-independent killing of xenogeneic targets by rat allospecific natural killer cells. *Transplantation*. 1997;63:119-123.

931. Silver ET, Lavender KJ, Gong DE, Hazes B, Kane KP. Allelic variation in the ectodomain of the inhibitory Ly-49G2 receptor alters its specificity for allogeneic and xenogeneic ligands. *J Immunol* . 2002;169:4752-4760.

932. Benoit LA, Shannon J, Chamberlain JW, Miller RG. Influence of xenogeneic beta2-microglobulin on functional recognition of H-2Kb by the NK cell inhibitory receptor Ly49C. *J Immunol* . 2005;175:3542-3553.

933. Benoit LA, Tan R. Xenogeneic beta 2-microglobulin substitution alters NK cell function. *J Immunol* . 2007;179:1466-1474.

934. Tran PD, Christiansen D, Winterhalter A, et al. Porcine cells express more than one functional ligand for the human lymphocyte activating receptor NKG2D. *Xenotransplant*. 2008;15:321-332.

935. Lilienfeld BG, Garcia-Borges C, Crew MD, Seebach JD. Porcine UL16-binding protein 1 expressed on the surface of endothelial cells triggers human NK cytotoxicity through NKG2D. *J Immunol* . 2006;177: 2146-2152.

936. Clark EA, Sturge JC. Phylogeny of NK cell reactivity against human and nonhuman primate lymphoblastoid cell lines: evolving and conserved target antigens. *J Immunol* . 1981;126:969-974.

937. Kim S, Poursine-Laurent J, Truscott SM, et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature*. 2005;436:709-713.

938. Joncker NT, Fernandez NC, Treiner E, Vivier E, Raulet DH. NK cell responsiveness is tuned commensurate with the number of inhibitory receptors for self-MHC class I: the rheostat model. *J Immunol* . 2009;182:4572-4580.

939. Tripathy SK, Keyel PA, Yang L, et al. Continuous engagement of a self-specific activation receptor induces NK cell tolerance. *J Exp Med*. 2008;205:1829-1841.

940. Coudert JD, Scarpellino L, Gros F, Vivier E, Held W. Sustained NKG2D engagement induces cross-tolerance of multiple distinct NK cell activation pathways. *Blood*. 2008;111:3571-3578.

941. Gritsch HA, Sykes M. Host marrow has a competitive advantage which limits donor hematopoietic repopulation in mixed xenogeneic chimeras. *Xenotransplant*. 1996;3:312-320.

942. Lapidot T, Pflumia F, Doedens M, Murdoch B, Williams DE, Dick JE. Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID mice. *Science*. 1992;255:1137-1143.

943. Yang YG, Sergio JJ, Swenson K, Glaser RM, Monroy R, Sykes M. Donor-specific growth factors promote swine hematopoiesis in SCID mice. *Xenotransplant*. 1996;3:92-101.

944. Simon AR, Warrens AN, Sykes M. Efficacy of adhesive interactions in pig-to-human xenotransplantation. *Immunol Today*. 1999;20:291-338.

945. Chen AM, Zhou Y, Swenson K, Sachs DH, Sykes M, Yang YG. Porcine stem cell engraftment and seeding of murine thymus with class II+ cells in mice expressing porcine cytokines: toward tolerance induction across discordant xenogeneic barriers. *Transplantation*. 2000;69: 2484-2490.

946. Lan P, Wang L, Diouf B, et al. Induction of human T cell tolerance to porcine xenoantigens through mixed hematopoietic chimerism. *Blood*. 2004;103:3964-3969.

947. Ide K, Wang H, Tahara H, et al. Role for CD47-SIRPalpha signaling in xenograft rejection by macrophages. *Proc Natl Acad Sci U S A* . 2007;104:5062-5066.

948. Wang C, Wang H, Ide K, et al. Human CD47 expression permits survival of porcine cells in immunodeficient mice that express SIRPalpha capable of binding to human CD47. *Cell Transplant*. 2011.

949. Lee LA, Gritsch HA, Sergio JJ, et al. Specific tolerance across a discordant xenogeneic transplantation barrier. *Proc Natl Acad Sci U S A* . 1994;91:10864-10867.

950. Zhao Y, Fishman JA, Sergio JJ, et al. Immune restoration by fetal pig thymus grafts in T cell-depleted, thymectomized mice. *J Immunol* . 1997;158:1641-1649.

951. Zhao Y, Swenson K, Sergio JJ, Arn JS, Sachs DH, Sykes M. Skin graft tolerance across a discordant xenogeneic barrier. *Nat Med*. 1996;2: 1211-1216.

952. Zhao Y, Sergio JJ, Swenson KA, Arn JS, Sachs DH, Sykes M. Positive and negative selection of functional mouse CD4 cells by porcine MHC in pig thymus grafts. *J Immunol* . 1997;159:2100-2107.

953. Zhao Y, Swenson K, Sergio JJ, Sykes M. Pig MHC mediates positive selection of mouse CD4+ T cells with a mouse MHC-restricted TCR in pig thymus grafts. *J Immunol* . 1998;161:1320-1326.

954. Zhao Y, Rodriguez-Barbosa JI, Zhao G, Shaffer J, Arn JS, Sykes M. Maturation and function of mouse T cells with a transgeneic TCR positively selected by highly disparate xenogeneic porcine MHC. *Cell Mol Biol* . 2007;47:217-228.

955. Nikolic B, Gardner JP, Scadden DT, Arn JS, Sachs DH, Sykes M. Normal development in porcine thymus grafts and specific tolerance of human T cells to porcine donor MHC. *J Immunol* . 1999;162:3402-3407.

956. Hongo D, Hadidi S, Damrauer S, et al. Porcine thymic grafts protect human thymocytes from HIV-1-induced destruction. *J Infect Dis*. 2007;196:900-910.

957. Shimizu I, Fudaba Y, Shimizu A, Yang YG, Sykes M. Comparison of human T cell repertoire generated in xenogeneic porcine and human thymus grafts. *Transplantation*. 2008;86:601-610.

958. Habiro K, Sykes M, Yang YG. Induction of human T-cell tolerance to pig xenoantigens via thymus transplantation in mice with an established human immune system. *Am J Transplant*. 2009;9:1324-1329.

959. Wu A, Yamada K, Neveille DM, et al. Xenogeneic thymic transplantation in a pig-to-baboon model. *Transplantation*. 2002;75:282-291.

960. Yamada K, Yazawa K, Shimizu A, et al. Marked prolongation of porcine renal xenograft survival in baboons through the use of alpha1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. *Nat Med*. 2005;11:32-34.

961. Yamada K, Yazawa K, Shimizu A, et al. Marked prolongation of porcine renal xenograft survival in baboons through the use of alpha1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. *Nat Med*. 2005;11:32-34.

962. Salaun J, Bandeira A, Khazaal I, et al. Thymic epithelium tolerizes for histocompatibility antigens. *Science*. 1990;247:1471-1474.

963. Ohki H, Martin C, Corbel C, Coltey M, Le Douarin NM. Tolerance induced by thymic epithelial grafts in birds. *Science*. 1986;237: 1032-1035.

964. Modigliani Y, Tomas-Vaslin V, Bandeira A, et al. Lymphocytes selected in allogeneic thymic epithelium mediate dominant tolerance toward tissue grafts of the thymic epithelium haplotype. *Proc Natl Acad Sci U S A* . 1995;92:7555-7559.

965. Modigliani Y, Pereira P, Thomas-Vaslin V, et al. Regulatory T cells in thymic epithelium-induced tolerance. I. Suppression of mature peripheral non-tolerant T cells. *Eur J Immunol* . 1995;25:2563-2571.

966. Jordan MS, Boesteanu A, Reed AJ, et al. Thymic selection of CD4+CD25+

regulatory T cells induced by an agonist self-peptide. *Nat Immunol*. 2001;2:301-306.

967. Taguchi O, Takahashi T, Seto M, Namikawa R, Matsuyama M, Nishizuka Y. Development of multiple organ-localized autoimmune diseases in nude mice after reconstitution of T cell function by rat fetal thymus graft. *J Exp Med*. 1986;164:60-71.

968. Nishigaki-Maki K, Takahashi T, Ohno K, et al. Autoimmune diseases developed in athymic nude mice grafted with embryonic thymus of xenogeneic origin. *Eur J Immunol* . 1999;29:3350-3359.

969. Xia G, Goebels J, Rutgeerts O, Vandeputte M, Waer M. Transplantation tolerance and autoimmunity after xenogeneic thymus transplantation. *J Immunol*. 2001;166:1843-1854.

970. Yan Y, Verbeken E, Vandeputte M, et al. Pathogenesis of autoimmunity after xenogeneic thymus transplantation. *Xenotransplantation*. 2001;8:S147-S147.

971. Zhao Y, Rodriguez-Barbosa JI, Shimizu A, Swenson K, Sachs DH, Sykes M. Despite efficient intrathymic negative selection of host-reactive T cells, autoimmune disease may develop in porcine thymusgrafted athymic mice: evidence for failure of regulatory mechanisms suppressing autoimmunity. *Transplantation*. 2002;75:1832-1840.

972. Fudaba Y, Onoe T, Chittenden M, et al. Abnormal regulatory and effector T cell function predispose to autoimmunity following xenogeneic thymic transplantation. *J Immunol*. 2008;181:7649-7659.

973. Anderson D, Billingham RE, Lampkin GH, Medawar PB. The use of skin grafting to distinguish between monozygotic and dizygotic twins in cattle. *Heredity*. 1951;5:379-397.

974. Bacchetta R, Vandekerckhove BAE, Touraine JL, et al. Chimerism and tolerance to host and donor in severe combined immunodeficiencies transplanted with fetal liver stem cells. *J Clin Invest*. 1993;91: 1067-1078.

975. Touraine JL. Treatment of human fetuses and induction of immunological tolerance in humans by in utero transplantation of stem cells into fetal recipients. *Acta Haematol*. 1996;96:115-119.

976. Flake AW, Roncarolo MG, Puck JM, et al. Treatment of X-linked severe combined immunodeficiency by in utero transplantation of paternal bone marrow. *New Engl J Med* . 1996;335:1806-1810.

977. Carrier E, Lee TH, Busch MP, Cowan MJ. Induction of tolerance in nondefective mice after in utero transplantation of major histocompatibility complex-mismatched fetal hematopoietic stem cells. *Blood*. 1995;86:4681-4690.

978. Kawashima I, Zanjani ED, Almaida-Porada G, Flake AW, Zeng H, Ogawa M. CD34+ human marrow cells that express low levels of Kit protein are enriched for long-term

marrow-engrafting cells. *Blood*. 1996;87:4136-4142.

979. Civin CI, Almeida-Porada G, Lee MJ, et al. Sustained, retransplantable, multilineage engraftment of highly purified adult human bone marrow stem cells in vivo. *Blood*. 1996;88:4102-4109.

980. Flake AW, Zanjani ED. In utero hematopoietic stem cell transplantation: ontogenic opportunities and biologic barriers. *Blood*. 1999; 94:2179-2191.

981. Donahue J, Gilpin E, Lee TH, Busch MP, Croft M, Carrier E. Microchimerism does not induce tolerance and sustains immunity after in utero transplantation. *Transplantation*. 2001;71:359-368.

982. Hedrick MH, Rice HE, MacGillivray TE, Bealer JF, Zanjani ED, Flake AW. Hematopoietic chimerism achieved by in utero hematopoietic stem cell injection does not induce donor-specific tolerance for renal allografts in sheep. *Transplantation*. 1994;58:110-111.

983. Mathes DW, Solari MG, Randolph MA, et al. Long-term acceptance of renal allografts following prenatal inoculation with adult bone marrow. *Transplantation*. 2005;80:1300-1308.

984. Billingham RE, Brent L, Medawar PB. “Actively acquired tolerance” of foreign cells. *Nature*. 1953;172:603-606.

985. Streilein JW. Neonatal tolerance of H-2 alloantigens. *Transplantation*. 1991;52:1-10.

986. Borenstein SH, Tao KS, West LJ, Chamberlain JW. Extrathymic deletion of CD8+ alloreactive T cells in a transgenic T cell receptor model of neonatal tolerance. *Transplantation*. 2001;72:1807-1816.

987. Russell PS, Chase CM, Sykes M, Ito H, Shaffer J, Colvin RB. Tolerance, mixed chimerism and chronic transplant arteriopathy. *J Immunol*. 2001;167:5731-5740.

988. Roser BJ. Cellular mechanisms in neonatal and adult tolerance. *Immunol Rev*. 1989;107:179-202.

989. Lubaroff DM, Silvers WK. The importance of chimerism in maintaining tolerance of skin allografts in mice. *J Immunol*. 1973;111:65-71.

990. Ridge JP, Fuchs EJ, Matzinger P. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. *Science*. 1996;271:1723-1726.

991. Starzl TE, Demetris AJ, Trucco M, et al. Chimerism and donorspecific nonreactivity 27 to 29 years after kidney allotransplantation. *Transplantation*. 1993;55:1272-1277.

992. Starzl TE, Demetris AJ, Murase N, Trucco M, Thomson AW, Rao AS. The lost chord:

microchimerism and allograft survival. *Immunol Today*. 1996;17:577-584.

993. Eynon EE, Parker DC. Parameters of tolerance induction by antigen targeted to B lymphocytes. *J Immunol*. 1993;151:2958-2964.

994. Fuchs EJ, Matzinger P. B cells turn off virgin but not memory T cells. *Science*. 1992;258:1156-1159.

995. Lombardi G, Hargreaves R, Sidhu S, et al. Antigen presentation by T cells inhibits IL-2 production and induces IL-4 release due to altered cognate signals. *J Immunol*. 1996;156:2769-2775.

996. Burlingham WJ, Grailer AP, Fechner JH Jr, et al. Microchimerism linked to cytotoxic T lymphocyte functional unresponsiveness (clonal anergy) in a tolerant renal transplant recipient. *Transplantation*. 1995; 59:1147-1155.

997. Wood K, Sachs DH. Chimerism and transplantation tolerance: cause and effect. *Immunol Today*. 1996;17:584-588.

998. Schlitt HJ, Hundreiser J, Ringe B, Pichlmayr R. Systemic microchimerism of donor-type associated with irreversible acute liver graft rejection eight years after transplantation. *New Engl J Med*. 1994;330: 646-647.

999. Schlitt HJ. Is microchimerism needed for allograft tolerance? *Transplant Proc*. 1997;29:82-84.

1000. Hisanaga M, Hundrieser J, Boker K, et al. Development, stability, and clinical correlations of allogeneic microchimerism after solid organ transplantation. *Transplantation*. 1996;61:40-45.

1001. Ko S, Deiwick A, Jager MD, et al. The functional relevance of passenger leukocytes and microchimerism for heart allograft acceptance in the rat. *Nat Med*. 1999;5:1292-1297.

1002. Shapiro R, Rao AS, Fontes P, et al. Combined simultaneous kidney/bone marrow transplantation. *Transplantation*. 1995;60:1421-1425.

1003. Rolles K, Burroughs AK, Davidson BR, Karatapanis S, Prentice HG, Hamon MD. Donor-specific bone marrow infusion after orthotopic liver transplantation. *Lancet*. 1994;343:263-265.

1004. Ricordi C, Karatzas T, Nery J, et al. High-dose donor bone marrow infusions to enhance allograft survival. The effect of timing. *Transplantation*. 1997;63:7-11.

1005. Garcia-Morales R, Carreno M, Mathew JM, et al. The effects of chimeric cells following donor bone marrow infusions as detected by PCR-flow assays in kidney transplant recipients. *J Clin Invest*. 1997;99:1118-1129.

1006. Rugeles MT, Aitouche A, Zeevi A, et al. Evidence for the presence of multilineage chimerism and progenitors of donor dendritic cells in the peripheral blood of bone marrow-augmented organ transplant recipients. *Transplantation*. 1997;64:735-741.

1007. Ciancio G, Burke GW, Garcia-Morales R, et al. Effect of living-related donor bone marrow infusion on chimerism and in vitro immunoregulatory activity in kidney transplant recipients. *Transplantation*. 2002;74:488-496.

1008. Tryphonopoulos P, Tzakis AG, Weppler D, et al. The role of donor bone marrow infusions in withdrawal of immunosuppression in adult liver allotransplantation. *Am J Transplant*. 1995;5:608-613.

1009. Mineo D, Ricordi C, Xu X, et al. Combined islet and hematopoietic stem cell allotransplantation: a clinical pilot trial to induce chimerism and graft tolerance. *Am J Transplant*. 2008;8:1262-1274.

1010. Ciancio G, Miller J, Garcia-Morales RO, et al. Six-year clinical effect of donor bone marrow infusions in renal transplant patients. *Transplantation*. 2001;71:827-835.

1011. Cirocco RE, Carreno MR, Mathew JM, et al. FoxP3 mRNA transcripts and regulatory cells in renal transplant recipients 10 years after donor marrow infusion. *Transplantation*. 2007;83:1611-1619.

1012. Mathew JM, Ciancio G, Burke GW, et al. Immune “tolerance profiles” in donor bone marrow infused kidney transplant patients using multiple ex vivo functional assays. *Hum Immunol*. 2010;71:566-576.

1013. Kawai T, Cosimi AB, Colvin RB, et al. Mixed allogeneic chimerism and renal allograft tolerance in cynomologous monkeys. *Transplantation*. 1995;59:256-262.

1014. Ratajczak MZ, Reca R, Wysoczynski M, Yan J, Ratajczak J. Modulation of the SDF-1-CXCR4 axis by the third complement component (C3)—implications for trafficking of CXCR4+ stem cells. *Exp Hematol*. 2006;34:986-995.

1015. Ramshaw HS, Crittenden RB, Dooner M, Peters SO, Rao SS, Quesenberry PJ. High levels of engraftment with a single infusion of bone marrow cells into normal unprepared mice. *Biol Blood Marrow Transplant* . 1995;1:74-80.

1016. Sykes M, Szot GL, Swenson K, Pearson DA, Wekerle T. Separate regulation of hematopietic and thymic engraftment. *Exp Hematol*. 1997;26:457-465.

1017. Yasukawa M, Ohminami H, Arai J, Kasahara Y, Ishida Y, Fujita S. Granule exocytosis, and not the Fas/Fas ligand system, is the main pathway of cytotoxicity mediated by alloantigen-specific CD4+ as well as CD8+ cytotoxic T lymphocytes in humans. *Blood*. 2000;95:2352-2355.

1018. Zheng J, Umikawa M, Zhang S, et al. Ex vivo expanded hematopoietic stem cells

overcome the MHC barrier in allogeneic transplantation. *Cell Stem Cell*. 2011;9:119-130.

1019. Boitano AE, Wang J, Romeo R, et al. Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. *Science*. 2010;329:1345-1348.

1020. Peugh WN, Wood KJ, Morris PJ. Genetic aspects of the blood transfusion effect. *Transplantation*. 1988;46:438-443.

1021. Fabre JW, Morris PJ. The effect of donor strain blood tretreatment on renal allograft rejection in rats. *Transplantation*. 1972;14:608-617.

1022. Salvatierra O, Vincenti F, Amend W. Four year experience with donor-specific blood transfusions. *Transplant Proc*. 1983;15:924-931.

1023. Opelz G, Vanrenterghem Y, Kirste G, et al. Prospective evaluation of pretransplant blood transfusions in cadaver kidney recipients. *Transplantation*. 1997;63:964-967.

1024. Bemelman F, Honey K, Adams E, Cobbold S, Waldmann H. Bone marrow transplantation induces either clonal deletion or infectious tolerance depending on the dose. *J Immunol*. 1998;160:2645-2648.

1025. Wood KJ, Evins J, Morris PJ. Suppression of renal allograft rejection in the rat by class I antigens on purified erythrocytes. *Transplantation*. 1985;39:56-62.

1026. Madsen JC, Superina RA, Wood KJ, Morris PJ. Immunological unresponsiveness induced by recipient cells transfected with donor MHC genes. *Nature*. 1988;332:161-164.

1027. Davies JD, Leong LYW, Mellor A, Cobbold SP, Waldmann H. T cell suppression in transplantation tolerance through linked recognition. *J Immunol*. 1996;156:3602-3607.

1028. Wong W, Morris PJ, Wood KJ. Pretransplant administration of a single donor class I major histocompatibility complex molecule is sufficient for the indefinite survival of fully allogeneic cardiac allografts. *Transplantation*. 1997;63:1490-1494.

1029. Wise MP, Bemelman F, Cobbold SP, Waldmann H. Linked suppression of skin graft rejection can operate through indirect recognition. *J Immunol*. 1998;161:5813-5816.

1030. Bushell A, Karim M, Kingsley CI, Wood KJ. Pretransplant blood transfusion without additional immunotherapy generates CD25+CD4+ regulatory T cells: a potential explanation for the blood-transfusion effect. *Transplantation*. 2003;76:449-455.

1031. Sykes M, Sachs DH, Nienhuis AW, Pearson DA, Moulton DA, Bodine DM. Specific prolongation of skin graft survival following retroviral transduction of bone marrow with an allogeneic MHC gene. *Transplantation*. 1993;55:197-202.

1032. Fry JW, Morris PJ, Wood KJ. Adenoviral transfer of a single donor specific MHC

class I gene to recipient bone marrow cells can induce specific immunological unresponsiveness in vivo. *Gene Therapy*. 2002;9:220-226.

1033. Emery DW, Sablinski T, Shimada H, et al. Expression of an allogeneic MHC DRB transgene, through retroviral transduction of bone marrow, induces specific reduction of alloreactivity. *Transplantation*. 1997;64:1414-1423.

1034. Sonntag KC, Emery DW, Yasumoto A, et al. Tolerance to solid organ transplants through transfer of MHC class II genes. *J Clin Invest*. 2001;107:65-71.

1035. Bagley J, Tian C, Sachs DH, Iacomini J. Induction of T-cell tolerance to an MHC class I alloantigen by gene therapy. *Blood*. 2002;99: 4394-4399.

1036. Tian C, Bagley J, Iacomini J. Persistence of antigen is required to maintain transplantation tolerance induced by genetic modification of bone marrow stem cells. *Am J Transplant*. 2006;6:2202-2207.

1037. Morelli AE, Thomson AW. Dendritic cells: regulators of alloimmunity and opportunities for tolerance induction. *Immunol Rev*. 2003; 196:125-146.

1038. Barratt-Boyes SM, Thomson AW. Dendritic cells: tools and targets for transplant tolerance. *Am J Transplant*. 2005;5:2807-2813.

1039. Ito T, Yang M, Wang YH, et al. Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. *J Exp Med*. 2007;204:105-115.

1040. Fugier-Vivier IJ, Rezzoug F, Huang Y, et al. Plasmacytoid precursor dendritic cells facilitate allogeneic hematopoietic stem cell engraftment. *J Exp Med*. 2005;201:373-383.

1041. Abe M, Wang Z, De Creus A, Thomson AW. Plasmacytoid dendritic cell precursors induce allogeneic T-cell hyporesponsiveness and prolong heart graft survival. *Am J Transplant*. 2005;5:1808-1819.

1042. Björck P, Coates PT, Wang Z, Duncan FJ, Thomson AW. Promotion of long-term heart allograft survival by combination of mobilized donor plasmacytoid dendritic cells and anti-CD154 monoclonal antibody. *J Heart Lung Transplant* . 2005;24:1118-1120.

1043. Fu F, Li Y, Qian S, et al. Costimulatory molecule-deficient dendritic cell progenitors (MHC class II+, CD80dim, CD86-) prolong cardiac allograft survival in nonimmunosuppressed recipients. *Transplantation*. 1996;62:659-665.

1044. Lutz MB, Suri RM, Niimi M, et al. Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo. *Eur J Immunol* . 2000;30:1813-1822.

1045. Lu L, Li W, Fu F, et al. Blockade of the CD40-CD40 ligand pathway potentiates the capacity of donor-derived dendritic cell progenitors to induce long-term cardiac allograft

survival. *Transplantation*. 1997;64:1808-1815.

1046. Taner T, Hackstein H, Wang Z, Morelli AE, Thomson AW. Rapamycintreated, alloantigen-pulsed host dendritic cells induce Ag-specific T cell regulation and prolong graft survival. *Am J Transplant*. 2005;5: 228-236.

1047. Sato K, Yamashita N, Yamashita N, Baba M, Matsuyama T. Regulatory dendritic cells protect mice from murine acute graft-versus-host disease and leukemia relapse. *Immunity*. 2003;18:367-379.

1048. Albert ML, Jegathesan M, Darnell RB. Dendritic cell maturation is required for the cross-tolerization of CD8+ T cells. *Nat Immunol*. 2001;2:1010-1017.

1049. Verhasselt V, Vosters O, Beuneu C, Nicaise C, Stordeur P, Goldman M. Induction of FOXP3-expressing regulatory CD4pos T cells by human mature autologous dendritic cells. *Eur J Immunol* . 2004;34:762-772.

1050. Menges M, Rossner S, Voigtlander C, et al. Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity. *J Exp Med*. 2002;195:15-21.

1051. Swiecki M, Colonna M. Unraveling the functions of plasmacytoid dendritic cells during viral infections, autoimmunity, and tolerance. *Immunol Rev*. 2010;234:142-162.

1052. Ochando JC, Homma C, Yang Y, et al. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat Immunol*. 2006;7:652-662.

1053. Lutz MB, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol*. 2002;23:445-449.

1054. Waldmann H. Reprogramming the immune system. *Immunol Rev*. 2002;185:227-235.

1055. Monaco AP, Wood ML, Russell PS. Studies on heterologous anti-lymphocyte serum in mice. III. Immunologic tolerance and chimerism produced across the H-2 Locus with adult thymectomy and antilymphocyte serum. *Ann N Y Acad Sci* . 1996;129:190-209.

1056. Fechner JH Jr, Vargo DJ, Geissler EK, et al. Split tolerance induced by immunotoxin in a rhesus kidney allograft model. *Transplantation*. 1997;63:1339-1345.

1057. Knechtle SJ, Vargo D, Fechner J, et al. FN18-CRM9 immunotoxin promotes tolerance in primate renal allografts. *Transplantation*. 1997;63:1-6.

1058. Nishimura H, Scalea J, Wang Z, et al. First experience with the use of a recombinant CD3 immunotoxin as induction therapy in pigto-primate xenotransplantation: the effect of T-cell depletion on outcome. *Transplantation*. 2011;92:641-647.

1059. Kirk AD, Hale DA, Mannon RB, et al. Results from a human renal allograft tolerance trial evaluating the humanized CD52-specific monoclonal antibody alemtuzumab (CAMPATH-1H). *Transplantation*. 2003;76:120-129.

1060. Kirk AD, Mannon RB, Kleiner DE, et al. Results from a human renal allograft tolerance trial evaluating T-cell depletion with alemtuzumab combined with deoxyspergualin. *Transplantation*. 2005;80: 1051-1059.

1061. Hanaway MJ, Woodle ES, Mulgaonkar S, et al. Alemtuzumab induction in renal transplantation. *N Engl J Med* . 2011;364:1909-1919.

1062. Calne R, Friend P, Moffatt S, et al. Prope tolerance, perioperative campath 1H, adn low-dose cyclosporin monotherapy in renal allograft recipients. *Lancet*. 1998;351:1702.

1063. Watson CJ, Bradley JA, Friend PJ, et al. Alemtuzumab (CAMPATH 1H) induction therapy in cadaveric kidney transplantation—efficacy and safety at five years. *Am J Transplant*. 2005;5:1347-1353.

1064. Barth RN, Janus CA, Lillesand CA, et al. Outcomes at 3 years of a prospective pilot study of Campath-1H and sirolimus immunosuppression for renal transplantation. *Transpl Int*. 2006;19:885-892.

1065. Pearl JP, Parris J, Hale DA, et al. Immunocompetent T-cells with a memory-like phenotype are the dominant cell type following antibody-mediated T-cell depletion. *Am J Transplant*. 2005;5:465-474.

1066. Trzonkowski P, Zilvetti M, Friend P, Wood KJ. Recipient memory-like lymphocytes remain unresponsive to graft antigens after CAMPATH-1H induction with reduced maintenance immunosuppression. *Transplantation*. 2006;82:1342-1351.

1067. De Serres SA, Mfarrej BG, Magee CN, et al. Immune profile of pediatric renal transplant recipients following alemtuzumab induction. *J Am Soc Nephrol* . 2012;23:174-182.

1068. Bloom DD, Chang Z, Fechner JH, et al. CD4+CD25+ FOXP3+ regulatory T cells increase de novo in kidney transplant patients after immunodepletion with Campath-1H. *Am J Transplant*. 2008;8:793-802.

1069. Hester J, Mills N, Shankar S, Carvalho-Gaspar M, Friend P, Wood KJ. Th17 cells in alemtuzumab-treated patients: the effect of longterm maintenance immunosuppressive therapy. *Transplantation*. 2011;91:744-750.

1070. Shaffer J, Villard J, Means TK, et al. Regulatory T-cell recovery in recipients of haploidentical nonmyeloablative hematopoietic cell transplantation with a humanized anti-CD2 mAb, MEDI-507, with or without fludarabine. *Exp Hematol*. 2007;35:1140-1152.

1071. Andreola G, Chittenden M, Shaffer J, et al. Mechanisms of donor-specific tolerance

in recipients of haploidentical combined bone marrow/kidney transplantation. *Am J Transplant*. 2011;11:1236-1247.

1072. Lopez M, Clarkson MR, Albin M, Sayegh MH, Najafian N. A novel mechanism of action for anti-thymocyte globulin: induction of CD4+CD25+Foxp^3+ regulatory T cells. *J Am Soc Nephrol*. 2006; 17:2844-2853.

1073. Feng G, Gao W, Strom TB, et al. Exogenous IFN-gamma ex vivo shapes the alloreactive T-cell repertoire by inhibition of Th17 responses and generation of functional Foxp3+ regulatory T cells. *Eur J Immunol* . 2008;38:2512-2527.

1074. Jones ND, Fluck NC, Mellor AL, Morris PJ, Wood KJ. The induction of transplantation tolerance by intrathymic (i.t.) delivery of allo antigen: a critical relationship between i.t. deletion, thymic export of new T cells and the timing of transplantation. *Int Immunol*. 1998;10:1637-1646.

1075. Sachs DH, Sykes M, Kawai T, Cosimi AB. Immuno-intervention for the induction of transplantation tolerance through mixed chimerism. *Semin Immunol*. 2011;23:165-173.

1076. Bretscher P, Cohn M. A theory of self-nonself discrimination. *Science*. 1970;169:1042-1049.

1077. Lafferty KJ, Cunningham AJ. A new analysis of allogeneic interactions. *Aust J Exp Biol Med Sci*. 197553:27-42.

1078. Schwartz RH. A cell culture model for T lymphocyte clonal anergy. *Science*. 1990;248:1349-1356.

1079. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol*. 2005;23:515-548.

1080. Sharpe AH. Mechanisms of costimulation. *Immunol Rev*. 2009;229:5-11.

1081. Bour-Jordan H, Esensten JH, Martinez-Llordella M, Penaranda C, Stumpf M, Bluestone JA. Intrinsic and extrinsic control of peripheral T-cell tolerance by costimulatory molecules of the CD28/ B7 family. *Immunol Rev*. 2011;241:180-205.

1082. Watts TH. TNF/TNFR family members in costimulation of T cell responses. *Annu Rev Immunol*. 2005;23:23-68.

1083. Larsen CP, Knechtle SJ, Adams A, Pearson T, Kirk AD. A new look at blockade of T-cell costimulation: a therapeutic strategy for long-term maintenance immunosuppression. *Am J Transplant*. 2006;6:876-883.

1084. Webber A, Hirose R, Vincenti F. Novel strategies in immunosuppression: issues in perspective. *Transplantation*. 2011;91:1057-1064.

1085. Sharma P, Wagner K, Wolchok JD, Allison JP. Novel cancer immunotherapy agents with survival benefit: recent successes and next steps. *Nat Rev Cancer* . 2011;11:805-812.

1086. Thompson CB. Distinct roles for the costimulatory ligands B7-1 and B7-2 in T helper cell differentiation? *Cell*. 1995;81:979-982.

1087. Bluestone JA. Is CTLA-4 a master switch for peripheral T cell tolerance? *J Immunol*. 1997;158:1989-1993.

1088. Schneider H, Downey J, Smith A, et al. Reversal of the TCR stop signal by CTLA-4. *Science*. 2006;313:1972-1975.

1089. Lenshow DJ, Zeng Y, Thistlethwaite JR, et al. Long-term survival of xenogeneic pancreatic islets induced by CTLA4Ig. *Science*. 1992; 257:789-792.

1090. Larsen CP, Elwood ET, Alexander DZ, et al. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature*. 1996;381:434-438.

1091. Pearson TC, Alexander DZ, Hendrix R, et al. CTLA4-Ig plus bone marrow induces long-term allograft survival and donor-specific unresponsiveness in the murine model. *Transplantation*. 1996;61:997-1004.

1092. Lin H, Bolling SF, Linsley PS, et al. Long-term acceptance of major histocompatibility complex mismatched cardiac allografts induced by CTLA4Ig plus donor-specific transfusion. *J Exp Med*. 1993;178: 1801-1806.

1093. Sayegh MH, Zheng XG, Magee C, Hancock WW, Turka LA. Donor antigen is necessary for the prevention of chronic rejection in CTLA4Igtreated murine cardiac allograft recipients. *Transplantation*. 1997; 64:1646-1650.

1094. Kirk AD, Harlan DM, Armstrong NN, et al. CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. *Proc Natl Acad Sci U S A* . 1997;94:8789-8798.

1095. Ford ML, Larsen CP. Translating costimulation blockade to the clinic: lessons learned from three pathways. *Immunol Rev*. 2009;229:294-306.

1096. Larsen CP, Pearson TC, Adams AB, et al. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am J Transplant*. 2005;5:443-453.

1097. Bluestone JA, St Clair EW, Turka LA. CTLA4Ig: bridging the basic immunology with clinical application. *Immunity*. 2006;24:233-238.

1098. Vincenti F, Larsen C, Durrbach A, et al. Costimulation blockade with belatacept in renal transplantation. *N Engl J Med* . 2005;353:770-781.

1099. Cardona K, Korbutt GS, Milas Z, et al. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat Med*. 2006;12:304-306.

1100. Grinyo J, Charpentier B, Pestana JM, et al. An integrated safety profile analysis of belatacept in kidney transplant recipients. *Transplantation*. 2010;90:1521-1527.

1101. Larsen CP, Alexander DZ, Hollenbaugh D, et al. CD40-GP39 interactions play a critical role during allograft rejection: suppression of allograft rejection by blockade of the CD40-gp39 pathway. *Transplantation*. 1996;61:4-9.

1102. Kirk AD, Burkly LC, Batty DS, et al. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. *Nat Med*. 1999;5:686-693.

1103. van Maurik A, Wood KJ, Jones ND. Impact of both donor and recipient strains on cardiac allograft survival after blockade of the CD40-CD154 costimulatory pathway. *Transplantation*. 2002;74:740-743.

1104. Markees TG, Phillips NE, Noelle RJ, et al. Prolonged survival of mouse skin allografts in recipients treated with donor splenocytes and antibody to CD40 ligand. *Transplantation*. 1997;64:329-335.

1105. Clarkson MR, Sayegh MH. T-Cell costimulatory pathways in allograft rejection and tolerance. *Transplantation*. 2005;80:555-563.

1106. Andre P, Prasad KS, Denis CV, et al. CD40L stabilizes arterial thrombi by a beta3 integrin—dependent mechanism. *Nat Med*. 2002;8:247-252.

1107. Noelle RJ, Roy M, Shepherd DM, Stamenkovic I, Ledbetter JA, Aruffo A. A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proc Natl Acad Sci U S A* . 1992;89:6550-6554.

1108. Kawabe T, Naka T, Yoshida K, et al. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity*. 1994;1:167-178.

1109. Xu J, Foy TM, Laman JD, et al. Mice deficient for the CD40 ligand. *Immunity*. 1994;1:423-431.

1110. Grewal IS, Foellmer HG, Grewal KD, et al. Requirement for CD40 ligand in costimulation induction, T cell activation, and experimental allergic encephalomyelitis. *Science*. 1996;273:1864-1867.

1111. Gurunathan S, Irvine KR, Wu CY, et al. CD40 ligand/trimer DNA enhances both humoral and cellular immune responses and induces protective immunity to infectious and tumor challenge. *J Immunol* . 1998;161:4563-4571.

1112. Liu Z, Tugulea S, Cortesini R, Lederman S, Suciu-Foca N. Inhibition of CD40 signaling pathway in antigen presenting cells by T suppressor cells. *Hum Immunol*. 1999;60:568-574.

1113. Parker DC, Greiner DL, Phillips NE, et al. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. *Proc Natl Acad Sci U S A* . 1995;92:9560-9564.

1114. Niimi M, Pearson TC, Larsen CP, et al. The role of the CD40 pathway in alloantigen-induced hyporesponsiveness in vivo. *J Immunol*. 1998;161:5331-5337.

1115. Ito H, Kurtz J, Shaffer J, Sykes M. CD4 T cell-mediated alloresistance to fully MHC-mismatched allogeneic bone marrow engraftment is dependent on CD40-CD40L interactions, and lasting T cell tolerance is induced by bone marrow transplantation with initial blockade of this pathway. *J Immunol*. 2001;166:2970-2981.

1116. Fehr T, Takeuchi Y, Kurtz J, Sykes M. Early regulation of CD8 T cell alloreactivity by CD4+CD25- T cells in recipients of anti-CD154 antibody and allogeneic BMT is followed by rapid peripheral deletion of donor-reactive CD8+ T cells, precluding a role for sustained regulation. *Eur J Immunol* . 2005;35:2679-2690.

1117. Larsen CP, Pearson TC. The CD40 pathway in allograft rejection, acceptance, and tolerance. *Curr Opin Immunol* . 1997;9:641-647.

1118. Larsen CP, Page A, Linzie KH, et al. An MHC-defined primate model reveals significant rejection of bone marrow after mixed chimerism induction despite full MHC matching. *Am J Transplant*. 2010; 10:2396-2409.

1119. Trambley J, Bingaman AW, Lin A, et al. Asialo GM1(+) CD8(+) T cells play a critical role in costimulation blockade-resistant allograft rejection. *J Clin Invest*. 1999;104:1715-1722.

1120. Jones ND, van Maurik A, Hara M, et al. CD40-CD40 ligand-independent activation of CD8+ T cells can trigger allograft rejection. *J Immunol*. 2000;165:1111-1118.

1121. Takeuchi Y, Ito H, Kurtz J, Wekerle T, Ho L, Sykes M. Earlier lowdose TBI or DST overcomes CD8+ T-cell-mediated alloresistance to allogeneic marrow in recipients of anti-CD40L. *Am J Transplant*. 2004;4:31-40.

1122. Kenyon NS, Chatzipetrou M, Masetti M, et al. Long-term survival and function of intrahepatic islet allografts in rhesus monkeys treated with humanized anti-CD154. *Proc Natl Acad Sci U S A*. 1999; 96: 8132-8137.

1123. Kirk, AD, Knechtle SJ, Sollinger HW, et al. Preliminary results of the use of humanized anti-CD 154 in human renal allotransplantation. *American Journal of Transplantation*. 2001;1:S191.

1124. Azimzadeh AM, Pfeiffer S, Wu G, et al. Alloimmunity in primate heart recipients with CD154 blockade: evidence for alternative costimulation mechanisms. *Transplantation*. 2006;81:255-264.

1125. Kanmaz T, Fechner JJ Jr, Torrealba J, et al. Monotherapy with the novel human anti-CD154 monoclonal antibody ABI793 in rhesus monkey renal transplantation model. *Transplantation*. 2004;77:914-920.

1126. Crow AR, Leytin V, Starkey AF, Rand ML, Lazarus AH. CD154 (CD40 ligand)-deficient mice exhibit prolonged bleeding time and decreased shear-induced platelet aggregates. *J Thromb Haemost*. 2003; 1:850-852.

1127. Haanstra KG, Sick EA, Ringers J, et al. Costimulation blockade followed by a 12-week period of cyclosporine A facilitates prolonged drug-free survival of rhesus monkey kidney allografts. *Transplantation*. 2005;79:1623-1626.

1128. Imai A, Suzuki T, Sugitani A, et al. A novel fully human anti-CD40 monoclonal antibody, 4D11, for kidney transplantation in cynomolgus monkeys. *Transplantation*. 2007;84:1020-1028.

1129. Cobbold S, Waldmann H. Skin allograft rejection by L3/T4+ and Lyt-2+ T cell subsets. *Transplantation*. 1986;41:634-639.

1130. Madsen JC, Peugh WN, Wood KJ, Morris PJ. The effect of anti-L3T4 monoclonal antibody treatment on first-set rejection of murine cardiac allografts. *Transplantation*. 1987;44:849-852.

1131. Shizuru JA, Gregory AK, Chao CT, Fathman CG. Islet allograft survival after a single course of treatment of recipient with antibody to L3T4. *Science*. 1987;237:278-280.

1132. Benjamin RJ, Cobbold SP, Clark MR, Waldmann H. Tolerance to rat monoclonal antibodies. Implications for serotherapy. *J Exp Med*. 1986;163:1539-1552.

1133. Benjamin RJ, Qin SX, Wise MP, Cobbold SP, Waldmann H. Mechanisms of monoclonal antibody-facilitated tolerance induction: a possible role for the CD4 (L3T4) and CD11a (LFA-1) molecules in self-non-self discrimination. *Eur J Immunol* . 1988;18:1079-1088.

1134. Gutstein NL, Wofsy D. Administration of F(ab')2 fragments of monoclonal antibody to L3T4 inhibits humoral immunity in mice without depleting L3T4+ cells. *J Immunol* . 1986;137:3414-3419.

1135. Qin S, Cobbold S, Tighe H, Benjamin R, Waldmann H. CD4 monoclonal antibody pairs for immunosuppression and tolerance induction. *Eur J Immunol* . 1987;17:1159-1165.

1136. Darby CR, Morris PJ, Wood KJ. Evidence that long-term cardiac allograft survival induced by anti-CD4 monoclonal antibody does not require depletion of CD4+ T cells. *Transplantation*. 1992;54:483-490.

1137. Pearson TC, Madsen JC, Larsen CP, Morris PJ, Wood KJ. Induction of transplantation tolerance in adults using donor antigen and anti-CD4 monoclonal antibody. *Transplantation*. 1992;54:475-483.

1138. Fischer A, Friedrich W, Fasth A, et al. Reduction of graft failure by a monoclonal antibody (anti-LFA-1 CD11a) after HLA nonidentical bone marrow transplantation in children with immunodeficiencies, osteopetrosis, and Fanconi's anemia: a European Group for Immunodeficiency/European Group for Bone Marrow Transplantation report. *Blood*. 1991;77:249-256.

1139. Cavazzana-Calvo M, Sarnacki S, Hadda E, et al. Prevention of bone marrow and cardiac graft rejection in an H-2 haplotye disparate mouse combination by an anti-LFA-1 antibody. *Transplantation*. 1995;59:1576-1582.

1140. Yang H, Issekutz TB, Wright JW Jr. Prolongation of rat islet allograft survival by treatment with monoclonal antibodies against VLA-4 and LFA-1. *Transplantation*. 1995;60:71-76.

1141. Nishihara M, Gotoh M, Ohzato H, et al. Awareness of donor alloantigens in antiadhesion therapy induces antigen-specific unresponsiveness to islet allografts. *Transplantation*. 1997;64:965-970.

1142. Le MB, Hourmant M, Rougier JP, et al. Effect of anti-LFA1 (CD11a) monoclonal antibodies in acute rejection in human kidney transplantation. *Transplantation*. 1991;52:291-296.

1143. Bashuda H, Seino K, Ra C, Yagita H, Okumura K. Lack of cognate help by CD4+ T cells and anergy of CD8+ T cells are the principal mechanisms for anti-leukocyte function-associated antigen- 1/intercellular adhesion molecule-1-induced cardiac allograft tolerance. *Transplantation*. 1997; 63:113-118.

1144. Nicolls MR, Coulombe M, Yang H, Bolwerk A, Gill RG. Anti-LFA-1 therapy induces long-term islet allograft acceptance in the absence of IFN-gamma or IL-4. *J Immunol*. 2000;164:3627-3634.

1145. Li B, Wang H, Dai J, et al. Construction and characterization of a humanized anti-human CD3 monoclonal antibody 12F6 with effective immunoregulation functions. *Immunology*. 2005;116:487-498.

1146. Thistlethwaite JR Jr, Stuart JK, Mayes JT, et al. Complications and monitoring of OKT3 therapy. *Am J Kidney Dis*. 1988;11:112-119.

1147. Gaston RS, Deierhoi MH, Patterson T, et al. OKT3 first-dose reaction: association with T cell subsets and cytokine release. *Kidney Int*. 1991;39:141-148.

1148. Chatenoud L. Immune therapy for type 1 diabetes mellitus-what is unique about anti-CD3 antibodies? *Nat Rev Endocrinol* . 2010;6: 149-157.

1149. Herold KC, Gitelman SE, Masharani U, et al. A single course of anti-CD3 monoclonal antibody hOKT3gamma1(Ala-Ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes. *Diabetes*. 2005;54:1763-1769.

1150. Keymeulen B, Vandemeulebroucke E, Ziegler AG, et al. Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. *N Engl J Med* . 2005;352:2598-2608.

1151. Bach JF. Anti-CD3 antibodies for type 1 diabetes: beyond expectations. *Lancet*. 2011;378:459-460.

1152. Dustin ML, Carpen O, Springer TA. Regulation of locomotion and cell-cell contact area by the LFA-1 and ICAM-1 adhesion receptors. *J Immunol* . 1992;148:2654-2663.

1153. Bachmann MF, McKall-Faienza K, Schmits R, et al. Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. *Immunity*. 1997;7:549-557.

1154. Van Seventer GA, Shimizu Y, Horgan KJ, Shaw S. The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. *The Journal of Immunology* . 1990;144:4579-4586.

1155. Badell IR, Russell MC, Thompson PW, et al. LFA-1-specific therapy prolongs allograft survival in rhesus macaques. *J Clin Invest*. 2010;120:4520-4531.

1156. Turgeon NA, Avila JG, Cano JA, et al. Experience with a novel efalizumab-based immunosuppressive regimen to facilitate single donor islet cell transplantation. *Am J Transplant*. 2010;10:2082-2091.

1157. Pearson TC, Darby CR, Bushell AR, West LJ, Morris PJ, Wood KJ. The assessment of transplantation tolerance induced by anti-CD4 monoclonal antibody in the murine model. *Transplantation*. 1993;55:361-367.

1158. Scully R, Qin S, Cobbold S, Waldmann H. Mechanisms in CD4 antibody-mediated transplantation tolerance: kinetics of induction, antigen dependency and role of regulatory T cells. *Eur J Immunol* . 1994;24:2383-2392.

1159. Bushell A, Morris PJ, Wood KJ. Transplantation tolerance induced by antigen pretreatment and depleting anti-CD4 antibody depends on CD4+ T cell regulation during the induction phase of the response. *Eur J Immunol* . 1995;25:2643-2649.

1160. Wong W, Morris PJ, Wood KJ. Syngeneic bone marrow expressing a single donor class I MHC molecule permits acceptance of a fully allogeneic cardiac allograft.

Transplantation. 1996;62:1462-1468.

1161. Qin S, Cobbold SP, Pope H, et al. “Infectious” transplantation tolerance. *Science*. 1993;259:974-977.

1162. Hamano K, Rawsthorne MA, Bushell AR, Morris PJ, Wood KJ. Evidence that the continued presence of the organ graft and not peripheral donor microchimerism is essential for maintenance of tolerance to alloantigen in vivo in anti-CD4 treated recipients. *Transplantation*. 1996;62:856-860.

1163. Kurtz J, Ito H, Wekerle T, Shaffer J, Sykes M. Mechanisms involved in the establishment of tolerance through costimulatory blockade and BMT: lack of requirement for CD40L-mediated signaling for tolerance or deletion of donor-reactive CD4+ cells. *Am J Transplant*. 2001;1:339-349.

1164. Parker DC, Greiner DL, Phillips NE, et al. Survival of mouse *pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. *Proc Natl Acad Sci U S A* . 1995;92:9560-9564.

1165. Markees TG, Phillips NE, Noelle RJ, et al. Prolonged survival of mouse skin allografts in recipients treated with donor splenocytes and antibody to CD40 ligand. *Transplantation*. 1997;64:329-335.

1166. Iwakoshi NN, Mordes JP, Markees TG, Phillips NE, Rossini AA, Greiner DL. Treatment of allograft recipients with donor-specific transfusion and anti-CD154 antibody leads to deletion of alloreactive CD8+ T cells and prolonged graft survival in a CTLA4-dependent manner. *J Immunol* . 2000;164:512-521.

1167. Takeuchi Y, Ito H, Kurtz J, Wekerle T, Ho L, Sykes M. Earlier low-dose TBI or DST overcomes CD8+ T-cell-mediated alloresistance to allogeneic marrow in recipients of anti-CD40L. *Am J Transplant* . 2004;4:31-40.

1168. Blazar BR, Taylor PA, Linsley PS, Vallera DA. In vivo blockade of CD28/CTLA4:B7/BB1 interaction with CTLA4-Ig reduces lethal murine graft-versus-host disease across the major histocompatibility complex barrier in mice. *Blood*. 1994;83:3815-3825.

1169. Blazar BR, Sharpe AH, Taylor PA, et al. Infusion of anti-B7.1 (CD80) and anti-B7.2 (CD86) monoclonal antibodies inhibits murine graftversus-host disease lethality in part via direct effects on CD4+ and CD8+ T cells. *J Immunol* . 1996;157:3250-3259.

1170. Blazar BR, Taylor PA, Panoskaltsis-Mortari A, et al. Blockade of CD40 ligand-CD40 interactions impairs CD4+ T cell-mediated alloreactivity by inhibiting mature donor T cell expansion and function after bone marrow transplantation. *J Immunol* . 1997;158:29-39.

1171. Taylor PA, Noelle RJ, Blazar BR. CD4(+)CD25(+) immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. *J Exp Med*. 2001;193:1311-1318.

1172. Guinan EC, Boussiotis VA, Neuberg D, et al. Transplantation of anergic histoincompatible bone marrow allografts. *New Engl J Med* . 1999;340:1704-1714.

1173. Davies JK, Gribben JG, Brennan LL, Yuk D, Nadler LM, Guinan EC. Outcome of alloanergized haploidentical bone marrow transplantation after ex vivo costimulatory blockade: results of 2 phase 1 studies. *Blood*. 2008;112:2232-2241.

1174. Blancho G, Gianello PR, Lorf T, Set al. Molecular and cellular events implicated in local tolerance to kidney allografts in miniature swine. *Transplantation*. 1997;63:26-33.

1175. Yamada K, Gianello PR, Ierino FL, et al. Influence of the thymus on transplantation tolerance in miniature swine. *Transplant Proc*. 1997;29:1076.

1176. Yamada K, Gianello PR, Iereno FL, et al. Role of the thymus in transplantation tolerance in miniature swine: I. Requirement of the thymus for rapid and stable induction of tolerance to class I-mismatched renal allografts. *J Exp Med*. 1997;186:497-506.

1177. Yamada K, Gianello PR, Ierono FL, et al. Role of the thymus in transplantation tolerance in miniature swine. II. Effect of steroids and age on the induction of tolerance to class I mismatched renal allografts. *Transplantation*. 1997;67:458-467.

1178. Ierino FL, Yamada K, Hatch T, Rembert J, Sachs DH. Peripheral tolerance to class I mismatched renal allografts in miniature swine: donor antigen-activated peripheral blood lymphocytes from tolerant swine inhibit antidonor CTL reactivity. *J Immunol* . 1999;162:550-559.

1179. Mezrich JD, Kesselheim JA, Johnston DR, Yamada K, Sachs DH, Madsen JC. The role of regulatory cells in miniature swine rendered tolerant to cardiac allografts by donor kidney cotransplantation. *Am J Transplant*. 2003;3:1107-1115.

1180. Wu A, Yamada K, Ierino FL, Vagefi PA, Sachs DH. Regulatory mechanism of peripheral tolerance: in vitro evidence for dominant suppression of host responses during the maintenance phase of tolerance to renal allografts in miniature swine. *Transpl Immunol*. 2003;11:367-374.

1181. Oluwole SF, Jin MX, Chowdhury NC, Ohajewkwe OA. Effectiveness of intrathymic inoculation of soluble antigens in the induction of specific unresponsiveness to rat islet allografts without transient recipient immunosuppression. *Transplantation*. 1994;58:1077-1081.

1182. Sayegh MH, Perico N, Gallon L, et al. Mechanisms of acquired thymic unresponsiveness to renal allografts. Thymic recognition of immunodominant allo-MHC peptides induces peripheral T cell anergy. *Transplantation*. 1994;58:125-132.

1183. Odorico JS, O'Connor T, Campos L, Barker CF, Posselt AM, Naji A. Examination of the mechanisms responsible for tolerance induction after intrathymic inoculation of

allogeneic bone marrow. *Ann Surg*. 1993;218:525-531.

1184. Agus DB, Surh CD, Sprent J. Reentry of T cells to the adult thymus is restricted to activated T cells. *J Exp Med*. 1991;173:1039-1046.

1185. Ali A, Garrovillo M, Oluwole OO, et al. Mechanisms of acquired thymic tolerance: induction of transplant tolerance by adoptive transfer of in vivo allomhc peptide activated syngeneic T cells. *Transplantation*. 2001;71:1442-1448.

1186. Gopinathan R, DePaz HA, Oluwole OO, et al. Role of reentry of in vivo alloMHC peptide-activated T cells into the adult thymus in acquired systemic tolerance. *Transplantation*. 2001;72:1533-1541.

1187. Bonasio R, Scimone ML, Schaerli P, et al. Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus. *Nat Immunol*. 2006;7:1092-1100.

1188. Hadeiba H, Lahl K, Edalati A, et al. Plasmacytoid dendritic cells transport peripheral antigens to the thymus to promote central tolerance. *Immunity*. 2012;36:438-450.

1189. Pearce NW, Spinelli A, Gurley KE, Hall BM. Specific unresponsiveness in rats with prolonged cardiac allograft survival after treatment with cyclosporine. V. Dependence of CD4+ suppressor cells on the presence of alloantigen and cytokines, including interleukin-2. *Transplantation*. 1993;55:374-379.

1190. Blancho G, Gianello P, Germana S, Baetscher M, Sachs DH, LeGuern C. Molecular identification of porcine interleukin 10: regulation of expression in a kidney allograft model. *Proc Natl Acad Sci U S A* . 1995;92:2800-2804.

1191. Odorico JS, O'Connor T, Campos L, Barker CF, Posselt AM, Naji A. Examination of the mechanisms responsible for tolerance induction after intrathymic inoculation of allogeneic bone marrow. *Ann Surg*. 1993;218:525-531.

1192. Griesemer AD, LaMattina JC, Okumi M, et al. Linked suppression across an MHC-mismatched barrier in a miniature swine kidney transplantation model. *J Immunol*. 2008;181:4027-4036.

1193. de Bruin RW, van Rossum TJ, Scheringa M, Bonthius F, Ijzermans JN, Marquet RL. Intrathymic injection of alloantigen may lead to hyperacute rejection and prolonged graft survival of heart allografts in the rat. *Transplantation*. 1996;60:1061-1063.

1194. Alfrey EJ, Wang X, Lee L, et al. Tolerance induced by direct inoculation of donor antigen into the thymus in low and high responder rodents. *Transplantation*. 1995;59:1171-1176.

1195. Hillebrands JL, Raue HP, Klatter FA, et al. Intrathymic immune modulation prevents acute rejection but not the development of graft arteriosclerosis (chronic rejection). *Transplantation*. 2001;71:914-924.

1196. Jonker M, van Den HY, Noort RC, et al. Immunomodulation by intrathymic injection of donor leukocytes in rhesus monkeys1. *Transplantation*. 2001;72:1432-1436.

1197. Allen MD, Weyhrich J, Gaur L, et al. Prolonged allogeneic and xenogeneic microchimerism in unmatched primates without immunosuppression by intrathymic implantation of CD34+ donor marrow cells. *Cell Immunol* . 1997;181:127-138.

1198. Brouard S, Mansfield E, Braud C, et al. Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. *Proc Natl Acad Sci U S A*. 2007;104:15448-15453.

1199. Newell KA, Asare A, Kirk AD, et al. Identification of a B cell signature associated with renal transplant tolerance in humans. *J Clin Invest*. 2010;120:1836-1847.

1200. Lozano JJ, Pallier A, Martinez-Llordella M, Danger R, et al. Comparison of transcriptional and blood cell-phenotypic markers between operationally tolerant liver and kidney recipients. *Am J Transplant*. 2011;11:1916-1926.

1201. Fuchimoto Y, Yamada K, Shimizu A, et al. Relationship between chimerism and tolerance in a kidney transplantation model. *J Immunol* . 2009;162:5704-5711.

1202. Sykes M, Preffer F, McAffee S, et al. Mixed lymphohematopoietic chimerism and graft-vs-lymphoma effects are achievable in adult humans following non-myeloablative therapy and HLA-mismatched donor bone marrow transplantation. *Lancet*. 1999;353:1755-1759.

1203. Spitzer TR, MCafee S, Sackstein R, et al. The intentional induction of mixed chimerism and achievement of anti-tumor responses following non-myeloablative conditioning therapy and HLA-matched and mismatched donor bone marrow transplantation for refractory hematologic malignancies. *Biol Blood Marrow Transplant* . 2000;6:309-320.

1204. Fudaba Y, Spitzer TR, Shaffer J, et al. Myeloma responses and tolerance following combined kidney and nonmyeloablative marrow transplantation: in vivo and in vitro analyses. *Am J Transplant*. 2006;6:2121-2133.

1205. Spitzer TR, Sykes M, Tolkoff-Rubin N, et al. Long-term follow-up of recipients of combined human leukocyte antigen-matched bone marrow and kidney transplantation for multiple myeloma with end-stage renal disease. *Transplantation*. 2011;91:672-676.

1206. Spitzer TR, MCafee S, Dey BR, et al. Non-myeloablative haploidentical stem cell transplantation using anti-CD2 monoclonal antibody (MEDI-507)-based conditioning for refractory hematologic malignancies. *Transplantation*. 2003;75:1748-1751.

1207. Locascio SA, Morokata T, Chittenden M, et al. Mixed chimerism, lymphocyte recovery, and evidence for early donor-specific unresponsiveness in patients receiving combined kidney and bone marrow transplantation to induce tolerance. *Transplantation*.

2010;90:1607-1615.

1208. Kawai T, Cosimi AB, Spitzer TR, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med* . 2008;358:353-361.

1209. Leventhal J, Abecassis M, Miller J, et al. Chimerism and tolerance without GVHD or engraftment syndrome in HLA-mismatched combined kidney and hematopoietic stem cell transplantation. *Sci Transl Med* . 2012;4:124ra28.

1210. Li HW, Sykes M. Emerging concepts in haematopoietic cell transplantation. *Nat Rev Immunol* . 2012;12:403-416.

1211. Strober S, Spitzer TR, Lowsky R, Sykes M. Translational studies in hematopoietic cell transplantation: treatment of hematologic malignancies as a stepping stone to tolerance induction. *Semin Immunol* . 2011;23:273-281.

1212. U.S. Department of Health & Human Services. The Organ Procurement and Transplantation Network. 2007. http://optn.transplant.hrsa.gov. Accessed September 4, 2012.

1213. Fudaba Y, Spitzer TR, Shaffer J, et al. Myeloma responses and tolerance following combined kidney and nonmyeloablative marrow transplantation: in vivo and in vitro analyses. *Am J Transplant* . 2006;6:2121-2133.

1214. Scandling JD, Busque S, Dejbakhsh-Jones S, et al. Tolerance and chimerism after renal and hematopoietic-cell transplantation. *N Engl J Med* . 2008;358:362-368.

1215. Montgomery RA, Lonze BE, King KE, et al. Desensitization in HLA-incompatible kidney recipients and survival. *N Engl J Med* . 2011; 365:318-326.

1216. Wong BS, Yamada K, Okumi M, et al. Allosensitization does not increase the risk of xenoreactivity to alpha1,3-galactosyltransferase gene-knockout miniature swine in patients on transplantation waiting lists. *Transplantation*. 2006;82:314-319.

1217. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR. The natural history of chronic allograft nephropathy. *N Engl J Med* . 2003;349:2326-2333.

1218. Bishara A, Brautbar C, Eid A, Scherman L, Ilan Y, Safadi R. Is presensitization relevant to liver transplantation outcome? *Hum Immunol* . 2002;63:742-750.

1219. Goggins WC, Fisher RA, Kimball PM, et al. The impact of a positive crossmatch upon outcome after liver transplantation. *Transplantation*. 1996;62:1794-1798.

1220. Hathaway M, Gunson BK, Keogh AC, Briggs D, McMaster P, Neuberger JM. A positive crossmatch in liver transplantation—no effect or inappropriate analysis? A prospective study. *Transplantation*. 1997;64:54-59.

1221. Navarro V, Herrine S, Katopes C, Colombe B, Spain CV. The effect of HLA class I (A and B) and class II (DR) compatibility on liver transplantation outcomes: an analysis of the OPTN database. *Liver Transpl* . 2006;12:652-658.

1222. Taylor AL, Gibbs P, Bradley JA. Acute graft versus host disease following liver transplantation: the enemy within. *Am J Transplant*. 2004;4:466-474.

1223. Snyder LD, Palmer SM. Immune mechanisms of lung allograft rejection. *Semin Respir Crit Care Med*. 2006;27:534-543.

1224. Auersvald LA, Rothstein DM, Oliveira SC, et al. Indefinite islet allograft survival in mice after a short course of treatment with anti-CD45 monoclonal antibodies. *Transplantation*. 1997;63:1355-1358.

1225. Hellerstrom C, Andersson A, Groth CG, et al. Experimental pancreatic transplantation in diabetes. *Diabetes Care*. 1988;11(suppl 1):45-53.

1226. Bretzel RG, Hering BJ, Federlin KF. Islet cell transplantation in diabetes mellitus —from bench to bedside. *Exp Clin Endocrinol Diabetes*. 1995;103(suppl 2):143-159.

1227. Alejandro R, Lehmann R, Ricordi C, et al. Long-term function (6 years) of islet allografts in type 1 diabetes. *Diabetes*. 1997;46:1983-1989.

1228. Brunicardi FC, Shackleton CR. Whole-organ versus islet pancreatic transplantation. *Curr Opin Gen Surg* . 1994;179-185.

1229. Paty BW, Lanz K, Kendall DM, Sutherland DE, Robertson RP. Restored hypoglycemic counterregulation is stable in successful pancreas transplant recipients for up to 19 years after transplantation. *Transplantation*. 2001;72:1103-1107.

1230. Cretin N, Buhler B, Fournier B, et al. Human islet allotransplantation: world experience and current status. *Dig Surg* . 1998;15:656-662.

1231. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med*. 2000;343:230-238.

1232. Ryan EA, Lakey JR, Rajotte RV, et al. Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. *Diabetes*. 2001;50:710-719.

1233. Ryan EA, Paty BW, Senior PA, et al. Five-year follow-up after clinical islet transplantation. *Diabetes*. 2005;54:2060-2069.

1234. Shapiro AM, Lakey JR, Paty BW, Senior PA, Bigam DL, Ryan EA. Strategic opportunities in clinical islet transplantation. *Transplantation*. 2005;79:1304-1307.

1235. Antin JH. Graft-versus-leukemia: no longer an epiphenomenon. *Blood*.

1993;82:2273-2277.

1236. McSweeney PA, Niederwieser D, Shizuru JA, et al. Hematopoietic cell transplantation in older patients with hematologic malignancies: replacing high-dose cytotoxic therapy with graft-versus- tumor effects. *Blood*. 2001;97:3390-3400.

1237. Giralt S, Thall PF, Khouri I, et al. Melphalan and purine analogcontaining preparative regimens: reduced- intensity conditioning for patients with hematologic malignancies undergoing allogeneic progenitor cell transplantation. *Blood*. 2001;97:631-637.

1238. Petersdorf E, Anasetti C, Martin PJ, et al. Genomics of unrelateddonor hematopoietic cell transplantation. *Curr Opin Immunol* . 2001;13:582-589.

1239. Ruggeri L, Capanni M, Casucci M, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood*. 1999;94:333-339.

1240. Bordignon C, Keever CA, Small TN, et al. Graft failure after T-celldepleted human leukocyte antigen identical marrow transplants for leukemia: II. In vitro analyses of host effector mechanisms. *Blood*. 1989;74:2237-2243.

1241. Martin PJ, Hansen JA, Buckner CD, et al. Effects of in vitro depletion of T cells in HLA-identical allogeneic marrow grafts. *Blood*. 1985;66:664-672.

1242. Kernan NA, Bordignon C, Heller G, et al. Graft failure after T-celldepleted human leukocyte antigen identical marrow transplants for leukemia: I. Analysis of risk factors and results of secondary transplants. *Blood*. 1989;74:2227-2236.

1243. Dey BR, MCafee S, Colby C, et al. Anti-tumor response despite loss of donor chimerism in patients treated with nonmyeloablative conditioning and allogeneic stem cell transplantation. *Br J Haematol* . 2005;128:351-359.

1244. Kraus AB, Shaffer J, Toh HC, et al. Early host CD8 T-cell recovery and sensitized anti-donor IL-2-producing and cytolytic T-cell responses associated with marrow graft rejection following nonmyeloablative bone marrow transplantation. *Exp Hematol*. 2003;31:609-621.

1245. Anasetti C, Amos D, Beatty PG, et al. Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. *N Engl J Med* . 1989;320:197-204.

1246. Martin PJ, Hansen JA, Torok-Storb B, et al. Graft failure in patients receiving T cell-depleted HLA-identical allogeneic marrow transplants. *Bone Marrow Transplant*. 1988;3:445-456.

1247. Martin PJ, Akatsuka Y, Hahne M, Sale G. Involvement of donor T-cell cytotoxic effector mechanisms in preventing allogeneic marrow graft rejection. *Blood*.

1998;92:2177-2181.

1248. Fleischhauer K, Kernan NA, O'Reilly RJ, Dupont B, Yang SY. Bone marrow-allograft rejection by T lymphocytes recognizing a single amino acid difference in HLA-B44. *N Engl J Med*. 1990;323: 1818-1822.

1249. Spellman S, Bray R, Rosen-Bronson S, et al. The detection of donordirected, HLA-specific alloantibodies in recipients of unrelated hematopoietic cell transplantation is predictive of graft failure. *Blood*. 2010;115:2704-2708.

1250. Schleuning M, Judith D, Jedlickova Z, et al. Calcineurin inhibitor-free GVHD prophylaxis with sirolimus, mycophenolate mofetil and ATG in Allo-SCT for leukemia patients with high relapse risk: an observational cohort study. *Bone Marrow Transplant*. 2009;43:717-723.

1251. Ballen KK, Koreth J, Chen YB, Dey BR, Spitzer TR. Selection of optimal alternative graft source: mismatched unrelated donor, umbilical cord blood, or haploidentical transplant. *Blood*. 2012;119:1972-1980.

1252. Liu H, Rich ES, Godley L, et al. Reduced-intensity conditioning with combined haploidentical and cord blood transplantation results in rapid engraftment, low GVHD, and durable remissions. *Blood*. 2011;118:6438-6445.

1253. Brunstein CG, Fuchs EJ, Carter SL, et al. Alternative donor transplantation after reduced intensity conditioning: results of parallel phase 2 trials using partially HLA-mismatched related bone marrow or unrelated double umbilical cord blood grafts. *Blood*. 2011;118: 282-288.

1254. Petersdorf E, Anasetti C, Martin PJ, et al. Genomics of unrelateddonor hematopoietic cell transplantation. *Curr Opin Immunol* . 2001; 13:582-589.

1255. Pedron B, Guerin-El K, Dalle VJH, et al. Contribution of HLA-A/B/C/DRB1/DQB1 common haplotypes to donor search outcome in unrelated hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* . 2011;17:1612-1618.

1256. Fefer A. Graft-versus-tumor responses. In: Thomas ED, Blume KG, Forman SJ, eds. *Hematopoietic Cell Transplantation*. 2nd ed. Malden, MA: Blackwell Science; 1999:316-326.

1257. Aversa F, Tabilio A, Velardi A, et al. Treatment of high-risk acute leukemia with T cell-depleted stem cells from related donors with one fully mismatched haplotype. *N Engl J Med*. 1998;339:1186-1193.

1258. Volpi I, Perruccio K, Tosti A, et al. Postgrafting administration of granulocyte colony-stimulating factor impairs functional immune recovery in recipients of human leukocyte antigen haplotype-mismatched hematopoietic transplants. *Blood*. 2001;97:2514-2521.

1259. Wolf D, von Lilienfeld-Toal M, Wolf AM, et al. Novel treatment concepts for graft-versus-host disease. *Blood*. 2012;119:16-25.

1260. Mapara MY, Kim YM, Wang SP, Bronson R, Sachs DH, Sykes M. Donor lymphocyte infusions mediate superior graft-versus-leukemia effects in mixed compared to fully allogeneic chimeras: a critical role for host antigen-presenting cells. *Blood*. 2002;100:1903-1909.

1261. Johnson BD, Becker EE, LaBelle JL, Truitt RL. Role of immunoregulatory donor T cells in suppression of graft-versus-host disease following donor leukocyte infusion therapy. *J Immunol* . 1999;163:6479-6487.

1262. Blazar BR, Lees CJ, Martin PJ, et al. Host T cells resist graft-versushost disease mediated by donor leukocyte infusions. *J Immunol* . 2000;165:4901-4909.

1263. Johnson BD, Becker EE, Truitt RL. Graft-vs.-host and graft-vs.-leukemia reactions after delayed infusions of donor T-subsets. Biol Blood Marrow Transplant. 1999;5:123-132.

1264. Kolb H, Holler E. Adoptive immunotherapy with donor lymphocyte transfusions. *Curr Opin Oncol* . 1997;9:139-145.

1265. Kim YM, Sachs T, Asavaroengchai W, Bronson R, Sykes M. Graftversus-host disease (GvHD) can be separated from graft-versus-lymphoma effects by controlling lymphocyte trafficking with FTY720. *J Clin Invest*. 2003;111:659-669.

1266. Mapara MY, Leng C, Kim YM, et al. Expression of chemokines in GVHD target organs is influenced by conditioning and genetic factors and amplified by GVHR. *Biol Blood Marrow Transplant*. 2006;12: 623-634.

1267. Palmer LA, Sale GE, Balogun JI, et al. Chemokine receptor CCR5 mediates alloimmune responses in graft-versus-host disease. *Biol Blood Marrow Transplant* . 2010;16:311-319.

1268. Wysocki CA, Burkett SB, Panoskaltsis-Mortari A, et al. Differential roles for CCR5 expression on donor T cells during graft-versushost disease based on pretransplant conditioning. *J Immunol* . 2004; 173:845-854.

1269. Krijanovski OI, Hill GR, Cooke KR, et al. Keratinocyte growth factor separates graft-versus-leukemia effects from graft-versus-host disease. *Blood*. 1999;94:825-831.

1270. Panoskaltsis-Mortari A, Taylor PA, Rubin JS, et al. Keratinocyte growth factor facilitates alloengraftment and ameliorates graft-versushost disease in mice by a mechanism independent of repair of conditioning-induced tissue injury. *Blood*. 2000;96:4350-4356.

1271. Shin HJ, Baker J, Leveson-Gower DB, Smith AT, Sega EI, Negrin RS. Rapamycin

and IL-2 reduce lethal acute graft-versus-host disease associated with increased expansion of donor type CD4+CD25+ Foxp3+ regulatory T cells. *Blood*. 2011;118:2342-2350.

1272. Brunstein CG, Miller JS, Cao Q, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood*. 2011;117:1061-1070.

1273. Hippen KL, Bucher C, Schirm DK, et al. Blocking IL-21 signaling ameliorates xenogeneic GVHD induced by human lymphocytes. *Blood*. 2012;119:619-628.

1274. Bonini C, Ferrari G, Verzeletti S, et al. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science*. 1997;276:1719-1724.

1275. Contassot E, Ferrand C, Angonin R, et al. Ganciclovir-sensitive acute graft-versus-host disease in mice receiving herpes simplex virus-thymidine kinase-expressing donor T cells in a bone marrow transplantation setting. *Transplantation*. 2000;69:503-508.

1276. Falkenburg JH, Wafelman AR, Smit WM, et al. Complete remission of accelerated phase chronic myeloid leukemia by treatment with leukemia-reactive cytotoxic T lymphocytes. *Blood*. 1999;94: 1201-1208.

1277. Fontaine P, Roy-Proulx G, Knafo L, Baron C, Roy DC, Perreault C. Adoptive transfer of minor histocompatibility antigen-specific T lymphocytes eradicates leukemia cells without causing graft-versus-host disease. *Nat Med*. 2001;7:789-794.

1278. Anderson BE, McNiff J, Yan J, et al. Memory CD4+ T cells do not induce graft-versus-host disease. *J Clin Invest*. 2003;112:101-108.

1279. Zheng H, Matte-Martone C, Li H, et al. Effector memory CD4+ T cells mediate graft-versus-leukemia without inducing graft-versus-host disease. *Blood*. 2008;111:2476-2484.

1280. Calne RY. Organ transplantation between widely disparate species. *Transplant Proc*. 1970;2:550-556.

1281. Sachs DH. The pig as a potential xenograft donor. *Vet Immunol Immunopathol*. 1994;43:185-191.

1282. Galili U, Wang L, LaTemple DC, Radic MZ. The natural anti-Gal antibody. *Subcell Biochem*. 1999;32:79-106.

1283. Auchincloss HJ, Sachs DH. Xenogeneic transplantation. *Annu Rev Immunol*. 1998;16:433-470.

1284. Yamada K, Sachs DH, DerSimonian H. Human anti-porcine xenogeneic T cell

response. Evidence for allelic specificity of mixed leukocyte reaction and for both direct and indirect pathways of recognition. *J Immunol*. 1995;155:5249-5256.

1285. Hering BJ, Walawalkar N. Pig-to-nonhuman primate islet xenotransplantation. *Transpl Immunol*. 2009;21:81-86.

1286. Buhler L, Yamada K, Alwayn I, et al. Miniature swine and hDAF pig kidney transplantation in baboons treated with a nonmyeloablative regimen and CD154 blockade. *Transplant Proc*. 2001;33:716.

1287. Bronson SK, Smithies O. Altering mice by homologous recombination using embryonic stem cells. *J Biol Chem*. 1994;269:27155-27158.

1288. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. *Nature*. 1997;385:810-813.

1289. Lai L, Kolber-Simonds D, Park KW, et al. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science*. 2002;295:1089-1092.

1290. Phelps CJ, Koike C, Vaught TD, et al. Production of alpha 1,3-galactosyltransferase-deficient pigs. *Science*. 2003;299:411-414.

1291. Dai Y, Vaught TD, Boone J, et al. Targeted disruption of the alpha1, 3-galactosyltransferase gene in cloned pigs. *Nat Biotechnol*. 2002;20: 251-255.

1292. Kolber-Simonds D, Lai L, Watt SR, et al. Production of alpha-1, 3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations. *Proc Natl Acad Sci U S A*. 2004;101:7335-7340.

1293. Kuwaki K, Tseng YL, Dor FJ, et al. Heart transplantation in baboons using alpha1,3-galactosyltransferase gene-knockout pigs as donors: initial experience. *Nat Med*. 2005;11:29-31.

1294. Yamada K, Yazawa K, Shimizu A, et al. Marked prolongation of porcine renal xenograft survival in baboons through the use of alpha1, 3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. *Nat Med*. 2005;11:32-34.

1295. Bartholomew AM, Powelson J, Sachs DH, et al. Tolerance in a concordant nonhuman primate xenograft model. *Transplantation*. 1999;68:1708-1716.

1296. Sablinski T, Emery DW, Monroy R, et al. Long-term discordant xenogeneic (porcine-to-primate) bone marrow engraftment in a monkey treated with porcine-specific growth factors. *Transplantation*. 1999;67:972-977.

1297. Tseng YL, Dor FJ, Kuwaki K, et al. Bone marrow transplantation from alpha1,3-

galactosyltransferase gene-knockout pigs in baboons. *Xenotransplantation*. 2004;11:361-370.

1298. Barth RN, Yamamoto S, LaMattina JC, et al. Xenogeneic thymokidney and thymic tissue transplantation in a pig-to-baboon model: I. Evidence for pig-specific T-cell unresponsiveness. *Transplantation*. 2003;75:1615-1624.

1299. Fishman JA, Patience C. Xenotransplantation: infectious risk revisited. *Am J Transplant*. 2004;4:1383-1390.

1300. Weiss RA. Retroviral zoonoses. *Nat Med*. 1998;4:391-392.

1301. Bach FH, Fishman JA, Daniels N, et al. Uncertainty in xenotransplantation: individual benefit versus collective risk. *Nat Med*. 1998;4:141-144.

1302. Paradis K, Langford G, Long Z, et al. Search for cross-species transmission of porcine endogenous retrovirus in patients treated with living pig tissue. The XEN 111 Study Group. *Science*. 1999;285:1236-1241.

1303. Denner J. Porcine endogenous retroviruses (PERVs) and xenotransplantation: screening for transmission in several clinical trials and in experimental models using non-human primates. *Ann Transplant*. 2003;8:39-48.

1304. Sykes M, Sandrin M, Cozzi E. International cooperation on xenotransplantation. *Nat Med*. 2004;10:119.

1305. Reemtsma K, McCracken BH, Schlegel JU. Renal heterotransplantation in man. *Ann Surg*. 1964;160:384.

1306. Deacon T, Schumacher J, Dinsmore J, et al. Histological evidence of fetal pig neural cell survival after transplantation into a patient with Parkinson's disease. *Nat Med*. 1997;3:350-353.

1307. Pierson RN III, Dorling A, Ayares D, et al. Current status of xenotransplantation and prospects for clinical application. *Xenotransplantation*. 2009;16:263-280.

1308. Lee SJ, Klein J, Haagenson M, et al. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. *Blood*. 2007;110:4576-4583.

1309. Morishima S, Ogawa S, Matsubara A, et al. Impact of highly conserved HLA haplotype on acute graft-versus-host disease. *Blood*. 2010;115:4664-4670.

1310. Petersdorf EW, Hansen JA. New advances in hematopoietic cell transplantation. *Curr Opin Hematol* . 2008;15:549-554.

1311. Petersdorf EW. Optimal HLA matching in hematopoietic cell transplantation. *Curr*

Opin Immunol. 2008;20:588-593.

1312. Susal C, Ovens J, Mahmoud K, et al. No association of kidney graft loss with human leukocyte antigen antibodies detected exclusively by sensitive Luminex single-antigen testing: a Collaborative Transplant Study report. *Transplantation*. 2011;91:883-887.

1313. Montgomery RA, Lonze BE, King KE, et al. Desensitization in HLA-incompatible kidney recipients and survival. *N Engl J Med* . 2011; 365:318-326.

1314. Kozlowski T, Andreoni K. Limitations of rituximab/IVIg desensitization protocol in kidney transplantation; is this better than a tincture of time? *Ann Transplant*. 2011;16:19-25.

1315. Pescovitz MD. Rituximab, an anti-cd20 monoclonal antibody: history and mechanism of action. *Am J Transplant*. 2006;6:859-866.

1316. Usuda M, Fujimori K, Koyamada N, et al. Successful use of anti-CD20 monoclonal antibody (rituximab) for ABO-incompatible living-related liver transplantation. *Transplantation*. 2005;79:12-16.

1317. Vo AA, Peng A, Toyoda M, et al. Use of intravenous immune globulin and rituximab for desensitization of highly HLA-sensitized patients awaiting kidney transplantation. *Transplantation*. 2010;89: 1095-1102.

1318. Wahrmann M, Haidinger M, Kormoczi GF, et al. Effect of the proteasome inhibitor bortezomib on humoral immunity in two presensitized renal transplant candidates. *Transplantation*. 2010;89:1385-1390.

1319. Zachary AA, Eng HS. Desensitization: achieving immune detente. *Tissue Antigens*. 2011;77:3-8.

1320. Li B, Hartono C, Ding R, et al. Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. *N Engl J Med* . 2001;344:947-954.

1321. Dadhania D, Muthukumar T, Ding R, et al. Molecular signatures of urinary cells distinguish acute rejection of renal allografts from urinary tract infection. *Transplantation*. 2003;75:1752-1754.

1322. Cornell LD, Colvin RB. Chronic allograft nephropathy. *Curr Opin Nephrol Hypertens*. 2005;14:229-234.

1323. Pescovitz MD, Thistlethwaite JR Jr, Auchincloss H Jr, et al. Effect of class II antigen matching on renal allograft survival in miniature swine. *J Exp Med*. 1984;160:1495-1508.

1324. Rosengard BR, Kortz EO, Ojikutu CA, et al. The failure of skin grafting to break

tolerance to class I disparate renal allografts in miniature swine despite inducing marked anti-donor cellular immunity. *Transplantation*. 1991;52:1044-1052.

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Chapter 47

Cancer Immunology

Hans Schreiber

INTRODUCTION

The goal of this chapter is to provide foundations and key facts in tumor immunology to students, immunologists, and oncologists in order to stimulate critical thinking and experimentation so that we may better prevent, cure, or at least control cancer. The study of cancer has had a tremendous impact on all fields of science (eg, molecular biology, virology, genetics, and immunology). For example, the discovery of the major histocompatibility complex (MHC) by Peter Gorer[7,8] clearly separated cancer immunity from autoimmunity. His successful search for specificity led subsequent generations of investigators to search for cancer-specific changes (eg, the consistent chromosome translocation in chronic myelogenous leukemia[9]). However, conclusive evidence that rejection antigens on cancer cells are cancer-specific came in 1995 when it was shown that they were encoded by somatic cancer-specific mutations.[10,11,12] Inherent in understanding the field of cancer immunology is the need to understand that immunology and cancer are two very large, different, and rapidly evolving complex fields of research. Thus, in order to make the immune system effective to prevent or treat cancer, an understanding of cancer and cancer models is required. The discussion of the important roles of cytokines in many aspects of tumor immunology is integrated in the various parts of this chapter.

CANCER

Despite all the progress in molecular biology and genetics, the pathologist using histologic or cytologic criteria[13] usually makes the only reliable diagnosis of cancer. Although *tumor* means “swelling,” the term is usually meant to include cancer cells and the stroma supporting the cancer cells; together they are often referred to as *neoplasm*, which literally means “new growth.” A neoplasm is an abnormal mass of cells that persists and proliferates after withdrawal of the stimulus that initiated its appearance. Leukemias are cancers caused by neoplastic proliferations of blood cells, but usually do not form tumor masses. There are two types of neoplasms: benign and malignant. The common term for all malignant neoplasms is *cancer*. Cancers of epithelial tissues (carcinomas) break through the basement membrane to invade adjacent tissues by infiltrative destructive growth. *Invasion* may or may not be followed by cancer cells entering the lymphatics, bloodstream, or fluid of the coelomic cavities to implant at sites discontinuous with the original tumor (*metastasis*; Greek for “emigration”). With extremely rare exceptions, metastasis defines a tumor as malignant; benign tumors do not metastasize. Invasion usually precedes metastases and suffices as diagnostic criterion of cancer, although this criterion cannot be used for leukemia and mesenchymal tumors. With the development of inbred mouse strains many decades ago, transplantability of tumors from one syngeneic animal to another became (and still is) a diagnostic criterion for the malignant phenotype of experimental tumors.

Cancer Cells

Many lines of evidence show that cancer is not a single disease. However, there are important principles that apply to many, if not all, cancers. There is substantial evidence that cancers in mice and humans are the result of multiple sequential mutations. As a result, certain molecules in cancer cells are mutant, up- or downregulated, or no longer expressed.

In addition, most if not all cancers show epigenetic changes in gene expression. An estimated 15% of the worldwide cancer incidence is attributed to infections,[14,15] but chemical and physical carcinogens are involved in the induction of most human cancers in industrialized countries.[16] Many of these carcinogens are mutagens.[17,18,19] While most mutations in cancer cells seem to be acquired (somatic mutations), increasing numbers of germline mutations are being discovered that make individuals prone to develop cancer.[20] A cancer may require as many as 10 or more mutations to develop full malignancy. The first stage of cancer development is called *tumor initiation*,[21] which is generally assumed to be irreversible due to somatic mutations or germline mutations in various oncogene, tumor suppressor gene, or deoxyribonucleic acid (DNA) repair pathways. Initiated cells do not form tumors. However, initiated cells clonally expand to premalignant lesions evolving over many years, often decades. This second protracted stage is driven by *tumor promotion* (ie, exposure to promoting conditions or chemicals[22,23,24] [see Cancer and Inflammation]). The most advanced stage of these premalignant lesions is referred to as intraepithelial neoplasia also *carcinoma in situ*, often abbreviated as, for example, CIN for cervical intraepithelial neoplasia, VIN for vulvar intraepithelial neoplasia, or PIN for prostatic sites. The premalignant process ends with invasion, the appearance of the first cancer cells. Remarkably, however, there is now clear molecular evidence for premalignant cells spreading to distant sites where these cells remain premalignant unless promoted to become malignant.[25,26,27]

A cancer cell that survives treatment may cause recurrence of the entire malignancy. Such cells are referred to as *cancer stem cells* or also tumor-propagating cells.[28,29,30] The elimination of these cancer stem cells is critical to prevent *relapse*. The sweeping assertion that these cells represent an extremely small percentage of the cancer cells in a tumor is not supported by rigorous experimentation or clinical experience. Some of the misconception comes from the observed rarity of only a few human tumor cells able to adapt to growth in a foreign (mouse) milieu.[30] Furthermore, flow cytometric analysis of cancer cells may detect a rare population that results from intraclonal and nonheritable heterogeneity in cancer cell populations.[31,32]

Tumor progression originally meant the change from a benign neoplasm to cancer[33,34] but is now usually used to describe the third phase of the multistep process. Invasive growth of a lesion usually ends with a highly aggressive, widely metastatic cancer that ultimately kills the host.[35,36] There is compelling evidence that most cancers are clonal in origin and that in cancer progression, new subpopulations of cells arise continuously due to Darwinian selection of genetic variants that have a growth advantage, escape homeostatic controls, or resist destruction by defense mechanisms or treatment.[37] During this evolution, sequential mutations result in changes in rate of growth, morphology, hormone dependence, enzyme and cytokine production, and expression of surface antigens. Importantly, by the time cancer is first detected in a patient, it measures usually at least 1 cm in average diameter, contains ~10^9 cancer cells, and has already undergone about 30 generations.[13] Thus, most of the diversity of a cancer has already occurred at time of detection with only 10 generations left before death of the individual unless treatment intervenes.

The term *primary* indicates the tumor from which cancer cells emigrate to *secondary* sites (ie, metastatic growth in tumor-draining [sentinel] lymph nodes or more distant organs). Experimentalists use the term *spontaneous metastases* to describe metastases that occur without experimental manipulations; *artificial metastases* are caused by cancer cells injected into systemic or portal veins of a tumorfree mouse to cause lung or liver metastases, respectively. Cancer cells can disseminate without further cell division (*microdissemination*) or they divide only minimally causing *micrometastases*, conditions that can only be recognized by microscopy and immunohistochemistry but are potential sources of relapse, the central problem of cancer therapy. Similarly, residual microscopic foci of cancer cells may remain at sites of incompletely excised cancer and cause *local recurrence*. Efforts are ongoing to develop sensitive markers and assays for determining the need for additional

therapy or determining the effectiveness of a therapy before relapse is detected clinically.

Cancer Stroma

Definition

Most of the cells in tumors may not be cancer cells but nonmalignant cells, referred to as *stromal cells*. Some of the most aggressive cancers, such as pancreatic cancer, mostly consist of nonmalignant stromal cells.[38] Virchow[39] believed that compression of the growing cancer cells induced a structural fibroblastic framework (now generally referred to as "stroma") in which the cancer cells grew. He thought that cancer cells and stroma both developed from the same primitive precursors. This concept changed with Ehrlich stating clearly that the host provided the stroma of solid tumors.[40] Borst[41,42] was the first to clearly point out the essential mutual relationship between cancer cells and tumor stroma by stating that the question of whether the epithelium or the connective tissue has the leading role in carcinogenesis was difficult to answer, because stroma of tumors is dependent on the presence of cancer cells, and cancer cells are dependent on stromal cells. Thus, cancer cells release factors that attract stromal precursor cells, and stromal cells in turn produce factors that support cancer cell growth.[43,44,45] Interestingly, Rous[46] emphasized the importance of vascularizing stroma for successful tumor transplantation and that immune reaction to nontumor cells led to rejection of the inoculum. Rous, however, was working with noninbred animals. It therefore remained unclear from his experiments whether an immune reaction just to stroma sufficed to cause tumor rejection until 1992[47] when similar experiments done in inbred mice showed that immune reaction to the stroma of transplanted tumor fragments led to the eradication of the inocula.

Components

Willis, in a careful survey of his own studies and published literature,[48] subdivided tumor stroma into just two major components: connective tissue, which usually represents the bulk of stroma, and vasculature, which is usually a smaller fraction. However, we should distinguish at least four critical components: 1) fibroblasts, 2) vasculature, 3) extracellular matrix (ECM), and 4) cells of myeloid and lymphoid lineage, such as macrophages, neutrophils, natural killer (NK) cells, and T and B cells, all derived from hematopoietic stem cells.

Fibroblasts are a prominent cell type in tumor stroma as well as in healing wounds and embryonic connective tissues. Stromal fibroblasts in cancers are metabolically active making matrix substances; the degree of activation of stromal fibroblasts correlates with aggressiveness of the cancer and inversely with survival of patients.[49,50] Unfortunately, we still lack reliable fibroblast-specific immunologic markers for these cells despite repeated assertions to the contrary.[51,52,53,54,55,56,57,58,59,60,61] Therefore, fibroblasts are still mostly defined by morphology and function.[62,63,64,65,66] Characteristically, they synthesize, secrete, and modulate proteins of the fibrous ECM, particularly alpha-collagen.[62,63,67] Macrophages are an essential and prominent part of stroma of every tumor. They lack Gr-1, express low levels of MHC class II, and express F4/80. These macrophages are "alternatively" activated (ie, typically have a tumor-promoting immunosuppressive M2 differentiation phenotype, particularly in hypoxic areas of the tumor[68]). Neutrophil granulocytes, also referred to as neutrophils or polymorphonuclear leukocytes, are an equally pivotal component of tumor stroma. Like the tumor-associated

macrophages, neutrophils in cancers are "alternatively" activated (ie, "N2"[69]). Unlike classically activated neutrophils, N2 do not cause tissue damage. These neutrophils express cluster of differentiation (CD)11b, Ly6-C, high levels of Gr-1 (defined by monoclonal antibody RB6-8C5[70]), and high levels of Ly6-G (recognized by the monoclonal antibody 1A8).[71] RB6-8C5 (anti-Gr-1) recognizes primarily Ly6-G[71] but apparently also binds weakly to Ly6-C,[71] although this is controversial.[72] In any case, RB6-8C5 (anti-Gr-1) not only eliminates

neutrophilic and eosinophilic granulocytes effectively (though only transiently),[43,44,45,70,73,74] but also markedly decreases cells of the subset of blood monocytes that express high levels of Ly6-C but lack Ly6-G.[74] Because of their suppressive effects on cultured T cells,[75] these Gr1+ CD11b+ leukocytes are also referred to as myeloid-derived suppressor cells (MDSCs).[76,77,78,79] MDSCs are increased in the circulation of tumor-bearing individuals but also found in the stroma of autochthonous[80] and transplanted tumors.[68]

Sources

It is presently unknown what percentage of bone marrow-derived stromal fibroblasts originates from hematopoietic versus mesenchymal lineage, what percentage of stromal fibroblasts is derived from circulation rather than local adjacent sources, and whether bone marrow-derived fibroblasts are functionally different.[81] As suggested over a century ago, some cells in the stroma come from progenitor cells entering the site of tumor growth either via the blood circulation or from adjacent normal tissues.[81,82,83,84,85,86,87,88,89,90,91,92,93,94,95,96,97,98,99,100,101,102] The relative contribution of the two sources (circulation or adjacent tissues) is still hotly debated,[103,104] but it is questionable whether circulating endothelial precursors contribute significantly to tumor vasculature in well-established tumors. Similarly, it needs to be determined whether local tissue reservoirs are a major source of alternatively activated M2 macrophages in autochthonous or transplanted cancers (> 2 weeks after transplantation) as it appears to be the case in other pathologic conditions of "type 2 inflammation."[105,106] Type 1 inflammation in tumors is an artifact clearly visible during the first 2 weeks after tumor transplantation.[68,107] An alternative major source of macrophages in tumor stroma may be monocytes or MDSCs from blood circulation.[68]

Function

Lack of tumor stroma drastically reduces tumorigenicity.[47,108,109] All cancers depend on stromal support and establish some type of paracrine loop.[43,44,45,52,110,111,112,113] Signals initiating the loop seem to be intrinsic to the cancer cells (ie, endogenous) and depend on oncogenic mutations in the cancer cells[114,115,116,117] (see Cancer and Inflammation). The proinflammatory mediators attract mesenchymal, endothelial, myeloid, and lymphoid progenitors to the stroma from adjacent and systemic reservoirs; the mediators also induce these cells to make factors that stimulate the growth of the cancer cells.[43,45,118,119,120,121,122,123,124,125] Cytokines released by transfected cancer cells can have powerful local effects on all components of tumor stroma including fibroblasts.[73,126,127,128,129,130,131,132,133,134,135,136,137,138,139,140,141,142,143]

Angiogenesis is a fundamental necessity for tumors to grow by allowing oxygenation and nutrients to diffuse from the blood into the mass. Myeloid cells, including mast cells, neutrophils, eosinophils, Gr-1+CD11b+MDSC/monocytes, and tumor-associated macrophages, can promote tumor angiogenesis.[144] However, cancer cells and tumor-associated fibroblasts can produce proangiogenic as well as growth stimulatory factors such as vascular endothelial growth factor (VEGF) and Bv8.[145] Several studies have shown the importance of neutrophils and granulocyte-colony-stimulating factor (G-CSF) production by cancer cells in causing refractoriness to anti-VEGF therapy.[43,44,45,146,147,148,149] Metalloprotease released from neutrophils and from Gr-1+CD11b+MDSC/monocytes catalyzes the release of preexistent VEGF and transforming growth factor (TGF)-β from the ECM and activates latent TGF-β.[150,151,152,153] Neutrophils are essential for mobilizing various types of stromal progenitor cells including the macrophages from bone marrow and other reservoirs in the body.[154] Finally, neutrophils and Gr-1+CD11b+ monocytes in the tumor stroma themselves can produce large amounts of TGF-β1.[69,80]

ECM is an essential stromal component providing the cancer cells not only with a scaffold for adherence and structure but also with growth and antiapoptotic signals, thereby preventing anoikis.[155,156,157,158,159,160] TGF-β1 stimulates fibroblasts to produce ECM proteins, including collagen, fibronectin, and proteoglycans, and TGF-β1 prevents ECM degradation.[161] Accordingly, transfecting cancer cells to produce TGF-β1 makes them more aggressive.[128] ECM is also a major reservoir for binding and releasing growth factors, chemokines, and cytokines.[162,163] Cancer cells may release the ECM proteoglycan versican that helps attract and activate myeloid cells via toll-like receptors (TLRs) to release interleukin (IL)-6.[164] At later stages, neoplasms often replace paracrine with autocrine loops, notably IL-6 activating signal transducer and activator of transcription (STAT)3.[123,165,166] However, all evidence suggests that even the most aggressive cancers still depend on some factors and ligands provided by tumor stroma.

Knowing how essential stroma is for cancers to grow, it is somewhat surprising that cancer cells would not generate their own stroma. Indeed, epithelial cancer cells can form a "pseudo-stroma" by assuming a mesenchymal phenotype at the invading edges of the cancer.[167,168] However, there is no evidence that this so-called epithelial-to-mesenchymal transition can replace the host-derived stroma (ie, the need of cancer cells to establish paracrine stimulatory loops with nonmalignant stroma[169]). Fusion of cancer cells with stromal cells, particularly macrophages, has been proposed as a major mechanism of cancer development and progression.[170,171,172,173,174,175] However, we still lack conclusive experimental evidence supporting this attractive hypothesis formulated over a century ago.[176,177]

Reaction to Cancer Cell Inoculation

Careful studies showed decades ago that the many cancer cells that die on inoculation play a critical role in the establishment of the cancer. It was found that adding lethally irradiated cancer cells to an inoculum of viable cancer cells at a 100:1 or larger ratio can increase the take of a cancer cell inoculum by more than a 100-fold.[178] The dead cancer

cells have potent thromboplastic activity,[179] and death of the cancer cells is required for this activity to form fibrin at the site of inoculation.[178] In addition, hypoxia induces VEGF-A and CXCL12 (stroma-derived factor [SDF]-1) in many types of cells, but many cancer cells produce VEGF even in normoxic conditions.[180] VEGF-A is a most potent inducer of vascular leakage of plasma proteins, including fibrinogen, that rapidly form a fibrin-fibronectin clot as a provisional tumor stroma.[181] Fibrin deposited at the site of inoculation serves as primitive ECM for cancer cells to escape anoikis. The ECM then undergoes major remodeling during the first 2 weeks after inoculation. Remodeling the ECM microenvironment requires the activity of ECM-degrading enzymes such as matrix metalloproteinases.[182] Thereby, these transplanted tumors acquire later the harder consistency typical for autochthonous tumors that evolve with stroma.

EXPERIMENTAL CANCER

Key Principles

Number of Cancer Cells Targeted

A critical determinant for any cancer therapy is the number of the targeted cancer cells that have proliferative potential ("cancer stem cells"). That number determines the likelihood of recurrence/relapse after most cancer cells have been destroyed by treatment. Even microscopic parts of a cancer left behind by a surgeon often lead to recurrence. Nevertheless, the size of tumor-stem cell population is not the only factor determining the likelihood of relapse as other cancer cells, even when dead or lethally irradiated, can increase the tumorigenicity of the remaining cancer cells by orders of magnitude.[178]

Most human tumors are not detected until they are 0.5 to 1 cm in diameter, have a volume of ~500 mm^3, and contain ~10^9 cancer cells.[13] In leukemia, malignant cells generally do not form tumors, but the patient has also ~10^9 cancer cells when the disease is clinically detected. It is not appropriate to adjust for the difference in host size when comparing cancer in man and mice. Not the size of the species but the size of the cancer cell population determines the chance of relapse because the latter correlates with the number of therapy-resistant variants causing relapse after therapy. Skipper, who pioneered combination chemotherapy of childhood leukemia in the mouse model of L1210 leukemia, targeted 10^9 cancer stem cells as the starting population. This was one major reason why the principles he established in an animal model were clinically relevant and led to the cure of most childhood leukemias. Cellular heterogeneity within a tumor becomes much more relevant when the tumor accumulates 1 billion (10^9) cancer cells, equivalent to a tumor with a diameter of 1 cm.[183,184,185] At this point, the accumulation of so many cancer cell variants makes it improbable that all cancer cells are susceptible to a single chemotherapeutic agent or specific T cell. Cancer cell variants can be considered analogous to drug-resistant bacteria or viruses (ie, the nature of the problem is fundamentally the same in both cases). Unfortunately, in most experimental studies on immunotherapy, almost fivefold smaller populations of cancer cells are being treated[4] (Fig. 47.1).

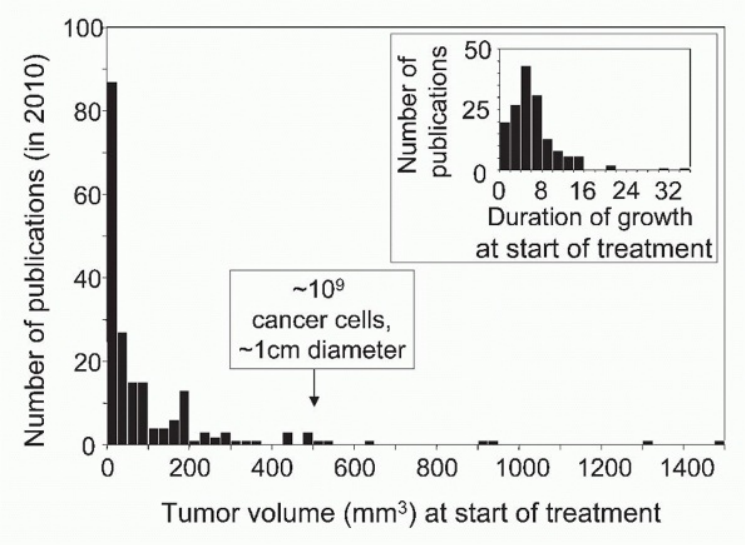

FIG. 47.1. Precipitous Drop in the Number of Publications Reporting the Effects of Experimental Cancer Immunotherapy with Increasing Size or Duration of Growth of Tumors Treated by Immunotherapy. A search of PubMed for year 2010 publications using the keywords "immunotherapy" AND "cancer" recovered 195 experimental studies listing tumor volume or size and 158 studies listing duration of growth at start of treatment. Seventy-five percent of tumors treated in these studies were smaller than 121 mm^3. Only 9 of the 158 tumors had grown for 2 weeks or longer. Modified from Wen et al.[4]

Duration of Growth

Duration of growth of a cancer greatly influences experimental results. Summarizing three decades of studies on immunity to cancer, Woglom concluded in 1929 that immunotherapy is futile against an established tumor, and "nothing may accordingly be hoped for at present in respect to a successful therapy from this direction."[186] Eighty-two years later, the outlook is not quite so grim, but unfortunately, the vast majority of research in animal models is still concentrating at treating malignant cell populations grown for an average of only 5 days after

cancer cell inoculation[4] (see Fig. 47.1). The word “established” is not a scientific term, yet is frequently used to describe neoplastic lesions caused by recently implanted cancer cells,[186] conveying the message that the malignancy being treated does not differ from what would be found in a human cancer patient. Just the opposite is true; transplanted tumors must grow for at least 2 weeks before they are histologically indistinguishable from autochthonous murine or human cancers.[107] Most human cancers have resided in the patient for months if not years before being detected and treated, whether primary, metastatic, dormant, or relapsing. An additional problem of experimental models is that growth of many serially transplanted “standard” cancer lines is so rapid that death may occur so early that treatment has to be started before solid tumors have established a microenvironment even vaguely comparable to that of an autochthonous tumor.

Measuring Growth and Destruction

Tumors are masses and have weight and volumes best approximated by the formula of an ellipsoid (V = πwh/6 or V = 0.5236 lwh or V-½ lwh); length 1, width w, and height h are the orthogonal diameters in the three perpendicular

axes. As was carefully documented, the third dimension, height (or depth), has an inordinately large effect on errors in volume[187] but can usually be determined accurately for subcutaneous tumors.[187,188,189] Describing tumors as areas is incorrect and illogical despite widely practiced misuse. By definition, areas have no volume nor contain a single cancer cell. Area measurements as commonly used by radiologists frequently disagree with volumetric measurements.[187,188,190] Depth of a tumor can greatly vary and determines malignancy and prognosis (eg, in melanoma).

There are important variables when extrapolating the number of cancer cells from a given tumor volume; for example, the human or murine adenocarcinoma of the pancreas usually consists predominantly (˜90%) of nonmalignant stromal cells yet is highly aggressive, whereas in most tumors stromal and cancer cells are more balanced in numbers. Furthermore, while it takes weeks for a tumor to disappear completely even after all cancer cells have been eradicated, it is usually impossible to determine from volume measurements how many viable cancer cells are left. Certain cancers release hormones that have systemic effects such as insulin; even microscopic growth can have major systemic effects.[191] While many experimental models utilize subcutaneous tumors, some cancers, particularly human tumor xenografts, may require transplantation to sites where it is more difficult to assess growth objectively.[192] Cancer cells have been transduced with luciferase to give a signal proportional to the tumor size when substrate is injected; this allows whole body scans with a luminometer, though sensitivity decreases for fewer than 1,000 cancer cells, and there is absorption by overlying tissues.[193]

Cure and Dormancy

The effects of the immune system on cancer can be read out in different ways. Certainly, *cure* of the cancer is the objective. There should be clear prolongation of survival and absence of relapse, but proving that all cancer cells have been eradicated is often difficult. Unfortunately, for most cancers, we lack assays sensitive enough to detect and quantify remaining *dormant* cancer cells. This information would indicate whether or not a patient requires further treatment. Only in a few types of cancers[194,195,196] does a negative diagnostic polymerase chain reaction analysis of the tissue reservoir from where a cancer may relapse suggest —though not prove— complete eradication. Experimental evidence for dormant cancer cells may come from provoking relapse by treating the animal with antibodies neutralizing factors or cells suspected of causing tumor dormancy. Experimentation should include waiting for minimally 30 days after the tumor has disappeared completely (it is best to wait several months or longer). A common unacceptable practice is using the word eradication or cure when there is no follow-up after the cancer becomes undetectable. *Relapse* of cancers may occur within days, weeks, months, or even years after complete disappearance, and is one of

the most important problems of cancer therapy. Nevertheless, experimentalists commonly describe treatments as effective even when followed by rapid relapse.[197] *Eradication* means tearing a tree out with its roots so it cannot regrow, and the term is synonymous with cure. Thus, using the term eradication is only appropriate if the host does not harbor dormant cancer cells, comparable to "sterilizing immunity" in infections.[198,199,200,201]

Inhibition, Arrest, Regression, and Equilibrium

Dependent on the extent and type of destruction as well as when it occurred, tumors may be found to have smaller or larger volumes after treatment compared to controls. It is important to determine whether the rate of tumor growth has been altered or the rate of growth remained unaltered but the onset of growth has changed. Altered rates of growth require an ongoing process, whereas altered onset of outgrowth is usually caused by an event that happened at times of inoculation. Slower growth rates (growth *inhibition*) should be distinguished from shrinkage (*regression*) of a cancer. A steady size of a treated tumor compared to controls is referred to as growth *arrest*, *equilibrium*, or *progression-free survival* in cancer patients, an important goal when cure cannot be achieved.[169,202]

Specificity Controls

It is completely inappropriate yet quite customary to study immunologic therapies specific for a self-antigen on a human cancer in mouse models (human tumor xenograft) when the murine host does not express the same target. These irrelevant models often yield impressive results and mislead the reader that the targeted molecule is tumor-specific when in the real situation it is not.[203,204] If someone subscribes to the widespread highly questionable perception that many, or even most, useful tumor antigens are self-antigens, then this investigator should also use appropriate experimentation. Either a mouse model must be used in which normal tissues express the target molecules closely resembling expression patterns in man or there should be a clear warning to the reader. For example, anti-carcinoembryonic antigen (CEA) immune responses caused toxicity in mice that expressed the target antigen also in normal tissues, as humans do,[205] predicting the severe toxicity later observed in a clinical study.[206] Side effects often become apparent only when the treatment is intense enough to provide clinical efficacy.[207] It is therefore questionable to dismiss results in proper animal models.[208,209]

Selection of Tumor Model

General Considerations

Experimentalists have to be able to translate their findings to clinicians and vice versa. There is no single human cancer, let alone single animal model, that can serve as appropriate model for all human cancers.[28] Organ site as well as histologic type of a cancer may greatly influence the results. Thus the complexity of cancer makes it extremely important that the experimental model used to study cancer immunity be relevant to the question asked; a single model, if carefully chosen, may be appropriate to answer a specific question. Nevertheless, it is essential that we uncover the broader principles underlying cancer-host interactions. For example, the extensive clinical and experimental research on immunotherapy of melanoma has failed to answer the

central question: why are we struggling to make similar advances in the immunotherapy of more common cancers such as breast, colon, prostate, and lung cancers? Weiss argued in 1980 that the failures of clinical immunotherapy were due to using irrelevant laboratory tumor models for extrapolating results for clinical application.[210] One of the major reasons for translating results from animal models to clinical cancer immunotherapy being frustratingly evasive is the disregard of using truly established tumors for experimental therapy (see Fig. 47.1).[4] Current experimentation in cancer immunology mostly uses young hosts to study the effects of the immune system on small, recently implanted inocula of cancer cells derived from tumors once induced in inbred mice (with homozygous genetic loci) and then passed in

vivo for decades from animal to animal, whereas human cancers are already well established when first detected, have a very high probability of heterozygous loci, have never been transplanted, and develop in mostly older individuals.

Modes of Induction

Spontaneous. The term *spontaneous cancer* is defined as cancers arising "in the absence of any experimental manipulation."[211] Spontaneous murine cancers develop without any known exposure to carcinogens or genetic (often transgenic) manipulations introducing oncogenes or eliminating tumor suppressor genes. For example, spontaneous tumors can be observed in many mouse strains with advancing age. All genes have a spontaneous rate of mutation, and genetic instability, though often a consequence, may not be a requirement for tumor development.[212,213] As would be expected from the sporadic occurrence of spontaneous mutations, the occurrence of spontaneous tumors is random and unpredictable.

Carcinogen-Induced (Physical, Chemical, or Viral) . Many of the physical and chemical carcinogens involved in the induction of cancers are mutagens.[16,17] Since repeated application of coal tar was used to induce the first chemically induced cancers,[214] a very large number of chemicals such as polycyclic hydrocarbons and nitroso compounds has been identified with remarkably potent cancer-inducing activity.[215] Cancers have been induced in many tissues and organs and several animal species. Ultraviolet light (UV) has potent skin cancer-inducing activity in man and mice,[216,217] and many well-defined models of UV-induced tumors are now available.[218,219] The potential of ionizing radiation to cause cancer in humans was recognized soon after Roentgen's discovery of x-rays in 1895.[220,221] But study of radiation carcinogenesis in animals mostly occurred after World War II when large-scale tumor-induction studies were carried out in many species over the succeeding three decades in response to the threat of irradiation from nuclear reactors or bombs.[221,222,223] Finally, many models of viral cancer induction have been developed after Rous[224] showed that viruses can also induce cancer in animals.

***Transgene-Induced*.** Experimental cancers are produced artificially by inserting oncogenes into the germline of mice or by manipulating the mouse genome to allow excision of tumor suppressor genes. Genes used for these manipulations are driven by tissue- or cell lineage-specific promoters that are either constitutively active or inducible locally or systemically. While these mice become prone to develop cancer, the tumors they develop are autochthonous but by no means "spontaneous" despite widespread misuse of terminology.

Autochthonous Tumors

Autochthonous tumors originate in the place where they were found (autochthonous, in Greek, means indigenous). The antonym is transplanted tumors. Autochthonous tumors can be spontaneous, carcinogen-induced, or transgeneinduced. Even when induced in the same experiment by the same mode of induction, autochthonous tumors will differ genetically, biologically, and antigenically from one another, because additional but individually differing genetic changes are required for each tumor to develop. Thus autochthonous tumors lack the uniformity of well-defined transplantable models. An advantage of autochthonous over transplanted tumors, however, is that the host's immune system has been neither artificially primed nor altered by an inoculum.

Unlike autochthonous tumors developing after exposure to physical, chemical, or viral carcinogens, transgeneinduced autochthonous tumors have the disadvantage that transgene expression in the thymus during development usually causes systemic tolerance to the transgenic proteins. Nevertheless, several excellent transgenic cancer models have been developed.[225,226] Some models are based on immunologic findings first made in patients.[12] For example, the R24C mutation in the cyclin-dependent kinase 4, first identified as tumor-specific antigen by T cells of a melanoma patient, causes familial melanoma when in the human germline.[227] Introducing this mutation in the germline makes mice highly susceptible

to develop melanoma[225]; tumor development, however, requires additional carcinogenic insults followed by prolonged chemical promotion.

Certain oncogenes such as SV40T are powerful because they inactivate several important suppressor pathways and may therefore require fewer additional mutations. In some transgenic cancer models, the transforming genetic event can be controlled, sometimes reversibly, by topical or systemic application of an inducer such as tamoxifen or tetracycline.[193] Alternatively, systemic or topical application of Cre-recombinase may excise a "floxed" blocking element or attenuator of gene expression.[228] These temporal controls appear to be helpful for answering important questions on tolerance, because expression at birth causes neonatal tolerance to a highly antigenic oncogene such as SV40 T antigen. An approach that more closely mimics the sporadic nature of human cancer relies on a spontaneous mutational event activating an introduced floxed oncogene. The sporadic nature of this event unfortunately also makes time and site of tumor development less predictable.[228]

Transplanted Tumors

Most current experimental work in tumor immunology uses transplantable tumors. However, there are substantial differences among transplantable tumors, and it is

important that the chosen cancer is appropriate for the particular question being asked. It is important to know that 24 hours after inoculation, the majority of the injected cancer cells usually have died,[178] leaving only a shallow outer rim of viable cancer cells at the oxygenated margin.[107] Histology or in vivo imaging reveals pronounced edema at the injection site. In fact, much of the "tumor growth" early after inoculation is due to tissue swelling caused by the inflammatory reaction. Cancer cells may well be invading surrounding normal tissues and be vascularized within 48 to 72 hours after transfer, thereby meeting standard criteria for malignancy. But experienced pathologists will immediately spot the abnormal inflammatory reaction to the transplantation injury and the necrotic center of the early inoculum. Thus, common terms such as "three-day established cancer" or "three-day established metastases" are highly misleading. Such necrosis would not be found in an autochthonous cancer of similar size. The inflammatory reaction progressively decreases with time, and at 14 days, the necrotic material has usually disappeared. The tumor then contains ~10^9 cancer cells, measures ~1 cm diameter, and is histologically indistinguishable from primary autochthonous tumors. Ideally, therapy uses tumors 2 weeks or more after inoculation (see Fig. 47.1).

Even though the terms *tumor transplantation* and *tumor injection* are old and commonly used, they are quite misleading, as they usually refer to inoculation of tumor fragments or a suspension of cancer cells. A common error is the belief that tumors can be injected or "transplanted" like a vascularized organ such as kidney or heart. Instead, much of the inoculum—whether a cancer cell suspension or fragments of a tumor—dies initially and needs about 2 weeks before it becomes histologically indistinguishable for autochthonous tumors.

Tumors can be induced in animals by injecting *cancer cell suspensions* prepared from cultures or by mincing tumors into 1-mm^3 pieces and injecting the *tumor fragments* using a 12-gauge trocar. Cancer cells in fragments are 10- to 100-fold more tumorigenic than stroma-free cancer cell suspensions.[47,108] It had long been known that certain cancers would only grow in mice when transplanted as tumor fragments. This was erroneously thought to be due to more cancer cells being inoculated with fragments.[229] Later analyses revealed that fragments contained fewer cancer cells than injected cell suspensions, yet produced a higher take or larger tumors earlier.[47,108] Another erroneous explanation was that the stroma of tumor fragments provided a physical barrier preventing cancer cells from migrating to draining lymph nodes and priming a protective T-cell response.[108,230] More likely, more cancer cells remain viable when embedded in tumor stroma (by preventing anoikis[160]) and therefore

release less antigen than suspended cancer cells, most of which die. In any case, as long as cancer cells express sufficient levels of antigen, professional antigen-presenting cells in the tumor stroma pick up the antigen and travel to the draining lymph nodes where they present the antigen to naïve T cells.[231]

It is important to know that whether suspensions of cancer cells or tumor fragments are being used, a threshold number of cells or fragments must be inoculated for tumors to develop. The threshold in T cell-deficient mice may be due to innate immunity or nonimmunologic mechanisms.[178,232] The increase in threshold in immunocompetent mice is probably due to adaptive immunity.

***Cell Lines from Tumors Serially Passed in Mice*.** When choosing tumors that have been serially passed in animals for transplantation, it is critical to be aware of how such tumors were altered by the serial transplantation. As was discovered decades ago, even a single in vivo passage of a cancer can select for heritable cancer variants.[45,219,233,234,235,236,237] Even half a century ago, investigators already noted that serial transplantations of these cancers "inevitably result in progression toward more rapid growth rate, loss of functional and histological differentiation, loss of responsiveness to extraneous stimuli"[238(p.522)] and a diminution of strain specificity, a problem shared by B16.[239] These serially transplanted cancers, such as B16, can easily be transferred in mice using cancer cell suspensions rather than tumor fragments. As a result of hundreds of passages in mice,[239] these cancer cells have become resistant to anoikis caused by lack of stroma by acquiring alternate signaling pathways that replace the prosurvival signals of ECM.[240] Many of these tumors grow and kill so fast that they must be treated early before they are truly established. Certainly, many of these tumors no longer resemble primary mouse or human tumors that virtually always grow at much slower rates and have been established for months or years before being treated.

B16 melanoma was derived in C57BL/6J mice in 1954 and is the parent of many available B16 sublines.[239] B16 had been transplanted serially through 328 mice for 13 years before it became available as a standard cancer cell line. Nevertheless, B16 still is arguably the "*Escherichia coli*" of tumor immunology with almost 1,000 entries in PubMed in 2011. The A/J-derived Sa1 originated in 1947 in an A albino mouse and was serially transplanted in A/J mice for 1,017 generations for 19 years. Similarly, the A/J-derived neuroblastoma 1300 derived in 1940 was serially transplanted for decades from mouse to mouse. The Lewis lung carcinoma, isolated by Lewis,[241] arose spontaneously in 1951 at the Wistar Institute in a black C57 mouse (not a C57BL/6 mouse), was serially transplanted extensively, and is still being used in numerous studies as a model for exploring the immune responses to lung cancers and their metastases. When genetically inbred mouse strains became available as sources for murine cancers over half a century ago, serial transplantation was necessary for maintaining a tumor. Dependable long-term cryopreservation did not become available until the late 1960s and early 1970s.[242,243,244] Even after dependable cryopreservation became available, investigators still continued to use serial transplantations to propagate newly derived cancers such as the BALB/c-derived CT26 colon cancer,[245] the C57BL/6-derived MC38 colon cancer,[246] and the BALB/cCr-derived RENCA renal cancer.[247] Many cell lines are renamed sublines of old parental cell lines. Thus, it is often overlooked that RMA, RMA-S, MBL-2, and EL-4 tumors are derived from the same single tumor line,[248] most likely

E.L.4 induced by 9:10-dimethylbenzanthracene in 1945 by Gorer[249] and serially transplanted for many decades from mouse to mouse. Importantly, referring to EL-4 as a C57BL/6 tumor is incorrect as this strain did not exist in 1945. Similarly, Neuro2a is a subline derived in 1969 from the A/J-derived neuroblastoma 1300 derived in 1940. Unfortunately, organizations providing these lines and/or publications often fail to cite the fact of long-term in vivo passage before cryopreservation.

Cell Lines from Autochthonous Mouse Tumors. Autochthonous cancers never need to be serially transplanted. Standard lines can be created by adapting the autochthonous cancer cells to culture and/or by freezing fragments or cells in liquid nitrogen.[218] To prove that antigens expressed by the malignant cancer are truly tumor-specific, nonmalignant cells, tissue and DNA from the host of tumor origin must be available.[218,219] This approach is commonly used by researchers studying the antigenicity and genetics of human cancers. Carefully controlled experimental cancers exist.[218,219]

Human Tumor Xenografts. For certain purposes, human tumor xenografts are useful models. Human cancer cell lines grown in vitro can cause tumors when injected into T-, B-, and/or NK-deficient mice. It is important to remember that the stroma of such cancers is entirely of mouse origin and the cancer cell-stromal loops are defective because of multiple mismatches in ligand-receptor signaling.[250] This problem can be partially alleviated by "humanizing" the recipient mouse. Humanizing usually refers to expressing certain human molecules such as receptors/cytokines in mice that lack murine T, B, and/or NK cells, and/or transferring human mesenchymal and/or hematopoietic cells to such mice. Interestingly, human T- and B-lymphocytes and fibroblasts contained in human tumor fragments may coengraft thereby "humanizing" the mouse[192] (eg, when injecting cell clusters or aggregates from human fresh ovarian cancers into nonobese diabetic-severe combined immunodeficiency IL2γR null mice). Progressive growth of these xenografts leads with great regularity to ascites formation, and pleural metastasis closely simulating classical tumor progression observed in patients with ovarian cancer.[192] Despite continuous improvement, mice can never be completely humanized. Only very few cancer cells of the xenografts may be able to progress in the chimeric milieu.[30] Nevertheless, once a tumor grows, its sensitivity to potential therapeutic agents might reveal the sensitivity of the original cancer growing in the patient.

Selection of Recipient/Host

The vast majority of human cancers develop in later midlife and old age,[251,252,253,254] and there is clear evidence that, at comparable ages, mice have difficulties rejecting immunogenic cancers.[255,256,257,258] Yet most experimental studies use young mice. Mice should have a clinically relevant tumor burden or be selected for treatment when the bulk of cancer cells has been removed to undetectable levels by surgery or chemotherapy, but dormant cancer cells stay behind to cause later relapse. T cell-deficient mice have been used extensively as models for adoptive transfer of T cells.[259] However, cancer patients are usually T cell competent and capable of generating regulatory T (T_{reg}) cells that may not allow an effective "take" of the transferred T cells unless the recipient is lymphodepleted.

CANCER ANTIGENS

No term in cancer immunology is more important and confusing than the term cancer antigen (or tumor antigen). Any molecule detected with T cells or antibody on the surface or within cancer cells is commonly referred to as cancer antigen. The usefulness of a cancer antigen for detection and destruction depends on its specificity (ie, that the antigen is not common to normal cells). The first well-defined tumor antigens encoded by the MHC were discovered by Gorer.[8] Immunity to MHC antigens will kill the cancer cells but also the host because of the ubiquitous expression of MHC on normal cells.

General Aspects

Early History

In the late 1800s,[260,261] it was discovered that in some instances tumors developing spontaneously in experimental animals could be transplanted into other animals of the same species and in this way could be propagated continuously. This finding provided an important experimental tool for cancer research.[40,262] Immediately, scientists began to investigate the

possibility of immunizing against such transplantable cancers. Rodents exposed to a small nonlethal challenge of certain tumors became immune to subsequent challenge with large transplants of the same tumor that regularly killed nonimmunized recipients. Also, complete removal of the transplanted tumors, after initial growth, immunized animals against that tumor. These early results seemed to suggest that immunization against cancer was possible. Furthermore, there were certain other spontaneous tumors that were not readily transplantable, and this was taken as evidence for "natural resistance" or "natural immunity" to the cancers. Many years later, it became clear that no such conclusions could be drawn from these early studies, because outbred, or incompletely inbred, rats or mice had been used. The problem became apparent when it was realized that the immunization with tumor would also immunize the host against normal tissue of the donor and that normal tissues of the donor could also immunize the host against the tumor.[186] These experiments brought the idea of tumorspecific antigens into disrepute but also started the search for antigens that caused rejection of normal transplanted tissue. This research eventually led to the discovery of the MHC and to the development of inbred mouse strains.[8,263,264] Once inbred mouse strains became available, it was found that cancers transplanted within an inbred mouse strain usually grew so well that the existence of tumor-specific antigens seemed very unlikely. In fact, transplantability of tumors in syngeneic animals became (and still is) a diagnostic criterion for the malignant phenotype of an experimental tumor. This criterion was especially useful because many rodent

tumors are cancers of nonepithelial origin (sarcomas) and also because a clear histologic demonstration of local invasive growth can be especially difficult in such cancers.

Proof of Specificity

After discovery of the MHC and the development of inbred mouse strains, the gloom over tumor immunology ended with the discovery that inbred mice could be immunized against syngeneic sarcomas induced by the chemical carcinogen methylcholanthrene (MCA). The first demonstration of induced immunity to a transplantable MCA-induced sarcoma was by Gross in 1943[265]; however, it was not until the 1950s that more complete experiments provided unequivocal evidence for "tumor-specific" rejection of transplanted cancers.[229,266,267,268,269,270] In particular, the experiments of Prehn and Main[268] in 1957 made it likely that the rejection antigens on the MCA-induced sarcomas were functionally tumor-specific, because transplantation assays could not detect these antigens in normal tissues of the mice used. However, irrefutable evidence for the existence of tumorspecific antigens in autochthonous unmanipulated cancers only came after a 50-year-long search[3,5,218,219,236,267,268,269,270,271,272,273,274,275,276,277,278,279,280,281,282] when in 1995, it was proven that tumor-specific antigens on cancer cells were encoded by somatic cancer-specific mutations absent in the normal cells of the host of tumor origin.[10,11,12]

Even though it is clear that cancer-specific antigens are encoded by somatic cancer-specific mutations, the terms "tumor-specific" or "cancer-specific" are frequently used inappropriately, sometimes even used in conjunction as "relatively" tumor-specific. The discussion is far from being semantic. In reality, an antigen either is or is not tumor-specific. Germline controls are absolutely critical for proving mutations are somatic and tumor-specific.[283,284,285,286] Unfortunately, germline controls are missing from virtually all tumors used for experimental work today. Nevertheless, mouse tumors with proper autologous controls have also been used and are available for distribution.[218,219] Notorious problems are genetic polymorphisms. For example, even after 20 backcross generations when a strain is arbitrarily pronounced "inbred" (because it is then more than 99.9% genetically identical), about 373 polymorphic proteinencoding loci remain allogeneic.[287] Any one of these loci could encode a pseudotumor-specific antigen when a tumor is transplanted into a mouse misperceived to be fully syngeneic. Mice respond preferentially to nonself- or mutant-self-antigens whether

caused by genetic polymorphism or tumor-specific somatic mutation.

Peptide Antigens and Major Histocompatibility Complex Affinity

T cell-mediated destruction of cancer cells requires the interaction of T-cell receptor (TCR), peptide, and MHC molecules. In this "three body problem," two affinities simultaneously determine the interaction[288,289]: the peptide to the MHC and that of the TCR to the peptide-MHC complex. Even when the complex cell-cell interaction is reduced to the three molecules interacting in vitro, biochemical analysis is still too complex for analyzing physiologic interactions. TCR affinity to peptide-MHC is therefore usually measured by plasmon resonance in the presence of saturating nonphysiologic amounts of the peptide, and TCR affinities measured this way range between (K_d) 1 to 100 μM.[288,289] This is a narrow range considering that affinities of peptides to the MHC range from 1 to more than 20,000 nM.[290] This difference points at the peptide-MHC affinity probably being the greatest variable and emphasizes the paramount importance of choosing target peptides with highest possible affinity to the presenting MHC. Proper selection is particularly important as the amount of peptide produced by the cancer cell may be relatively small and always must compete for binding with all other peptides naturally present in the cancer or in the cells cross-presenting the antigen. Affinity of a peptide to a given MHC molecule is best measured empirically and many empirical affinities are already available (eg, http://tools.immuneepitope.org or www.syfpeithi.de).[290,291,292] However, even when a peptide binds with high affinity to MHC, it will only be expressed on the cell surface when it is naturally processed and present in sufficient amount.[293,294]

Antigens Revealed by "Reverse Immunology"

"Reverse immunology" is the attempt to predict T-cell epitopes within a given amino acid sequence. Traditionally, "reverse immunology" has focused on finding optimal T-cell antigens, properly referred to as peptide epitopes, on infectious agents for generating vaccines. More recently, self- or mutant proteins recognized by T cells or antibodies from cancer patients have been analyzed in the search for peptide epitopes that may be effective targets of T-cell immunity. Computerized algorithms (eg, http://tools.immuneepitope.org[295,296] and www.syfpeithi.de[297]) have been developed to predict the affinity of peptide-MHC binding, appropriate proteasomal cleavage, and transport by the transporter associated with antigen processing (TAP).[290,291,292,297] Though these algorithms are useful, it appears that these tools cannot replace empirical biochemical measurements. Many of the antigens proposed by reverse immunology have affinities to MHC insufficient to serve as effective targets when tested in appropriate animal models. Together, high peptide-MHC affinity is essential but not sufficient to predict that a given peptide serves as an efficient target.

Complete sequencings of cancer cell genomes from individual patients is becoming increasingly affordable and has revealed up to many thousands of mutations per cancer cell.[298,299,300] However < 1% of these mutations cause amino acid substitutions. For example, of the 33,345 nucleic acid base substitutions found in a human melanoma, most of the mutations were intergenic, intronic, noncoding, silent, or truncating. Only 187 caused amino acid substitutions in coding genes,[300] and only a few of these 187 coding substitutions are expected to lead to mutant peptides that serve as effective targets. To be effective, the mutant peptide 1) must bind with a high affinity (IC_{50} in the nM range) to the particular MHC molecules of that individual and 2) be naturally processed, 3) escape destruction by proteasome cleavage, and 4) be present in sufficient amounts. Only a few of the myriad of tumor-specific mutations identified in human or murine cancers give rise to antigens that fulfill these requirements. However, the frequency of unique tumor-specific targets in

a cancer cell may be larger than predicted when considering only mutations in coding sequences, because mutations in intron sequences can be translated and also encode tumorspecific antigens.[11] These mutations are over 50-fold more frequent than those in

protein-encoding loci.

Antigens Recognized by Patients' T cells

In the search for the best antigens to eradicate the cancer, T cells infiltrating tumors (TILs) have been recovered, expanded, and used therapeutically. However, TILs are removed from cancers that have not been destroyed. It is, therefore, a widespread misperception that the TILs were necessarily fighting cancer growth. Three questions become obvious: 1) Are strong antigens retained because the host has been tolerized during very early stage of cancer growth? 2) Can T cells specific for the strongest antigens be recovered from the TILs? 3) Can T cells specific for weak antigens promote rather than inhibit tumor growth?

It is possible that 1) potent rejection antigens are retained because they tolerize the host very early during cancer development,[228,301] 2) a few T cells have not been tolerized and can be propagated for therapy, and 3) tolerization is reversible, and competent effector cells can be obtained from TILs. Clinically, the patients who fare best have T cells in their peripheral blood that are specific for antigens encoded by somatic tumor-specific mutations.[12,302,303] Whether the same T cells were infiltrating these cancers as TILs is unknown. In any case, it is likely that only a few (if any) of an endless array of antigens recognized on cancer cells by antibodies or tumor-infiltrating T cells may have significance as targets or diagnostic markers.

Cancer-Specific Antigens (Encoded by Mutant Genes)

Prevalence

All cancers in man and mouse that have been analyzed carefully express bona fide tumor-specific antigens that could be targeted by T cells. Because tumor-specific antigens arise from mutations,[10,11,12] they are usually unique. Each patient's cancer seems to have a unique set of mutations, and unique antigens can provoke powerful immune responses. In mice, immunization with one tumor protects only against the same tumor (Fig. 47.2).[5,274] Shared cancer-specific antigens do exist when the same mutations occur in several cancers, but by comparison, such antigens are relatively rare. Most cancer-specific mutations affect intracellular proteins that may be recognized by T cells as mutant peptide-MHC complex on the surface of viable cancer cells. Very few cancer-specific mutations affect surface proteins, such as the mutant epidermal growth factor receptor (EGFR), a shared tumor-specific antigen on glioma cells.[304,305]

Oncogenicity

A major misconception is that unique cancer-specific antigens are caused by random mutations and are incidental to the oncogenic process. For example, a group of experts

stated in 2009[306(p.5326)] that unique antigens come “from random mutations presumed to be present in all patients,” implying mutations encoding unique tumor antigens have no functional significance in the malignant process. Nothing is further from the truth. Of course, all mutations caused by chemical and physical carcinogens are random, but virtually all of them are lost during cancer evolution except those that promote the neoplastic process. When it was first shown in 1995 that cancers harbor cancer-specific antigens caused by somatic tumor-specific mutations,[10,11,12] every one of the three antigens represented a mutation in a tumor suppressor locus.[307,308,309,310,311] For example, the mutation in the cycline-dependent kinase 4 reduces the binding to its inhibitor and tumor-suppressor protein p16INK4a,[12] and the same mutation is found in the germline in cases of familial melanoma.[227] The fact that selection for a tumorspecific antigen commonly represents a mutation in genes with functional significance in the malignancy of that cancer (oncogenes or tumor suppressor genes) has been shown in many other studies.[219,312,313,314,315] Remarkably, T cells against unique tumor antigens identify mostly novel, functionally important tumor-specific mutations and oncogenic proteins that would not have been easily

detected by other technologies.[10,11,12,219,307,308,309,316] Importantly, several of these unique tumor-specific antigens are excellent targets because they cannot be lost by immune selection. The reason is that some of these mutant proteins are not only oncogenic but are also needed to provide an essential household function no longer provided by the second allele due to Knudson-type loss[317] or mutational inactivation.[302,307,308,309]

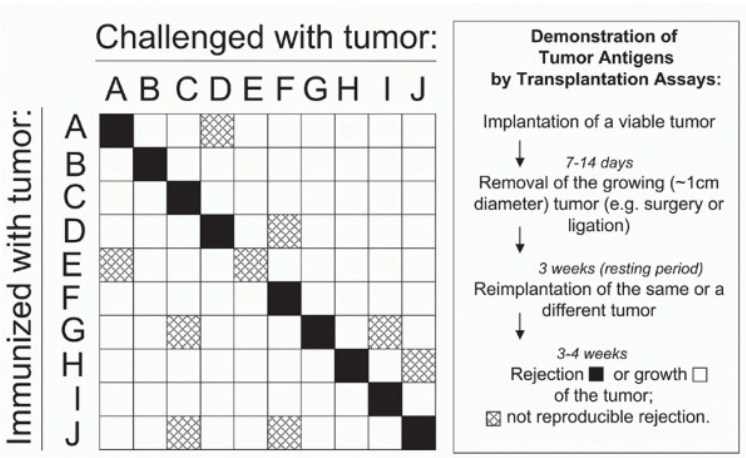

FIG. 47.2. Demonstration of the Individual (Unique) Specificity of Rejection Antigens on Independently Derived Methylcholanthrene-Induced Tumors in Transplantation Experiments. For details, see Basombrio.[3] Modified from Basombrio and Prehn.[5]

Therapeutic Significance

Another misperception surrounding unique tumor-specific antigens is that they have remained unexploited clinically, because truly personalized therapy would be required. Again, nothing is further from the truth. One of the most effective immunologic treatments of cancer today is the adoptive cell transfer of autologous tumor-infiltrating lymphocytes from patients with metastatic melanoma: response rates are in the range of 50% to 70% of the patients, and a few of these patients are cured.[318] This truly personalized therapy involves reinfusion of the patients' own lymphocytes isolated from the patients' own melanomas and expanded in vitro. Because the TILs response is dominated by T cells to unique tumor-specific antigens,[303] it is likely that the success of the reinfused T cells depends on their reactivity to unique tumor-specific antigens. Similarly, clinical studies show that therapeutic vaccinations with autologous cancer cells are likely to be much more effective,[319,320] confirming decades of experimental work.[229,234,268,269,271,321,322] Self-antigens may serve as useful and effective targets in a very few instances (eg, CD20 and CD19), but industry supported by government panels and organizations focuses almost exclusively on self-antigens,[306,323,324] so far with very modest results.[325] Finally, it is a misperception that immunizing each patient to the unique antigen of the individual's tumor is impractical because of cost and should not be pursued. This is incorrect compared with other highly individualized treatments such as those used in renal transplantation. Thus, individualizing cancer therapy could be affordable as other strategies used in the clinics.

Shared Tumor-Specific Antigens

Ideally, antigens targeted on cancers would be expressed exclusively on malignant cells but be shared by cancers of the same type or at least subtype. Several cancer-specific mutations have been identified that are shared between cancers,[9,304,326,327] but very few of them have so far been found to be an effective immunologic target. The main reason probably is that, aside from specificity, additional requirements for effective recognition by T cells are 1)

high-affinity binding to the patients' MHC molecules and 2) expression of the protein at sufficient amounts to compete with other peptide for presentation. Unique tumor-specific antigens must fulfill the same requirements but, because these unique mutations are more abundant, chances are much higher to yield an effective antigen. A few examples of shared tumor-specific antigens are discussed in the following.

Mutant Epidermal Growth Factor Receptor **.** See further under Immunotherapy.

Fusion Proteins. Fusion proteins found in cancer cells are the result of internal deletions (see mutant receptor EGFRvIII under Immunotherapy) or chromosomal translocations.[9,326] New antigenic determinants can result from the juxtaposition of previously distant amino acid sequences, resulting in a mutant peptide sequence at the breakpoint and possibly a change in conformational structure. The same chromosomal breakpoints consistently recur in different individuals with the same cancer, therefore result in shared tumor-specific antigens. Fusion proteins encoded by these translocations are usually essential for cancer maintenance, making them ideal targets for pharmacologic and immunologic intervention because tumor cells may not easily escape therapy by losing expression of the fusion proteins.[328] Pharmacologic approaches are exemplified by the drug imatinib targeted to the fusion protein of the 9;22 translocation in chronic myelogenous leukemia.[9,294,329] The antikinase drug imatinib does not selectively inhibit the catalytic activity of the BCRABL fusion protein, and this results in toxicity to normal cells in the patient. The intracellular BCR/ABL fusion proteins can be recognized specifically by antibody but only in fixed cells.[330] BCR/ABL fusion peptides can also be recognized by human CD4+ T cells in the context of MHC class II[331] and serve as targets for CD8+ human cytolytic T cells.[332] Why do we then still lack effective immunologic therapies for targeting this and other fusion proteins? As explained previously, the predicted epitopes may 1) not be generated naturally by cells,[293] 2) lack sufficient affinity to the particular MHC of that patient, or 3) not be produced in sufficient amounts.

Mutant RAS**.** Point mutations in oncogenes can also be shared by several cancers and could encode useful antigens. For example, a valine for glycine substitution at position 12 of RAS is one of the most common mutations in human cancers and can be recognized by human CD4+ T cells.[333] The region of the mutant RAS protein from which

the peptide was derived is identical for all the three members of the *RAS* proto-oncogene family, namely, H-RAS, K-RAS, and N-RAS, which have different prevalence in different cancers. In addition, about 90% of pancreatic adenocarcinomas, a very aggressive human cancer, have one of three to four different single amino acid substitutions in codon 12 of the cellular Kirsten *RAS* gene.[334] Despite remarkable clinical and experimental efforts,[335,336,337,338,339,340] targeting mutant RAS-derived epitopes has so far met with little if any success, the likely reasons being the same as those mentioned under the section titled Fusion Proteins. Immunizing cancer-prone mice harboring “initiated” mutant ras-expressing cells with mutant ras peptide resulted in mutant ras-specific CD4+ T cells, antibodies to the mutant ras protein, and more tumors and faster tumor development.[341] Possibly, the antibody, produced in response to antigen, activated myeloid cells via FcR-γ to become tumor-promoting, and/or antibody carrying TGF-β downregulated CD8+ effector responses.[342,343]

Self-Antigens (Encoded by Normal Genes)

All antigens listed in this section are encoded by nonmutant cellular genes and expressed not only by cancer but also by at least some normal adult cells. Therefore, these antigens are not tumor-specific and are commonly referred to as *tumor-associated* antigens. The level of expression of these antigens can vary from widespread expression to restriction to a small population or a subset of normal cells. However, self-antigens should not be called “quasi-tumor-specific” because even very low levels and/or selective expression of a self-antigen may cause severe even lethal toxicity when targeted.[204,206]

Self-antigens expressed by tumor cells are used for destruction, inhibition, or detection of cancerous growth. When used as targets for destruction, T cells or antibodies must eliminate the cancer cells while not destroying normal cells expressing the same self-antigen, or destruction of selfantigen -expressing cells must be tolerated. Thus, no serious toxicity must occur even when the immunity is strong enough to destroy the cancer cells.[195,196] Antibodies against growth factors or their receptors can inhibit growth of cancer cells without destroying them. When self-antigens are used for diagnosis, background levels of antigen generated by normal cells complicate use of these antigens for early detection of cancer. However, changes in the amount of circulating self-antigens may indicate relapse of cancer after therapy.

All normal individuals have so-called natural autoantibodies as well as T cells to a wide spectrum of self-antigens without causing pathology, but those B-cell receptors and TCRs have usually very low affinity. In fact, any immune receptor binds with some affinity to any particular antigen[344] and immune receptors may bind to several molecularly unrelated structures,[345] making the discussion of specificity seemingly useless. However, the antibody response of an individual to self- and nonself-antigens differs in affinity by many orders of magnitude, and therapeutically effective antiself-antibodies or TCRs are commonly raised in a nonself, usually xenogeneic, setting. Whether removing natural mechanisms that prevent autoimmunity is a general approach to achieve truly effective antitumor immunity needs to be substantiated.

The problem of B-cell unresponsiveness can be overcome by immunizing mice with xenogeneic human antigen. Misleadingly, these high-affinity antixenogeneic antibodies are usually advertised as “fully human” when they were made in mice in which the murine immunoglobulin (Ig) gene locus had been inactivated and replaced with the human Ig gene locus.[346] The toxicities of such “fully human” antibody will still be those of high-affinity antiself antibody: severe to lethal toxicity. These reactions may occur when the variable region of such antibodies (eg, anti-CEA or anti-human epidermal growth factor receptor [HER]-2[204,206]) is fused with the transmembrane and signaling domains and then transduced as chimeric antibody receptors (CARs) into T cells.[347]

T-cell tolerance to self can be overcome by making T cells in a host that is allogeneic to the MHC class I molecule presenting the targeted peptide.[348] However, recent studies exposed that such allo-human leukocyte antigen (HLA)-restricted high-affinity T cells can have severe “off-target” reactivity likely to cause toxicity when used in patients.[349] T cells transduced with these TCRs kill each other (fratricide) when the self-antigen (eg, wild-type p53 or survivin) is expressed by the T cells.[350,351] High-affinity TCR cells can also be generated in knockout mice lacking the targeted selfpeptide (eg, wild-type p53 peptide sequences[352]), but T cells expressing these TCRs are lethal when given to normal mice because wild-type p53 is expressed in bone marrow and can also be expressed by most other normal cells.[350]

Overexpressed Molecules

***Growth Factors and Their Receptors*.** See Immunotherapy.

Survivin. Although survivin is overexpressed in many cancer cells, it is a problematic antigen particularly as a vaccine because it is widely expressed on lymphocytes causing fratricide.[351] Not surprisingly, this antigen has been ineffective in vaccine trials.

p53. Research using p53 as target for immunotherapy is exemplary of the problems and persistent misconceptions of research on tumor-specific (unique or shared) and selfantigens. Mutations in the p53 suppressor gene are among the most common found in human and experimental cancers.[353,354] These mutations tend to cluster in evolutionarily conserved regions of the gene, but the exact locations are highly diverse in individual cancers.[355] Targeting these mutations would require an individualized therapy considered by many impractical. Therefore, researchers have attempted to exploit the fact that mutant p53 is

usually overexpressed in cancers. Thus, major efforts have been made to target conserved, nonmutated regions of the p53 protein hoping for “relative tumor specificity” based on the usual belittlement for low-level expression of this protein by every normal cell including lymphocytes. As a result, T cells expressing highaffinity anti-p53 TCRs commit fratricide unless the anti-p53 TCRs are transduced into T cells from p53 knockout mice.

However adoptive transfer of these T cells causes lethal hematopoietic ablation in normal mice.[350] Mortality can be prevented by reconstitution of the recipients with bone marrow from p53 knockout mice,[350] an option unavailable in humans. Not surprisingly, there is no evidence for clinical efficacy of targeting wild-type p53 in humans.

***Prostate-Specific Membrane Antigen*.** Prostate-specific membrane antigen (PSMA) is a type II transmembrane glycoprotein more correctly referred to as glutamate carboxypeptidase II. PSMA is an inappropriate misnomer because it is anything but specific for prostate; it is also expressed by duodenal mucosal cells, proximal renal tubule cells, and a subpopulation of neuroendocrine cells in colonic crypts,[356] and has important functions in the brain.[357] Vaccination of mice with human PSMA induces antitumor effects without causing toxicity but because the mouse model lacks expression of human PSMA on normal tissues, the findings in this inappropriate model are meaningless.[358] Similarly, a “fully humanized” anti-PSMA antibody had potent antitumor xenograft activity in mice that lacked expression of this antigen on normal cells.[359] Therefore, this antibody may have severe, possibly lethal, toxicity in patients.

Differentiation/Lineage-Specific Molecules

Some antigens expressed on tumor cells are also expressed during at least some stage of differentiation on nonmalignant cells of the lineage from which the tumor developed. Differentiation antigens may therefore help to determine the organ or cell type of origin (lineage) of a cancer.[360] For example, B-cell tumors express surface Ig, and T-cell leukemias can be separated into helper and suppressor cell leukemias using T-cell subset-specific monoclonal antibodies to surface and intracellular antigens including transcription factors. For metastatic cancer of unknown origin, differentiation antigens can be important indicators of the histologic and organ site of the primary tumor.[361] Careful diagnostic delineation of different subtypes of cancer is important because different tumor subtypes may have different prognoses and may be susceptible to different therapies. However, the use of differentiation markers for histologic or cytologic tumor classification has pitfalls.[361] Lineage-specific antigens represent a very diverse group of proteins: glycoproteins (including mucins) and glycolipids (carbohydrate, peptide, glycopeptide, or glycolipid epitopes). Several of these antigens are being explored as potential immunotherapeutic targets. However, the normal cell types that express these antigens will determine toxicity and usefulness of any antibody or agent for which these are targets.

Cluster of Differentiation 20 and Cluster of Differentiation 19 **.** CD20 is a signature B-cell differentiation antigen targeted by a genetically engineered monoclonal antibody that is relatively effective in the treatment of B-cell lymphoma. Lineage restriction of this marker limits the cytocidal effects to long-term depletion of normal B cells. This depletion is usually well tolerated because patients can be protected by intravenous administration of IgG. While antibodies to CD20 antibodies are therapeutically effective, relapse is common. CD19, but not CD20, appears to be expressed on the more immature malignant cells causing the relapse. Therefore, anti-CD19 Fv has been used in the killing of these cancer cells, either as a fusion protein with an anti-CD3 Fv to engage T cells[195] or as chimeric antibody receptor inserted into the patient's T cells.[196] These treatments may prevent relapse.

***Melanocyte-Specific Differentiation Antigens*.** Several differentiation antigens (such as tyrosinase, the related brown locus protein or tyrosinase-related protein 1 (Trp-1), gp100, and Melan A/MART-1[362,363]) appear to be restricted to melanocytes, and all of them are being explored as immunotherapeutic targets in melanoma.[364,365,366] Immune recognition of the

melanocyte differentiation antigens can lead to rejection of a tumor challenge but this self-antigen-specific immunotherapy not only targets the tumor cells but also normal cells expressing the shared antigens,[365,367] resulting in the depigmentation of normal skin (vitiligo) and possibly other, more serious toxicities (see following discussion). For example, mice immunized with syngeneic Trp-1 in various adjuvant settings fail to produce a T- or B-cell response.[368] Xenogeneic or altered Trp-1, however, induces responses that cause vitiligo and protect against challenge with melanoma cells.[368] Passive transfer of Trp-1-specific antibodies from these mice causes vitiligo and protects against metastatic spread of melanoma cells in mice when given at the time of seeding of the malignant cells.[367] Similar antibodies may have analogous beneficial effects in human melanoma patients,[369] but evidence is lacking for such antibodies eliminating bulky human melanoma. Trp-1-deficient mice respond to Trp-1 as a foreign antigen and Trp-1-specific CD4+ T cells from these mice can eradicate large B16 melanoma 10 to 14 days after inoculation,[370,371] cause autoimmune vitiligo, and damage the retina.[370] Designating these T cells as “tumor-specific” when they are clearly not is incorrect and misleading.[370] Trp-1 is expressed in all neurocrest-derived pigmented cells, not only those of the skin but also those of the eye (uvea), inner ear, and brain (substantia nigra, forebrain, and midbrain).[372,373,374,375] Other targets include gangliosides GD2 and GD3 that are also not only overexpressed in melanoma but are also found in other cells of neurocrest origin and in other tissues.[376,377]

***Prostate-Specific Antigen*.** Prostate-specific antigen (PSA), also called kallikrein-3, is a chymotrypsin-like protease that digests semenogelin I and II to release motile sperm.[378] Elevation of PSA above the normal range occurs in inflammation (prostatitis) and benign hypertrophy of the prostate as well as in prostate cancer. Using PSA levels for early detection of prostate cancer is controversial. The U.S. Preventive Services Task Force no longer recommends this test in healthy man.[379] However, detection of any PSA following complete surgical removal of the prostate indicates residual tumor cells and/or recurrence.[380] Prostate-specific phosphatase is selectively expressed and secreted by the epithelial cells of the prostate gland.[378,381] Sipuleucel-T, a vaccine targeting prostate-specific phosphatase

and approved by the U.S. Food and Drug Administration for the treatment of castration-resistant prostate cancer, results in only a very modest improvement in overall survival.[323] Furthermore, a recent analysis of internal documents that became available after the U.S. Food and Drug Administration approval questions the adequacy of the trial and correctness of the conclusions drawn.[382]

***Epithelial Cell Adhesion Molecule*.** Normal and malignant cells of epithelial origin express the transmembrane glycoprotein epithelial cell adhesion molecule (Epcam).[383] Anti-Epcam antibody 17-1A[384] showed promising results in patients with colon cancer in several early studies,[385] whereas subsequent randomized clinical trials consistently failed to show any benefit.[386] A high-affinity engineered anti-Epcam antibody caused cases of acute pancreatitis and was discontinued.[387] Other anti-Epcam antibodies in the form of bi- and trifunctional constructs may be more effective, but finding efficacy while keeping lethal toxicity under control presents a major hurdle.

Tumor Antigens Caused by Altered Glycosylation

Aberrant glycosylation and the overexpression of certain carbohydrate moieties is a consistent feature of cancers,[388,389] and tumor-associated oligosaccharides are actively investigated as targets for immunotherapy.

***Mucins*.** Mucins are normally heavily glycosylated glycoproteins (ie, containing complex O-glycans) that protect the luminal mucous epithelial surfaces. Mucins of cancer cells often show decreased expression of the complex O-glycans and increased expression of short

oligosaccharides, the TF, sialyl-Tn, and Tn antigens[390] (see following discussion). Thus, human adenocarcinomas of the pancreas, breast, and colon express mucins[391] that can be recognized on cancer cells by MHC-unrestricted cytolytic T cells. These T cells apparently react specifically with repeated epitopes on the protein core of the mucin molecules,[392] exposed because of deficient glycosylation in the malignant cells.[393] The epitopes are expressed at high density, do not require processing, have a stable conformation, and can directly bind to certain TCRs without being presented by MHC molecules.[394] Hypoglycosylated MUC1 is expressed on about two-thirds of newly diagnosed cancers. However, multiple types of vaccines using the nonglycosylated tandem repeat peptides or tumor-associated saccharide antigens conjugated to carriers failed to immunize effectively.[395,396] This finding is consistent with multiple lines of evidence that humans and MUC1-transgenic mice are tolerant to the unglycosylated long MUC1 peptide. Numerous approaches have been developed capable of overcoming this tolerance such as using short and long synthetic glycopeptides or plant-expressed MUC1.[397,398,399,400] Many different mucin genes have been identified[401] and antigens have been defined, but evidence for clinical efficacy with controllable toxicity is lacking.

***T and Tn Antigens*.** Tn antigen on human erythocytes, first described by Moreau et al. in 1957,[402] is the cause of a hemolytic autoimmune disorder, Tn syndrome.[403] Tn ("T antigen nouvelle") is a Ser/Thr-O-linked *N* -acetylgalactosamine monosaccharide distinct from the disaccharide T (or TF) antigen galactose-β1-3-N-acetylgalactose O-linked to a Ser or Thr (Galβ1-3GalNAcα1-Ser/Thr) described earlier by Huebner, Thomsen, and Friedenreich.[404] Human cancers very frequently express TF and Tn antigens.[405,406] Recently it was shown that the Tn syndrome is caused by somatic mutations in the chaperone COSMC.[407] While TF antigen is an oncofetal antigen highly expressed in the embryo and fetus,[390] there is no evidence that Tn is also an oncofetal antigen.[404] Mutational deletion of the chaperone COSMC leads to expression of the Tn antigen on virtually every embryonic cell and leads to early embryonic death.[408] Most adults naturally have anti-Tn as well as anti-TF antibodies due to antigenic stimulation by Tn and TF antigens expressed on the bacterial flora.[409,410] Tn antigen is also expressed on human immunodeficiency virus-1 and pathogenic parasites. Thus, evidence is lacking for any expression of Tn antigen on adult or embryonic human or murine cells except by patients suffering from Tn syndrome and cancers that may or may not show a tumor-specific deletion of COSMC. There is no evidence that Tn is an effective therapeutic target. This may be due to anti-Tn antibodies being usually IgM or IgA of very low affinity, though recently developed IgG antibodies reduced slightly the growth rate of human cancer cells in vitro and in vivo.[411]

***Glycopeptide Antigens Resulting from Tumor-Specific COSMC Mutations*.** The remarkable characteristic of this new class of antigens is the exquisite tumor specificity of the antigen[412,413,414] even though it is encoded by normal genes. Appearance of the antigen, however, depends on a tumor-specific somatic mutation that destroys the chaperone COSMC that is essential for any functioning of the core 1β (1-3) galactosyl-transferase, T-synthase. COSMC protects the newly synthesized T synthase from aggregation and subsequent endoplasmic reticulum-associated degradation.[404] T-synthase is essential for extending O-linked glycosylation beyond a single O-linked *N*-acetylgalactosamine (ie, Tn antigen). Importantly, the antigen recognized by the high-affinity, tumor-specific antibody does not bind Tn alone but contains the Tn hapten (ie, the single O-linked *N*- acetylgalactosamine on a threonine or serine). X-ray crystallography shows that the antibody completely envelops the carbohydrate moiety while interacting with the unique sequence of the peptide moiety in a shallow groove.[413] Because Tn does not exist on normal embryonic or adult human or murine cells, there is no central or peripheral tolerance to these antigens allowing high-affinity destructive immune reactions to occur. This also explains the severity of the autoimmune disease associated with the Tn syndrome caused by somatic (not germline) mutations of the

COSMC gene.[407] Importantly, somatic tumor-specific mutations disabling the X chromosome encoded COSMC gene (for which one copy is naturally silenced) seem to be more common than originally assumed and occur in a wide variety of spontaneous or virally induced human and murine cancers.[415] Nevertheless, it must be shown that high-affinity receptors when used as CARs on T cells or linked to anti-CD3 are effective against tumors but have little if any toxicity to

normal tissues. This may be expected from the fact that the epitopes depend on somatic tumor-specific mutations.

Oncofetal, Carcinoembryonic, and Cancer-Germline Antigens

Over 50 years ago, human cancer cells were found to express antigens that serologically cross-react with normal embryonic tissue.[416] Since then, it has been postulated repeatedly that certain normal genes that are completely silent in all nonmalignant cells may be activated exclusively in malignant cells.[417] Alternatively, it has been postulated that cancer cells may express proteins (or immature forms of a protein) that are only expressed in fetal but not in nonmalignant adult cells.[418] There certainly is profound hope for finding a universal tumor rejection antigen that can be used for vaccination, prevention of cancer, and antitumor therapy. However, repeated claims of selective activation of normal genes or selective expression of immature forms of these proteins in cancer cells leading to tumor-specific antigens have not been substantiated. Expression of the same antigen by at least one normal cell type in the adult was later often discovered.[419,420,421,422]

***Cancer-Germline/Cancer-Testis Antigens*.** Changes in gene methylation lead to the expression of a large number of genes encoding a group of antigens referred to “cancer-testis,” “cancer-germline,” or “cancer-spermatogonal” antigens. Many cancer-testis antigens have been described in humans such as MAGE, BAGE, GAGE, LAGE/NY-ESO-1, SAGE, HAGE, and BORIS.[422,423,424,425,426,427,428,429,430] All cancer-testis antigens are expressed at high levels in spermatocytes in the testis. And most, if not all, are also expressed in the thymus.[430,431,432] Many, but not all, cancer-testis antigens are encoded on the X chromosome.[433] Most of cancer-testis antigens can be recognized by autologous cytotoxic T-lymphocyte (CTLs), but there is no evidence for these antigens being generally effective targets for cancer cell destruction in human.[434,435] P1A was the first cancer-testis antigen to be identified in the mouse mast cell tumor line P815.[436,437] P1A can be induced in a broad range of tumors of diverse histologic origins with the demethylating agent 5-aza-2′-deoxycytidine.[438] Various normal tissues including thymus and premeiotic spermatocytes also express P1A.[437,439] While active immunization has only modest protective effects against cancer cell inoculation,[440] adoptively transferred T cells (monoclonal or polyclonal) targeting only the P1A antigen shrink large tumors (>1 cm in diameter) for several weeks followed by relapse caused by epitope-loss variants.[441] For eradication, cancer cells must first be transfected to express costimulatory molecules, which probably leads to the induction of T cells to recognize other tumor antigens.[442] Remarkably, autoimmunity has not been reported to be associated with a response to this antigen. In humans, the HLA-A2-restricted NY-ESO1 peptide has very poor affinity to its presenting MHC class I molecule, but high-affinity TCRs to this antigen have been generated and their efficacy in therapy is being explored.[443] While many cancer-testis antigens have been described and studied extensively for two decades, their usefulness as targets eradicating human cancers still remains to be shown. Despite these uncertainties, occasional successes have been observed. The hypothetical explanation: the relatively few T cells induced against the cancer-testis antigen served to elicit additional antitumor T-cell clones directed against other antigens that are actually responsible for the tumor regression by a poorly understood phenomenon referred to as epitope spreading.[434,444]

Numerous cancer-testis antigens have been detected using sera of cancer patients for

identifying antigens by recombinant expression cloning (SEREX). Importantly, sera from mice infected with cytopathic or noncytopathic viruses or injected with tumor cell lysates also show an autoantibody response of broad specificity, and intriguingly the majority of the identified autoantigens have been previously described as autoantigens in humans.[445,446] This suggests that human SEREX antigens may have to be regarded as afterglows of infection-associated immunopathology and/or tissue damage. This is consistent with the assumption that the human adult IgG autoantibody repertoire is the result of lifelong encounters with bacterial and viral agents and tissue damage. Together, usefulness of the SEREX-defined selfantigens remains to be demonstrated.

***Carcinoembryonic Antigen*.** CEA is a 200-kDa membraneassociated glycoprotein that is expressed not only in fetal but also in adult nonmalignant tissues such as normal colonic mucosa, lung, and lactating breast tissue.[447,448] It is released into surrounding fluids. At one time, it was hoped that CEA could be used as a marker for early diagnosis of gastrointestinal and other malignancies[449]; however, elevated serum levels of CEA are also found in the absence of malignancy (eg, in smokers and in inflammatory bowel diseases such as ulcerative colitis). Though serum levels of CEA are not useful for detecting early cancer, the level of CEA in the blood can be used to monitor the effects of therapy to indicate whether a cancer has been successfully eradicated or has recurred.[450] Using CEA as immunotherapeutic target is highly problematic because of severe toxicity. Mice expressing the target antigen in normal tissues closely resembling expression in humans showed severe toxicity from anti-CEA T-cell responses,[205] a result that predicted the severe adverse effects later observed in patients treated with anti-CEA CARs.[206]

***Alpha-Fetoprotein*.** Alpha-fetoprotein (AFP) was the first defined oncofetal protein.[451] Though produced by fetal liver and yolk sac cells, it is also present in small amounts in cells and the serum of normal adults. The amount of this protein is elevated in some patients with cancer of the liver or testis and also in some patients with various nonmalignant liver diseases. Therefore, similar to CEA, using AFP as a marker for the early diagnosis of cancer is of questionable use.[452] Nevertheless, assays of AFP can detect primary liver cancer at a time when the cancer is treatable, and AFP assays are also used for monitoring patients after therapy.[452] The use of AFP as target for active or passive immunotherapy is complicated by lack of specificity.[417,453]

Clonal Antigens

Clonal antigens are expressed only on the clone of cells from which the cancer originated.[454] Except for idiotypes of surface Ig-positive B-cell and T-cell malignancies, there

are presently no candidates for other clonal antigens. Immunization of animals against the idiotype induces an idiotype-specific transplantation immunity against the growth of cancers (myeloma, lymphoma, and leukemia) expressing the idiotype.[455,456] Furthermore, idiotype-specific antibodies prevented the growth of surface idiotype-positive murine malignant B cells in mice and guinea pigs in vivo and in vitro,[457,458,459,460] and occasionally induced the cancers to go into a long-lasting dormant state.[461] Finally, idiotypespecific monoclonal antibodies have caused several partial remissions and one complete remission in patients with B-cell lymphoma,[462] and targeting the idiotype on B-cell malignancies continues to be explored.[463,464,465] It is necessary to generate different monoclonal antibodies or idiotypic vaccines for each individual cancer to be treated, but the advantage of using such clonal antigens over less restricted tumor-associated antigens is that eliminating the few normal cells bearing the same antigen would probably not adversely affect the patient.

Viral Antigens (Encoded by Viral Genes)

Cancer-causing tumor viruses such as SV40, polyoma viruses, human papillomavirus (HPV), hepatitis B virus, hepatitis C virus, human T-lymphotropic virus 1, Epstein-Barr virus (EBV),

and other herpes viruses and their antigens are discussed in other parts of the chapter. For discussion of a 70-kDa glycoprotein (gp70)[419,420,466,467] and other important antigens[468,469,470] encoded by ribonucleic acid (RNA) tumor viruses (eg, murine leukemia virus, maize streak virus,[471,472,473,474] and mouse mammary tumor virus[475]), see Coffin[476] and Schreiber.[477]

IMMUNOGENICITY OF AUTOCHTHONOUS CANCERS

There is the widely held misconception that human cancers are usually less immunogenic than are murine tumors.[478] However, convincing evidence indicates that many, probably all, human cancers are antigenic. Similarly, even the most antigenic murine UV-induced "regressor" tumors grow progressively and invariably kill the primary host[218,479] (Fig. 47.3). Only transplantation of primary murine tumors into young, syngeneic, immunocompetent, tumor-free recipients reveals their antigenicity and immunogenicity. UV-induced regressors are so immunogenic that they are rejected by naïve immunocompetent hosts. Many experimental cancers require preimmunization to be rejected upon transplantation, and the degree of *immunogenicity* usually refers to the relative strength of antigen-specific protection a tumor can induce against rechallenge with that tumor. Obviously, the resistance of the host to its autochthonous tumor cannot be great at the time it is clinically apparent and progressively growing.[228] Only weak reactions can be detected[321] because resistance of the autologous host depends on vaccinations

with the autochthonous tumor following complete tumor removal.[269] *Antigenicity* refers to the antigens on a cancer cell. However, a cancer may have strong antigens yet fail to induce a response (ie, lack immunogenicity). A cancer may also be antigenic and immunogenic but resistant to destruction by the immune response it induces. Thus immunogenicity, antigenicity, and *immunosensitivity* must be distinguished.

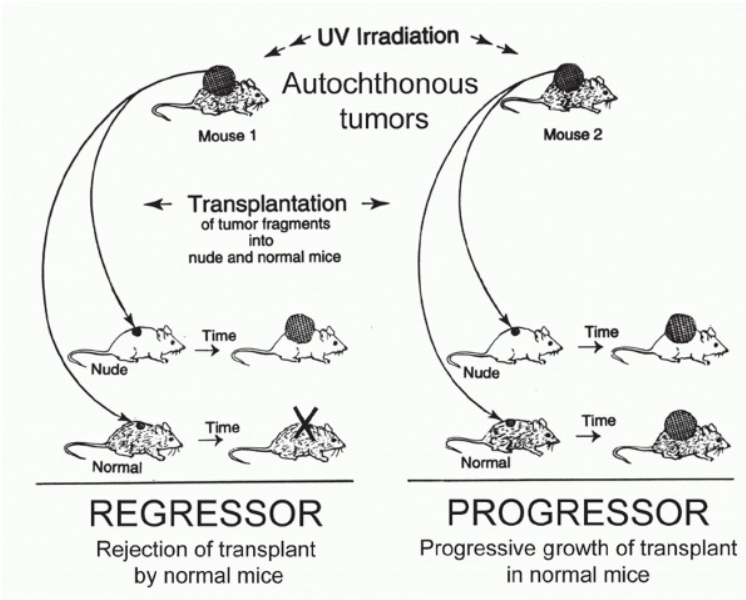

FIG. 47.3. Autochthonous Cancers are Indistinguishable in the Primary Host Regardless of Whether They are Highly Antigenic or Not. Only subsequent transplantation into young syngeneic immunocompetent hosts defines these tumors as regressors or progressors, and shows their antigenicity and immunogenicity.

Progressively growing autochthonous cancers differ greatly in immunogenicity as determined by responses of syngeneic, tumor-free hosts. Autochthonous tumors induced in mice with UV are among the most immunogenic cancers; transplantation resistance to these so-called

regressor tumors appears to be absolute rather than relative. Rejection by normal syngeneic mice is observed without prior immunization, even when the largest testable doses of tumor cells or fragments are used.[275] Most MCA-induced fibrosarcomas, unless induced in immunodeficient mice (see following discussion),[480] display an intermediate degree of immunogenicity in normal mice.[268,269,481] This is shown by the fact that induction of immunologic resistance to most chemically induced tumors requires prior immunization because the initial graft of the tumor generally produces progressive lethal growth.[268] Importantly, failure of a cancer to induce a tumor-destructive immune response does not mean it lacks either antigenicity or immunogenicity. (See below discussion of sporadic SV40-induced tumors under Immune Surveillance of Cancer[228,301].)

Age and Latency

Experimentally, the length of the latency period of a tumor is usually inversely proportional to the dose of carcinogen. In humans, the doses of (ie, the levels of unintentional exposure to) carcinogens and promoters are believed to be usually relatively low. This means usually a long time is needed for the initiated cells to accumulate the multiple genetic events essential for a premalignant or cancerous lesion. Once the neoplastic cells have reached sufficient numbers to stimulate a response, the host may be too old to respond vigorously. The overwhelming majority of human cancers arise in individuals past 60 years of age,[254] and it has been shown repeatedly in humans and mice that the immune response to new antigens declines with age[258,482,483,484]; this should include antigens on the developing tumors. Thus we do not know the extent of which cancers arising in old individuals may have been selected or retained antigenicity and immunogenicity. In stark contrast, most experimental cancers are induced at an age correlating to that of young middle-aged humans. If 2 years of mouse life roughly correlate to 60 years of human life, then most experimental cancers mirror cancers developing in humans 30 years old or less. Tumors induced by MCA or transgenic oncogenes are usually produced in mice less than 1 year old.[485,486,487,488,489] The length of the latency period of these MCA-induced tumors correlates inversely with the degree of immunogenicity.[229,490,491,492] There is no such correlation in UV-induced tumors[275,479] that begin to develop past 9 months of age, mostly in mice more than 1 year old. Old mice beginning at about 9 months of age fail to reject various types of highly antigenic cancer cells that are regularly rejected by young mice.[255,256,257,258] Even though UV irradiation is immunosuppressive, young UV-irradiated mice remain immunocompetent long enough to select for antigen loss variants.[235] This indicates that advanced age must contribute to allowing the developing cancers to remain immunogenic. An intriguing question is how many of the tumors that spontaneously arise in older animals would grow in younger syngeneic hosts.[480] Considerable but only indirect evidence makes it very unlikely that all tumors would grow.

Spontaneous versus Induced

“Spontaneous” cancers that develop without any known exposure to carcinogens tend to be less immunogenic than cancers induced by DNA tumor viruses or by deliberate exposure to carcinogen.[266,267,493,494,495] Unfortunately, serially transplanted tumors were used for most of these comparisons, and transplantation may have selected for less immunogenic variants, thereby confusing the results. If spontaneous murine cancers more closely resembled human cancers, this could suggest that human cancers are poorly immunogenic.[495] However, most human cancers are not “spontaneous” but induced by environmental carcinogens, have never been transplanted, and develop mostly in old individuals that may not select for cancer variants. Most, if not all, carcinogens are mutagens[17] and probably always cause the expression of tumor-specific antigens. However, it is not clear why tumors induced with the same dose of chemical or physical carcinogen may exhibit quite different degrees of immunogenicity.[268,479] One reason might be that the actual

local dose of carcinogen that is delivered to a particular target cell or target tissue may vary greatly from animal to animal. Another reason might be that mutations are selected that favor malignant behavior irrespective of the degree of immunogenicity of that mutant protein. This is consistent with the observation that considerable differences in immunogenicity of primary UV-induced tumors only become apparent *after* transplantation into secondary hosts[218,479] (see Fig. 47.3).

"Regressors" from Immunocompromised Hosts

Immunocompetence of the host may not affect the cancer incidence but still influence the immunogenicity of the developing tumor. Conversely, immunosuppression or immune deficiency of the host during carcinogenesis should allow growth of highly immunogenic tumors in the absence of such selection.

Clinical Evidence

Clinical support comes from the appearance of highly antigenic EBV-associated lymphomas in immunosuppressed transplant recipients that are virtually unknown to occur in immunocompetent humans.[502] In addition, renal transplant patients experience a reduced risk of developing UV-induced skin cancer after immunosuppressive medications are stopped.[503] It is tempting to suggest that many of these

cancers developing in transplant patients are actually "regressor" tumors that have arisen only because host defenses were damaged.

Experimental Evidence

In normal adult mice, Moloney sarcoma virus[496] induces tumors larger than 2 cm in diameter that then regress while tumors continue to grow and kill immunodeficient adult or newborn mice.[497,498,499] The first experimental evidence for UV-induced regressors came from Kripke in 1974,[275] showing that UV irradiation made mice immunodeficient and induced cancers that were often "regressors," meaning that they were rejected with any testable size inoculum by naïve syngeneic immunocompetent mice. Tumor transplants will grow for about a week and then disappear, though small numbers of the same tumor cells will grow and kill athymic nude mice. The same regressors grew after inoculation of few cancer cells in UV-irradiated mice. So, it may be highly relevant that some carcinogens are immunosuppressive.[500,501] Repeated exposures of mice to UV induce persistent immune suppression,[502,503,504] leading to the development of highly immunogenic regressor tumors. In contrast, the single injection of MCA induces tumors in 100% of mice and only a short-lived state of immune suppression,[501] thus allowing a fully immunocompetent host to select for less immunogenic variants. The concept that immunocompetence of a host influences the immunogenicity of the developing tumor has been tested experimentally by Roberts and Daynes[480] comparing the MCA-induced tumors occurring in immunodeficient UV-irradiated mice with cancers induced in normal mice. Indeed, cancers induced with MCA in mice immunosuppressed with UV were frequently regressor tumors, whereas none of tumors induced with MCA in immunocompetent mice were regressors.[480] Decades later, this concept was confirmed with tumors induced with MCA in nude, severe combined immunodeficiency, or Rag-/- mice.[487,505,506]

Selection by the Immunocompetent Host

Immunocompetent hosts can select for cancer variants. Thus, when regressors are transplanted into normal immunocompetent hosts, heritable progressor variants can escape. Some of these variants show antigen loss,[218,219,507,508,509] but many others retain their antigenicity and grow faster than the parental tumor when their growth is compared in T, B, or NK cell-deficient hosts,[43,45,218,307,505,508,510] suggesting that mechanisms other than antigenicity must participate determining the differences in growth behavior between

regressors and progressors.

"Regressors" from Immunocompetent Hosts

Human cancers are sporadic (ie, occur at irregular times and locations); this is in part due to the occasional genetic events that can cause malignant transformation in a single cell that expands to become a detectable tumor. A murine model of sporadic genetic events is the recombinational activation of a normally silenced transgene encoding the strongly oncogenic and strongly antigenic molecule SV40 T, thereby causing sporadic appearance of cancers.[228,301] These tumors are regressors. The tumors "snuck" through immune surveillance and immunoselection despite expressing such a powerful antigen and oncogene, but not unnoticed. The developing cancers did induce antibodies and proliferation of antigen-specific T cells during their early dormant phase of growth, but developing tumor-specific T cells were anergized in that they failed to produce interferon (IFN)γ and kill tumor cells in vivo. In contrast, titers of the T antigen-specific IgG response increased with progressive tumor growth.

EFFECTOR MECHANISMS IN CANCER IMMUNITY

Because cancer is not a single disease, it is not surprising that findings using one tumor model may not apply to other tumor models. Considering the antigenic diversity found in tumors, it is also not surprising that different types of innate and adaptive, humoral and cell-mediated immunity have been shown to play different roles in the destruction or enhancement of malignant cells in one or another of the numerous tumor models.

Assays to Study Effector Mechanisms in Vivo

In principle, five different assays have been used to evaluate the importance of different effector mechanisms in vivo. The first type of assay involves transfer of effector cells, cytokines, or antibodies into sublethally irradiated, cyclophosphamide-pretreated, or normal animals challenged with tumor cells. There are certain limitations of this assay. Effector cells or molecules may not reach or localize in the tumor unless both the effector cells and cancer cells are injected intravenously, and both may be trapped in the lungs. Furthermore, if transferred cells or reagents are effective, the assay does not rule out that other effector mechanisms of the host may have been activated by the procedure. In a second procedure, called the *Winn assay*,[229,269,511] tumor cells are mixed with effector cells or serum in vitro; the mixture is injected subcutaneously into an animal to determine whether tumor growth in vivo is prevented. Tumor cells may be killed within minutes before or shortly after the injection, though the readout takes much longer. Therefore, the Winn assay is, in part, an in vitro cytotoxicity assay, even though the host is used as a readout for viable cancer cells. A third method involves elimination of specific lymphocyte subsets or cytokines in vivo by treatment with antibodies specific for different lymphocyte subsets or cytokines. Failure of the host to resist a tumor challenge indicates that the particular subsets or cytokines are an essential component of the host resistance. A fourth method is to use mice genetically deficient in a certain effector mechanism, cell, or cytokine. An analysis of tumor variants that have escaped tumor destruction by the host provides a fifth way for determining the importance of immunologic effectors in vivo.[43,44,45,218,235,236,507,512] The phenotypic changes observed in these variants may indicate which effector mechanism was responsible for the selection. Therefore, the type of phenotypic change

may give insight into the relative importance of a naturally occurring defense mechanism that may function in immunocompetent mice (analogous to deducing the action of an antibiotic from the type of change found in the bacterium that has become drug resistant). Host-selected variants from experimentally induced regressor tumors are used extensively for this approach.

Antibodies and B Cells

The role of B cells in regulating tumor immunity continues to be studied extensively yet remains poorly understood. In a tumor model of leukemia in which CD4+ helper T cells were

required for successful treatment, B cells were necessary for efficient T-cell priming.[513] Conversely, in other tumor models, elimination of CD4+T cells promoted tumor rejection by CD8+ cells, and an absence of B cells improved CTL responses and tumor rejection.[514,515,516] Antibodies and B cells have been associated with enhanced papilloma formation and autochthonous cancer development.[341,489,517,518,519] The mechanism recently postulated is that regulatory B cells help the macrophage become tumor-promoting,[520,521] or that they suppress surveillance by T cells that normally eliminate neoplastic cells with oncogenic mutations.[519] There are other possible mechanisms.[342,343] Also, none of these experiments identified the antigens involved, except one study showing that induction of mutant-ras specific-antibodies correlates with much more effective papilloma development in an autochthonous tumor model.[341]

Human antisera and monoclonal antibodies reactive with autologous tumors have been isolated.[420,522] However, a strong humoral response to tumor antigens does not correlate with demonstrable resistance of the host to the tumors. TL+ leukemias induce high titers of TL-specific antibodies that are cytotoxic to TL+ leukemia cells in vitro in the presence of heterologous complement,[420] but TL+ leukemias grow equally well in immunized mice having high titers of TL antigen-specific antibody and in nonimmunized mice.[523,524] Similarly, humoral immune response to MCA-induced sarcomas does not provide protective immunity against a tumor transplant.[525] Obviously, the presence of antibodies for these kinds of tumors has no relevance in predicting whether the host will reject the tumor.

Normal or malignant cells of hematopoietic origin are generally lysed quite effectively by antibody and heterologous complement in vitro; however, normal cells such as fibroblasts or malignant cells derived from solid tissues may be much less affected, even when expressing high levels of antigen. The reasons for this striking difference are still unclear. In vitro, some tumor cells are killed by a process involving coating with antibody, opsonization, and subsequent phagocytosis by macrophages; this process may be enhanced by the presence of heterologous complement. Alternatively, antibody-coated tumor cells may be killed in the absence of phagocytosis by antibody-dependent cell-mediated cytotoxicity when cocultured with macrophages, NK cells, or neutrophils. The general relevance of these mechanisms for killing tumor cells in vivo is unclear. Exogenous antibodies that block growth factors and/or their receptors or activate costimulatory molecules are useful for some kinds of immunotherapy (see Immunotherapy).

T-Lymphocytes

It has been demonstrated convincingly that T cell-mediated immunity is critical for rejection of virally[526,527,528] and chemically induced tumors,[229,269,529] or for the rejection of allogeneic[530] and UV-induced tumors.[218,275,503,508] For example, in the model of murine MCA-induced tumors, it was shown that intravenous injection of immune cells, but not of immune serum, could transfer systemic tumor-specific immunity into sublethally x-irradiated mice.[229] These findings were consistent with earlier work with allogeneic tumors showing that lymphocytes not serum were effective in transferring transplant resistance.[531,532,533] In another study, transfer of immunity to a plasma-cell tumor was abolished by pretreatment of the immune cells by anti-T-cell antibodies and complement.[529]

The relative importance of various T-cell subsets in tumor rejection (ie, T_C1 [type I CD8+ cytotoxic T cells]; T_C2 [type II CD8+ cytotoxic T cells]; T_H1 [type I CD4+ helper T cells]; T_H2 [type II CD4+ helper T cells], T_C17 [IL-17-producing CD8+ T cells], etc.) have been the subject of repeated, and probably unnecessary, controversies.[534,535] Different tumors are dissimilar enough so that differences would be expected in T-cell subset requirements. Also different therapeutic settings may require different subsets (eg, to prevent cancer development or destroy premalignant lesions), established solid cancers, malignant effusions, microdisseminated cancer cells, or leukemic cells. Dependent on their subtype, T cells

produce and induce various cytokines and chemokines that may destroy tumors by direct effects on tumor vasculature or recruit neutrophils, macrophages, and NK and other innate effector cells that are needed for tumor eradication.[536] Many recent studies employ overexpressed model antigens and TCR-transgenic T cells not available in humans; thus, results need to be confirmed using non-TCR-transgenic models and genuine tumor-specific antigens. Interestingly however, adoptive transfer of TCR-transgenic CD8+ T cells specific for a model antigen can eliminate large established tumors[536,537,538,539,540] or artificial pulmonary metastases[541] without the T cells needing perforin.[536,541] Release of IFNγ and tumor necrosis factor (TNF) by the T cells seems to be critical; receptors for both cytokines must be expressed on bone marrow- and non-bone marrow-derived tumor stroma to kill T cell-resistant cancer variants as bystanders or relapse may occur.[537,538,540,542] CD4+ T-cell subsets can influence antitumor immunity, and truly tumor-specific CD4+ T cell-recognized tumor antigens exist.[10] CD4+ T cells seem to be critical at the effector phase of CD8+ T cells for cancer cell destruction in vivo,[543,544] for sustaining CD8+ T-cell memory,[545] and for the survival of adoptively transferred CD8+ T cells.[546,547] However, CD4+ T cells do not necessarily require recruitment of CD8+ T cells for eliminating cancer cells in vivo,[10,371,548,549,550,551] even when the cancer cells are MHC class II negative and killing as well as presentation must be indirect.[10,549,552,553] Destruction of the cancer cells

in vivo requires IFNγ, but occurs even when cancer cells lack the receptor for it.[552] Thus the effects of CD4+ T cell-released IFNγ must be on stroma, most likely tumor vasculature.[554,555]

Certain CD4+ subsets may also suppress tumor rejection, because elimination of the CD4+ T-cell subset may increase tumor resistance in certain tumor models.[514,556] Now termed T_{reg} cells, these Foxp3+CD25+ T cells mostly suppress the induction of immune response to new antigens, reduce anticancer immunity particularly to self-antigens, and suggest poor prognosis when prevalent in excised cancers.[557] However, it is uncertain whether these T_{reg}s can prevent adoptively transferred tumor-specific memory T cells from eradicating cancers.

Natural Killer, Lymphokine-Activated Killer, and Natural Killer T Cells

NK cells are distinct subpopulations of lymphocytes that, without prior sensitization and without the requirement for MHC restriction, can kill some cancer cells, particularly while circulating in the bloodstream, as well as nonmalignant nonself cells[558,559,560,561,562,563,564,565] (see Chapter on NK cells in this book). NK cells occur as 1) "resting" NK cells that nevertheless kill very sensitive targets such as YAC, 2) "activated NK" cells induced within hours by IFNαβ to become cytolytic but without proliferation (many conditions and microbial agents rapidly induce IFNαβ), and 3) lymphokine (IL-2)- activated killer (LAK) cells developing after days of *culture* in high doses of IL-2 and requiring proliferation.

NK cells can "recognize" the absence of self[566] (ie, the missing MHC allele fails to provide an inhibitory signal to prevent the activation of NK cells to kill the target[567]). Therefore, cancer cells that fail to express at least one of the MHC class I alleles of the host are killed more effectively.[568,569] Transformed cells often have decreased or lost MHC class I surface expression or have induced expression of ligands for activating receptors on NK cells and thus are targets of NK cells. In vivo, NK cells or NKG2D-mediated effects inhibited autochthonous and transplanted tumor formation and tumor recurrence, and reduced metastatic dissemination of intravenously injected cancer cells.[570,571,572,573,574] However, NK cells were effective usually only at incipient stages.[575,576] In patients, intratumoral NK cells may reduce metastatic seeding, thereby leading to longer survival,[577]

and NK cells may be a key factor in the occasional cure of acute myeloid leukemia and childhood acute lymphoblastic leukemia by allotransplantation.[578]

Activation of peripheral blood cells in vitro with high doses of IL-2 induces LAK cell.[579] Cancer cells, even when resistant to NK cells, are usually susceptible to killing by LAK cells in vitro, whereas most nonmalignant target cells have been reported to be resistant to killing by LAK cells.[580] Intravenous injection of LAK cells early after intravenous seeding of cancer cells into mice reduces the metastatic tumor cell growth in the lungs; however, with this procedure, both LAK and cancer cells are trapped in the lungs.[581,582] Antitumor responses have also been reported in humans after adoptive transfer of LAK cells in patients with renal cell carcinoma and melanoma.[583] This selectivity is difficult to explain, considering the general susceptibility of cancer cells to LAK cells in vitro. In more recent studies, adoptive transfer of NK cells activated in vitro with IL-2 failed to cause tumor regression in patients with melanoma.[584] The cells that mediate the killing in vitro of a broad range of malignant cells are more than 90% activated CD16+/CD3- NK cells,[585,586] but which cells have antitumor activity in vivo is not fully established. Even though murine LAK cells can be generated from nude mouse spleen cells,[587] it has not been demonstrated that LAK cells from nude mice or normal mice have similar therapeutic effects against tumor cells in vivo. Other cell types, such as CD3+ lymphocytes, which are regularly present in every preparation of LAK cells, may contribute significantly to the killing of tumor cells in vivo, particularly because activated CD8+ T cells express the stimulatory lectin-like NKG2D receptor and can kill tumor cells expressing the ligands for the receptor.[588]

Many surface receptors originally discovered in NK cells are expressed by subsets of T cells such as NKT cells, defined by the invariant Vα14/24Jα18 TCR α chain.[589] NKT cells, when treated with IL-12 at the time of cancer cell inoculation or after 1 day, inhibited tumor development and metastasis.[590,591] Also, the development of tumors induced by MCA was found to be reduced in some but not other studies.[486,489,592] In any case, clinical studies are exploring the usefulness of NKT cells in certain cancers.[593,594,595,596] NKT cell infiltration of neuroblastoma is associated with a favorable outcome; NKT cells may target the tumor-promoting stromal macrophages rather than the neuroblastoma cells directly.[597]

Together, previous studies have failed to provide evidence that NK, LAK, or NKT cells can eliminate well-established solid tumors casting continuing skepticism about the clinical usefulness of these cells in the therapy of advanced solid tumors.[575,576,598] A recent study, however, shows even very large solid tumors being eradicated by NK cells alone when the NK cells are properly activated by IL-15 in vivo at the site of the tumor.[599]

Macrophages and Granulocytes

Neutrophils rapidly appear at the site of injury and respond very rapidly to diverse chemotactic and inflammatory stimuli.[200] Furthermore, neutrophils often appear to pave the way for the influx of inflammatory monocytes that then mature to “angry” classically activated macrophages (or M1 macrophages) (eg, after “classical” activation by intraperitoneal/pleural injection of thioglycolate[105,106,600,601,602]). However, such inflammatory stimuli are usually absent in cancers. As a result, neutrophils can be relatively rare (0.1% to 0.4% of the total cells in solid tumors).[603] Macrophages, however, are regularly found in the microenvironment of solid tumors and are usually at least 10 times more abundant than neutrophils,[604] sometimes a third or more of the tumor mass. These tumor-associated macrophages differ from “angry,” tissue-destructive, “classically activated” macrophages (or M1) but are “alternatively” activated macrophages (M2).

“Alternatively” activated macrophages seem to be derived from resident tissue macrophages that proliferate.[105,106] Cancers may not recruit tissue-destructive inflammatory cells (N1 neutrophils and M1 macrophages); apparently, cancer cells lack the signals necessary for

activating innate immunity,[605] but cancer cells transfected to express CD95L induce cancer cell-destructive neutrophils.[606] Therapeutic interventions may aim at converting cells of innate immunity to a tumor-inhibitory “type 1” response pattern. IL-4, the prototypical “type 2” cytokine, can inhibit growth of cancer cells transfected to produce this cytokine by activating granulocytes[73,127,137,138] and can activate macrophages to kill cancer cells in vitro,[607] but may also interfere with eventual clearance leading to outgrowth of escape variants.[142] IL-4-secreting tumors are heavily infiltrated by eosinophils,[73,127] and many clinical and experimental studies reported eosinophilic tumor infiltrates.[535] Nevertheless, the contribution of eosinophils to tumor destruction and tumor growth is still incompletely understood.[138,535,608]

Macrophages and neutrophils from normal donors are generally not cytotoxic to tumor cells or normal cells in vitro; however, macrophages and neutrophils can be activated by bacterial products and IFNγ (or other cytokines) in vitro to cause selective cytolysis or cytostasis of malignant cells.[609,610,611,612,613,614,615,616,617] Fully activated macrophages require long-term 16- to 72-hour assays to demonstrate in vitro tumoricidal activity in isotope-release assays or cytostatic activity in growth-inhibition assays. Some of the cytolytic or cytostatic effects of macrophages on tumor cells involve cell contact and/or the secretion of various cytotoxic substances, but phagocytosis may also play an important role.[618] TNF-α[619,620] produced by thioglycolate-activated macrophages can account for all of the classical tumoricidal effects of macrophages against some cancer cells in vitro.[621,622,623,624] However, as might be expected because of the plethora of cytotoxic molecules that can be released by activated macrophages,[200,625] other mechanisms such as reactive nitrogen intermediates can also be important mediators of killing of cancer cells in vitro.[626,627] Systemic activation of macrophages reduces metastatic seeding,[628] while a (nonactivated) colony-stimulating factor 1-dependent macrophage subset seems to help metastatic seeding.[629] Because of the rather selective cytotoxicity of thioglycolate-activated macrophages against malignant cells, numerous studies have considered the potential role of this cell type in cancer (see Cancer and Inflammation, Immunotherapy, Factors Limiting Tumor Immunity for discussion of myeloid-derived suppressor cells). As would be expected, these in vitro-activated leukocytes mixed with cancer cells and injected into an animal prevent cancer development from the inoculum “in vivo” (a Winn assay, see previous discussion).[630] Interestingly, dependent on the role neutrophils have in a particular cancer model, their transient elimination in vivo can either abrogate or enable T cell-mediated transplantation resistance.[44,631] However, there is a major difficulty of effectively eliminating macrophages or neutrophils long-term in vivo. Therefore, there is, at present, no critical evidence to establish or refute the idea[632] that macrophages and or granulocytes activated in vivo destroy nascent tumors and therefore play a role in immune surveillance.

FACTORS LIMITING CANCER IMMUNITY

Various mechanisms allow cancers to escape innate or adaptive host immunity, principally by 1) inducing a protective and supportive stromal microenvironment, 2) increasing resistance to direct attack, and 3) inducing T-cell anergy. Often, cancer cells seem to use a combination of the three, but numerous lines of evidences indicate that the first is key and virtually always part of tumor progression and escape. Unfortunately, most experimental tumor immunology depends on observations using cancers serially transplanted for decades. This is surprising, knowing that a single transplantation of an autochthonous tumor into an immunocompetent host dependably results in heritable variants that avoid destruction by the host[43,44,45,218,219,508] (see above under Experimental Cancer, Key Principles, and Selection of Tumor Model).

Tumor Microenvironment

Many escape variants keep their antigens but induce a stroma more effectively than the

parental cells for better support of growth and protection against destruction by the host (also see Cancer Stroma). Experiments have shown that stroma is critical for preventing or permitting immunologic destruction of cancer,[47] and it is likely that cancer stroma is also an important factor in causing very early cancers to resist therapeutic immunization.[633] Local factors must explain why tumor-bearing mice, while failing to reject a primary tumor transplant, reject a later implant of small numbers of the same cancer cells at second sites, a phenomenon called concomitant immunity (see discussion below under Cancer and Inflammation, Facilitation of Inhibition of Metastasis). Local factors particular to the tumor environment must also explain why mice bearing malignant grafts fail to reject the established tumors but reject nonmalignant grafts expressing the same rejection antigen[634]; T cells in these tumorbearing mice are neither clonally exhausted nor systemically anergic.[635] Antigenic stroma, as it exists in nonmalignant allografts[635] or when strong tumor-specific antigens are crosspresented by tumor stroma,[537,538,539,540,542] can help T cells to eradicate cancers. In addition, recent work in two tumor models suggest that lack of “help” at the site of cancer growth may be an important reason for the failure of cancer cells to be rejected by CD8+ T cells, thereby stressing the importance of CD4+ T cells cooperating with CD8+ T cells in the effector phase.[543,544] The situation might be somewhat analogous to transgenic mice that express allo-MHC class I molecules as self-antigen on islet cells and have autoreactive T cells that infiltrate the islets,[636] but even after priming, the autoreactive cells fail to destroy the islet cells unless local help is provided in the form of IL-2.[636] Antigen-specific T cells can infiltrate even tumors growing in immunologically privileged sites, but proper differentiation of the infiltrating T cells is prevented.[301,637,638,639] Several different models have shown that the

milieu of large tumors can inhibit the function of adoptively transferred T cells.[640,641,642,643,644,645,646] Lack of costimulatory molecules or expression of Fas ligand by the cancer cells may lead to peripheral anergy.[647,648,649] Suppression in the tumor microenvironment may also be the result of macrophages long known for abrogating immune responses when prevalent in culture.[650] In the stroma, the macrophages are alternatively activated and produce indolamine-2,3-dioxygenase, inducible nitric oxide synthetase, lactate dehydrogenase-A, and myeloid cell-derived arginase.[651,652,653] Autochthonous cancers transplanted once and reisolated are usually heritable variants that grow more progressively, more effectively attract macrophages and neutrophils that are pro-angiogenic (see Cancer Stroma), and more rapidly induce a protective and supportive stromal microenvironment.[43,44,45] Treatment with RB6-8C5 allows mice to reject a lethal tumor cell inoculum,[43,44,45] thereby indicating the profound role that Gr1+ CD11b+ leukocytes have in helping tumors to escape. The significance of tumor-infiltrating T_{reg} cells is discussed separately in the following. Finally, tumor-bearing mice and cancer patients may have alterations in the signal-transduction machinery in T cells, particularly in those infiltrating the tumors but also in T cells from draining lymph nodes or, at later stages, even in circulating T cells.[654,655,656] NK cells can be similarly affected.[654] A decrease in NF-κB p65 at an early stage is followed by loss of TCRζ chain and p56 lck after continued tumor growth.[657,658,659,660,661,662] Activated macrophages can secrete substances that induce these structural abnormalities.[663] As another example of subversion of host defenses in the tumor microenvironment, tumors are a privileged site for bacterial growth.[664,665] Together, numerous immunosuppressive mechanisms have been shown to be functioning in the tumor microenvironment and the tumor draining lymph node. Their relative significance is difficult to judge because there is rarely a real “positive control” (ie, destruction of a truly long established large solid tumor in the absence of the implicated mechanism).

Resistance to Direct Attack

Cancer variants can become resistant to direct attack by T cells by 1) loss/downregulation of

MHC class I and II molecules, 2) losing expression of the rejection antigens, or 3) increased resistance to the killing pathways.

Selective loss or downregulation of MHC class I molecules or the associated antigen-processing machinery allows cancer cells to resist direct attack by T cells.[237,666,667,668,669,670,671,672,673,674,675,676,677,678,679,680,681,682,683,684,685,686,687,688,689,690,691,692] These mechanisms allow cancer cells to escape while retaining the antigen that might be essential for cancer cell survival and malignant behavior.[10,302,307,693] As noticed already decades ago, MHC-heterozygous F1 tumors can escape destruction when transplanted into either parent by loss of the mismatched MHC antigens.[694,695] This agrees with the commonly observed loss of the mismatched HLA after haploidentical hematopoietic stem cell transplantation.[696,697] MHC homozygous tumors rarely lose MHC antigens and require MHC compatible strains for serial transplantation, and they were therefore an essential tool to define the MHC.[698] Two cancers are known to be naturally transmitted within a species as cells.[699] The devil facial tumor disease, first observed in 1996, is a lethal cancer cell line serially transmitted by facial bites during fights among Tasmanian devils.[700,701] These marsupial carnivores in Tasmania have very little MHC diversity and are in rapid decline due to spread of the cancer. Canine transmissible venereal tumor, first described in 1876,[260] is passed between dogs through coitus and bites.[702,703,704] The tumor cells express very little β2-microglobulin and MHC class I during the progressive growth phase followed by regression after ˜6 months unless the dog is immunosuppressed. Some cancer cells express low levels of MHC, but levels can be upregulated by cytokines. At least some of these changes seem to be due to epigenetic mechanisms.[705,706] In other cancer cells, irreversible loss of MHC class I expression is caused by several molecular mechanisms, including mutations in the gene coding for β2-microglobulin.[672,674,675,707] Loss of a single HLA class I allele was found more commonly than loss of all class I alleles.[706] The E1A gene of the adenovirus (Ad) strain Ad12 suppresses MHC class I expression in transformed cells that are tumorigenic and escape T cell-mediated destruction,[708] but there was no correlation between the level of MHC class I expression and tumorigenicity of Ad2 and Ad5 transformed cells. Oncogenes such as myc can cause locusspecific suppression of MHC class I antigen expression.[709] Importantly, MHC class I molecules have now been shown to act as tumor suppressor genes.[710] Thus, some of the observed changes in MHC expression may not be the result of immunoselection.

Cancers also escape by loss of expression of the rejection antigens.[218,219,236,507,508] Even when the antigen is necessary for cancer cell survival,[10,302,307,693] point mutations might preserve the oncogenic potential while rendering the protein nonantigenic. Mutations in SV40 large T that preserve the transformed phenotype in vitro have been selected for with CTL clones, but, in vivo, evidence is lacking for tumorigenicity or selection of such clones.[711] Interestingly, mutational changes in E6 and E7, the transforming genes of HPV, have not been observed in cervical cancer while total or allelic loss of HLA class I expression is commonly observed in this cancer.[712] Immune selection has been reported in patients at later stages of malignancy and after therapy.[713,714,715] Partial immune suppression may lead to a higher yield of antigen loss variants.[235,716] UV-irradiated mice show a partial immune deficiency so that the generation of cytolytic T cells is delayed.[235,717] Incomplete therapy of bacterial infections with antibiotic drugs also favors the outgrowth of variant bacterial strains that show heritable resistance to these drugs; by analogy, partial or incomplete immunotherapy of cancer-bearing individuals may lead to selection of antigenloss variants. When targeting cross-presented tumor antigen on tumor stroma only, T cells do not select for cancer cell variants.[169] Thus, direct (or very short-range) cell-cell interactions appear to be essential for selecting antigen or MHC-loss variants. Antigenic modulation, a reversible antibody-induced loss of surface antigen,[523,524,718,719] and cellular resistance

to perforin/granzyme,[720] FAS/CD95,[721] and other pathways to evade destruction are not discussed.

Anergy/Unresponsiveness/Neonatal Tolerance

Antigens newly expressed on cancers in the adult may be ignored at the earliest stages of autochthonous cancer development because of insufficient amounts of antigen.[191,228,301,722] But once the tumor grows and the amount of antigen increases, the host responds.[723] Thus, immunologic ignorance is unlikely in cancer patients. Tumor transplantation induces acute inflammation that may influence the immune response to the transplanted tumors.[724,725] By contrast, the antigens on sporadic autochthonous tumors fail to induce tumor destruction.[228,301] Importantly, these cancers grow progressively though they are not resistant to direct immune attack (eg, by adoptively transferred T cells). Furthermore, tumor antigen-specific CD8+ T cells are remarkably increased in number in mice bearing autochthonous cancers but failed to lyse antigen-specific targets in vivo and the production of IFNγ was low whereas the production of TGF-β1 was increased and high titers of IgG antibody specific for the rejection antigen were observed.[228,301] Thus, antigen-specific B and T cells in mice bearing the autochthonous tumors responded to the rejection antigen but in a way that resulted in failure to reject the cancers. This altered responsiveness[726,727] must be distinguished from "peripheral" or "extrathymic" tolerance that occurred under certain conditions when an antigen was presented to an adult in highly immunogenic form with the consequence of temporary expansion of mature T cells followed by clonal elimination.[728] The important distinction is that clonal deletion would require adoptive T cell transfer whereas retention of antigen-specific T cells in the host bearing autochthonous cancers may allow rescue or induction of a tumor-specific destructive T-cell response.

Thymic tolerance to self may explain why oncofetal and carcinoembryonic or oncospermatogonal self-antigens, all expressed in the thymus, induce weaker protection than is found in animals immunized with tumor-specific antigens.[364,453,729,730] Nevertheless, at least certain cancers seemingly do not follow the rules that prevent immune recognition of normal self-tissues. Thus, some tumor-bearing hosts may readily recognize normal differentiation antigens on certain cancers such as melanomas. In this type of cancer, it appears to be possible to uncouple the mechanisms of autoimmunity from tumor immunity.[731,732] However, it is unclear how effectively these T cells can control tumor growth.[733] Epigenetic memory can prevent self-reactive CD8+ T cells from escaping their tolerant fate.[734] Because the thymus "selects the useful, neglects the useless and destroys the harmful" T cells,[735(p.57)] only self-antigen-reactive T cells with too low avidity to cause destruction escape deletion and prominently infiltrate tumors.[736,737]

Age

As already discussed (see Immunogenicity of Autochthonous Tumors), most cancers developing in older individuals may be fully sensitive to tumor-specific T cells but for age-related reasons fail to induce an effective immune response. Immunotherapy in older individuals may require rescuing the age-dependent immune deficiencies of the host environment[738] as well as the T cells[484] because adoptively transferred lymphocytes from young, but not old, immunized mice eradicate large solid tumors.[258]

Regulatory T Cells/B Cells/Antibodies/Blocking Factors

Passively administered antibody can prevent the rejection of tumor allografts (homografts).[739,740,741] These antibodies are also referred to as "enhancing antibodies." "Blocking factors" are complexes of antigen and antibodies that can suppress cell-mediated immunity in vitro.[742,743] Complexes of tumor antigen and antibody can induce suppressor T

cells[744] (now generally referred to as T_{reg}s) that can suppress specific immune responses in vivo. Treatments of recipients with donor-type cells as antigen and homologous antibody to donor-type cells enhances acceptance of rat renal allografts.[745,746] The mechanisms responsible for these findings are incompletely understood. The induction of T_{reg}s required active TGF-β; latent TGF-β linked to IgG causes the suppression of sensitization/proliferation of CD8+ effector T cells. Recent and earlier experiments have pointed at a joint role of CD4+ T cells and B cells/antibodies in preventing the rejection of transplanted tumors[514,515] and enhancing the development of autochthonous cancers in mice.[341,517,520] Eliminating B cells or CD4+ T cells can have virtually identical effects in preventing tumor rejection in the same tumor model,[514,515] pointing at the fact that B cells are required for multiple functions of CD4+ T cells and vice versa[747,748,749,750] and that T_{reg}s may not necessarily be generated or act independent of B cells and antibodies.[751,752] "Suppressor T cells" can suppress tumor rejection.[514,556,753,754,755] Residual cancer cells remaining after incomplete tumor removal can be sufficient for continuing the suppression.[756] However, unlike what has been reported,[756] suppression is short-lived after complete tumor removal and may give way to specific immunity without further immunization.[757] Unlike CD4+ helper T cells, T_{reg}s usually express CD25 and Foxp3.[758,759,760,761,762] The Foxp3+ CD25+ T cells are essential in preventing autoimmune destruction of the host[759] and suggest, as discussed previously, poor prognosis when prevalent in excised cancers.[763] T_{reg}s are sensitive to low-dose gamma irradiation and cyclophosphamide pretreatment.[764,765] Preirradiation, cyclophosphamide pretreatment, T-cell deficiency, or CD4+ T-cell depletion of the tumor-bearing host facilitates tumor destruction by adoptively transferred immune T cells.[766,767] However, these treatments also cause homeostasis-driven expansion and activation of the transferred T cells,[768] which may be sufficient or at least synergize with the effects of T_{reg}s depletion. However, whether T_{reg}s can suppress memory T cells needs further study. In any case, these proposed mechanisms and observed effects confirm the early pivotal discovery that low dose whole-body x-irradiation and/or chemotherapy are an essential adjunct to adoptive tumor immunotherapy in mice.[766,767] "Nonmyeloablative lymphodepleting chemotherapy" is now standard treatment in melanoma patients before adoptive T-cell transfer.[769]

Myeloid-Derived Suppressor Cells

MDSCs[75,76,77,78,79] are a subset of monocytes[68] that express CD11b and Ly6C (resulting in intermediate levels of Gr-1 (RB6/8C5) staining), but lack Ly6G (defined by 1A8) (see Components, under Cancer Stroma). They differ from alternatively activated tumor-associated M2 macrophages in well-established tumors 3 weeks after transplantation and from neutrophils. Neutrophils express high levels of Ly6G and Gr-1 and intermediate levels of Ly6C. Cells of myeloid origin are increased in numbers in the peripheral blood of tumor-bearing mice and cancer patients, especially in later stages. In the mouse, MDSCs come from bone marrow as well as spleen, which is a natural extramedullary blood-forming organ in this species. MDSCs and blood monocytes seem to be the predominant precursors of intratumoral macrophages not only in transplanted but also autochthonous tumors.[68,770] A vast number of studies indicate that MDSCs isolated from the peripheral blood of cancer-bearing individuals are profoundly immunosuppressive in vitro. How this correlates to immune suppression in vivo remains an open question.[77,78]

IMMUNE SURVEILLANCE OF CANCER

Types of Surveillance Mechanisms

The term surveillance should be restricted to its meaning (ie, protection from cancer

development[771]). Early in the last century, Ehrlich wrote, “I am convinced that the development of aberrant [mutant] cells occurs very frequently during the extremely complicated fetal and postnatal development but that these foci luckily remain completely latent in most humans because of protective mechanisms in the host. If these mechanisms were not existent, cancer would probably develop with an incredible frequency. „261(p.95),262(pp.289-290) Ehrlich's statement was based on experimental and clinical findings which included *Xeroderma pigmentosum*, an inherited defect in repair of DNA damage caused by UV leading to countless skin cancers. Ehrlich distinguished two basic types of surveillance: 1) immunologic responses (acquired or natural) and 2) cellular (cell-intrinsic) resistance. The latter, attributed by Ehrlich to deprivation of nutrients (athrepsia), is now best explained as cell-intrinsic controls such as DNA repair, checkpoint functions, programmed cell death, and epigenetic controls. With great foresight, Ehrlich emphasized his “firm conviction” that cell-intrinsic protective mechanisms were critical for resistance to cancer development, but discussing these cell-intrinsic mechanisms[772,773,774] is beyond the scope of this chapter.

Today, the microenvironment produced by stroma should be added as third important type of surveillance. Numerous cytokines and cell-cell interactions in the stromal microenvironment can be highly effective in preventing tumor development from transformed cells.[139,555,775,776,777,778,779] Diet, host microbial flora, and chemicals affect stroma and microenvironment, and can thereby promote or prevent cancer development depending on the agent and the condition. However, these factors have little or no effect once full-blown cancer has developed. This means therapy directed at stroma to prevent cancer or to cure cancer must be fundamentally different, though recent reviews do not make this distinction.[780] Of the three principle types of surveillance mechanisms, focus here is on the immune system that can be a powerful restraint against the development of certain cancers.

Adaptive Immunosurveillance

The concept of surveillance by adaptive immunity was especially attractive in the 1950s and 1960s because it provided evolutionary significance to T cell-mediated cellular immunity, which previously seemed to have no use other than to cause rejection of experimental allografts.[781,782,783] Thus, Burnet's hypothesis of immunologic surveillance suggested that the primary reason for development of T cell-mediated immunity during the evolution of vertebrates was defense against altered self- or neoplastic cells.[784] We now know that T cell-mediated immunity is necessary for resistance to many viral and other infections and therefore may be important in the host response to cancers induced by these agents.

Congenital Immunodeficiency Diseases

Patients with certain congenital immunodeficiency diseases may have a markedly (several thousand-fold) increased incidence of cancer,[785] but mostly of lymphopoietic and reticuloendothelial or certain virally induced cancers (Table 47.1).[786,787] Most common forms of cancer (eg, lung, breast, colon, and prostate) do not occur earlier or at a significantly higher rate than in the general immunocompetent population. It is therefore possible that the congenital abnormality itself contributed to the high incidence of lymphopoietic and reticuloendothelial cancers. Mice with congenital immunodeficiency diseases also have a higher incidence of cancers of hematopoietic origin.[788,789,790,791]

Virally Induced Cancers

While gene-targeted mice have demonstrated the influences of innate immunity on tumor induction,[792] these defects are usually absent in human transplant patients that therefore have intact innate immunity to exert cancer surveillance. This would also explain why patients do not show an increased incidence of nonvirally related cancers, and we need more information how innate effector mechanism might influence cancer incidence in humans.

It is important to realize that resistance to tumor induction by oncogenic viruses is also

genetically determined, as illustrated by the example of the lymphotropic herpes virus saimiri.[793] In its natural host, the Old World squirrel monkey, the virus is an innocuous inhabitant probably due to Darwinian selection. However, some New World monkeys (such as the marmoset or owl monkey) do not harbor the virus, and experimental inoculation of the virus regularly causes malignant lymphomas. The susceptible monkeys do respond immunologically to the virally encoded antigens but too late and only at a time when lymphoma development has already occurred. These results suggest that viruses with oncogenic potential survive because lethal tumors would eliminate the virus along with the host. A further example is lymphotropic EBV, which causes a self-limiting lymphoproliferative disease called mononucleosis in humans, the natural host of EBV.[771] Infection usually occurs at very early age usually without any symptoms or, rarely, at later ages (10 to 35 years old) when it may cause disease (mononucleosis). After infection, EBV becomes latent. About 90% of adults are latently infected. Only immunosuppressed individuals appear to develop EBV-associated lymphoproliferative disease and malignant lymphomas. EBV-encoded, CTL-recognized antigens must be important for host recognition and tumor rejection because lymphomas expressing these antigens in immunocompetent individuals have not been found. Instead, EBV-associated lymphomas express the full array of CTL-recognized antigens only in immunosuppressed patients.[794]

TABLE 47.1 Cancers with Increased Incidence in Patients with Immunodeficiency

Type of Immunodeficiency	Cancer	Cause/Carcinogen	Reference
Primary (inherited)	B-cell lymphoma	EBV[a]	786,1100
	Hepatocellular carcinoma, biliary tumors	HBV	787,1101
	Gastric cancer*	*Helicobacter pylori*	1102
	Hematologic malignancies	Germline	785,786,1103
Secondary (drug-induced) (patients with or without allograft)	B-cell lymphoma	EBV	909,1103,1104
	Kaposi sarcoma	HHV-8	1105
	Skin cancer (nonmelanoma)	HPV, UV	806,808,818,819
	Vulvar, oral, or anal carcinoma	HPV	1106
	Merkel cell carcinoma	MCV	796,1107,1108

	Cervical carcinoma	HPV	909
Acquired immunodeficiency syndrome	B-cell lymphoma	EBV	909
	Kaposi sarcoma	HHV-8	909
	Hodgkin lymphoma	EBV	
	Anal, vulvar, or oral carcinoma	HPV	909
	Merkel cell carcinoma	MCV	796,1108,1109
	Cervical carcinoma	HPV	909
	Hepatocellular carcinoma	HBV, HCV	909
	Skin cancer (nonmelanoma)	HPV, UV	909

EBV, Epstein-Barr virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HHV, human herpesvirus; HPV, human papillomavirus; MCV, Merkel cell polyomavirus; UV, ultraviolet light.

* Patients with primary hypogammaglobulinemia.

In principle, adaptive T cell-mediated immunity may protect against the development of primary virus-associated cancers by either preventing or shortening the duration of infection with the oncogenic viruses or by eliminating virally transformed cells expressing the virus. As example for the latter, cells malignantly transformed by oncogenic DNA viruses often do not produce (in contrast to oncogenic RNA viruses) viruses; only the tumor formation, not infection by these viruses, may be prevented by the immune system. Such a resistance to tumor induction by DNA tumor viruses is consistent with the fact that polyomavirus is a common harmless passenger virus in adult mice and is commonly found in wild mice without inducing malignancies.[795] But even a generally harmless polyomavirus when it integrates and acquires the mutations needed to inactivate viral production may induce cancer, as in the Merkel cell carcinoma in immunosuppressed humans.[796] By contrast, in the case of high-risk human papilloma viruses 16 and 18, T cell-mediated responses may prevent or at least shorten the duration of viral infection. This may reduce the chance that the initially mostly episomal virus integrates its oncogenic E6/E7 sequences into the host's epithelial cells[15,797,798] (also see Immunoprevention).

Cells transformed by certain DNA or RNA viruses can be very immunogenic.[771] For example, SV40 and polyomaviruses usually do not induce tumors in adult animals[799] or humans[800] because the viruses induce rejection antigens on the transformed cells that are immunogenic

enough to elicit rejection without prior immunization. Therefore, the use of immunoincompetent animals, such as neonatal animals or nude mice, is required for tumor induction, a finding that led to a breakthrough in studying the tumorigenicity of viruses and of cells transformed by viruses in vitro. It is important to note that this immunologic resistance of the natural host is directed against the oncogenes expressed by the virally transformed cells, not against the virus itself. Interestingly, when the oncogenic SV40 large-T antigen is expressed as a sporadic extremely rare event in single cells (rather than as part of a systemic infection alerting the host to respond), this strongly antigenic and oncogenic protein can cause cancer in mice.[228,301] Transplants of these autochthonous cancers are regularly rejected by naïve syngeneic normal hosts, indicating that during development, the autochthonous cancers managed to sneak through all host controls in the euthymic normal individual without losing their highly antigenicity and immunogenicity.[228,301] Even a progressor tumor may be highly antigenic, immunogenic, and sensitive to immune destruction (immunosensitive), yet induce, for example, a strong T_{reg} cell and B cell "type 2" immune response that downregulates destructive T-cell responses and promotes tumor growth.

MHC genes may regulate immune responses to cancer cells or cancer-causing viruses, and since the discovery that the MHC profoundly influences the susceptibility of mice to leukemia caused by Gross virus,[801,802] investigators have searched for a possible association between MHC type and cancer susceptibility in humans and mice.[803] However, no firm association between HLA haplotype and the occurrence of any major human cancer has been established except for virally induced nasopharyngeal, cervical, and liver cancers.[804,805]

Ultraviolet Light-Induced Cancers

Renal transplant patients show a 65- to 250-fold increase in skin cancers virtually restricted to UV-exposed sites of the body.[806,807] The increase is independent of whether the immunosuppressive agent is mutagenic or not,[808] although cyclosporine enhances transformation in vitro.[809] Skin cancers in transplant patients are often more aggressive with a mortality of 7% to 9% due to metastases.[810] The increased incidence in transplant patients could be in part due to an increase of mutations due to impaired repair of DNA damaged by UV.[811,812] Immunosuppressive drugs can decrease local production of IFNγ ,[813] and this decrease may favor tumor development.[555,814,815,816,817]

UV-signature mutations in oncogenes and suppressor genes play a critical role for skin cancer development. Nevertheless, viruses of the beta HPV group may play an essential role as cocarcinogens.[818,819] This would make these cancers virally related and more similar to the other cancers increased in transplant patients. In any case, two-thirds of these transplant patients experience reduced skin cancer development after immunosuppressive medications are stopped[820]; it appears that, similar to murine UV-induced tumors,[218,479] many human UV-induced cancers are actually "regressor" tumors and have arisen only because host defenses had been damaged.

Chemically Induced Cancers

We lack evidence that the incidence of chemically induced cancers is increased in immunosuppressed humans.[821,822] Experimentally, loss of adaptive immunity increases the incidence of virally induced cancers in virtually all studies and all species tested; however, the increased incidence or a shortened latency of chemically induced cancers due to lack of T cells has been found only in some experiments and not in others.[487,488,489,506,792,823,824,825,826,827,828] Transporter associated with antigen processing-deficient mice are defective in presenting intracellular mutant peptides (the key targets of adaptive immunity to nonviral cancers) to αβ TCR-bearing T cells (the key effectors of adaptive T-cell immunity). Nevertheless, transporter associated with antigen processing

knockout mice do not have an increased or accelerated incidence of chemically induced cancers whether or not these mice are also nullizygous for p53.[829] Clearly, immunosurveillance by adaptive T-cell immunity is effective against the development of several virally induced cancers, but it is still uncertain how effectively adaptive T-cell immunity prevents the development of forms of cancers that are induced by chemical or physical carcinogens.

Innate Immunosurveillance

The innate immune system uses nonrearranging germline receptors to trigger responses of cellular effectors that can recognize and kill cancer cells or normal cells. Many studies using gene-targeted mice have demonstrated the important influence of various components of innate immunity on tumor induction.[792] When it comes to the mechanisms of protection against cancer, the effects of innate immunity are not easily delineated from cancer cell-intrinsic and/or microenvironmental/stromal control mechanisms mentioned briefly at the beginning of this section. For example, mice lacking the transcriptional activator interferon regulatory factor (IRF)-1 show several immunologic disorders, most notably a severe defect in the development of NK cells,[830] but the tumor-prone phenotype of these mice was shown to be directly attributable to the cell-intrinsic lack of protective IRF-1.[815] IRF-1 regulates DNA repair and is also an activator of IFNα and IFNβ transcription, and is required for double-stranded RNA induction of these genes. Many cell types secrete IFNα and IFNβ. Furthermore, IFNα/β signaling contributes to p53-mediated tumor suppression.[831] Thus, cooperation between innate and cell-intrinsic mechanisms is likely to be common. IFNγ induces the IRF-1 that functions as a tumor suppressor,[139,814] and mice with a defective IFNγ signaling pathway (IFNγ receptor- or STAT1-deficient mice) have an increased incidence of MCA-induced tumors.[485,487,777] The spontaneous[485,487] but not MCA-induced[777] tumor incidence was increased when these mice also lacked p53. Thus, the IFNγ pathway may help eliminate somatic cells harboring spontaneous DNA damage. Stimulation of the IFNγ pathway by IL-12 reduces oncogene-driven tumor development in HER-2/neu transgenic mice.[832] This surveillance effect of IFNγ may be complemented by cytolytic γδ T cells or NKT cells. These cells may use their NKG2D receptors to eliminate somatic cells exposed to the initiating chemical carcinogen followed by the tumor promoter TPA.[573,574,827]

While gene-targeted mice have demonstrated the influences of innate immunity on tumor induction,[792] these defects are usually absent in human transplant patients that therefore have intact innate immunity to exert cancer surveillance. This would also explain why patients do not show an increased incidence of nonvirally related cancers; we need more information about how innate effector mechanism might influence cancer incidence in humans.

CANCER AND INFLAMMATION

Key Principles

Rudolf Virchow was among the first to stress the importance of local irritation for neoplastic proliferation, but central parts of his “Reiztheorie” (irritation theory) were incorrect.[833,834] Instead Virchow's contemporary, Carl Thiersch, may have been the first to state clearly that epithelial cancer cells invaded adjacent tissues and were not derived from connective tissue or inflammatory cells.[835] (An exception may be gastric cancer induced in *Helicobacter felis*-infected mice and proposed to originate from bone marrow-derived

cells recruited to sites of injury and inflammation[836].) In this section, we will discuss the evidence that local inflammatory reactions can stimulate or destroy cancerous growth, and that stroma plays an integral role in permitting or forbidding these interactions.

It seems confusing that in one and the same organ (eg, urinary bladder), inflammation can promote cancer development in humans as well as prevent cancer relapse. Schistosomiasis,

a parasitic helminth infection, increases the incidence of cancer whereas instillation of live Bacille Calmette-Guérin (BCG) prevents relapse of superficial bladder cancer. Schistosomiasis causes a chronic, growth-promoting, IL-4-driven "type 2" inflammation characterized by tissue repair and angiogenesis, alternatively activated neutrophils (N2) and macrophages (M2), and T_H2 and T_{reg} leukocytes driven by active TGF-β and STAT3 signaling. In contrast, BCG causes an acute, bacteriocidal, tissue-destructive "type 1" inflammation with classically activated neutrophils (N1) and macrophages (M1), and T_C1, T_H1, T_H17, and other killer leukocytes driven by STAT1, IFNγ, TNF, and IL-17. Dependent upon the antigen, cell, and cytokine involvement, the result may be either inhibition or stimulation of growth of the premalignant or malignant cells by acquired and/or innate immunity (Fig. 47.4). There is little knowledge of how differences in amount, kinetics, and local concentration of cytokines determine these different biologic outcomes.[619,837,838,839,840,841,842]

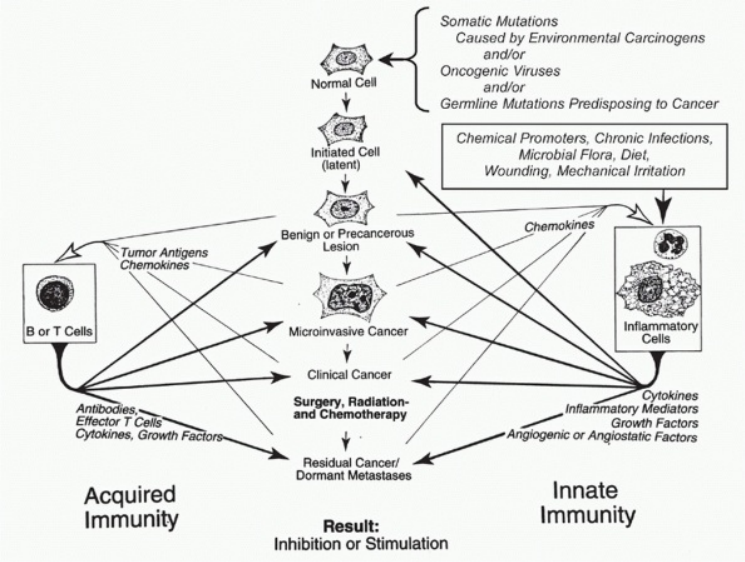

FIG. 47.4. Both Acquired and Innate Immunity can Stimulate or Inhibit All Stages of Cancer Development and Progression.

Exogenous Promotion

It is generally assumed that most human and animals harbor initiated mutant cells from exposure to low levels of chemical or physical carcinogens. Experimentally, this can be mirrored by exposing a small area of mouse skin to very small doses of the polycyclic hydrocarbon carcinogen 7,12-dimethylbenz[*a*]anthracene. No cancers develop over the life of the animal unless this site is exposed to prolonged inflammation caused by wounding or a nonmutagenic proinflammatory promoter[23,34,843,844,845] (eg, croton oil, a now obsolete laxative,[21,22] or its active component, phorbol ester [12-*O*-tetradecanoylphorbol-13-acetate, also referred to as phorbol-12-myristate-13-acetate][846]). Phorbol esters are strong inducers of NF-κB in keratinocytes and many other cell types. NF-κB, as a crucial communicator of innate

immunity and inflammation, normally receives exogenous signals from the proinflammatory cytokines TNF-α and IL-1β, TLRs signaling tissue damage and microorganisms. The major force opposing NF-κB is p53 that is upregulated by sensing DNA damage caused by the carcinogens (Fig. 47.5).[119] Upregulated p53 induces apoptosis and blocks cell proliferation and the replication of cells with damaged DNA. Evasion from p53-induced cell death is essential for mutant cells to survive and for cancers to develop.[774] The prosurvival signal

exerted by NF-κB is therefore essential for the premalignant cells to escape apoptosis-mediated cell death.[119] Wounding, used by Rous,[844] also seems to be effective if there is a prolonged healing.[847,848,849] Tumors have been compared with “wounds that do not heal.”[850(p.1650)] Mouse strains that have low or poor inflammatory reaction to wounding or phorbol esters are more resistant to tumor promotion.[851]

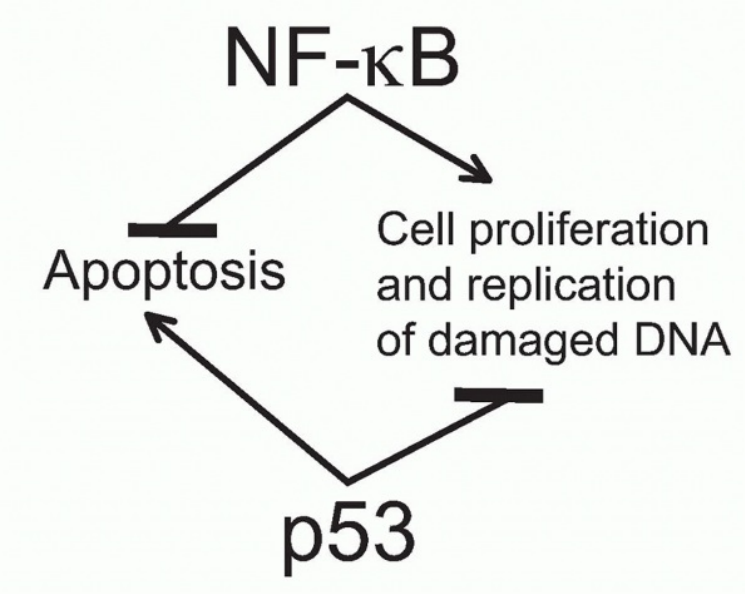

FIG. 47.5. Opposing Effects of p53 and NF-kB. p53 not only inhibits cell cycle progression, cellular proliferation, and the replication of deoxyribonucleic acid, but also leads to apoptosis. By contrast, NF-κB promotes cellular proliferation and blocks apoptosis. Thus, any substance that chronically upregulates the NF-κB pathway may promote cancer development and therefore be dangerous.

Until recently, few experimental studies have critically examined whether the microbial flora and chronic infections can exert substantial tumor-promoting effects, as suggested by clinical evidence. In one early study, the sensitivity to chemical carcinogen of germfree or specific pathogenfree rats was compared with that of chronically infected animals.[852] It was shown that chronic respiratory infection enhanced cancer development. Clearly, the microbial flora is a major regulator of innate and adaptive immunity,[853] and certain bacteria that are part of the normal gut flora exert a protective effect against inflammatory bowel disease that is strongly associated with the development of colon cancer.[854,855]

Switch to Paracrine Loops

When the chronic application of an exogenous chemical promoter is stopped during experimental skin carcinogenesis, some papillomas regress spontaneously while others persist and continue to grow.[848] Tumor persistence and progression appears to depend on heritable changes in premalignant and cancer cells to establish a “paracrine stimulatory loop”[43,44,45]: cancer cells producing chemokines that attract and activate leukocytes. These leukocytes in turn produce cytokines and growth factors that stimulate tumor angiogenesis or the growth of the cancer cells.[110,629,856] Thus, several experiments have shown that, during tumor progression, cancer cells can switch from being inhibited to being stimulated by inflammatory cells[43,44,45] or cytokines (eg, TGF-β or IL-6).[857,858,859,860,861] It has also now become clear that cause of the heritable switch is cancer cell-intrinsic (ie, the expression of mutant oncoproteins that causes an activation of NF-κB signaling pathways).[114,115,862,863] This allows the neoplastic cells not only to escape apoptosis-

mediated cell death but also to produce proinflammatory signals that initiate the paracrine loop.[80,114,115,116,862,864,865,866,867,868,869,870] Interestingly, the signals coming from the same oncogenic mutation can diverge into separate proinflammatory NF-κB and transforming RAS/PI3K signaling cascades.[862] The K-RAS pathway can lead to the secretion of MCP-1 (CCL2), KC (CXCL-1), MIP-2 (CXCL-2), and IL-8 (CXCL-8).[864,870] These more recent insights give a mechanistic basis for much earlier data showing that cancer cells release numerous inflammatory mediators and growth factors such as MCP-1 (CCL2), KC (CXCL-1), MIP-2 (CL-2), SDF-1 (CXCL12), IL-1β, IL-6, IL-8 (CXCL-8), IL-10, MIF, CXCL5, NFκB, TGF-β, osteopontin, versican, PDGF, FGF-2, VEGF, G-CSF, and granulocyte-macrophage colony-stimulating factor (GM-CSF).[164,780,869,871,872,873,874,875,876,877,878,879,880,881,882,883,884]

Regression Following Acute Inflammation

By the mid-1800s, physicians observed that occasional cancer patients who developed erysipelas, a serious acute bacterial infection of the skin, had remarkable regression, sometimes cures, of cancers that would have been inoperable today.[885] This led to intentional infection of patients,[886,887] later with cloned *Streptococcus pyogenes*, the causative agent of erysipelas.[888,889] Stunning successes occurred, in some cases regression,[888,889] and a decade later in 1893, the work was confirmed in the United States.[890] The variability of success was suspected to be a function of the different types of cancers treated, and the successes in occasional patients were marred by serious complications including death in the preantibiotic era. Today, topical use of live bacteria remains restricted to treating residual superficial bladder cancer, which typically recurs following surgery.[891,892] Repeated instillation of the live BCG mycobacteria into the bladder by a catheter after surgery has become a treatment of choice for this cancer. The repeated local infection with BCG leads to prolonged acute inflammation in the bladder wall. This is invariably associated with local production of type I and type II IFNs and a significantly reduced cancer recurrence. Consistent with this idea, topical application of imiquimod, a TLR7 agonist, can induce immunologic destruction of premalignant actinic keratoses and basal cell and squamous cell carcinomas.[893,894] Imiquimod also enhances IFNγ production and T-cell effector function when topically applied to UV-induced skin cancers in transplant recipients.[895]

Increased Incidence Following Chronic Inflammation

Chronic “type 2” inflammation by infectious agents is associated with development of some kinds of cancers[896] (eg, hepatitis B and C virus and hepatocellular carcinoma, *Helicobacter pylori* and gastric cancer, HPV infection and cervical cancer, and schistosomiasis and bladder cancer). Also, chronic tissue damage caused by physical or chemical agents is associated with development of some cancers (eg, reflux esophagitis and esophageal cancer), chronic pancreatitis and pancreatic carcinoma and inflammatory disorders of unknown etiology (such as ulcerative colitis or Crohn disease), and colon cancer.[118,780,897] Substantial clinical, epidemiologic, and preclinical data indicates that nonsteroidal anti-inflammatory drugs (eg, aspirin) are effective in reducing colorectal polyps and colon cancer.[898,899] Western diet is another highly significant risk factor to developing colon cancer.[900,901] Diet has major effects on shaping the intestinal microbiome[902] and may be associated with chronic inflammation,[853] but the mechanisms of this important link remain to be discovered. Chronic inflammatory responses to impaired glycosylation of intestinal mucosa has been suggested to be tumor promoting.[903] At least some patients with ulcerative colitis have impaired glycosylation due to lost T synthase function as a result of somatic mutations in COSMC in colonic Tn antigen-positive crypt cells.[904] Such mutations have also been found in human colon cancer.[412] Mice selectively deficient in T synthase in colonic

mucosa express the Tn antigen and develop massive inflammatory infiltrates and abscesses in their distal colon, similar to patients with the cancer-predisposing ulcerative colitis.[904]

Prognostic Significance of Tumor Infiltrates

Melanoma

Despite some claims,[905] the infiltration of lymphocytes into melanoma remains a histopathologic variable of unproved prognostic significance. In a recent study, absence of TILs in melanomas was associated with a sixfold higher probability of metastases in the sentinel lymph nodes[906]; however, depth of invasion of the primary melanoma, not the TILs status, was prognostic of survival of the patients.

Colorectal and Ovarian Cancers

Relapse of colorectal cancer (stage Duke B or C) ranges between about 25% to 70% in patients who had the cancer surgically removed. Histopathologic and gene expression analysis of leukocyte infiltrates in primary colorectal cancers can apparently predict recurrence as well as survival. Cancers with dense CD45R+ CD8+ T-cell infiltrates have a better prognosis at any stage, whereas patients with low numbers of T cells regardless of stage have more recurrences.[907] Inflammatory cells, such as macrophages or lymphocytes, may look morphologically identical, but secrete different cytokines that have opposite effects on tumor growth. This may be the reason why gene expression analyses can serve as a better indicator of the functional properties of immune infiltrates in cancers.[908] Current work suggests that these analyses may provide better prognostic indicators in colorectal cancers than conventional TNM staging. Different expression profiles may reflect differences in cancer cell-intrinsic oncogenes/regulatory pathway usage. This may result in different cytokines, chemokines, and other factors being released that attract different types of lymphocytes and innate immune cells. The incidence of primary colon cancer virtually remains unchanged in renal transplant patients compared to virally related cancers.[909]

In ovarian cancers, the presence of intratumoral T cells is correlated with delayed recurrence of cancer and delayed death.[910] Absence of intratumoral T cells is correlated with increased levels of VEGF. Furthermore, recruitment of T_{reg} cells predicts reduced survival.[557,763] Again, differences between different ovarian cancers could be due to the use of different oncogenes/regulatory pathways resulting in different aggressiveness. Ovarian cancers are not increased in immunosuppressed organ transplant patients.[909]

Prognostic Significance of "Regression" and Vitiligo

Much research continues to be stimulated by the observation that regression of melanomas can occur, spontaneously fostering the idea that immunosurveillance or immunologic destruction is responsible.[911,912] However, regression of a melanoma is usually partial, rarely total, and usually involves its horizontally growing (radial), intraepidermal part while the major predictor of survival is the extent of vertical growth (ie, depth of invasion). The prognosis is excellent for melanomas <1 mm in depth (thickness) but much worse if the cancer is ≥1 mm in thickness. In a recent analysis of over 2,000 melanomas, regression neither predicted the likelihood of lymph node metastases nor survival of the patient.[913] Possibly, partial destruction of a malignant lesion may result from infiltrative growth into the surrounding tissue and destruction of that part of the tumor's blood supply.

Vitiligo (a depigmentation of normal skin) has many causes including an autoimmune etiology.[914] Though vitiligo is frequently induced experimentally or clinically as during treatment targeting melanocyte differentiation antigens,[364,731,733,915,916] it is not predictive nor needed for success of eradicating the melanoma.[917,918]

Inflammatory Responses to Tumor Growth

Experimental as well as clinical studies are now producing a rather coherent mechanistic

picture of the significance of systemic inflammatory responses in tumor growth. About 30% of patients with solid tumor have elevated granulocytes in their peripheral blood (> 8,000/μl), and granulocytosis is common in tumor-bearing mice.[871,883,919,920,921,922,923,924,925] It is long known that systemic effects, such as splenic enlargement,[926] frequently accompany local growth of autochthonous or transplanted murine tumors. Although many tumors commonly used in experiments have been serially passed hundreds or more times through euthymic mice,[239] a similar enlargement is also observed in response to growth of primary autochthonous tumors in nontransgenic mice.[927] Enlargement is mostly caused by increased myeloid hematopoiesis with macrophages, monocytes, and neutrophils accounting for more than half of the splenocytes.[770,928] G-CSF,

GM-CSF, and/or IL-6,[871,876,878,883,929,930] released by cancers, may be particularly important in inducing the systemic effects. STAT3 is persistently activated in many types of cancers, particularly in cancer cells at the invasive edge of tumors adjacent to inflammatory cells.[849] IL-6 preferentially activates STAT3,[931] causing cancer cells to resist apoptosis, sustain angiogenesis, and suppress acute STAT1-driven inflammation[849,931,932] and destructive T-cell responses.

G-CSF plays a central role in neutrophil production. Importantly, G-CSF also induces stem cell mobilization by upregulating CXCR4 and decreasing its ligand SDF-1 (CXCL12) in the bone marrow,[154,933] spleen, and local[86,88] reservoirs for progenitors. Degradation of SDF-1 in the progenitor/stem cell reservoirs is probably a result of G-CSF-induced granulocytosis that causes an increase of the neutrophil-derived proteases (TIMP-free matrix metalloproteinase-9 and neutrophil elastase, specific products of neutrophils that degrade SDF-1[934,935,936]). Mobilization of progenitors expressing CXCR4, the receptor for SDF-1, includes the mesenchymal, hematopoietic, and angiopoietic progenitors and T_{reg} cells.[146,937] Attraction of these cells into the tumor stroma of a neoplastic lesion occurs along a chemotactic gradient and depends on high local concentrations of SDF-1 produced by cancer cells[879,880,938] and tumor-stromal myofibroblasts.[939,940]

Given these mechanisms, it is not surprising that elevated neutrophil blood counts, neutrophil/lymphocyte ratio, matrix metalloproteinase-9, and plasma levels of C-reactive protein have been described as prognostic indicators of recurrence and reduced survival.[941,942,943,944,945] However, systemic responses to cancer vary greatly between individuals depending on stage and type of cancer. Even when they are not detectable, the mechanistic loops outlined previously are likely needed locally for every malignant growth.

Facilitation or Inhibition of Metastasis

A systemic increase in circulating factors such as IL-6 and progenitor cells may facilitate metastasis by creating “metastatic niches” (ie, a stroma in which disseminated cancer cells can successfully engraft).[629,946,947,948,949,950,951,952,953,954] Experimentally, there is little evidence that facilitation of metastasis (or secondary tumors developing at distant sites) is antigen-specific.[757,884,955] However, a primary transplanted tumor may suppress the growth of a second inoculation with the same tumor.[261] The phenomenon can be caused by immunologic as well as nonimmunologic mechanisms. The phenomenon is therefore properly referred to as “concomitant tumor resistance.”[956] Following surgical removal of the primary tumor, accelerated seeding and/or growth of metastases will occur, obviously a troubling observation for patients undergoing cancer surgery. This phenomenon, first described a hundred years ago,[957] occurs in different species and in several tumor models.[958] As would be expected, the primary tumor during its growth must inhibit metastasis possibly by usurping most available progenitor cells and/or consuming growth factors and nutrients (similar to what Ehrlich described nutrient deprivation or athrepsia[261]) and/or by producing antimitotic factors.[959] There is little evidence whether or not cancer patients inhibit metastasis by

antigen-specific immunity. Concomitant immunity refers to the observation that an individual bearing a primary transplanted tumor may be resistant to secondary challenge with the same tumor at a different location because of an antigen-specific immune response.[958] However, this old extensively studied observation[958,960,961,962] may well be the result of artificially priming the host by tumor transplantation.

IMMUNOPREVENTION

There is convincing evidence that immunosurveillance can prevent or reduce the incidence of cancers associated with certain viruses. Therefore, active immunization against viral capsid proteins may prevent infection and thereby cancer induction. While this is expected, it is still too early to confirm this with results. However, a federally funded, extensive vaccination program began in Australia in April of 2007 with a quadrivalent HPV vaccine to provide protection against the high-risk HPV types 16 and 18, which cause cervical cancer, and also low-risk types 6 and 11, which cause genital warts.[963,964] There has been a highly significant decline in the diagnoses of genital warts and a significant decrease in high-grade cervical abnormalities only 3 years after implementation of the program.[963] The latency period for cervical cancer is 15 to 25 years; therefore, evidence for a decline in cancer incidence cannot be expected until about 2027. Furthermore, only 70% of cervical cancers are caused by the HPV types that are in the present vaccine; thus, this vaccine can be expected to prevent only about 70% of cervix cancers. The effect might be somewhat higher because of cross-reactivity with the other HPV types, which may provide cross-protection with viral types 31, 33, and 45. Therapeutic HPV vaccines for treating already existent persistent infections or advanced cervical lesions are needed.[965] The current vaccine has no effect against HPV infection once it has been acquired and must therefore be given before onset of sexual activity. Remarkably, there is a therapeutic vaccine that seems to be effective against HPV-induced vulvar preneoplastic lesions.[966] The same vaccine has not been shown to be effective against high-grade premalignant cervical lesions. In general, premalignant lesions often persist for a very long time; destroying these lesions should prevent the development of cancer.

After introduction of hepatitis B virus vaccines, a decline in the incidence and prevalence of hepatitis B virus infection occurred[967]; this should eventually lead to a decline of chronic hepatitis and hepatocellular carcinoma. Several strategies for vaccination against hepatitis C virus, human T-lymphotropic virus 1, and human herpesvirus 8 are being developed. Also, developing vaccines against *Helicobacter pylori* and *Schistosoma* infections remains extremely important. The major influences of diet and microbial flora on the incidence of colorectal cancer and probably other cancers suggest new approaches for immunoprevention of these cancers.

It will be important to determine whether cancer can also be prevented by active immunization of cancer-prone individuals with predisposing inherited or acquired antigens resulting from mutation (eg, in K-ras[968]). An ever-increasing

number of predisposing inherited or acquired mutations are being identified; however, inducing immune responses against them may be problematic. For example, active immunization against an oncogenic viral protein became ineffective in preventing cancer when the immunization was begun in the later part of the latency period[228,799,969,970] or after the oncogenic protein was expressed in premalignant host tissues[301,722,971] for reasons that are poorly understood. Finally, we need to avoid stimulating cancer development when vaccinating cancer-prone individuals.[341]

IMMUNOTHERAPY

General Aspects

Multiple immunotherapeutic strategies involving innate or acquired immunity have been developed to control cancer; they include 1) local application of a live bacterial vaccine, BCG

(see discussion under Cancer and Inflammation); 2) use of cytokines; 3) active immunization; 4) passive therapy with antibodies; and 5) adoptive transfer of effector cells. Some of these strategies are being combined with other forms of cancer treatment. It is important to realize that chemo- and radiation therapy can synergize with or antagonize innate or adaptive immunity dependent on timing and sequence of the treatments. These important issues have been described and discussed elsewhere[539,972,973,974,975,976] and previously in this chapter. But why are so few immunotherapies effective or the treatment of choice, except for melanoma and superficial bladder cancer? Extensive recent reviews of immunotherapy of cancers expose some complementary but several differing viewpoints.[306,323,324,977,978,979,980] There is also an astounding repetition of findings made decades ago, and a tendency of overstating the translational potential of new findings.[4,143] As already discussed (see Fig. 47.1), the focus of current experimental research remains treating very small transplanted lesions in mice < 2 weeks after cancer cell inoculation.[4,633,981] Very few publications report immunotherapy in animals with cancers of clinically relevant size (≥ 10^9 cancer cells and ~1 cm average diameter) and duration of growth (> 14 days). Adoptive T-cell therapy and anti-CD20 antibody treatments were singled out as experimental therapies effective in causing regression at this stage (Fig. 47.6). Indeed, adoptive transfer of T cells (with endogenous or transduced TCRs) may be effective with longer established tumor loads.[977,982,983] Because both of these therapies can also be effective in humans, there is no "disconnect" between preclinical models and clinical experience.[4,107] Relapse after therapy, the main problem of cancer treatments, is rarely

considered; in fact, most animal studies break off before relapse could occur.

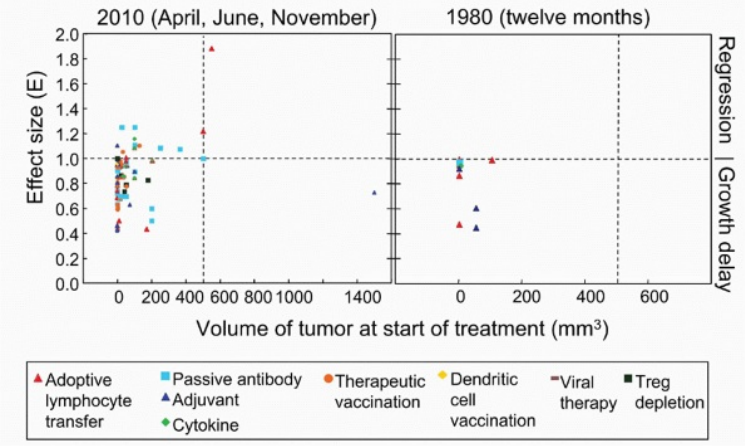

FIG. 47.6. Analysis of All Experimental Cancer Immunotherapy Publications Listed in PubMed for April, June, and November of 2010 and of the Entire Year 1980. Most experimental immunotherapies published treat small tumors (< 500 mm^3) yet succeed only at slowing or delaying tumor growth. In several 2010 reports, larger tumors are being treated than in 1980, and a few reports indicate tumor regression. An effect size (E) of 1 indicates the treatment arrested tumor growth. An E < 1 indicates that the treated tumor still grew progressively but slower than control. An E > 1 indicates tumor regression. Regression of tumors > 200 mm^3 is observed only after passive antibody or adoptive T-cell therapy. Modified from Wen et al.[4]

Therapeutic Vaccinations

Active immunization of cancer-bearing mice or humans is a heroic approach that was abandoned in the clinical management of infectious diseases, except for rabies, which has very long incubation period.[984,985] Cancer cells have a much slower generation time than

most infectious organisms, and most of the bulk of the tumor load can usually be removed by other therapy (eg, surgery). At the time when the antigen load is lowest, the suppressive environment may be removed, and conceivably active immunization might lead to an effective immune response. However, persistent antigen appears to be a major reason why cancers and chronic infectious diseases are ineffectively treated by therapeutic vaccination. Possibly, combining active immunization with blockade of immunoinhibitory pathways may lead to more effective therapeutic vaccinations.[965]

Certain cancer cells may be fully sensitive to tumor-specific T cells but for various reasons fail to induce a response. The reasons for the poor immunogenicity may vary; therefore, different methods must be used for different cancers to immunize effectively.[492] Currently, most methods have been developed in tumor-free mice (preventive/prophylactic vaccination).[986] Immunization with small numbers of viable tumor cells may cause cancer, but, if not, it can stimulate solid long-lived memory immunity that prevents growth. Dead and disrupted tumor cells, membrane fractions, or cell extracts may enhance the growth of the cancer, although they stimulate “immune” (ie, lymphocyte) responses. Destroying the proliferative potential of the tumor cells, while leaving the cells viable and metabolically active, may result in a prolonged exposure to the antigen that allows T-cell immunity to develop.[987] There are no general rules for accomplishing this, but exposing cancer cells to gamma radiation or certain cytostatic chemicals, such as mitomycin C, can work. However, chemotherapy makes cancer cells more immunogenic when they can undergo autophagy.[986] In any case, chemotherapy of transplantable tumors is more efficient in immunocompetent than in immunodeficient mice.[988]

Because these methods alone are often insufficient to elicit a cytolytic T-cell response to cancer cells, many strategies including genetic engineering have been designed to increase the immunogenicity of the tumor-cell inoculum and/or stimulate innate immunity at the site of vaccinations by the use of chemical and/or bacterial agents. However, irrespective of which particular genetic engineering of the tumor cells is used, rejection of the modified tumor cells is often followed by T cell-mediated immunity against the unmodified tumor cells. Methods include infecting cancer cells with certain viruses[989,990,991]; somatic cell fusion with various nontumorigenic cells[992,993,994]; transfection of self- or foreign MHC class I or class II molecules[995,996,997]; hapten conjugation[998,999]; exposure to mutagens[1000]; transfection of tumor cells to express the B7 ligand that can provide a costimulating signal to T cells[1001,1002]; attracting secondary lymphoid structures to the cancer[1003]; targeting herpesvirus entry mediator pathways[965]; combining tumor cells with killed bacteria, such as *Corynebacterium parvum*[1004]; recombinant vaccines of antigen expressed by vaccinia, listeria, or virus-like particles[2,1005]; transfection of tumor cells to produce certain cytokines such as IL-2, IFNγ, IL-4, IL-6, IL-7, G-CSF, GM-CSF, or TNF-α[134,138,141,535,1006]; injecting naked DNA constructs encoding the tumor antigen (whereby the gene for GM-CSF may also be used to recruit dendritic cells)[456,1007,1008,1009]; vaccination with anti-idiotypic antibodies, which bear the internal image of a tumor antigen[1010]; inhibiting extracellular adenosine triphosphate-degrading enzymes to increase autophagy[986]; transfection of tumor cells to express antisense RNA of a required growth factor thereby inducing terminal differentiation[1011]; peptide vaccines combined with blocking IFNγ action[1012,1013] with peptides long enough not to tolerize[987,1013,1014]; loading peptides to heat-shock protein[1015]; siRNA-mediated inhibition of nonsense-mediated messenger RNA decay,[1016] which may trigger innate immunity[1017] or the expression of new epitopes to which the host is not tolerant; and finally dendritic cells[323,1018,1019] that can be loaded with 1) synthetic antigenic peptides, 2) recombinant proteins, 3) native peptides stripped from tumor cell surfaces, 4) tumor-derived, peptide-loaded heat-shock proteins, 5) tumor-derived messenger RNA, or 6) by fusion of tumor cells. One advantage of the latter three strategies is that immunity to (unique) individually distinct tumor antigens, as well as tumor-associated antigen

may be induced without having to identify the antigens. The limitation is that the antigen dose cannot be standardized. Vaccines are also being used that target the mutant epidermal growth factor receptor EGFRvIII, a truly tumor-specific antigen on the surface of human malignant glioma cells capable of inducing strong B- and T-cell responses. The goal is to prolong patients' relapse-free survival because this cancer, the most common primary brain malignancy, is untreatable by conventional therapy.[305] The mutation in the EGFRvIII occurs in about 40% of the glioblastomas and represents an internal deletion in the gene encoding the receptor.[1020] About half of the patients with this variant receptor have the same deletion, which generates a new amino acid at the fusion point of the resulting fusion protein and new antigenic determinant recognized specifically by a monoclonal antibody.[304]

A healthy skepticism is needed to stimulate experiments determining whether therapeutic effects of active immunization are found when the experimental tumor is not in early stages of malignant growth (see Figs. 47.1 and 47.7). Until then, it remains uncertain whether any of these procedures will be effective against longer established or advanced stages of cancer including microdisseminated cancer cells.[382,985]

Therapy with Engineered Antibodies

Targeting Cancer Cells Directly

General Considerations. The major alternatives to therapeutic vaccinations are antibody therapy and adoptive transfer of tumor-specific T cells. As already mentioned, there is little evidence that antibody produced by the host

in response to autochthonous or transplanted cancers has any beneficial effects on the growing tumor. However, B-cell tolerance to self-antigens on human cancers can be overcome by immunizing mice with xenogeneic human antigen.[1021] Thus, high-affinity antixenogeneic antibodies to self-antigens such as human Epcam, FAP, CD20, CD19, HER-2, and various other receptors and growth factors have been made.[1022] Misleadingly, some of these high-affinity antixenogeneic antibodies are usually advertised as “fully human” when they were made in mice in which the murine Ig gene locus had been inactivated and replaced with the human Ig gene locus.[346] The toxicities of such “fully human” antibodies will still be those of high-affinity anti-self-antibody. Severe toxicity and lethal reactions may only occur once the destructive capabilities of the antibody are increased further by engineering (see following discussion).[204,206]

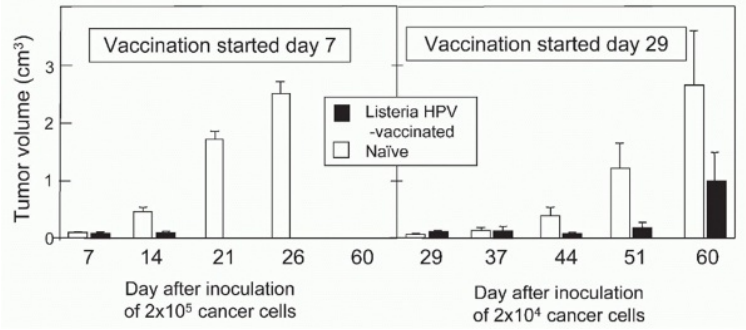

FIG. 47.7. Tumors can Escape from Therapeutic Vaccinations Once Truly Established. Then, therapeutic vaccinations become ineffective even against small tumors. Note the volumes of the tumors at start of therapeutic vaccinations, at day 7, or at day 29, respectively, are virtually the same size. However, only when vaccinations against were started early after TC1 cancer cell inoculation, tumors are eradicated and outgrowth prevented. †, mice in the naïve group were sacrificed. For human papillomavirus-16 E7 listeria, vaccine LLO-E7 leads to production of a fusion protein containing the complete E7 sequence.[1,2] TC-1 cancer cells are HPV16 E6 and E7 and

Ha-Ras transduced cell.[6] Unpublished; courtesy of Zhen-Kun Pan and Yvonne Paterson, University of Pennsylvania, Philadelphia, PA, USA.

Some antibodies can be relatively effective and useful in the therapy of selected human cancers, but rarely curative. To increase the clinical efficacy, scientists have searched to combine the specificity conferred by antibodies with the destructive potential of T cells. Two ideas, published decades ago,[347,1023,1024] led to reagents that are in clinical use[195,196,1025] and have little toxicity, but only if appropriate targets are used.

The first approach makes bispecific molecules linking the cancer-reactive antibody (or its variable regions) with an antibody (or its variable regions) binding to and activating T cells (eg, anti-CD3).[1023,1024] The second approach uses CARs consisting of the cancer-reactive variable region fused with transmembrane and signaling domains that are then transduced into T cells.[347]

Considerable technologic efforts are being made to enhance the ability of antibodies to kill tumor cells by using them as carriers for cytokines or cytotoxic agents, such as radiochemicals or natural toxins.[1026,1027,1028,1029,1030,1031] The recombinant antibody-cytokine or antibody-toxin fusion proteins may be useful to concentrate these agents in the stroma surrounding the tumor cells, but some of these coupled antibodies may have serious toxicity unless selective delivery of the conjugates to the tumor is achieved,[1032] a problem shared with bispecific or trispecific antibodies that bind effector cells as well as tumor antigens.[1033]

Cluster of Differentiation 20 and Cluster of Differentiation 19 **.** Monoclonal antibodies against B-cell lineage-specific differentiation antigens such as CD20 or CD19 are highly effective in preclinical models and can destroy large established B-cell lymphomas in patients,[1034] though relapse is common. However, when anti-CD20 or anti-CD19 are combined with anti-CD3 antibody or their variable regions are chimerized and used as CARs, patients with chronic lymphocytic leukemia or other B-cell malignancies may be cured.[195,196] Depletion of normal B cells occurs regularly but can be tolerated by giving intravenous Ig to the patient. Unfortunately, the antigens CD20 and CD19 are currently the exceptions rather than the rule. Other self-antigens exclusively expressed on cancer cells and only dispensable normal cells of the patient have not been identified.

HER-2. Anti-HER-2 antibodies are effective and useful in the therapy of some cancers (see following discussion). Treatment may be tolerated relatively well because the most

important effect may be inhibition by blocking signaling rather than destruction of cells.[1035,1036] Thus, antibodies to HER-2 (anti-HER-2/erb-B2/neu antibody; trastuzumab) can inhibit tumor growth independent of perforin or FasL in mice but inhibition requires type I and II IFNs released by NK and CD8 T cells.[1037] The role of antibody-dependent cell-mediate cytotoxicity in the trastuzumab efficacy in HER2-positive breast cancer patients is unclear, because a recent large study[1038] failed to confirm the previously published association between therapeutic effectiveness of anti-HER-2 antibodies and FcR genetic polymorphisms reflecting high- or low-affinity Fc receptors. In mice, the effects of anti-HER-2 antibodies seem to depend also on the participation of innate and adaptive immunity of the host that may be abrogated when the antibody is combined with chemotherapy[1039]; the relevance of this finding for the clinic is still unknown. However, HER-2 is expressed at low levels in several vital human tissues, and severe toxicity and death have occurred when the antibody receptor was inserted as antigen-receptor for T cells as CARs.[204]

GD2 and Carcinoembryonic Antigen **.** Some therapeutic effects have also been observed in patients treated with other murine monoclonal antibodies,[1040,1041] and anti-GD2 ganglioside-specific CARs had moderate antitumor effects in patients with

neuroblastoma.[1042] GD2 is expressed in the human cerebellum and peripheral nerves; serious toxicity as observed with anti-HER-2 CARs may have not been observed with anti-GD2 CARs because of a lesser therapeutic potency of the latter. Certainly, another CAR targeting CEA expressed on cancers and normal epithelium of the gut caused severe toxicity in patients.[206]

Other Receptors and Growth Factors. Antibodies to the IL-2 receptors can cause tumor regression in patients with cutaneous T-cell lymphoma.[1043] Other examples of overexpressed growth factors and their receptors are VEGF and receptors and epidermal growth factor and its receptor-1 (EGFR-1). Treatments with antibodies to these factors and receptors are fraught with complications (eg, anti-VEGF [bevacizumab] therapy for example has been linked to cardiac failures, digestive tract perforations, and bleedings in the lungs[1044]).

Targeting Immunoregulatory Molecules

Major clinical and preclinical efforts utilize monoclonal antibodies specific for regulatory and activation molecules expressed on T cells (anti-CTLA4, anti-PD-1 and anti-CD137 [4-1BB][1045,1046,1047]) and on cancer and normal host cells (eg, antibodies to ligands of PD-1, PD-L1, and L2[1048]). Monoclonal antibodies to the regulatory molecule CTLA-4 counteract tolerance and have antitumor effects in some but not in all tumor models.[1045,1049,1050] Severe autoimmunity may be a complication of this treatment. This subject is extensively reviewed elsewhere.[980,1051,1052]

Cytokines

The action of cytokines is essential for immunotherapy. Cytokines act in diffusion-limited spaces, but, when they act systemically, they may be toxic or lethal as in the Shwartzman-like shock reaction caused by systemic release of TNF-α[837,1053,1054] Mice surviving systemic TNF-α show a remarkable necrosis in the center of very large solid tumors of many different types of cancers.[619] Unlike the tumor neovasculature, the preexisting vessels at the tumor margin are resistant to TNF-α, and residual cancer at the viable margins gives rise to relapse. “Cytokine storm” caused by systemic high-dose anti-CD3 antibodies is mediated largely by TNF-α and can be lethal. Intratumoral injections of genetic vectors encoding TNF-α or other cytokines have some restricted applicability.[1055] Intravenous infusions of high doses of IL-2 induce clinical responses in a very small number of patients with a few types of cancers but have substantial side effects that need close medical supervision.[1056] Infusions of IL-2 are also used to amplify the effects of adoptively transferred T cells; the effect of IL-2 is indirect through action on T cells, NK cells, endothelial cells, and by inducing other cytokines. IL-2 effects on endothelial cells may cause vascular leakage syndrome that may allow better extravasation of transferred T cells into tumors. Clinical trials with another powerful cytokine, IL-15, are beginning in patients with cancer and human immunodeficiency virus.[1057]

Adoptive T-Cell Therapy

Seminal studies by Mitchison showed adoptive transfer of lymphocytes, not serum, produced resistance to allogeneic tumor transplants.[531,532,533] This was confirmed using syngeneic tumor transplants.[229,269,321,1058] Lymphocytes, either injected systemically or mixed with the cancer cell suspension and inoculated subcutaneously (Winn-type assay; see Assays to Study Effector Mechanisms in Vivo), protected against outgrowth of cancers while serum was ineffective. However, lymphocytes were as ineffective as serum in a therapeutic setting. Adoptive T-cell therapy of cancers was pioneered by Fefer, later working with Cheever, Greenberg, and Gillis, using established maize streak virus-induced tumors or Friend leukemia as a models.[471,766,767,1059,1060,1061,1062] Critical for the advance was recognizing the need for transient lymphodepletion of the recipient and transiently culturing and expanding the T cells in vitro with the help of IL-2 before transfer to recipients.

The use of TILs for adoptive T-cell therapy was pioneered by Rosenberg and coworkers at the National Institutes of Health.[1063] In preclinical models, TILs were effective even when given about 2 weeks after intravenous cancer cell inoculation.[1063] Adoptive cell transfer of autologous tumor-infiltrating lymphocytes was effective in ~50% of the patients with metastatic melanoma, but only few of the patients qualified for the treatment.[318,1064] Melanomainfiltrating lymphocytes were expanded in vitro with IL-2 and then infused into patients who receive IL-2 as well. Because the TIL response is dominated by T cells to unique tumor-specific antigen,[303] it is likely that the success of the reinfused T cells depends on their reactivity to unique tumor-specific antigens. Unfortunately, it is unclear how effective this personalized therapy is in patients with cancers other than melanoma.

Allogeneic EBV nuclear antigen-specific T cells sharing major MHC allele restriction with the patient can be used

to treat successfully posttransplant lymphoproliferative disease[1065] and EBV-positive lymphomas.[1066,1067] Such antigen-specific allogeneic T cells proved to be safe[1068] and effective.[1069] However, detailed knowledge of the molecular biology and immunology of EBV were critical to establish procedures that drive out EBV-specific T cells while losing alloresponsiveness.[1070] The donors did not need to be immunized with the patient's cancer cells. Instead, donor T cells containing EBV-specific precursors were primed specifically to EBV antigens using autologous EBV antigen-positive stimulators in vitro. Thus, donor T cells were never primed to the patient's alloantigens and were exclusively EBV-specific, although, as mentioned previously, donors and recipients need to be partially matched so that the EBV-specific T cells will be appropriately restricted. However for other cancers, immunization of the donor with the patient's cancer cells will be essential but not acceptable until proven to be without hazard to the donor and recipient. Much more work remains.

Rather than immunizing T-cell donors or cancer patients, current preclinical and clinical research focuses on the transfer of TCR or CAR genes into a patient's own T cells.[1071,1072,1073] These gene-modified T cells thereby gain specificity for cancer antigens and are reinfused. Recent studies in humans and mice are promising but also raise serious concerns.[204,208,1074,1075,1076,1077]

Preventing Relapse and Targeting Variants

As mentioned previously, experimental and human tumors have multiple tumor-specific CTL-recognized epitopes that are lost independently by selection with T cells in vitro.[218,278] However, in vivo, the host fails to recognize all antigens simultaneously on a cancer cell.[716,1078,1079] Recognition of a second antigen occurred only after the first antigen was lost by most of the tumor cells,[716,1079] even though the subdominant antigen can serve as effective target.[1080] This suggested that an immunodominant antigen prevents sensitization to other tumor antigens.[237,716,1079] A hierarchy in the immune response to multiple independent antigens has also been described in the study of immune responses to multiple minor histocompatibility antigens expressed on a single cell.[1081] The mechanism for the priority of the first response is unclear.[342,1082] However, understanding how to break the hierarchy could help prevent immune selection and tumor escape. For example, studies in vitro using CTL clones suggest that the rate of mutation resulting in the loss of a single antigen from the tumor cells is less than 10^{-6}. Even if the frequency were as high as 10^{-4}, only one tumor cell that had lost four independent antigens would be expected in 10^{16} tumor cells (ie, in a tumor larger than the human body).[278,1083] Thus, if the immune response of the host could be manipulated so that multiple antigens were attacked simultaneously, no escape of tumors should occur. Experimental evidence suggests that immunization with tumor-cell variants, selected in vitro and expressing selective antigenic components, can overcome immunodominance and prevent tumor escape.[1084] This will be important for

constructing vaccines.[1085]

Targeting Tumor Stroma and Bystander Killing

When complete surgical excision is no longer feasible, cancers usually contain variants resistant to numerous types of therapy. Whether killing of the overwhelming majority of antigen-positive cancer cells will result in the death of the few antigen-negative tumor cells "as bystanders" was studied over three decades ago with contradictory results.[1086,1087,1088] It is now clear that bystander killing of cancer variants can occur through stromal destruction and lead to the eradication of well-established solid tumors. This requires targeting tumorspecific antigens released from the cancer cells in the form of exosomes, microvesicles, and membrane fragments[1089,1090] that are cross-presented by stromal cells in the immediate tumor microenvironment. Antigen-specific, adoptively transferred T cells eradicate large tumors including variants by also targeting and destroying the tumor antigen cross-presenting stroma.[169,537,538,540,542] Pretreatment of the cancer by chemo-or radiotherapy can temporarily increase stromal loading and thereby increase the efficacy of cross-presentation and tumor eradication by subsequent adoptive T-cell therapy.[539] Thus, the therapy-resistant variants "caught" in the destroyed stroma are killed or die as bystanders in the necrotic stroma. Because cross-presentation depends on vicinity of stroma to the cancer cells, systemic toxicity has not been observed. No other antigens or molecules exist that are specific for stromal cells in cancers and not present on normal cells in the rest of the body. In contrast, other most intriguing and diverse approaches targeting antigens on stroma that are not cancerspecific[1091,1092,1093,1094,1095,1096] must overcome likely limitations in terms of toxicity and/or efficacy.[143]

EPILOGUE

Cancer immunology is at the interphase of two extraordinarily complex fields of research. Few fields of immunology have stimulated more emotional discussions. A healthy skepticism about the validity and relevance of experiments and concepts stimulates more experiments.[800] Differences in opinion do not harm scientific progress and should not be branded as attempts to "destroy the field." Any scientific field can only be strengthened by experiments in the search for truth.[1097] Opinions, but not truth, can be refuted.[1098] Many perceived "truths" have been refuted in the past, and this will likely also apply to parts of what is said in this chapter. The value of a scientist depends not on possessing real or perceived truth but his or her honest search for a better resolution of questions asked. Possession acquiesces and makes us proud and insensitive to opposing views of colleagues, but search for truth increases our strength to perfect our knowledge[1099] and find answers to the many important questions remaining in tumor immunology.

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REFERENCES

1. Gunn GR, Zubair A, Peters C, Pan ZK, Wu TC, Paterson Y. Two Listeria monocytogenes vaccine vectors that express different molecular forms of human papilloma virus-16 (HPV-16) E7 induce qualitatively different T cell immunity that correlates with their ability to induce regression of established tumors immortalized by HPV-16. *J Immunol*. 2001;167:6471-6479.

2. Guirnalda P, Wood L, Paterson Y. Listeria monocytogenes and its products as agents for cancer immunotherapy. *Adv Immunol*. 2012;113: 81-118.

3. Basombrio MA. Search for common antigenicities among twenty-five sarcomas induced by methylcholanthrene. *Cancer Res*. 1970;30: 2458-2462.

4. Wen FT, Thisted RA, Rowley DA, Schreiber H. A systematic analysis of experimental immunotherapies on tumors differing in size and duration of growth. *Oncoimmunology*. 2012;1:172-178.

5. Basombrio MA, Prehn RT. Studies on the basis for diversity and time of appearance of antigens in chemically induced tumors. *Natl Cancer Inst Monogr*. 1972;35:117-124.

6. Lin KY, Guarnieri FG, Staveley-O'Carroll KF, et al. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res*. 1996;56:21-26.

7. Gorer PA. The genetic and antigenic basis of tumour transplantation. *J Pathol Bacteriol*. 1937;44:691-697.

8. Gorer PA. Some recent work on tumor immunity. *Adv Cancer Res*. 1956;4:149-186.

9. Rowley JD. A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature*. 1973; 243:290-293.

10. Monach PA, Meredith SC, Siegel CT, Schreiber H. A unique tumor antigen produced by a single amino acid substitution. *Immunity*. 1995;2:45-59.

11. Coulie PG, Lehmann F, Lethe B, et al. A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma. *Proc Natl Acad Sci U S A*. 1995;92:7976-7980.

12. Wölfel T, Hauer M, Schneider J, et al. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. *Science*. 1995;269:1281-1284.

13. Stricker TP, Kumar V. Neoplasia. In: Kumar V, Abbas AK, Fausto N, Aster J, eds. *Robbins & Cotran Pathologic Basis of Disease*. 8th ed. Philadelphia, PA: WB Saunders; 2009:259-330.

14. Mueller NE, Birmann BM, Parsonnet J, Schiffman MA, Stuver SO. *Infectious Agents. Cancer Epidemiology and Prevention*. New York, NY: Oxford University Press; 2006:507-548.

15. Zur Hausen H. HPV vaccines: what remains to be done? Interview by Lauren Constable. *Expert Rev Vaccines*. 2011;10:1505-1507.

16. Schottenfeld D, Fraumeni JF Jr. *Cancer Epidemiology and Prevention*. New York, NY: Oxford University Press; 2006.

17. Williams GM, Iatropoulos MJ, Weisburger JH. Chemical carcinogen mechanisms of action and implications for testing methodology. *Exp Toxicol Pathol*. 1996;48:101-111.

18. Ward JF. Radiation mutagenesis: the initial DNA lesions responsible. *Radiat Res*. 1995;142:362-368.

19. Uren AG, Kool J, Berns A, van Lohuizen M. Retroviral insertional mutagenesis: past,

present and future. *Oncogene*. 2005;24:7656-7672.

20. Fletcher O, Houlston RS. Architecture of inherited susceptibility to common cancer. *Nat Rev Cancer* . 2010;10:353-361.

21. Shubik P. Current status of chemical carcinogenesis. *Proc Natl Acad Sci U S A*. 1972;69:1052-1055.

22. Berenblum I, Shubik P. A new, quantitative, approach to the study of the stages of chemical cartinogenesis in the mouse's skin. *Br J Cancer* . 1947;1:383-391.

23. Berenblum I, Shubik P. The persistence of latent tumour cells induced in the mouse's skin by a single application of 9:10-dimethyl-1:2- benzanthracene. *Br J Cancer* . 1949;3:384-386.

24. Berenblum I. A re-evaluation of the concept of cocarcinogenesis. *Prog Exp Tumor Res*. 1969;11:21-30.

25. Franklin WA, Gazdar AF, Haney J, et al. Widely dispersed p53 mutation in respiratory epithelium. A novel mechanism for field carcinogenesis. *J Clin Invest*. 1997;100:2133-2137.

26. Husemann Y, Geigl JB, Schubert F, et al. Systemic spread is an early step in breast cancer. *Cancer Cell* . 2008;13:58-68.

27. Zeki SS, Graham TA, Wright NA. Stem cells and their implications for colorectal cancer. *Nat Rev Gastroenterol Hepatol* . 2011;8:90-100.

28. Skipper HE. Cancer chemotherapy is many things: G.H.A. Clowes Memorial Lecture. *Cancer Res*. 1971;31:1173-1180.

29. Skipper HE, Schabel FM. Quantitative and cytokinetikc studies in experimental tumor models. In: Holland JF, ed. *Cancer Medicine*. Philadelphia, PA: Lea and Febiger; 1973:629-650.

30. Kelly PN, Dakic A, Adams JM, Nutt SL, Strasser A. Tumor growth need not be driven by rare cancer stem cells. *Science*. 2007;317:337.

31. Taupier MA, Kearney JF, Leibson PJ, Loken MR, Schreiber H. Nonrandom escape of tumor cells from immune lysis due to intraclonal fluctuations in antigen expression. *Cancer Res*. 1983;43:4050-4056.

32. Gupta PB, Fillmore CM, Jiang G, et al. Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells. *Cell*. 2011;146:633-644.

33. Rous P, Beard JW. The progression to carcinoma of virus-induced rabbit papillomas (Shope). *J Exp Med*. 1935;62:523-528.

34. Shubik P. Reflections on the implications of multistage carcinogenesis for the nature of neoplasia. *Food Chem Toxicol* . 2002;40:739-742.

35. Foulds L. The experimental study of tumor progression. A review. *Cancer Res*. 1954;14:327-339.

36. Nowell PC. The clonal evolution of tumor cell populations. *Science*. 1976;194:23-29.

37. Bodmer W. The somatic evolution of cancer. The Harveian Oration of 1996. *J R Coll Physicians Lond*. 1997;31:82-89.

38. Neesse A, Michl P, Frese KK, et al. Stromal biology and therapy in pancreatic cancer. *Gut*. 2011;60:861-868.

39. Virchow R. Zur Entwickelungsgeschichte des Krebses nebst Bemerkungen über Fettbildung im thierischen Körper und pathologische Resorption. *Archiv für pathologische Anatomie und Physiologie und für klinische Medicin*. 1847;1:94-204.

40. Ehrlich P. Experimentelle Studien an Mäusetumoren. *Z Krebsforsch*. 1907;5:59-81.

41. Borst M. *Allgemeine Pathologie der malignen Geschwülste*. Leipzig, Germany: S. Hirzel; 1924.

42. Borst M. *Die Lehre von den Geschwülsten*. Volume 1. Wiesbaden: Verlag von J.F. Bergmann; 1902.

43. Pekarek LA, Starr BA, Toledano AY, Schreiber H. Inhibition of tumor growth by elimination of granulocytes. *J Exp Med*. 1995;181:435-440.

44. Seung LP, Rowley DA, Dubey P, Schreiber H. Synergy between T-cell immunity and inhibition of paracrine stimulation causes tumor rejection. *Proc Natl Acad Sci U S A* . 1995;92:6254-6258.

45. Seung LP, Seung SK, Schreiber H. Antigenic cancer cells that escape immune destruction are stimulated by host cells. *Cancer Res*. 1995;55:5094-5100.

46. Rous P. An experimental comparison of transplanted tumor and a transplanted normal tissue capable of growth. *J Exp Med*. 1910;12:344-366.

47. Singh S, Ross SR, Acena M, Rowley DA, Schreiber H. Stroma is critical for preventing or permitting immunological destruction of antigenic cancer cells. *J Exp Med*. 1992;175:139-146.

48. Willis RA. The stroma of tumours. In: Willis RA, ed. *The Spread of Tumours in the Human Body*. London, United Kingdom: Butterworth & Co Ltd; 1952:115-123.

49. Chang HY, Nuyten DS, Sneddon JB, et al. Robustness, scalability, and integration of a wound-response gene expression signature in predicting breast cancer survival. *Proc Natl Acad Sci U S A*. 2005;102: 3738-3743.

50. Chang HY, Sneddon JB, Alizadeh AA, et al. Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds. *PLoS Biol*. 2004; 2:E7.

51. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest*. 2002;110:341-350.

52. Bhowmick NA, Chytil A, Plieth D, et al. TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science*. 2004;303:848-851.

53. Rosty C, Ueki T, Argani P, et al. Overexpression of S100A4 in pancreatic ductal adenocarcinomas is associated with poor differentiation and DNA hypomethylation. *Am J*

Pathol. 2002;160:45-50.

54. Mazzucchelli L. Protein S100A4: too long overlooked by pathologists? *Am J Pathol*. 2002;160:7-13.

55. Ito K, Chen J, El Chaar M, et al. Renal damage progresses despite improvement of renal function after relief of unilateral ureteral obstruction in adult rats. *Am J Physiol Renal Physiol*. 2004;287:F1283-1293.

56. Inoue T, Plieth D, Venkov CD, Xu C, Neilson EG. Antibodies against macrophages that overlap in specificity with fibroblasts. *Kidney Int*. 2005;67:2488-2493.

57. Le Hir M, Hegyi I, Cueni-Loffing D, Loffing J, Kaissling B. Characterization of renal interstitial fibroblast-specific protein 1/S100A4- positive cells in healthy and inflamed rodent kidneys. *Histochem Cell Biol*. 2005;123:335-346.

58. Osterreicher CH, Penz-Osterreicher M, Grivennikov SI, et al. Fibroblastspecific protein 1 identifies an inflammatory subpopulation of macrophages in the liver. *Proc Natl Acad Sci U S A*. 2011;108:308-313.

59. Schwann T. *Microscopical Researches into the Accordance in the Structure and Growth of Animals and Plants*. London, United Kingdom: Sydenham Society; 1947.

60. Paget J. *Lectures on Surgical Pathology Delivered at the Royal College of Surgeons of England, London*. London, United Kingdom: Longmans & others; 1863.

61. Dunphy JE. The fibroblast—a ubiquitous ally for the surgeon. *New Engl J Med*. 1963;268:1368-1377.

62. Bucher O. *Histologie und mikroskopische Anatomie des Menschen*. 4th ed. Bern, Switzerland: Medizinischer Verlag Hans Huber; 1965.

63. Ham AW, Cormack DH. *Histology*. 8th ed. Philadelphia, PA: JB Lippincott Co; 1979.

64. Kierszenbaum AL. *Histology and Cell Biology: An Introduction to Pathology*. St Louis, MO: Mosby; 2002:106-108.

65. Junqueira LC, Carneiro J. *Basic Histology. Text and Atlas*. New York, NY: Lange; 2003:95-125.

66. Gottrup F, Storgård Jensen S, Andreasen JO. Wound healing subsequent to injury. In: Andreasen JO, Andreasen FM, Andersson L, eds. *Textbook and Color Atlas of Traumatic Injuries to Teeth*. 4th ed. Oxford, United Kingdom: Blackwell Munksgaard; 2007:1-61.

67. Clark JG, Hilderbran JN. Fibroblast procollagen production rates in vitro based on [3H]hydroxyproline production and procollagen hydroxyproline specific activity. *Anal Biochem*. 1984;140:478-485.

68. Movahedi K, Laoui D, Gysemans C, et al. Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. *Cancer Res*. 2010;70:5728-5739.

69. Fridlender ZG, Sun J, Kim S, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: “N1” versus “N2” TAN. *Cancer Cell*. 2009;16:183-194.

70. Coffman RL, Carty J. A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-gamma. *J Immunol*. 1986; 136:949-954.

71. Fleming TJ, Fleming ML, Malek TR. Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte- differentiation antigen (Gr-1) detects members of the Ly-6 family. *J Immunol*. 1993;151:2399-2408.

72. Nagendra S, Schlueter AJ. Absence of cross-reactivity between murine Ly-6C and Ly-6G. *Cytometry A*. 2004;58:195-200.

73. Tepper RI, Coffman RL, Leder P. An eosinophil-dependent mechanism for the antitumor effect of interleukin-4. *Science*. 1992;257:548-551.

74. Daley JM, Thomay AA, Connolly MD, Reichner JS, Albina JE. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J Leukoc Biol*. 2008;83:64-70.

75. Bronte V, Wang M, Overwijk WW, et al. Apoptotic death of CD8+ T lymphocytes after immunization: induction of a suppressive population of Mac-1+/Gr-1+ cells. *J Immunol*. 1998;161:5313-5320.

76. Gabrilovich DI, Bronte V, Chen SH, et al. The terminology issue for myeloid- derived suppressor cells. *Cancer Res*. 2007;67:425, author reply 426.

77. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*. 2009;9:162-174.

78. Ostrand-Rosenberg S. Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity. *Cancer Immunol Immunother*. 2010;59:1593-1600.

79. Peranzoni E, Zilio S, Marigo I, et al. Myeloid-derived suppressor cell heterogeneity and subset definition. *Curr Opin Immunol*. 2010;22:238-244.

80. Yang L, Huang J, Ren X, et al. Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. *Cancer Cell*. 2008;13:23-35.

81. Reilkoff RA, Bucala R, Herzog EL. Fibrocytes: emerging effector cells in chronic inflammation. *Nat Rev Immunol*. 2011;11:427-435.

82. Hansemann D. Das Krebsstroma und die Grawitz'sche Theorie der Schlummerzellen. *Virchows Arch*. 1893;133:147-165.

83. Gothert JR, Gustin SE, Van Eekelen AM, et al. Genetically tagging endothelial cells in vivo: bone marrow-derived cells do not contribute to tumor endothelium. *Blood*. 2004;104:1769-1777.

84. Larrivee B, Niessen K, Pollet I, et al. Minimal contribution of marrowderived endothelial precursors to tumor vasculature. *J Immunol*. 2005; 175:2890-2899.

85. Udagawa T, Puder M, Wood M, Schaefer BC, D'Amato RJ. Analysis of tumor-associated stromal cells using SCID GFP transgenic mice: contribution of local and bone marrow-derived host cells. *FASEB J*. 2006;20:95-102.

86. Purhonen S, Palm J, Rossi D, et al. Bone marrow-derived circulating endothelial precursors do not contribute to vascular endothelium and are not needed for tumor

growth. *Proc Natl Acad Sci U S A* . 2008;105:6620-6625.

87. Aschoff L. Das reticulo-endotheliale system. *Ergebnisse der Inneren Medizin und Kinderheikunde*. 1924;26:1-118.

88. Crisan M, Yap S, Casteilla L, et al. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell*. 2008;3: 301-313.

89. Caplan AI. All MSCs are pericytes? *Cell Stem Cell*. 2008;3:229-230.

90. Rouget CMB. Sur la contractilité capillaires sanguins. *Comptes rendus de l'Académie des Sciences*. 1879;88:916-918.

91. Lamagna C, Bergers G. The bone marrow constitutes a reservoir of pericyte progenitors. *J Leukoc Biol*. 2006;80:677-681.

92. Grillo HC. Origin of fibroblasts in wound healing. An autoradiographic study of inhibition of cellular proliferation by local x-irradiation. *Ann Surg*. 1963;157:453-467.

93. Macdonald RA. Origin of fibroblasts in experimental healing wounds: autoradiographic studies using tritiated thymidine. *Surgery*. 1959;46:376-382.

94. Ross R, Everett NB, Tyler R. Wound healing and collagen formation. VI. The origin of the wound fibroblast studied in parabiosis. *J Cell Biol*. 1970;44:645-654.

95. Maximov A. Culture of blood leukocytes. From lymphocytes and monocytes to connective tissue. *Arch Exp Zellforsch*. 1928;5:169-268.

96. Bucala R, Spiegel LA, Chesney J, Hogan M, Cerami A. Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. *Mol Med*. 1994;1:71-81.

97. Phillips RJ, Burdick MD, Hong K, et al. Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. *J Clin Invest*. 2004;114:438-446.

98. Ishii G, Ito TK, Aoyagi K, et al. Presence of human circulating progenitor cells for cancer stromal fibroblasts in the blood of lung cancer patients. *Stem Cells*. 2007;25:1469-1477.

99. Sangai T, Ishii G, Kodama K, et al. Effect of differences in cancer cells and tumor growth sites on recruiting bone marrow-derived endothelial cells and myofibroblasts in cancer-induced stroma. *Int J Cancer* . 2005;115:885-892.

100. Direkze NC, Hodivala-Dilke K, Jeffery R, et al. Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts. *Cancer Res*. 2004;64:8492-8495.

101. LaRue AC, Masuya M, Ebihara Y, et al. Hematopoietic origins of fibroblasts: I. In vivo studies of fibroblasts associated with solid tumors. *Exp Hematol*. 2006;34:208-218.

102. De Wever O, Demetter P, Mareel M, Bracke M. Stromal myofibroblasts are drivers of invasive cancer growth. *Int J Cancer* . 2008;123:2229-2238.

103. Kerbel RS, Benezra R, Lyden DC, et al. Endothelial progenitor cells are cellular hubs essential for neoangiogenesis of certain aggressive adenocarcinomas and metastatic transition but not adenomas. *Proc Natl Acad Sci U S A* . 2008;105:E54, author reply E55.

104. Salven P, Purhonen S, Derrick Rossi D, Ylä-Herttuala S, Alitalo K, Weissman IL. Reply to Kerbel et al.: EPCs are again claimed to be essential in yet other models despite the irreproducibility of the original experiments introducing them. *Proc Natl Acad Sci U S A*. 2008;105:E55.

105. Jenkins SJ, Ruckerl D, Cook PC, et al. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science*. 2011;332:1284-1288.

106. Randolph GJ. Immunology. No need to coax monocytes. *Science*. 2011;332:1268-1269.

107. Schreiber K, Rowley DA, Riethmuller G, Schreiber H. Cancer immunotherapy and preclinical studies: why we are not wasting our time with animal experiments. *Hematol Oncol Clin North Am*. 2006;20:567-584.

108. Ochsenbein AF, Klenerman P, Karrer U, et al. Immune surveillance against a solid tumor fails because of immunological ignorance. *Proc Natl Acad Sci U S A* . 1999;96:2233-2238.

109. Lin EY, Nguyen AV, Russell RG, Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med*. 2001;193:727-740.

110. Rettig MB, Ma HJ, Vescio RA, et al. Kaposi's sarcoma-assoicated herpesvirus infection of bone marrow dendritic cells from multiple myeloma patients. *Science*. 1997;276:1851.

111. Sawyers CL. Where lies the blame for resistance—tumor or host? *Nat Med*. 2007;13:1144-1145.

112. Binder M, Lechenne B, Ummanni R, et al. Stereotypical chronic lymphocytic leukemia B-cell receptors recognize survival promoting antigens on stromal cells. *PLoS One*. 2010;5:e15992.

113. Nieman KM, Kenny HA, Penicka CV, et al. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nat Med*. 2011;17:1498-1503.

114. Russell JP, Shinohara S, Melillo RM, Castellone MD, Santoro M, Rothstein JL. Tyrosine kinase oncoprotein, RET/PTC3, induces the secretion of myeloid growth and chemotactic factors. *Oncogene*. 2003;22:4569-4577.

115. Russell JP, Engiles JB, Rothstein JL. Proinflammatory mediators and genetic background in oncogene mediated tumor progression. *J Immunol* . 2004;172:4059-4067.

116. Borrello MG, Alberti L, Fischer A, et al. Induction of a proinflammatory program in normal human thyrocytes by the RET/PTC1 oncogene. *Proc Natl Acad Sci U S A* . 2005;102:14825-14830.

117. Pufnock JS, Rothstein JL. Oncoprotein signaling mediates tumorspecific inflammation and enhances tumor progression. *J Immunol* . 2009;182:5498-5506.

118. Schreiber H, Rowley DA. Inflammation and cancer. In: Gallin JI, Snyderman R, eds. *Inflammation: Basic Principles and Clinical Correlates*. 3rd ed. Philadelphia, PA: Lippincott Williams & Wilkins; 1999:1117-1129.

119. Philip M, Rowley DA, Schreiber H. Inflammation as a tumor promoter in cancer induction. *Semin Cancer Biol* . 2004;14:433-439.

120. Coussens LM, Werb Z. Inflammation and cancer. *Nature*. 2002;420: 860-867.

121. Bissell MJ, Radisky D. Putting tumours in context. *Nat Rev Cancer* . 2001;1:46-54.

122. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol* . 2009;182:4499-4506.

123. Grivennikov S, Karin E, Terzic J, et al. IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell* . 2009;15:103-113.

124. Stuelten CH, Barbul A, Busch JI, et al. Acute wounds accelerate tumorigenesis by a T cell-dependent mechanism. *Cancer Res* . 2008;68: 7278-7282.

125. Owen JL, Iragavarapu-Charyulu V, Gunja-Smith Z, Herbert LM, Grosso JF, Lopez DM. Up-regulation of matrix metalloproteinase-9 in T lymphocytes of mammary tumor bearers: role of vascular endothelial growth factor. *J Immunol* . 2003;171:4340-4351.

126. Watanabe Y, Kuribayashi K, Miyatake S, et al. Exogenous expression of mouse interferon gamma cDNA in mouse neuroblastoma C1300 cells results in reduced tumorigenicity by augmented anti-tumor immunity. *Proc Natl Acad Sci U S A* . 1989;86:9456-9460.

127. Tepper RI, Pattengale PK, Leder P. Murine interleukin-4 displays potent anti-tumor activity in vivo. *Cell* . 1989;57:503-512.

128. Torre-Amione G, Beauchamp RD, Koeppen H, et al. A highly immunogenic tumor transfected with a murine transforming growth factor type beta 1 cDNA escapes immune surveillance. *Proc Natl Acad Sci U S A*. 1990;87:1486-1490.

129. Fearon ER, Pardoll DM, Itaya T, et al. Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. *Cell* . 1990;60:397-403.

130. Gansbacher B, Bannerji R, Daniels B, Zier K, Cronin K, Gilboa E. Retroviral vector-mediated gamma-interferon gene transfer into tumor cells generates potent and long lasting antitumor immunity. *Cancer Res* . 1990;50:7820-7825.

131. Blankenstein T, Li WQ, Uberla K, et al. Retroviral interleukin 5 gene transfer into interleukin 5-dependent growing cell lines results in autocrine growth and tumorigenicity. *Eur J Immunol* . 1990;20: 2699-2705.

132. Vink A, Coulie P, Warnier G, et al. Mouse plasmacytoma growth in vivo: enhancement by interleukin 6 (IL-6) and inhibition by antibodies directed against IL-6 or its receptor. *J Exp Med* . 1990;172: 997-1000.

133. Teng MN, Park BH, Koeppen HK, Tracey KJ, Fendly BM, Schreiber H. Long-term inhibition of tumor growth by tumor necrosis factor in the absence of cachexia or T-cell immunity. *Proc Natl Acad Sci U S A* . 1991;88:3535-3539.

134. Colombo MP, Ferrari G, Stoppacciaro A, et al. Granulocyte colonystimulating factor gene transfer suppresses tumorigenicity of a murine adenocarcinoma in vivo. *J Exp Med* .

1991;173:889-897.

135. Blankenstein T, Qin ZH, Uberla K, et al. Tumor suppression after tumor cell-targeted tumor necrosis factor alpha gene transfer. *J Exp Med*. 1991;173:1047-1052.

136. Blankenstein T, Rowley DA, Schreiber H. Cytokines and cancer: experimental systems. *Curr Opin Immunol* . 1991;3:694-698.

137. Platzer C, Richter G, Uberla K, Hock H, Diamantstein T, Blankenstein T. Interleukin-4-mediated tumor suppression in nude mice involves interferon-gamma. *Eur J Immunol* . 1992;22:1729-1733.

138. Noffz G, Qin Z, Kopf M, Blankenstein T. Neutrophils but not eosinophils are involved in growth suppression of IL-4-secreting tumors. *J Immunol* . 1998;160:345-350.

139. Wu TH, Pabin CN, Qin Z, et al. Long-term suppression of tumor growth by TNF requires a Stat1- and IFN regulatory factor 1- dependent IFN-gamma pathway but not IL-12 or IL-18. *J Immunol* . 2004;172: 3243-3251.

140. Blankenstein T. The role of tumor stroma in the interaction between tumor and immune system. *Curr Opin Immunol* . 2005;17:180-186.

141. Schuler T, Kornig S, Blankenstein T. Tumor rejection by modulation of tumor stromal fibroblasts. *J Exp Med* . 2003;198:1487-1493.

142. Olver S, Groves P, Buttigieg K, et al. Tumor-derived interleukin-4 reduces tumor clearance and deviates the cytokine and granzyme pro-file of tumor-induced CD8+ T cells. *Cancer Res* . 2006;66:571-580.

143. Engels B, Rowley DA, Schreiber H. Targeting stroma to treat cancers. *Semin Cancer Biol* . 2012;22:41-49.

144. Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. *Nat Rev Cancer* . 2008;8:618-631.

145. Barnas JL, Simpson-Abelson MR, Brooks SP, Kelleher RJ Jr, Bankert RB. Reciprocal functional modulation of the activation of T lymphocytes and fibroblasts derived from human solid tumors. *J Immunol* . 2010;185:2681-2692.

146. Pahler JC, Tazzyman S, Erez N, et al. Plasticity in tumor-promoting inflammation: impairment of macrophage recruitment evokes a compensatory neutrophil response. *Neoplasia*. 2008;10:329-340.

147. Shojaei F, Wu X, Malik AK, et al. Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells. *Nat Biotechnol* . 2007;25:911-920.

148. Shojaei F, Ferrara N. Refractoriness to antivascular endothelial growth factor treatment: role of myeloid cells. *Cancer Res* . 2008;68:5501-5504.

149. Shojaei F, Wu X, Qu X, et al. G-CSF-initiated myeloid cell mobilization and angiogenesis mediate tumor refractoriness to anti-VEGF therapy in mouse models. *Proc Natl Acad Sci U S A*. 2009;106: 6742-6747.

150. Bergers G, Brekken R, McMahon G, et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol* . 2000;2:737-744.

151. Belotti D, Paganoni P, Manenti L, et al. Matrix metalloproteinases (MMP9 and MMP2) induce the release of vascular endothelial growth factor (VEGF) by ovarian carcinoma cells: implications for ascites formation. *Cancer Res*. 2003;63:5224-5229.

152. Yang L, DeBusk LM, Fukuda K, et al. Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell*. 2004;6:409-421.

153. Mott JD, Werb Z. Regulation of matrix biology by matrix metalloproteinases. *Curr Opin Cell Biol*. 2004;16:558-564.

154. Petit I, Szyper-Kravitz M, Nagler A, et al. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat Immunol*. 2002;3:687-694.

155. Meredith JE Jr, Fazeli B, Schwartz MA. The extracellular matrix as a cell survival factor. *Mol Biol Cell*. 1993;4:953-961.

156. Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol*. 1994;124:619-626.

157. Blohm U, Potthoff D, van der Kogel AJ, Pircher H. Solid tumors “melt” from the inside after successful CD8 T cell attack. *Eur J Immunol*. 2006;36:468-477.

158. Kim SH, Turnbull J, Guimond S. Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. *J Endocrinol*. 2011;209:139-151.

159. Nelson CM, Bissell MJ. Of extracellular matrix, scaffolds, and signaling: tissue architecture regulates development, homeostasis, and cancer. *Annu Rev Cell Dev Biol*. 2006;22:287-309.

160. Ingber DE. Can cancer be reversed by engineering the tumor microenvironment? *Semin Cancer Biol*. 2008;18:356-364.

161. Menke A, Adler G. TGFbeta-induced fibrogenesis of the pancreas. *Int J Gastrointest Cancer*. 2002;31:41-46.

162. Folkman J, Klagsbrun M, Sasse J, Wadzinski M, Ingber D, Vlodavsky I. A heparin-binding angiogenic protein—basic fibroblast growth factor—is stored within basement membrane. *Am J Pathol*. 1988;130:393-400.

163. Nathan C, Sporn M. Cytokines in context. *J Cell Biol*. 1991;113: 981-986.

164. Kim S, Takahashi H, Lin WW, et al. Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. *Nature*. 2009;457:102-106.

165. Niu G, Heller R, Catlett-Falcone R, et al. Gene therapy with dominant- negative Stat3 suppresses growth of the murine melanoma B16 tumor in vivo. *Cancer Res*. 1999;59:5059-5063.

166. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer*. 2009;9:798-809.

167. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol*. 2003;15:740-746.

168. Guarino M, Rubino B, Ballabio G. The role of epithelial-mesenchymal transition in cancer pathology. *Pathology*. 2007;39:305-318.

169. Zhang B, Zhang Y, Bowerman NA, et al. Equilibrium between host and cancer caused by effector T cells killing tumor stroma. *Cancer Res*. 2008;68:1563-1571.

170. Goldenberg DM, Pavia RA, Tsao MC. In vivo hybridisation of human tumour and normal hamster cells. *Nature*. 1974;250:649-651.

171. Rachkovsky M, Sodi S, Chakraborty A, et al. Melanoma x macrophage hybrids with enhanced metastatic potential. *Clin Exp Metastasis*. 1998;16:299-312.

172. Pawelek JM. Tumour cell hybridization and metastasis revisited. *Melanoma Res*. 2000;10:507-514.

173. Duelli D, Lazebnik Y. Cell fusion: a hidden enemy? *Cancer Cell* . 2003; 3:445-448.

174. Vignery A. Macrophage fusion: are somatic and cancer cells possible partners? *Trends Cell Biol*. 2005;15:188-193.

175. Lu X, Kang Y. Cell fusion as a hidden force in tumor progression. *Cancer Res*. 2009;69:8536-8539.

176. Aichel O. *Eine neue Hypothese über Ursachen und Wesen bösartiger Geschwülste*. München, Germany: JF Lehmann; 1908.

177. Aichel O. *Über Zellverschmelzung mit qualitativ abnormer Chromosomen Verteilung als Ursache der Geschwulstbidung*. Leipzig, Germany: Wilhelm Engelmann; 1911.

178. Peters LJ, Hewitt HB. The influence of fibrin formation on the transplantability of murine tumour cells: implications for the mechanism of the Revesz effect. *Br J Cancer* . 1974;29:279-291.

179. Rak J, Milsom C, Yu J. Tissue factor in cancer. *Curr Opin Hematol* . 2008;15:522-528.

180. Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol*. 2002;20:4368-4380.

181. Dvorak HF, Harvey VS, Estrella P, Brown LF, McDonagh J, Dvorak AM. Fibrin containing gels induce angiogenesis. Implications for tumor stroma generation and wound healing. *Lab Invest*. 1987;57:673-686.

182. Goetz JG, Minguet S, Navarro-Lerida I, et al. Biomechanical remodeling of the microenvironment by stromal caveolin-1 favors tumor invasion and metastasis. *Cell*. 2011;146:148-163.

183. Skipper HE. The forty-year-old mutation theory of Luria and Delbruck and its pertinence to cancer chemotherapy. *Adv Cancer Res* . 1983;40:331-363.

184. Goldie JH, Coldman AJ. A model for tumor response to chemotherapy: an integration of the stem cell and somatic mutation hypotheses. *Cancer Invest* . 1985;3:553-564.

185. Spratt JS, Meyer JS, Spratt JA. Rates of growth of human solid neoplasms: part I. *J Surg Oncol* . 1995;60:137-146.

186. Woglom WH. Immunity to transplantable tumors. *Cancer Rev* . 1929;4:129-214.

187. Rockwell SC, Kallman RF, Fajardo LF. Characteristics of a serially transplanted mouse mammary tumor and its tissue-culture-adapted derivative. *J Natl Cancer Inst* . 1972;49:735-749.

188. Dethlefsen LA, Prewitt JM, Mendelsohn ML. Analysis of tumor growth curves. *J Natl Cancer Inst* . 1968;40:389-405.

189. Tomayko MM, Reynolds CP. Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother Pharmacol* . 1989;24:148-154.

190. Jennings SG, Winer-Muram HT, Tarver RD, Farber MO. Lung tumor growth: assessment with CT—comparison of diameter and cross-sectional area with volume measurements. *Radiology* . 2004;231:866-871.

191. Speiser DE, Miranda R, Zakarian A, et al. Self antigens expressed by solid tumors do not efficiently stimulate naive or activated T cells: Implications for immunotherapy. *J Exp Med* . 1997;186:645-653.

192. Bankert RB, Balu-Iyer SV, Odunsi K, et al. Humanized mouse model of ovarian cancer recapitulates patient solid tumor progression, ascites formation, and metastasis. *PLoS One* . 2011;6:e24420.

193. Anders K, Buschow C, Herrmann A, et al. Oncogene-targeting T cells reject large tumors while oncogene inactivation selects escape variants in mouse models of cancer. *Cancer Cell* . 2011;20:755-767.

194. Palandri F, Amabile M, Rosti G, et al. Imatinib therapy for chronic myeloid leukemia patients who relapse after allogeneic stem cell transplantation: a molecular analysis. *Bone Marrow Transplant* . 2007;39:189-191.

195. Bargou R, Leo E, Zugmaier G, et al. Tumor regression in cancer patients by very low doses of a T cell-engaging antibody. *Science* . 2008;321: 974-977.

196. Kalos M, Levine BL, Porter DL, et al. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med* . 2011;3:95ra73.

197. Frankel TL, Burns WR, Peng PD, et al. Both CD4 and CD8 T cells mediate equally effective in vivo tumor treatment when engineered with a highly avid TCR targeting tyrosinase. *J Immunol* . 2010;184:5988-5998.

198. Li Y, Karlin A, Loike JD, Silverstein SC. A critical concentration of neutrophils is required for effective bacterial killing in suspension. *Proc Natl Acad Sci U S A* . 2002;99:8289-8294.

199. Li Y, Karlin A, Loike JD, Silverstein SC. Determination of the critical concentration of neutrophils required to block bacterial growth in tissues. *J Exp Med* . 2004;200:613-622.

200. Nathan C. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol*. 2006;6:173-182.

201. Budhu S, Loike JD, Pandolfi A, et al. CD8+ T cell concentration determines their efficiency in killing cognate antigen-expressing syngeneic mammalian cells in vitro and in mouse tissues. *J Exp Med*. 2010;207:223-235.

202. Ferrara N. Role of myeloid cells in vascular endothelial growth factor- independent tumor angiogenesis. *Curr Opin Hematol* . 2010;17: 219-224.

203. Zhao Y, Wang QJ, Yang S, et al. A herceptin-based chimeric antigen receptor with modified signaling domains leads to enhanced survival of transduced T lymphocytes and antitumor activity. *J Immunol*. 2009;183:5563-5574.

204. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther*. 2010;18:843-851.

205. Bos R, van Duikeren S, Morreau H, et al. Balancing between antitumor efficacy and autoimmune pathology in T-cell-mediated targeting of carcinoembryonic antigen. *Cancer Res*. 2008;68:8446-8455.

206. Parkhurst MR, Yang JC, Langan RC, et al. T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. *Mol Ther*. 2011;19:620-626.

207. Palmer DC, Chan CC, Gattinoni L, et al. Effective tumor treatment targeting a melanoma/melanocyte-associated antigen triggers severe ocular autoimmunity. *Proc Natl Acad Sci U S A*. 2008;105:8061-8066.

208. Bendle GM, Linnemann C, Hooijkaas AI, et al. Lethal graft-versushost disease in mouse models of T cell receptor gene therapy. *Nat Med*. 2010;16:565-570, 1p following 70.

209. Rosenberg SA. Of mice, not men: no evidence for graft-versus-host disease in humans receiving T-cell receptor-transduced autologous T cells. *Mol Ther*. 2010;18:1744-1745.

210. Weiss DW. Animal models of cancer immunotherapy: questions of relevance. *Cancer Treat Rep* . 1980;64:481-485.

211. Klein G, Klein E. Immune surveillance against virus-induced tumors and nonrejectability of spontaneous tumors: contrasting consequences of host versus tumor evolution. *Proc Natl Acad Sci U S A* . 1977;74: 2121-2125.

212. Tomlinson I, Bodmer W. Selection, the mutation rate and cancer: ensuring that the tail does not wag the dog. *Nat Med*. 1999;5:11-12.

213. Bodmer W, Bielas JH, Beckman RA. Genetic instability is not a requirement for tumor development. *Cancer Res*. 2008;68:3558-3560; discussion 3560-3561.

214. Yamagiva Kal, K. Experimental study of the pathogenesis of cancer. *J Cancer Res* . 1918;3:1-21.

215. Heidelberger C. Chemical carcinogenesis. *Annu Rev Biochem*. 1975; 44:79-121.

216. Findlay G. Ultra-violet light and skin cancer. *Lancet*. 1928;2:1070-1073.

217. Blum H. *Carcinogenesis by Ultraviolet Light*. Princeton, NJ: Princeton University Press; 1959.

218. Ward PL, Koeppen H, Hurteau T, Schreiber H. Tumor antigens defined by cloned immunological probes are highly polymorphic and are not detected on autologous normal cells. *J Exp Med*. 1989;170: 217-232.

219. Dubey P, Hendrickson RC, Meredith SC, et al. The immunodominant antigen of an ultraviolet-induced regressor tumor is generated by a somatic point mutation in the DEAD box helicase p68. *J Exp Med*. 1997;185:695-705.

220. Röntgen WC. *Eine neue Art von Strahlen*. Würzburg, Germany: Stahel; 1885-1886.

221. Little JB. Radiation carcinogenesis. *Carcinogenesis*. 2000;21:397-404.

222. Kaplan HS. On the natural history of the murine leukemias: presidential address. *Cancer Res*. 1967; 27:1325-40.

223. Fry R, Storer J. External radiation carcinogenesis. In: Lett J, ed. *Advances in Radiation Biology*. New York: Academic Press; 1987;13:31-90.

224. Rous P. A transmissible avian neoplasm. (Sarcoma of the common fowl.). *J Exp Med*. 1910;12:696-705.

225. Sotillo R, Garcia JF, Ortega S, et al. Invasive melanoma in Cdk4- targeted mice. *Proc Natl Acad Sci U S A* . 2001;98:13312-13317.

226. Hruban RH, Adsay NV, Albores-Saavedra J, et al. Pathology of genetically engineered mouse models of pancreatic exocrine cancer: consensus report and recommendations. *Cancer Res*. 2006;66:95-106.

227. Zuo L, Weger J, Yang Q, et al. Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma. *Nature Genet*. 1996; 12:97-99.

228. Willimsky G, Blankenstein T. Sporadic immunogenic tumors avoid destruction by inducing T-cell tolerance. *Nature*. 2005;437:141-146.

229. Old LJ, Boyse EA, Clarke DA, Carswell EA. Antigenic properties of chemically-induced tumors. *Ann NY Acad Sci* . 1962;101:80-106.

230. Ochsenbein AF, Sierro S, Odermatt B, et al. Roles of tumour localization, second signals and cross priming in cytotoxic T-cell induction. *Nature*. 2001;411:1058-1064.

231. Spiotto MT, Yu P, Rowley DA, et al. Increasing tumor antigen expression overcomes “ignorance” to solid tumors via crosspresentation by bone marrow-derived stromal cells. *Immunity*. 2002;17:737-747.

232. Greenberg AH, Greene M. Non-adaptive rejection of small tumour inocula as a model of immune surveillance. *Nature*. 1976;264: 356-359.

233. Graffi A, Pasternak G, Horn KH. The production of resistance against isologous

transplants of ultraviolet-induced sarcoma in mice [in German]. *Acta Biol Med Ger*. 1964;12:726-728.

234. Pasternak G. Immunologic properties of chemically and physically induced tumors [in German]. *Arch Geschwulstforsch*. 1967;29:113-141.

235. Urban JL, Holland JM, Kripke ML, Schreiber H. Immunoselection of tumor cell variants by mice suppressed with ultraviolet radiation. *J Exp Med*. 1982;156:1025-1041.

236. Uyttenhove C, Maryanski J, Boon T. Escape of mouse mastocytoma P815 after nearly complete rejection is due to antigen-loss variants rather than immunosuppression. *J Exp Med*. 1983;157:1040-1052.

237. Seung S, Urban JL, Schreiber H. A tumor escape variant that has lost one major histocompatibility complex class I restriction element induces specific CD8+ T cells to an antigen that no longer serves as a target. *J Exp Med*. 1993;178:933-940.

238. Murphy ED. Characteristic tumors. In: Green EL, ed. *Biology of the Laboratory Mouse*. 2nd ed. New York, NY: McGraw-Hill; 1966:521-522.

239. Green EL. *Handbook on Genetically Standardized Jax Mice*. 2nd ed. Bar Harbor, Maine: The Jackson Laboratory; 1968.

240. Goundiam O, Nagel MD, Vayssade M. Growth and survival signalling in B16F10 melanoma cells in 3D culture. *Cell Biol Int*. 2010;34: 385-391.

241. Sugiura K, Stock CC. Studies in a tumor spectrum III. The effect of phosphoramides on the growth of a variety of mouse and rat tumors. *Cancer Res*. 1954:38-51.

242. Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature*. 1949;164:666.

243. Albright JF, Makinodan T, Mazur P. Preservation of antibody-producing cells at low temperature: a method of storage that allows complete recovery of activity. *Proc Soc Exp Biol Med*. 1963;114:489-493.

244. Mazur P. Cryobiology: the freezing of biological systems. *Science*. 1970;168:939-949.

245. Griswold DP, Corbett TH. A colon tumor model for anticancer agent evaluation. *Cancer*. 1975;36:2441-2444.

246. Eisenthal A, Kashtan H, Rabau M, Ramakrishna V, Chaitchik S, Skornick Y. Antitumor effects of recombinant interleukin-6 expressed in eukaryotic cells. *Cancer Immunol Immunother*. 1993;36:101-107.

247. Murphy GP, Hrushesky WJ. A murine renal cell carcinoma. *J Natl Cancer Inst*. 1973;50:1013-1025.

248. van Hall T, van Bergen J, van Veelen PA, et al. Identification of a novel tumor-specific CTL epitope presented by RMA, EL-4, and MBL-2 lymphomas reveals their common origin. *J Immunol*. 2000;165: 869-877.

249. Gorer PA. Studies in antibody response of mice to tumour inoculation. *Br J Cancer*. 1950;4:372-379.

250. Fidler IJ, Fogler WE, Kleinerman ES, Saiki I. Abrogation of species specificity for activation of tumoricidal properties in macrophages by recombinant mouse or human interferon-gamma encapsulated in liposomes. *J Immunol*. 1985;135:4289-4296.

251. Peters RL, Rabstein LS, Spahn GJ, Madison RM, Huebner RJ. Incidence of spontaneous neoplasms in breeding and retired breeder BALB-cCr mice throughout the natural life span. *Int J Cancer*. 1972;10:273-282.

252. Bode G, Hartig F, Hebold G, Czerwek H. Incidence of spontaneous tumors in laboratory rats. *Exp Pathol*. 1985;28:235-243.

253. Mahler JF, Stokes W, Mann PC, Takaoka M, Maronpot RR. Spontaneous lesions in aging FVB/N mice. *Toxicol Pathol*. 1996;24:710-716.

254. Harding C, Pompei F, Wilson R. Peak and decline in cancer incidence, mortality, and prevalence at old ages. *Cancer*. 2012;118:1371-1386.

255. Spellman CW, Daynes RA. Immunoregulation by ultraviolet light- III. Enhancement of suppressor cell activity in older animals. *Exp Gerontol*. 1978;13:141-146.

256. Flood PM, Urban JL, Kripke ML, Schreiber H. Loss of tumor-specific and idiotype-specific immunity with age. *J Exp Med*. 1981;154: 275-290.

257. Flood PM, Liu X, Alexander R, Schreiber H, Haque S. Loss of resistance to a highly immunogenic tumor with age corresponds to the decline of CD8 T cell activity. *J Immunother*. 1998;21:307-316.

258. Schreiber K, Arina A, Engels B, et al. Spleen cells from young but not old immunized mice eradicate large established cancers. *Clin Cancer Res*. 2012:18(9):2526-2533.

259. Kast WM, Offringa R, Peters PJ, et al. Eradication of adenovirus E1-induced tumors by E1A-specific cytotoxic T lymphocytes. *Cell*. 1989;59:603-614.

260. Nowinsky M. Zur Frage über die Impfung der krebsigen Geschwülste. *Centralblatt für die Medicinischen Wissenschaften*. 1876;14:790-791.

261. Ehrlich P. Experimentelle Carcinomstudien an Mäusen Arbeiten aus dem Königlichen. *Institut für Experimentelle Therapy zu Frankfurt aM*. 1906;Heft 1:76-102.

262. Ehrlich P. Über den jetzigen Stand der Karzinomforschung. *Ned Tijdschr Geneeskd*. 1909;5:273-290.

263. Andervont HB. The use of pure strain animals in studies on natural resistance to transplantable tumors. *Pub Health Rep*. 1937;52: 1885-1895.

264. Altman PL, Katz DD, eds. *Inbred and Genetically Defined Strains of Laboratory Animals Part I Mouse and Rat*. Bethesda, MD: Federation of American Societies for Experimental Biology. 1979.

265. Gross L. Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. *Cancer Res*. 1943;3:326-333.

266. Foley EJ. Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. *Cancer Res*. 1953;13:835-837.

267. Baldwin RW. Immunity to methylcholanthrene-induced tumors-inbred rats following atrophy and regression of implanted tumors. *Br J Cancer*. 1955;9:652-665.

268. Prehn RT, Main JM. Immunity to methylcholanthrene-induced sarcomas. *J Natl Cancer Inst*. 1957;18:769-778.

269. Klein G, Sögren HO, Klein E, Hellström KE. Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochthonous host. *Cancer Res*. 1960;20:1561-1572.

270. Globerson A, Feldman M. Antigenic specificity of benzo(a) pyreneinduced sarcomas. *J Natl Cancer Inst* . 1964;32:1229-1243.

271. Pasternak G, Graffi A, Horn KH. The demonstration of specific antigenecity in UV-induced mouse sarcoma [in German]. *Acta Biol Med Ger*. 1964;13:276-279.

272. Burnet FM. A certain symmetry: histocompatibility antigens compared with immunocyte receptors. *Nature*. 1970;226:123-126.

273. Embleton MJ, Heidelberger C. Antigenicity of clones of mouse prostate cells transformed in vitro. *Int J Cancer* . 1972;9:8-18.

274. Basombrio MA, Prehn RT. Antigenic diversity of tumors chemically induced within the progeny of a single cell. *Int J Cancer* . 1972;10:1-8.

275. Kripke ML. Antigenicity of murine skin tumors induced by ultraviolet light. *J Natl Cancer Inst*. 1974;53:1333-1336.

276. Hood L, Huang HV, Dreyer WJ. The area-code hypothesis: the immune system provides clues to understanding the genetic and molecular basis of cell recognition during development. *J Suprastruct*. 1977;7:531-559.

277. Lampson LA, Levy R. A role for clonal antigens in cancer diagnosis and therapy. *J Natl Cancer Inst*. 1979;62:217-220.

278. Wortzel RD, Philipps C, Schreiber H. Multiple tumour-specific antigens expressed on a single tumour cell. *Nature*. 1983;304:165-167.

279. Knuth A, Danowski B, Oettgen HF, Old LJ. T-cell-mediated cytotoxicity against autologous malignant melanoma: analysis with interleukin 2-dependent T-cell cultures. *Proc Natl Acad Sci U S A* . 1984;81: 3511-3515.

280. Srivastava PK, Old LJ. Individually distinct transplantation antigens of chemically induced mouse tumors. *Immunol Today*. 1988;9:78-83.

281. Van den Eynde B, Hainaut P, Herin M, et al. Presence on a human melanoma of multiple antigens recognized by autologous CTL. *Intl J Cancer*. 1989;44:634-640.

282. Kono K, Petersson M, Ciupitu AM, Wen T, Klein G, Kiessling R. Methylcholanthrene-induced mouse sarcomas express individually distinct major histocompatibility complex class I-associated peptides recognized by specific CD8+ T-cell lines. *Cancer Res*. 1995;55: 5648-5655.

283. Philipps C, McMillan M, Flood PM, et al. Identification of a unique tumor-specific

antigen as a novel class I major histocompatibility molecule. *Proc Natl Acad Sci U S A* . 1985;82:5140-5144.

284. Lee DR, Rubocki RJ, Lie WR, Hansen TH. The murine MHC class I genes, H-2Dq and H-2Lq, are strikingly homologous to each other, H-2Ld, and two genes reported to encode tumor-specific antigens. *J Exp Med*. 1988;168:1719-1739.

285. Mandelboim O, Berke G, Fridkin M, Feldman M, Eisenstein M, Eisenbach L. CTL induction by a tumour-associated antigen octapeptide derived from a murine lung carcinoma. *Nature*. 1994;369:67-71.

286. Berke G, Krutovskikh V, Yamasaki H. Connexin 37 gene is not mutated in lung carcinomas 3LL and CMT. *Cancer Lett* . 2003;195:67-72.

287. Bailey DW. How pure are inbred strains of mice? *Immunol Today*. 1982;3:210-214.

288. Davis MM, Boniface JJ, Reich Z, et al. Ligand recognition by alpha beta T cell receptors. *Annu Rev Immunol* . 1998;16:523-544.

289. Williams CB, Engle DL, Kersh GJ, Michael White J, Allen PM. A kinetic threshold between negative and positive selection based on the longevity of the T cell receptor-ligand complex. *J Exp Med*. 1999;189: 1531-1544.

290. Moutaftsi M, Salek-Ardakani S, Croft M, et al. Correlates of protection efficacy induced by vaccinia virus-specific CD8+ T-cell epitopes in the murine intranasal challenge model. *Eur J Immunol* . 2009;39:717-722.

291. Assarsson E, Sidney J, Oseroff C, et al. A quantitative analysis of the variables affecting the repertoire of T cell specificities recognized after vaccinia virus infection. *J Immunol*. 2007;178:7890-7901.

292. Sidney J, Southwood S, Oseroff C, del Guercio MF, Sette A, Grey HM. Measurement of MHC/peptide interactions by gel filtration. *Curr Protoc Immunol*. 2001;Chapter 18:Unit 18 3.

293. Popovic J, Li LP, Kloetzel PM, Leisegang M, Uckert W, Blankenstein T. The only proposed T-cell epitope derived from the TEL-AML1 translocation is not naturally processed. *Blood*. 2011;118(4):946-954.

294. Schreiber H, Rowley JD, Rowley DA. Targeting mutations predictably. *Blood*. 2011;118:830-831.

295. Sette A, Rappuoli R. Reverse vaccinology: developing vaccines in the era of genomics. *Immunity*. 2010;33:530-541.

296. Kim Y, Sette A, Peters B. Applications for T-cell epitope queries and tools in the Immune Epitope Database and Analysis Resource. *J Immunol Methods*. 2011;374:62-69.

297. Singh-Jasuja H, Emmerich NP, Rammensee HG. The Tubingen approach: identification, selection, and validation of tumor-associated HLA peptides for cancer therapy. *Cancer Immunol Immunother* . 2004;53:187-195.

298. Wood LD, Parsons DW, Jones S, et al. The genomic landscapes of human breast and colorectal cancers. *Science*. 2007;318:1108-1113.

299. Weir BA, Woo MS, Getz G, et al. Characterizing the cancer genome in lung adenocarcinoma. *Nature*. 2007;450:893-898.

300. Pleasance ED, Cheetham RK, Stephens PJ, et al. A comprehensive catalogue of somatic mutations from a human cancer genome. *Nature*. 2010;463:191-196.

301. Willimsky G, Czeh M, Loddenkemper C, et al. Immunogenicity of premalignant lesions is the primary cause of general cytotoxic T lymphocyte unresponsiveness. *J Exp Med*. 2008;205:1687-1700.

302. Karanikas V, Colau D, Baurain JF, et al. High frequency of cytolytic T lymphocytes directed against a tumor-specific mutated antigen detectable with HLA tetramers in the blood of a lung carcinoma patient with long survival. *Cancer Res*. 2001;61:3718-3724.

303. Lennerz V, Fatho M, Gentilini C, et al. The response of autologous T cells to a human melanoma is dominated by mutated neoantigens. *Proc Natl Acad Sci U S A*. 2005;102:16013-10618.

304. Humphrey PA, Wong AJ, Vogelstein B, et al. Anti-synthetic peptide antibody reacting at the fusion junction of deletion-mutant epidermal growth factor receptors in human glioblastoma. *Proc Natl Acad Sci U S A*. 1990;87:4207-4211.

305. Sampson JH, Archer GE, Mitchell DA, Heimberger AB, Bigner DD. Tumor-specific immunotherapy targeting the EGFRvIII mutation in patients with malignant glioma. *Semin Immunol*. 2008;20:267-275.

306. Cheever MA, Allison JP, Ferris AS, et al. The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. *Clin Cancer Res*. 2009;15:5323-5337.

307. Beck-Engeser GB, Monach PA, Mumberg D, et al. Point mutation in essential genes with loss or mutation of the second allele: relevance to the retention of tumor-specific antigens. *J Exp Med*. 2001;194: 285-300.

308. Takagi M, Absalon MJ, McLure KG, Kastan MB. Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. *Cell*. 2005;123:49-63.

309. Ofir-Rosenfeld Y, Boggs K, Michael D, Kastan MB, Oren M. Mdm2 regulates p53 mRNA translation through inhibitory interactions with ribosomal protein L26. *Mol Cell*. 2008;32:180-189.

310. Acquaviva J, Chen X, Ren R. IRF-4 functions as a tumor suppressor in early B-cell development. *Blood*. 2008;112:3798-3806.

311. Poi MJ, Yen T, Li J, et al. Somatic INK4a-ARF locus mutations: a significant mechanism of gene inactivation in squamous cell carcinomas of the head and neck. *Mol Carcinog*. 2001;30:26-36.

312. Parkhurst MR, Salgaller ML, Southwood S, et al. Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. *J Immunol*. 1996;157:2539-2548.

313. He X. Unwinding a path to nuclear beta-catenin. *Cell*. 2006;127: 40-42.

314. Yang L, Lin C, Liu ZR. Phosphorylations of DEAD box p68 RNA helicase are associated with cancer development and cell proliferation. *Mol Cancer Res* . 2005;3:355-363.

315. Suzuki HI, Yamagata K, Sugimoto K, Iwamoto T, Kato S, Miyazono K. Modulation of microRNA processing by p53. *Nature*. 2009;460: 529-533.

316. Baurain JF, Colau D, van Baren N, et al. High frequency of autologous anti-melanoma CTL directed against an antigen generated by a point mutation in a new helicase gene. *J Immunol* . 2000;164: 6057-6066.

317. Knudson AG Jr. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A*. 1971;68:820-823.

318. Dudley ME, Yang JC, Sherry R, et al. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol* . 2008;26: 5233-5239.

319. Neller MA, Lopez JA, Schmidt CW. Antigens for cancer immunotherapy. *Semin Immunol* . 2008;20:286-295.

320. Lucas S, Coulie PG. About human tumor antigens to be used in immunotherapy. *Semin Immunol* . 2008;20:301-307.

321. Yoshida TO, Southam CM. Attempts to find cell associated immune reaction against autochthonous tumors. *Jpn J Exp Med* . 1963;33: 369-383.

322. Rosenau W, Moon HD. Cellular reactions to methylcholanthreneinduced sarcomas transplanted to isogenic mice. *Lab Invest*. 1966;15: 1212-1224.

323. Hoos A, Britten CM, Huber C, O'Donnell-Tormey J. A methodological framework to enhance the clinical success of cancer immunotherapy. *Nat Biotechnol* . 2011;29:867-870.

324. Fox BA, Schendel DJ, Butterfield LH, et al. Defining the critical hurdles in cancer immunotherapy. *J Transl Med* . 2011;9:214.

325. Higano CS, Schellhammer PF, Small EJ, et al. Integrated data from 2 randomized, double-blind, placebo-controlled, phase 3 trials of active cellular immunotherapy with sipuleucel-T in advanced prostate cancer. *Cancer* . 2009;115:3670-3679.

326. Solomon E, Borrow J, Goddard AD. Chromosome aberrations and cancer. *Science*. 1991;254:1153-1160.

327. Kato Y, Jin G, Kuan CT, McLendon RE, Yan H, Bigner DD. A monoclonal antibody IMab-1 specifically recognizes IDH1R132H, the most common glioma-derived mutation. *Biochem Biophys Res Commun*. 2009;390:547-551.

328. Worley BS, van den Broeke LT, Goletz TJ, et al. Antigenicity of fusion proteins from sarcoma-associated chromosomal translocations. *Cancer Res* . 2001;61:6868-6875.

329. Schindler T, Bornmann W, Pellicena P, Miller WT, Clarkson B, Kuriyan J. Structural mechanism for STI-571 inhibition of abelson tyrosine kinase. *Science*. 2000;289:1938-1942.

330. van Denderen J, Hermans A, Meeuwsen T, et al. Antibody recognition of the tumor-specific bcr-abl joining region in chronic myeloid leukemia. *J Exp Med*. 1989;169:87-98.

331. Pawelec G, Max H, Halder T, et al. BCR/ABL leukemia oncogene fusion peptides selectively bind to certain HLA-DR alleles and can be recognized by T cells found at low frequency in the repertoire of normal donors. *Blood*. 1996;88:2118-2124.

332. Clark RE, Dodi IA, Hill SC, et al. Direct evidence that leukemic cells present HLA-associated immunogenic peptides derived from the BCR-ABL b3a2 fusion protein. *Blood*. 2001;98:2887-2893.

333. Jung S, Schluesener HJ. Human T lymphocytes recognize a peptide of single point-mutated, oncogenic ras proteins. *J Exp Med*. 1991;173: 273-276.

334. Hruban RH, van Mansfeld AD, Offerhaus GJ, et al. K-ras oncogene activation in adenocarcinoma of the human pancreas. A study of 82 carcinomas using a combination of mutant-enriched polymerase chain reaction analysis and allele-specific oligonucleotide hybridization. *Am J Pathol*. 1993;143:545-554.

335. Skipper J, Stauss HJ. Identification of two cytotoxic T lymphocyte-recognized epitopes in the Ras protein. *J Exp Med*. 1993;177: 1493-1498.

336. Fossum B, Gedde-Dahl T III, Breivik J, et al. p21-ras-peptide-specific T-cell responses in a patient with colorectal cancer. CD4+ and CD8+ T cells recognize a peptide corresponding to a common mutation (13Gly→Asp). *Int J Cancer*. 1994;56:40-45.

337. Gjertsen MK, Bakka A, Breivik J, et al. Vaccination with mutant ras peptides and induction of T-cell responsiveness in pancreatic carcinoma patients carrying the corresponding RAS mutation. *Lancet*. 1995; 346:1399-1400.

338. Gjertsen MK, Bakka A, Breivik J, et al. Ex vivo ras peptide vaccination in patients with advanced pancreatic cancer: results of a phase I/II study. *Int J Cancer*. 1996;65:450-453.

339. Linard B, Bezieau S, Benlalam H, et al. A ras-mutated peptide targeted by CTL infiltrating a human melanoma lesion. *J Immunol*. 2002; 168:4802-4808.

340. Kubuschok B, Neumann F, Breit R, et al. Naturally occurring T-cell response against mutated p21 ras oncoprotein in pancreatic cancer. *Clin Cancer Res*. 2006;12:1365-1372.

341. Siegel CT, Schreiber K, Meredith SC, et al. Enhanced growth of primary tumors in cancer-prone mice after immunization against the mutation region of an inherited oncoprotein. *J Exp Med*. 2000;191: 1945-1956.

342. Rowley DA, Stach RM. B lymphocytes secreting IgG linked to latent transforming growth factor- beta prevent primary cytolytic T lymphocyte responses. *Int Immunol*. 1998;10:355-363.

343. Stach RM, Rowley DA. A first or dominant immunization. II. Induced immunoglobulin carries transforming growth factor beta and suppresses cytolytic T cell responses to unrelated alloantigens. *J Exp Med*. 1993;178:841-852.

344. Talmage DW. Immunological specificity, unique combinations of selected natural globulins provide an alternative to the classical concept. *Science*. 1959;129:1643-1648.

345. Richards FF, Konigsberg WH, Rosenstein RW, Varga JM. On the specificity of antibodies. *Science*. 1975;187:130-137.

346. Mendez MJ, Green LL, Corvalan JR, et al. Functional transplant of megabase human immunoglobulin loci recapitulates human antibody response in mice. *Nat Genet*. 1997;15:146-156.

347. Gross G, Waks T, Eshhar Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibodytype specificity. *Proc Natl Acad Sci U S A* . 1989;86:10024-10028.

348. Sadovnikova E, Stauss HJ. Peptide-specific cytotoxic T lymphocytes restricted by nonself major histocompatibility complex class I molecules: reagents for tumor immunotherapy. *Proc Natl Acad Sci U S A* . 1996;93:13114-13118.

349. Falkenburg WJ, Melenhorst JJ, van de Meent M, et al. Allogeneic HLA-A*02-restricted WT1-specific T cells from mismatched donors are highly reactive but show off-target promiscuity. *J Immunol*. 2011; 187:2824-2833.

350. Lauwen MM. *p53 Specific (Auto)Immunity in Mice* [PhD]. Leiden, Netherlands: Leiden University; 2008.

351. Leisegang M, Wilde S, Spranger S, et al. MHC-restricted fratricide of human lymphocytes expressing survivin-specific transgenic T cell receptors. *J Clin Invest*. 2010;120:3869-3877.

352. Theobald M, Biggs J, Hernandez J, Lustgarten J, Labadie C, Sherman LA. Tolerance to p53 by A2.1-restricted cytotoxic T lymphocytes. *J Exp Med*. 1997;185:833-841.

353. Levine AJ. The p53 tumor suppressor gene and gene product. *Princess Takamatsu Symp*. 1989;20:221-230.

354. Goh AM, Coffill CR, Lane DP. The role of mutant p53 in human cancer. *J Pathol*. 2011;223:116-126.

355. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science*. 1991;253:49-53.

356. Silver DA, Pellicer I, Fair WR, Heston WD, Cordon-Cardo C. Prostate-specific membrane antigen expression in normal and malignant human tissues. *Clin Cancer Res*. 1997;3:81-85.

357. Sacha P, Zamecnik J, Barinka C, et al. Expression of glutamate carboxypeptidase II in human brain. *Neuroscience*. 2007;144:1361-1372.

358. Kuratsukuri K, Sone T, Wang CY, Nishisaka N, Jones RF, Haas GP. Inhibition of prostate-specific membrane antigen (PSMA)-positive tumor growth by vaccination with either full-length or the C-terminal end of PSMA. *Int J Cancer* . 2002;102:244-249.

359. Ma D, Hopf CE, Malewicz AD, et al. Potent antitumor activity of an auristatin-conjugated, fully human monoclonal antibody to prostatespecific membrane antigen. *Clin Cancer Res*. 2006;12:2591-2596.

360. Morawietz L, Floore A, Stork-Sloots L, et al. Comparison of histopathological and gene expression-based typing of cancer of unknown primary. *Virchows Arch*. 2010;456:23-29.

361. Natoli C, Ramazzotti V, Nappi O, et al. Unknown primary tumors. *Biochim Biophys Acta*. 2011;1816:13-24.

362. Coulie PG, Brichard V, Van Pel A, et al. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J Exp Med*. 1994;180:35-42.

363. Kawakami Y, Eliyahu S, Delgado CH, et al. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc Natl Acad Sci U S A*. 1994;91:3515-3519.

364. Colella TA, Bullock TN, Russell LB, et al. Self-tolerance to the murine homologue of a tyrosinase-derived melanoma antigen: implications for tumor immunotherapy. *J Exp Med*. 2000;191:1221-1232.

365. Yee C, Thompson JA, Roche P, et al. Melanocyte destruction after antigen-specific immunotherapy of melanoma: direct evidence of T cell-mediated vitiligo. *J Exp Med*. 2000;192:1637-1644.

366. Rosenberg SA, Dudley ME. Adoptive cell therapy for the treatment of patients with metastatic melanoma. *Curr Opin Immunol* . 2009;21: 233-240.

367. Hara I, Takechi Y, Houghton AN. Implicating a role for immune recognition of self in tumor rejection: passive immunization against the brown locus protein. *J Exp Med*. 1995;182:1609-1614.

368. Naftzger C, Takechi Y, Kohda H, Hara I, Vijayasaradhi S, Houghton AN. Immune response to a differentiation antigen induced by altered antigen: a study of tumor rejection and autoimmunity. *Proc Natl Acad Sci U S A*. 1996;93:14809-14814.

369. Livingston PO. Approaches to augmenting the immunogenicity of melanoma gangliosides: from whole melanoma cells to ganglioside- KLH conjugate vaccines. *Immunol Rev*. 1995;145:147-166.

370. Muranski P, Boni A, Antony PA, et al. Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood*. 2008;112:362-373.

371. Xie Y, Akpinarli A, Maris C, et al. Naive tumor-specific CD4(+) T cells differentiated in vivo eradicate established melanoma. *J Exp Med*. 2010; 207:651-667.

372. Tief K, Schmidt A, Beermann F. New evidence for presence of tyrosinase in substantia nigra, forebrain and midbrain. *Brain Res Mol Brain Res*. 1998;53:307-310.

373. Tachibana M. Sound needs sound melanocytes to be heard. *Pigment Cell Res*. 1999;12:344-354.

374. Murisier F, Beermann F. Genetics of pigment cells: lessons from the tyrosinase gene family. *Histol Histopathol* . 2006;21:567-578.

375. Lu H, Li L, Watson ER, et al. Complex interactions of Tyrp1 in the eye. *Mol Vis*. 2011;17:2455-2468.

376. Reisfeld RA. Antigen repertoire of human melanoma. *Prog Clin Biol Res*. 1988;256:343-360.

377. Garin-Chesa P, Beresford HR, Carrato-Mena A, et al. Cell surface molecules of human melanoma. Immunohistochemical analysis of the gp57, GD3, and mel-CSPG antigenic systems. *Am J Pathol*. 1989;134:295-303.

378. Bilhartz DL, Tindall DJ, Oesterling JE. Prostate-specific antigen and prostatic acid phosphatase: biomolecular and physiologic characteristics. *Urology*. 1991;38:95-102.

379. Chou R, Croswell JM, Dana T, et al. Screening for prostate cancer: a review of the evidence for the U.S. Preventive Services Task Force. *Ann Intern Med*. 2011;155:762-771.

380. Walczak JR, Carducci MA. Prostate cancer: a practical approach to current management of recurrent disease. *Mayo Clin Proc*. 2007;82:243-249.

381. Kutscher W, Wolbergs H. Prostataphophatase. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie*. 1935;236:237-240.

382. Huber ML, Haynes L, Parker C, Iversen P. Interdisciplinary critique of sipuleucel-T as immunotherapy in castration-resistant prostate cancer. *J Natl Cancer Inst*. 2012;104:273-279.

383. Cimino A, Halushka M, Illei P, Wu X, Sukumar S, Argani P. Epithelial cell adhesion molecule (EpCAM) is overexpressed in breast cancer metastases. *Breast Cancer Res Treat*. 2010;123:701-708.

384. Herlyn DM, Steplewski Z, Herlyn MF, Koprowski H. Inhibition of growth of colorectal carcinoma in nude mice by monoclonal antibody. *Cancer Res*. 1980;40:717-721.

385. Riethmuller G, Holz E, Schlimok G, et al. Monoclonal antibody therapy for resected Dukes' C colorectal cancer: seven-year outcome of a multicenter randomized trial. *J Clin Oncol*. 1998;16:1788-1794.

386. Fields AL, Keller A, Schwartzberg L, et al. Adjuvant therapy with the monoclonal antibody Edrecolomab plus fluorouracil-based therapy does not improve overall survival of patients with stage III colon cancer. *J Clin Oncol*. 2009;27:1941-1947.

387. de Bono JS, Tolcher AW, Forero A, et al. ING-1, a monoclonal antibody targeting Ep-CAM in patients with advanced adenocarcinomas. *Clin Cancer Res*. 2004;10:7555-7565.

388. Hakomori S, Wang SM, Young WW Jr. Isoantigenic expression of Forssman glycolipid in human gastric and colonic mucosa: its possible identity with “A-like antigen” in human cancer. *Proc Natl Acad Sci U S A*. 1977;74:3023-3027.

389. Lloyd KO. Philip Levine award lecture. Blood group antigens as markers for normal differentiation and malignant change in human tissues. *Amer J Clin Path*. 1987;87:129-139.

390. Yu LG. The oncofetal Thomsen-Friedenreich carbohydrate antigen in cancer progression. *Glycoconj J*. 2007;24:411-420.

391. Kim YS, Gum J Jr, Brockhausen I. Mucin glycoproteins in neoplasia. *Glycoconj J*.

1996;13:693-707.

392. Barnd DL, Lan MS, Metzgar RS, Finn OJ. Specific, major histocompatibility complex-unrestricted recognition of tumor-associated mucins by human cytotoxic T cells. *Proc Natl Acad Sci U S A*. 1989;86:7159-7163.

393. Finn OJ, Gantt KR, Lepisto AJ, Pejawar-Gaddy S, Xue J, Beatty PL. Importance of MUC1 and spontaneous mouse tumor models for understanding the immunobiology of human adenocarcinomas. *Immunol Res*. 2011;50:261-268.

394. Alajez NM, Schmielau J, Alter MD, Cascio M, Finn OJ. Therapeutic potential of a tumor-specific, MHC-unrestricted T-cell receptor expressed on effector cells of the innate and the adaptive immune system through bone marrow transduction and immune reconstitution. *Blood*. 2005;105:4583-4589.

395. Slovin SF, Ragupathi G, Musselli C, et al. Fully synthetic carbohydratebased vaccines in biochemically relapsed prostate cancer: clinical trial results with alpha-N-acetylgalactosamine-O-serine/threonine conjugate vaccine. *J Clin Oncol* . 2003;21:4292-4298.

396. Beatson RE, Taylor-Papadimitriou J, Burchell JM. MUC1 immunotherapy. *Immunotherapy*. 2010;2:305-327.

397. Gad M, Jensen T, Gagne R, et al. MUC1-derived glycopeptide libraries with improved MHC anchors are strong antigens and prime mouse T cells for proliferative responses to lysates of human breast cancer tissue. *Eur J Immunol* . 2003;33:1624-1632.

398. Sorensen AL, Reis CA, Tarp MA, et al. Chemoenzymatically synthesized multimeric Tn/STn MUC1 glycopeptides elicit cancer-specific anti- MUC1 antibody responses and override tolerance. *Glycobiology*. 2006; 16:96-107.

399. Ryan SO, Vlad AM, Islam K, Gariepy J, Finn OJ. Tumor-associated MUC1 glycopeptide epitopes are not subject to self-tolerance and improve responses to MUC1 peptide epitopes in MUC1 transgenic mice. *Biol Chem*. 2009;390:611-618.

400. Pinkhasov J, Alvarez ML, Rigano MM, et al. Recombinant plant- expressed tumour-associated MUC1 peptide is immunogenic and capable of breaking tolerance in MUC1.Tg mice. *Plant Biotechnol J*. 2011;9: 991-1001.

401. Gendler SJ, Spicer AP. Epithelial mucin genes. *Annu Rev Physiol* . 1995; 57:607-634.

402. Moreau R, Dausset J, Bernard J, Moullec J. Acquired hemolytic anemia with polyagglutinability of erythrocytes by a new factor present in normal blood [in French]. *Bull Mem Soc Med Hop Paris*. 1957;73:569-587.

403. Dausset J, Moullec J, Bernard J. Acquired hemolytic anemia with polyagglutinability of red blood cells due to a new factor present in normal human serum (Anti-Tn). *Blood*. 1959;14:1079-1093.

404. Ju T, Otto VI, Cummings RD. The Tn antigen-structural simplicity and biological complexity. *Angew Chem Int Ed Engl* . 2011;50: 1770-1791.

405. Springer GF, Desai PR, Banatwala I. Blood group MN specific substances and

precursors in normal and malignant human breast tissues. *Naturwissenschaften*. 1974;61:457-458.

406. Springer GF, Desai PR, Banatwala I. Blood group MN antigens and precursors in normal and malignant human breast glandular tissue. *J Natl Cancer Inst* . 1975;54:335-339.

407. Ju T, Cummings RD. Protein glycosylation: chaperone mutation in Tn syndrome. *Nature*. 2005;437:1252.

408. Wang Y, Ju T, Ding X, et al. Cosmc is an essential chaperone for correct protein O-glycosylation. *Proc Natl Acad Sci U S A* . 2010;107: 9228-9233.

409. Springer GF. T and Tn, general carcinoma autoantigens. *Science*. 1984;224:1198-1206.

410. Springer GF, Tegtmeyer H. Origin of anti-Thomsen-Friedenreich (T) and Tn agglutinins in man and in White Leghorn chicks. *Br J Haematol*. 1981;47:453-460.

411. Welinder C, Baldetorp B, Borrebaeck C, Fredlund BM, Jansson B. A new murine IgG1 anti-Tn monoclonal antibody with in vivo anti-tumor activity. *Glycobiology*. 2011;21:1097-1107.

412. Schietinger A, Philip M, Yoshida BA, et al. A mutant chaperone converts a wild-type protein into a tumor-specific antigen. *Science*. 2006;314:304-308.

413. Brooks CL, Schietinger A, Borisova SN, et al. Antibody recognition of a unique tumor-specific glycopeptide antigen. *Proc Natl Acad Sci U S A*. 2010;107:10056-10061.

414. Steentoft C, Schjoldager KT, Clo E, et al. Characterization of an immunodominant cancer-specific O-glycopeptide epitope in murine podoplanin (OTS8). *Glycoconj J*. 2010;27:571-582.

415. Ju T, Lanneau GS, Gautam T, et al. Human tumor antigens Tn and sialyl Tn arise from mutations in Cosmc. *Cancer Res*. 2008;68: 1636-1646.

416. Hirszfeld L, Halber W, Rosenblat J. Untersuchungen über Verwandtschaftsreaktionen zwischen Embryonal- und Krebsgewebe. II. Menschenembryo und Menschenkrebs. *Z Immunitätsforsch*. 1932; 75:209-216.

417. Medawar PB, Hunt R. The significance of embryonic reexpression in cancer. *Cancer Res*. 1976;36:3453-3454.

418. Coggin JH Jr. Oncofetal antigens. *Nature*. 1986;319:428.

419. Chen YT, Obata Y, Stockert E, Takahashi T, Old LJ. Tla-region genes and their products. *Immunol Res*. 1987;6:30-45.

420. Old LJ. Cancer immunology: the search for specificity—G. H. A. Clowes Memorial lecture. *Cancer Res*. 1981;41:361-375.

421. Wu M, van Kaer L, Itohara S, Tonegawa S. Highly restricted expression of the thymus leukemia antigens on intestinal epithelial cells. *J Exp Med*. 1991;174:213-218.

422. van der Bruggen P, Traversari C, Chomez P, et al. A gene encoding an antigen

recognized by cytolytic T lymphocytes on a human melanoma. *Science*. 1991;254:1643-1647.

423. Traversari C, van der Bruggen P, Luescher IF, et al. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. *J Exp Med*. 1992;176:1453-1457.

424. Amar-Costesec A, Godelaine D, Van den Eynde B, Beaufay H. Identification and characterization of the tumor-specific P1A gene product. *Biol Cell*. 1994;81:195-203.

425. Chomez P, Williams R, De Backer O, Boon T, Vennstrom B. The SMAGE gene family is expressed in post-meiotic spermatids during mouse germ cell differentiation. *Immunogenetics*. 1996;43:97-100.

426. De Smet C, De Backer O, Faraoni I, Lurquin C, Brasseur F, Boon T. The activation of human gene MAGE-1 in tumor cells is correlated with genome- wide demethylation. *Proc Natl Acad Sci U S A* . 1996;93:7149-7153.

427. De Plaen E, Lurquin C, Lethe B, et al. Identification of genes coding for tumor antigens recognized by cytolytic T lymphocytes. *Methods*. 1997;12:125-142.

428. Martelange V, De Smet C, De Plaen E, Lurquin C, Boon T. Identification on a human sarcoma of two new genes with tumor-specific expression. *Cancer Res*. 2000;60:3848-3855.

429. Ghochikyan A, Mkrtichyan M, Loukinov D, et al. Elicitation of T cell responses to histologically unrelated tumors by immunization with the novel cancer-testis antigen, brother of the regulator of imprinted sites. *J Immunol*. 2007;178:566-573.

430. Jones TA, Ogunkolade BW, Szary J, et al. Widespread expression of BORIS/CTCFL in normal and cancer cells. *PLoS One*. 2011;6:e22399.

431. Derbinski J, Schulte A, Kyewski B, Klein L. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat Immunol*. 2001;2:1032-1039.

432. Gotter J, Brors B, Hergenhahn M, Kyewski B. Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters. *J Exp Med*. 2004;199:155-166.

433. Caballero OL, Chen YT. Cancer/testis (CT) antigens: potential targets for immunotherapy. *Cancer Sci* . 2009;100:2014-2021.

434. Lurquin C, Lethe B, De Plaen E, et al. Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. *J Exp Med*. 2005;201:249-257.

435. Carrasco J, Van Pel A, Neyns B, et al. Vaccination of a melanoma patient with mature dendritic cells pulsed with MAGE-3 peptides triggers the activity of nonvaccine anti-tumor cells. *J Immunol*. 2008;180:3585-3593.

436. Van den Eynde B, Lethe B, Van Pel A, De Plaen E, Boon T. The gene coding for a major tumor rejection antigen of tumor P815 is identical to the normal gene of syngeneic DBA/2 mice. *J Exp Med*. 1991;173:1373-1384.

437. Uyttenhove C, Godfraind C, Lethe B, et al. The expression of mouse gene P1A in testis does not prevent safe induction of cytolytic T cells against a P1A-encoded tumor antigen. *Int J Cancer* . 1997;70:349-356.

438. Guo ZS, Hong JA, Irvine KR, et al. De novo induction of a cancer/testis antigen by 5-aza-2'-deoxycytidine augments adoptive immunotherapy in a murine tumor model. *Cancer Res*. 2006;66:1105-1113.

439. Sarma S, Guo Y, Guilloux Y, Lee C, Bai XF, Liu Y. Cytotoxic T lymphocytes to an unmutated tumor rejection antigen P1A: normal development but restrained effector function in vivo. *J Exp Med*. 1999;189:811-820.

440. Brandle D, Bilsborough J, Rulicke T, Uyttenhove C, Boon T, Van den Eynde BJ. The shared tumor-specific antigen encoded by mouse gene P1A is a target not only for cytolytic T lymphocytes but also for tumor rejection. *Eur J Immunol* . 1998;28:4010-4019.

441. Bai XF, Liu J, Li O, Zheng P, Liu Y. Antigenic drift as a mechanism for tumor evasion of destruction by cytolytic T lymphocytes. *J Clin Invest*. 2003;111:1487-1496.

442. Sarma S, Bai XF, Liu JQ, May KF Jr, Zheng P, Liu Y. On the role of unmutated antigens in tumor rejection in mice with unperturbed T-cell repertoires. *Cancer Res*. 2003;63:6051-6055.

443. Robbins PF, Morgan RA, Feldman SA, et al. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol*. 2011;29:917-924.

444. Corbiere V, Chapiro J, Stroobant V, et al. Antigen spreading contributes to MAGE vaccination-induced regression of melanoma metastases. *Cancer Res*. 2011;71:1253-1262.

445. Ludewig B, Krebs P, Metters H, Tatzel J, Tureci O, Sahin U. Molecular characterization of virus-induced autoantibody responses. *J Exp Med*. 2004;200:637-646.

446. Preiss S, Kammertoens T, Lampert C, Willimsky G, Blankenstein T. Tumor-induced antibodies resemble the response to tissue damage. *Int J Cancer* . 2005;115:456-462.

447. Thompson JA. Molecular cloning and expression of carcinoembryonic antigen gene family members. *Tumour Biol* . 1995;16:10-16.

448. Hammarstrom S. The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. *Semin Cancer Biol* . 1999;9:67-81.

449. Gold P, Freedman SO. Specific carcinoembryonic antigens of the human digestive system. *J Exp Med*. 1965;122:467-481.

450. Tan E, Gouvas N, Nicholls RJ, Ziprin P, Xynos E, Tekkis PP. Diagnostic precision of carcinoembryonic antigen in the detection of recurrence of colorectal cancer. *Surg Oncol*. 2009;18:15-24.

451. Abelev GI, Perova SD, Khramkov NI, Postnikova ZA, Irlin IS. Production of embryonal a-globulin by transplantable mouse hepatomas. *Transplantation*. 1963;1:174-180.

452. Haydon GH, Hayes PC. Screening for hepatocellular carcinoma. *Eur J Gastroenterol Hepatol*. 1996;8:856-860.

453. Medawar PB, Hunt R. Can fetal antigens be used for prophylactic immunization? *Ciba Found Symp*. 1983;96:160-181.

454. Lampson LA, Levy R. A role for clonal antigens in cancer diagnosis and therapy. *J Natl Cancer Inst*. 1979;62:217-220.

455. Lynch RG, Graff RJ, Sirisinha S, Simms ES, Eisen HN. Myeloma proteins as tumor-specific transplantation antigens. *Proc Natl Acad Sci U S A*. 1972;69:1540-1544.

456. Syrengelas AD, Chen TT, Levy R. DNA immunization induces protective immunity against B-cell lymphoma. *Nat Med*. 1996;2: 1038-1041.

457. Beatty PG, Kim BS, Rowley DA, Coppleson LW. Antibody against the antigen receptor of a plasmacytoma prolongs survival of mice bearing the tumor. *J Immunol*. 1976;116:1391-1396.

458. Stevenson FK, Elliott EV, Stevenson GT. Some effects on leukaemic B lymphocytes of antibodies to defined regions of their surface immunoglobulin. *Immunol*. 1977;32:549-557.

459. Schreiber H, Leibson P. Suppression of myeloma growth in vitro by anti-idiotypic antibodies: inhibition of DNA synthesis and colony formation. *J Natl Cancer Inst*. 1978;60:225-233.

460. Krolick KA, Uhr JW, Slavin S, Vitetta ES. In vivo therapy of a murine B cell tumor (BCL1) using antibody-ricin A chain immunotoxins. *J Exp Med*. 1982;155:1797-1809.

461. Uhr JW, Picker LJ, Scheuermann RH, Tucker TF, Vitetta ES, Yefenof E. Cancer dormancy: isolation and characterization of dormant lymphoma cells. *45th Annual Symposium on Fundamental Cancer Research*. 1992;1992:29-30.

462. Miller RA, Maloney DG, Warnke R, Levy R. Treatment of B-cell lymphoma with monoclonal anti-idiotype antibody. *N Engl J Med*. 1982;306:517-522.

463. Weng WK, Czerwinski D, Levy R. Humoral immune response and immunoglobulin G Fc receptor genotype are associated with better clinical outcome following idiotype vaccination in follicular lymphoma patients regardless of their response to induction chemotherapy. *Blood*. 2007;109:951-953.

464. Ai WZ, Tibshirani R, Taidi B, Czerwinski D, Levy R. Anti-idiotype antibody response after vaccination correlates with better overall survival in follicular lymphoma. *Blood*. 2009;113:5743-5746.

465. Houot R, Levy R. Vaccines for lymphomas: idiotype vaccines and beyond. *Blood Rev*. 2009;23:137-142.

466. Stoye JP, Coffin JM. Polymorphism of murine endogenous proviruses revealed by using virus class-specific oligonucleotide probes. *J Virol*. 1988;62:168-175.

467. Nowinski RC, Stone MR, Tam MR, Lostrom ME, Burnette WN, O'Donnell PV. Mapping of viral proteins with monoclonal antibodies. In: Kennett RH, McKearn TJ,

Bechton KB, eds. *Monoclonal Antibodies Hybridomas: A New Dimension in Biological Analyses*. New York, NY: Plenum Press; 1980:295-316.

468. Huang AY, Gulden PH, Woods AS, et al. The immunodominant major histocompatibility complex class I-restricted antigen of a murine colon tumor derives from an endogenous retroviral gene product. *Proc Natl Acad Sci U S A* . 1996;93:9730-9735.

469. Uenaka A, Ono T, Akisawa T, Wada H, Yasuda T, Nakayama E. Identification of a unique antigen peptide pRL1 on BALB/c RL male 1 leukemia recognized by cytotoxic T lymphocytes and its relation to the Akt oncogene. *J Exp Med*. 1994;180:1599-1607.

470. de Bergeyck V, De Plaen E, Chomez P, Boon T, Van Pel A. An intracisternal A-particle sequence codes for an antigen recognized by syngeneic cytolytic T lymphocytes on a mouse spontaneous leukemia. *Eur J Immunol*. 1994;24:2203-2212.

471. Fefer A, McCoy JL, Glynn JP. Induction and regression of primary moloney sarcoma virus-induced tumors in mice. *Cancer Res*. 1967;27:1626-1631.

472. Plata F, Lilly F. Viral specificity of H-2-restricted T killer cells directed against syngeneic tumors induced by Gross, Friend, or Rauscher leukemia virus. *J Exp Med*. 1979;150:1174-1186.

473. Plata F, Langlade-Demoyen P, Abastado JP, Berbar T, Kourilsky P. Retrovirus antigens recognized by cytolytic T lymphocytes activate tumor rejection in vivo. *Cell*. 1987;48:231-240.

474. Klarnet JP, Kern DE, Okuno K, Holt C, Lilly F, Greenberg PD. FBL-reactive CD8+ cytotoxic and CD4+ helper T lymphocytes recognize distinct Friend murine leukemia virus-encoded antigens. *J Exp Med*. 1989;169:457-467.

475. Golovkina TV, Chervonsky A, Dudley JP, Ross SR. Transgenic mouse mammary tumor virus superantigen expression prevents viral infection. *Cell*. 1992;69:637-645.

476. Coffin JM. Endogenous retrovirus. In: Weiss R, Teich N, Varmus HE, eds. *Molecular Biology of Tumor Viruses*. Pt. III. New York: Cold Spring Harbor Laboratory; 1982:1109-1203.

477. Schreiber H. Chapter 48. Tumor immunology. In: Paul W, ed. *Fundamental Immunology*. 5th ed. Philadelphia, PA: Lippincott- Williams & Wilkins; 2003:1557-1592.

478. Rapp HJ. Appropriateness of animal models for the immunology of human cancer. *Cancer Res*. 1979;39:4285-4287.

479. Kripke ML. Latency, histology, and antigenicity of tumors induced by ultraviolet light in three inbred mouse strains. *Cancer Res*. 1977; 37:1395-1400.

480. Roberts LK, Daynes RA. Modification of the immunogenic properties of chemically induced tumors arising in hosts treated concomitantly with ultraviolet light. *J Immunol*. 1980;125:438-447.

481. Leffell MS, Coggin JH Jr. Common transplantation antigens on methylcholanthrene-induced murine sarcomas detected by three assays of tumor rejection. *Cancer Res*. 1977;37:4112-4119.

482. Miller RA. The aging immune system: primer and prospectus. *Science*. 1996;273:70-

74.

483. Makinodan T, Kay MM. Age influence on the immune system. *Adv Immunol*. 1980;29:287-330.

484. Urban JL, Schreiber H. Rescue of the tumor-specific immune response of aged mice in vitro. *J Immunol*. 1984;133:527-534.

485. Kaplan DH, Shankaran V, Dighe AS, et al. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc Natl Acad Sci U S A*. 1998;95:7556-7561.

486. Smyth MJ, Thia KY, Street SE, et al. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med*. 2000;191:661-668.

487. Shankaran V, Ikeda H, Bruce AT, et al. IFN gamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*. 2001;410:1107-1111.

488. Koebel CM, Vermi W, Swann JB, et al. Adaptive immunity maintains occult cancer in an equilibrium state. *Nature*. 2007;450:903-907.

489. Kammertoens T, Qin Z, Briesemeister D, Bendelac A, Blankenstein T. Methylcholanthrene-induced carcinogenesis is promoted by B-cells and IL-4 but there is no evidence for a role of T / NKT-cells and their effector molecules (Fas-ligand, TNF-alpha, Perforin). *Int J Cancer*. 2011;131(7):1499-1508.

490. Johnson S. The effect of thymectomy and of the dose of 3-methylcholanthrene on the induction and antigenic properties of sarcomas in C56Bl mice. *Br J Cancer*. 1968;22:93-104.

491. Prehn RT, Bartlett GL. Surveillance, latency and the two levels of MCA-induced tumor immunogenicity. *Int J Cancer*. 1987;39:106-110.

492. Bartlett GL. Effect of host immunity on the antigenic strength of primary tumors. *J Natl Cancer Inst*. 1972;49:493-504.

493. Baldwin RW. Tumour-specific immunity against spontaneous rat tumours. *Intl J Cancer*. 1966;1:257-264.

494. Hewitt HB, Blake ER, Walder AS. A critique of the evidence for active host defence against cancer, based on personal studies of 27 murine tumours of spontaneous origin. *Br J Cancer*. 1976;33:241-259.

495. Hewitt HB. The choice of animal tumors for experimental studies of cancer therapy. *Adv Cancer Res*. 1978;27:149-1200.

496. Moloney JB. A virus-induced rhabdomyosarcoma of mice. *Natl Cancer Inst Monogr*. 1966;22:139-142.

497. Fefer A, McCoy JL, Glynn JP. Antigenicity of a virus-induced murine sarcoma (Moloney). *Cancer Res*. 1967;27:962-967.

498. Fefer A, McCoy JL, Glynn JP. Induction and regression of primary moloney sarcoma virus-induced tumors in mice. *Cancer Res*. 1967;27:1626-1631.

499. Fefer A, McCoy JL, Perk K, Glynn JP. Immunologic, virologic, and pathologic studies of regression of autochthonous Moloney sarcoma virus-induced tumors in mice. *Cancer Res*. 1968;28:1577-1585.

500. Malmgren RA, Bennison BE, McKinely TW Jr. Reduced antibody titers in mice treated with carcinogenic and cancer chemotherapeutic agents. *Proc Soc Exp Biol Med*. 1952;79:484-488.

501. Saboori AM, Newcombe DS. Environmental chemicals with immunotoxic properties. In: Newcombe DS, Rose NR, Bloom JC, eds. *Clinical Immunotoxicology*. New York, NY: Raven Press; 1992:365-400.

502. Kripke ML, Fisher MS. Immunologic parameters of ultraviolet carcinogenesis. *J Natl Cancer Inst*. 1976;57:211-215.

503. Spellman CW, Daynes RA. Ultraviolet light, tumors, and suppressor T cells. *Hum Path*. 1981;12:299-301.

504. Kripke ML. Immunological unresponsiveness induced by ultraviolet radiation. *Immunol Rev*. 1984;80:87-102.

505. Svane IM, Engel AM, Nielsen MB, Ljunggren HG, Rygaard J, Werdelin O. Chemically induced sarcomas from nude mice are more immunogenic than similar sarcomas from congenic normal mice. *Eur J Immunol*. 1996;26:1844-1850.

506. Engel AM, Svane IM, Rygaard J, Werdelin O. MCA sarcomas induced in scid mice are more immunogenic than MCA sarcomas induced in congenic, immunocompetent mice. *Scand J Immunol*. 1997;45: 463-470.

507. Urban JL, Burton RC, Holland JM, Kripke ML, Schreiber H. Mechanisms of syngeneic tumor rejection. Susceptibility of host-selected progressor variants to various immunological effector cells. *J Exp Med*. 1982;155:557-573.

508. Ward PL, Koeppen HK, Hurteau T, Rowley DA, Schreiber H. Major histocompatibility complex class I and unique antigen expression by murine tumors that escaped from CD8+ T-cell-dependent surveillance. *Cancer Res*. 1990;50:3851-3858.

509. Schreiber K, Wu TH, Kast WM, Schreiber H. Tracking the common ancestry of antigenically distinct cancer variants. *Clin Cancer Res*. 2001;7:871s-875s.

510. Wu TH, Schreiber K, Arina A, et al. Progression of cancer from indolent to aggressive despite antigen retention and increased expression of interferon-gamma inducible genes. *Cancer Immun*. 2011;11:2.

511. Winn HJ. Immune mechanisms in homotransplantation. II. Quantitative assay of the immunologic activity of lymphoid cells stimulated by tumor homografts. *J Immunol*. 1961;86:228-239.

512. Urban JL, Schreiber H. Selection of macrophage-resistant progressor tumor variants by the normal host. Requirement for concomitant T cell-mediated immunity. *J Exp Med*. 1983;157:642-656.

513. Schultz KR, Klarnet JP, Gieni RS, HayGlass KT, Greenberg PD. The role of B cells for in vivo T cell responses to a Friend virus-induced leukemia. *Science*. 1990;249:921-923.

514. Koeppen HK, Singh S, Stauss HJ, Park BH, Rowley DA, Schreiber H. CD4-positive and B lymphocytes in transplantation immunity. I. Promotion of tumor allograft rejection through elimination of CD4- positive lymphocytes. *Transplantation*. 1993;55:1349-1355.

515. Monach PA, Schreiber H, Rowley DA. CD4+ and B lymphocytes in transplantation immunity. II. Augmented rejection of tumor allografts by mice lacking B cells. *Transplantation*. 1993;55:1356-1361.

516. Qin Z, Richter G, Schuler T, Ibe S, Cao X, Blankenstein T. B cells inhibit induction of T cell-dependent tumor immunity. *Nat Med*. 1998;4: 627-630.

517. de Visser KE, Korets LV, Coussens LM. De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. *Cancer Cell*. 2005;7:411-423.

518. Ammirante M, Luo JL, Grivennikov S, Nedospasov S, Karin M. B-cellderived lymphotoxin promotes castration-resistant prostate cancer. *Nature*. 2010;464:302-305.

519. Schioppa T, Moore R, Thompson RG, et al. B regulatory cells and the tumor-promoting actions of TNF-alpha during squamous carcinogenesis. *Proc Natl Acad Sci U S A*. 2011;108:10662-10667.

520. Andreu P, Johansson M, Affara NI, et al. FcRgamma activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell* . 2010;17:121-134.

521. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol* . 2010;11: 889-896.

522. Real FX, Mattes MJ, Houghton AN, Oettgen HF, Lloyd KO, Old LJ. Class 1 (unique) tumor antigens of human melanoma. Identification of a 90,000 dalton cell surface glycoprotein by autologous antibody. *J Exp Med*. 1984;160:1219-1233.

523. Old LJ, Boyse EA, Stockert E. Antigenic properties of experimental leukemias. I. Serological studies in vitro with spontaneous and radiation- induced leukemias. *J Natl Cancer Inst*. 1963;31:977-986.

524. Boyse EA, Old LJ, Luell S. Antigenic properties of experimental leukemias. II. Immunological studies *in vivo* with C57Bl/6 radiationinduced leukemias. *J Natl Cancer Inst*. 1963;31:987-995.

525. Brown JP, Woodbury RG, Hart CE, Hellström I, Hellström KE. Quantitative analysis of melanoma-associated antigen p97 in normal and neoplastic tissues. *Proc Natl Acad Sci U S A*. 1981;78:539-543.

526. Leclerc JC, Gomard E, Levy JP. Cell-mediated reaction against tumors induced by oncornaviruses. I. Kinetics and specificity of the immune response in murine sarcoma virus (MSV)-induced tumors and transplanted lymphomas. *Intl J Cancer* . 1972;10:589-601.

527. Tevethia SS, Blasecki JW, Vaneck G, Goldstein AL. Requirement of thymus-derived theta-positive lymphocytes for rejection of DNA virus (SV 40) tumors in mice. *J Immunol* . 1974;113:1417-1423.

528. Cheever MA, Thompson DB, Klarnet JP, Greenberg PD. Antigendriven long term-cultured T cells proliferate in vivo, distribute widely, mediate specific tumor therapy, and

persist long-term as functional memory T cells. *J Exp Med*. 1986;163:1100-1112.

529. Rouse BT. Anti-theta serum-induced suppession of the cellular transfer of tumour-specific immunity to a syngeneic plasma cell tumour. *Nat New Biol* . 1972;238:116-117.

530. Algire GH, Weaver JM, Prehn RT. Growth of cells in vivo in diffusion chambers. I. Survival of homografts in immunized mice. *J Natl Cancer Inst*. 1954;15:493-507.

531. Mitchison NA. Passive transfer of transplantation immunity. *Nature*. 1953;171:267-268.

532. Mitchison NA. Passive transfer of transplantation immunity. *Proc R Soc Lond B Biol Sci*. 1954;142:72-87.

533. Mitchison NA. Studies on the immunological response to foreign tumor transplants in the mouse. I. The role of lymph node cells in conferring immunity by adoptive transfer. *J Exp Med*. 1955;102: 157-177.

534. Robins RA. T-cell responses at the host: tumour interface. *Biochim Biophy Acta*. 1986;865:289-305.

535. Ellyard JI, Simson L, Parish CR. Th2-mediated anti-tumour immunity: friend or foe? *Tissue Antigens*. 2007;70:1-11.

536. Garcia-Hernandez Mde L, Hamada H, Reome JB, Misra SK, Tighe MP, Dutton RW. Adoptive transfer of tumor-specific Tc17 effector T cells controls the growth of B16 melanoma in mice. *J Immunol* . 2010;184:4215-4227.

537. Spiotto MT, Rowley DA, Schreiber H. Bystander elimination of antigen loss variants in established tumors. *Nat Med*. 2004;10:294-298.

538. Spiotto MT, Schreiber H. Rapid destruction of the tumor microenvironment by CTLs recognizing cancer-specific antigens cross-presented by stromal cells. *Cancer Immun*. 2005;5:8.

539. Zhang B, Bowerman NA, Salama JK, et al. Induced sensitization of tumor stroma leads to eradication of established cancer by T cells. *J Exp Med*. 2007;204:49-55.

540. Zhang B, Karrison T, Rowley DA, Schreiber H. IFN-gamma- and TNF-dependent bystander eradication of antigen-loss variants in established mouse cancers. *J Clin Invest*. 2008;118:1398-1404.

541. Dobrzanski MJ, Reome JB, Hollenbaugh JA, Hylind JC, Dutton RW. Effector cell-derived lymphotoxin alpha and Fas ligand, but not perforin, promote Tc1 and Tc2 effector cell-mediated tumor therapy in established pulmonary metastases. *Cancer Res*. 2004;64:406-414.

542. Gerbitz A, Sukumar M, Helm F, et al. Stromal interferon-gamma signaling and cross-presentation are required to eliminate antigen-loss variants of B cell lymphomas in mice. *PLoS One*. 2012;7:e34552.

543. Schietinger A, Philip M, Liu RB, Schreiber K, Schreiber H. Bystander killing of cancer requires the cooperation of CD4(+) and CD8(+) T cells during the effector phase. *J Exp Med*. 2010;207:2469-2477.

544. Bos R, Sherman LA. CD4+ T-cell help in the tumor milieu is required for recruitment and cytolytic function of CD8+ T lymphocytes. *Cancer Res*. 2010;70:8368-8377.

545. Matloubian M, Concepcion RJ, Ahmed R. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J Virol*. 1994;68:8056-8063.

546. Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N Engl J Med*. 1995;333:1038-1044.

547. Sun JC, Williams MA, Bevan MJ. CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nat Immunol*. 2004;5:927-933.

548. Fujiwara H, Fukuzawa M, Yoshioka T, Nakajima H, Hamaoka T. The role of tumor-specific Lyt-1+2- T cells in eradicating tumor cells in vivo. I. Lyt-1+2- T cells do not necessarily require recruitment of host's cytotoxic T cell precursors for implementation of in vivo immunity. *J Immunol*. 1984;133:1671-1676.

549. Greenberg PD, Kern DE, Cheever MA. Therapy of disseminated murine leukemia with cyclophosphamide and immune Lyt-1^{+},2^{-} T cells. Tumor eradication does not require participation of cytotoxic T cells. *J Exp Med*. 1985;161:1122-1134.

550. Frey AB. Rat mammary adenocarcinoma 13762 expressing IFN-gamma elicits antitumor CD4+ MHC class II-restricted T cells that are cytolytic in vitro and tumoricidal in vivo. *J Immunol*. 1995;154:4613-4622.

551. Nishimura T, Iwakabe K, Sekimoto M, et al. Distinct role of antigenspecific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. *J Exp Med*. 1999;190:617-627.

552. Mumberg D, Monach PA, Wanderling S, et al. CD4+ T cells eliminate MHC class II-negative cancer cells in vivo by indirect effects of IFNg. *Proc Natl Acad Sci U S A*. 1999;96:8633-8638.

553. Perez-Diez A, Joncker NT, Choi K, et al. CD4 cells can be more efficient at tumor rejection than CD8 cells. *Blood*. 2007;109:5346-5354.

554. Qin Z, Blankenstein T. CD4+ T cell—mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. *Immunity*. 2000;12:677-686.

555. Briesemeister D, Sommermeyer D, Loddenkemper C, et al. Tumor rejection by local interferon gamma induction in established tumors is associated with blood vessel destruction and necrosis. *Int J Cancer*. 2011;128:371-378.

556. Awwad M, North RJ. Immunologically mediated regression of a murine lymphoma after treatment with anti-L3T4 antibody. A consequence of removing L3T4+ suppressor T cells from a host generating predominantly Lyt-2+ T cell- mediated immunity. *J Exp Med*. 1988;168:2193-2206.

557. Nishikawa H, Sakaguchi S. Regulatory T cells in tumor immunity. *Int J Cancer*. 2010;127:759-767.

558. Takasugi M, Mickey MR, Terasaki PI. Reactivity of lymphocytes from normal persons

on cultured tumor cells. *Cancer Res*. 1973;33: 2898-2902.

559. Sendo F, Aoki T, Boyse EA, Buafo CK. Natural occurrence of lymphocytes showing cytotoxic activity to BALB/c radiation-induced leukemia RL male 1 cells. *J Natl Cancer Inst*. 1975;55:603-609.

560. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. *Intl J Cancer* . 1975;16: 216-229.

561. Kiessling R, Klein E, Pross H, Wigzell H. “Natural” killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur J Immunol* . 1975;5: 117-121.

562. Kiessling R, Klein E, Wigzell H. “Natural” killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur J Immunol* . 1975;5:112-117.

563. Joihdal M, Press H. Surface markers on human B and T lymphocytes VI. Cytotoxicity against cell lines as a functional marker for lymphocyte subpopulations. *Int J Cancer* . 1975;15:596.

564. Matthews N, Maclaurin BP, Clarke GN. Characterization of the normal lymphocyte population cytolytic to Burkitt's lymphoma cells of the EB2 cell line. *Aust J Exp Biol Med Sci*. 1976;53:389-398.

565. Trinchieri G. Biology of natural killer cells. *Adv Immunol*. 1989;47: 187-376.

566. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature*. 1986;319:675-678.

567. Ciccone E, Pende D, Viale O, et al. Involvement of HLA class I alleles in natural killer (NK) cell-specific functions: expression of HLA-Cw3 confers selective protection from lysis by alloreactive NK clones displaying a defined specificity (specificity 2). *J Exp Med*. 1992;176: 963-971.

568. Snell GD. Histocompatibility genes of the mouse. II. Production and analysis of isogenic resistant lines. *J Natl Cancer Inst* . 1958;21:843-877.

569. Cudkowicz G, Cosgrove GE. Immunologically competent cells in adult mouse liver: studies with parent-to-hybrid radiation chimeras (26113). *Proc Soc Exp Biol Med*. 1960;105:366-371.

570. Talmadge JE, Meyers KM, Prieur DJ, Starkey JR. Role of natural killer cells in tumor growth and metastasis: C57BL/6 normal and beige mice. *J Natl Cancer Inst* . 1980;65:929-935.

571. Hanna N, Fidler IJ. Role of natural killer cells in the destruction of circulating tumor emboli. *J Natl Cancer Inst* . 1980;65:801-809.

572. Hanna N, Burton RC. Definitive evidence that natural killer (NK) cells inhibit experimental tumor metastases in vivo. *J Immunol*. 1981; 127:1754-1758.

573. Gasser S, Orsulic S, Brown EJ, Raulet DH. The DNA damage pathway regulates

innate immune system ligands of the NKG2D receptor. *Nature*. 2005;436:1186-1190.

574. Guerra N, Tan YX, Joncker NT, et al. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity*. 2008;28:571-580.

575. Terme M, Ullrich E, Delahaye NF, Chaput N, Zitvogel L. Natural killer cell-directed therapies: moving from unexpected results to successful strategies. *Nat Immunol*. 2008;9:486-494.

576. Stojanovic A, Cerwenka A. Natural killer cells and solid tumors. *J Innate Immun*. 2011;3:355-364.

577. Coca S, Perez-Piqueras J, Martinez D, et al. The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. *Cancer*. 1997;79:2320-2328.

578. Romagne F, Vivier E. Natural killer cell-based therapies. *F1000 Med Rep*. 2011;3:9.

579. Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA. Lymphokineactivated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. *J Exp Med*. 1982;155:1823-1841.

580. Grimm EA, Rosenberg SA. The human lymphokine-activated killer cell phenomenon. In: Pick E, ed. *Lymphokines*. Vol 9. New York, NY: Academic Press; 1984:279-311.

581. Eberlein TJ, Rosenstein M, Rosenberg SA. Regression of a disseminated syngeneic solid tumor by systemic transfer of lymphoid cells expanded in interleukin 2. *J Exp Med*. 1982;156:385-397.

582. Mazumder A, Rosenberg SA. Successful immunotherapy of natural killer-resistant established pulmonary melanoma metastases by the intravenous adoptive transfer of syngeneic lymphocytes activated in vitro by interleukin 2. *J Exp Med*. 1984;159:495-507.

583. Rosenberg SA, Lotze MT, Muul LM, et al. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N Engl J Med*. 1987;316:889-897.

584. Parkhurst MR, Riley JP, Dudley ME, Rosenberg SA. Adoptive transfer of autologous natural killer cells leads to high levels of circulating natural killer cells but does not mediate tumor regression. *Clin Cancer Res*. 2011;17:6287-6297.

585. Phillips JH, Lanier LL. Dissection of the lymphokine-activated killer phenomenon. Relative contribution of peripheral blood natural killer cells and T lymphocytes to cytolysis. *J Exp Med*. 1986;164:814-825.

586. Ortaldo JR, Mason A, Overton R. Lymphokine-activated killer cells. Analysis of progenitors and effectors. *J Exp Med*. 1986;164: 1193-1205.

587. Yang JC, Mule JJ, Rosenberg SA. Murine lymphokine-activated killer (LAK) cells: phenotypic characterization of the precursor and effector cells. *J Immunol*. 1986;137:715-722.

588. Diefenbach A, Jensen ER, Jamieson AM, Raulet DH. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature*. 2001;413:165-171.

589. Narni-Mancinelli E, Vivier E, Kerdiles YM. The “T-cell-ness” of NK cells: unexpected similarities between NK cells and T cells. *Int Immunol*. 2011;23:427-431.

590. Cui J, Shin T, Kawano T, et al. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science*. 1997;278:1623-1626.

591. Kawano T, Cui J, Koezuka Y, et al. Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells. *Proc Natl Acad Sci U S A* . 1998;95:5690-5693.

592. Smyth MJ, Crowe NY, Godfrey DI. NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. *Int Immunol*. 2001;13:459-463.

593. Dhodapkar MV, Richter J. Harnessing natural killer T (NKT) cells in human myeloma: progress and challenges. *Clin Immunol*. 2011; 140:160-166.

594. Motohashi S, Okamoto Y, Yoshino I, Nakayama T. Anti-tumor immune responses induced by iNKT cell-based immunotherapy for lung cancer and head and neck cancer. *Clin Immunol*. 2011;140:167-176.

595. Metelitsa LS. Anti-tumor potential of type-I NKT cells against CD1dpositive and CD1d-negative tumors in humans. *Clin Immunol*. 2011; 140:119-129.

596. Dellabona P, Casorati G, de Lalla C, Montagna D, Locatelli F. On the use of donor-derived iNKT cells for adoptive immunotherapy to prevent leukemia recurrence in pediatric recipients of HLA haploidentical HSCT for hematological malignancies. *Clin Immunol*. 2011;140: 152-159.

597. Song L, Asgharzadeh S, Salo J, et al. Valpha24-invariant NKT cells mediate antitumor activity via killing of tumor-associated macrophages. *J Clin Invest*. 2009;119:1524-1536.

598. Introna M, Mantovani A. Natural killer cells in human solid tumors. *Cancer Metastasis Rev*. 1983;2:337-350.

599. Liu RB, Engels B, Arina A, et al. Densely cranulated murine NK cells eradicate large solid tumors. *Cancer Res*. 2012;72(8):1964-1974.

600. Pereira HA, Shafer WM, Pohl J, Martin LE, Spitznagel JK. CAP37, a human neutrophil-derived chemotactic factor with monocyte specific activity. *J Clin Invest*. 1990;85:1468-1476.

601. Kudo C, Yamashita T, Terashita M, Sendo F. Modulation of in vivo immune response by selective depletion of neutrophils using a monoclonal antibody, RP-3. II. Inhibition by RP-3 treatment of mononuclear leukocyte recruitment in delayed-type hypersensitivity to sheep red blood cells in rats. *J Immunol*. 1993;150:3739-3746.

602. Conlan JW, North RJ. Neutrophils are essential for early anti-Listeria defense in the liver, but not in the spleen or peritoneal cavity, as revealed by a granulocyte-depleting monoclonal antibody. *J Exp Med*. 1994; 179:259-268.

603. Nozawa H, Chiu C, Hanahan D. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. *Proc Natl Acad Sci U S A* .

2006;103:12493-12498.

604. Mantovani A, Bottazzi B, Colotta F, Sozzani S, Ruco L. The origin and function of tumor-associated macrophages. *Immunol Today*. 1992;13:265-270.

605. Fearon DT, Locksley RM. The instructive role of innate immunity in the acquired immune response. *Science*. 1996;272:50-53.

606. Chen JJ, Sun Y, Nabel GJ. Regulation of the proinflammatory effects of Fas ligand (CD95L). *Science*. 1998;282:1714-1717.

607. Ellyard JI, Quah BJ, Simson L, Parish CR. Alternatively activated macrophage possess antitumor cytotoxicity that is induced by IL-4 and mediated by arginase-1. *J Immunother*. 2010;33:443-452.

608. Lotfi R, Lee JJ, Lotze MT. Eosinophilic granulocytes and damage-associated molecular pattern molecules (DAMPs): role in the inflammatory response within tumors. *J Immunother*. 2007;30:16-28.

609. Pace JL, Russell SW. Activation of mouse macrophages for tumor cell killing. I. Quantitative analysis of interactions between lymphokine and lipopolysaccharide. *J Immunol*. 1981;126:1863-1867.

610. Nathan C, Murray W, Wiebe ME, Rubin BY. Identification of interferon-g as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J Exp Med*. 1983;158:670-681.

611. Inoue T, Sendo F. In vitro induction of cytotoxic polymorphonuclear leukocytes by supernatant from a concanavalin A-stimulated spleen cell culture. *J Immunol*. 1983;131:2508-2514.

612. Nathan CF, Karnovsky ML, David JR. Alterations of macrophage functions by mediators from lymphocytes. *J Exp Med*. 1971;133:1356- 1376.

613. Hibbs JB Jr, Lambert LH Jr, Remington JS. Possible role of macrophage mediated nonspecific cytotoxicity in tumour resistance. *Nat New Biol*. 1972;235:48-50.

614. Hibbs JB Jr, Lambert LH Jr, Remington JS. Control of carcinogenesis: a possible role for the activated macrophage. *Science*. 1972;177: 998-1000.

615. Evans R, Alexander P. Mechanism of immunologically specific killing of tumour cells by macrophages. *Nature*. 1972;236:168-170.

616. Meltzer MS. Macrophage activation for tumor cytotoxicity: characterization of priming and trigger signals during lymphokine activation. *J Immunol*. 1981;127:179-183.

617. Lichtenstein A. Granulocytes as possible effectors of tumor immunity. *Immunol Allergy Clin North Am*. 1990;731.

618. Munn DH, Cheung NK. Phagocytosis of tumor cells by human monocytes cultured in recombinant macrophage colony-stimulating factor. *J Exp Med*. 1990;172:231-237.

619. Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci U S A*. 1975;72:3666-3670.

620. Pennica D, Nedwin GE, Hayflick JS, et al. Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature*. 1984;312:724-729.

621. Urban JL, Shepard HM, Rothstein JL, Sugarman BJ, Schreiber H. Tumor necrosis factor: a potent effector molecule for tumor cell killing by activated macrophages. *Proc Natl Acad Sci U S A*. 1986;83:5233-5237.

622. Philip R, Epstein LB. Tumour necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, gammainterferon and interleukin-1. *Nature*. 1986;323:86-89.

623. Ziegler-Heitbrock HW, Möller A, Linke RP, Haas JG, Reiber EP, Riethmüller G. Tumor necrosis factor as effector molecule in monocyte mediated cytotoxicity. *Cancer Res*. 1986;46:5947-5952.

624. Feinman R, Henriksen-DeStefano D, Tsujimoto M, Vilcek J. Tumor necrosis factor is an important mediator of tumor cell killing by human monocytes. *J Immunol*. 1987;138:635-640.

625. Nathan CF, Murray HW, Cohn ZA. The macrophage as an effector cell. *N Engl J Med*. 1980;303:622-626.

626. Hibbs JB Jr, Taintor RR, Vavrin Z. Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science*. 1987;235:473-476.

627. Keller R, Geiges M, Keist R. L-arginine-dependent reactive nitrogen intermediates as mediators of tumor cell killing by activated macrophages. *Cancer Res*. 1990;50:1421-1425.

628. Fidler IJ. Therapy of cancer metastasis by systemic activation of macrophages. *Adv Pharmacol*. 1994;30:271-326.

629. Lin EY, Nguyen AV, Russell RG, Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med*. 2001;193:727-740.

630. Fujii Y, Kimura S, Arai S, Sendo F. In vivo antitumor effect of lymphokine- activated rodent polymorphonuclear leukocytes. *Cancer Res*. 1987;47:6000-6005.

631. Midorikawa Y, Yamashita T, Sendo F. Modulation of the immune response to transplanted tumors in rats by selective depletion of neutrophils in vivo using a monoclonal antibody: abrogation of specific transplantation resistance to chemical carcinogen-induced syngeneic tumors by selective depletion of neutrophils in vivo. *Cancer Res*. 1990; 50:6243-6247.

632. Adams DO, Snyderman R. Do macrophages destroy nascent tumors? *J Natl Cancer Inst*. 1979;62:1341-1345.

633. Golumbek PT, Lazenby AJ, Levitsky HI, et al. Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. *Science*. 1991;254:713-716.

634. Perdrizet GA, Ross SR, Stauss HJ, Singh S, Koeppen H, Schreiber H. Animals bearing malignant grafts reject normal grafts that express through gene transfer the same antigen. *J Exp Med*. 1990;171:1205-1220.

635. Wick M, Dubey P, Koeppen H, et al. Antigenic cancer cells can grow progressively in immune hosts without evidence for T cell exhaustion or systemic anergy. *J Exp Med*. 1997;186:229-237.

636. Heath WR, Allison J, Hoffmann MW, et al. Autoimmune diabetes as a consequence of locally produced interleukin-2. *Nature*. 1992;359: 547-549.

637. Ksander BR, Streilein JW. Failure of infiltrating precursor cytotoxic T cells to acquire direct cytotoxic function in immunologically privileged sites. *J Immunol*. 1990;145:2057-2063.

638. Ksander BR, Acevedo J, Streilein JW. Local T helper cell signals by lymphocytes infiltrating intraocular tumors. *J Immunol*. 1992;148: 1955-1963.

639. Streilein JW, Ksander BR, Taylor AW. Immune deviation in relation to ocular immune privilege. *J Immunol*. 1997;158:3557-3560.

640. Hanson HL, Donermeyer DL, Ikeda H, et al. Eradication of established tumors by CD8+ T cell adoptive immunotherapy. *Immunity*. 2000;13:265-276.

641. Blohm U, Roth E, Brommer K, Dumrese T, Rosenthal FM, Pircher H. Lack of effector cell function and altered tetramer binding of tumorinfiltrating lymphocytes. *J Immunol*. 2002;169:5522-5530.

642. Bendle GM, Holler A, Pang LK, et al. Induction of unresponsiveness limits tumor protection by adoptively transferred MDM2-specific cytotoxic T lymphocytes. *Cancer Res*. 2004;64:8052-8056.

643. Anderson MJ, Shafer-Weaver K, Greenberg NM, Hurwitz AA. Tolerization of tumor-specific T cells despite efficient initial priming in a primary murine model of prostate cancer. *J Immunol*. 2007;178: 1268-1276.

644. Janicki CN, Jenkinson SR, Williams NA, Morgan DJ. Loss of CTL function among high-avidity tumor-specific CD8+ T cells following tumor infiltration. *Cancer Res*. 2008;68:2993-3000.

645. Bai A, Higham E, Eisen HN, Wittrup KD, Chen J. Rapid tolerization of virus-activated tumor-specific CD8+ T cells in prostate tumors of TRAMP mice. *Proc Natl Acad Sci U S A*. 2008;105:13003-13008.

646. Olurinde MO, Shen CH, Drake A, Bai A, Chen J. Persistence of tumorinfiltrating CD8 T cells is tumor-dependent but antigen-independent. *Cell Mol Immunol*. 2011;8:415-423.

647. O'Connell J, O'Sullivan GC, Collins JK, Shanahan F. The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. *J Exp Med*. 1996;184:1075-1082.

648. Hahne M, Rimoldi D, Schroter M, et al. Melanoma cell expression of Fas(Apo-1/CD95) ligand: implications for tumor immune escape. *Science*. 1996;274:1363-1366.

649. Strand S, Hofmann WJ, Hug H, et al. Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells—a mechanism of immune evasion? *Nat Med*. 1996;2:1361-1366.

650. Unanue ER, Allen PM. The basis for the immunoregulatory role of macrophages and

other accessory cells. *Science*. 1987;236: 551-557.

651. Munn DH, Mellor AL. The tumor-draining lymph node as an immune- privileged site. *Immunol Rev*. 2006;213:146-158.

652. Munder M. Arginase: an emerging key player in the mammalian immune system. *Br J Pharmacol*. 2009;158:638-651.

653. Singer K, Gottfried E, Kreutz M, Mackensen A. Suppression of T-cell responses by tumor metabolites. *Cancer Immunol Immunother* . 2011;60:425-431.

654. Matsuda M, Petersson M, Lenkei R, et al. Alterations in the signaltransducing molecules of T cells and NK cells in colorectal tumor-infiltrating, gut mucosal and peripheral lymphocytes: correlation with the stage of the disease. *Int J Cancer* . 1995;61:765-772.

655. Whiteside TL. Signaling defects in T lymphocytes of patients with malignancy. *Cancer Immunol Immunother* . 1999;48:346-352.

656. Frey AB, Monu N. Signaling defects in anti-tumor T cells. *Immunol Rev*. 2008;222:192-205.

657. Mizoguchi H, O'Shea JJ, Longo DL, Loeffler CM, McVicar DW, Ochoa AC. Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. *Science*. 1992;258:1795-1798.

658. Nakagomi H, Petersson M, Magnusson I, et al. Decreased expression of the signal-transducing zeta chains in tumor-infiltrating T-cells and NK cells of patients with colorectal carcinoma. *Cancer Res*. 1993;53:5610-5612.

659. Rabinowich H, Suminami Y, Reichert TE, et al. Expression of cytokine genes or proteins and signaling molecules in lymphocytes associated with human ovarian carcinoma. *Intl J Cancer* . 1996;68:276-284.

660. Franco JL, Ghosh P, Wiltrout RH, et al. Partial degradation of T-cell signal transduction molecules by contaminating granulocytes during protein extraction of splenic T cells from tumor-bearing mice. *Cancer Res*. 1995;55:3840-3846.

661. Ling W, Rayman P, Uzzo R, et al. Impaired activation of NFkappaB in T cells from a subset of renal cell carcinoma patients is mediated by inhibition of phosphorylation and degradation of the inhibitor, IkappaBalpha. *Blood*. 1998;92:1334-1341.

662. Ghosh P, Sica A, Young HA, et al. Alterations in NF kappa B/Rel family proteins in splenic T-cells from tumor-bearing mice and reversal following therapy. *Cancer Res*. 1994;54:2969-2972.

663. Aoe T, Okamoto Y, Saito T. Activated macrophages induce structural abnormalities of the T cell receptor-CD3 complex. *J Exp Med*. 1995;181:1881-1886.

664. Spitalny GL, North RJ. Subversion of host defense mechanisms by malignant tumors: an established tumor as a privileged site for bacterial growth. *J Exp Med*. 1977;145:1264-1277.

665. Pawelek JM, Low KB, Bermudes D. Tumor-targeted Salmonella as a novel anticancer vector. *Cancer Res*. 1997;57:4537-4544.

666. Garrido F, Festenstein H, Schirrmacher V. Further evidence for depression of H-2 and Ia-like specificities of foreign haplotypes in mouse tumour cell lines. *Nature*. 1976;261:705-707.

667. Pellegrino MA, Ferrone S, Reisfeld RA, Irie RF, Golub SH. Expression of histocompatibility (HLA) antigens on tumor cells and normal cells from patients with melanoma. *Cancer*. 1977;40:36-41.

668. Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv Immunol*. 2000;74:181-273.

669. Garrido F, Algarra I, Garcia-Lora AM. The escape of cancer from T lymphocytes: immunoselection of MHC class I loss variants harboring structural-irreversible “hard” lesions. *Cancer Immunol Immunother* . 2010;59:1601-1606.

670. Trowsdale J, Travers P, Bodmer WF, Patillo RA. Expression of HLA-A, -B, and -C and beta 2-microglobulin antigens in human choriocarcinoma cell lines. *J Exp Med*. 1980;152:11s-17s.

671. Travers PJ, Arklie JL, Trowsdale J, Patillo RA, Bodmer WF. Lack of expression of HLA-ABC antigens in choriocarcinoma and other human tumor cell lines. *Natl Cancer Inst Monogr*. 1982;60:175-180.

672. Wang Z, Cao Y, Albino AP, Zeff RA, Houghton A, Ferrone S. Lack of HLA class I antigen expression by melanoma cells SK-MEL-33 caused by a reading frameshift in beta 2-microglobulin messenger RNA. *J Clin Invest*. 1993;91:684-692.

673. Garcia-Lora A, Algarra I, Gaforio JJ, Ruiz-Cabello F, Garrido F. Immunoselection by T lymphocytes generates repeated MHC class I- deficient metastatic tumor variants. *Intl J Cancer*. 2001;91:109-119.

674. Paschen A, Arens N, Sucker A, et al. The coincidence of chromosome 15 aberrations and beta2-microglobulin gene mutations is causative for the total loss of human leukocyte antigen class I expression in melanoma. *Clin Cancer Res*. 2006;12:3297-3305.

675. Maleno I, Aptsiauri N, Cabrera T, et al. Frequent loss of heterozygosity in the beta2-microglobulin region of chromosome 15 in primary human tumors. *Immunogenetics*. 2011;63:65-71.

676. Ruiter DJ, Bergman W, Welvaart K, et al. Immunohistochemical analysis of malignant melanomas and nevocellular nevi with monoclonal antibodies to distinct monomorphic determinants of HLA antigens. *Cancer Res*. 1984;44:3930-3935.

677. Natali PG, Giacomini P, Bigotti A, et al. Heterogeneity in the expression of HLA and tumor-associated antigens by surgically removed and cultured breast carcinoma cells. *Cancer Res*. 1983;43:660-668.

678. Benitez R, Godelaine D, Lopez-Nevot MA, et al. Mutations of the beta2-microglobulin gene result in a lack of HLA class I molecules on melanoma cells of two patients immunized with MAGE peptides. *Tissue Antigens*. 1998;52:520-529.

679. Stein B, Momburg F, Schwarz V, Schlag P, Moldenhauer G, Moller P. Reduction or

loss of HLA-A, B, C antigens in colorectal carcinoma appears not to influence survival. *Br J Cancer*. 1988;57:364-368.

680. Smith ME, Bodmer WF, Bodmer JG. Selective loss of HLA-A,B,C locus products in colorectal adenocarcinoma. *Lancet*. 1988;1:823-824.

681. Kaklamanis L, Gatter KC, Hill AB, et al. Loss of HLA class-I alleles, heavy chains and beta 2-microglobulin in colorectal cancer. *Intl J Cancer*. 1992;51:379-385.

682. Torres LM, Cabrera T, Concha A, Oliva MR, Ruiz-Cabello F, Garrido F. HLA class I expression and HPV-16 sequences in premalignant and malignant lesions of the cervix. *Tissue Antigens*. 1993;41:65-71.

683. Brady CS, Bartholomew JS, Burt DJ, et al. Multiple mechanisms underlie HLA dysregulation in cervical cancer. *Tissue Antigens*. 2000;55: 401-411.

684. Ljunggren HG, Karre K. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. *J Exp Med*. 1985;162:1745-1759.

685. Ljunggren HG, Paabo S, Cochet M, Kling G, Kourilsky P, Karre K. Molecular analysis of H-2-deficient lymphoma lines. Distinct defects in biosynthesis and association of MHC class I heavy chains and beta 2-microglobulin observed in cells with increased sensitivity to NK cell lysis. *J Immunol*. 1989;142:2911-2917.

686. Lassam N, Jay G. Suppression of MHC class I RNA in highly oncogenic cells occurs at the level of transcription initiation. *J Immunol*. 1989;143:3792-3797.

687. Ljunggren HG, Stam NJ, Ohlen C, et al. Empty MHC class I molecules come out in the cold. *Nature*. 1990;346:476-480.

688. Hosken NA, Bevan MJ. Defective presentation of endogenous antigen by a cell line expressing class I molecules. *Science*. 1990;248:367-370.

689. Restifo NP, Esquivel F, Asher AL, et al. Defective presentation of endogenous antigens by a murine sarcoma. Implications for the failure of an anti-tumor immune response. *J Immunol*. 1991;147:1453-1459.

690. Seliger B. Different regulation of MHC class I antigen processing components in human tumors. *J Immunotoxicol*. 2008;5:361-367.

691. Ferrone S, Marincola FM. Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. *Immunol Today*. 1995;16:487-494.

692. Aptsiauri N, Cabrera T, Garcia-Lora A, Lopez-Nevot MA, Ruiz- Cabello F, Garrido F. MHC class I antigens and immune surveillance in transformed cells. *Int Rev Cytol*. 2007;256:139-189.

693. Chiari R, Foury F, De Plaen E, Baurain JF, Thonnard J, Coulie PG. Two antigens recognized by autologous cytolytic T lymphocytes on a melanoma result from a single point mutation in an essential housekeeping gene. *Cancer Res*. 1999;59:5785-5792.

694. Mitchison NA. Antigens of heterozygous tumours as material for the study of cell heredity. *Proc Royal Physical Soc Edinburgh*. 1956; 25:45-48.

695. Klein E, Klein G, Révész L. Permanent modification (mutation?) of a histocompatibility gene in a heterozygous tumor. *J Natl Cancer Inst* . 1957;19:95-114.

696. Vago L, Perna SK, Zanussi M, et al. Loss of mismatched HLA in leukemia after stem-cell transplantation. *N Engl J Med* . 2009;361:478-488.

697. Villalobos IB, Takahashi Y, Akatsuka Y, et al. Relapse of leukemia with loss of mismatched HLA resulting from uniparental disomy after haploidentical hematopoietic stem cell transplantation. *Blood*. 2010;115:3158-3161.

698. Snell GD. Methods for the study of histocompatibility genes. *J Genet*. 1948;49:87-108.

699. Murgia C, Pritchard JK, Kim SY, Fassati A, Weiss RA. Clonal origin and evolution of a transmissible cancer. *Cell*. 2006;126:477-487.

700. Pearse AM, Swift K. Allograft theory: transmission of devil facial-tumour disease. *Nature*. 2006;439:549.

701. McCallum H. Tasmanian devil facial tumour disease: lessons for conservation biology. *Trends Ecol Evol* . 2008;23:631-637.

702. Cohen D. The biological behaviour of the transmissible venereal tumour in immunosuppressed dogs. *Eur J Cancer* . 1973;9:253-258.

703. Cohen D. The canine transmissible venereal tumor: a unique result of tumor progression. *Adv Cancer Res* . 1985;43:75-112.

704. Rebbeck CA, Thomas R, Breen M, Leroi AM, Burt A. Origins and evolution of a transmissible cancer. *Evolution*. 2009;63:2340-2349.

705. Campoli M, Ferrone S. HLA antigen changes in malignant cells: epigenetic mechanisms and biologic significance. *Oncogene*. 2008;27: 5869-5885.

706. Garrido F, Cabrera T, Aptsiauri N. “Hard” and “soft” lesions underlying the HLA class I alterations in cancer cells: implications for immunotherapy. *Int J Cancer* . 2010;127:249-256.

707. D'Urso CM, Wang ZG, Cao Y, Tatake R, Zeff RA, Ferrone S. Lack of HLA class I antigen expression by cultured melanoma cells FO-1 due to a defect in B2m gene expression. *J Clin Invest*. 1991;87:284-292.

708. Schrier PI, Bernards R, Vaessen RT, Houweling A, van der Eb AJ. Expression of class I major histocompatibility antigens switched off by highly oncogenic adenovirus 12 in transformed rat cells. *Nature*. 1983;305:771-775.

709. Versteeg R, Kruse-Wolters KM, Plomp AC, et al. Suppression of class I human histocompatibility leukocyte antigen by c-myc is locus specific. *J Exp Med*. 1989;170:621-635.

710. Garrido C, Paco L, Romero I, et al. MHC class I molecules act as tumor suppressor genes regulating the cell cycle gene expression, invasion, and intrinsic tumorigenicity of melanoma cells. *Carcinogenesis*. 2012;33:687-693.

711. Lill NL, Tevethia MJ, Hendrickson WG, Tevethia SS. Cytotoxic T lymphocytes (CTL)

against a transforming gene product select for transformed cells with point mutations within sequences encoding CTL recognition epitopes. *J Exp Med*. 1992;176:449-457.

712. Garrido F, Ruiz-Cabello F, Cabrera T, et al. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol Today*. 1997;18:89-95.

713. Lehmann F, Marchand M, Hainaut P, et al. Differences in the antigens recognized by cytolytic T cells on two successive metastases of a melanoma patient are consistent with immune selection. *Eur J Immunol* . 1995;25:340-347.

714. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat Immunol*. 2002;3:999-1005.

715. Khong HT, Wang QJ, Rosenberg SA. Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression. *J Immunother*. 2004;27:184-190.

716. Urban JL, Kripke ML, Schreiber H. Stepwise immunologic selection of antigenic variants during tumor growth. *J Immunol*. 1986;137: 3036-3041.

717. Daynes RA, Fernandez PA, Woodward JG. Cell-mediated immune response to syngeneic ultraviolet-induced tumors. II. The properties and antigenic specificities of cytotoxic T lymphocytes generated in vitro following removal from syngeneic tumor-immunized mice. *Cell Immunol*. 1979;45:398-414.

718. Boyse EA, Old LJ. Some aspects of normal and abnormal cell surface genetics. *Annu Rev Genet*. 1969;3:269-290.

719. Takahashi T, Old LJ, McIntire KR, Boyse EA. Immunoglobulin and other surface antigens of cells of the immune system. *J Exp Med*. 1971;134:815-832.

720. Tschopp J, Nabholz M. Perforin-mediated target cell lysis by cytolytic T lymphocytes. *Annu Rev Immunol*. 1990;8:279-302.

721. Otten HG, van Ginkel WG, Hagenbeek A, Petersen EJ. Prevalence and clinical significance of resistance to perforin- and FAS-mediated cell death in leukemia. *Leukemia*. 2004;18:1401-1405.

722. Ye X, McCarrick J, Jewett L, Knowles BB. Timely immunization subverts the development of peripheral nonresponsiveness and suppresses tumor development in simian virus 40 tumor antigen-transgenic mice. *Proc Natl Acad Sci U S A* . 1994;91:3916-3920.

723. Spiotto MT, Reth MA, Schreiber H. Genetic changes occurring in established tumors rapidly stimulate new antibody responses. *Proc Natl Acad Sci U S A* . 2003;100:5425-5430.

724. Staveley-O'Carroll K, Sotomayor E, Montgomery J, et al. Induction of antigen-specific T cell anergy: an early event in the course of tumor progression. *Proc Natl Acad Sci U S A*. 1998;95:1178-1183.

725. Shrikant P, Mescher MF. Control of syngeneic tumor growth by activation of CD8+ T cells: efficacy is limited by migration away from the site and induction of nonresponsiveness. *J Immunol*. 1999;162: 2858-2866.

726. Lo D, Burkly LC, Widera G, et al. Diabetes and tolerance in transgenic mice expressing class II MHC molecules in pancreatic beta cells. *Cell*. 1988;53:159-168.

727. Qin SX, Cobbold S, Benjamin R, Waldmann H. Induction of classical transplantation tolerance in the adult. *J Exp Med*. 1989;169:779-794.

728. Webb S, Morris C, Sprent J. Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. *Cell*. 1990;63: 1249-1256.

729. Ramarathinam L, Sarma S, Maric M, et al. Multiple lineages of tumors express a common tumor antigen, P1A, but they are not cross-protected. *J Immunol*. 1995;155:5323-5329.

730. Srivastava PK. Do human cancers express shared protective antigens? or the necessity of remembrance of things past. *Sem Immunol*. 1996; 8:295-302.

731. Bowne WB, Srinivasan R, Wolchok JD, et al. Coupling and uncoupling of tumor immunity and autoimmunity. *J Exp Med*. 1999;190: 1717-1722.

732. Clynes R, Takechi Y, Moroi Y, Houghton A, Ravetch JV. Fc receptors are required in passive and active immunity to melanoma. *Proc Natl Acad Sci U S A*. 1998;95:652-656.

733. Lee PP, Yee C, Savage PA, et al. Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. *Nat Med*. 1999;5:677-685.

734. Schietinger A, Delrow JJ, Basom RS, Blattman JN, Greenberg PD. Rescued tolerant CD8 T cells are preprogrammed to reestablish the tolerant state. *Science*. 2012;335:723-727.

735. von Boehmer H, Teh HS, Kisielow P. The thymus selects the useful, neglects the useless and destroys the harmful. *Immunol Today*. 1989;10:57-61.

736. Gervois N, Guilloux Y, Diez E, Jotereau F. Suboptimal activation of melanoma infiltrating lymphocytes (TIL) due to low avidity of TCR/MHC-tumor peptide interactions. *J Exp Med*. 1996;183:2403-2407.

737. Naeher D, Daniels MA, Hausmann B, Guillaume P, Luescher I, Palmer E. A constant affinity threshold for T cell tolerance. *J Exp Med*. 2007;204:2553-2559.

738. Zhang X, Fujii H, Kishimoto H, LeRoy E, Surh CD, Sprent J. Aging leads to disturbed homeostasis of memory phenotype CD8(+) cells. *J Exp Med*. 2002;195:283-293.

739. Kaliss N, Molomut N. The effect of prior injections of tissue antiserums on the survival of cancer homoiografts in mice. *Cancer Res*. 1952;12:110-112.

740. Kaliss N, Kandutsch AA. Acceptance of tumor homografts by mice injected with antiserum. I. Activity of serum fractions. *Proc Soc Exp Biol Med*. 1956;91:118-121.

741. Kaliss N. Immunological enhancement of tumor homografts in mice: a review. *Cancer Res*. 1958;18:992-1003.

742. Sjogren HO, Hellstrom I, Bansal SC, Hellstrom KE. Suggestive evidence that the “blocking antibodies” of tumor-bearing individuals may be antigen—antibody complexes. *Proc Natl Acad Sci U S A*. 1971;68:1372-1375.

743. Hellström KE, Hellström I. Lymphocyte-mediated cytotoxicity and blocking serum activity to tumor antigens. *Adv Immunol*. 1974;18: 209-277.

744. Gershon RK, Mokyr MB, Mitchell MS. Activation of suppressor T cells by tumour cells and specific antibody. *Nature*. 1974;250: 594-596.

745. Stuart FP, Saitoh T, Fitch FW. Rejection of renal allografts: specific immunologic suppression. *Science*. 1968;160:1463-1465.

746. Rowley DA, Stuart FP, Fitch FW. Prevention of renal allograft rejection without immune suppression: a model to revisit. *Clin Transplant*. 2011;25:104-110.

747. Ron Y, De Baetselier P, Gordon J, Feldman M, Segal S. Defective induction of antigen-reactive proliferating T cells in B cell-deprived mice. *Eur J Immunol* . 1981;11:964-968.

748. Cobbold SP, Jayasuriya A, Nash A, Prospero TD, Waldmann H. Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo. *Nature*. 1984;312:548-551.

749. Ron Y, Sprent J. T cell priming in vivo: a major role for B cells in presenting antigen to T cells in lymph nodes. *J Immunol*. 1987;138: 2848-2856.

750. Cerny A, Hugin AW, Hardy RR, et al. B cells are required for induction of T cell abnormalities in a murine retrovirus-induced immunodeficiency syndrome. *J Exp Med*. 1990;171:315-320.

751. Gershon RK, Cohen P, Hencin R, Liebhaber SA. Suppressor T cells. *J Immunol*. 1972;108:586-590.

752. Gershon RK, Kondo K. Infectious immunological tolerance. *Immunology*. 1971;21:903-914.

753. Fujimoto S, Greene MI, Sehon AH. Regulation of the immune response to tumor antigens. I. Immunosuppressor cells in tumorbearing hosts. *J Immunol*. 1976;116:791-799.

754. Berendt MJ, North RJ. T-cell-mediated suppression of anti-tumor immunity. An explanation for progressive growth of an immunogenic tumor. *J Exp Med*. 1980;151:69-80.

755. Dye ES, North RJ. T cell-mediated immunosuppression as an obstacle to adoptive immunotherapy of the P815 mastocytoma and its metastases. *J Exp Med*. 1981;154:1033-1042.

756. Bursuker I, North RJ. Generation and decay of the immune response to a progressive fibrosarcoma. *J Exp Med*. 1984;159:1312-1321.

757. Mullen CA, Rowley DA, Schreiber H. Highly immunogenic regressor tumor cells can prevent development of postsurgical tumor immunity. *Cell Immunol*. 1989;119:101-113.

758. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of selftolerance causes various autoimmune diseases. *J Immunol*. 1995;155: 1151-1164.

759. Brunkow ME, Jeffery EW, Hjerrild KA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet*. 2001; 27:68-73.

760. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003;299:1057-1061.

761. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*. 2003;4:330-336.

762. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol*. 2003;4:337-342.

763. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*. 2004;10:942-949.

764. North RJ. Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. *J Exp Med*. 1982;155:1063-1074.

765. North RJ. Gamma-irradiation facilitates the expression of adoptive immunity against established tumors by eliminating suppressor T cells. *Cancer Immunol Immunother*. 1984;16:175-181.

766. Glynn JP, Halpern BL, Fefer A. An immunochemotherapeutic system for the treatment of a transplanted Moloney virus-induced lymphoma in mice. *Cancer Res*. 1969;29:515-520.

767. Fefer A. Immunotherapy and chemotherapy of Moloney sarcoma virus-induced tumors in mice. *Cancer Res*. 1969;29:2177-2183.

768. Cho BK, Rao VP, Ge Q, Eisen HN, Chen J. Homeostasis-stimulated proliferation drives naive T cells to differentiate directly into memory T cells. *J Exp Med*. 2000;192:549-556.

769. Dudley ME, Wunderlich JR, Yang JC, et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol*. 2005;23:2346-2357.

770. Cortez-Retamozo V, Etzrodt M, Newton A, et al. Origins of tumorassociated macrophages and neutrophils. *Proc Natl Acad Sci U S A*. 2012;109:2491-2496.

771. Klein G. Immunological surveillance against neoplasia. *Harvey Lect*. 1975;69:71-102.

772. Klein G, Klein E. Surveillance against tumors—is it mainly immunological? *Immunol Lett*. 2005;100:29-33.

773. Klein G, Imreh S, Zabarovsky ER. Why do we not all die of cancer at an early age? *Adv Cancer Res*. 2007;98:1-16.

774. Kelly GL, Strasser A. The essential role of evasion from cell death in cancer. *Adv Cancer Res*. 2011;111:39-96.

775. Braun AC. Plant tumors as an experimental model. *Harvey Lect*. 1960;56:191-210.

776. Mintz B, Illmensee K. Normal genetically mosaic mice produced from malignant teratocarcinoma cells. *Proc Natl Acad Sci U S A* . 1975;72:3585-3589.

777. Qin Z, Kim HJ, Hemme J, Blankenstein T. Inhibition of methylcholanthrene- induced carcinogenesis by an interferon gamma receptordependent foreign body reaction. *J Exp Med*. 2002;195:1479-1490.

778. Kenny PA, Bissell MJ. Tumor reversion: correction of malignant behavior by microenvironmental cues. *Int J Cancer* . 2003;107:688-695.

779. Proia DA, Kuperwasser C. Stroma: tumor agonist or antagonist. *Cell Cycle*. 2005;4:1022-1025.

780. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature*. 2008;454:436-444.

781. Burnet M. Cancer; a biological approach. I. The processes of control. *Br Med J* . 1957;1:779-786.

782. Thomas L. Discussion of cellular and humoral aspects of the hypersensitive states. In: Lawrence HS. ed. *Cellular and Humoral Aspects of the Hypersensitive States; a symposium held at the New York Academy of Medicine*. New York, NY: Hoeber-Harper; 1959:529-532.

783. Burnet M. Immunological factors in the process of carcinogenesis. *Br Med Bull*. 1964;20:154-158.

784. Burnet FM. The concept of immunological surveillance. *Prog Exp Tumor Res*. 1970;13:1-27.

785. Good R. Relations between immunity and malignancy. *Proc Natl Acad Sci U S A*. 1972;69:1026-1030.

786. Oertel SH, Riess H. Immunosurveillance, immunodeficiency and lymphoproliferations. *Recent Results Cancer Res* . 2002;159:1-8.

787. Hayward AR, Levy J, Facchetti F, et al. Cholangiopathy and tumors of the pancreas, liver, and biliary tree in boys with X-linked immunodeficiency with hyper-IgM. *J Immunol*. 1997;158:977-983.

788. Holland JM, Mitchell TJ, Gipson LC, Whitaker MS. Survival and cause of death in aging germfree athymic nude and normal inbred C3Hf/He mice. *J Natl Cancer Inst* . 1978;61:1357-1361.

789. Haliotis T, Ball JK, Dexter D, Roder JC. Spontaneous and induced primary oncogenesis in natural killer (NK)-cell- deficient beige mutant mice. *Int J Cancer* . 1985;35:505-513.

790. Chiu PP, Ivakine E, Mortin-Toth S, Danska JS. Susceptibility to lymphoid neoplasia in immunodeficient strains of nonobese diabetic mice. *Cancer Res*. 2002;62:5828-5834.

791. Tsuji H, Ishii-Ohba H, Noda Y, Kubo E, Furuse T, Tatsumi K. Rag-dependent and Rag-independent mechanisms of Notch1 rearrangement in thymic lymphomas of Atm(-/-)

and scid mice. *Mutat Res*. 2009;660:22-32.

792. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. Natural innate and adaptive immunity to cancer. *Annu Rev Immunol* . 2011;29: 235-271.

793. Deinhardt FW, Falk LA, Wolfe LG. Simian herpesviruses and neoplasia. *Adv Cancer Res*. 1974;19:167-205.

794. Nagy N, Klein G, Klein E. To the genesis of Burkitt lymphoma: regulation of apoptosis by EBNA-1 and SAP may determine the fate of Ig-myc translocation carrying B lymphocytes. *Semin Cancer Biol* . 2009;19:407-410.

795. Tooze J. *Origins of Contemporary Tumor Virus Research* . New York, NY: Cold Spring Harbor Laboratory; 1973:1-73.

796. Chang Y, Moore PS. Merkel cell carcinoma: a virus-induced human cancer. *Annu Rev Pathol*. 2012;7:123-144.

797. Vinokurova S, Wentzensen N, Kraus I, et al. Type-dependent integration frequency of human papillomavirus genomes in cervical lesions. *Cancer Res* . 2008;68:307-313.

798. Muderspach L, Wilczynski S, Roman L, et al. A phase I trial of a human papillomavirus (HPV) peptide vaccine for women with highgrade cervical and vulvar intraepithelial neoplasia who are HPV 16 positive. *Clin Cancer Res* . 2000;6:3406-3416.

799. Deichman GI. Immunological aspects of carcinogenesis by deoxyribonucleic acid tumor viruses. *Adv Cancer Res* . 1969;12:101-136.

800. zur Hausen H. SV40 in human cancers—an endless tale? *Int J Cancer* . 2003;107:687.

801. Lilly F, Boyse EA, Old LJ. Genetic basis of susceptibility to viral leukaemogenesis. *Lancet*. 1964;2:1207-1209.

802. Lilly F. The inheritance of susceptibility to the Gross leukemia virus in mice. *Genetics*. 1966;53:529-539.

803. Demant P. Cancer susceptibility in the mouse: genetics, biology and implications for human cancer. *Nat Rev Genet* . 2003;4:721-734.

804. Chang ET, Adami HO. The enigmatic epidemiology of nasopharyngeal carcinoma. *Cancer Epidemiol Biomarkers Prev* . 2006;15: 1765-1777.

805. Wank R, Thomssen C. High risk of squamous cell carcinoma of the cervix for women with HLA-DQw3. *Nature*. 1991;352:723-725.

806. Hardie IR, Strong RW, Hartley LC, Woodruff PW, Clunie GJ. Skin cancer in Caucasian renal allograft recipients living in a subtropical climate. *Surgery*. 1980;87:177-183.

807. Robinson JK. Sun exposure, sun protection, and vitamin D. *JAMA*. 2005;294:1541-1543.

808. Bouwes Bavinck JN, Hardie DR, Green A, et al. The risk of skin cancer in renal transplant recipients in Queensland, Australia. A follow-up study. *Transplantation*.

1996;61:715-721.

809. Hojo M, Morimoto T, Maluccio M, et al. Cyclosporine induces cancer progression by a cell-autonomous mechanism. *Nature*. 1999;397: 530-534.

810. Berg D, Otley CC. Skin cancer in organ transplant recipients: epidemiology, pathogenesis, and management. *J Am Acad Dermatol* . 2002;47: 1-17, quiz 18-20.

811. Brash DE, Rudolph JA, Simon JA, et al. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc Natl Acad Sci U S A* . 1991;88:10124-10128.

812. Berg RJ, van Kranen HJ, Rebel HG, et al. Early p53 alterations in mouse skin carcinogenesis by UVB radiation: immunohistochemical detection of mutant p53 protein in clusters of preneoplastic epidermal cells. *Proc Natl Acad Sci U S A* . 1996;93:274-278.

813. Williams RO, Mauri C, Mason LJ, et al. Therapeutic actions of cyclosporine and anti-tumor necrosis factor alpha in collagen-induced arthritis and the effect of combination therapy. *Arthritis Rheum*. 1998; 41:1806-1812.

814. Tanaka N, Ishihara M, Kitagawa M, et al. Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcription factor IRF-1. *Cell*. 1994;77:829-839.

815. Nozawa H, Oda E, Nakao K, et al. Loss of transcription factor IRF-1 affects tumor susceptibility in mice carrying the Ha-ras transgene or nullizygosity for p53. *Genes Dev*. 1999;13:1240-1245.

816. de Gruijl FR, Rebel H. Early events in UV carcinogenesis—DNA damage, target cells and mutant p53 foci. *Photochem Photobiol*. 2008;84: 382-387.

817. Benjamin CL, Ullrich SE, Kripke ML, Ananthaswamy HN. p53 tumor suppressor gene: a critical molecular target for UV induction and prevention of skin cancer. *Photochem Photobiol*. 2008;84:55-62.

818. Queille S, Luron L, Spatz A, et al. Analysis of skin cancer risk factors in immunosuppressed renal transplant patients shows high levels of UV-specific tandem CC to TT mutations of the p53 gene. *Carcinogenesis*. 2007;28:724-731.

819. Viarisio D, Mueller-Decker K, Kloz U, et al. E6 and E7 from beta HPV38 cooperate with ultraviolet light in the development of actinic keratosis-like lesions and squamous cell carcinoma in mice. *PLoS Pathog*. 2011;7:e1002125.

820. Otley CC, Coldiron BM, Stasko T, Goldman GD. Decreased skin cancer after cessation of therapy with transplant-associated immunosuppressants. *Arch Dermatol*. 2001;137:459-463.

821. Kinlein LJ. Immunologic factors including AIDS. In: Schottenfield D, Fraumeni JF Jr, eds. *Cancer Epidemiology and Prevention* . 2nd ed. New York, NY: Oxford University Press; 1996:532-545.

822. Serraino D, Piselli P. Cancer cohort consortium approach: cancer epidemiology in immunosuppressed groups. In: Verma M, ed. *Methods in Molecular Biology, Cancer Epidemiology*. Totowa, NJ: Humana Press; 2009:409-419.

823. Stutman O. Tumor development after 3-methylcholanthrene in immunologically deficient athymic nude mice. *Science*. 1974;183:534- 536.

824. Stutman O. Chemical carcinogenesis in nude mice: comparison between nude mice from homozygous matings and heterozygous matings and effect of age and carcinogen dose. *J Natl Cancer Inst* . 1979;62: 353-358.

825. Engel AM, Svane IM, Mouritsen S, Rygaard J, Clausen J, Werdelin O. Methylcholanthrene-induced sarcomas in nude mice have short induction times and relatively low levels of surface MHC class I expression. *APMIS*. 1996;104:629-639.

826. Outzen HC, Custer RP, Eaton GJ, Prehn RT. Spontaneous and induced tumor incidence in germfree “nude” mice. *J Reticuloendothel Soc*. 1975;17:1-9.

827. Girardi M, Oppenheim DE, Steele CR, et al. Regulation of cutaneous malignancy by gamma delta T Cells. *Science*. 2001;294:605-609.

828. Blankenstein T, Qin Z. Chemical carcinogens as foreign bodies and some pitfalls regarding cancer immune surveillance. *Adv Cancer Res* . 2003;90:179-207.

829. Johnsen AK, France J, Nagy N, et al. Systemic deficits in transporter for antigen presentation (TAP)-1 or proteasome subunit LMP2 have little or no effect on tumor incidence. *Int J Cancer* . 2001;91:366-372.

830. Ogasawara K, Hida S, Azimi N, et al. Requirement for IRF-1 in the microenvironment supporting development of natural killer cells. *Nature*. 1998;391:700-703.

831. Takaoka A, Hayakawa S, Yanai H, et al. Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. *Nature*. 2003;424:516-523.

832. Boggio K, Nicoletti G, Di Carlo E, et al. Interleukin 12-mediated prevention of spontaneous mammary adenocarcinomas in two lines of Her-2/neu transgenic mice. *J Exp Med*. 1998;188:589-596.

833. Virchow R. *Pathogenie der neoplastischen Geschwülste, Fünfte Vorlesung* In: *Die krankhaften*. Geschwülste. *Dreissig Vorlesungen*. Berlin: Verlag von August Hirschwald; 1863:72-101.

834. Virchow R. Reizung und Reizbarkeit. *Arch Pathol Anat Klin Med* . 1858;14:1-63.

835. Thiersch C. *Der Epithelialkrebs namentlich der Haut* . Leipzig, Germany: Engelmann; 1865.

836. Houghton J, Stoicov C, Nomura S, et al. Gastric cancer originating from bone marrow-derived cells. *Science*. 2004;306:1568-1571.

837. Rothstein JL, Schreiber H. Synergy between tumor necrosis factor and bacterial products causes hemorrhagic necrosis and lethal shock in normal mice. *Proc Natl Acad Sci U S A*. 1988;85:607-611.

838. Kindler V, Sappino AP, Grau GE, Piguet PF, Vassalli P. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. *Cell*. 1989;56:731-740.

839. Suganuma M, Okabe S, Marino MW, Sakai A, Sueoka E, Fujiki H. Essential role of tumor necrosis factor alpha (TNF-alpha) in tumor promotion as revealed by TNF-alpha-deficient mice. *Cancer Res*. 1999;59:4516-4518.

840. Moore BB, Arenberg DA, Stoy K, et al. Distinct CXC chemokines mediate tumorigenicity of prostate cancer cells. *Am J Pathol*. 1999;154: 1503-1512.

841. Beattie EC, Stellwagen D, Morishita W, et al. Control of synaptic strength by glial TNFalpha. *Science*. 2002;295:2282-2285.

842. Stellwagen D, Malenka RC. Synaptic scaling mediated by glial TNF-alpha. *Nature*. 2006;440:1054-1059.

843. Berenblum I. The mechanism of carcinogenesis: a study of the significance of cocarcinogenic action. *Cancer Res*. 1941;1:807-814.

844. Rous P, Kidd JG. Conditional neoplasms and subthreshold neoplastic states. A study of the tar tumors of rabbits. *J Exp Med*. 1941;73: 365-390.

845. MacKenzie I, Rous P. The experimental disclosure of latent neoplastic changes in tarred skin. *J Exp Med*. 1941;73:391-415.

846. Hecker E. Phorbol esters from croton oil. Chemical nature and biological activities. *Naturwissenschaften*. 1967;54:282-284.

847. Cannon RE, Spalding JW, Trempus CS, et al. Kinetics of wound-induced v-Ha-ras transgene expression and papilloma development in transgenic Tg.AC mice. *Mol Carcinog*. 1997;20:108-114.

848. Yuspa SH. The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis—thirty-third G.H.A. Clowes Memorial Award Lecture. *Cancer Res*. 1994;54:1178-1189.

849. Bromberg J, Wang TC. Inflammation and cancer: IL-6 and STAT3 complete the link. *Cancer Cell*. 2009;15:79-80.

850. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med*. 1986;315:1650-1659.

851. DiGiovanni J. Genetic factors controlling responsiveness to skin tumor promotion in mice. *Prog Clin Biol Res*. 1995;391:195-212.

852. Schreiber H, Nettesheim P, Lijinsky W, Richter CB, Walburg HE Jr. Induction of lung cancer in germfree, specific-pathogen-free, and infected rats by N-nitrosoheptamethyleneimine: enhancement by respiratory infection. *J Natl Cancer Inst*. 1972;49:1107-1114.

853. Jarchum I, Pamer EG. Regulation of innate and adaptive immunity by the commensal microbiota. *Curr Opin Immunol*. 2011;23:353-360.

854. Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature*. 2008;453:620-625.

855. Strober W. Inside the microbial and immune labyrinth: gut microbes: friends or fiends? *Nat Med*. 2010;16:1195-1197.

856. Coussens LM, Werb Z. Inflammatory cells and cancer: think different! *J Exp Med*. 2001;193:F23-F26.

857. Huang F, Hsu S, Yan Z, Winawer S, Friedman E. The capacity for growth stimulation by TGF beta 1 seen only in advanced colon cancers cannot be ascribed to mutations in APC, DCC, p53 or ras. *Oncogene*. 1994;9:3701-3706.

858. Mintz B, Silvers WK. Accelerated growth of melanomas after specific immune destruction of tumor stroma in a mouse model. *Cancer Res*. 1996;56:463-466.

859. Okamoto M, Lee C, Oyasu R. Interleukin-6 as a paracrine and autocrine growth factor in human prostatic carcinoma cells in vitro. *Cancer Res*. 1997;57:141-146.

860. Lu C, Kerbel RS. Interleukin-6 undergoes transition from paracrine growth inhibitor to autocrine stimulator during human melanoma progression. *J Cell Biol*. 1993;120:1281-1288.

861. Strieter RM. Chemokines: not just leukocyte chemoattractants in the promotion of cancer. *Nat Immunol*. 2001;2:285-286.

862. Wixted JH, Rothstein JL, Eisenlohr LC. Identification of functionally distinct TRAF proinflammatory and PI3K/MEK transforming activities emanating from the RET/PTC fusion oncoprotein. *J Biol Chem*. 2011;287:3691-3703.

863. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature*. 2006;441:431-436.

864. Sparmann A, Bar-Sagi D. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer Cell*. 2004;6: 447-458.

865. Phillips RJ, Mestas J, Gharaee-Kermani M, et al. Epidermal growth factor and hypoxia-induced expression of CXC chemokine receptor 4 on non-small cell lung cancer cells is regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin signaling pathway and activation of hypoxia inducible factor-1alpha. *J Biol Chem*. 2005;280:22473-22481.

866. Shchors K, Shchors E, Rostker F, Lawlor ER, Brown-Swigart L, Evan GI. The Myc-dependent angiogenic switch in tumors is mediated by interleukin 1beta. *Genes Dev*. 2006;20:2527-2538.

867. Soucek L, Lawlor ER, Soto D, Shchors K, Swigart LB, Evan GI. Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. *Nat Med*. 2007;13:1211-1218.

868. Clark CE, Hingorani SR, Mick R, Combs C, Tuveson DA, Vonderheide RH. Dynamics of the immune reaction to pancreatic cancer from inception to invasion. *Cancer Res*. 2007;67:9518-9527.

869. Ancrile B, Lim KH, Counter CM. Oncogenic Ras-induced secretion of IL6 is required for tumorigenesis. *Genes Dev*. 2007;21:1714-1719.

870. Ji H, Houghton AM, Mariani TJ, et al. K-ras activation generates an inflammatory response in lung tumors. *Oncogene*. 2006;25: 2105-2112.

871. Hibberd AD, Metcalf D. Proliferation of macrophage and granulocyte precursors in response to primary and transplanted tumors. *Isr J Med Sci*. 1971;7:202-210.

872. Metcalf D. The serum factor stimulating colony formation in vitro by murine plasmacytoma cells: response to antigens and mineral oil. *J Immunol*. 1974;113:235-243.

873. Bottazzi B, Polentarutti N, Acero R, et al. Regulation of the macrophage content of neoplasms by chemoattractants. *Science*. 1983; 220:210-212.

874. Matsushima K, Larsen CG, DuBois GC, Oppenheim JJ. Purification and characterization of a novel monocyte chemotactic and activating factor produced by a human myelomonocytic cell line. *J Exp Med*. 1989;169:1485-1490.

875. Yoshimura T, Robinson EA, Tanaka S, Appella E, Kuratsu J, Leonard EJ. Purification and amino acid analysis of two human glioma-derived monocyte chemoattractants. *J Exp Med*. 1989;169:1449-1459.

876. Van Meir E, Sawamura Y, Diserens AC, Hamou MF, de Tribolet N. Human glioblastoma cells release interleukin 6 in vivo and in vitro. *Cancer Res*. 1990;50:6683-6688.

877. Bottazzi B, Erba E, Nobili N, Fazioli F, Rambaldi A, Mantovani A. A paracrine circuit in the regulation of the proliferation of macrophages infiltrating murine sarcomas. *J Immunol*. 1990;144:2409-2412.

878. Pekarek LA, Weichselbaum RR, Beckett MA, Nachman J, Schreiber H. Footprinting of individual tumors and their variants by constitutive cytokine expression patterns. *Cancer Res*. 1993;53:1978-1981.

879. Zou W, Machelon V, Coulomb-L'Hermin A, et al. Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells. *Nat Med*. 2001;7:1339-1346.

880. Scotton CJ, Wilson JL, Scott K, et al. Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer. *Cancer Res*. 2002;62:5930-5938.

881. Wilson JM, Coletta PL, Cuthbert RJ, et al. Macrophage migration inhibitory factor promotes intestinal tumorigenesis. *Gastroenterology*. 2005;129:1485-1503.

882. Rodrigues LR, Teixeira JA, Schmitt FL, Paulsson M, Lindmark- Mansson H. The role of osteopontin in tumor progression and metastasis in breast cancer. *Cancer Epidemiol Biomarkers Prev*. 2007;16:1087-1097.

883. DuPre SA, Hunter KW, Jr. Murine mammary carcinoma 4T1 induces a leukemoid reaction with splenomegaly: association with tumor-derived growth factors. *Exp Mol Pathol*. 2007;82:12-24.

884. McAllister SS, Gifford AM, Greiner AL, et al. Systemic endocrine instigation of indolent tumor growth requires osteopontin. *Cell*. 2008;133:994-1005.

885. Bruns P. Die Heilwirkung des Erysipels auf Geschwülste. *Beitr Klin Chir*. 1888;3:443-466.

886. Busch H. Niederrheinische Gesellschaft für Natur- und Heilkunde in Bonn. 14. März 1866. *Berliner Klinische Wochenschrift*. 1866:245-246.

887. Busch H. Niederrheinische Gesellschaft für Natur- und Heilkunde in Bonn. Aus der Sitzung der medicinschen Section vom 13. November 1867. *Berliner Klinische Wochenschrift*. 1868;5:137-138.

888. Fehleisen F. Über die Züchtung der Erysipel-Kokken auf künstlichen Nährböden und die Übertragbarkeit auf den Menschen. *Deutsche Med Wschr*. 1882;8:553-554.

889. Fehleisen F. *Die Aetiologie des Erysipels. Berlin: Theodor Fischer's medicinische* . Buchhandlung; 1883.

890. Coley WB. The treatment of malignant tumors by repeated inoculations of erysipelas: with a report of ten original cases. *Am J Med Sci* . 1893;105:487-511.

891. Morales A, Eidinger D, Bruce AW. Intracavitary bacillus Calmette- Guerin in the treatment of superficial bladder tumors. *J Urol* . 1976;116:180-183.

892. Alexandroff AB, Jackson AM, O'Donnell MA, James K. BCG immunotherapy of bladder cancer: 20 years on. *Lancet*. 1999;353:1689-1694.

893. Hurwitz DJ, Pincus L, Kupper TS. Imiquimod: a topically applied link between innate and acquired immunity. *Arch Dermatol*. 2003;139: 1347-1350.

894. Peris K, Micantonio T, Fargnoli MC, Lozzi GP, Chimenti S. Imiquimod 5% cream in the treatment of Bowen's disease and invasive squamous cell carcinoma. *J Am Acad Dermatol*. 2006;55: 324-327.

895. Huang SJ, Hijnen D, Murphy GF, et al. Imiquimod enhances IFN-gamma production and effector function of T cells infiltrating human squamous cell carcinomas of the skin. *J Invest Dermatol*. 2009;129: 2676-2685.

896. Parkin DM. The global health burden of infection-associated cancers in the year 2002. *Int J Cancer* . 2006;118:3030-3044.

897. Rook GA, Dalgleish A. Infection, immunoregulation, and cancer. *Immunol Rev*. 2011;240:141-159.

898. Giovannucci E, Egan KM, Hunter DJ, et al. Aspirin and the risk of colorectal cancer in women. *N Engl J Med* . 1995;333:609-614.

899. Flossmann E, Rothwell PM. Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. *Lancet*. 2007;369:1603-1613.

900. Weisburger JH. Antimutagens, anticarcinogens, and effective worldwide cancer prevention. *J Environ Pathol Toxicol Oncol* . 1999; 18:85-93.

901. Meyerhardt JA. Beyond standard adjuvant therapy for colon cancer: role of nonstandard interventions. *Semin Oncol*. 2011;38:533-541.

902. De Filippo C, Cavalieri D, Di Paola M, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci U S A*. 2010;107: 14691-14696.

903. Finn OJ. Premalignant lesions as targets for cancer vaccines. *J Exp Med*.

2003;198:1623-1626.

904. Fu J, Wei B, Wen T, et al. Loss of intestinal core 1-derived O-glycans causes spontaneous colitis in mice. *J Clin Invest*. 2011;121:1657-1666.

905. Clark WH Jr, Elder DE, Guerry Dt, et al. Model predicting survival in stage I melanoma based on tumor progression. *J Natl Cancer Inst* . 1989;81:1893-1904.

906. Taylor RC, Patel A, Panageas KS, Busam KJ, Brady MS. Tumorinfiltrating lymphocytes predict sentinel lymph node positivity in patients with cutaneous melanoma. *J Clin Oncol*. 2007;25:869-875.

907. Mlecnik B, Tosolini M, Kirilovsky A, et al. Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. *J Clin Oncol*. 2011;29:610-618.

908. Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science*. 2006;313:1960-1964.

909. Grulich AE, van Leeuwen MT, Falster MO, Vajdic CM. Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis. *Lancet*. 2007;370:59-67.

910. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* . 2003;348:203-213.

911. Gromet MA, Epstein WL, Blois MS. The regressing thin malignant melanoma: a distinctive lesion with metastatic potential. *Cancer*. 1978;42:2282-2292.

912. Mackensen A, Carcelain G, Viel S, et al. Direct evidence to support the immunosurveillance concept in a human regressive melanoma. *J Clin Invest*. 1994;93:1397-1402.

913. Burton AL, Gilbert J, Farmer RW, et al. Regression does not predict nodal metastasis or survival in patients with cutaneous melanoma. *Am Surg*. 2011;77:1009-1013.

914. Yaghoobi R, Omidian M, Bagherani N. Vitiligo: a review of the published work. *J Dermatol*. 2011;38:419-431.

915. Overwijk WW, Theoret MR, Finkelstein SE, et al. Tumor regression and autoimmunity after reversal of a functionally tolerant state of selfreactive CD8+ T cells. *J Exp Med*. 2003;198:569-580.

916. Engelhorn ME, Guevara-Patino JA, Noffz G, et al. Autoimmunity and tumor immunity induced by immune responses to mutations in self. *Nat Med*. 2006;12:198-206.

917. Zhang S, Bernard D, Khan WI, Kaplan MH, Bramson JL, Wan Y. CD4+ T-cell-mediated anti-tumor immunity can be uncoupled from autoimmunity via the STAT4/STAT6 signaling axis. *Eur J Immunol* . 2009;39:1252-1259.

918. Bouwhuis MG, ten Hagen TL, Eggermont AM. Immunologic functions as prognostic indicators in melanoma. *Mol Oncol*. 2011;5: 183-189.

919. Delmonte L, Liebelt RA. Granulocytosis-promoting extract of mouse tumor tissue: partial purification. *Science*. 1965;148:521-523.

920. Milas L, Basic I. Stimulated granulocytopoiesis in mice bearing fibrosarcoma. *Eur J Cancer*. 1972;8:309-313.

921. Balducci L, Hardy C. High proliferation of granulocyte-macrophage progenitors in tumor-bearing mice. *Cancer Res*. 1983;43:4643-4647.

922. Lee MY, Kaushansky K, Judkins SA, Lottsfeldt JL, Waheed A, Shadduck RK. Mechanisms of tumor-induced neutrophilia: constitutive production of colony-stimulating factors and their synergistic actions. *Blood*. 1989;74:115-122.

923. Cardiff RD, Leder A, Kuo A, Pattengale PK, Leder P. Multiple tumor types appear in a transgenic mouse with the ras oncogene. *Am J Pathol*. 1993;142:1199-1207.

924. Scholl SM, Pallud C, Beuvon F, et al. Anti-colony-stimulating factor-1 antibody staining in primary breast adenocarcinomas correlates with marked inflammatory cell infiltrates and prognosis. *J Natl Cancer Inst* . 1994;86:120-126.

925. Uemura Y, Kobayashi M, Nakata H, Harada R, Kubota T, Taguchi H. Effect of serum deprivation on constitutive production of granulocyte- colony stimulating factor and granulocyte macrophage-colony stimulating factor in lung cancer cells. *Int J Cancer* . 2004;109: 826-832.

926. Woglom WH. The size of the spleen in immune mice. *J Cancer Res* . 1919;4:281-323.

927. Schreiber K, Schreiber H. Unpublished results. 2008.

928. Radoja S, Rao TD, Hillman D, Frey AB. Mice bearing late-stage tumors have normal functional systemic T cell responses in vitro and in vivo. *J Immunol*. 2000;164:2619-2628.

929. Jameson JL, Johnson BE. Paraneoplastic syndromes: endocrinologic/hematologic. In: Fauci AS, ed. *Harrison's Principles of Internal Medicine* . 17th ed. New York, NY: McGraw-Hill Medical; 2008:617-623.

930. Levey DL, Srivastava PK. Alterations in T cells of cancer-bearers: whence specificity? *Immunol Today*. 1996;17:365-368.

931. Regis G, Pensa S, Boselli D, Novelli F, Poli V. Ups and downs: the STAT1:STAT3 seesaw of interferon and gp130 receptor signalling. *Semin Cell Dev Biol* . 2008;19:351-359.

932. Yu H, Jove R. The STATs of cancer—new molecular targets come of age. *Nat Rev Cancer*. 2004;4:97-105.

933. Dar A, Kollet O, Lapidot T. Mutual, reciprocal SDF-1/CXCR4 interactions between hematopoietic and bone marrow stromal cells regulate human stem cell migration and development in NOD/SCID chimeric mice. *Exp Hematol*. 2006;34:967-975.

934. Heissig B, Hattori K, Dias S, et al. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell*. 2002;109:625-637.

935. Valenzuela-Fernandez A, Planchenault T, Baleux F, et al. Leukocyte elastase negatively regulates stromal cell-derived factor-1 (SDF-1)/CXCR4 binding and functions by amino-terminal processing of SDF-1 and CXCR4. *J Biol Chem*. 2002;277:15677-15689.

936. Jin F, Zhai Q, Qiu L, et al. Degradation of BM SDF-1 by MMP-9: the role in G-CSF-induced hematopoietic stem/progenitor cell mobilization. *Bone Marrow Transplant*. 2008;42:581-588.

937. Zou L, Barnett B, Safah H, et al. Bone marrow is a reservoir for CD4+CD25+ regulatory T cells that traffic through CXCL12/CXCR4 signals. *Cancer Res*. 2004;64:8451-8455.

938. Kryczek I, Wei S, Keller E, Liu R, Zou W. Stroma-derived factor (SDF-1/CXCL12) and human tumor pathogenesis. *Am J Physiol Cell Physiol*. 2007;292:C987-C995.

939. Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell*. 2005;121:335-348.

940. Orimo A, Weinberg RA. Stromal fibroblasts in cancer: a novel tumorpromoting cell type. *Cell Cycle*. 2006;5:1597-1601.

941. Schmidt H, Suciu S, Punt CJ, et al. Pretreatment levels of peripheral neutrophils and leukocytes as independent predictors of overall survival in patients with American Joint Committee on Cancer Stage IV Melanoma: results of the EORTC 18951 Biochemotherapy Trial. *J Clin Oncol*. 2007;25:1562-1569.

942. Allin KH, Nordestgaard BG, Flyger H, Bojesen SE. Elevated pretreatment levels of plasma C-reactive protein are associated with poor prognosis after breast cancer: a cohort study. *Breast Cancer Res*. 2011;13:R55.

943. Sharaiha RZ, Halazun KJ, Mirza F, et al. Elevated preoperative neutrophil:lymphocyte ratio as a predictor of postoperative disease recurrence in esophageal cancer. *Ann Surg Oncol*. 2011;18:3362-3369.

944. Al-Batran SE, Pauligk C, Wirtz R, et al. The validation of matrix metalloproteinase- 9 mRNA gene expression as a predictor of outcome in patients with metastatic gastric cancer. *Ann Oncol*. 2012;23(7): 1699-1705.

945. Richards CH, Leitch EF, Horgan PG, Anderson JH, McKee RF, McMillan DC. The relationship between patient physiology, the systemic inflammatory response and survival in patients undergoing curative resection of colorectal cancer. *Br J Cancer*. 2010;103:1356-1361.

946. Kaplan RN, Riba RD, Zacharoulis S, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature*. 2005;438:820-827.

947. Wels J, Kaplan RN, Rafii S, Lyden D. Migratory neighbors and distant invaders: tumor-associated niche cells. *Genes Dev*. 2008;22:559-574.

948. Malanchi I, Santamaria-Martinez A, Susanto E, et al. Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature*. 2012;481:85-89.

949. Oskarsson T, Massague J. Extracellular matrix players in metastatic niches. *EMBO J*. 2011;31:254-256.

950. Pantel K, Alix-Panabieres C, Riethdorf S. Cancer micrometastases. *Nat Rev Clin Oncol*. 2009;6:339-351.

951. DeMichele A, Martin AM, Mick R, et al. Interleukin-6 -174G→C polymorphism is associated with improved outcome in high-risk breast cancer. *Cancer Res*. 2003;63:8051-8056.

952. Knupfer H, Preiss R. Significance of interleukin-6 (IL-6) in breast cancer (review). *Breast Cancer Res Treat* . 2007;102:129-135.

953. Heikkila K, Ebrahim S, Lawlor DA. Systematic review of the association between circulating interleukin-6 (IL-6) and cancer. *Eur J Cancer* . 2008;44:937-945.

954. Studebaker AW, Storci G, Werbeck JL, et al. Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner. *Cancer Res*. 2008;68:9087-9095.

955. Mullen CA, Urban JL, Van Waes C, Rowley DA, Schreiber H. Multiple cancers. Tumor burden permits the outgrowth of other cancers. *J Exp Med*. 1985;162:1665-1682.

956. Prehn RT. Two competing influences that may explain concomitant tumor resistance. *Cancer Res*. 1993;53:3266-3269.

957. Tyzzer EE. Factors in the production and growth of tumor metastases. *J Med Res*. 1913;28:309-321.

958. Gorelik E. Concomitant tumor immunity and the resistance to a second tumor challenge. *Adv Cancer Res* . 1983;39:71-120.

959. Ruggiero RA, Bruzzo J, Chiarella P, et al. Tyrosine isomers mediate the classical phenomenon of concomitant tumor resistance. *Cancer Res*. 2011;71:7113-7124.

960. Russel B. The nature of resistance to the inoculation of cancer. In: *Third Scientific Report on the Investigations of the Imperial Cancer Research Fund*. London: Taylor & Francis. 1908;341-358.

961. Bashford EF, Murray JA, Cramer W. Natural and induced resistance of mice to the growth of cancer. *Proc R Soc Lond B Biol Sci* . 1907;79: 164-187.

962. Gershon RK, Carter RL, Kondo K. On concomitant immunity in tumour-bearing hamsters. *Nature*. 1967;213:674-676.

963. Brotherton JM, Fridman M, May CL, Chappell G, Saville AM, Gertig DM. Early effect of the HPV vaccination programme on cervical abnormalities in Victoria, Australia: an ecological study. *Lancet*. 2011;377:2085-2092.

964. Donovan B, Franklin N, Guy R, et al. Quadrivalent human papillomavirus vaccination and trends in genital warts in Australia: analysis of national sentinel surveillance data. *Lancet Infect Dis*. 2011;11:39-44.

965. Lasaro MO, Sazanovich M, Giles-Davis W, et al. Active immunotherapy combined with blockade of a coinhibitory pathway achieves regression of large tumor masses in

cancer-prone mice. *Mol Ther*. 2011;19:1727-1736.

966. Kenter GG, Welters MJ, Valentijn AR, et al. Vaccination against HPV- 16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med*. 2009;361:1838-1847.

967. Aspinall EJ, Hawkins G, Fraser A, Hutchinson SJ, Goldberg D. Hepatitis B prevention, diagnosis, treatment and care: a review. *Occup Med (Lond)*. 2011;61:531-540.

968. Kamisawa T, Takuma K, Tabata T, Egawa N, Yamaguchi T. Longterm follow-up of chronic pancreatitis patients with K-ras mutation in the pancreatic juice. *Hepatogastroenterology*. 2011;58:174-176.

969. Deichman GI, Kluchareva TE. Prevention of tumour induction in Sv 40-infected hamsters. *Nature*. 1964;202:1126-1128.

970. Deichman GI, Kluchareva TE. Immunological determinants of oncogenesis in hamsters infected with Sv40 virus. *Virology*. 1964;24: 131-137.

971. Schell TD, Knowles BB, Tevethia SS. Sequential loss of cytotoxic T lymphocyte responses to simian virus 40 large T antigen epitopes in T antigen transgenic mice developing osteosarcomas. *Cancer Res*. 2000;60:3002-3012.

972. Nowak AK, Lake RA, Marzo AL, et al. Induction of tumor cell apoptosis in vivo increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host tumor-specific CD8 T cells. *J Immunol*. 2003;170:4905-4913.

973. Uno T, Takeda K, Kojima Y, et al. Eradication of established tumors in mice by a combination antibody-based therapy. *Nat Med*. 2006;12: 693-698.

974. Lee Y, Auh SL, Wang Y, et al. Therapeutic effects of ablative radiation on local tumor require CD8+ T cells: changing strategies for cancer treatment. *Blood*. 2009;114:589-595.

975. Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. Immunological aspects of cancer chemotherapy. *Nat Rev Immunol*. 2008;8:59-73.

976. Ma Y, Conforti R, Aymeric L, et al. How to improve the immunogenicity of chemotherapy and radiotherapy. *Cancer Metastasis Rev*. 2011;30:71-82.

977. June CH. Principles of adoptive T cell cancer therapy. *J Clin Invest*. 2007;117:1204-1212.

978. Dougan M, Dranoff G. Immune therapy for cancer. *Annu Rev Immunol*. 2009;27:83-117.

979. Weber J, Atkins M, Hwu P, Radvanyi L, Sznol M, Yee C. White paper on adoptive cell therapy for cancer with tumor-infiltrating lymphocytes: a report of the CTEP subcommittee on adoptive cell therapy. *Clin Cancer Res*. 2011;17:1664-1673.

980. Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. *Nature*. 2011;480:480-489.

981. Ali OA, Emerich D, Dranoff G, Mooney DJ. In situ regulation of DC subsets and T cells mediates tumor regression in mice. *Sci Transl Med*. 2009;1:8ra19.

982. Schmitt TM, Ragnarsson GB, Greenberg PD. T cell receptor gene therapy for cancer. *Hum Gene Ther*. 2009;20:1240-1248.

983. Jorritsma A, Schotte R, Coccoris M, de Witte MA, Schumacher TN. Prospects and limitations of T cell receptor gene therapy. *Curr Gene Ther*. 2011;11:276-287.

984. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med*. 2004;10:909-915.

985. Eggermont AM. Therapeutic vaccines in solid tumours: can they be harmful? *Eur J Cancer*. 2009;45:2087-2090.

986. Michaud M, Martins I, Sukkurwala AQ, et al. Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. *Science*. 2011;334:1573-1577.

987. Weijzen S, Meredith SC, Velders MP, Elmishad AG, Schreiber H, Kast WM. Pharmacokinetic differences between a T cell-tolerizing and a T cell- activating peptide. *J Immunol*. 2001;166:7151-7157.

988. Casares N, Pequignot MO, Tesniere A, et al. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *J Exp Med*. 2005;202:1691-1701.

989. Lindenmann J, Klein PA. Viral oncolysis: increased immunogenicity of host cell antigen associated with influenza virus. *J Exp Med*. 1967;126:93-108.

990. Austin FC, Boone CW. Virus augmentation of the antigenicity of tumor cell extracts. *Adv Cancer Res*. 1979;30:301-345.

991. Haas C, Schirrmacher V. Immunogenicity increase of autologous tumor cell vaccines by virus infection and attachment of bispecific antibodies. *Cancer Immunol Immunother* . 1996;43:190-194.

992. Liang W, Cohen EP. Resistance to murine leukemia in mice rejecting syngeneic somatic hybrid cells. *J Immunol*. 1976;116:623-626.

993. Guo Y, Wu M, Chen H, et al. Effective tumor vaccine generated by fusion of hepatoma cells with activated B cells. *Science*. 1994;263: 518-520.

994. Gong J, Chen D, Kashiwaba M, Kufe D. Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. *Nat Med*. 1997;3:558-561.

995. Baskar S. Gene-modified tumor cells as cellular vaccine. *Cancer Immunol Immunother*. 1996;43:165-173.

996. DeBruyne L. Treatment of malignancy by direct gene transfer of a foreign MHC class I molecule. *Cancer Immunol Immunother* . 1996;43:180-189.

997. Baskar S, Glimcher L, Nabavi N, Jones RT, Ostrand-Rosenberg S. Major histocompatibility complex class II+B7-1+ tumor cells are potent vaccines for stimulating tumor rejection in tumor-bearing mice. *J Exp Med*. 1995;181:619-629.

998. Mitchison NA. Immunologic approach to cancer. *Trans Proc*. 1970; 2:92-103.

999. Sato T. Active specific immunotherapy with hapten-modified autologous melanoma cell vaccine. *Cancer Immunol Immunother* . 1996;43:174-179.

1000. Van Pel A, Georlette M, Boon T. Tumor cell variants obtained by mutagenesis of a Lewis lung carcinoma cell line: immune rejection by syngeneic mice. *Proc Natl Acad Sci U S A*. 1979;76:5282-5285.

1001. Chen L, Ashe S, Brady WA, et al. Costimulation of antitumor immunity by the B7 counter-receptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell*. 1992;71:1093-1102.

1002. Townsend SE, Allison JP. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. *Science*. 1993;259:368-370.

1003. Yu P, Lee Y, Liu W, et al. Priming of naive T cells inside tumors leads to eradication of established tumors. *Nat Immunol* . 2004;5:141-149.

1004. Dye ES, North RJ, Mills CD. Mechanisms of anti-tumor action of Corynebacterium parvum. I. Potentiated tumor-specific immunity and its therapeutic limitations. *J Exp Med*. 1981;154:609-620.

1005. Schiller JT, Lowy DR. Vaccines to prevent infections by oncoviruses. *Annu Rev Microbiol*. 2010;64:23-41.

1006. Blankenstein T. Increasing tumour immunogenicity by genetic modification. *Eur J Cancer*. 1994;30A:1182-1187.

1007. Dyall R, Bowne WB, Weber LW, et al. Heteroclitic immunization induces tumor immunity. *J Exp Med*. 1998;188:1553-1561.

1008. Liu MA. DNA vaccines: an historical perspective and view to the future. *Immunol Rev*. 2011;239:62-84.

1009. Fu YX, Watson G, Jimenez JJ, Wang Y, Lopez DM. Expansion of immunoregulatory macrophages by granulocyte-macrophage colonystimulating factor derived from a murine mammary tumor. *Cancer Res*. 1990;50:227-234.

1010. Mittelman A, Wang X, Matsumoto K, Ferrone S. Antiidiotypic response and clinical course of the disease in patients with malignant melanoma immunized with mouse antiidiotypic monoclonal antibody MK2-23. *Hybridoma*. 1995;14:175-181.

1011. Trojan J, Johnson TR, Rudin SD, Ilan J, Tykocinski ML, Ilan J. Treatment and prevention of rat glioblastoma by immunogenic C6 cells expressing antisense insulin-like growth factor I RNA. *Science*. 1993;259:94-97.

1012. Cho HI, Lee YR, Celis E. Interferon gamma limits the effectiveness of melanoma peptide vaccines. *Blood*. 2011;117:135-144.

1013. Melief CJ, van der Burg SH. Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. *Nat Rev Cancer* . 2008;8:351-360.

1014. Toes RE, Offringa R, Blom RJ, Melief CJ, Kast WM. Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction. *Proc Natl Acad Sci U S A*. 1996;93:7855-7860.

1015. Mo A, Musselli C, Chen H, et al. A heat shock protein based polyvalent vaccine targeting HSV-2: CD4(+) and CD8(+) cellular immunity and protective efficacy. *Vaccine*. 2011;29:8530-8541.

1016. APastor F, Kolonias D, Giangrande PH, Gilboa E. Induction of tumour immunity by targeted inhibition of nonsense-mediated mRNA decay. *Nature*. 2010;465:227-230.

1017. Sander LE, Davis MJ, Boekschoten MV, et al. Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. *Nature*. 2011;474:385-389.

1018. Schuler G. Dendritic cells in cancer immunotherapy. *Eur J Immunol* . 2010;40:2123-2130.

1019. Palucka K, Ueno H, Banchereau J. Recent developments in cancer vaccines. *J Immunol*. 2011;186:1325-1331.

1020. Humphrey PA, Gangarosa LM, Wong AJ, et al. Deletion-mutant epidermal growth factor receptor in human gliomas: effects of type II mutation on receptor function. *Biochem Biophys Res Commun*. 1991;178:1413-1420.

1021. Herlyn D, Koprowski H. IgG2a monoclonal antibodies inhibit human tumor growth through interaction with effector cells. *Proc Natl Acad Sci U S A*. 1982;79:4761-4765.

1022. Campoli M, Ferris R, Ferrone S, Wang X. Immunotherapy of malignant disease with tumor antigen-specific monoclonal antibodies. *Clin Cancer Res*. 2010;16:11-20.

1023. Staerz UD, Kanagawa O, Bevan MJ. Hybrid antibodies can target sites for attack by T cells. *Nature*. 1985;314:628-631.

1024. Liu MA, Kranz DM, Kurnick JT, Boyle LA, Levy R, Eisen HN. Heteroantibody duplexes target cells for lysis by cytotoxic T lymphocytes. *Proc Natl Acad Sci U S A* . 1985;82:8648-8652.

1025. Wang J, Press OW, Lindgren CG, et al. Cellular immunotherapy for follicular lymphoma using genetically modified CD20-specific CD8+ cytotoxic T lymphocytes. *Mol Ther*. 2004;9:577-586.

1026. Krolick KA, Villemez C, Isakson P, Uhr JW, Vitetta ES. Selective killing of normal or neoplastic B cells by antibodies coupled to the A chain of ricin. *Proc Natl Acad Sci U S A* . 1980;77:5419-5423.

1027. Gilliland DG, Steplewski Z, Collier RJ, Mitchell KF, Chang TH, Koprowski H. Antibody-directed cytotoxic agents: use of monoclonal antibody to direct the action of toxin A chains to colorectal carcinoma cells. *Proc Natl Acad Sci U S A* . 1980;77:4539-4543.

1028. Pirker R, FitzGerald DJ, Hamilton TC, et al. Characterization of immunotoxins active against ovarian cancer cell lines. *J Clin Invest*. 1985;76:1261-1267.

1029. Gillies SD, Young D, Lo KM, Foley SF, Reisfeld RA. Expression of genetically engineered immunoconjugates of lymphotoxin and a chimeric anti-ganglioside GD2 antibody. *Hybridoma*. 1991;10:347-356.

1030. Carnemolla B, Borsi L, Balza E, et al. Enhancement of the antitumor properties of interleukin-2 by its targeted delivery to the tumor blood vessel extracellular matrix. *Blood*.

2002;99:1659-1665.

1031. FitzGerald DJ, Wayne AS, Kreitman RJ, Pastan I. Treatment of hematologic malignancies with immunotoxins and antibody-drug conjugates. *Cancer Res*. 2011;71:6300-6309.

1032. Schrama D, Reisfeld RA, Becker JC. Antibody targeted drugs as cancer therapeutics. *Nat Rev Drug Discov* . 2006;5:147-159.

1033. Weiner LM, Surana R, Wang S. Monoclonal antibodies: versatile platforms for cancer immunotherapy. *Nat Rev Immunol* . 2010;10:317-327.

1034. Grillo-Lopez AJ, White CA, Dallaire BK, et al. Rituximab: the first monoclonal antibody approved for the treatment of lymphoma. *Curr Pharm Biotechnol*. 2000;1:1-9.

1035. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* . 2001;344:783-792.

1036. Shepard HM, Brdlik CM, Schreiber H. Signal integration: a framework for understanding the efficacy of therapeutics targeting the human EGFR family. *J Clin Invest*. 2008;118:3574-3581.

1037. Stagg J, Loi S, Divisekera U, et al. Anti-ErbB-2 mAb therapy requires type I and II interferons and synergizes with anti-PD-1 or anti-CD137 mAb therapy. *Proc Natl Acad Sci U S A*. 2011;108:7142-7147.

1038. Hurvitz S, Betting D, Stern H, et al. Analysis of Fc receptor IIA & IIIA polymorphisms: correlation with outcome in trastuzumab-treated HER2/Neu amplified early and metastatic breast cancer patients. In: *Thirty-Second Annual CTRC-AACR San Antonio Breast Cancer Symposium*. San Antonio, TX: 2009.

1039. Park S, Jiang Z, Mortenson ED, et al. The therapeutic effect of anti- HER2/neu antibody depends on both innate and adaptive immunity. *Cancer Cell* . 2010;18:160-170.

1040. Houghton AN, Mintzer D, Cordon-Cardo C, et al. Mouse monoclonal IgG3 antibody detecting GD3 ganglioside: a phase I trial in patients with malignant melanoma. *Proc Natl Acad Sci U S A*. 1985;82: 1242-1246.

1041. Goodman GE, Hellström I, Brodzinsky L, et al. Phase I trial of murine monoclonal antibody L6 in breast, colon, ovarian, and lung cancer. *J Clin Oncol* . 1990;8:1083-1092.

1042. Pule MA, Savoldo B, Myers GD, et al. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med*. 2008;14: 1264-1270.

1043. Waldmann TA. Multichain interleukin-2 receptor: a target for immunotherapy in lymphoma. *J Natl Cancer Inst* . 1989;81:914-923.

1044. Gressett SM, Shah SR. Intricacies of bevacizumab-induced toxicities and their management. *Ann Pharmacother* . 2009;43:490-501.

1045. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science*. 1996;271:1734-1736.

1046. Hardy B, Kovjazin R, Raiter A, Ganor N, Novogrodsky A. A lymphocyte- activating monoclonal antibody induces regression of human tumors in severe combined immunodeficient mice. *Proc Natl Acad Sci U S A*. 1997;94:5756-5760.

1047. Melero I, Shuford WW, Newby SA, et al. Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. *Nat Med*. 1997;3:682-685.

1048. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A* . 2002;99:12293-12297.

1049. Shrikant P, Khoruts A, Mescher MF. CTLA-4 blockade reverses CD8+ T cell tolerance to tumor by a CD4+ T cell- and IL-2-dependent mechanism. *Immunity*. 1999;11:483-493.

1050. Sotomayor EM, Borrello I, Tubb E, Allison JP, Levitsky HI. In vivo blockade of CTLA-4 enhances the priming of responsive T cells but fails to prevent the induction of tumor antigen-specific tolerance. *Proc Natl Acad Sci U S A* . 1999;96:11476-11481.

1051. Ascierto PA, Simeone E, Sznol M, Fu YX, Melero I. Clinical experiences with anti-CD137 and anti-PD1 therapeutic antibodies. *Semin Oncol*. 2010;37:508-516.

1052. Agarwala SS, Ribas A. Current experience with CTLA4-blocking monoclonal antibodies for the treatment of solid tumors. *J Immunother* . 2010;33:557-569.

1053. Shwartzman G. Studies on bacillus typhosus toxic substances: I. Phenomenon of local skin reactivity to B. typhosus culture filtrate. *J Exp Med*. 1928;48:247-268.

1054. Thomas L, Good RA. Studies on the generalized Shwartzman reaction: I. General observations concerning the phenomenon. *J Exp Med*. 1952;96:605-624.

1055. Weichselbaum RR, Kufe D. Translation of the radio- and chemo-inducible TNFerade vector to the treatment of human cancers. *Cancer Gene Ther* . 2009;16:609-619.

1056. Turcotte S, Rosenberg SA. Immunotherapy for metastatic solid cancers. *Adv Surg* . 2011;45:341-360.

1057. Steel JC, Waldmann TA, Morris JC. Interleukin-15 biology and its therapeutic implications in cancer. *Trends Pharmacol Sci* . 2012; 33:35-41.

1058. Rosenau W. Tumor-specific inhibition of growth of methylcholanthrene- induced sarcomas in vivo and in vitro by sensitized isologous lymphoid cells. *J Natl Cancer Inst* . 1966;36:825-836.

1059. Fefer A. Adoptive tumor immunotherapy in mice as an adjunct to whole-body x-irradiation and chemotherapy. A review. *Isr J Med Sci* . 1973;9:350-365.

1060. Cheever MA, Kempf RA, Fefer A. Tumor neutralization, immunotherapy, and chemoimmmunotherapy of a Friend leukemia with cells secondarily sensitized in vitro. *J Immunol* . 1977;119:714-718.

1061. Greenberg PD, Cheever MA, Fefer A. Eradication of disseminated murine leukemia by chemoimmunotherapy with cyclophosphamide and adoptively transferred immune syngeneic Lyt-1+2- lymphocytes. *J Exp Med* . 1981;154:952-963.

1062. Cheever MA, Greenberg PD, Fefer A, Gillis S. Augmentation of the anti-tumor therapeutic efficacy of long-term cultured T lymphocytes by in vivo administration of purified interleukin 2. *J Exp Med*. 1982;155:968-980.

1063. Rosenberg SA, Spiess P, Lafreniere R. A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. *Science*. 1986;233:1318-1321.

1064. Rosenberg SA, Packard BS, Aebersold PM, et al. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. *N Engl J Med*. 1988;319:1676-1680.

1065. Papadopoulos EB, Ladanyi M, Emanuel D, et al. Infusions of donor leukocytes to treat Epstein-Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. *N Engl J Med* . 1994;330:1185-1191.

1066. Cohen JI, Bollard CM, Khanna R, Pittaluga S. Current understanding of the role of Epstein-Barr virus in lymphomagenesis and therapeutic approaches to EBV-associated lymphomas. *Leuk Lymphoma*. 2008;49(suppl 1):27-34.

1067. Barker JN, Doubrovina E, Sauter C, et al. Successful treatment of EBV-associated posttransplantation lymphoma after cord blood transplantation using third-party EBV-specific cytotoxic T lymphocytes. *Blood*. 2010;116:5045-5049.

1068. Sun Q, Burton R, Reddy V, Lucas KG. Safety of allogeneic Epstein- Barr virus (EBV)-specific cytotoxic T lymphocytes for patients with refractory EBV-related lymphoma. *Br J Haematol* . 2002;118:799- 808.

1069. Fujita Y, Rooney CM, Heslop HE. Adoptive cellular immunotherapy for viral diseases. *Bone Marrow Transplant* . 2008;41:193-198.

1070. Smith C, Khanna R. Generation of cytotoxic T lymphocytes for immunotherapy of EBV-associated malignancies. *Methods Mol Biol* . 2010;651:49-59.

1071. Bendle GM, Haanen JB, Schumacher TN. Preclinical development of T cell receptor gene therapy. *Curr Opin Immunol* . 2009;21: 209-214.

1072. Jena B, Dotti G, Cooper LJ. Redirecting T-cell specificity by introducing a tumor-specific chimeric antigen receptor. *Blood*. 2010;116:1035- 1044.

1073. Zhang L, Morgan RA. Genetic engineering with T cell receptors. *Adv Drug Deliv Rev*. 2012;64(8):756-762.

1074. Morgan RA, Dudley ME, Wunderlich JR, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science*. 2006;314:126-129.

1075. Johnson LA, Morgan RA, Dudley ME, et al. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood*. 2009;114:535-546.

1076. Engels B, Chervin AS, Sant AJ, Kranz DM, Schreiber H. Long-term persistence of CD4(+) but rapid disappearance of CD8(+) T cells expressing an MHC class I-restricted TCR of nanomolar affinity. *Mol Ther*. 2012;20:652-660.

1077. Parkhurst MR, Joo J, Riley JP, et al. Characterization of genetically modified T-cell receptors that recognize the CEA:691-699 peptide in the context of HLA-A2.1 on human colorectal cancer cells. *Clin Cancer Res*. 2009;15:169-180.

1078. Wortzel RD, Urban JL, Philipps C, Fitch FW, Schreiber H. Independent immunodominant and immunorecessive tumor-specific antigens on a malignant tumor: antigenic dissection with cytolytic T cell clones. *J Immunol*. 1983;130:2461-2466.

1079. Urban JL, Van Waes C, Schreiber H. Pecking order among tumorspecific antigens. *Eur J Immunol* . 1984;14:181-187.

1080. Feltkamp MC, Vreugdenhil GR, Vierboom MP, et al. Cytotoxic T lymphocytes raised against a subdominant epitope offered as a synthetic peptide eradicate human papillomavirus type 16-induced tumors. *Eur J Immunol* . 1995;25:2638-2642.

1081. Wettstein PJ, Bailey DW. Immunodominance in the immune response to “multiple” histocompatibility antigens. *Immunogenetics*. 1982;16:47-58.

1082. Rowley DA, Stach RM. A first or dominant immunization. I. Suppression of simultaneous cytolytic T cell responses to unrelated alloantigens. *J Exp Med*. 1993;178:835-840.

1083. Wortzel RD, Urban JL, Schreiber H. Malignant growth in the normal host after variant selection in vitro with cytolytic T-cell lines. *Proc Natl Acad Sci U S A* . 1984;81:2186-2190.

1084. Van Waes C, Monach PA, Urban JL, Wortzel RD, Schreiber H. Immunodominance deters the response to other tumor antigens thereby favoring escape: prevention by vaccination with tumor variants selected with cloned cytolytic T cells in vitro. *Tissue Antigens*. 1996;47:399-407.

1085. Grufman P, Sandberg JK, Wolpert EZ, Karre K. Immunization with dendritic cells breaks immunodominance in CTL responses against minor histocompatibility and synthetic peptide antigens. *J Leukoc Biol*. 1999;66:268-271.

1086. Klein E, Klein G. Specificity of homograft rejection in vivo, assessed by inoculation of artificially mixed compatible and incompatible tumor cells. *Cell Immunol*. 1972;5:201-208.

1087. Weissman IL. Tumor immunity in vivo: evidence that immune destruction of tumor leaves “bystander” cells intact. *J Natl Cancer Inst* . 1973;51:443-448.

1088. Prehn RT. Destruction of tumor as an “innocent bystander” in an immune response specifically directed against nontumor antigens. *Isr J Med Sci* . 1973;9:375-379.

1089. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol* . 2009;9:581-593.

1090. Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol*. 2009;19:43-51.

1091. Luo Y, Zhou H, Krueger J, et al. Targeting tumor-associated macrophages as a novel strategy against breast cancer. *J Clin Invest*. 2006;116:2132-2141.

1092. Loeffler M, Kruger JA, Niethammer AG, Reisfeld RA. Targeting tumor-associated

fibroblasts improves cancer chemotherapy by increasing intratumoral drug uptake. *J Clin Invest*. 2006;116: 1955-1962.

1093. Maciag PC, Seavey MM, Pan ZK, Ferrone S, Paterson Y. Cancer immunotherapy targeting the high molecular weight melanomaassociated antigen protein results in a broad antitumor response and reduction of pericytes in the tumor vasculature. *Cancer Res*. 2008;68: 8066-8075.

1094. Liao D, Luo Y, Markowitz D, Xiang R, Reisfeld RA. Cancer associated fibroblasts promote tumor growth and metastasis by modulating the tumor immune microenvironment in a 4T1 murine breast cancer model. *PLoS One*. 2009;4:e7965.

1095. Kraman M, Bambrough PJ, Arnold JN, et al. Suppression of antitumor immunity by fibroblast activation protein-a-expressing stromal cells. *Science*. 2010;30:827-830.

1096. Beatty GL, Chiorean EG, Fishman MP, et al. CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans. *Science*. 2011;331:1612-1616.

1097. Ehrlich P. Vorwort. In: Ehrlich P, ed. *Gesammelte Arbeiten zur Immunitätsforschung*. Berlin, Germany: August Hirschwald; 1904:I-VIII.

1098. Plato. *Symposion*. 402 BC.

1099. Lessing GE. *Eine Duplik*. Braunschweig, Germany: Buchhandlung des Fürstlichen Waisenhauses; 1778.

1100. Filipovich AH, Heinitz KJ, Robison LL, Frizzera G. The Immunodeficiency Cancer Registry. A research resource. *Am J Pediatr Hematol Oncol*. 1987;9:183-184.

1101. Filipovich AH, Mathur A, Kamat D, Kersey JH, Shapiro RS. Lymphoproliferative disorders and other tumors complicating immunodeficiencies. *Immunodeficiency*. 1994;5:91-112.

1102. Kinlen LJ, Webster AD, Bird AG, et al. Prospective study of cancer in patients with hypogammaglobulinaemia. *Lancet*. 1985;1:263-266.

1103. Morgan GJ, Linet MS, Rabkin CS. Chapter 27: immunologic factors. In: Schottenfeld D, Fraumeni JF, eds. *Cancer Epidemiology and Prevention*. 3rd ed. New York, NY: Oxford University Press; 2006:549-561.

1104. Vajdic CM, van Leeuwen MT. Cancer incidence and risk factors after solid organ transplantation. *Int J Cancer*. 2009;125:1747-1754.

1105. Penn I. The occurrence of cancer in immune deficiencies. *Curr Probl Cancer*. 1982;6:1-64.

1106. Fairley CK, Sheil AG, McNeil JJ, et al. The risk of ano-genital malignancies in dialysis and transplant patients. *Clin Nephrol*. 1994;41: 101-105.

1107. Douds AC, Mellotte GJ, Morgan SH. Fatal Merkel-cell tumour (cutaneous neuroendocrine carcinoma) complicating renal transplantation. *Nephrol Dial Transplant*. 1995;10:2346-2348.

1108. Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in

human Merkel cell carcinoma. *Science*. 2008;319:1096-1100.

1109. Engels EA, Frisch M, Goedert JJ, Biggar RJ, Miller RW. Merkel cell carcinoma and HIV infection. *Lancet*. 2002;359:497-498.

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Chapter 48

Inborn Errors of Immunity

Jean-Laurent Casanova

Mary Ellen Conley

Luigi D. Notarangelo

INTRODUCTION

Louis Pasteur established the germ theory of infectious diseases whilst working near Alès, between 1865 and 1870, following his discovery that the two most prevalent diseases of silkworm were caused by different microbes.[1] This groundbreaking discovery apparently settled a long discussion between two conflicting theories. The first, in which diseases were considered to be intrinsic, had long held sway over the second, in which diseases were considered to be extrinsic. It rapidly became clear, with the successive identification of numerous microbes, culminating in Koch's discovery of *Mycobacterium tuberculosis* in 1882,[2] that this new paradigm accounted for the heavy burden of childhood fever and death that had prevailed throughout human history. About half the children born died of fever before the age of 15, and this death toll could be attributed to microbes.[3] This theory did not explain why rare children survived infectious diseases and assumed that healthy children had remained free from infection. However, the edifice on which this theory was constructed collapsed between 1905 and 1915, with the gradual realization that most infected individuals remained asymptomatic, often throughout their lifetime. Asymptomatic individuals were found to harbor latent microbes—nonreplicating microbes in a dormant state— such as *M. tuberculosis*.[4] Even more strikingly, actively replicating microbes were found to cause silent infections in other individuals, such infections being termed “unapparent infections” by Charles Nicolle.[5] The question of interindividual variability in the course of infection therefore became, and has since remained, a key question in the fields of infectious disease and immunology, and is, arguably, one of the most important questions in biology and medicine. The problem in itself suggests that there are, after all, some intrinsic determinants of disease. The first explanation put forward followed on naturally from another ground-breaking discovery by Pasteur in 1880 to 1881: the prevention of infectious diseases and the foundations of immunology, with the use of attenuated microbes to vaccinate against fowl cholera and sheep anthrax.[6] This led to the hypothesis that related, less virulent microbes or smaller amounts of the same microbes had previously immunized the individuals who remained healthy in the course of infection with a microbe virulent enough to kill other individuals. This powerful idea can be seen as an immunologic or somatic theory of infectious diseases. We now know that this acquired immunity (often referred to as adaptive immunity) emerged twice in the evolution of vertebrates, by convergent evolution, that it is lymphoid, and that it involves both genetic and epigenetic components. However, although this theory was considered plausible in adults, and perhaps in teenagers, especially during secondary infections or reactivation from latency, it was less convincing for primary infections in early childhood, which comprised the majority of cases. In this context, a few human geneticists looking at the problem from the complementary standpoints of clinical and population genetics, including Archibald Garrod and Karl Pearson in particular, collected evidence between 1910 and 1930 for strong, germline, genetic determinism, controlling innate immunity against microbes.[7,8] As stated by Garrod, “It is, of necessity, no easy matter to distinguish between immunity which is inborn and that which has been acquired.”[7]

The development of new vaccines and the discovery of sulfonamides and antibiotics during the 1930s rendered these questions obsolete; it became less pressing to understand a problem that everyone thought would soon be resolved. Paradoxically, antibiotics themselves triggered renewed interest in the question in the early 1950s, when a small group of pediatricians in Europe and America noted that rare children suffered from multiple, recurrent infectious diseases, each of which was treated with antibiotics, lacked a major leukocyte subset or gammaglobulins, and shared this phenotype with relatives, consistent with an inheritable trait.[9,10,11,12,13,14] This was the birth of the field of primary immunodeficiency (PID), a term apparently first coined in 1971[15,16] that remains more frequently used than inborn errors of immunity, which was first used in 1966.[16,17] These children had major immunologic abnormalities, such as neutropenia,[11,14] alymphocytosis,[12,13] or agammaglobulinemia.[9,10] Moreover, their infectious and immunologic phenotypes often

followed a Mendelian pattern of segregation within their families. It is, however, important to bear in mind that the definition of PID established in the 1950s was based on an artificial, as opposed to natural phenotype, because these children with multiple life-threatening infections would have died during their first episode of infection before the advent of antibiotics. Moreover, although the infectious phenotype of these patients led to their investigation and the pattern of inheritance was suggestive of a Mendelian trait, PIDs were classified and defined solely on the basis of the immunologic phenotype. There had been previous descriptions of inherited disorders associated with infectious diseases, such ataxia-telangiectasia in 1926 and 1941,[18,19] Wiskott-Aldrich syndrome in 1937,[20,21] and epidermodysplasia verruciformis in 1933[22] (and its viral etiology in 1946[23]), but

these disorders were not recognized as PIDs until 1964,[24,25] 1959,[26,27] and 2002,[28] respectively. They were long considered from other angles, in the absence of detectable immunologic abnormalities. Autoimmune polyendocrinopathy syndrome with mucocutaneous candidiasis was probably first described clinically between the 1920s and 1940s, but the autoantibodies involved were not described until the 1970s.[29,30] More surprisingly, Chediak-Higashi syndrome (CHS) was first described as an inherited predisposition to infections with detectable leukocyte abnormalities in 1943 to 1954,[31,32,33] but was not recognized as a PID until 1962.[34]

Severe congenital neutropenia (SCN) and CHS were among the first PIDs to be described, but these and other inborn errors of myeloid cells, such as chronic granulomatous disease (CGD), which was first described in 1966, and complement defects, first described in 1963 (hereditary angioneurotic edema due to an absence of C1 esterase inhibitor),[35] were not included in the first classifications of PIDs, which were restricted to the lymphoid arm of immunity, in 1968,[36] 1970,[37,38] 1971,[15] 1972,[39] 1973,[40] and 1974.[41] Over this period, the fields of immunology and PIDs evolved in parallel, focusing on acquired or adaptive immunity, which is sometimes referred to as adaptive or lymphoid immunity. The lymphoid imprint was so strong that the first report on PIDs even distinguished explicitly between these disorders and myeloid abnormalities. Unsurprisingly, given that these PIDs affected the development or function of B- and/or T-cell immunity, these patients displayed multiple, recurrent infectious diseases that were often opportunistic (ie, not seen in patients with apparently intact immunity). Patients with T- and B-cell PIDs also displayed noninfectious, autoimmune, allergic, and in some cases, cancer phenotypes. Despite the broad infectious phenotypes of the patients with complement and phagocyte disorders identified, the first mention of quantitative and qualitative disorders of phagocytes did not occur until 1974, when it appeared, somewhat amusingly, in a preliminary report,[42] but not in the official report[43] of the second World Health Organization (WHO) international workshop on PIDs. The weaknesses of this definition and classification of PIDs were apparent to some investigators, including Gatti, who ironically compared this system to the ancient Chinese classification for animals.[44] The classification nonetheless evolved, as complement disorders were mentioned in 1976 but phagocyte defects were not,[45] and qualitative phagocyte disorders (including CGD and CHS) appeared in the third (1978),[46] fourth (1983),[47] fifth (1986),[48] sixth (1989),[49] and seventh (1992)[50] WHO classifications, although SCN did not. The full range of PIDs was not covered until the eighth WHO classification in 1999,[51] and has since been dealt with in the 10th (2003),[52] 11th (2004),[53] 12th (2006),[54] 13th (2007),[55] 14th (2009),[56] and the most recent (15th[57]) WHO International Union of Immunological Societies reports. Meanwhile, two prominent reviews published in 1984 and 1995 made no reference to phagocyte defects.[58,59,60] Some aspects of the history of the field have been covered in at least two reviews.[61,62]

The common PID classification and the underlying definition of PIDs proved increasingly inadequate and unable to describe the situation in reality, which extended well beyond phagocytic disorders, from the 1990s onwards. The definition and classification of PIDs have always been engaged in an eternal game of catch-up with the reality of the situation on the ground, and this gap between the conservative words used to describe these conditions and the continual discovery of new PIDs, ever increasing the known spectrum of these diseases, has been maintained.[63,64] Phenotypic studies in this field have progressed in at least two ways. First, multiple and diverse new phenotypes have progressively been attributed to PIDs, including, of course, autoimmunity,[30] malignancy,[65] and allergy,[66] but also various other phenotypes, such as autoinflammation,[67] angioedema,[68] hemophagocytosis,[69] and thrombotic microangiopathies.[70] The underlying mutations involve multiple circuits affecting both myeloid and lymphoid cells. Second, patients with a single infectious disease, and often with a single infectious episode, have been shown to display PIDs.[71] Again, the underlying mutations affect both lymphoid and myeloid cells, but they may also, in some cases, concern nonhematopoietic cells. PIDs were initially associated with multiple, recurrent, and often

opportunistic infections with an early onset and fatal outcome. They were familial, recessive traits. Exceptions to these rules gradually emerged, beginning with the description of patients with autosomal recessive (AR) defects in the terminal components of complement (C5 to C9), who are specifically susceptible to *Neisseria*,[72] patients with X-linked recessive (XR) lymphoproliferative syndrome, who are susceptible to Epstein-Barr virus (EBV),[73] and patients with AR epidermodysplasia verruciformis, resulting in a selective predisposition to infection with skin-tropic, oncogenic papillomaviruses.[74] These studies paved the way for the discovery of new PIDs underlying particular infectious diseases in children who were otherwise healthy and normally resistant to other infectious diseases. Children with mycobacterial diseases were found to carry inborn errors of interferon (IFN)γ immunity.[75] Mutations in the toll-like receptor (TLR) and interleukin (IL)-1R pathway are associated with pyogenic bacterial diseases, whereas mutations in the TLR3 pathway are associated with herpes simplex encephalitis.[76] Finally, inborn errors of IL-17 immunity underlie chronic mucocutaneous candidiasis.[77,78] These discoveries indicated that otherwise healthy children with a single infectious disease can display inborn errors of immunity to primary (in cases of acute disease) or recurrent/latent (for chronic disease) infection.[71,79]

The definition of PIDs is evolving, thanks largely to the clinical delineation and genetic dissection of new phenotypes. These advances are also leading to changes in the classification of PIDs. PIDs were initially classified into two groups (defects of humoral and cell-mediated immunity), then into four major groups (T, B, complement, and phagocyte disorders). The 2011 WHO classification includes up to 10 (somewhat overlapping) categories of PIDs, despite the contentious omission of certain types of PID.[79a] However, there is no consensus about the definition and classification of PIDs.[16] The classification of PIDs principally on the basis of immunologic phenotypes entails a risk of clinical and genetic overlap and of some disorders being ignored. A classification based on clinical phenotype would be more useful at the patient's bedside, and a classification based

on genotype would be more useful at the research bench. However, the apparent lack of a solution to this problem is not a major concern. Hopefully, the classification will improve with the characterization of more PIDs, the deciphering of their genotypes, the dissection of their molecular, cellular, and immunologic phenotypes. and the description of their clinical phenotypes. Like a jigsaw puzzle, every piece should start to fit into place as we approach the solution. This task may not be as vast as it might initially appear, as there are no more than 25,000 genes for 7 billion individuals reproducing every two or three decades. However, we may never reach the asymptote, as there is no such thing as a distinct disease entity, precisely because of the tremendous germline and somatic genetic variability that makes each disease in each patient unique: there are only patients. There is therefore unlikely to be any satisfactory definition and classification of inborn errors of immunity in the near future. This does not matter so long as rapid progress is made in this field, in terms of awareness, diagnosis, treatment, and above all, in explorations of the genotype and intermediate phenotypes of known and new clinical phenotypes.

Why is it so important for the field of PIDs to thrive? This progress is above all important for the patients. As in other areas of medicine, strategies improving our understanding of pathogenesis constitute the only rational approach to improving the clinical management of patients, in terms of the quality of diagnosis, prognosis, and treatment. The availability of blood samples has made it possible to carry out very careful analyses of the relationships between genotypes, cellular phenotypes, immunologic phenotypes, and clinical phenotypes, as for inborn errors of erythrocytes and platelets, perhaps more thoroughly than in other fields of human genetics and pediatrics. For example, the discovery of new PIDs over the last two decades has made it clear that the 10 conventional warning signs used in PID awareness campaigns are completely out of date and require revision.[80] Another clinical lesson learned in the last 50 years is that most PID-causing genes are associated with high levels of clinical heterogeneity. Remarkable examples include mutations in the *NEMO* gene, the effects of which range from death in utero to mild immunodeficiency in adults, reflecting the severity of the biochemical deficit caused by the morbid alleles,[81] and mutations in *RAG* genes, the impact of which ranges from life-threatening severe combined immunodeficiency in infancy to combined immunodeficiency in adults.[57] Several genes have even been found to harbor loss-of-function (LOF) and gain-of-function (GOF) mutations. These genes include *WASP*, LOF mutations, which underlie Wiskott-Aldrich syndrome (WAS), and GOF mutations, which underlie SCN,[82] and *STAT1*, LOF mutations, which underlie mycobacterial or viral diseases, and GOF mutations, which underlie chronic mucocutaneous candidiasis (CMC).[78] Similarly, most, if not all of the known clinical phenotypes are associated with high levels of locus and allelic genetic heterogeneity. For example, several agammaglobulinemia-causing autosomal genes were identified following the discovery of *BTK* mutations in boys with XR agammaglobulinemia.[83] A large proportion of the patients with each PID, particularly for the most recently described conditions, do not carry mutations in known morbid genes. It is

therefore highly likely that new genetic etiologies will be discovered in the future. The clinical implications of research in this field actually extend well beyond diagnosis and pathogenesis, as the first cases of successful immunoglobulin (Ig) substitution,[9] bone marrow transplantation,[84] transfusion-based enzymatic replacement,[85] and gene therapy[86] all concerned children with PIDs. These children were also among the first to benefit from PEGylated enzymatic replacement[87] and treatment with recombinant cytokines.[88]

Perhaps of greater relevance to this book, this field has had extraordinary immunologic implications. One of the pioneers in this field, Robert Good, often referred to PIDs as "experiments of nature," reviving a line of investigation that began with Harvey and was followed by Osler, Garrod, McQuarrie, and Burnet, among others.[89,90] Indeed, physicians and scientists can learn much by deciphering the enigmas posed by the experiment of nature represented by each patient with a PID. No matter how rare a disorder, it can provide considerable insight into the fundamental laws operating in living organisms. More conventional experiments, designed by humans and carried out in animal models, benefit from being carefully thought out and executed in a controlled manner. However, they suffer from the limitations inherent to their experimental nature. Experimental protocols differ from natural processes in many ways (inbred animals, conditions of infection, microbes). Human genetics provides us with a unique opportunity to define the function of host genes in natural, as opposed to experimental, conditions: in a natural ecosystem governed by natural selection.[91,92] Immunity *in natura* can be defined by the careful dissection of PIDs and by other related approaches, such as epidemiologic and evolutionary genetics. The differences between mice and humans are often discussed, and rightly so, as these two species differ in many ways, despite the similar architecture of their immune systems. Beyond these multiple, and in some cases large, differences between humans and mice, there may be major differences between the processes used to study phenotypes, infectious and otherwise, in the two models. For example, experimental infections in mice generally involve inoculation with microbes that have not coevolved with these rodents, via artificial routes of infection and at high doses. By contrast, most human infections are natural, although some experimental infections, such as those caused by live vaccines, have played an important role in the development of this field. The experimental triggers of autoimmunity in mice are also different from those operating in natural conditions in humans. The genetic dissection of PIDs thus provides us with a unique opportunity to cast new light on the function of human genes in a natural ecosystem. Over the years, these observations have provided invaluable insights sometimes at odds with the mouse model.

It will not be possible to cover the entire field in this chapter. More than 200 inborn errors of immunity have been characterized genetically.[57,93] Many other PIDs have been described clinically but have no known genetic etiology. Doctors in this field also know that a large fraction of the patients under their care suffer from exceedingly rare

disorders, sometimes apparently limited to a single family, not reported in the medical literature. Moreover, many phenotypes might be due to new PIDs. These phenotypes form a large reservoir for the future dissection of new inborn errors of immunity, as previously illustrated by autoinflammation, hemophagocytosis, angioedema, thrombotic microangiopathies, and isolated infectious diseases.[64] Finally, we know that most known PIDs are associated with tremendous locus and allelic heterogeneity. With about 25,000 known genes and only 8000 known inborn errors, including about 300 PIDs, we can predict that 10,000 inborn errors of immunity, defined by a specific causal relationship between a genotype and a phenotype, currently await discovery. An estimate of 1000 to 2000 PID-causing loci is not unrealistic. Even with the only 300 or so known PIDs, it is clear that we cannot cover even a substantial fraction of these diseases here. We will therefore focus our review on seven topics, focusing in more detail on a specific syndrome or disease for each and discussing its genetic basis. We will pay particular attention to the immunologic lessons that can be drawn from these experiments of nature.

INBORN ERRORS OF T-CELL DEVELOPMENT OR FUNCTION

In 1950, Glanzman and Riniker described two infants who presented with severe infections, diarrhea, failure to thrive, and disseminated candidiasis, leading to early death. Severe lymphoid depletion tissue was found at the postmortem examination.[94] Similar cases of early death due to severe infection (often with weakly virulent microbes), lymphopenia, and agammaglobulinemia were reported a few years later in Switzerland.[95] Both male and female patients were affected by this disease, which was named "Swiss-type agammaglobulinemia "[96] to distinguish it from Bruton agammaglobulinemia, which affects principally male patients and is characterized chiefly by recurrent bacterial infections.[97] The cases described by Glanzman and Riniker[94] and by Hitzig et al.[95,96] constitute the first description of humans with severe combined immunodeficiency (SCID), a group of conditions characterized by a lack of autologous T cells and extreme susceptibility to infections caused by a broad range of pathogens, including weakly virulent agents. The genetic heterogeneity of SCID became

apparent in the 1960s, with the identification of families in which the disease was inherited as an X-linked trait.[98] In the 1970s and 1980s, the immunologic phenotype of SCID was shown to be heterogeneous, through the demonstration that all patients had low levels of autologous T-lymphocytes but that only some of these patients displayed an associated decrease in the numbers of B and/or natural killer (NK) lymphocytes.[99] This has led to the identification of various immunological subtypes of SCID: a) with a complete absence of T, B, and NK lymphocytes (T-B- NK- SCID); b) T- B+ NK- SCID; c) T- B+ NK+ SCID; and d) T- B- NK+ SCID. Finally, genetic studies over the last three decades have shown that SCID is also highly heterogeneous in terms of its genetics[57,100] (Table 48.1; Fig. 48.1).

SCID has a prevalence of 1:50,000 to 1:100,000 live births. The study of SCID has played an essential role in the identification of the key mechanisms governing human T-cell development. In several cases, SCID-causing gene defects were identified in patients before the generation of animal models. Moreover, comparison of humans and mice with mutations in orthologous genes associated with SCID has often revealed differences in the immunologic phenotype in these two species, indicating the existence of speciesspecific differences in the mechanisms governing lymphoid development. Finally, the heterogeneity of mutations in individual SCID-causing genes is associated with variability of the clinical and immunologic phenotype, which may include manifestations other than SCID, such as various forms of immune deficiency and immune dysregulation, particularly in patients with hypomorphic mutations allowing residual development of T- and/or B-lymphocytes.[101]

Molecular Mechanisms Accounting for Severe Combined Immunodeficiency

Defective thymus organogenesis is associated with a profound impairment of T-cell development. The genetic defects affecting the development of the thymic epithelium are extrahematopoietic in nature, but they may greatly disturb T-cell development, in some cases leading to a clinical and immunologic SCID phenotype. *DiGeorge syndrome* (DGS) results from defects of the third and fourth branchial pouches and is characterized by impaired thymic development, congenital heart disease, hypoparathyroidism, and facial dysmorphisms. Most patients carry a heterozygous interstitial deletion of chromosome 22q11.[102] The molecular mechanism by which haploinsufficiency for the genes of the DGS critical region underlies defective thymic development remains unclear. The severity of the thymic development defect varies considerably between patients. Most patients with DGS have moderate T-cell lymphopenia, but a few have complete thymic aplasia (complete DGS) and no circulating T-lymphocytes.[102,103,104,105] Another PID in which thymogenesis is impaired is caused by mutations of the gene encoding FOXN1, a transcription factor required for the development and maturation of the thymus stroma and eccrine glands.[106,107] Biallelic *FOXN1* mutations cause an AR SCID phenotype in which a lack of circulating T-lymphocytes is associated with alopecia.[108,109,110] This disease is the human equivalent of the *nude* phenotype in mice, for which the underlying gene was identified before the discovery of human patients.[111]

The proliferation and survival of lymphoid progenitor cells are also essential for the generation of a normal number of mature lymphocytes. Some forms of SCID are associated with high rates of apoptosis. In 1972, while investigating polymorphic enzymes as genetic markers, Giblett discovered that the erythrocytes of two infants with SCID displayed no adenosine deaminase (ADA) activity.[112] ADA converts adenosine to inosine (and deoxyadenosine to deoxyinosine).[113] In patients with AR *ADA deficiency*, the accumulation of toxic phosphorylated derivatives of deoxyadenosine causes cell death, resulting in extreme lymphopenia, with an almost total absence of T, B, and NK lymphocytes.[112,114,115] A few years after describing ADA deficiency, Giblett discovered

that a deficiency of another enzyme involved in purine metabolism, purine nucleoside phosphorylase, was also associated with impaired lymphoid development.[116]

TABLE 48.1 Inborn Errors of T-Cell Development

Disease	Gene Defect	T Cells	B Cells	NK Cells	Inheritance	Pathogenesis	Associated Features Other than Immunodeficiency
FOXN1 deficiency	*FOXN1*	↓↓↓	N	N	AR	Defective maturation of thymic	Alopecia

						epithelium	
DiGeorge syndrome	22q11del*	↓↓	N	N	Sporadic	Defective thymus organogenesis	Hypoparathyroidism, congenital heart disease
Reticular dysgenesis	*AK2*	↓↓↓	N or ↓	↓↓	AR	Increased apoptosis	Neutropenia, sensorineural deafness
Adenosine deaminase deficiency†	*ADA*	↓↓	↓↓	↓↓	AR	Increased apoptosis	Bone abnormalities, neurologic problems, liver and lung involvement
Nucleoside phosphorylase deficiency	*PNP*	↓↓	N	N	AR	Increased apoptosis	Neurologic problems, autoimmunity
X-linked SCID†	*IL2RG*	↓↓↓	N	↓↓↓	XL	Defective signaling through gc	
JAK3 deficiency	*JAK3*	↓↓↓	N	↓↓↓	AR	Defective signaling through gc	
IL-7R deficiency†	*IL7R*	↓↓↓	N	N	AR	Defective signaling through IL-7R	
RAG1 deficiency†	*RAG1*	↓↓↓	↓↓↓	N	AR	Defective V(D)J recombination	
RAG2 deficiency†	*RAG2*	↓↓↓	↓↓↓	N	AR	Defective V(D)J recombination	
Artemis deficiency†	*DCLRE1C*	↓↓↓	↓↓↓	N	AR	Defective V(D)J recombination	Developmental defects, radiation sensitivity
DNA ligase IV deficiency†	*LIG4*	↓↓	↓↓	N	AR	Defective V(D)J recombination	Microcephaly, dysmorphisms, radiation sensitivity
Cernunnos/XLF deficiency	*NHEJ1*	↓↓	↓↓	N	AR	Defective V(D)J recombination	Microcephaly, radiation sensitivity
DNA-PKcs deficiency	*PRKDC*	↓↓↓	↓↓↓	N	AR	Defective	V(D)J recombination
CD3γ deficiency	*CD3G*	N or ↓	N	N	AR	Defective TCR signaling	Most often, autoimmunity
CD3δ deficiency	*CD3D*	↓↓↓	N	N	AR	Defective signaling through pre-TCR	
CD3ε deficiency	*CD3E*	↓↓↓	N	N	AR	Defective signaling through pre-TCR	

CD3ζ deficiency	*CD3Z*	↓↓↓	N	N	AR	Defective signaling through pre-TCR	
CD45 deficiency	*CD45*	↓↓↓	N	N	AR	Defect of signaling	
ZAP-70 deficiency†	*ZAP70*	↓↓↓ of CD8+ cells	N	N	AR	Defect of signaling (impaired positive selection of CD8+ cells)	
MHC class II deficiency	*CIITA, RFX5, RFXANK, RFXAP*	↓↓ of CD4+ cells	N	N	AR	Defect of positive selection of CD4+ cells	
Coronin-1A deficiency	*CORO1A*	↓↓↓	N	N	AR	Defect of thymocyte egress, impaired T lymphocyte survival	
ORAI1 deficiency	*ORAI1*	N (but defective function)	N	N	AR	Defective calcium flux	Autoimmunity, myopathy
STIM1 deficiency	*STIM1*	N (but defective function)	N	N	AR	Defective calcium flux	Autoimmunity, myopathy
MAGT1 deficiency	*MAGT1*	↓↓ of CD4+ cells, reduced proliferation	N	N	XL	Defective magnesium flux	Chronic viral infections, lymphoma
DOCK8 deficiency	*DOCK8*	↓	↓	↓	AR	Defective activation	Severe atopy, severe cutaneous viral infections
CD40L deficiency	*CD40LG*	N	N	N	XL	Impaired CD40L-mediated costimulation of B and dendritic cells by activated CD4+ cells	Neutropenia, *Cryptosporidium* infection, biliary tract disease, neuroectodermal tumors; IgM levels are often elevated

AR: autosomal recessive; CD, cluster of differentiation; DNA, deoxyribonucleic acid; gc, germinal center; Ig, immunoglobulin; IL, interleukin; MHC, major histocompatibility complex;

N, normal; NK, natural killer; PNP, purine nucleoside phosphorylase; RAG, recombinase-activating gene; SCID, severe combined immunodeficiency; TCR, T-cell receptor; XL,

X-linked; XLF, XRCC4-Like factor.

* DiGeorge syndrome is most often associated with 22q11 chromosomal deletion; however, other cytogenetic and genetic abnormalities have been also reported in a minority of cases.

† Hypomorphic mutations in these genes have been associated with Omenn syndrome or other conditions with immune dysregulation.

SCID may also be caused by impaired cytokine signaling by T-cell precursors in the thymus. In 1993, two groups demonstrated that the newly cloned *IL2RG* gene encoding the γ chain of the IL-2 receptor was mutated in male patients with XR SCID, the most common form of SCID in western countries.[117,118] XR SCID is characterized by a T-B+ NK- phenotype, indicating that *IL2RG* mutations are deleterious to the development of both T- and NK lymphocytes. This was a surprising discovery, because disruption of the *Il2* gene in mice had been shown to be associated with immune dysregulation rather than SCID.[119] It soon became clear that the IL-2Rγ chain was also common to other cytokine receptors, including those for IL-4, IL-7, IL-9, IL-15, and IL-21,[120,121,122,123,124,125] leading to its being renamed the common γ chain, γc. In all these receptors, the γc is coupled to the intracellular kinase JAK3, allowing intracellular signaling.[126] These discoveries have helped define the molecular basis of XR SCID and to identify other genetic defects causing SCID in humans. In 1995, two groups established that an AR variant of T- B+ NK- SCID (a phenocopy of XR SCID) was due to mutations of the *JAK3* gene.[127,128] Furthermore, the observation of severely impaired T-cell development in *Il7*$^{-/-}$ and *Il7r*$^{-/-}$ mice[129,130] led to the discovery of *IL7R* mutations in patients with AR T- B+ NK+ SCID.[131,132] IL-7 provides important signals for the survival and proliferation of thymocytes and peripheral T cells and regulates the rearrangement of T-cell receptor (TCR) genes,[133] thus accounting for the absence of circulating T cells in patients with SCID due to *IL2RG*,*JAK3*,and *IL7R* mutations. Interestingly, B-cell development is unaffected in these forms of SCID, but abolished in *Il2rg*$^{-/-}$, *Jak3*$^{-/-}$, and *Il7r*$^{-/-}$ mice.[134,135,136,137] These data highlight significant species-specific differences in the molecular mechanisms governing lymphoid development. Circulating NK lymphocytes are absent in patients with XR SCID and with JAK3 deficiency, but not in those with IL-7R deficiency, suggesting that impaired signaling through another γc-dependent cytokine may occur. The cytokine concerned is probably IL-15, because IL-15-mediated signaling is essential for human NK cell development in vitro[138] and *Il15*$^{-/-}$ and *Il15r*$^{-/-}$ mice lack NK cells in vivo.[139,140]

SCID can also result from impaired TCR gene rearrangement due to abnormalities in VDJ recombination, a process essential for the expression of both the pre-TCR and the mature TCR.[141,142] The lymphoid-specific recombinaseactivating gene (RAG) 1 and RAG2 proteins initiate VDJ recombination by recognizing recombination-specific sequences flanking the variable (V), diversity (D), and joining (J) elements of the TCR and introducing deoxyribonucleic acid (DNA) double-strand breaks.[143,144] These breaks are eventually repaired through the ubiquitously expressed nonhomologous end-joining (NHEJ) pathway.[145,146] RAG1, RAG2, and the NHEJ pathway are also involved in the rearrangement of Ig genes, which is required for expression of the pre-B-cell receptor (BCR) and the BCR.[141,143] Mutations of the *RAG1* and *RAG2* genes,[147] and of the genes encoding Artemis,[148] DNA ligase IV,[149,150,151,152] and DNA-PKcs[153] (all components of the NHEJ pathway) result in T- B- NK+ SCID. Mutations of Cernunnos/XLF, another component of the

NHEJ pathway, severely impair, but do not completely abolish T- and B-cell development.[154,155] The genes encoding Artemis[148] and Cernunnos/XLF[154] were cloned from humans before they were cloned from mice, the availability of fibroblastic cell lines from SCID patients being essential to this achievement. NHEJ is involved in general mechanisms of DNA repair, including those occurring in nonlymphoid cells. Thus, patients with defects of this pathway also display an unusually high level of cellular sensitivity to radiation, have a higher than normal risk of malignancy, and may present with neurologic problems.[146,156] Finally, hypomorphic mutations of genes involved in V(D) J recombination and NHEJ have been identified in patients with “leaky” forms of SCID, in whom the residual development of T- (and in some cases, B-) lymphocytes was associated with autoimmunity and a high risk of lymphoid malignancies.[157,158,159]

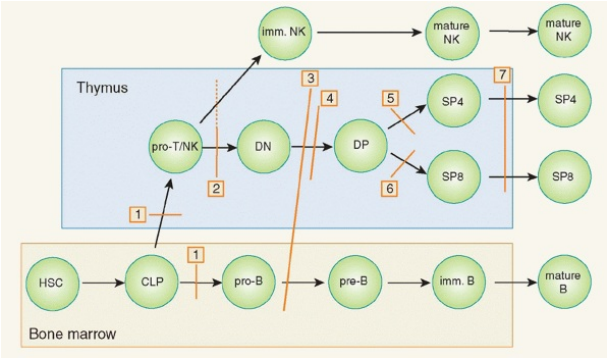

FIG 48.1. Schematic Representation of Developmental Blocks in Combined Immunodeficiencies. Discrete stages in T-, B-, and natural killer (NK)-cell development are shown. *Red bars* identify stages at which developmental arrests are caused by specific gene defects. *Numbers in boxes* identify the molecular defects accounting for the developmental blocks. In particular: 1): adenosine deaminase deficiency; 2) defects of cytokine-mediated signaling (*IL2RG*, *IL7R*, *JAK3* deficiency); 3) defects of V(D)J recombination (*RAG1*, *RAG2*, *Artemis*, *LIG4*, *Cernunnos*, and *DNA-PKcs* deficiency); 4) defects of pre-T-cell receptor signaling (*CD3D*, *CD3E*, *CD3Z* deficiency); 5) defects of positive selection of cluster of differentiation (CD)4+ thymocytes (major histocompatibility complex class II deficiency); 6) defects of positive selection of CD8+ thymocytes (ZAP-70 deficiency); and 7) defects of thymocyte egress from the thymus (Coronin-1A deficiency). The *hatched bar* indicates that NK-cell development is affected by mutations of *IL2RG* and *JAK3* gene (that interfere with interleukin-15-mediated signaling) but not by *IL7R* mutations. Not shown is severe combined immunodeficiency due to *FOXN1* mutations (that impair thymus organogenesis) and reticular dysgenesis, due to mutations of the *AK2* gene, in which the exact stage at which T-cell development is blocked is not known. CLP, common lymphoid progenitor; DN, double negative; DP, double positive; HSC, hematopoietic stem cell; imm., immature; SP4, single positive CD4; SP8, single positive CD8.

Signaling through the pre-TCR is essential to promote the progression from cluster of differentiation (CD)4-CD8- double-negative (DN) thymocytes to CD4+ CD8+ double-positive cells. This signaling is mediated by the CD3 complex, mimicking the requirement of this complex for TCR signaling in mature T cells. Null mutations of the *CD3D*, *CD3E*, and *CD3Z* genes interfere with this process and result in AR SCID.[160,161,162,163] By contrast, *CD3G* mutations are more often associated with a milder phenotype including autoimmunity.[164]

Other Combined Immune Deficiencies Due to Late Defects in T-Cell Development and Function

The positive selection of CD4+ and CD8+ thymocytes requires the interaction of thymocytes expressing a functional TCR with thymic epithelial cells (and dendritic cells) expressing self-antigens bound to human leukocyte antigen (HLA) class II and class I molecules, respectively.[165,166] Several gene defects account for *HLA class II deficiency* in humans,[167,168,169,170] a condition characterized by severe CD4+ lymphocyte depletion and normal CD8+ T-cell development.[171] Conversely, mutations of *ZAP-70*, a tyrosine kinase that binds to the CD3ζ chain and promotes TCR-mediated signaling,[172] cause immunodeficiency with a lack of CD8+ cells.[173,174,175] The CD4+ lymphocytes of affected patients develop normally but are nonfunctional and fail to

proliferate in response to mitogens and antigens. ZAP-70 deficiency in humans results in a phenotype different from that of *Zap70*$^{-/-}$ mice, which lack both CD4+ and CD8+ lymphocytes.[176] The prolonged expression of SYK in human (but not in mouse) double-positive thymocytes seems to compensate for the lack of ZAP-70 protein, allowing the generation of CD4+ T cells.[177,178] However, mature CD4+ lymphocytes from ZAP-70-deficient patients have no SYK and are therefore functionally impaired.

The egress of mature thymocytes from the thymus to the periphery requires rearrangement of the actin cytoskeleton. Coronin-1A is involved in this process.[179] Accordingly, *Coronin-1A deficiency* causes the retention of mature thymocytes and a failure to generate peripheral T cells, although impaired survival of newly generated T lymphocytes is probably another very important mechanism of disease.[180,181]

Finally, mature T cells in the periphery respond to mitogenic signals by releasing calcium from

endoplasmic reticulum stores and opening calcium release-activating channels on the cell membrane, allowing calcium influx to occur.[182] Mutations of *STIM1* (which encodes a sensor of endoplasmic reticulum calcium stores) and of *ORAI1* (which encodes calcium release-activating channels) cause a severe immunodeficiency in which T-lymphocyte generation in the thymus is not affected, but the function of peripheral T cells is impaired.[181] Both conditions are also characterized by myopathy and the impairment of immune homeostasis, with autoimmunity.[183,184]

Clinical and Immunologic Features and Treatment

Infants with SCID are susceptible to severe infections from shortly after birth. Infections may be due to bacteria, viruses, or fungi. Infections with weakly virulent microorganisms (eg, *Pneumocystis jiroveci*, cytomegalovirus) are common and frequently cause interstitial pneumonia and chronic diarrhea, leading to failure to thrive.[101,185,186]

Lymphopenia is present in 60% to 70% of affected infants.[186] T-cell lymphopenia is even more common. In 30% to 50% of cases, a variable number of T-lymphocytes of maternal origin (that have crossed the placenta and not been rejected by the fetus with SCID) are detected.[187] Maternal T-lymphocytes may cause alloreactive signs resembling graft-versus-host disease, with infiltration and damage to the liver, the skin, the gut, and the bone marrow.[187,188] All patients lack autologous T cells.

Alternatively, less severe (hypomorphic) mutations in SCID-causing genes may be permissive for residual T-cell development, but result in a lower level of TCR diversity in circulating T cells.[101] The oligoclonal expansion of these T-lymphocytes is often associated with tissue damage, as in infants with Omenn syndrome.[189] This damage resembles the severe inflammatory lesions resulting from the homeostatic proliferation of a few T-cell clonotypes in mice.[190] In other patients, the mutations may underlie milder forms of combined immunodeficiency.[159,191] However, the severity of the clinical and immunologic phenotype is not determined solely by the nature of the mutation, because different phenotypes (eg, SCID and Omenn syndrome) have been reported in different individuals harboring the same mutation and even in individuals from the same family.[192]

Lymphocyte and lymphocyte subset counts are the mainstay of SCID diagnosis and may provide useful information about the underlying genetic defect.[101] The presence of T cells does not exclude a diagnosis of SCID, as the T cells detected may be of maternal origin. However, the maternal T cells have a very limited in vitro proliferative response to mitogens (eg, phytohemagglutinin).[186] Infants with SCID are unable to mount specific antibody responses.[186] Genetic diagnosis is guided by the NK- and B-cell phenotype and the mode of inheritance. A SCID screening test for neonates has recently become available. This test is based on the polymerase chain reaction-mediated amplification of TCR excision circles (TRECs) from a dried blood spot collected at birth.[193,194] TRECs are a byproduct of TCRα rearrangement that persist in newly developed T-lymphocytes expressing the αβ form of the TCR,[195] but are diluted out during the subsequent proliferation of cells in the periphery. TREC quantification therefore provides important information about thymic function.

Infants with SCID require the regular administration of Igs and antimicrobial prophylaxis. When indicated, blood products should be irradiated before transfusion to prevent transfusional graft-versus-host disease, and should be obtained from cytomegalovirus-negative donors to prevent the risk of transmission. The diagnosis and treatment of infection should be prompt and aggressive. However, in the absence of prophylaxis, SCID is inevitably fatal within a few months, and patients do not survive beyond a few years in the absence of transplantation.[185] Indeed, hematopoietic stem cell transplantation (HSCT) is the mainstay of SCID treatments, and optimal results (> 90% survival) are obtained if the donor is an HLA-matched sibling.[196,197] If no such donor is available, HSCT can be performed with a matched unrelated donor or with a haploidentical parent as the donor. However, if a haploidentical parent acts as the donor, mature T-lymphocytes must be removed from the graft to prevent graft-versus-host disease. Excellent results (> 95% survival) have been reported for SCID patients treated by HSCT before the age of 3.5 months,[197] highlighting the importance of neonatal screening for SCID. HSCT results are less satisfactory for combined immunodeficiency with the residual presence of autologous T-lymphocytes, because there is a risk of graft rejection and of treatmentrelated toxicity, due to the need for chemotherapy for ablation of the immune system of the host.[196] In particular, the outcome of HSCT is far from good in patients with major histocompatibility complex (MHC) class II deficiency, because the transplantation of hematopoietic stem cells does not correct the lack of MHC class II antigen expression on thymic epithelial cells.[198] Enzyme replacement therapy may lead to detoxification and immune reconstitution in infants with ADA deficiency.[199,200] Finally, gene therapy has been successfully used in infants with SCID due

to ADA deficiency[201] or to a γc defect.[85,202,203] However, 5 of 20 infants with X-linked SCID treated by gene therapy developed leukemic proliferation due to insertional mutagenesis (ie, insertion of the transgene near an oncogene, mostly LMO2, leading to transcriptional activation of the oncogene).[204,205]

Thus, studies of patients with SCID have laid the foundations for an understanding of the molecular and cellular mechanisms governing lymphoid development in humans. Identification of the molecular basis of SCID in humans has preceded the generation of gene-targeted mice on a number of occasions. Furthermore, important differences have emerged between the immunologic phenotypes of humans and mice, with mutations in orthologous genes associated with SCID. Finally, the early identification of SCID patients with molecular and immunologic tools has important clinical implications, optimizing survival after HSCT. Despite these advances, the gene responsible for disease remains unidentified in most cases of combined immunodeficiency and a small minority (< 5%) of infants with SCID. The deep sequencing of human exomes and genomes should lead to the identification of still more genes causing T-cell deficiencies in humans.

INBORN ERRORS OF B-CELL DEVELOPMENT OR FUNCTION

Antibody deficiencies constitute the largest group of currently recognized PIDs (Table 48.2).[206,207,208] This is partly because these disorders were among the first to be recognized to cause inborn errors in immunity and because they are among the easiest to detect with routine laboratory studies. Antibody deficiencies, regardless of their etiology, are associated with recurrent or persistent infections with encapsulated bacteria, particularly *Streptococcus pneumoniae* and *Haemophilus influenzae*. Patients develop infections typical of these organisms, including otitis, sinusitis, and pneumonia, but they are also likely to experience more severe, invasive infections, such as sepsis, meningitis, joint infections, and cellulitis. Other infections and clinical findings are more specific to particular antibody deficiencies.

X-linked Agammaglobulinemia

X-linked agammaglobulinemia (XLA), sometimes called Bruton agammaglobulinemia or congenital agammaglobulinemia, was first described in 1952, when Colonel Bruton reported the case of an 8-year-old boy with recurrent pneumococcal sepsis and no globulin fraction on serum electrophoresis.[209] The patient's clinical course was improved by treatment with exogenous gammaglobulin. Once techniques had been developed for the evaluation of B cells in the peripheral blood, it became clear that patients with XLA had very few or no B cells in the bloodstream.[210,211,212,213] This is the most consistent feature in patients with XLA. Although 10% to 15% of patients have higher than expected serum Ig concentrations, all patients have < 2% CD19+ lymphocytes in the peripheral blood (normal range 5% to 20% CD19+ cells). Patients are treated by gammaglobulin replacement and the aggressive use of antibiotics.

In addition to infections caused *S. pneumoniae* and *H. influenzae*, patients with XLA are more vulnerable to enteroviral infections, particularly enteroviral encephalitis and vaccine-associated polio.[214,215,216,217,218] Mycoplasma and ureaplasma infections are also more common and may cause arthritis, prostatitis, and meningitis, as well as pneumonia.[219,220] The incidence of *Giardia* infection is also high in patients with XLA; some of these patients have protein-losing enteropathy and low serum IgG as the first sign of infection.[221] Although significant complications and early death are still sometimes reported for patients with XLA,[216] many patients now survive into adulthood.[222]

In 1993, two groups, using two separate approaches, showed that XLA was due to mutations in a previously unrecognized cytoplasmic tyrosine kinase, now called Bruton tyrosine kinase (BTK).[223,224] One group identified the gene on the basis of linkage analysis[223]; the other isolated a cDNA encoding a tyrosine kinase from B-cell precursors.[224] This gene mapped to the XLA critical region on the long arm of the X chromosome, making it an excellent candidate gene.[224] BTK is an enzyme specific to hematopoietic cells that is expressed throughout B-cell differentiation.[225] It is found in the platelets and myeloid cells, but not in T cells or plasma cells.[226] However, the clinical effects of BTK deficiency appear to be limited to the B-cell lineage. Severe neutropenia is seen in some patients with XLA, particularly those who are very young.[218,227] However, very low neutrophil counts are also seen in patients with defects specific to the B-cell lineage, such as μ heavy chain deficiency.[228] This neutropenia may be due to bone marrow suppression by viruses in the absence of natural antibody.

BTK has an amino-terminal pleckstrin homology domain, a proline-rich region, and an SH3 domain, followed by an SH2 and a carboxy-terminal kinase domain.[224] It is activated and phosphorylated within minutes of pre-BCR or BCR activation.[229,230] Activated BTK and

PLCγ2 then bind to the scaffold protein BLNK, allowing BTK to phosphorylate PLCγ2, resulting in biphasic calcium flux.[231]

Over 600 different mutations have been identified in the BTK gene,[232,233] most of which result in an absence of detectable BTK in monocytes and platelets.[234,235,236] Amino acid substitutions, particularly those that do not affect the stability of the protein, tend to result in a milder phenotype characterized by an older age at diagnosis, higher serum Ig concentrations, and the presence of B cells in small, but detectable numbers.[237,238,239] However, the genotype/phenotype correlation is not strong.

Mutations of the BTK gene account for 85% of cases of early onset of infection and isolated defects in B-cell development.[240] A similar clinical phenotype is seen in patients with mutations in genes encoding components of the pre-BCR and BCR, including the μ heavy chain,[228,241,242,243] the surrogate light chain protein λ5,[244] the signal transduction molecules Igα and Igβ (also called CD79a and CD79b),[242,245,246] and the downstream scaffold protein BLNK.[243,247]

Bone marrow studies have shown B-cell development to be blocked at the pro-B cell to pre-B cell transition, the stage at which the pre-BCR is first expressed, in patients with mutations affecting BTK, the μ heavy chain, λ5, Igα, Igβ, or BLNK. BTK gene mutations cause a leaky defect in B-cell differentiation. Most patients with XLA have a small number of both pre-B cells in the bone marrow and immature

B cells in the bloodstream.[248,249,250] As in healthy controls, the number of B cells produced decreases with age,[251] and most adults with XLA have less than 0.02% B cells in the blood. Mutations in the other genes cause a more severe block in B-cell development. Null mutations in the μ heavy chain, Igα, and Igβ genes result in a complete absence of CD19+ lymphocytes from the blood (< 0.01%). A patient with a hypomorphic mutation in Igβ and young children with mutations in λ5 or BLNK have been reported to have a small number of B cells (< 0.1% CD19+ cells). By contrast, mice with BTK, λ5, or BLNK defects have been reported to have 50%, 20%, or 10%, respectively, the normal number of B cells in the blood.

TABLE 48.2 Antibody Deficiencies

Disease	Gene Defect	B cells	T cells	Serum Igs	Inheritance	Pathogenesis	Associated Features or Comments
Agammaglobulinemia with absent or very low B cells							
X-linked agammaglobulinemia	*BTK*	↓↓↓	N	All isotypes decreased	XL	Defective signaling through the BCR	Detectable serum Igs in some patients
μ heavy chain deficiency	*IGHM*	↓↓↓↓	N	All isotypes decreased	AR	Defective signaling through the BCR	
λ5 deficiency	*IGLL1*	↓↓↓↓	N	All isotypes decreased	AR	Defective signaling through the BCR	
Igα deficiency	*CD79A*	↓↓↓↓	N	All isotypes decreased	AR	Defective signaling through the BCR	
Igβ deficiency	*CD79B*	↓↓↓↓	N	All isotypes decreased	AR	Defective signaling through the BCR	
BLNK deficiency	*BLNK*	↓↓↓↓	N	All	AR	Defective	

				isotypes decreased		signaling through the BCR	
Hypogammaglobulinemia with normal or low B cells							
CVID	*Multifactorial*	N or ↓	N or ↓	↓IgG and IgA; IgM may be normal	Usually sporadic	variable	Autoimmune cytopenias; granulomatous disease; lymphoma
IgA deficiency	*Multifactorial*	N	N	↓ IgA; normal IgG and IgM	Usually sporadic	variable	Sometimes assoicated with IgG subclass deficiency
ICOS deficiency	*ICOS*	N	N	↓ IgG and IgA; IgM may be normal	AR	Defective T-cell activation	
CD19 deficiency	*CD19*	N	N	↓ IgG; ↓ or normal gA and IgM	AR	Poor amplification of BCR signal	May have glomerulonephritis
CD81 deficiency	*CD81*	N	N	↓ IgG; ↓ or normal gA and IgM	AR	Poor amplification of BCR signal	May have glomerulonephritis; coreceptor with CD19
CD20 deficiency	*MS4A1*	N	N	↓ IgG; normal IgA and IgM	AR	Defective B-cell activation	Poor antibody response to T cell-independent antigens
TACI	*TNFRSF13B*	N	N	variable	AD or AR	Defective B-cell activation and survival	Susceptibility gene not diseasecausing; associated with autoimmunity
BAFF-R	*TNFRSF13C*	↓	N	↓ IgG and IgM; normal IgA	AR	Defective B-cell activation and survival	
Class switch recombination defects							
X-linked hyper-IgM syndrome	*CD40LG*	N	N	↓ IgG and IgA; IgM may be normal or ↑↑↑	XL	Impaired CD40L-mediated costimulation of B and dendritic cells by activated CD4+ cells	Neutropenia, *Cryptosporidium* infection, biliary tract disease, neuroectodermal tumors
CD40 deficiency	*CD40*	N	N	↓ IgG and IgA; IgM may be normal or ↑↑↑	AR	Impaired CD40L-mediated costimulation of B and dendritic cells by activated CD4+ cells	Neutropenia, *Cryptosporidium* infection, biliary tract disease, neuroectodermal tumors
AID deficiency	*AICDA*	N	N	↓ IgG and IgA; IgM	AR or AD	Defective initiation of	Lymphadenopathy, increased

				↑↑↑		somatic hypermutation and class switch recombination	incidence of autoimmune disease
UNG deficiency	*UNG*	N	N	↓ IgG and IgA; IgM ↓↓↓	AR	Defective somatic hypermutation and class switch recombination	Lymphoid hyperplasia

AD, autosomal deficient; AID, activation-induced cytidine deaminase; AR, autosomal recessive; BCR, B-cell receptor; CD, cluster of differentiation; CVID, common variable immunodeficiency; Ig, immunoglobulin; N, normal; UNG, uracil-DNA glycosylase; XL, X-linked.

Hyperimmunoglobulin M Syndromes/Class Switch Recombination Defects

Hyper IgM syndrome is characterized by normal or high serum IgM concentrations, with IgG and IgA either present at very low concentrations or undetectable.[216,252,253] However, with the identification of the genetic etiologies of the most common forms of hyper-IgM syndrome, it has become clear that not all patients have high IgM concentrations. This has led to the suggestion of "class switch recombination defects" as a more appropriate designation for this group of disorders.[253] We will use these terms interchangeably here. Approximately 65% of patients with hyper-IgM syndrome have the XR form of the disease, which is caused by mutations in the gene encoding the tumor necrosis factor (TNF) family member CD40 ligand (CD40L).[254,255,256] These patients tend to have more severe illness than patients with XLA.[216,252] The median age at diagnosis is 12 months, and patients generally have little or no detectable IgA and IgG in the serum, frequently accompanied by neutropenia and/or opportunistic infections. The numbers of T cells and B cells are usually within normal limits. Infections with encapsulated bacteria, *Pneumocystis*, cytomegalovirus, parvovirus, *Cryptosporidium*, and *Histoplasma* are problematic in patients with CD40L deficiency. Sclerosing cholangitis secondary to *Cryptosporidium* infection[252] and neuroendocrine or hepatobiliary carcinomas[257,258] have been reported in a significant number of patients. Treatment consists of gammaglobulin replacement, the aggressive use of antibiotics, granulocyte-colony stimulating factor (G-CSF) in cases of persistent neutropenia and, in some patients, HSCT.[259]

CD40L (also called CD154, gp39, TRAP, and TNFSF5) is a type II transmembrane protein transiently expressed on the surface of activated T cells[260,261,262] and platelets. The binding of CD40L to its cognate receptor on B cells, CD40, induces B-cell activation, short- and long-term B-cell proliferation, and in the presence of cytokines, isotype switching[260,262,263,264] through the induction of activation-induced cytosine deaminase (AID) production. By deaminating cytosine residues in VH and switch regions, AID performs the first step in both class switch recombination and somatic hypermutation.[265,266] However, CD40 is also expressed on monocytes,[267] dendritic cells,[268] activated platelets,[269] epithelial cells,[270] and endothelial cells.[271] The stimulation of these target cells via CD40L elicits an inflammatory response, with release of cytokines, including IL-12 in particular.[267,272] The failure to elicit this inflammatory response accounts for the viral, fungal, and parasitic diseases seen in patients with CD40L deficiency.

Several other genetic disorders resulting in hyper-IgM syndrome have been reported. A small number of patients with AR defects in CD40 have been described.[273] These patients have a clinical phenotype identical to that seen in patients with mutations in CD40L. Approximately 10% to 15% of patients with class switch recombination defects have mutations in *AICDA*, which encodes the B cell-specific protein AID described previously.[266,274,275] Defects in UNG, an enzyme responsible for eliminating the uracil molecules generated by AID activity, also account for a small number of cases.[276] Patients with mutations affecting AID or UNG do not have the viral, parasitic, and fungal infections or neutropenia seen in patients with CD40L deficiency, but they are more likely to have lymphadenopathy and autoimmune disease.[277]

Common Variable Immunodeficiency

Shortly after Bruton described the first case of agammaglobulinemia in a young boy, other authors reported adult patients, of both sexes, with severe

hypogammaglobulinemia.[278,279,280] Many of these patients appeared to have acquired, rather than congenital immunodeficiency, and both clinical and

laboratory findings were highly variable.[281,282] This disorder therefore came to be called acquired hypogammaglobulinemia or common variable immunodeficiency (CVID). Hypogammaglobulinemia can be seen in other immunodeficiencies and may result from infections, drug reactions, protein-losing conditions, or cancers, and it is therefore important to rule out these other possibilities before diagnosing CVID.[283]

Patients of any age may be diagnosed as having CVID, but onset occurs most frequently between the ages of 10 and 40 years.[284,285,286,287,288,289] Respiratory tract infections, diarrhea, and autoimmune disorders are the most common findings on presentation. Laboratory analyses show low serum IgG and IgA concentrations, but IgM concentration may be low or within the normal range. B cells are easily detected in the blood of most but not all patients.[290] However, most patients lack CD27+ memory B cells.[291,292] About 10% of patients have a family history of immunodeficiency or autoimmune disease.[286] Patients with CVID or IgA deficiency are more likely to have certain uncommon MHC haplotypes.[293,294,295] The precise gene within this locus responsible for susceptibility remains unclear.[296] Recent genome-wide association studies have suggested that some copy number variants are more common in CVID.[297]

In the last 10 years, several genetic disorders resulting in clinical signs consistent with CVID have been reported. A small number of CVID patients with homozygous mutations in *ICOS* have been identified.[298,299] Heterozygous and homozygous mutations affecting TAC1, which belongs to the TNF receptor family (also known as TNFRSF13B), have been reported in 10% of patients with CVID and 1% of healthy controls.[300,301,302,303,304] CVID patients with TACI alterations are more likely to have autoimmunity and splenomegaly than other patients with CVID.[300,304] Homozygous mutations of the gene encoding another TNF receptor family member, BAFF-R, have been described in a brother and sister in whom immunodeficiency was first recognized after the age of 50 years.[290] Homozygous mutations of the genes encoding CD19 or another component of the CD19 complex, CD81, have been reported in fewer than 10 patients.[305,306,307,308] A child with a homozygous mutation of the gene encoding CD20 and a low serum IgG concentration has been reported.[309] The clinical and laboratory findings for all the individuals with these mutations are highly variable, and it is not clear that any of these genetic alterations are sufficient to cause clinical symptoms when present on their own. In most patients, a combination of genetic and environmental factors is likely to play a role.

INBORN ERRORS OF PHAGOCYTE DEVELOPMENT OR FUNCTION

A number of inborn errors affect the polymorphonuclear or mononuclear phagocytes of the myeloid lineage (Table 48.3). Granulocyte disorders mostly affect neutrophils, whereas mononuclear phagocyte disorders affect monocytes, macrophages, or dendritic cells. Some disorders affect phagocytes and other leukocytes; they may also sometimes affect nonhematopoietic cells. Some of these disorders will be covered elsewhere in this chapter. The disorders mostly or exclusively affecting phagocytes have traditionally been grouped together under the umbrella of “phagocyte disorders.” We will not review all these disorders here, as this group is rapidly expanding. Instead, we will focus on two of these disorders that have played an important role in the history of this field, in which considerable progress has been made in recent decades. These two disorders—severe congenital neutropenia (SCN) and chronic granulomatous disease (CGD), first described by Rolf Kostmann, and CGD, first described by Robert Good— elegantly illustrate the quantitative and qualitative defects of phagocytes. Studies of these disorders have also provided substantial immunologic insight into granulocyte function and the respiratory burst of phagocytes. Many other equally fascinating quantitative defects, such as AR IRF8 deficiency, in which circulating monocytes and dendritic cells do not develop, have been described but will not be discussed here.[310] Similarly, we will not consider qualitative disorders here, such as specific granule deficiency, which is characterized by a lack of specific granules, making it impossible to distinguish between neutrophil, basophil, and eosinophil granulocytes, and is caused by AR C/EBP-ε deficiency.[311] A list of phagocyte disorders is provided in Table 48.3, under the caveat that the classification of these disorders is imperfect.

SCN is characterized by absolute neutrophil counts of < 500/μl from early infancy, often falling below 200/μl, with normal counts of other leukocytes.[312,313,314,315] Patients with SCN have normal counts of basophil and eosinophil granulocytes. Myeloid maturation in the bone marrow is arrested at the promyelocyte or myelocyte stage of development. Differential diagnoses include complex and often syndromic PIDs with neutropenia, such as warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis syndrome, cartilage hair hypoplasia, CHS, dyskeratosis congenita, Fanconi anemia, reticular dysgenesis, and

Schwachman-Diamond syndrome. Several other inborn errors of immunity do not affect the myeloid lineage directly but often result in transient or chronic neutropenia, due to infections or the use of particular drugs. Cyclic neutropenia (CN) is characterized by regular oscillations of the number of peripheral neutrophils, with a nadir at about 200/μl and a period of about 21 days. SCN is also known as Kostmann syndrome, and CN is sometimes referred to as cyclic hematopoiesis. Both disorders were long absent from PID classifications, principally because patients with diseases of myeloid cells were seen by hematologists, whereas patients with lymphoid diseases were seen by immunologists. CN is marked by fever, oral ulcers, and bacterial infections during the nadir. The prognosis on G-CSF treatment is excellent[316]; these patients do not develop myelodysplasia or acute myeloid leukemia (AML).[315] The clinical features of SCN are more severe, with mucocutaneous and deep-seated bacterial and fungal infections. The bacterial infections are caused by various Gramnegative and Gram-positive species, including staphylococci in particular. The fungi responsible for infections are equally diverse and include *Candida albicans*. Without treatment, the outcome in infancy or early childhood is poor. Before the advent of G-CSF treatment, half the patients died from sepsis in the first year of life, the other half dying during early childhood. Myelodysplastic syndromes (MDSs) and AML were rare. Antibacterial and antifungal prophylaxis cannot provide long-term protection. HSCT has been successful in some patients. Spectacular progress was achieved in 1989, with the discovery that recombinant G-CSF could restore normal counts of circulating granulocytes, in at least some patients.[87] G-CSF treatment should be tailored to the individual patient. Most SCN patients and almost all CN patients respond to G-CSF treatment, although SCN patients require higher doses. It was subsequently shown that a substantial fraction of SCN patients treated with G-CSF developed MDS or AML, these conditions now constituting a more common cause of death in these patients than sepsis.[317,318] No such effect was observed in CN patients. The cumulative incidence of MDS/AML is about 20% after 10 years on G-CSF treatment. Somatic mutations of the G-CSF receptor and monosomy 7 often precede MDS/AML. It is unclear whether G-CSF treatment itself favors these complications or whether treatment reveals natural complications of the underlying disorders. Patients who do not respond to G-CSF, or are undergoing malignant transformation, should be treated with HSCT.[319,320,321]

TABLE 48.3 Inborn Errors of Phagocytes

Disease	Affected Cells	Affected Function	Associated Features	Inheritance	Genetic Defect/Presumed Pathogenesis	OMIM Number
1. Defects of neutrophil differentiation						
a. Severe congenital neutropenia 1 (ELANE deficiency)	N	Myeloid differentiation	Subgroup with myelodysplasia	AD	*ELANE:* misfolded protein response	202700
b. SCN2* (GFI 1 deficiency)	N	Myeloid differentiation	B/T lymphopenia	AD	*GFI1*: loss of repression of ELANE	613107
c. SCN3 (Kostmann disease)	N	Myeloid differentiation	Cognitive and neurologic defects in some patients	AR	*HAX1*: control of apoptosis	610738
d. SCN4 (G6PC3 deficiency)	N + F	Myeloid differentiation, chemotaxis, O_2^- production	Structural heart defects, urogenital abnormalities, and venous angiectasias of trunks and limbs	AR	*G6PC3*: abolished enzymatic activity of glucose-6-phosphatase, aberrant glycosylation, and enhanced apoptosis of N and F	612541
e. Glycogen storage disease type	N + M	Myeloid differentiation, chemotaxis,	Fasting hypoglycemia, lactic acidosis, hyperlipidemia,	AR	*G6PT1*: Glucose-6-phosphate transporter 1	232220

1b		O_2^- production	hepatomegaly			
f. Cyclic neutropenia	N	?	Oscillations of other leukocytes and platelets	AD	*ELANE*: misfolded protein response	162800
g. X-linked neutropenia*/ myelodysplasia	N + M	Mitosis	Monocytopenia	XL	*WAS:* Regulator of actin cytoskeleton (loss of autoinhibition)	300299
h. P14 deficiency*	N + L Mel	Endosome biogenesis	Neutropenia Hypogammaglobulinemia ↓CD8 cytotoxicity Partial albinism Growth failure	AR	*ROBLD3*: Endosomal adaptor protein 14	610389
i. Barth syndrome	N	Myeloid differentiation	Cardiomyopathy, growth retardation	XL	Tafazzin (*TAZ*) gene: Abnormal lipid structure of mitochondrial membrane	302060
j. Cohen syndrome	N	Myeloid differentiation	Retinopathy, developmental delay, facial dysmorphisms	AR	*COH1* gene: Pg unknown	216550
k. Poikiloderma with neutropenia	N	Myeloid differentiation, O_2^- production	Poikiloderma, MDS	AR	*C16orf57* gene: Pg unknown	604173
2. Defects of motility						
a. LAD type 1	N + M + L + NK	Adherence, chemotaxis, endocytosis, T/NK cytotoxicity	Delayed cord separation, skin ulcers Periodontitis Leukocytosis	AR	*INTGB2*: Adhesion protein (CD18)	116920
b. LAD type 2*	N + M	Rolling, chemotaxis	Mild LAD type 1 features plus hh-blood group plus mental and growth retardation	AR	*FUCT1*: GDP-Fucose transporter	266265
c. LAD type 3	N + M + L + NK	Adherence, chemotaxis	LAD type 1 plus bleeding tendency	AR	*KINDLIN3*: Rap1-activation of β1-3 integrins	612840
d. Rac 2 deficiency*	N	Adherence, chemotaxis O_2^- production	Poor wound healing, leukocytosis	AD	*RAC2*: Regulation of actin cytoskeleton	602049
e. β-actin deficiency*	N + M	Motility	Mental retardation, short stature	AD	*ACTB*: Cytoplasmic Actin	102630
f. Localized juvenile periodontitis	N	Formylpeptide-induced chemotaxis	Periodontitis only	AR	*FPR1*: Chemokine receptor	136537
g. Papillon-Lefèvre syndrome	N + M	Chemotaxis	Periodontitis, palmoplantar hyperkeratosis in some patients	AR	*CTSC*: Cathepsin C activation of serine proteases	245000
h. Specific granule deficiency*	N	Chemotaxis	Neutrophils with bilobed nuclei	AR	*C/EBPE*: Myeloid transcription factor	245480

i. Shwachman-Diamond syndrome	N	Chemotaxis	Pancytopenia, exocrine pancreatic insufficiency, chondrodysplasia	AR	*SBDS:* Defective ribosome synthesis	260400
3. Defects of respiratory burst						
a. XL CGD	N + M	Killing (faulty O_2^- production)	McLeod phenotype in patients with deletions extending into the contiguous Kell locus	XL	*CYBB*: Electron transport protein (gp91phox)	306400
b-e. Autosomal CGDs	N + M	Killing (faulty O_2^- production)		AR	*CYBA*: Electron transport protein (p22phox) *NCF1*: Adapter protein (p47phox) *NCF2*: Activating protein (p67phox) *NCF4*: Activating protein (p40 phox)	233690 233700 233710 601488
4. MSMD						
a. IL-12 and IL-23 receptor β1 chain deficiency	L + NK	IFNγ secretion	Susceptibility to *Mycobacteria* and *Salmonella*	AR	*IL12RB1*: IL-12 and IL-23 receptor β1 chain	601604
b. IL-12p40 deficiency	M	IFNγ secretion	Susceptibility to *Mycobacteria* and *Salmonella*	AR	*IL12B:* subunit of IL-12/IL-23	161561
c. IFNγ receptor 1 deficiency	M + L	IFNγ binding and signaling	Susceptibility to *Mycobacteria* and *Salmonella*	AR, AD	*IFNGR1*: IFNγR ligand binding chain	107470
d. IFNγ receptor 2 deficiency	M + L	IFNγ signaling	Susceptibility to *Mycobacteria* and *Salmonella*	AR	*IFNGR2*: IFNγR accessory chain	147569
e. STAT1 deficiency (AD form)*	M + L	IFNγ signaling	Susceptibility to *Mycobacteria, Salmonella*	AD	*STAT1*	**600555**
f. Macrophage gp91 phox deficiency*	Mf only	Killing (faulty O_2^- production)	Isolated susceptibility to mycobacteria	XL	*CYBB:* Electron transport protein (gp 91 phox)	**306400**
g. IRF8 deficiency (AD form)*	CD1c+MDC	Differentiation of CD1c+MDC subgroup	Susceptibility to *Mycobacteria*	AD	*IRF8:* IL12 production by CD1c+MDC	**601565**
5. Other defects						
a. IRF8 deficiency (AR form)*	Monocytes peripheral DC	Cytopenias	Susceptibility to *Mycobacteria*, *Candida*, myeloproliferation	AR	*IRF8*: IL-12 production	
b. GATA2 deficiency (mono MAC syndrome)	Monocytes peripheral DC + NK + B	Multilineage cytopenias	Susceptibility to *Mycocbacteria*, papillomaviruses, histoplasmosis, alveolar proteinosis, MDS/AML/CMML	AD	*GATA-2*: loss of stem cells	137295
c. Pulmonary alveolar	Alveolar macrophages	GM-CSF signaling	Alveolar proteinosis	Biallelic mutations in	*CSF2RA*	306250

proteinosis*	pseudoautosomal gene

ACTB, Actin beta; AD, autosomal dominant inheritance; AML, acute myeloid leukemia; AR, autosomal recessive inheritance; B, B lymphocytes; CD, cluster of differentiation; CEBPE, CCAAT/Enhancer-binding protein epsilon; CGD, chronic granulomatous disease; CMML, chronic myelomonocytic leukemia; CTSC, cathepsin C; CYBA, cytochrome b alpha subunit; CYBB, cytochrome b beta subunit; DC, dendritic cell; ELANE elastase neutrophil-expressed; F, fibroblasts; FPR1, formylpeptide receptor 1; FUCT1, fucose transporter 1; GATA2, GATA binding protein 2; GFI1, growth factor independent 1; GM-CSF, granulocyte macrophage-colony stimulating factor; HAX1, HLCS1-associated protein X1; IFN, interferon; IFNGR1, interferon-gamma receptor subunit 1; IFNGR2, interferon-gamma receptor subunit2; IL-12B, interleukin-12 beta subunit; IL-12RB1, interleukin-12 receptor beta 1; IFR8, interferon regulatory factor 8; ITGB2, integrin beta-2; L, lymphocytes; LAD, leukocyte adhesion deficiency; M, monocytes-macrophages; MDC, myeloid dendritic cell; MDS, myelodysplasia; Mel, melanocytes; Mf, macrophages; MSMD, Mendelian susceptibility to mycobacterial disease; N, neutrophils; NCF1, neutrophil cytosolic factor 1; NCF2, neutrophil cytosolic factor 2; NCF4, neutrophil cytosolic factor 4; NK, natural killer; OMIM, Online Mendelian Inheritance in Man; ROBLD3: roadblock domain containing 3; SBDS, Shwachman-Bodian-Diamond syndrome; STAT, signal transducer and activator of transcription; XL, X-linked inheritance.

* Ten or fewer unrelated cases reported in the literature.

P.1247
P.1248
P.1249
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Multiple genetic etiologies of SCN have been identified. The first was responsible for autosomal deficient (AD) SCN, whereas more recent genome-wide linkage approaches have discovered causes of AR SCN. Heterozygous mutations of *ELA2* are responsible for about 60% of SCN cases and most cases of CN.[322,323,324] Penetrance appears to be complete. In most, but not all cases, the mutations cause either SCN or CN, although both phenotypes have been observed in a few rare kindreds. The mechanism of disease remained unclear for many years, as *ELA2* encodes a cytoplasmic neutrophil elastase, a serine protease synthesized at the promyelocyte stage and stored in the primary granules of neutrophils. ELA2-deficient mice are not neutropenic but they do have impaired host defenses.[325] By contrast, humans with heterozygous *ELA2* mutations display impaired neutrophil granulocyte differentiation, due to high levels of intramedullary apoptosis. These mutations were recently shown to activate unfolded protein responses (UPRs) due to the detection of misfolded ELA2 proteins in the secretory pathway, resulting in cellular apoptosis.[326,327] Thus, dominant-negative *ELA2* mutations seem to cause SCN or CN not because ELA2 is involved in granulocyte differentiation, but simply because the resulting misfolded proteins, which are selectively expressed in granulocytes, activate the apoptosis-inducing UPR pathway in a “normal” manner. The degree of UPR activation seems to depend on the *ELA2* mutation, possibly determining the type of phenotypic expression: CN (mild mutations) or SCN (more severe mutations). Rare cases of SCN are due to heterozygous dominant-negative mutations of the gene encoding GFI1,[328] which encodes a transcriptional repressor of myeloid genes. GFI1 knockout mice are severely neutropenic and display an accumlulation of immature monocytes in the blood.[329,330] A mouse model of the human mutation also displays specific neutropenia.[331] However, the molecular mechanisms by which *GFI1* mutations cause AD SCN remain unclear.[314] Interestingly, GOF mutations in the WASP gene have been shown to cause X-linked dominant SCN.[81] The underlying mechanism involves unregulated actin polymerization, leading to defects of mitosis and cytokinesis that result in the apoptosis of granulocyte precursors.[332] Some of the patients with these mutations have platelet and lymphocyte abnormalities.

The original kindred described by Kostmann displayed AR inheritance.[11,14,333] The underlying genetic defect was not identified until 2007, when causal mutations were discovered in the *HAX1* gene, which encodes a mitochondrial protein.[334] This is the most common genetic etiology of AR SCN. The mutations concerned are LOF and cause the mitochondrial membrane potential to dissipate, leading to the release of proapoptotic molecules into the cytoplasm, potentially accounting for SCN pathogenesis.[335] However, the mechanism underlying SCN in HAX1-deficient patients remains unclear.[314] Apoptosis rates are high in the neurons and lymphocytes of HAX1-deficient mice.[336] HAX1-deficient patients from the kindred originally studied by Kostmann also displayed some neurologic signs.[333] Moreover, *HAX1* mutations affecting only isoform A were found to underlie SCN, whereas mutations affecting isoforms A and B (lacking part of exon 2 and expressed in neurons) were found to cause both SCN and various forms of neurologic impairment.[337,338] Another genetic etiology of AR SCN has been identified in a few kindreds with mutations of the *G6PC3* gene, encoding a member of the endoplasmic reticulum-resident glucose-6-

phosphatase family.[339] As in patients with *ELA2* mutations, these patients display signs of endoplasmic reticulum stress and UPR pathway activation, possibly due to impairment of the glycosylation of proteins transported in the secretory pathway. The knockout mice also display neutropenia.[340] As this enzyme is ubiquitous, the other clinical features recorded in patients, including developmental defects of the heart or urogenital tract, are not particularly surprising. Overall, what have we learned from these experiments of nature? A number of genes cause CN and SCN, and there are probably multiple mechanisms of disease, converging on the apoptosis of myeloid precursors of neutrophil granulocytes. These studies have identified key players in neutrophil differentiation, in some cases even before the description of their impact in mice (HAX1), which differs for at least the two most common genetic etiologies of SCN (HAX1- and ELA2-deficient mice do not display neutropenia). These studies also identified the first gene for which LOF and GOF mutations underlie two different immunologic disorders: XR WAS and X-linked dominant SCN. These investigations are not yet complete, not only because the pathogenesis of SCN remains unclear for several genetic defects (HAX1, G6PC3), but also because no genetic etiology has yet been discovered for some patients with SCN, suggesting that new morbid genes remain to be discovered.

CGD has also played an important historical role, as it was the first functional defect of phagocytes to be identified. It is also the most common and best characterized functional defect of phagocytes. It was first described clinically in individual patients in the late 1950s,[341,342,343,344] and the first series of affected boys was studied in 1965.[344] The phagocyte

phenotype was demonstrated in 1966, in a study showing the impaired killing of staphylococci by granulocytes,[17,345] in which the terms “CGD” and “inborn error” were first used. Granulocytes from CGD patients were found to kill streptococci normally, these bacteria posing no particular threat to these patients.[346] Shortly thereafter, in 1967, a lack of respiratory burst in the granulocytes of patients was reported.[347,348] The concomitant identification of a respiratory burst phenotype in the mothers of affected boys confirmed that most known cases resulted from XR inheritance.[349] The nitroblue tetrazolium reduction assay was soon developed,[350] and an AR form of CGD was discovered.[350,351] A deficiency of phagocyte nicotinamide adenine dinucleotide phosphate activity was then demonstrated.[352] The first review on CGD, an insightful and comprehensive paper, was published by Good et al. in 1968.[353] These early studies led to the demonstration that patients lacked a particular enzyme, phagocytic nicotinamide adenine dinucleotide phosphate oxidase (abbreviated to Phox). This discovery preceded the identification of the underlying genetic lesions, as reviewed in detail elsewhere.[354,355,356,357,358,359] Phox has five components, including membrane-bound gp91 and p22, which form the flavocytochrome b558. The other three components are cytosolic, and the complex forms after phagocyte activation, as occurs during phagocytosis. This complex is more potent in granulocytes than in other phagocytes. Upon formation of the Phox complex, reactive oxygen species, including superoxide and hydrogen peroxide (the levels of which can be assessed in various assays, including the nitroblue tetrazolium reduction assay) are released into the phagosomes, where they directly and indirectly contribute to the killing of ingested microbes. The patients have an abolished (CGD) or impaired (variant CGD) granulocyte respiratory burst. Mutations in any of the five components of Phox can be found in patients with XR or AR CGD. Mutations of the X-linked *CYBB* (encoding gp91, two-thirds of patients), *CYBA* (encoding p22), *NCF1* (encoding p47, the most common AR form), and *NCF2* (encoding p67) genes were identified in the 1980s, and it was not until 2010 that a patient with p40 deficiency was discovered.[360] The *CYBB* gene, encoding one of the two membrane-bound components, was the first human morbid gene to be identified, based on its chromosomal location.[361] Also interesting from a genetic standpoint, most, if not all mutations of *NCF1* result from a rare gene conversion event between the gene and a nearby homologous pseudogene.[362]

CGD affects about 1/100,000 individuals. Typically, it is symptomatic in early childhood, whereas variant CGD may manifest later in life.[363,364,365,366] Affected children display bacterial and fungal infections, but with different frequencies of the causal microbes. Most, but not all pathogens causing infections in CGD patients produce catalase, which was long thought to be an essential virulence factor in this setting.[367] Some bacteria, such as *Staphylococcus* and *Serratia*, are major threats, whereas closely related bacteria, such as *Streptococcus* and *Klebsiella*, which pose a threat to many other PID patients, are almost completely innocuous in CGD patients. *Aspergillus* species pose a major threat to CGD patients, whereas other fungi, such as *Candida*, are rarely pathogenic in these patients. The defect in host defense is profound, as illustrated by the occasional detection of very weakly virulent microbes that appear to pose a threat only to CGD patients.[368] Patients with AR CGD have a residual respiratory burst, albeit weaker than that of variant CGD patients, and

this may account for their somewhat milder clinical phenotype and better prognosis.[369] Interestingly, an impaired respiratory burst has been demonstrated in the macrophages and B cells, but not in the monocytes and granulocytes, of two kindreds with unique missense mutations in *CYBB*.[370] These two kindreds display a pure Mendelian susceptibility to mycobacterial disease (MSMD) phenotype, consistent with the susceptibility of CGD patients to Bacillus Calmette-Guérin and *M. tuberculosis*.[371] The results obtained for these two kindreds suggest that the respiratory burst in macrophages is essential for protective immunity against tuberculous mycobacteria. They also suggest that the respiratory burst in other phagocytes contributes to other manifestations of CGD, whether infectious or otherwise. Indeed, CGD patients also have inflammatory lesions, consisting of granulomas, that can affect almost all tissues and organs. The treatment of these lesions with steroids may precipitate the development of new infections. The prevention of infection in CGD patients has gradually been improved, initially by the use of oral trimetoprim-metronidazole, then by the subcutaneous administration of IFNγ,[372] and finally by the addition of oral itraconazole to the treatment regimen.[373] Most patients now survive until adulthood, provided that inflammatory and infectious complications are treated aggressively. The course of the disease is heterogeneous in CGD patients. Some patients do very well and survive without infection throughout adulthood, thanks to antibacterial and antifungal prophylaxis. Others experience invasive fungal disease and chronic inflammation, which may require transfusions of leukocytes and steroids, respectively, or, in some cases, HSCT. The success of HSCT has raised questions as to whether this procedure should be proposed to a larger number of patients early in life.[359] Gene therapy trials were initiated but were stopped early due to insertional mutagenesis precipitating myeloid transformation in some cases.[366,374] In any case, the investigation of CGD as an experiment of nature has resulted in the dissection of a fundamental mechanism by which phagocytes control microbes. The respiratory burst has been dissected in human phagocytes, largely through studies of patients with CGD. Mice lacking one or other of the components of Phox were engineered in the 1990s and their biochemical and infectious phenotypes matched those of human patients. There is little doubt that other functional defects of phagocytes will be discovered in the near future. Indeed, CGD patients are normally resistant to a large number of microbes, implying that other microbicidal pathways also operate in phagocytes.

SYNDROMIC IMMUNODEFICIENCIES

There are several PIDs in which physical or laboratory findings unrelated to the greater susceptibility to infection are

a prominent part of the clinical picture (Table 48.4).Some of these disorders are caused by mutations in genes that are broadly expressed and others are caused by defects affecting hematopoietic cells alone but involving processes not specific to immune defense. Interestingly, defects in the same signal transduction pathway or biologic process may cause different clinical manifestations.

Wiskott-Aldrich Syndrome and Defects in Cytoskeletal Organization

The first cases of WAS were reported in 1937 by Wiskott, a German physician who described three brothers with bloody diarrhea, draining otitis, and severe eczema.[21] In 1954, Aldrich et al. described a family in which 17 affected males had symptoms similar to those of the patients reported by Wiskott, with an XR pattern of inheritance.[20] All the patients reported by both Wiskott and Aldrich died before the age of 3 years. Over time, it became clear that a low platelet number (thrombocytopenia) and unusually small platelets were the most frequent findings in WAS.[375,376] The immune deficiency consists of recurrent infections, low serum IgM and high serum IgA and IgE concentrations, and impaired antibody production in response to carbohydrate antigens, particularly pneumococcal polysaccharides. Eczema is the least consistent component of the disease. Autoimmune disease and malignancies, such as EBV-related B-cell lymphoma in particular, are also more common in patients with WAS.[377,378]

In 1994, the gene responsible for WAS, a previously unknown gene, was identified by positional cloning. This gene encodes a hematopoietic cell-specific cytoplasmic protein, WASP, that acts as a scaffold, promoting rearrangement of the actin cytoskeleton in response to cell activation and assembly of the proteins involved in signal transduction.[375,379,380] The carboxy-terminal VCA domain (verprolin homology, cofilin homology, acidic region) is the most strongly conserved domain in protein family members. Other recognized domains of WASP include the amino-terminal WH1 domain, which is followed by the GTPase-binding domain (GBD) and a proline-rich region proximal to the VCA domain. In resting cells, WASP is in an autoinhibited conformation in which the VCA domain binds to a hydrophobic pocket within the GBD.[381,382] When a GTPase, generally CDC42, binds to the GDB domain, the VCA domain is released and WASP forms a dimer in which a pair of VCA domains bind the

actin-related proteins 2 and 3 complex.[380] The actin-related proteins 2/3 complex recruits monomeric actin and stimulates the assembly of branched actin filaments.[383]

WASP forms part of the immune synapse in activated T cells, B cells, and dendritic cells.[384,385,386,387,388] It participates in the recruitment of downstream signaling molecules and cell

motility. In the absence of WASP, T-cell proliferation, differentiation, and survival are impaired, and the number of T cells tends to decrease with age.[389] WASP is also important for maintenance of the function and normal numbers of circulating invariant NKT cells and regulatory T cells.[390,391,392,393]

TABLE 48.4 Syndromic Immunodeficiencies

Disease	Gene Defect	B Cells	T Cells	Serum Igs	Inheritance	Pathogenesis	Associated Features or Comments
Wiskott-Aldrich syndrome	*WASP*	Progressive ↓	N	↓ IgM, ↑ IgA and IgE, IgG variable	XL	Defective cytoskeletal response to activation	Thrombocytopenia with small platelets; eczema; autoimmunity; lymphoma
AT	*ATM*	May be ↓	May be ↓	↓ IgA and IgE; IgG and IgM variable	AR	Defective phosphorylation of targets required for DNA repair	Ataxia; telangiectasia; malignancy; progeria
AT-like disease	*MRE11*	May be ↓	May be ↓	Variable	AR	Defective repair of DNA double-strand breaks	
Nijmegen breakage syndrome	*NBS1*	May be ↓	May be ↓	Variable	AR	Defective repair of DNA double-strand breaks	Microcephaly; bird-like facies; growth failure
Nijmegen breakage-like disease	*RAD50*	↓↓↓↓	N	All isotypes decreased	AR	Defective repair of DNA double-strand breaks	Microcephaly; bird-like facies; growth failure
Hyper-IgE syndrome	*STAT3*	N	N	Elevated IgE	AD	Impaired response to cytokine and activation signals	Eczema; staphylococcal abscesses; delayed shedding of primary teeth

AD, autosomal dominant; AR, autosomal recessive; AT, ataxia telangiectasia; DNA, deoxyribonuclec aicd; Ig, immunoglobulin; N, normal.

Lymphocytes with normal WASP expression have a strong selective advantage in proliferation and survival over WASP-negative cells. This has been demonstrated in heterozygous carriers of a WASP mutation (the mothers of affected boys),[394,395] in chimeric mice treated with a mixture of wildtype and WASP-deficient bone marrow,[396] and in patients with WAS with a second mutation of the WASP gene.[397,398,399,400] In as many as 11% of patients with WAS, there is evidence of either a reversion of the primary mutation to the wild-type sequence, or of a second mutation compensating for the disease-causing mutation in at least some cells.[400,401] Gene reversion is seen most frequently in T cells, but it has been detected in NK cells and B cells. The first example of somatic gene reversion improving the clinical course of an inherited disorder was documented in 1994 for another PID, ADA deficiency.[402,403] Such reversion now appears to be less rare than initially thought, at least in WAS patients.

A wide diversity of *WASP* mutations has been observed in affected patients, with some

genotype/phenotype correlation.[404] However, this correlation is not strong enough to predict clinical outcome. Amino acid substitutions and some splice variants are more likely to be associated with mild disease, particularly if some WASP protein is detectable on western blots. A subset of patients with mutations in WASP have thrombocytopenia or even intermittent thrombocytopenia as the only manifestation of their disease.[405,406] These patients usually display alterations to the WHI domain. The WHI domain binds a protein called the WASP-interacting protein (WIP), which stabilizes WASP and contributes to actin polymerization.[407,408] A nonsense mutation in *WIP*, introducing a premature stop codon, has been identified in a single patient with no WASP protein in monocytes and a clinical phenotype very similar to that of patients with WAS.[409]

Activating mutations in WASP have been reported in a small number of patients with severe congenital neutropenia but not thrombocytopenia.[81,410,411] Additional clinical manifestations vary in the 17 reported patients: some displayed low levels of proliferation in response to CD3 cross-linking, some had small numbers of monocytes, and one patient had large platelets. Several had low CD4+ T-cell counts. The activating mutations result in amino acid substitutions in the GBD of WASP, preventing the autoinhibitory binding of the VCA domain to the GBD and resulting in the disorganization of actin polymerization.[332] These mutations led to the first description of two unrelated phenotypes caused by GOF and LOF mutations in a gene responsible for immunodeficiency.

Another two immunodeficiencies resulting in abnormal regulation of the actin cytoskeleton have recently been described. DOCK8 is a GTP exchange factor that binds to CDC42. Homozygous or compound heterozygous mutations in the gene encoding this protein result in a syndrome with some similarities to both hyper-IgE syndrome (HIES) and WAS.[412,413] Patients with DOCK8 deficiency have low serum IgM and markedly high IgE concentrations, like patients with WAS. However, one of the most striking characteristics of their immunodeficiency is severe viral infections of the skin caused by herpes simplex virus (HSV), varicella virus, molluscum contagiosum virus, and papillomaviruses. The incidence of cancer is also higher than normal in these patients. Thrombocytopenia is not reported in this syndrome.

Heterozygous dominant-negative mutations of *Rac2*, which encodes a GTPase similar to CDC42, have been reported in three patients with an early onset of severe bacterial infections, high neutrophil counts, impaired respiratory burst, and poor chemotaxis.[414,415,416] One of these patients was identified during newborn screening for SCID based on low levels of T cell.[416]

Ataxia Telangiectasia and Defects in the Deoxyribonucleic Double-Strand Break Response

An unusual combination of ocular telangiectasia and cerebellar ataxia was first described in French medical journals in 1926 by Syllaba and Henner[19] and then in 1941 by Louis-Bar.[18] In the mid-1950s, Broder and Sedgwick named this disorder ataxia telangiectasia (AT) and noted the high incidence of pulmonary infections in affected patients.[24] AT is a complex, highly variable, progressive disorder characterized by the onset of an unsteady gait after the first year of life, telangiectasia first noted when the child is 2 to 8 years old, unusual eye movements, drooling, recurrent respiratory infections, premature aging, infertility, an affable personality, and a high risk of cancer.[25] Patients are usually wheelchair-bound by adolescence and often die from cancer or pulmonary disease before the age of 20 years.[417] Lymphocytes from patients with AT are particularly susceptible to radiation damage and display frequent translocations involving chromosomes 7 and 14, involving the sites encoding the antigen receptor genes.[418,419,420]

AT is caused by homozygous or compound heterozygous mutations of *ATM* (ataxia telangiectasia mutated),[421,422] a large gene consisting of 66 exons. Most of the mutations in patients with classical AT are premature stop codons or frameshift mutations resulting in a null phenotype.[423] The protein encoded by *ATM* consists of 3056 amino acids, the 350 most carboxy-terminal residues displaying sequence similarities to the catalytic domain of phosphatidylinositol 3-kinases. However, ATM functions as a serine/threonine kinase. The amino-terminal end of ATM binds to several substrates, including BRACA1 and p53.[424] ATM is predominantly a nuclear protein that responds rapidly to doublestrand DNA breaks and oxidative stress.[425,426]

In response to double-strand DNA breaks, a complex consisting of Mre11, Rad50, and Nbs1 (MRN complex) binds to DNA ends and recruits ATM.[427] ATM is then able to phosphorylate Rad50, which acts as a scaffold for downstream targets. ATM also phosphorylates a wide array of target proteins involved in DNA double-strand break repair, cell cycle control, and stress responses.[425] ATM is also activated, in a pathway independent of the MRN complex,

by high levels of reactive oxygen species production.[426]

Mutations of the genes encoding each of the components of the MRN complex are associated with clinical disorders with some, but not all, of the features of AT. Homozygous mutations in *Nbs1* (also called nibrin) cause Nijmegen breakage syndrome,[427,428,429] a disorder associated with profound microcephaly, chromosomal breakage, and a high risk of cancer, but not ataxia or telangiectasia.[430] A syndrome that has been referred to as AT-like disorder is caused by hypomorphic mutations of the Mre11 gene.[431,432,433] Affected patients have ataxia and some have microcephaly, but no telangiectasia is observed. A single patient has been reported with microcephaly and hypomorphic mutations of the Rad50 gene.[434] All three disorders are associated with abnormally high sensitivity to ionizing radiation.

Hyperimmunoglobulin E Syndrome

Autosomal dominant HIES, also called Job syndrome, is a multisystem disorder characterized by eczema-like skin rashes, recurrent skin and pulmonary abscesses, pneumonia, hyperextensible joints, a high incidence of bone fractures and scoliosis, late shedding of primary teeth, and markedly high serum concentrations of IgE with eosinophilia.[435,436,437,438] Heterozygous dominant-negative mutations of the gene encoding the (signal transducer and activator of transcription) STAT3 are responsible for this disorder.[439,440]

Like other members of the STAT family, STAT3 is activated by various cytokines and growth factors using the JAK-STAT pathway. The receptors containing the widely expressed ligand binding chain gp130, including receptors for IL-6, IL-11, IL-27, OSM, CNTF, and cardiotrophin-1 all activate STAT3. These receptors activate STAT3, which forms dimers that enter the nucleus and enhance the transcription of genes encoding mediators of inflammation and immunity.[441] Paradoxically, STAT3 may have both pro- and anti-inflammatory effects, depending on the cell type and activating signal.[442] In mice, homozygous null mutations of *STAT3* are lethal early in embryogenesis.[443] In patients with autosomal dominant HIES, the mutations of *STAT3* cluster in the regions of the gene encoding the SH2 and DNA-binding domains.[444]

Homozygous mutations of the gene encoding the JAK Tyk2 may also result in an immunodeficiency characterized by rashes, unusual susceptibility to viral and fungal infections, and high serum IgE concentration.[445] However, the phenotype of patients with *Tyk2* mutations is variable, and one patient was found to be particularly susceptible to mycobacteria and viruses but to have none of the cardinal features of HIES: high serum IgE concentration, rashes, and staphylococcal disease.[446] As noted previously, mutations in *DOCK8* also result in high serum IgE concentration and susceptibility to infections.

INBORN ERRORS OF IMMUNITY TO A NARROW RANGE OF INFECTIONS

PIDs were originally defined by the identification of immunologic phenotypes. As the first immunologic abnormalities detected were profound, such as neutropenia and agammaglobulinemia, PIDs were, unsurprisingly, initially associated with multiple, recurrent, and often opportunistic infections. The first exception to this rule was provided by the description of XR lymphoproliferative disease (XLP), which manifests as various EBV-driven clinical phenotypes in previously healthy individuals (Table 48.5). The clinical features of this disease, including hemophagocytosis, lymphoma, and agammaglobulinemia, were described between 1974 and 1976, and all were attributed to an XR inheritance of susceptibility to EBV in a large kindred.[447,448,449,450] The variety of phenotypes triggered by EBV was confirmed by studies of additional kindreds.[451] The morbid gene, *SAP*, was identified in 1998 and shown to be specifically expressed in T cells.[452,453] A related XR disorder associated principally with hemophagocytosis was found to be due to mutations in *XIAP*.[72,454,455] The pathogenesis of XLP was fully deciphered only recently, with the discovery that SAP-deficient cytotoxic T cells from healthy female carriers could not respond to EBV-infected B cells.[456] Moreover, somatic reversion of SAP mutations in cytotoxic CD8+ T cells was documented in XLP patients with the mildest forms of disease.[456a] Other well-known examples of narrow vulnerability to infection are provided by AR deficiencies of any component of the complement membrane attack complex (C5 to C9), and XR deficiencies of properdin and factor H. Patients with these conditions display selective susceptibility to recurrent, invasive *Neisseria* infections. The first AR deficiencies of C6, C7, and C8 were reported in 1974 to 1976,[457,458,459,460,461] whereas the first XR properdin deficiency was not reported until 1982,[462] and AR deficiencies of factors I[463] and D[464] were reported even later. Properdin and factors I and D act by stabilizing the alternative pathway. Remarkably, alternative and terminal complement defects are almost exclusively associated with

gonorrheal and meningococcal diseases.[71,465,466] A third example, although it should really be considered the first in terms of chronology, is provided by epidermodysplasia verruciformis (EV), a rare, lifelong disorder characterized by an abnormal and selective susceptibility to disseminated and persistent warts following the infection of keratinocytes with weakly virulent skin-tropic human β-papillomaviruses.[73] EV is also associated with an increase in the risk of nonmelanoma skin carcinomas, with no other clinical signs in most patients.[467] The viral etiology of EV was demonstrated between 1946 and 1959, and specific human papillomavirus genotypes were implicated in this disease in 1978.[23,468] EV-causing human papillomaviruses are now known to cause asymptomatic infections in the general population. Cockayne first suggested in 1933 that EV could be inherited as an AR condition.[22] EV may therefore be seen as one of the first PIDs described, although the lack of a detectable immunologic phenotype long prevented this disease from being considered as such. The first genetic etiologies of EV were identified in 2002, with the identification of mutations in *EVER1* (*TMC6*) and *EVER2* (*TMC8*).[28] Both *EVER1* and *EVER2* are strongly expressed in circulating lymphocytes, including CD4+ and CD8+ T cells, B cells, and NK cells. These two genes are also expressed in keratinocytes, in which the EVER proteins form a complex with the zinc transporter ZnT1. Inactivating

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P.1256

mutations in *EVER1* or *EVER2* affect intracellular zinc distribution and the activity of transcription factors stimulated by zinc.[469] The remaining 25% or so of patients with EV and no demonstrated mutations in *EVER1* or *EVER2* include patients from families displaying AR,[470] XR,[471] and AD inheritance.[472] A third genetic etiology, with mutations in *RHOH* underlying a specific T-cell deficiency, was recently discovered, suggesting that the pathogenesis of EV in EVER-deficient patients might involve T cells.[472a] These pioneering studies together set the stage for the discovery of new inborn errors of immunity underlying life-threatening infections in previously and otherwise healthy children.

TABLE 48.5 Inborn Errors of Immunity Against a Single Infection

Disease	Affected Cell	Functional Defect	Associated Features	Inheritance	Genetic Defect/Presumed Pathogenesis	OMIM Number
1. Anhidrotic EDA-ID						
a. EDA-ID, XL (NEMO deficiency)	Lymphocytes + monocytes	NF-κB signaling pathway	Anhidrotic ectodermal dysplasia + specific antibody deficiency (lack of Ab response to polysaccharides) + various infections (mycobacteria and pyogens)	XL	Mutations of NEMO (*IKBKG*), a modulator of NF-κB activation	300291, 300584, 300301
b. EDA-ID, autosomal-dominant*	Lymphocytes + monocytes	NF-κB signaling pathway	Anhidrotic ectodermal dysplasia + T-cell defect + various infections	AD	Gain-of-function mutation of *IKBA*, resulting in impaired activation of NF-κB	612132
2. IRAK4 deficiency	Lymphocytes + monocytes	TIR-IRAK signaling pathway	Bacterial infections (pyogens)	AR	Mutation of *IRAK4*, a component of TLR- and IL-1R-signaling pathway	607676
3. MyD88 deficiency	Lymphocytes + monocytes	TIR-MyD88 signaling pathway	Bacterial infections (pyogens)	AR	Mutation of *MYD88*, a component of the TLR and IL-1R signaling pathway	612260
4. Warts, hypogammaglobulinemia, infections, myelokathexis syndrome	Granulocytes + lymphocytes	Increased response of the CXCR4 chemokine	Hypogammaglobulinemia, reduced B-cell numbers, severe reduction of neutrophil count,	AD	GOF mutations of *CXCR4*, the receptor for CXCL12	193670

		receptor to its ligand CXCL12 (SDF-1)	warts/HPV infection			
5. Epidermodysplasia verruciformis	Keratinocytes + leukocytes		HPV (group B1) infections and cancer of the skin	AR	Mutations of *EVER1*, *EVER2*	226400
6. HSE*						
a. TLR3 deficiency*	CNS resident cells and fibroblasts	TLR3-dependent IFNα, -β, and -l induction UNC-93B-dependent	HSE	AD	Mutations of *TLR3*	613002
b. UNC93B1 deficiency	CNS resident cells and fibroblasts		HSE	AR	Mutations of *UNC93B1*	610551
c. TRAF3 deficiency	CNS resident cells and fibroblasts	IFNα, -β, and -l induction TRAF3-dependent	HSE	AD	Mutation of *TRAF3*	
7. Predisposition to fungal diseases*	Mononuclear phagocytes	IFNα, -β, and -l induction CARD9 signaling pathway	Invasive candidiasis and peripheral dermatophytosis	AR	Mutations of *CARD9*	212050
8. CMC						
a. IL-17RA deficiency*	Epithelial cells, fibroblasts, mononuclear phagocytes	IL-17RA signaling pathway	CMC	AR	Mutation in *IL17RA*	605461
b. IL-17F deficiency*	T cells	IL-17F-containing dimers	CMC	AD	Mutation in *IL17F*	606496
c. STAT1 GOF	T cells	GOF STAT1 mutations that impair the development of IL-17-producing T cells	CMC	AD	Mutations in *STAT1*	Not in OMIM yet
9. Trypanosomiasis*		APOL-I	Trypanosomiasis	AD	Mutation in *APOL-I*	603743

AD, autosomal dominant inheritance; AR, autosomal recessive inheritance; CMC, chronic mucocutaneous candidiasis; EDA-ID, anhidrotic ectodermal dysplasia with immunodeficiency; GOF, gain-of-function; HPV, human papilloma virus; HSE, HSV-1 encephalitis; IFN, interferon; IL, interleukin; OMIM, Online Mendelian Inheritance in Man; STAT, signal transducer and activator of transcription; TIR, toll and interleukin-1 receptor; TLR, Toll-like receptor; XL, X-linked inheritance.

* Ten or fewer unrelated cases reported in the literature.

The idea that PIDs may underlie infectious diseases in patients normally resistant to most other microbes gained ground from the mid-1990s onwards, with the identification of AR IFN-γR1 deficiency as the first genetic basis of MSMD (Fig. 48.2).[473,474] Mutations in two X-linked (*NEMO*, *CYBB*) and six autosomal (*IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *STAT1*, *IRF8*) genes have since been discovered.[74,310,370,475] MSMD was first described in the 1950s, as unexplained infections caused by the Bacillus Calmette-Guérin vaccine.[476,477,478] The high levels of locus and allelic heterogeneity have resulted in the definition of 15 different disorders, accounting for only about half the known cases. These defects are physiologically related, as they all result in an impairment of IFNγ immunity. Mutations affecting *IFNGR1*, *IFNGR2*, and *STAT1* impair cellular responses to IFNγ. Mutations affecting *IL12B* and *IL12RB1* impair the IL-12-dependent induction of IFNγ, accounting for MSMD, and the IL-23-dependent induction of IL-17, accounting for the mild CMC documented in some of these patients.[479,480] MSMD-causing mutations in *NEMO* impair the T cell- and CD40L-dependent induction of IL-12 by dendritic cells.[481] MSMD-causing mutations in *CYBB* impair the respiratory burst in macrophages.[370] In both these cases, subtle mutations in genes for which null alleles are known to cause more complex PIDs—anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) and CGD, respectively—have highlighted the effects of specific alleles on a specific signaling pathway (*NEMO*) or a specific cell (*CYBB*).[482] Heterozygous mutations in the IRF8 gene prevent the development of IL-12-producing CD1c+ CD11c+ dendritic cells.[310] Patients with MSMD display clinical disease caused by weakly virulent mycobacteria, such as Bacillus Calmette-Guérin vaccines and environmental mycobacteria. They also often suffer from nontyphoidal, extraintestinal salmonellosis. They are highly prone to tuberculosis upon exposure to *M. tuberculosis*. AR IL-12Rβ1

deficiency was the first genetic etiology of the severe forms of pediatric tuberculosis to be identified.[483,484,485,486] Such identification of the defects underlying MSMD has been very beneficial to patients. Poor producers of IFNγ, due to IL-12 deficiency, for example, benefit from preventive or curative treatment with recombinant IFNγ. Conversely, patients whose cells do not respond to IFNγ, such as those with complete IFNγR1 deficiency, should undergo HSCT. These studies have also had important immunologic implications. These experiments of nature have shown that IFNγ is essential for immunity against mycobacteria and a few other intracellular bacteria, fungi, and parasites that infect macrophages. Surprisingly, however, these patients are not prone to infections with other intracellular agents, including most viruses, in particular. The rare viral illnesses occurring in these patients may have been favored by mycobacterially induced immunosuppression. IFNγ plays a more important role as a macrophage-activating factor than as an antiviral molecule.[487] The "Th1" arm of immunity, if such a lineage exists in humans, is not as broadly potent in host defense as reported for the mouse model. The dissection of MSMD has, therefore, revealed the narrow role of the "Th1" branch of T helper cell immunity. These studies have also suggested that mycobacterial diseases in other medical settings may result from the impairment of IFNγ immunity. Finally, these studies lend weight to the idea that otherwise healthy children with other infectious diseases may suffer from single-gene inborn errors of immunity.[70]

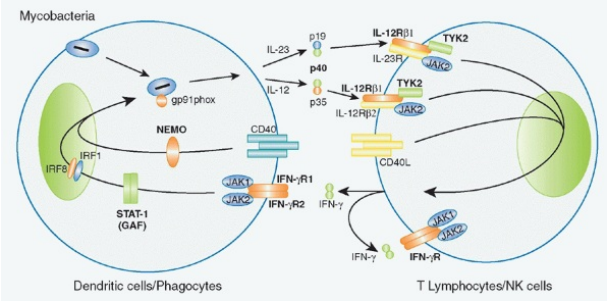

FIG 48.2. Inborn Errors in the Interleukin (IL)-12/23-Interferon (IFN)γ Pathway Underlie Mendelian Susceptibility to Mycobacterial Diseases (MSMD). Schematic diagram of cytokine production and cooperation between phagocytes/dendritic myeloid cells and natural killer/T lymphocytes. The IL-12/IFNγ circuit, the CD40/CD40L pathway, and the oxidative burst (mediated in part by CYBB-encoded gp91, a component of the nicotinamide adenine dinucleotide phosphate phagocyte oxydase) are crucial for protective immunity against mycobacterial infection in humans. Mutations in IFNGR1 or IFNGR2, encoding the ligand-binding and associated chains of the IFNγR, impair cellular responses to IFNγ. Likewise, heterozygous dominant-negative mutations in STAT1 impair IFNγ but not IFNα/β responses. Mutations in IL-12p40 or IL-12Rb1 impair IL-12-dependent induction of IFNγ. Mutations in CYBB that selectively impair the respiratory

burst in monocyte-derived macrophages are associated with MSMD. Heterozygous dominant-negative mutations in IRF8 impair the development of IL-12-producing CD1cCD11c dendritic cells. Proteins for which mutations in the corresponding genes have been identified and associated with MSMD are shown in *red*. The allelic heterogeneity is described in Table 48.1.

No germline mutation affecting the "Th2" arm of immunity has yet been identified, but mutations impairing the recently described "Th17" arm of immunity have been identified during investigations of CMC disease (CMCD), which was clinically described in the late 1960s and shown to display AR or AD inheritance in the early 1970s.[488,489,490] CMC is characterized by persistent or recurrent infections of the nails, skin, and oral and genital mucosae with the fungus *C. albicans*. It is common in patients with various inherited or acquired T-cell deficits that are also associated with other infections.[491] In patients with AD HIES caused by dominant-negative *STAT3* mutations, the two principal infectious threats are CMC and staphylococcal diseases.[65,492] These patients have a small proportion of IL-17-producing T cells.[479,493,494] However, this does not formally demonstrate a role for impaired IL-17 immunity in the CMC observed in these patients, as this immunologic phenotype may be silent or even be responsible for other phenotypes, infectious or otherwise. Interestingly, however, patients with autoimmune polyendocrinopathy type I suffer from a single infectious disease, CMC, which is accompanied by numerous autoimmune signs.[495] They have high titers of neutralizing auto-Abs against IL-17 cytokines.[496,497] Again, this observation alone does not prove that impaired IL-17 immunity underlies CMC in such patients, as the auto-Abs may be clinically silent or may attenuate autoimmune signs. However, these clinical observations, together with the results of studies in mice, suggest that impaired IL-17 immunity may generally underlie CMC.[491] These studies paved the way for the discovery of patients with isolated CMC (CMCD) due to inborn errors of IL-17F or IL-17RA immunity.[76] These patients were otherwise healthy and normally resistant to other infections, with the exception of a few cutaneous staphylococcal infections. One patient displayed AR complete IL-17RA deficiency, whereas patients from another kindred displayed AD partial IL-17F deficiency. These findings provided the first genetic etiologies of CMCD and suggested that IL-17A and IL-17F were essential for mucocutaneous immunity against *C. albicans*, but otherwise largely redundant in host defense, at odds with findings for the mouse model. Moreover, the use of a genome-wide approach led to the discovery of GOF mutations of *STAT1* in other patients with CMCD, some of whom also displayed features of autoimmunity.[77,498] Previously described *STAT1* mutations were LOF (null or hypomorphic) and associated with AD or AR predisposition to viral and/or mycobacterial diseases.[499,500,501] By contrast, the CMCD-causing *STAT1* mutations are GOF. They prevent the nuclear dephosphorylation of activated STAT1-containing complexes, thereby increasing transcriptional activity in response to IFNγ, IFNα/β, IFNλ, and IL-27, the biologic functions of which are highly dependent on STAT1. Cytokines that activate STAT3 predominantly and STAT1 to a lesser extent, including IL-6, IL-21, and IL-23, also trigger enhanced STAT1-dependent responses in these patients. Patients with GOF mutations in *STAT1* have small proportions of IL-17 T cells, because IFNγ, IFNα/β, IFNλ, and IL-27 are inhibitors of IL-17 T-cell differentiation via STAT1, or because IL-6, IL-21, and IL-23 are inducers of IL-17 T cells via STAT3 but not via STAT1, or both.[77] Impaired IL-17 immunity therefore underlies CMCD in these patients. Surprisingly, some CMC patients with *STAT1* mutations were recently reported to suffer from reactivations of viral disease.[501a] This situation is reminiscent of the observation that patients with HIES that are heterozygous for *STAT3* develop shingles due to impaired T-cell memory.[502] Overall, *STAT1* LOF alleles underlie viral diseases that occur during primary infection due to the impairment of antiviral IFN activity. By contrast, GOF *STAT1* mutations may confer a predisposition to the reactivation viral diseases (including some caused by the same herpes viruses) due to insufficient T-cell memory. In any case, studies of CMCD revealed that the "Th17" arm of immunity is apparently redundant for host defense against most microbes in humans, at odds with findings for the mouse model. Studies of MSMD and CMC have indicated that helper T-cell immunity is unlikely to be restricted to the "Th1" and "Th2" arms, even with the addition of the third partner "Th17." Indeed, mutations of IFNγ and IL-17 immunity underlie predispositions to only a few of the millions of microbes in the environment, including hundreds of known pathogens.

Another step forward came with the investigation of children with invasive pneumococcal disease (IPD). Patients with inborn errors of IL-17 or IFNγ immunity typically display recurrent or persistent infectious diseases, consistent with the production of these cytokines principally by T cells. Only patients with IL-12 and IL-12R deficiencies

seem to display a single episode of mycobacterial disease, implying that these cytokines are necessary for immunity against primary infection with mycobacteria but not for immunity to

latent or secondary infection.[78] IPD was long known to be favored by inborn errors of pneumococcal opsonization, such as defects of the classical complement pathway or of antibody responses to capsular glycans, and asplenia.[503] Patients with any of these PIDs are actually susceptible to a wide range of encapsulated bacteria. Interest in the investigation of IPD was stimulated in 2001 by the genetic dissection of a rare PID, known as EDA-ID.[504,505] IPD is, by far, the most common infection in these patients, but susceptibility to other pyogenic bacteria, mycobacteria, and some fungi and viruses has been recorded. Inflammation is weak or delayed in the course of infection. The only consistent immunologic abnormality is a lack of antibodies directed against glycans. This finding is consistent with the observation that IPD is often recurrent in these patients, with recurrences in some cases even being caused by the same serotype. Inborn errors of NF-κB were identified as responsible for the disease: patients with XR-EDA-ID carry hypomorphic mutations in *NEMO*,[506] whereas patients with AD-EDA-ID carry hypermorphic mutations in *IKBA*.[507] The key role of NF-κB in developmental processes accounts for the developmental features of the disease in affected children. Impaired immunity has a broad basis, consistent with the broad role of the multiple TNF, IL-1, and TLR receptors downstream from NF-κB. The *NEMO* mutations underlying EDA-ID are hypomorphic; null alleles cause the death in utero of male embryos and incontinentia pigmenti in women.[508] These hypomorphic mutations are associated with immunologic and infectious clinical phenotypes, some underlying IPD[509] and others underlying mycobacterial diseases[481] in otherwise healthy patients. *NEMO* mutations probably define the broadest phenotypic diversity in the field of PIDs, ranging from death in utero or lifethreatening neonatal disease to a mild and transient B-cell deficiency in adults. The mechanisms underlying IPD and other infections in patients with *NEMO* or *IKBA* mutations are progressively being deciphered through the identification of patients with germline mutations in genes encoding products acting upstream or downstream from NEMO. Children with isolated IPD provide the best example, as some have been shown to suffer from IRAK-4 or MyD88 deficiency.[510,511] These children have no developmental phenotype and are normally resistant to viruses, fungi, and mycobacteria. They suffer from IPD and, more rarely, from invasive staphylococcal diseases. Gram-negative infections are rare, with the exception of those caused by *Pseudomonas* and, more rarely, *Shigella*.[512,513,514] The patients display weak, delayed biologic and clinical signs of inflammation during infection. The patients' cells do not respond to agonists of most TLRs (other than TLR3 agonists) and IL-1Rs (including IL-1, IL-18, and IL-33). Surprisingly, these patients are susceptible to a few pyogenic bacteria, but normally resistant to other bacteria and parasites, fungi, and viruses. The clinical status of patients improves spontaneously from adolescence onwards, with no deaths or invasive infections observed in the absence of prophylaxis. This implies that TLRs and IL-1Rs are not required for protective T- and B-cell immunity to these infectious agents. This experiment of nature has not only confirmed that IPD may have a genetic basis, as previously shown in patients with inborn errors of opsonization, but has also revealed that TLR and IL-1R immunity is largely redundant in host defense. This is at odds with theoretical models attributing a broad role in host defense to TLRs, as microbial sensors or pathogenassociated molecular pattern receptors, and with experiments conducted in MyD88-deficient mice, which were found to be susceptible to more than 35 of the pathogens tested.[75] However, this finding is entirely consistent with evolutionary genetic studies, which have shown surfaceexpressed human TLRs to be subject to weaker selection pressure than intracellular TLRs.[515] Impaired TLR immunity may even play no more than a modest role in the development of infections in MyD88- and IRAK-4-deficient patients. It is not inconceivable that impaired IL-1 immunity alone accounts for the development of the few pyogenic bacterial infections observed in these patients.

TLR3, the only TLR that does not signal via MyD88 and IRAK-4 but instead uses TRIF as its sole adaptor, was serendipitously found to be essential for protective immunity against HSV-1 in the central nervous system (CNS), in the course of primary infection, in at least some children (Fig. 48.3).[75] The other intracellular TLRs—TLR7, TLR8, and TLR9—which are stimulated by nucleic acids and generally thought to play an important role in antiviral defense, were found to be redundant in humans against most viruses.[512] However, the strong purifying selection operating on these genes suggests that past pathogens or other physiologic processes have exerted selection pressure on these four receptors.[515] The role of TLR3 in host defense was deciphered by investigations of children with HSV-1 encephalitis (HSE).[516] This disease is the most common sporadic viral encephalitis in western countries. In this terrible disease, the virus is restricted to the CNS. It is absent from the bloodstream and does not spread to other organs. HSE is neurotropic in terms of both the route it follows and its destination: it reaches the CNS via cranial nerves. Patients with the most severe myeloid and lymphoid PIDs, including children with no T cells, display no particular susceptibility to HSE. The disease is sporadic in the vast majority of cases, with only four multiplex kindreds reported in 60 years, but there is a high frequency of parental consanguinity (14% in the French survey) in these cases, suggesting that HSE may be due

to single-gene inborn errors of immunity displaying incomplete clinical penetrance.[516] The first genetic etiology of HSE was identified as AR UNC-93B deficiency, resulting in an impairment of cellular responses to the four intracellular TLRs, including TLR3.[517] Involvement of the TLR3 pathway was then suspected, because IRAK-4- and MyD88-deficient patients, whose cells do not respond to TLR7-9, are not prone to HSE. TLR3 was formally implicated in the disease when AD and AR TLR3 deficiencies were discovered in other patients with HSE.[518,519] The subsequent identification of children with AR or AD TRIF deficiency confirmed the role of TLR3-TRIF and further suggested that childhood HSE might result from a collection of highly diverse

but immunologically related single-gene lesions.[520] HSE-causing heterozygous mutations of TRAF3 further highlighted the potential role of subtle mutations in pleiotropic genes in narrow clinical phenotypes. In AD TRAF3 deficiency, the mutation is dominant-negative and impaired TLR3 responses account for HSE, whereas the other cellular phenotypes, such as impaired responses to members of the TNF receptor superfamily, are clinically silent.[521] There is a broad immunologic phenotype but a narrow infectious phenotype, because the thresholds for clinical consequences differ between cellular phenotypes. Patients with *NEMO* mutations are broadly susceptible to viral infections, including HSE, reflecting the impairment of antiviral IFN production in response to the stimulation of multiple receptors, including TLR3, in their cells.[522] The target genes involved in HSE, downstream from TLR3, were identified as antiviral IFN genes in 2003, when patients with LOF mutations of *STAT1* were found to be prone to multiple viral diseases, including HSE.[500,523,524] All these genetic etiologies display complete penetrance at the cellular level (in fibro-blasts) but incomplete clinical penetrance, accounting for the sporadic nature of HSE. Interestingly, these defects also predispose subjects to childhood HSE, in the course of primary infection, but do not seem to impair immunity against latent HSV-1 infection, in the CNS and elsewhere. The molecular dissection of HSE also led to its cellular dissection. The TLR3 pathway was recently shown to be largely redundant for poly(I:C) responses in keratinocytes and leukocytes, whereas it is essential in CNS-resident cells, including astrocytes, neurons, and oligodendrocytes.[524a] However, anti-HSV1 immunity has been shown to be critically dependent on the TLR3-dependent production of IFNα/β in neurons and oligodendrocytes only. These findings strongly suggest that HSE results from a CNS-intrinsic defect of antiviral immunity. HSE probably results from a collection of single-gene inborn errors of TLR3 intrinsic immunity operating in CNS-resident cells, including neurons and oligodendrocytes in particular. Overall, the genetic dissection of HSE has shed new light on host defenses, revealing that the TLR3 pathway is responsible for ensuring CNS-intrinsic

protective immunity against HSV-1 during primary, but not latent infection.

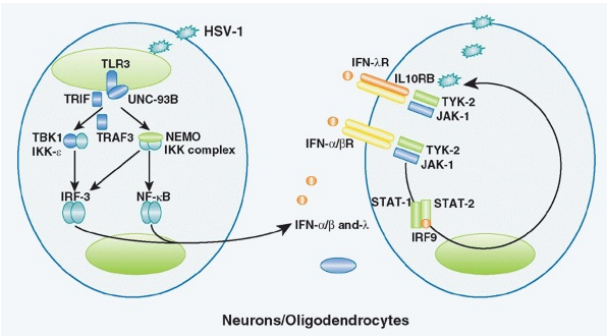

FIG 48.3. Inborn Errors of Toll-Like Receptor (TLR)3-Dependent, Interferon (IFN)α/β and -l Immunity Underlie Childhood Herpes Simplex Virus (HSV) 1 Encephalitis (HSE). Schematic representation of the production of and response to IFNα/β and IFNλ in anti-HSV-1 immunity in the central nervous system, based on the genetic dissection of children with HSE. Like most viruses, HSV-1 produces double-stranded ribonucleic acid (dsRNA) intermediates during its replication. TLR3 is an endosomal transmembrane receptor for dsRNA. The recognition of dsRNA by TLR3 induces activation of the IRF-3 and NF-kB pathways via TRIF, leading to IFNα/β and/or IFNλ production. TLR3, UNC-93B, TRIF, TRAF3, TBK1, and NEMO deficiencies are all associated with impaired IFNα/β and/or IFNλ production and predisposition to HSE in the course of primary infection by HSV-1. The binding of IFNα/β and IFNλ to their receptors induce the phosphorylation of JAK1 and TYK-2, activating the signal transduction proteins signal transducer and activator of transcription (STAT)-1, STAT-2, and IRF9. This complex is translocated as a heterotrimer to the nucleus, where it acts as a transcriptional activator, binding to specific deoxyribonucleic acid response elements in

the promoter region of IFN-inducible genes. STAT-1 and TYK2 deficiencies are associated with impaired IFNα/β responses and, for STAT1, impaired IFNλ responses and predisposition to HSE. Proteins for which genetic mutations have been identified and associated with susceptibility to isolated HSE are shown in *blue*. Proteins for which genetic mutations have been identified and associated with susceptibility to mycobacterial, bacterial, and viral diseases, including HSE, are shown in *green*. Proteins for which genetic mutations have been identified but not associated with susceptibility to infectious diseases are shown in *red*. This figure will be revised as new results are obtained with the genetic and immunologic dissection of children with HSE and other viral diseases.

INBORN ERRORS OF TOLERANCE

The second most common phenotype associated with PIDs is autoimmunity (Table 48.6). The random process of assembling V, D, and J elements of TCR and Ig genes through the process of VDJ recombination inevitably generates TCRs and BCRs recognizing self-antigens. Tonal signaling in response to self-antigens is essential to promote the progression of T- and B-cell development in the thymus and bone marrow, respectively. However, the prevention of autoimmunity requires T- and B-lymphocytes with high affinity for self-antigens to be deleted or kept in check. The mechanism underlying immune tolerance in the T-lymphocyte compartment include deletion of self-reactive clones,[525,526] conversion of autoreactive T-lymphocytes to self-antigen-specific regulatory T (T_{reg}) cells, and the apoptosis of self-reactive T cells. Autoreactive B-lymphocytes are eliminated from the bone marrow through receptor editing (whereby the reexpression of *RAG* genes promotes sequential rearrangements of the immunoglobulin genes, thus modifying antigen specificity).[527] Furthermore, B cellactivating factor levels regulate the survival of autoreactive B-lymphocytes in the periphery.[528] These processes define central and peripheral mechanisms of tolerance, depending on the developmental stage at which they occur.

As discussed previously, autoimmune manifestations have frequently been reported in patients with combined immunodeficiency and residual T- and B-cell development, but not in infants with SCID. In addition, some rare monogenic disorders associated mostly, if not exclusively, with autoimmunity highlight the critical role played by tolerogenic mechanisms in immune homeostasis and function (see Table 48.6).[92]

Autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy (*APECED*, also known as autoimmune polyglandular syndrome type 1) is an AR disorder characterized by multiple autoimmune endocrine manifestations (hypoparathyroidism, Addison disease), CMC, and nail dystrophy. The syndrome was probably first described in the 1920s to 1940s.[30] In 1981, Neufeld et al. collated information for 295 patients with autoimmune Addison disease as a component of polyglandular autoimmune syndrome, and established that APECED/autoimmune polyglandular syndrome

type 1 consisted of a combination of at least two of the following three featurs: Addison disease, hypoparathyroidism, and CMC.[529] Other autoimmune manifestations are also common (hepatitis, ovarian failure).[530]

TABLE 48.6 Inborn Errors of Tolerance

Disease	Gene Defect	Inheritance	Immunologic Phenotype	Pathogenesis	Associated Features
APECED	*AIRE*	AR	None	Defect of central tolerance	Autoimmune endocrinopathies, candidiasis
IPEX	*FOXP3*	XL	↓↓↓ FOXP3+ T_{reg} cells	Defect of T_{reg}	Autoimmune enteropathy, IDDM, skin rash
ALPS					
ALPS-FAS	*FAS (TNFRSF6)*	AD, rarely AR	↑ TCRαβ+CD4-CD8- cells (DN T)	Impaired apoptosis via FAS	Splenomegaly, adenopathies, autoimmunity, increased risk of

					lymphoma
ALPS-FASLG	*FASLG (TNFSF6)*	AD, AR	↑ TCRαβ+ CD4-CD8- cells (DN T)	Impaired apoptosis via FAS	Splenomegaly, adenopathies, autoimmunity, SLE
ALPS-CASP10	*CASP10*	AD	↑ TCRαβ+ CD4-CD8- cells (DN T)	Impaired apoptosis (intrinsic pathway)	Splenomegaly, adenopathies, autoimmunity
Caspase-8 deficiency	*CASP8*	AD	Slight ↑ of DN T cells	Impaired apoptosis (intrinsic pathway)	Recurrent infections, splenomegaly, adenopathies
N-RAS defect	*NRAS*	Sporadic	DN T cells are ↑ or N	Activating N-RAS mutations that impair apoptosis	Splenomegaly, adenopathies, lymphoma
K-RAS defect	*KRAS*	Sporadic	DN T cells are ↑ or N	Activating N-RAS mutations that impair apoptosis	Splenomegaly, adenopathies, lymphoma
FADD deficiency	*FADD*	AR	↑ DN T cells	Impaired apoptosis	Recurrent infections, liver dysfunction, encephalopathy
CD25 deficiency	*IL2RA*	AR	T cells ↓ or N	Impaired homeostasis of T cells	Recurrent infections, autoimmunity (IPEX-like)
ITCH deficiency	*ITCH*	AR	None known	Not known	Autoimmunity, developmental delay, microcephaly, lung disease

AD, autosomal deficient; ALPS, autoimmune lymphoproliferative syndrome; APECED, autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy; AR, autosomal recessive; CD, cluster of differentiation; DN, double negative; FADD, Fas-associated protein with death domain; IDDM, insulin-dependent diabetes mellitus; IPEX, immune dysregulation polyendocrinopathy enteropathy X-linked; N, normal; SLE, systemic lupus erythematosus; T_{reg}, regulatory T; XL, X-linked.

Although it was obvious that identification of the gene underlying APECED could shed light on the mechanisms governing immune tolerance, it was not until 1997 that two groups independently and simultaneously established that the disease was due to mutations of the autoimmune regulator (*AIRE*) gene.[531,532] This discovery constituted a major breakthrough in the understanding of central T-cell tolerance and led to fundamental studies in mice. AIRE is a transcription factor expressed by terminally differentiated medullary thymic epithelial cells, in which it regulates the expression of tissue-specific antigens.[533] AIRE-dependent tissue-specific antigens associated with HLA molecules are presented to developing T cells. Through this mechanism, self-reactive T-lymphocytes are deleted from the thymus.[534] Consistently, mutations of *AIRE* cause autoimmunity that is not restricted to endocrine glands and other tissues, but also includes the production of autoantibodies against various cytokines, including IL-17A, IL-17F, and IL-22, which play a key role in defense against *Candida* spp.[535,536] This accounts for the occurrence of CMC in patients with APECED. The autoimmune phenotype of autoimmune polyendocrinopathy type I patients might well be both broader and more profound in the absence of neutralization of these cytokines, which have been shown to play an important role in autoimmunity in the mouse model. Conversely, it is remarkable that the most severe autoimmune disorder known occurs despite the neutralization of these cytokines, indicating the IL-17-independent nature of at least a few

autoimmune diseases in humans.

In 1959, Russell et al. described a naturally occurring mutant mouse strain (the "scurfy" mouse) with an XR phenotype characterized by enteropathy, runting, dermatitis, and early lethality.[537] In 1982, Powell et al. described a large family in which 19 male subjects presented early-onset enteropathy, dermatitis, and endocrine abnormalities. Most of the affected male subjects died before the age of 3 years.[538] Other kindreds with a similar phenotype were subsequently described and the disease was named *immune dysregulation polyendocrinopathy enteropathy X-linked* (*IPEX*) syndrome. Linkage analysis mapped the *scurfy* locus in mice and the IPEX locus in humans to the same syntenic region of the X chromosome.[539,540] The demonstration that disruption of the *Foxp3* gene is responsible for the *scurfy* phenotype in mice[541] led to a candidate gene approach culminating in the identification of *FOXP3* gene mutations in patients with IPEX.[542] FOXP3 is a transcription factor required for the development and function of T_{reg} cells.[543,544] In patients with IPEX, activated autoreactive T-lymphocytes infiltrate target tissues and secrete cytokines, causing tissue damage. The patients lack CD4+ $CD25^{hi}$ FOXP3+ (T_{reg}) cells. T_{reg} cells may be generated in the thymus (natural T_{reg}) or in the periphery (induced T_{reg}), and these cells have suppressive activity.[545] Patients with IPEX require immune suppression, but the only definitive treatment currently available is allogeneic HSCT.[546]

Inborn Errors of Interleukin-2 Responses

T_{reg} cells express the high-affinity IL-2R. IL-2 plays an important role in both T-lymphocyte proliferation and immune homeostasis, by increasing *FOXP3* expression and enhancing the suppressive activity of T_{reg} cells.[547] Mutations of the *IL2RA (CD25)* gene, which encodes the α chain of the IL-2 receptor, are inherited as an AR trait and cause IPEX-like signs of immune dysregulation and lymphoproliferation (lymphadenopathy, hepatosplenomegaly), associated with susceptibility to severe infections.[548,549] The presence of circulating FOXP3+ T_{reg} cells in CD25-deficient patients[549] indicates that CD25 is dispensable for the generation of natural T_{reg} cells, for which a low IL-2R signaling threshold may suffice.[550] However, signaling through the high-affinity IL-2R is important for the generation of Foxp3+-induced T_{reg} lymphocytes from naive peripheral T-lymphocytes.[551] Moreover, CD25 deficiency leads to an impairment of the survival and fitness of mature natural T_{reg}s in mice.[547,552]

The binding of IL-2 to IL-2R activates the JAK-STAT signaling pathway, resulting in STAT5B phosphorylation, dimerization, and nuclear translocation, with the expression of IL-2-dependent genes, including FOXP3, Bcl-2, and CD25.[553] Furthermore, STAT5B is also activated in response to signaling through the growth hormone receptor, inducing the expression of insulin growth factor-1, a critical growth mediator.[553] In 2003, Kofoed et al. described a patient with short stature and growth hormone insensitivity, severe infections due to viruses and *P. jiroveci*, and lymphoid interstitial pneumonitis with a homozygous missense mutation of *STAT5B*.[554] Additional patients with a similar phenotype combining short stature, immunodeficiency, and autoimmunity have been reported,[555] thus substantiating the role of STAT5B-mediated signaling in immune function and homeostasis.

Inborn Errors of Apoptosis

A syndrome consisting of generalized lymphadenopathy, autoimmune cytopenia, and hypergammaglobulinemia was described in 1967 by Canale and Smith.[556] In 1992, Sneller et al. reported other patients with this constellation of symptoms and showed that these patients had larger than normal numbers of circulating T cells expressing the αβ form of TCR, but lacking the expression of CD4 and CD8 on their surface.[557] These cells have since been named DN T cells, and the disease (originally named Canale-Smith syndrome) has been renamed *autoimmune lymphoproliferative syndrome (ALPS)*. Sneller et al.[557] recognized the similarity of the ALPS phenotype to a mouse model of genetically determined lymphoproliferation (*lpr/lpr* mice) shown to result from mutations of the *Fas* gene.[558]

The apoptosis of autoreactive lymphocytes plays an important role in the preservation of immune homeostasis. Interactions between FAS ligand, produced by activated lymphocytes, and FAS (CD95) trigger an intracellular signaling pathway that ultimately results in the activation of caspases and cell death.[559] *FAS* mutations are the leading cause of ALPS in humans.[560,561,562] These patients have a higher

than normal risk of developing cancers (especially B-cell lymphomas), which are observed in 10% of patients with *FAS* mutations.[563]

ALPS is mostly inherited as an AD trait and is due to dominant-negative mutations inhibiting FAS-mediated signaling and caspase activation.[560] ALPS may more rarely be AR and due to biallelic *FAS* mutations completely abolishing the production or function of FAS. Furthermore, somatic mutations in the *FAS* gene may also cause ALPS.[564] Finally, combination of a germline *FAS* mutation affecting one allele and somatic mutations affecting the other allele have recently been reported in some patients with progressive development of the disease phenotype.[565] Other rare causes of ALPS include mutations of the gene encoding the FAS-associated death domain protein,[566] FAS ligand,[567,568] caspase-8,[569] and caspase-10.[570] The apoptosis of chronically activated T-lymphocytes can also be induced through a FAS-independent mechanism involving the release of cytochrome *c* and the activation of caspase-9.[571] This "intrinsic" (or mitochondrial) pathway of apopotosis is elicited in response to cell damage and cytokine (eg, IL-2) deprivation. GOF mutations of *N-RAS* may disrupt this pathway and cause ALPS.[572] Somatic mutations of *K-RAS* may also cause ALPS,[573,574] but this variant does not result in an increase in the number of DN T-lymphocytes.

ALPS is diagnosed on the basis of clinical features, the demonstration of a larger than normal number of DN T cells, and defective apoptosis in response to FAS stimulation or activation of the intrinsic pathway.[562,575] Treatment is based on the use of immunosuppressive drugs and surveillance for lymphoma.[561,563]

The study of patients with ALPS has provided an important confirmation that mechanisms for controlling T-cell survival are important in immune homeostasis. However, it has also raised interesting questions that remain unanswered: why do patients with ALPS suffer principally from autoimmune cytopenia, whereas AIRE and FOXP3 defects are mostly associated with organ-specific autoimmunity? Why is the phenotype of *lpr* mice (increased in the risk of kidney disease) different from that of FAS-mutated patients? Deep sequencing of the exomes and genomes of patients with unexplained autoimmunity will undoubtedly shed new light on the mechanisms governing central and peripheral tolerance in B cells and T cells.

INBORN ERRORS OF LYMPHOCYTE CYTOTOXICITY UNDERLYING HEMOPHAGOCYTOSIS

In 1952, Farquhar and Claireaux described the cases of a brother and sister who presented with high fever, progressive pancytopenia, hepatosplenomegaly, and bruising.[576] Unfortunately, a rapid progression to death was observed in both cases. Postmortem examination revealed a prominent infiltrate of lymphocytes, plasma cells, and histiocytes in the liver, spleen, and bone marrow. Other similar cases were subsequently reported. In some cases, the disease occurred in multiple family members and was thought to be intrinsic, whereas the sporadic presentation in other patients was thought to be a consequence of infections or cancers. This led to a distinction being made between primary and secondary forms of hemophagocytic lymphohistocytosis, although it is now clear that many sporadic cases are actually genetic in origin and that episodes of familial cases can be triggered by infection (Table 48.7; Fig. 48.4).

Familial hemophagocytic lymphohistiocytosis (FHL) is a genetically determined condition characterized by impaired lymphocyte-mediated cytotoxicity.[577,578,579,580,581] In patients with FHL, an inability to clear viral infections results in the persistent activation of CD8+ and NK lymphocytes, high levels of IFNγ production, macrophage activation, and the uncontrolled release of proinflammatory cytokines (TNFα, IL-6).[582] Clinical features of FHL include episodes of high fever, pancytopenia, liver and spleen enlargement, and neurologic signs. The study of patients with hemophagocytic lymphohistiocytosis has made an important contribution to definition of the role of lymphocyte cytotoxicity in immune homeostasis.

The elimination of virus-infected cells is dependent on CD8+ cytotoxic T-lymphocytes (CTLs) and NK lymphocytes, which release cytotoxic proteins (granzyme B, granulolysin) into target cells through cell membrane pores formed by perforin multimers, causing the activation of caspases and apoptosis.[583] The cytotoxic proteins are contained in endosomal lytic granules. Following the interaction of CTL or NK cells with the target cell, rearrangement of the cytoskeleton guides the transport of the lytic granules toward the immunologic synapse.[584] During this process, the small GTPase Rab27a promotes docking of the lytic granules, Munc 13-4 induces priming of the cytolytic granules, and syntaxin-11 and Munc 18-2 (also known as syntaxin-binding protein) promote the fusion of the lytic granules with the cell membrane. The lytic proteins are then released by exocytosis through pores formed by perforin (see Fig. 48.3).[583]

Mutations in the genes encoding Rab27a, Munc13-4, syntaxin-11, Munc 18-2, and perforin cause AR FHL. In addition, patients with Rab27a deficiency (also known as Griscelli syndrome type 2) show partial albinism, because the Rab27a protein is also important for melanin transport in melanocytes, as seen in patients with CHS (see following discussion).

An important step in the diagnosis of FHL is the demonstration of defective cell-mediated cytotoxicity.[585,586] NK cells present in peripheral blood mononuclear cell preparations from healthy controls kill ^{51}Cr-labeled K562 erythroleukemic cells; a profound defect in this killing is observed in FHL patients. Moreover, during cytotoxic processes, degranulation is accompanied by the surface expression of proteins (such as CD107a) that are normally sequestered in endosomal granules. FHL-associated defects in the docking, priming, and fusion of cytolytic granules impair the expression of CD107a in response to the in vitro activation of CTLs and NK lymphocytes.[587] Finally, flow cytometry can be used to detect defects of perforin expression.[585,586]

FHL is fatal in the absence of treatment, which is based on the prompt recognition and specific therapy of underlying

infections associated with chemotherapy and immunosuppressive regimens.[588] However, although such treatment may induce remission, relapses are the rule. The only curative approach to FHL is thus HSCT. Optimal results have recently been obtained with reduced-intensity conditioning regimens.[589]

TABLE 48.7 Inborn Errors of Lymphocyte Cytotoxicity

Disease	Circulating T Cells	Circulating B Cells	Serum Ig	Associated Features	Inheritance	Genetic Defect/Presumed Pathogenesis	OMIM Number
1. Immunodeficiency with hypopigmentation							
a. Chediak-Higashi syndrome	Normal	Normal	Normal	Partial albinism, recurrent infections, late-onset primary encephalopathy, increased lymphoma risk;n eutropenia, giant lysosomes, low NK and CTL activities, elevation of acute-phase markers	AR	Mutations in *LYST*, impaired lysosomal trafficking	214500
b. Griscelli syn-drome, type2	Normal	Normal	Normal	Partial albinism, elevation of acute-phase markers, encephalopathy in some patients; low NK and CTL activities	AR	Mutations in *RAB27A* encoding a GTPase that promotes docking of secretory vesicles to the cell membrane	607624
c. Hermansky-Pudlak syndrome, type 2	Normal	Normal	Normal	Partial albinism, increased bleeding; neutropenia, low NK and CTL activity	AR	Mutations in the *AP3B1* gene, encoding for the b subunit of the AP-3 complex	608233
2. FHL syndromes							
a. Perforin deficiency, FHL2	Normal	Normal	Normal	Severe inflammation, persistent fever, cytopenias, splenomegaly; hemophagocytosis, decreased to absent NK and CTL activities	AR	Mutations in *PRF1*; perforin, a major cytolytic protein	603553
b. UNC13D (Munc13-4) deficiency, FHL3	Normal	Normal	Normal	Severe inflammation, persistent fever, splenomegaly, hemophagocytosis, decreased NK and CTL	AR	Mutations in *UNC13* (as named in OMIM) required to prime vesicles for fusion; note	608898

				activities		that also in OMIM the “official” name is UNC13D deficiency with the alternative title of MUNC13D deficiency	
c. Syntaxin 11 deficiency, FHL4	Normal	Normal	Normal	Severe inflammation, persistent fever, splenomegaly; hemophagocytosis, decreased to absent NK activity	AR	Mutations in *STX11*, required for fusion of secretory vesicles with the cell membrane and release of contents	603552
d. STXBP2 (Munc 18-2) deficiency, FHL5	Normal	Normal	Normal or low	Severe inflammation, fever, splenomegaly, hemophagocytosis possible bowel disease; decreased NK and CTL activities with partial restoration after IL-2 stimulation	AR	Mutations in *STXBP2*, required for fusion of secretory vesicles with the cell membrane and release of contents	613101
3. Lymphoproliferative syndromes							
a. SH2D1A deficiency, XLP1	Normal	Normal or reduced	Normal or low	Clinical and immunologic abnormalities triggered by EBV infection, including hepatitis, hemophagocytic syndrome, aplastic anaemia, and lymphoma Dysgammaglobulinemia or hypogammaglobulinemia, low to absent NKT cells	XL	Mutations in *SH2D1A* encoding an adaptor protein regulating intracellular signals	308240
b. XIAP deficiency, XLP2	Normal	Normal or reduced	Normal or low	Clinical and immunologic abnormalities triggered by EBV infection, including splenomegaly, hepatitis, hemophagocytic syndrome, colitis	XL	Mutations in *XIAP* encoding an inhibitor of apoptosis	300635

AR, autosomal recessive; CTL, cytotoxic T-lymphocyte; EBV, Epstein-Barr virus; FHL, familial hemophagocytic lymphohistiocytosis; Ig, immunoglobulin; IL, interleulin; NK, natural killer; OMIM, Online Mendelian Inheritance in Man; XL, X-linker; XLP, XR lymphoproliferative disease.

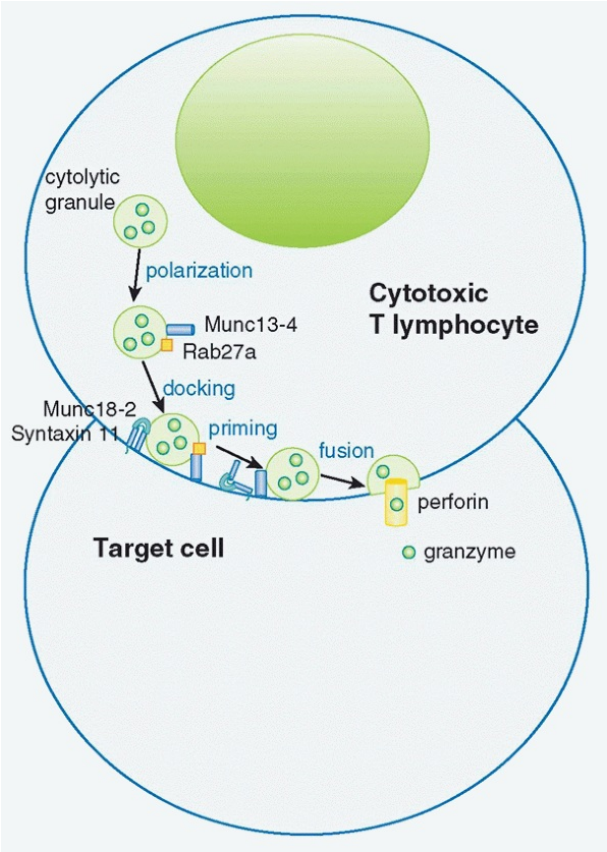

FIG 48.4. Schematic Representation of Cytolytic Granules Trafficking and Release. Upon recognition of target cells, cytotoxic T-lymphocytes mobilize cytolytic granules toward the point of contact with the target cell. The Rab27a protein mediates docking of the cytolytic granule; the syntaxin-11/Munc18-2 complex also participates at this process. Then, Munc13-4 favors priming of the granule, allowing a shift of syntaxin-11 from a closed to an open conformation. Eventually, the membrane of the cytolytic granule fuses with the cell membrane and granzyme, contained in the cytolytic granules is delivered to the target cell through pores formed by perforin.

CHS associates features of FHL with partial albinism, peripheral neuropathy, and the presence of giant lysosomes, which are easy to identify in leukocytes.[590] In this disease, the defect lies in the sorting of proteins to secretory endosomes.[591,592] This defect affects not only cytotoxic lymphocytes (accounting for FHL-like clinical features), but also melanocytes, which are unable to transfer melanin to keratinocytes and other epithelial cells, thus accounting for albinism.

X-Linked Lymphoproliferative Disease

In 1975, Purtilo et al. described a large family in which 6 of 18 male subjects died from a lymphoproliferative disease; infectious mononucleosis due to EBV infection preceded clinical signs of the disease and death in at least three of these cases. At the postmortem examination, lymphocytic and histiocytic infiltration was apparent in multiple organs.[448] This constituted the first report of XLP in humans.

In normal individuals, EBV causes a self-limiting disease: infectious mononucleosis. The virus establishes a latent infection in B-lymphocytes, salivary glands, and some epithelial cells, but is kept under control by CD8+ CTL and NK lymphocytes.[593] By contrast, EBV infection may lead to life-threatening complications in patients with genetic defects in affecting the mechanism controlling EBV infection. Male subjects with XLP are uniquely susceptible to EBV,[594] although EBV is also a common trigger of the acute disease phenotype in patients with FHL. Most male subjects with XLP carry mutations in the *SH2D1A* gene encoding a small adaptor molecule: SLAM-associated protein (SAP).[595,596,597,598] Various molecules of the SLAM family are expressed on the surface of CTL and NK cells. Through SAP, they trigger activatory signaling pathways that ultimately lead to the killing of EBV-infected cells.[597] In the absence of SAP, these activatory pathways are inhibited, and EBV infection remains uncontrolled.[599] The persistence of the virus within B-lymphocytes triggers the continuous activation of virus-specific CD8+ CTLs, which display hyperproliferation and

release large amounts of IFNγ. This ultimately leads to a macrophage activation syndrome, as described for patients with FHL. Alternatively, EBV may cause neoplastic degeneration of the infected B-lymphocytes (lymphoma). SAP is also important for the function of follicular helper T cells (T_{FH}), which interact with B-lymphocytes in secondary lymphoid organs and secrete IL-21, promoting B-cell activation, germinal center reaction, memory B-cell development, and plasmablast differentiation.[600] Consistent with these findings, male subjects with XLP and SAP defects often develop hypogammaglobulinemia with a lack of memory B-lymphocytes. Finally, SAP is also important for the development of NKT lymphocytes, which are typically absent in SAP-deficient patients.[601] The cellular pathogenesis of XLP in SAP-deficient patients was recently clarified by two studies. First, CTL from female carriers expressing the mutant SAP were shown not to recognize and kill EBV-infected target cells, whereas CTL from the same individuals expressing the wild-type SAP did.[456] Second, somatic reversions of the germline SAP mutations in CTL from affected male subjects were associated with the control of EBV infection.[456a] These data therefore implicate CTL, as opposed to other lymphocytes that also normally express SAP, in the pathogenesis of XLP.

A minority of patients with XLP carry defects in another gene (*BIRC4*) encoding the X-linked inhibitor of apoptosis.[602] Consistently lymphocytes from patients with *BIRC4* mutations are particularly susceptible to apoptotic stimuli. However, unlike SAP deficiency, X-linked inhibitor of apoptosis defects are not associated with lymphoma and are more often present with hemophagocytic lymphohistiocytosis, even in the absence of EBV infection.[455]

XLP is a severe disease. Most patients die from fulminant hepatitis or B-cell lymphoma, and survivors often display

hypogammaglobulinemia.[594] XLP is diagnosed on the basis of an analysis of SAP expression[603,604] and is ultimately confirmed by mutation analysis. HSCT is the only curative treatment available and should probably be attempted before primary EBV infection, if possible, in genetically affected individuals.[605]

It should be stressed that we owe our full appreciation of the consequences of SAP deficiency to the study of XLP patients, because mice are not susceptible to EBV infection. XLP demonstrates the importance of studying human disease in natura. *Sap*$^{-/-}$ mice have been generated by gene targeting,[606] but they cannot fully replicate the human disease, because EBV does not infect murine cells. Other viruses, such as lymphocytic choriomeningitis virus in particular, have been used to model XLP in mice. This has made it possible to show that CD4+ and CD8+ lymphocytes proliferate in an unrestrained manner in *Sap*$^{-/-}$ mice infected with lymphocytic choriomeningitis virus and that this proliferation is associated with a marked increase in the production of IFNγ.[606] These are important observations, but they fall short of the broad and dramatic phenotype of XLP patients following EBV infection.

CONCLUSION

We have attempted to cover the diversity of inborn errors of immunity by highlighting disorders affecting different branches of the host response and resulting in various phenotypes, infectious and otherwise. The field is so large and diverse that we could easily have selected other topics. For example, we did not review the inherited disorders of complement, despite their recently discovered surprising association with hemolytic uremic syndrome and related disorders.[607] It is also clear that the rapidly expanding and fascinating group of autoinflammatory disorders merits more attention.[66] Patients with these diseases do not display autoimmunity, in the classical sense of the term, because they have no detectable autoreactive T cells and B cells. Most patients suffer from enhanced IL-1- or TNF-mediated inflammation. Similarly, we did not discuss the inborn errors of enhanced IFNα/β production, resulting in Aicardi-Goutière syndrome and systemic lupus erythematosus.[608] This chapter focuses on pediatric PIDs, discussing only briefly the most common form of PID in adults, common variable immunodeficiency, which has taken much longer to dissect than anticipated, possibly because patients with this disease have more than one genetic lesion.[82] The possibly polygenic basis of some immunologic diseases is not reviewed here, and we have not even touched on autoimmune diseases that are almost Mendelian, such as type I diabetes, which is closely associated with HLA-II alleles, such as HLA-DQ8, in particular.[609] Finally, we do not consider here the ground-breaking discoveries of Mendelian resistance to certain infectious diseases.[610] Patients with AR DARC, CCR5, or FUT2 deficiencies are intrinsically resistant to *Plasmodium vivax*, human immunodeficiency virus, and norovirus, respectively. Individuals carrying at least one functional allele at any of these loci therefore display AD susceptibility to the corresponding pathogen. In these cases, the PIDs are represented by the wild-type alleles, which are currently more common, but the

selective pressure exerted by the pathogens may gradually inverse the allele frequencies.[611] These are only some of the topics not covered here due to space constraints. We have undoubtedly omitted many other fascinating lines of research in the field of PIDs and we apologize to our colleagues for these omissions. The field of PIDs is one of the most rapidly expanding fields of research in immunology. New phenotypes are continually being explored. As host defenses are mediated not only by leukocytes, but by almost all cells and tissues, a myriad of diseases, infectious and otherwise, may result from genetic lesions affecting host defense genes. We anticipate that thousands of PIDs will be deciphered in the future.

In any case, we have attempted to highlight the main clinical and immunologic implications of these studies. Over the last 60 years, more than 200 disorders have been clinically described, immunologically deciphered, and genetically dissected. New therapeutic approaches have been pioneered in patients with PIDs, including IgG substitution, enzyme replacement, recombinant cytokine, HSCT, and gene therapy. This field has, arguably, been one of the most successful in pediatrics and clinical immunology. The immunologic lessons learned from these experiments of nature are also of considerable interest. Some ground-breaking immunologic discoveries have resulted directly from the dissection of PIDs, such as the discovery that AIRE was responsible for autoimmune polyendocrinopathy type I.[612] Many other genes first discovered in patients with PIDs, only some of which have been discussed here, have paved the way to new avenues of immunologic investigation. PIDs have also provided considerable insight in situations in which the morbid genes were first described in the mouse model. Indeed, whether identified by genome-wide or candidate gene approaches, these genes have, in some cases, been assigned a new function, or at least had their known function redefined, in the human model. There are grounds for optimism, as the increasingly careful and widespread clinical care of 7 billion patients will provide an extraordinary wealth of phenotypic description. Moreover, spectacular technologic progress in genetics is making it possible to study the genome of these patients rapidly, searching for morbid lesions at high speed. Finally, tremendous progress in basic immunology, in the mouse model in particular, has provided investigators with the concepts and tools they require to connect PID genotypes and phenotypes experimentally. The identification of causal relationships between gene lesions and clinical phenotypes is based on the molecular and cellular dissection of immunologic pathogenesis. These studies will be of benefit to patients, while providing new insight into the function of host defense genes. We like to think that PIDs is a new frontier in basic immunology and that some of the most extraordinary, paradigm-shifting, immunologic discoveries in the near future will be generated by the investigation of human patients.

REFERENCES

1. Pasteur L. *Etudes Sur la Maladie des Vers à Soie. La Pébrine et la Flacherie* . Paris, France: Gauthiers-Villars; 1870.

2. Koch R. Die Aetiologie der Tuberkulose. *Berl Klin Wochenschr* . 1882;19:221-230.

3. Cairns J. *Matters of Life and Feath* . Princeton, NJ: Princeton University Press; 1997.

4. von Pirquet C. Frequency of tuberculosis in childhood. *JAMA*. 1909;LII:675-678.

5. Nicolle C. Les infections inapparentes. *Scientia*. 1933:181-271.

6. Pasteur L. *Maladies Virulentes, Virus-Vaccins, Prophylaxie de la Rage*. Paris, France: Masson et Cie; 1922-1939.

7. Garrod AE. *The Inborn Factors in Disease*. Gloucestershire, England: Clarendon Press; 1931.

8. Pearson K. Tuberculosis, heredity and environment. *Eugenics Laboratory Lecture Series*. 1912;VIII:694.

9. Bruton OC. Agammaglobulinemia. *Pediatrics*. 1952;9:722-728.

10. Janeway CA, Apt L, Gitlin D. Agammaglobulinemia. *Trans Assoc Am Physicians* . 1953;66:200-202.

11. Kostmann R. Infantile genetic agranulocytosis. *Acta Pediatr Scand* . 1956;45:1-78.

12. Donohue WL. Alymphocytosis. *Pediatrics*. 1953;11:129-139.

13. Glanzmann E, Riniker P. Essential lymphocytophthisis; new clinical aspect of infant

pathology. *Ann Paediatr*. 1950;175:1-32.

14. Kostmann R. Hereditär reticulos - en ny systemsjukdom. *Svenska Läkartdin*. 1950;47:2861.

15. Fudenberg H, et al. Primary immunodeficiencies. Report of a World Health Organization Committee. *Pediatrics*. 1971;47:927-946.

16. Conley ME, Notarangelo LD, Casanova JL. Definition of primary immunodeficiency in 2011: a “trialogue” among friends. *Ann N Y Acad Sci* . 2011;1238:1-6.

17. Holmes B, Quie PG, Windhorst DB, Good RA. Fatal granulomatous disease of childhood. An inborn abnormality of phagocytic function. *Lancet*. 1966;1:1225-1228.

18. Louis-Bar D. Sur un syndrome progressif comprenant des télangiectasies capillaires cutanées et conjonctivales symétriques à disposition naevoïdes et des troubles cérébelleux. *Confin Neurol (Basle)*. 1941;4:32.

19. Syllaba L, Henner K. Contribution à l'indépendance de l'athétose double idiopathique et congénitale: atteinte familiale, syndrome dystrophique, sgne du réseau vasculaire conjonctival, intégrité psychique. *Revue Neurologique (Paris)*. 1926;1:541-562.

20. Aldrich RA, Steinberg AG, Campbell DC. Pedigree demonstrating a sex-linked recessive condition characterized by draining ears, eczematoid dermatitis and bloody diarrhea. *Pediatrics*. 1954;13:133-139.

21. Wiskott A. Familiärer, angeborener Morbus Werlhofii? *Monatsschr Kinderheilkd*. 1937;68:212-216.

22. Cockayne EA. In: *Inherited Abnormalities of the Skin and its Appendages* . Oxford, United Kingdom: Oxford University Press; 1933:156.

23. Lutz W. A propos de l'épidermodysplasie verruciforme. *Dermatologica*. 1946;92:30-43.

24. Boder E, Sedgwick RP. Ataxia-telangiectasia; a familial syndrome of progressive cerebellar ataxia, oculocutaneous telangiectasia and frequent pulmonary infection. *Pediatrics*. 1958;21:526-554.

25. Peterson RD, Kelly WD, Good RA. Ataxia-telangiectasia. Its association with a defective thymus, immunological-deficiency disease, and malignancy. *Lancet*. 1964;1:1189-1193.

26. Krivit W, Good RA. Aldrich's syndrome (thrombocytopenia, eczema, and infection infants); studies of the defense mechanisms. *AMA J Dis Child*. 1959;97:137-153.

27. West CD, Hong R, Holland NH. Immunoglobulin levels from the newborn period to adulthood and in immunoglobulin deficiency states. *J Clin Invest*. 1962;41:2054-2064.

28. Ramoz N, et al. Mutations in two adjacent novel genes are associated with epidermodysplasia verruciformis. *Nat Genet*. 2002;32:579-581.

29. Thorpe E, Handley H. Chronic tetany and chronic mycelial stomatitis in a child aged four and one-half years. *Am J Dis Child*. 1929;28:328-338.

30. Cheng MH, Anderson MS. Monogenic autoimmunity. *Annu Rev Immunol*. 2012;30:393-427.

31. Béguez César A. Neutropenia cronica maligna familiar con granulaciones atipicas de los leucocitos. *Bol Soc Cubana Pediat*. 1943;15:900.

32. Chediak MM. New leukocyte anomaly of constitutional and familial character. *Rev Hematol*. 1952;7:362-367.

33. Higashi O. Congenital gigantism of peroxidase granules; the first case ever reported of qualitative abnormity of peroxidase. *Tohoku J Exp Med*. 1954;59:315-332.

34. Page AR, Berendes H, Warner J, Good RA. The Chediak-Higashi syndrome. *Blood*. 1962;20:330-343.

35. Donaldson VH, Evans RR. A biochemical abnormality in herediatry angioneurotic edema: absence of serum inhibitor of C' 1-esterase. *Am J Med*. 1963;35:37-44.

36. Seligmann M, Fudenberg HH, Good RA. A proposed classification of primary immunologic deficiencies. *Am J Med*. 1968;45817-825.

37. Gatti RA, Good RA. The immunological deficiency diseases. *Med Clin North Am*. 1970;54:281-307.

38. Fudenberg HH, et al. Classification of the primary immune deficiencies: WHO recommendation. *N Engl J Med* . 1970;283:656-657.

39. Rosen FS. Primary immunodeficiency diseases. *R I Med J*. 1972;55:190- 192.

40. Cooper MD, et al. Classification of primary immunodeficiencies. *N Engl J Med* . 1973;288:966-967.

41. Rosen FS. Primary immunodeficiency. *Pediatr Clin North Am* . 1974;21:533-549.

42. Belohradsky BH, et al. Meeting report of the Second International Workshop on Primary Immunodeficiency Diseases in Man. *Clin Immunol Immunopathol* . 1974;2:281-295.

43. Cooper MD, et al. Meeting report of the Second International Workshop on Primary Immunodeficiency Disease in Man held in St. Petersburg, Florida, February, 1973. *Clin Immunol Immunopathol*. 1974;2:416-445.

44. Gatti RA. On the classification of patients with primary immunodeficiency disorders. *Clin Immunol Immunopathol* . 1974;3:243-247.

45. Good RA, Hansen MA. Primary immunodeficiency diseases. *Adv Exp Med Biol* . 1976;73(pt B):155-178.

46. Immunodeficiency: report of a WHO scientific group. *World Health Organ Tech Rep Ser* . 1978;3-80.

47. Primary immunodeficiency diseases. Report prepared for the WHO by a scientific group on immunodeficiency. *Clin Immunol Immunopathol* . 1983;28:450-475.

48. Primary immunodeficiency diseases. Report of a World Health Organization scientific group. *Clin Immunol Immunopathol* . 1986;40:166-196.

49. Primary immunodeficiency diseases. Report of a WHO sponsored meeting. *Immunodefic Rev*. 1989;1:173-205.

50. Primary immunodeficiency diseases. Report of a WHO scientific group. *Immunodefic Rev*. 1992;3:195-236.

51. Primary immunodeficiency diseases. Report of an IUIS Scientific Committee. International Union of Immunological Societies. *Clin Exp Immunol*. 1999;118(suppl 1):1-28.

52. Chapel H, Geha R, Rosen F. Primary immunodeficiency diseases: an update. *Clin Exp Immunol*. 2003;132:9-15.

53. Notarangelo L, et al. Primary immunodeficiency diseases: an update. *J Allergy Clin Immunol*. 2004;114:677-687.

54. Notarangelo L, et al. Primary immunodeficiency diseases: an update from the International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee Meeting in Budapest, 2005. *J Allergy Clin Immunol* . 2006;117:883-896.

55. Geha RS, et al. Primary immunodeficiency diseases: an update from the International

Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee. *J Allergy Clin Immunol* . 2007;120:776-794.

56. Notarangelo LD, et al. Primary immunodeficiencies: 2009 update. *J Allergy Clin Immunol*. 2009;124:1161-1178.

57. Al-Herz W, et al. Primary immunodeficiency diseases: an update on the classification from the International Union of Immunological Societies Committee for Primary Immunodeficiency. *Frontiers in Immunology*. 2011;2:54.

58. Rosen FS, Cooper MD, Wedgwood RJ. The primary immunodeficiencies. (2). *N Engl J Med*. 1984;311:300-310.

59. Rosen FS, Cooper MD, Wedgwood RJ. The primary immunodeficiencies (1). *N Engl J Med*. 1984;311:235-242.

60. Rosen FS, Cooper MD, Wedgwood RJ. The primary immunodeficiencies. *N Engl J Med*. 1995;333:431-440.

61. Stiehm ER, Johnston RB Jr. A history of pediatric immunology. *Pediatr Res* . 2005:57(3):458-467.

62. Burgio GR, Notarangelo LD, Duse M, Ugazio AG. Primary immunodeficiencies: milestones in the history of pediatric immunology. *Pediatr Hematol Oncol* . 1991;8:203-214.

63. Notarangelo LD, Casanova JL. Primary immunodeficiencies: increasing market share. *Curr Opin Immunol* . 2009;21(5):461-465.

64. Casanova JL, Abel L. Primary immunodeficiencies: a field in its infancy. *Science*. 2007;317:617-619.

65. Shapiro RS. Malignancies in the setting of primary immunodeficiency: implications for hematologists/oncologists. *Am J Hematol* . 2011;86:48-55.

66. Minegishi Y. Hyper-IgE syndrome. *Curr Opin Immunol* . 2009;21:487-492.

67. Masters SL, Simon A, Aksentijevich I, Kastner DL. Horror autoinflammaticus: the molecular pathophysiology of autoinflammatory disease. *Annu Rev Immunol* . 2009;27:621-668.

68. Cicardi M, Zanichelli A. Angioedema due to C1 inhibitor deficiency in 2010. *Intern Emerg Med*. 2010;5:481-486.

69. Pachlopnik Schmid J, et al. Inherited defects in lymphocyte cytotoxic activity. *Immunol Rev*. 2010;235:10-23.

70. Zipfel PF, Heinen S, Skerka C. Thrombotic microangiopathies: new insights and new challenges. *Curr Opin Nephrol Hyperten* . 2010;19:372-378.

71. Alcais A, et al. Life-threatening infectious diseases of childhood: singlegene inborn errors of immunity? *Ann N Y Acad Sci* . 2010;1214:18-33.

72. Mathew S, Overturf GD. Complement and properidin deficiencies in meningococcal disease. *Pediatr Infect Dis J* . 2006;25:255-256.

73. Filipovich AH, Zhang K, Snow AL, Marsh RA. X-linked lymphoproliferative syndromes: brothers or distant cousins? *Blood*. 2010;116:3398-3408.

74. Orth G. Host defenses against human papillomaviruses: lessons from epidermodysplasia verruciformis. *Curr Top Microbiol Immunol* . 2008; 321:59-83.

75. Casanova JL, Abel L. Genetic dissection of immunity to mycobacteria: the human model. *Annu Rev Immunol* . 2002;20:581-620.

76. Casanova JL, Abel L, Quintana-Murci L. Human TLRs and IL-1Rs in host defense: natural insights from evolutionary, epidemiological, and clinical genetics. *Annu Rev*

Immunol. 2011;29:447-491.

77. Puel A, et al. Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science*. 2011;332:65-68.

78. Liu L, et al. Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis. *J Exp Med*. 2011;208:1635-1648.

79. Bousfiha A, et al. Primary immunodeficiencies of protective immunity to primary infections. *Clin Immunol*. 2010;135:204-209.

79a. Al-Herz W, Notarangelo LD. Classification of primary immunodeficiency disorders: one-fits-all does not help anymore. *Clin Immunol*. 2012;144:24-25.

80. Arkwright PD, Gennery AR. Ten warning signs of primary immunodeficiency: a new paradigm is needed for the 21st century. *Ann N Y Acad Sci* . 2011;1238:7-14.

81. Picard C, Casanova JL, Puel A. Infectious diseases in patients with IRAK-4, MyD88, NEMO, or IkappaBalpha deficiency. *Clin Microbiol Rev*. 2011;24:490-497.

82. Devriendt K, et al. Constitutively activating mutation in WASP causes X-linked severe congenital neutropenia. *Nat Genet*. 2001;27:313-317.

83. Conley ME, et al. Primary B cell immunodeficiencies: comparisons and contrasts. *Annu Rev Immunol*. 2009;27:199-227.

84. Gatti RA, Meuwissen HJ, Allen HD, Hong R, Good RA. Immunological reconstitution of sex-linked lymphopenic immunological deficiency. *Lancet*. 1968;2:1366-1369.

85. Polmar SH, et al. Enzyme replacement therapy for adenosine deaminase deficiency and severe combined immunodeficiency. *N Engl J Med* . 1976;295:1337-1343.

86. Cavazzana-Calvo M, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science*. 2000;288:669-672.

87. Hershfield MS, et al. Treatment of adenosine deaminase deficiency with polyethylene glycol-modified adenosine deaminase. *N Engl J Med* . 1987;316:589-596.

88. Bonilla MA, et al. Effects of recombinant human granulocyte colonystimulating factor on neutropenia in patients with congenital agranulocytosis. *N Engl J Med* . 1989;320:1574-1580.

89. Good RA. Experiments of nature in immunobiology. *N Engl J Med* . 1968;279:1344-1345.

90. Good RA. In: Kagen BM, Stiehm ER, eds. *Immunologic Incompetence*. Amsterdam, The Netherlands: Year Book Medical Publishing; 1971:149-177.

91. Casanova JL, Abel L. The human model: a genetic dissection of immunity to infection in natural conditions. *Nat Rev Immunol* . 2004;4:55-66.

92. Quintana-Murci L, Alcais A, Abel L, Casanova JL. Immunology in natura: clinical, epidemiological and evolutionary genetics of infectious diseases. *Nat Immunol* . 2007;8:1165-1171.

93. Ochs H, Smith CIE, Puck J. *Primary Immunodeficiencies: A Molecular and Genetic Approach*. 3rd ed. Oxford, United Kingdom: Oxford University Press; 2012.

94. Glanzmann E, Riniker P. Essential lymphocytophthisis; new clinical aspect of infant pathology. *Ann Paediatr* . 1950;175:1-32.

95. Hitzig WH, Biro Z, Bosch H, Huser HJ. Agammaglobulinemia & alymphocytosis with atrophy of lymphatic tissue [in German]. *Helv Paediatr Acta* . 1958;13:551-585.

96. Hitzig WH, Kay HE, Cottier H. Familial lymphopenia with agammaglobulinemia: an attempt at treatment by implantation of fetal thymus. *Lancet*. 1965;1:151-154.

97. Bruton OC. Agammaglobulinemia. *Pediatrics*. 1952;9:722-728.

98. Rosen FS, Gotoff SP, Craig JM, Ritchie J, Janeway CA. Further observations on the Swiss type of agammaglobulinemia (alymphocytosis). The effect of syngeneic bone-marrow cells. *N Engl J Med* . 1966;274:18-21.

99. Cooper MD, et al. Meeting report of the Second International Workshop on Primary Immunodeficiency Disease in Man held in St. Petersburg, Florida, February, 1973. *Clin Immunol Immunopathol*. 1974;2:416-445.

100. Fischer A, et al. Severe combined immunodeficiency. A model disease for molecular immunology and therapy. *Immunol Rev*. 2005; 203:98-109.

101. van der Burg M, Gennery AR. Educational paper. The expanding clinical and immunological spectrum of severe combined immunodeficiency. *Eur J Pediatr* . 2011;170:561-571.

102. McDonald-McGinn DM. Chromosome 22q11.2 deletion syndrome (DiGeorge syndrome/velocardiofacial syndrome). *Medicine (Baltimore)*. 2011;90:1-18.

103. Lima KAT, Foelling I, Natvig S, Ryder LP, Olaussen RW. Low thymic output in the 22q11.2 deletion syndrome measured by CCR9+CD45RA+ T cell counts and T cell receptor rearrangement excision circles. *Clin Exp Immunol*. 2010;161:98-107.

104. Markert ML, Alexieff MJ, Li J, et al. Review of 54 patients with complete DiGeorge anomaly enrolled in protocols for thymus transplantation: outcome of 44 consecutive transplants. *Blood*. 2007;109:4539-4547.

105. Janda A, et al. Multicenter survey on the outcome of transplantation of hematopoietic cells in patients with the complete form of DiGeorge anomaly. *Blood*. 2010;116:2229-2236.

106. Su DM, Navarre S, Oh WJ, Condie BG, Manley NR. A domain of Foxn1 required for crosstalk-dependent thymic epithelial cell differentiation. *Nat Immunol*. 2003;4:1128-1135.

107. Nowell CS, Tetélin S, Jin X, et al. Foxn1 regulates lineage progression in cortical and medullary thymic epithelial cells but is dispensable for medullary sublineage divergence. *PLoS Genet*. 2011;7:e1002348.

108. Frank J, et al. Exposing the human nude phenotype. *Nature*. 1999;398:473-474.

109. Markert ML, Neven B, Devlin BH, et al. First use of thymus transplantation therapy for FOXN1 deficiency (nude/SCID): a report of 2 cases. *Blood*. 2011;117:688-696.

110. Vigliano I, Fusco A, Vitiello L, et al. FOXN1 mutation abrogates prenatal T-cell development in humans. *J Med Genet*. 2011;48:413-416.

111. Nehls M, Pfeifer D, Schorpp M, Hedrich H, Boehm T. New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature*. 1994;372:103-107.

112. Giblett ER, Anderson JE, Cohen F, Pollara B, Meuwissen HJ. Adenosine-deaminase deficiency in two patients with severely impaired cellular immunity. *Lancet*. 1972;2:1067-1069.

113. Hirschhorn R. Inherited enzyme deficiencies and immunodeficiency: adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) deficiencies. *Clin Immunol Immunopathol*. 1986;40: 157-165.

114. Thompson LF, et al. Metabolites from apoptotic thymocytes inhibit thymopoiesis in adenosine deaminase-deficient fetal thymic organ cultures. *J Clin Invest*. 2000;106:1149-1157.

115. Cassani B, et al. Altered intracellular and extracellular signaling leads to impaired T-cell functions in ADA-SCID patients. *Blood*. 2008; 111:4209-4219.

116. Giblett ER, Ammann AJ, Wara DW, Sandman R, Diamond LK. Nucleoside-phosphorylase deficiency in a child with severely defective T-cell immunity and normal B-

cell immunity. *Lancet*. 1975;1: 1010-1013.

117. Noguchi M, et al. Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell*. 1993;73:147-157.

118. Puck JM, et al. The interleukin-2 receptor gamma chain maps to Xq13.1 and is mutated in X-linked severe combined immunodeficiency, SCIDX1. *Hum Mol Genet*. 1993;2:1099-1104.

119. Schorle H, Holtschke T, Hunig T, Schimpl A, Horak I. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature*. 1991;352:621-624

120. Asao H, et al. Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex. *J Immunol*. 2001;167:1-5.

121. Giri JG, et al. Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO J*. 1994;13:2822-2830.

122. Kimura Y, et al. Sharing of the IL-2 receptor gamma chain with the functional IL-9 receptor complex. *Int Immunol*. 1995;7:115-120.

123. Kondo M, et al. Sharing of the interleukin-2 (IL-2) receptor gamma chain between receptors for IL-2 and IL-4. *Science*. 1993;262:1874-1877.

124. Noguchi M, et al. Interleukin-2 receptor gamma chain: a functional component of the interleukin-7 receptor. *Science*. 1993;262:1877-1880.

125. Russell SM, et al. Interleukin-2 receptor gamma chain: a functional component of the interleukin-4 receptor. *Science*. 1993;262:1880-1883.

126. Russell SM, et al. Interaction of IL-2R beta and gamma c chains with Jak1 and Jak3: implications for XSCID and XCID. *Science*. 1994;266:1042-1045.

127. Macchi P, et al. Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID). *Nature*. 1995;377:65-68.

128. Russell SM, et al. Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. *Science*. 1995;270:797-800.

129. Peschon JJ, et al. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med*. 1994;180:1955-1960.

130. von Freeden-Jeffry U, et al. Lymphopenia in interleukin (IL)-7 genedeleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med*. 1995;181:1519-1526.

131. Puel A, Ziegler SF, Buckley RH, Leonard WJ. Defective IL7R expression in T(-)B(+)NK(+) severe combined immunodeficiency. *Nat Genet*. 1998;20:394-397.

132. Giliani S, et al. Interleukin-7 receptor alpha (IL-7Ralpha) deficiency: cellular and molecular bases. Analysis of clinical, immunological, and molecular features in 16 novel patients. *Immunol Rev*. 2005;203: 110-126.

133. Rochman Y, Spolski R, Leonard WJ. New insights into the regulation of T cells by gamma© family cytokines. *Nat Rev Immunol*. 2009;9: 480-490.

134. Peschon JJ, et al. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med*. 1994;180:1955-1960.

135. Cao X, et al. Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. *Immunity*. 1995;2:223-238.

136. Nosaka T, et al. Defective lymphoid development in mice lacking Jak3. *Science*. 1995;270:800-802.

137. Park SY, et al. Developmental defects of lymphoid cells in Jak3 kinasedeficient mice. *Immunity*. 1995;3:771-782.

138. Cavazzana-Calvo M, et al. Role of interleukin-2 (IL-2), IL-7, and IL-15 in natural killer cell differentiation from cord blood hematopoietic progenitor cells and from gamma c transduced severe combined immunodeficiency X1 bone marrow cells. *Blood*. 1996;88:3901-3909.

139. Kennedy MK, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J Exp Med*. 2000; 191:771-780.

140. Lodolce JP, et al. IL-15 recepto r maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity*. 1998;9:669-676.

141. Willerford DM, Swat W, Alt FW. Developmental regulation of V(D)J recombination and lymphocyte differentiation. *Curr Opin Genet Dev* . 1996;6:603-609.

142. Krangel MS. Mechanics of T cell receptor gene rearrangement. *Curr Opin Immunol* . 2009;21:133-139.

143. Oettinger MA, Schatz DG, Gorka C, Baltimore D. RAG-1 and RAG- 2, adjacent genes that synergistically activate V(D)J recombination. *Science*. 1990;248:1517-1523.

144. Difilippantonio MJ, McMahan CJ, Eastman QM, Spanopoulou E, Schatz DG. RAG1 mediates signal sequence recognition and recruitment of RAG2 in V(D)J recombination. *Cell*. 1996;87:253-262.

145. Soulas-Sprauel P, et al. V(D)J and immunoglobulin class switch recombinations: a paradigm to study the regulation of DNA end-joining. *Oncogene*. 2007;26:7780-7791.

146. de Villartay JP. V(D)J recombination deficiencies. *Adv Exp Med Biol* . 2009;650:46-58.

147. Schwarz K, et al. RAG mutations in human B cell-negative SCID. *Science*. 1996;274:97-99.

148. Moshous D, et al. Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency. *Cell*. 2001;105:177-186.

149. O'Driscoll M, et al. DNA ligase IV mutations identified in patients exhibiting developmental delay and immunodeficiency. *Mol Cell*. 2001; 8:1175-1185.

150. Buck D, et al. Severe combined immunodeficiency and microcephaly in siblings with hypomorphic mutations in DNA ligase IV. *Eur J Immunol* . 2006;36:224-235.

151. Enders A, et al. A severe form of human combined immunodeficiency due to mutations in DNA ligase IV. *J Immunol*. 2006;176:5060-5068.

152. van der Burg M, et al. A new type of radiosensitive T-B-NK+ severe combined immunodeficiency caused by a LIG4 mutation. *J Clin Invest*. 2006;116:137-145.

153. van der Burg M, et al. A DNA-PKcs mutation in a radiosensitive T-B- SCID patient inhibits Artemis activation and nonhomologous endjoining. *J Clin Invest*. 2009;119:91-98.

154. Buck D, et al. Cernunnos, a novel nonhomologous end-joining factor, is mutated in human immunodeficiency with microcephaly. *Cell*. 2006;124:287-299.

155. Ahnesorg P, Smith P, Jackson SP. XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining. *Cell*. 2006;124:301-313.

156. Slatter MA, Gennery AR. Primary immunodeficiencies associated with DNA-repair disorders. *Expert Rev Mol Med* . 2010;12:e9.

157. Villa A, et al. Partial V(D)J recombination activity leads to Omenn syndrome. *Cell*. 1998;93:885-896.

158. Moshous D, et al. Partial T and B lymphocyte immunodeficiency and predisposition to lymphoma in patients with hypomorphic mutations in Artemis. *J Clin Invest*. 2003;111:381-387.

159. Felgentreff K, et al. Clinical and immunological manifestations of patients with atypical severe combined immunodeficiency. *Clin Immunol*. 2011;141:73-82.

160. de Saint Basile G, et al. Severe combined immunodeficiency caused by deficiency in either the delta or the epsilon subunit of CD3. *J Clin Invest*. 2004;114:1512-1517.

161. Rieux-Laucat F, et al. Inherited and somatic CD3zeta mutations in a patient with T-cell deficiency. *N Engl J Med*. 2006;354:1913-1921.

162. Roberts JL, et al. T-B+NK+ severe combined immunodeficiency caused by complete deficiency of the CD3zeta subunit of the T-cell antigen receptor complex. *Blood*. 2007;109:3198-3206.

163. Dadi HK, Simon AJ, Roifman CM. Effect of CD3delta deficiency on maturation of alpha/beta and gamma/delta T-cell lineages in severe combined immunodeficiency. *N Engl J Med*. 2003;349:1821-1828.

164. Arnaiz-Villena A, et al. Brief report: primary immunodeficiency caused by mutations in the gene encoding the CD3-gamma subunit of the T-lymphocyte receptor. *N Engl J Med*. 1992;327:529-533.

165. Starr TK, Jameson SC, Hogquist KA. Positive and negative selection of T cells. *Ann Rev Immunol*. 2003;21:139-176.

166. Ziegler A, Muller CA, Bockmann RA, Uchanska-Ziegler B. Low-affinity peptides and T-cell selection. *Trends Immunol*. 2009;30:53-60.

167. Steimle V, Otten LA, Zufferey M, Mach B. Complementation cloning of an MHC class II transactivator mutated in hereditary MHC class II deficiency (or bare lymphocyte syndrome). *Cell*. 1993;75:135-146.

168. Villard J, et al. Mutation of RFXAP, a regulator of MHC class II genes, in primary MHC class II deficiency. *N Engl J Med*. 1997;337:748-753.

169. Masternak K, et al. A gene encoding a novel RFX-associated transactivator is mutated in the majority of MHC class II deficiency patients. *Nat Genet*. 1998;20:273-277.

170. Steimle V, et al. A novel DNA-binding regulatory factor is mutated in primary MHC class II deficiency (bare lymphocyte syndrome). *Genes Dev*. 1995;9:1021-1032.

171. Villard J, Masternak K, Lisowska-Grospierre B, Fischer A, Reith W. MHC class II deficiency: a disease of gene regulation. *Medicine (Baltimore)*. 2001;80:405-418.

172. van Oers NS, Killeen N, Weiss A. ZAP-70 is constitutively associated with tyrosine-phosphorylated TCR zeta in murine thymocytes and lymph node T cells. *Immunity*. 1994;1:675-685.

173. Arpaia E, Shahar M, Dadi H, Cohen A, Roifman CM. Defective T cell receptor signaling and CD8+ thymic selection in humans lacking zap- 70 kinase. *Cell*. 1994;76:947-958.

174. Elder ME, et al. Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase. *Science*. 1994;264:1596-1599.

175. Chan AC, et al. ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency. *Science*. 1994;264:1599-1601.

176. Negishi I, et al. Essential role for ZAP-70 in both positive and negative selection of thymocytes. *Nature*. 1995;376:435-438.

177. Chu DH, et al. Pre-T cell receptor signals are responsible for the down-regulation of Syk protein tyrosine kinase expression. *J Immunol*. 1999;163:2610-2620.

178. Palacios EH, Weiss A. Distinct roles for Syk and ZAP-70 during early thymocyte development. *J Exp Med*. 2007;204:1703-1715.

179. Foger N, Rangell L, Danilenko DM, Chan AC. Requirement for coronin 1 in T

lymphocyte trafficking and cellular homeostasis. *Science*. 2006;313:839-842.

180. Shiow LR, et al. The actin regulator coronin 1A is mutant in a thymic egress-deficient mouse strain and in a patient with severe combined immunodeficiency. *Nat Immunol*. 2008;9:1307-1315.

181. Feske S, et al. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature*. 2006;441:179-185.

182. Feske S. Calcium signalling in lymphocyte activation and disease. *Nat Rev Immunol*. 2007;7:690-702.

183. McCarl CA, et al. ORAI1 deficiency and lack of store-operated Ca2+ entry cause immunodeficiency, myopathy, and ectodermal dysplasia. *J Allergy Clin Immunol*. 2009;124:1311-1318.

184. Picard C, et al. STIM1 mutation associated with a syndrome of immunodeficiency and autoimmunity. *N Engl J Med*. 2009;360: 1971-1980.

185. Stephan JL, et al. Severe combined immunodeficiency: a retrospective single-center study of clinical presentation and outcome in 117 patients. *J Pediatr*. 1993;123:564-572.

186. Buckley RH, et al. Human severe combined immunodeficiency: genetic, phenotypic, and functional diversity in one hundred eight infants. *J Pediatr*. 1997;130:378-387.

187. Muller SM, et al. Transplacentally acquired maternal T lymphocytes in severe combined immunodeficiency: a study of 121 patients. *Blood*. 2001;98:1847-1851.

188. Palmer K, et al. Unusual clinical and immunologic manifestations of transplacentally acquired maternal T cells in severe combined immunodeficiency. *J Allergy Clin Immunol*. 2007;120:423-428.

189. Villa A, Notarangelo LD, Roifman CM. Omenn syndrome: inflammation in leaky severe combined immunodeficiency. *J Allergy Clin Immunol*. 2008;122:1082-1086.

190. Milner JD, Ward JM, Keane-Myers A, Paul WE. Lymphopenic mice reconstituted with limited repertoire T cells develop severe, multiorgan, Th2-associated inflammatory disease. *Proc Natl Acad Sci U S A*. 2007;104:576-581

191. Schuetz C, et al. An immunodeficiency disease with RAG mutations and granulomas. *N Engl J Med*. 2008;358:2030-2038.

192. Corneo B, et al. Identical mutations in RAG1 or RAG2 genes leading to defective V(D)J recombinase activity can cause either T-B-severe combined immune deficiency or Omenn syndrome. *Blood*. 2001;97: 2772-2776.

193. Routes JM, et al. Statewide newborn screening for severe T-cell lymphopenia. *JAMA*. 2009;302:2465-2470.

194. Puck JM. Neonatal screening for severe combined immunodeficiency. *Curr Opin Pediatr*. 2011;23:667-673.

195. Douek DC, et al. Changes in thymic function with age and during the treatment of HIV infection. *Nature*. 1998;396:690-695.

196. Gennery AR, et al. Transplantation of hematopoietic stem cells and long-term survival for primary immunodeficiencies in Europe: entering a new century, do we do better? *J Allergy Clin Immunol*. 2010;126: 602-610.

197. Buckley RH. Transplantation of hematopoietic stem cells in human severe combined immunodeficiency: longterm outcomes. *Immunol Res*. 2011;49:25-43.

198. Picard C, Fischer A. Hematopoietic stem cell transplantation and other management strategies for MHC class II deficiency. *Immunol Allergy Clin North Am*. 2010;30:173-178.

199. Hershfield MS, et al. Treatment of adenosine deaminase deficiency with polyethylene glycol-modified adenosine deaminase. *N Engl J Med*. 1987;316:589-596.

200. Chan B, et al. Long-term efficacy of enzyme replacement therapy for adenosine deaminase (ADA)-deficient severe combined immunodeficiency (SCID). *Clin Immunol*. 2005;117:133-143.

201. Aiuti A, et al. Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *N Engl J Med*. 2009;360:447-458.

202. Hacein-Bey-Abina S, et al. Efficacy of gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med*. 2010;363:355-364.

203. Gaspar HB, et al. Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet*. 2004;364:2181-2187.

204. Hacein-Bey-Abina S, et al. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest*. 2008;118:3132-3142.

205. Howe SJ, et al. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest*. 2008;118:3143-3150.

206. Luzi G, Businco L, Aiuti F. Primary immunodeficiency syndromes in Italy: a report of the national register in children and adults. *J Clin Immunol*. 1983;3:316-320.

207. Matamoros Flori N, Mila Llambi J, Espanol Boren T, Raga Borja S, Fontan Casariego G. Primary immunodeficiency syndrome in Spain: first report of the National Registry in Children and Adults. *J Clin Immunol*. 1997;17:333-339.

208. Zelazko M, et al. Primary immunodeficiency diseases in Latin America: first report from eight countries participating in the LAGID. Latin American Group for Primary Immunodeficiency Diseases. *J Clin Immunol*. 1998;18:161-166.

209. Bruton OC, Apt L, Gitlin D, Janeway CA. Absence of serum gamma globulins. *AMA Am J Dis Child*. 1952;84:632-636.

210. Siegal FP, Pernis B, Kunkel HG. Lymphocytes in human immunodeficiency states: a study of membrane-associated immunoglobulins. *Eur J Immunol* . 1971;1:482-486.

211. Cooper MD, Lawton AR, Bockman DE. Agammaglobulinaemia with B lymphocytes. Specific defect of plasma-cell differentiation. *Lancet*. 1971;2:791-794.

212. Geha RS, Rosen FS, Merler E. Identification and characterization of subpopulations of lymphocytes in human peripheral blood after fractionation on discontinuous gradients of albumin. The cellular defect in X-linked agammaglobulinemia. *J Clin Invest*. 1973;52:1726-1734.

213. Preud'Homme JL, Griscelli C, Seligmann M. Immunoglobulins on the surface of lymphocytes in fifty patients with primary immunodeficiency diseases. *Clin Immunol Immunopathol*. 1973;1:241-256.

214. McKinney RE Jr, Katz SL, Wilfert CM. Chronic enteroviral meningoencephalitis in agammaglobulinemic patients. *Rev Infect Dis*. 1987;9:334-356.

215. Quartier P, et al. Enteroviral meningoencephalitis in X-linked agammaglobulinemia: intensive immunoglobulin therapy and sequential viral detection in cerebrospinal fluid by polymerase chain reaction. *Pediatr Infect Dis J* . 2000;19:1106-1108.

216. Winkelstein JA, et al. X-linked agammaglobulinemia: report on a United States registry of 201 patients. *Medicine (Baltimore)*. 2006;85:193-202.

217. Davis LE, Bodian D, Price D, Butler IJ, Vickers JH. Chronic progressive poliomyelitis secondary to vaccination of an immunodeficient child. *N Engl J Med*. 1977;297:241-245

218. Conley ME, Howard V. Clinical findings leading to the diagnosis of X-linked agammaglobulinemia. *J Pediatr*. 2002;141:566-571.

219. Roifman CM, et al. Increased susceptibility to Mycoplasma infection in patients with hypogammaglobulinemia. *Am J Med*. 1986;80:590-594.

220. Furr PM, Taylor-Robinson D, Webster AD. Mycoplasmas and ureaplasmas in patients with hypogammaglobulinaemia and their role in arthritis: microbiological observations over twenty years. *Ann Rheum Dis*. 1994;53:183-187.

221. LoGalbo PR, Sampson HA, Buckley RH. Symptomatic giardiasis in three patients with X-linked agammaglobulinemia. *J Pediatr* . 1982;101: 78-80.

222. Howard V, et al. The health status and quality of life of adults with X-linked agammaglobulinemia. *Clin Immunol*. 2006;118:201-208.

223. Vetrie D, et al. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. *Nature*. 1993;361:226-233.

224. Tsukada S, et al. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell*. 1993;72: 279-290.

225. de Weers M, et al. The Bruton's tyrosine kinase gene is expressed throughout B cell differentiation, from early precursor B cell stages preceding immunoglobulin gene rearrangement up to mature B cell stages. *Eur J Immunol* . 1993;23:3109-3114.

226. Smith CI, et al. Expression of Bruton's agammaglobulinemia tyrosine kinase gene, BTK, is selectively down-regulated in T lymphocytes and plasma cells. *J Immunol*. 1994;152:557-565.

227. Farrar JE, Rohrer J, Conley ME. Neutropenia in X-linked agammaglobulinemia. *Clin Immunol Immunopathol*. 1996;81:271-276.

228. Lopez Granados E, et al. Clinical and molecular analysis of patients with defects in micro heavy chain gene. *J Clin Invest*. 2002;110:1029-1035.

229. Saouaf SJ, et al. Temporal differences in the activation of three classes of non-transmembrane protein tyrosine kinases following B-cell antigen receptor surface engagement. *Proc Natl Acad Sci U S A* . 1994;91: 9524-9528.

230. Guo B, Kato RM, Garcia-Lloret M, Wahl MI, Rawlings DJ. Engagement of the human pre-B cell receptor generates a lipid raft-dependent calcium signaling complex. *Immunity*. 2000;13:243-253.

231. Rawlings DJ. Bruton's tyrosine kinase controls a sustained calcium signal essential for B lineage development and function. *Clin Immunol*. 1999;91:243-253.

232. Conley ME, et al. Genetic analysis of patients with defects in early B-cell development. *Immunol Rev*. 2005;203:216-234.

233. Valiaho J, Smith CI, Vihinen M. BTKbase: the mutation database for X-linked agammaglobulinemia. *Hum Mutat*. 2006;27:1209-1217.

234. Gaspar HB, Lester T, Levinsky RJ, Kinnon C. Bruton's tyrosine kinase expression and activity in X-linked agammaglobulinaemia (XLA): the use of protein analysis as a diagnostic indicator of XLA. *Clin Exp Immunol*. 1998;111:334-338.

235. Futatani T, et al. Deficient expression of Bruton's tyrosine kinase in monocytes from X-linked agammaglobulinemia as evaluated by a flow cytometric analysis and its clinical application to carrier detection. *Blood*. 1998;91:595-602.

236. Futatani T, Watanabe C, Baba Y, Tsukada S, Ochs HD. Bruton's tyrosine kinase is present in normal platelets and its absence identifies patients with X-linked agammaglobulinaemia and carrier females. *Br J Haematol* . 2001;114:141-149.

237. Broides A, Yang W, Conley ME. Genotype/phenotype correlations in X-linked agammaglobulinemia. *Clin Immunol*. 2006;118:195-200.

238. Plebani A, et al. Clinical, immunological, and molecular analysis in a large cohort of patients with X-linked agammaglobulinemia: an Italian multicenter study. *Clin Immunol*. 2002;104:221-230.

239. Lopez-Granados E, Perez de Diego R, Ferreira Cerdan A, Fontan Casariego G,

Garcia Rodriguez MC. A genotype-phenotype correlation study in a group of 54 patients with X-linked agammaglobulinemia. *J Allergy Clin Immunol* . 2005;116:690-697

240. Conley ME, Mathias D, Treadaway J, Minegishi Y, Rohrer J. Mutations in btk in patients with presumed X-linked agammaglobulinemia. *Am J Hum Genet*. 1998;62:1034-1043.

241. Yel L, et al. Mutations in the mu heavy-chain gene in patients with agammaglobulinemia. *N Engl J Med* . 1996;335:1486-1493.

242. Ferrari S, et al. Molecular analysis of the pre-BCR complex in a large cohort of patients affected by autosomal-recessive agammaglobulinemia. *Genes Immun*. 2007;8:325-333.

243. van Zelm MC, et al. Gross deletions involving IGHM, BTK, or Artemis: a model for genomic lesions mediated by transposable elements. *Am J Hum Genet*. 2008;82:320-332.

244. Minegishi Y, et al. Mutations in the human lambda5/14.1 gene result in B cell deficiency and agammaglobulinemia. *J Exp Med*. 1998;187: 71-77.

245. Minegishi Y, et al. Mutations in Igalpha (CD79a) result in a complete block in B-cell development. *J Clin Invest*. 1999;104:1115-1121.

246. Dobbs AK, et al. Cutting edge: a hypomorphic mutation in Igbeta (CD79b) in a patient with immunodeficiency and a leaky defect in B cell development. *J Immunol* . 2007;179:2055-2059.

247. Minegishi Y, et al. An essential role for BLNK in human B cell development. *Science*. 1999;286:1954-1957.

248. Conley ME. B cells in patients with X-linked agammaglobulinemia. *J Immunol* . 1985;134:3070-3074.

249. Nomura K, et al. Genetic defect in human X-linked agammaglobulinemia impedes a maturational evolution of pro-B cells into a later stage of pre-B cells in the B-cell differentiation pathway. *Blood*. 2000;96: 610-617.

250. Noordzij JG, et al. Composition of precursor B-cell compartment in bone marrow from patients with X-linked agammaglobulinemia compared with healthy children. *Pediatr Res.* 2002;51:159-168.

251. Nuñez C, et al. B cells are generated throughout life in humans. *J Immunol* . 1996;156:866-872.

252. Levy J, et al. Clinical spectrum of X-linked hyper-IgM syndrome. *J Pediatr* . 1997;131:47-54.

253. Durandy A, Taubenheim N, Peron S, Fischer A. Pathophysiology of B-cell intrinsic immunoglobulin class switch recombination deficiencies. *Adv Immunol* . 2007;94:275-306.

254. Allen RC, et al. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science.* 1993;259:990-993.

255. Korthäuer U, et al. Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. *Nature*. 1993;361: 539-541.

256. Conley ME, et al. Hyper IgM syndrome associated with defective CD40-mediated B cell activation. *J Clin Invest*. 1994;94:1404-1409.

257. Hayward AR, et al. Cholangiopathy and tumors of the pancreas, liver, and biliary tree in boys with X-linked immunodeficiency with hyper-IgM. *J Immunol* . 1997;158:977-983.

258. Erdos M, et al. Neuroendocrine carcinoma associated with X-linked hyper-immunoglobulin M syndrome: report of four cases and review of the literature. *Clin Immunol* . 2008;129:455-461.

259. Gennery AR, et al. Treatment of CD40 ligand deficiency by hematopoietic stem cell transplantation: a survey of the European experience, 1993-2002. *Blood*. 2004;103:1152-1157.

260. Armitage RJ, et al. Molecular and biological characterization of a murine ligand for CD40. *Nature*. 1992;357:80-82.

261. Graf D, Korthauer U, Mages HW, Senger G, Kroczek RA. Cloning of TRAP, a ligand for CD40 on human T cells. *Eur J Immunol* . 1992;22:3191-3194.

262. Hollenbaugh D, et al. The human T cell antigen gp39, a member of the TNF gene family, is a ligand for the CD40 receptor: expression of a soluble form of gp39 with B cell co-stimulatory activity. *EMBO J*. 1992;11:4313-4321.

263. Gauchat JF, Aversa G, Gascan H, de Vries JE. Modulation of IL-4 induced germline epsilon RNA synthesis in human B cells by tumor necrosis factor-alpha, anti-CD40 monoclonal antibodies or transforming growth factor-beta correlates with levels of IgE production. *Int Immunol*. 1992;4:397-406.

264. Shapira SK, Vercelli D, Jabara HH, Fu SM, Geha RS. Molecular analysis of the induction of immunoglobulin E synthesis in human B cells by interleukin 4 and engagement of CD40 antigen. *J Exp Med*. 1992;175: 289-292.

265. Muramatsu M, *et al*. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell*. 2000;102:553-563.

266. Revy P, et al. Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell*. 2000;102:565-575

267. Shu U, et al. Activated T cells induce interleukin-12 production by monocytes via CD40-CD40 ligand interaction. *Eur J Immunol* . 1995;25:1125-1128.

268. Caux C, et al. B70/B7-2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. *J Exp Med*. 1994;180:1841-1847.

269. Henn V, Steinbach S, Buchner K, Presek P, Kroczek RA. The inflammatory action of CD40 ligand (CD154) expressed on activated human platelets is temporally limited by coexpressed CD40. *Blood*. 2001;98:1047-1054.

270. Galy AH, Spits H. CD40 is functionally expressed on human thymic epithelial cells. *J Immunol*. 1992;149:775-782.

271. Henn V, et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature*. 1998;391:591-594.

272. Cella M, et al. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med*. 1996;184:747-752.

273. Ferrari S, et al. Mutations of CD40 gene cause an autosomal recessive form of immunodeficiency with hyper IgM. *Proc Natl Acad Sci U S A* . 2001;98:12614-12619.

274. Minegishi Y, et al. Mutations in activation-induced cytidine deaminase in patients with hyper IgM syndrome. *Clin Immunol*. 2000;97: 203-210.

275. Lee WI, et al. Molecular analysis of a large cohort of patients with the hyper immunoglobulin M (IgM) syndrome. *Blood*. 2005;105: 1881-1890.

276. Imai K, et al. Human uracil-DNA glycosylase deficiency associated with profoundly impaired immunoglobulin class-switch recombination. *Nat Immunol*. 2003;4:1023-1028.

277. Quartier P, et al. Clinical, immunologic and genetic analysis of 29 patients with autosomal recessive hyper-IgM syndrome due to activation- induced cytidine deaminase deficiency. *Clin Immunol*. 2004;110: 22-29.

278. Wall RL, Saslaw S. Adult agammaglobulinemia. *AMA Arch Intern Med*. 1955;95:33-36.

279. Grant GH, Wallace WD. Agammaglobulinaemia. *Lancet*. 1954;267: 671-673.

280. Sanford JP, Favour CB, Tribeman MS. Absence of serum gamma globulins in an adult. *N Engl J Med* . 1954;250:1027-1029.

281. Hermans PE, Diaz-Buxo JA, Stobo JD. Idiopathic late-onset immunoglobulin deficiency. Clinical observations in 50 patients. *Am J Med*. 1976;61:221-237.

282. Geha RS, Schneeberger E, Merler E, Rosen FS. Heterogeneity of “acquired” or common variable agammaglobulinemia. *N Engl J Med* . 1974;291:1-6.

283. Conley ME, Notarangelo LD, Etzioni A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). *Clin Immunol*. 1999;93:190-197.

284. Hausser C, Virelizier JL, Buriot D, Griscelli C. Common variable hypogammaglobulinemia in children. Clinical and immunologic observations in 30 patients. *Am J Dis Child*. 1983;137:833-837.

285. Conley ME, Park CL, Douglas SD. Childhood common variable immunodeficiency with autoimmune disease. *J Pediatr* . 1986;108: 915-922.

286. Cunningham-Rundles C, Bodian C. Common variable immunodeficiency: clinical and immunological features of 248 patients. *Clin Immunol*. 1999;92:34-48.

287. Quinti I, et al. Long-term follow-up and outcome of a large cohort of patients with common variable immunodeficiency. *J Clin Immunol*. 2007;27:308-316.

288. Wehr C, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. *Blood*. 2008;111:77-85.

289. Oksenhendler E, et al. Infections in 252 patients with common variable immunodeficiency. *Clin Infect Dis*. 2008;46:1547-1554.

290. Warnatz K, et al. B-cell activating factor receptor deficiency is associated with an adult-onset antibody deficiency syndrome in humans. *Proc Natl Acad Sci U S A* . 2009;106:13945-13950.

291. Brouet JC, Chedeville A, Fermand JP, Royer B. Study of the B cell memory compartment in common variable immunodeficiency. *Eur J Immunol* . 2000;30:2516-2520.

292. Chapel H, et al. Common variable immunodeficiency disorders: division into distinct clinical phenotypes. *Blood*. 2008;112:277-286.

293. Schaffer FM, et al. Individuals with IgA deficiency and common variable immunodeficiency share polymorphisms of major histocompatibility complex class III genes. *Proc Natl Acad Sci U S A* . 1989;86: 8015-8019.

294. Howe HS, So AK, Farrant J, Webster AD. Common variable immunodeficiency is associated with polymorphic markers in the human major histocompatibility complex. *Clin Exp Immunol*. 1991;83: 387-390.

295. Olerup O, Smith CI, Hammarstrom L. Different amino acids at position 57 of the HLA-DQ beta chain associated with susceptibility and resistance to IgA deficiency. *Nature*. 1990;347:289-290.

296. Kralovicova J, Hammarstrom L, Plebani A, Webster AD, Vorechovsky I. Fine-scale mapping at IGAD1 and genome-wide genetic linkage analysis implicate HLA-DQ/DR as a major susceptibility locus in selective IgA deficiency and common variable immunodeficiency. *J Immunol* . 2003;170:2765-2775.

297. Orange JS, et al. Genome-wide association identifies diverse causes of common variable immunodeficiency. *J Allergy Clin Immunol* . 2011;127:1360-1367.

298. Grimbacher B, et al. Homozygous loss of ICOS is associated with adultonset common variable immunodeficiency. *Nat Immunol* . 2003;4: 261-268.

299. Takahashi N, et al. Impaired CD4 and CD8 effector function and decreased memory T cell populations in ICOS-deficient patients. *J Immunol* . 2009;182:5515-5527.

300. Salzer U, et al. Mutations in TNFRSF13B encoding TACI are associated with common variable immunodeficiency in humans. *Nat Genet*. 2005;37:820-828.

301. Castigli E, et al. TACI is mutant in common variable immunodeficiency and IgA deficiency. *Nat Genet*. 2005;37:829-834.

302. Pan-Hammarström Q, et al. Reexamining the role of TACI coding variants in common variable immunodeficiency and selective IgA deficiency. *Nat Genet*. 2007;39:429-430.

303. Castigli E, et al. Reexamining the role of TACI coding variants in common variable immunodeficiency and selective IgA deficiency. *Nat Genet*. 2007;39:430-431.

304. Zhang L, et al. Transmembrane activator and calcium-modulating cyclophilin ligand interactor mutations in common variable immunodeficiency: clinical and immunologic outcomes in heterozygotes. *J Allergy Clin Immunol* . 2007;120:1178-1185.

305. van Zelm MC, et al. An antibody-deficiency syndrome due to mutations in the CD19 gene. *N Engl J Med* . 2006;354:1901-1912.

306. Kanegane H, et al. Novel mutations in a Japanese patient with CD19 deficiency. *Genes Immun.* 2007;8:663-670.

307. Vince N, et al. Defects in the CD19 complex predispose to glomerulonephritis, as well as IgG1 subclass deficiency. *J Allergy Clin Immunol* . 2011;127:538-541.

308. van Zelm MC, et al. CD81 gene defect in humans disrupts CD19 complex formation and leads to antibody deficiency. *J Clin Invest*. 2010;120:1265-1274.

309. Kuijpers TW, et al. CD20 deficiency in humans results in impaired T cell-independent antibody responses. *J Clin Invest*. 2010;120:214-222.

310. Hambleton S, et al. IRF8 mutations and human dendritic-cell immunodeficiency. *N Engl J Med*. 2011;110(12):750-758.

311. Lekstrom-Himes JA, Dorman SE, Kopar P, Holland SM, Gallin JI. Neutrophil-specific granule deficiency results from a novel mutation with loss of function of the transcription factor CCAAT/enhancer binding protein epsilon. *J Exp Med*. 1999;189:1847-1852.

312. Boxer LA, Newburger PE. A molecular classification of congenital neutropenia syndromes. *Pediatr Blood Cancer* . 2007;49:609-614.

313. Berliner N. Lessons from congenital neutropenia: 50 years of progress in understanding myelopoiesis. *Blood*. 2008;111:5427-5432.

314. Klein C. Genetic defects in severe congenital neutropenia: emerging insights into life and death of human neutrophil granulocytes. *Annu Rev Immunol* . 2011;29:399-413.

315. Dale DC, Welte K. Cyclic and chronic neutropenia. *Cancer Treat Res* . 2011;157:97-108.

316. Hammond WP, Price TH, Souza LM, Dale DC. Treatment of cyclic neutropenia with granulocyte colony-stimulating factor. *N Engl J Med* . 1989;320:1306-1311.

317. Freedman MH, et al. Myelodysplasia syndrome and acute myeloid leukemia in patients with congenital neutropenia receiving G-CSF therapy. *Blood*. 2000;96:429-436.

318. Rosenberg PS, et al. The incidence of leukemia and mortality from sepsis in patients with severe congenital neutropenia receiving long-term G-CSF therapy. *Blood*. 2006;107:4628-4635.

319. Connelly JA, Choi SW, Levine JE. Hematopoietic stem cell transplantation for severe congenital neutropenia. *Curr Opin Hematol* . 2012;19:44-51.

320. Zeidler C, et al. Stem cell transplantation in patients with severe congenital

neutropenia without evidence of leukemic transformation. *Blood*. 2000;95:1195-1198.

321. Choi SW, et al. Stem cell transplantation in patients with severe congenital neutropenia with evidence of leukemic transformation. *Bone Marrow Transplant*. 2005;35:473-477.

322. Horwitz M, Benson KF, Person RE, Aprikyan AG, Dale DC. Mutations in ELA2, encoding neutrophil elastase, define a 21-day biological clock in cyclic haematopoiesis. *Nat Genet*. 1999;23:433-436.

323. Horwitz MS, et al. Neutrophil elastase in cyclic and severe congenital neutropenia. *Blood*. 2007;109:1817-1824.

324. Dale DC, et al. Mutations in the gene encoding neutrophil elastase in congenital and cyclic neutropenia. *Blood*. 2000;96:2317-2322.

325. Belaaouaj A, et al. Mice lacking neutrophil elastase reveal impaired host defense against gram negative bacterial sepsis. *Nat Med*. 1998;4: 615-618.

326. Grenda DS, et al. Mutations of the ELA2 gene found in patients with severe congenital neutropenia induce the unfolded protein response and cellular apoptosis. *Blood*. 2007;110:4179-4187.

327. Köllner I, et al. Mutations in neutrophil elastase causing congenital neutropenia lead to cytoplasmic protein accumulation and induction of the unfolded protein response. *Blood*. 2006;108:493-500.

328. Person RE, et al. Mutations in proto-oncogene GFI1 cause human neutropenia and target ELA2. *Nat Genet*. 2003;34:308-312.

329. Karsunky H, et al. Inflammatory reactions and severe neutropenia in mice lacking the transcriptional repressor Gfi1. *Nat Genet*. 2002;30: 295-300.

330. Hock H, et al. Intrinsic requirement for zinc finger transcription factor Gfi-1 in neutrophil differentiation. *Immunity*. 2003;18:109-120.

331. Zarebski A, et al. Mutations in growth factor independent-1 associated with human neutropenia block murine granulopoiesis through colony stimulating factor-1. *Immunity*. 2008;28:370-380.

332. Moulding DA, et al. Unregulated actin polymerization by WASp causes defects of mitosis and cytokinesis in X-linked neutropenia. *J Exp Med*. 2007;204:2213-2224.

333. Carlsson G, Fasth A. Infantile genetic agranulocytosis, morbus Kostmann: presentation of six cases from the original “Kostmann family” and a review. *Acta Paediatr*. 2001;90:757-764.

334. Klein C, et al. HAX1 deficiency causes autosomal recessive severe congenital neutropenia (Kostmann disease). *Nat Genet*. 2007;39:86-92.

335. Han J, et al. Deregulation of mitochondrial membrane potential by mitochondrial insertion of granzyme B and direct Hax-1 cleavage. *J Biol Chem*. 2010;285:22461-22472.

336. Chao JR, et al. Hax1-mediated processing of HtrA2 by Parl allows survival of lymphocytes and neurons. *Nature*. 2008;452:98-102.

337. Ishikawa N, et al. Neurodevelopmental abnormalities associated with severe congenital neutropenia due to the R86X mutation in the HAX1 gene. *J Med Genet*. 2008;45:802-807.

338. Germeshausen M, et al. Novel HAX1 mutations in patients with severe congenital neutropenia reveal isoform-dependent genotype-phenotype associations. *Blood*. 2008;111:4954-4957.

339. Boztug K, et al. A syndrome with congenital neutropenia and mutations in G6PC3. *N Engl J Med*. 2009;360:32-43.

340. Cheung YY, et al. Impaired neutrophil activity and increased susceptibility to bacterial infection in mice lacking glucose-6-phosphatasebeta. *J Clin Invest*. 2007;117:784-793.

341. Landing BH, Shirkey HS. A syndrome of recurrent infection and infiltration of viscera by pigmented lipid histiocytes. *Pediatrics*. 1957;20:431-438.

342. Bridges RA, Berendes H, Good RA. A fatal granulomatous disease of childhood; the clinical, pathological, and laboratory features of a new syndrome. *AMA J Dis Child*. 1959;97:387-408.

343. Berendes H, Bridges RA, Good RA. A fatal granulomatosus of childhood: the clinical study of a new syndrome. *Minn Med*. 1957;40: 309-312.

344. Carson MJ, Chadwick DL, Brubaker CA, Cleland RS, Landing BH. Thirteen boys with progressive septic granulomatosis. *Pediatrics*. 1965;35:405-412.

345. Quie PG, White JG, Holmes B, Good RA. In vitro bactericidal capacity of human polymorphonuclear leukocytes: diminished activity in chronic granulomatous disease of childhood. *J Clin Invest*. 1967;46:668-679.

346. Kaplan EL, Laxdal T, Quie PG. Studies of polymorphonuclear leukocytes from patients with chronic granulomatous disease of childhood: bactericidal capacity for streptococci. *Pediatrics*. 1968;41: 591-599.

347. Holmes B, Page AR. Good RA. Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocytic function. *J Clin Invest*. 1967;46:1422-1432.

348. Baehner RL, Nathan DG. Leukocyte oxidase: defective activity in chronic granulomatous disease. *Science*. 1967;155:835-836.

349. Windhorst DB, Holmes B, Good RA. A newly defined X-linked trait in man with demonstration of the Lyon effect in carrier females. *Lancet*. 1967;1:737-739.

350. Baehner RL, Nathan DG. Quantitative nitroblue tetrazolium test in chronic granulomatous disease. *N Engl J Med* . 1968;278:971-976.

351. Quie PG, Kaplan EL, Page AR, Gruskay FL, Malawista SE. Defective polymorphonuclear-leukocyte function and chronic granulomatous disease in two female children. *N Engl J Med* . 1968;278:976-980.

352. Baehner RL, Karnovsky ML. Deficiency of reduced nicotinamide-adenine dinucleotide oxidase in chronic granulomatous disease. *Science*. 1968;162:1277-1279.

353. Good RA, et al. Fatal (chronic) granulomatous disease of childhood: a hereditary defect of leukocyte function. *Semin Hematol*. 1968;5: 215-254.

354. Segal AW. The NADPH oxidase and chronic granulomatous disease. *Mol Med Today*. 1996;2:129-135.

355. Heyworth PG, Cross AR, Curnutte JT. Chronic granulomatous disease. *Curr Opin Immunol*. 2003;15:578-584.

356. Babior BM. NADPH oxidase. *Curr Opin Immunol* . 2004;16:42-47.

357. Dinauer MC. Chronic granulomatous disease and other disorders of phagocyte function. *Hematology Am Soc Hematol Educ Program*. 2005;89-95.

358. Holland SM. Chronic granulomatous disease. *Clin Rev Allergy Immunol* . 2010;38:3-10.

359. Kang EM, et al. Chronic granulomatous disease: overview and hematopoietic stem cell transplantation. *J Allergy Clin Immunol* . 2011;127:1319-1326.

360. Matute JD, et al. A new genetic subgroup of chronic granulomatous disease with autosomal recessive mutations in p40 phox and selective defects in neutrophil NADPH

oxidase activity. *Blood*. 2009;114: 3309-3315.

361. Royer-Pokora B, et al. Cloning the gene for an inherited human disorder —chronic granulomatous disease—on the basis of its chromosomal location. *Nature*. 1986;322:32-38.

362. Roesler J, et al. Recombination events between the p47-phox gene and its highly homologous pseudogenes are the main cause of autosomal recessive chronic granulomatous disease. *Blood*. 2000;95:2150-2156.

363. Segal BH, Leto TL, Gallin JI, Malech HL, Holland SM. Genetic, biochemical, and clinical features of chronic granulomatous disease. *Medicine (Baltimore)*. 2000;79:170-200.

364. Winkelstein JA, et al. Chronic granulomatous disease. Report on a national registry of 368 patients. *Medicine (Baltimore)*. 2000;79:155-169.

365. van den Berg JM, et al. Chronic granulomatous disease: the European experience. *PLoS One*. 2009;4:e5234.

366. Seger RA. Advances in the diagnosis and treatment of chronic granulomatous disease. *Curr Opin Hematol* . 2010. Epub ahead of print.

367. Reichenbach J, et al. Actinomyces in chronic granulomatous disease: an emerging and unanticipated pathogen. *Clin Infect Dis*. 2009;49: 1703-1710.

368. Greenberg DE, et al. A novel bacterium associated with lymphadenitis in a patient with chronic granulomatous disease. *PLoS Pathog*. 2006;2:e28.

369. Kuhns DB, et al. Residual NADPH oxidase and survival in chronic granulomatous disease. *N Engl J Med* . 2010;363:2600-2610.

370. Bustamante J, et al. Germline CYBB mutations that selectively affect macrophages in kindreds with X-linked predisposition to tuberculous mycobacterial disease. *Nat Immunol*. 2011;12:213-221.

371. Bustamante J, et al. BCG-osis and tuberculosis in a child with chronic granulomatous disease. *J Allergy Clin Immunol* . 2007;120:32-38.

372. Marciano BE, et al. Long-term interferon-gamma therapy for patients with chronic granulomatous disease. *Clin Infect Dis*. 2004;39:692- 699.

373. Gallin JI, et al. Itraconazole to prevent fungal infections in chronic granulomatous disease. *N Engl J Med* . 2003;348:2416-2422.

374. Stein S, et al. Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. *Nat Med*. 2010;16:198-204.

375. Thrasher AJ, Burns SO. WASP: a key immunological multitasker. *Nat Rev Immunol* . 2010;10:182-192.

376. Notarangelo LD, Miao CH, Ochs HD. Wiskott-Aldrich syndrome. *Curr Opin Hematol* . 2008;15:30-36.

377. Perry GS 3rd, et al. The Wiskott-Aldrich syndrome in the United States and Canada (1892-1979). *J Pediatr* . 1980;97:72-78.

378. Sullivan KE, Mullen CA, Blaese RM, Winkelstein JA. A multiinstitutional survey of the Wiskott-Aldrich syndrome. *J Pediatr* . 1994;125: 876-885.

379. Derry JM, Ochs HD, Francke U. Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. *Cell*. 1994;79:922.

380. Padrick SB, Rosen MK. Physical mechanisms of signal integration by WASP family proteins. *Annu Rev Biochem*. 2010;79:707-735.

381. Kim AS, Kakalis LT, Abdul-Manan N, Liu GA, Rosen MK. Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein. *Nature*. 2000;404:151-158.

382. Prehoda KE, Scott JA, Mullins RD, Lim WA. Integration of multiple signals through cooperative regulation of the N-WASP-Arp2/3 complex. *Science*. 2000;290:801-806.

383. Padrick SB, Doolittle LK, Brautigam CA, King DS, Rosen MK. Arp2/3 complex is bound and activated by two WASP proteins. *Proc Natl Acad Sci U S A* . 2011;108:E472-E479.

384. Dupré L, et al. Wiskott-Aldrich syndrome protein regulates lipid raft dynamics during immunological synapse formation. *Immunity*. 2002;17:157-166.

385. Hao JJ, Carey GB, Zhan X. Syk-mediated tyrosine phosphorylation is required for the association of hematopoietic lineage cell-specific protein 1 with lipid rafts and B cell antigen receptor signalosome complex. *J Biol Chem*. 2004;279:33413-33420.

386. Sims TN, et al. Opposing effects of PKCtheta and WASp on symmetry breaking and relocation of the immunological synapse. *Cell*. 2007;129:773-785.

387. Pulecio J, et al. Expression of Wiskott-Aldrich syndrome protein in dendritic cells regulates synapse formation and activation of naive CD8+ T cells. *J Immunol*. 2008;181:1135-1142.

388. Bouma G, et al. Cytoskeletal remodeling mediated by WASp in dendritic cells is necessary for normal immune synapse formation and T-cell priming. *Blood*. 2011;118:2492-2501.

389. Ochs HD, et al. The Wiskott-Aldrich syndrome: studies of lymphocytes, granulocytes, and platelets. *Blood*. 1980;55:243-252.

390. Locci M, et al. The Wiskott-Aldrich syndrome protein is required for iNKT cell maturation and function. *J Exp Med*. 2009;206:735-742.

391. Humblet-Baron S, et al. Wiskott-Aldrich syndrome protein is required for regulatory T cell homeostasis. *J Clin Invest*. 2007;117:407-418.

392. Maillard MH, et al. The Wiskott-Aldrich syndrome protein is required for the function of CD4(+)CD25(+)Foxp3(+) regulatory T cells. *J Exp Med*. 2007;204:381-391.

393. Astrakhan A, Ochs HD, Rawlings DJ. Wiskott-Aldrich syndrome protein is required for homeostasis and function of invariant NKT cells. *J Immunol*. 2009;182:7370-7380.

394. Prchal JT, et al. Wiskott-Aldrich syndrome: cellular impairments and their implication for carrier detection. *Blood*. 1980;56:1048-1054.

395. Gealy WJ, Dwyer JM, Harley JB. Allelic exclusion of glucose-6-phosphate dehydrogenase in platelets and T lymphocytes from a Wiskott- Aldrich syndrome carrier. *Lancet*. 1980;1:63-65.

396. Westerberg LS, et al. WASP confers selective advantage for specific hematopoietic cell populations and serves a unique role in marginal zone B-cell homeostasis and function. *Blood*. 2008;112:4139-4147.

397. Ariga T, et al. Spontaneous in vivo reversion of an inherited mutation in the Wiskott-Aldrich syndrome. *J Immunol*. 2001;166:5245-5249.

398. Wada T, et al. Second-site mutation in the Wiskott-Aldrich syndrome (WAS) protein gene causes somatic mosaicism in two WAS siblings. *J Clin Invest*. 2003;111:1389-1397.

399. Lutskiy MI, Beardsley DS, Rosen FS, Remold-O'Donnell E. Mosaicism of NK cells in a patient with Wiskott-Aldrich syndrome. *Blood*. 2005;106:2815-2817.

400. Boztug K, et al. Multiple independent second-site mutations in two siblings with somatic mosaicism for Wiskott-Aldrich syndrome. *Clin Genet*. 2008;74:68-74.

401. Davis BR, Candotti F. Revertant somatic mosaicism in the Wiskott- Aldrich syndrome. *Immunol Res*. 2009;44:127-131.

402. Hirschhorn R, Yang DR, Israni A, Huie ML, Ownby DR. Somatic mosaicism for a newly identified splice-site mutation in a patient with adenosine deaminase-deficient immunodeficiency and spontaneous clinical recovery. *Am J Hum Genet*. 1994;55:59-68.

403. Hirschhorn R, et al. Spontaneous in vivo reversion to normal of an inherited mutation in a patient with adenosine deaminase deficiency. *Nat Genet*. 1996;13:290-295.

404. Jin Y, et al. Mutations of the Wiskott-Aldrich syndrome protein (WASP): hotspots, effect on transcription, and translation and phenotype/genotype correlation. *Blood*. 2004;104:4010-4019.

405. Zhu Q, et al. Wiskott-Aldrich syndrome/X-linked thrombocytopenia: WASP gene mutations, protein expression, and phenotype. *Blood*. 1997;90:2680-2689.

406. Notarangelo LD, et al. Missense mutations of the WASP gene cause intermittent X-linked thrombocytopenia. *Blood*. 2002;99:2268-2269.

407. Ramesh N, Anton IM, Hartwig JH, Geha RS. WIP, a protein associated with Wiskott-Aldrich syndrome protein, induces actin polymerization and redistribution in lymphoid cells. *Proc Natl Acad Sci U S A* . 1997;94:14671-14676.

408. de la Fuente MA, et al. WIP is a chaperone for Wiskott-Aldrich syndrome protein (WASP). *Proc Natl Acad Sci U S A* . 2007;104: 926-931.

409. Lanzi G, et al. A novel primary human immunodeficiency due to deficiency in the WASP-interacting protein WIP. *J Exp Med*. 2012;209:29-34.

410. Ancliff PJ, et al. Two novel activating mutations in the Wiskott- Aldrich syndrome protein result in congenital neutropenia. *Blood*. 2006;108:2182-2189.

411. Beel K, et al. A large kindred with X-linked neutropenia with an I294T mutation of the Wiskott-Aldrich syndrome gene. *Br J Haematol* . 2009;144:120-126.

412. Zhang Q, et al. Combined immunodeficiency associated with DOCK8 mutations. *N Engl J Med*. 2009;361:2046-2055.

413. Engelhardt KR, et al. Large deletions and point mutations involving the dedicator of cytokinesis 8 (DOCK8) in the autosomal-recessive form of hyper-IgE syndrome. *J Allergy Clin Immunol*. 2009;124: 1289-1302.

414. Ambruso DR, et al. Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation. *Proc Natl Acad Sci U S A* . 2000;97:4654-4659.

415. Williams DA, et al. Dominant negative mutation of the hematopoieticspecific Rho GTPase, Rac2, is associated with a human phagocyte immunodeficiency. *Blood*. 2000;96:1646-1654.

416. Accetta D, et al. Human phagocyte defect caused by a Rac2 mutation detected by means of neonatal screening for T-cell lymphopenia. *J Allergy Clin Immunol* . 2011;127:535-538.

417. Micol R, et al. Morbidity and mortality from ataxia-telangiectasia are associated with ATM genotype. *J Allergy Clin Immunol* . 2011;128: 382-389.

418. Gotoff SP, Amirmokri E, Liebner EJ. Ataxia telangiectasia. Neoplasia, untoward response to x-irradiation, and tuberous sclerosis. *Am J Dis Child*. 1967;114:617-625.

419. McCaw BK, Hecht F, Harnden DG, Teplitz RL. Somatic rearrangement of chromosome 14 in human lymphocytes. *Proc Natl Acad Sci U S A* . 1975;72:2071-2075.

420. Polani PE. DNA repair defects and chromosome instability disorders. *Ciba Found Symp*. 1979;81-131.

421. Savitsky K, et al. A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science*. 1995;268:1749-1753.

422. Savitsky K, et al. The complete sequence of the coding region of the ATM gene reveals similarity to cell cycle regulators in different species. *Hum Mol Genet*. 1995;4:2025-2032.

423. Gilad S, et al. Predominance of null mutations in ataxia-telangiectasia. *Hum Mol Genet*. 1996;5:433-439.

424. Fernandes N, et al. DNA damage-induced association of ATM with its target proteins requires a protein interaction domain in the N terminus of ATM. *J Biol Chem*. 2005;280:15158-15164.

425. Bensimon A, Aebersold R, Shiloh Y. Beyond ATM: the protein kinase landscape of the DNA damage response. *FEBS Lett*. 2011;585: 1625-1639.

426. Guo Z, Kozlov S, Lavin MF, Person MD, Paull TT. ATM activation by oxidative stress. *Science*. 2010;330:517-521.

427. Carney JP, et al. The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. *Cell*. 1998;93:477-486.

428. Varon R, et al. Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome. *Cell*. 1998;93:467-476.

429. Matsuura S, et al. Positional cloning of the gene for Nijmegen breakage syndrome. *Nat Genet*. 1998;19:179-181.

430. Taalman RD, et al. Further delineation of the Nijmegen breakage syndrome. *Am J Med Genet*. 1989;32:425-431.

431. Stewart GS, et al. The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder. *Cell*. 1999;99:577-587.

432. Fernet M, et al. Identification and functional consequences of a novel MRE11 mutation affecting 10 Saudi Arabian patients with the ataxia telangiectasia-like disorder. *Hum Mol Genet*. 2005;14:307-318.

433. Matsumoto Y, et al. Two unrelated patients with MRE11A mutations and Nijmegen breakage syndrome-like severe microcephaly. *DNA Repair (Amst)* . 2011;10:314-321.

434. Waltes R, et al. Human RAD50 deficiency in a Nijmegen breakage syndrome- like disorder. *Am J Hum Genet*. 2009;84:605-616.

435. Davis SD, Schaller J, Wedgwood RJ. Job's syndrome. Recurrent, “cold,” staphylococcal abscesses. *Lancet*. 1966;1:1013-1015.

436. Buckley RH, Wray BB, Belmaker EZ. Extreme hyperimmunoglobulinemia E and undue susceptibility to infection. *Pediatrics*. 1972;49: 59-70.

437. Grimbacher B, et al. Hyper-IgE syndrome with recurrent infections —an autosomal dominant multisystem disorder. *N Engl J Med* . 1999;340:692-702.

438. Freeman AF, et al. Coronary artery abnormalities in Hyper-IgE syndrome. *J Clin Immunol*. 2011;31:338-345.

439. Minegishi Y, et al. Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. *Nature*. 2007;448: 1058-1062.

440. Holland SM, et al. STAT3 mutations in the hyper-IgE syndrome. *N Engl J Med* . 2007;357(16):1608-1619.

441. Jones SA, Scheller J, Rose-John S. Therapeutic strategies for the clinical blockade of IL-6/gp130 signaling. *J Clin Invest*. 2011;121:3375-3383.

442. Tangye SG, Cook MC, Fulcher DA. Insights into the role of STAT3 in human lymphocyte differentiation as revealed by the hyper-IgE syndrome. *J Immunol*. 2009;182:21-28.

443. Takeda K, et al. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc Natl Acad Sci U S A* . 1997;94: 3801-3804.

444. Heimall J, et al. Paucity of genotype-phenotype correlations in STAT3 mutation positive hyper IgE syndrome (HIES). *Clin Immunol*. 2011;139:75-84.

445. Minegishi Y, et al. Human tyrosine kinase 2 deficiency reveals its requisite roles in multiple cytokine signals involved in innate and acquired immunity. *Immunity*. 2006;25:745-755.

446. Kilic SS, et al. A patient with tyrosine kinase 2 deficiency without hyper-IgE syndrome. *J Pediatr* . 2012;160(6):1055-1057.

447. Purtilo DT, Cassel C, Yang JP. Letter: fatal infectious mononucleosis in familial lymphohistiocytosis. *N Engl J Med* . 1974;291:736.

448. Purtilo DT, Cassel CK, Yang JP, Harper R. X-linked recessive progressive combined variable immunodeficiency (Duncan's disease). *Lancet*. 1975;1:935-940.

449. Purtilo DT. Pathogenesis and phenotypes of an X-linked recessive lymphoproliferative syndrome. *Lancet*. 1976;2:882-885.

450. Purtilo DT, et al. Variable phenotypic expression of an X-linked recessive lymphoproliferative syndrome. *N Engl J Med* . 1977;297:1077-1080.

451. Hamilton JK, et al. X-linked lymphoproliferative syndrome registry report. *J Pediatr* . 1980;96:669-673.

452. Coffey AJ, et al. Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. *Nature Genet*. 1998;20:129-135.

453. Sayos J, et al. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature*. 1998;395:462-469.

454. Rigaud S, et al. XIAP deficiency in humans causes an X-linked lymphoproliferative syndrome. *Nature*. 2006;444:110-114.

455. Marsh RA, et al. XIAP deficiency: a unique primary immunodeficiency best classified as X-linked familial hemophagocytic lymphohistiocytosis and not as X-linked lymphoproliferative disease. *Blood*. 2010;116: 1079-1082.

456. Palendira U, et al. Molecular pathogenesis of EBV susceptibility in XLP as revealed by analysis of female carriers with heterozygous expression of SAP. *PLoS Biol*. 2011;9:e1001187.

456a. Palendira U, et al. Expansion of somatically reverted memory CD8+ T cells in patients with X-linked lymphoproliferative disease caused by selective pressure from Epstein-Barr virus. *J Exp Med*. 2012;209:913-924.

457. Wellek B, Opferkuch W. A case of deficiency of the seventh component of complement in man. Biological properties of a C7-deficient serum and description of a C7-inactivating principle. *Clin Exp Immunol*. 1975;19:223-235.

458. Lim D, et al. Absence of the sixth component of complement in a patient with repeated episodes of meningococcal meningitis. *J Pediatr* . 1976;89:42-47.

459. Petersen BH, Graham JA, Brooks GF. Human deficiency of the eighth component of complement. The requirement of C8 for serum Neisseria gonorrhoeae bactericidal activity. *J Clin Invest*. 1976;57:283-290.

460. Leddy JP, Frank MM, Gaither T, Baum J, Klemperer MR. Hereditary deficiency of the sixth component of complement in man. I. Immunochemical, biologic, and family studies. *J Clin Invest*. 1974;53:544-553.

461. Boyer JT, Gall EP, Norman ME, Nilsson UR, Zimmerman TS. Hereditary deficiency of the seventh component of complement. *J Clin Invest*. 1975;56:905-913.

462. Sjoholm AG, Braconier JH, Soderstrom C. Properdin deficiency in a family with fulminant meningococcal infections. *Clin Exp Immunol*. 1982;50:291-297.

463. Teisner B, et al. Factor I deficiency and C3 nephritic factor: immunochemical findings and association with Neisseria meningitidis infection in two patients. *Scand J Immunol*. 1984;20:291-297.

464. Hiemstra PS, et al. Complete and partial deficiencies of complement factor D in a Dutch family. *J Clin Invest*. 1989;84:1957-1961.

465. Ram S, Lewis LA, Rice PA. Infections of people with complement deficiencies and patients who have undergone splenectomy. *Clin Microbiol Rev*. 2010;23:740-780.

466. Pettigrew HD, Teuber SS, Gershwin ME. Clinical significance of complement deficiencies. *Ann N Y Acad Sci* . 2009;1173:108-123.

467. Lutzner MA. Epidermodysplasia verruciformis. An autosomal recessive disease characterized by viral warts and skin cancer. A model for viral oncogenesis. *Bull Cancer*. 1978;65:169-182.

468. Orth G, et al. Characterization of two types of human papillomaviruses in lesions of epidermodysplasia verruciformis. *Proc Natl Acad Sci U S A* . 1978;75:1537-1541.

469. Lazarczyk M, et al. Regulation of cellular zinc balance as a potential mechanism of EVER-mediated protection against pathogenesis by cutaneous oncogenic human papillomaviruses. *J Exp Med*. 2008;205(1):35-42.

470. Orth G. Genetics of epidermodysplasia verruciformis: insights into host defense against papillomaviruses. *Semin Immunol*. 2006;18:362-374.

471. Androphy EJ, Dvoretzky I, Lowy DR. X-linked inheritance of epidermodysplasia verruciformis. Genetic and virologic studies of a kindred. *Arch Dermatol*. 1985;121:864-868.

472. McDermott DF, et al. Autosomal dominant epidermodysplasia verruciformis lacking a known EVER1 or EVER2 mutation. *Pediatr Dermatol* . 2009;26:306-310.

472a. Crequer A, et al. Human RHOH deficiency causes T cell defects and susceptibility to EV-HPV infections. *J Clin Invest*. 2012; Aug 1 [Epub ahead of print]

473. Newport MJ, et al. A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. *N Engl J Med*. 1996;335:1941-1949.

474. Jouanguy E, et al. Interferon-gamma-receptor deficiency in an infant with fatal bacille Calmette-Guérin infection. *N Engl J Med*. 1996;335:1956-1961.

475. Filipe-Santos O, et al. Inborn errors of IL-12/23- and IFN-gammamediated immunity: molecular, cellular, and clinical features. *Semin Immunol*. 2006;18:347-361.

476. Mimouni J. Notre expérience de trois années de vaccination à Constantine; étude de 25 cas de complications. *Alger Médicale*. 1951;55:1138-1147.

477. Casanova JL, et al. Idiopathic disseminated bacillus Calmette- Guérin infection: a French national retrospective study. *Pediatrics*. 1996;98:774-778.

478. Casanova JL, Jouanguy E, Lamhamedi S, Blanche S, Fischer A. Immunological conditions of children with BCG disseminated infection. *Lancet*. 1995;346:581.

479. de Beaucoudrey L, et al. Mutations in STAT3 and IL12RB1 impair the development of human IL-17-producing T cells. *J Exp Med*. 2008;205:1543-1550.

480. de Beaucoudrey L, et al. Revisiting human IL-12Rbeta1 deficiency: a survey of 141 patients from 30 countries. *Medicine (Baltimore)*. 2010;89:381-402.

481. Filipe-Santos O, et al. X-linked susceptibility to mycobacteria is caused by mutations in NEMO impairing CD40-dependent IL-12 production. *J Exp Med*. 2006;203:1745-1759.

482. Bustamante J, Picard C, Boisson-Dupuis S, Abel L, Casanova JL. Genetic lessons learned from X-linked Mendelian susceptibility to mycobacterial diseases. *Ann N Y Acad Sci*. 2011;1246:92-101.

483. Tabarsi P, et al. Lethal tuberculosis in a previously healthy adult with IL-12 receptor deficiency. *J Clin Immunol*. 2011;31:537-539.

484. Boisson Dupuis S, et al. IL-12Rβ1 deficiency in two of fifty children with severe tuberculosis from Iran, Morocco, and Turkey. *PLoS One*. 2011;6(4):e18524.

485. Altare F, et al. Interleukin-12 receptor beta1 deficiency in a patient with abdominal tuberculosis. *J Infect Dis*. 2001;184:231-236.

486. Alcais A, Fieschi C, Abel L, Casanova JL. Tuberculosis in children and adults: two distinct genetic diseases. *J Exp Med*. 2005;202:1617-1621.

487. Zhang SY, et al. Inborn errors of interferon (IFN)-mediated immunity in humans: insights into the respective roles of IFN-alpha/beta, IFN-gamma, and IFN-lambda in host defense. *Immunol Rev*. 2008;226:29-40.

488. Canales L, Middlemas RO III, Louro JM, South MA. Immunological observations in chronic mucocutaneous candidiasis. *Lancet*. 1969;2: 567-571.

489. Chilgren RA, Quie PG, Meuwissen HJ, Hong R. Chronic mucocutaneous candidiasis, deficiency of delayed hypersensitivity, and selective local antibody defect. *Lancet*. 1967;2:688-693.

490. Wells RS, Higgs JM, Macdonald A, Valdimarsson H, Holt PJ. Familial chronic muco-cutaneous candidiasis. *J Med Genet*. 1972;9:302-310.

491. Puel A, et al. Inborn errors of mucocutaneous immunity to Candida albicans in humans: a role for IL-17 cytokines? *Curr Opin Immunol*. 2010;22:467-474.

492. Freeman AF, Holland SM. Clinical manifestations of hyper IgE syndromes. *Dis Markers*. 2010;29:123-130.

493. Ma CS, et al. Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J Exp Med*. 2008;205:1551-1557.

494. Milner JD, et al. Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature*. 2008;452:773-776.

495. Husebye ES, Anderson MS. Autoimmune polyendocrine syndromes: clues to type 1 diabetes pathogenesis. *Immunity*. 2010;32:479-487.

496. Puel A, et al. Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I. *J Exp Med*. 2010;207:291-297.

497. Kisand K, et al. Chronic mucocutaneous candidiasis in APECED or thymoma patients correlates with autoimmunity to Th17-associated cytokines. *J Exp Med*. 2010;207:299-308.

498. van de Veerdonk FL, et al. STAT1 mutations in autosomal dominant chronic mucocutaneous candidiasis. *N Engl J Med*. 2011;365:54-61.

499. Dupuis S, et al. Impairment of mycobacterial but not viral immunity by a germline human STAT1 mutation. *Science*. 2001;293:300-303.

500. Dupuis S, et al. Impaired response to interferon-alpha/beta and lethal viral disease in human STAT1 deficiency. *Nat Genet*. 2003;33:388-391.

501. Chapgier A, et al. A partial form of recessive STAT1 deficiency in humans. *J Clin Invest*. 2009;119:1502-1514.

501a. Toth B, et al. Herpes in STAT1 deficiency. *Lancet*. 2012;379:2500.

502. Siegel AM, et al. A critical role for STAT3 transcription factor signaling in the development and maintenance of human T cell memory. *Immunity*. 2011;35:806-818.

503. Picard C, Puel A, Ku CL, Casanova JL. Primary immunodeficiencies associated with pneumococcal disease. *Curr Opin Allergy Clin Immunol* . 2003;3:451-459.

504. Abinun M. Ectodermal dysplasia and immunodeficiency. *Arch Dis Child*. 1995;73:185.

505. Abinun M, Spickett G, Appleton AL, Flood T, Cant AJ. Anhidrotic ectodermal dysplasia associated with specific antibody deficiency. *Eur J Pediatr* . 1996;155:146-147.

506. Döffinger R, et al. X-linked anhidrotic ectodermal dysplasia with immunodeficiency is caused by impaired NF-kappaB signaling. *Nat Genet*. 2001;27:277-285.

507. Courtois G, et al. A hypermorphic IkappaBalpha mutation is associated with autosomal dominant anhidrotic ectodermal dysplasia and T cell immunodeficiency. *J Clin Invest*. 2003;112:1108-1115.

508. Smahi A, et al. Genomic rearrangement in NEMO impairs NF-kappaB activation and is a cause of incontinentia pigmenti. The International Incontinentia Pigmenti (IP) Consortium. *Nature*. 2000;405:466-472.

509. Ku CL, et al. IRAK4 and NEMO mutations in otherwise healthy children with recurrent invasive pneumococcal disease. *J Med Genet*. 2007;44:16-23.

510. Picard C, et al. Pyogenic bacterial infections in humans with IRAK-4 deficiency. *Science*. 2003;299:2076-2079.

511. von Bernuth H, et al. Pyogenic bacterial infections in humans with MyD88 deficiency. *Science*. 2008;321:691-696.

512. Ku CL, *et al*. Selective predisposition to bacterial infections in IRAK-4 deficient children: IRAK-4 dependent TLRs are otherwise redundant in protective immunity. *J Exp Med*. 2007;204:2407-2422.

513. Picard C, et al. Clinical features and outcome of patients with IRAK-4 and MyD88 deficiency. *Medicine (Baltimore)*. 2010;89:403-425.

514. Yang K, et al. Human TLR-7-, -8-, and -9-mediated induction of IFN-alpha/beta and -lambda Is IRAK-4 dependent and redundant for protective immunity to viruses. *Immunity*. 2005;23:465-478.

515. Barreiro LB, et al. Evolutionary dynamics of human toll-like receptors and their different contributions to host defense. *PLoS Genet*. 2009;5:e1000562.

516. Abel L, et al. Age-dependent Mendelian predisposition to HSV-1 encephalitis in childhood. *J Pediatr*. 2010;157(4):623-629.

517. Casrouge A, et al. Herpes simplex virus encephalitis in human UNC- 93B deficiency. *Science*. 2006;314:308-312.

518. Zhang SY, et al. TLR3 deficiency in patients with herpes simplex encephalitis. *Science*. 2007;317:1522-1527.

519. Guo Y, et al. Herpes simplex virus encephalitis in a patient with complete TLR3 deficiency: TLR3 is otherwise redundant in protective immunity. *J Exp Med*. 2011;208:2083-2098.

520. Sancho-Shimizu V, et al. Herpes simplex encephalitis in children with autosomal recessive and dominant TRIF deficiency. *J Clin Invest*. 2011;121:4889-4902.

521. Perez de Diego R, et al. Human TRAF3 adaptor molecule deficiency leads to impaired toll-like receptor 3 response and susceptibility to herpes simplex encephalitis. *Immunity*. 2010;33:400-411.

522. Audry M, et al. NEMO is a key component of NF-kappaB- and IRF-3- dependent TLR3-mediated immunity to herpes simplex virus. *J Allergy Clin Immunol* . 2011;128:610-

617.

523. Sancho-Shimizu V, et al. Genetic susceptibility to herpes simplex virus 1 encephalitis in mice and humans. *Curr Opin Allergy Clin Immunol* . 2007;7:495-505.

524. Sancho-Shimizu V, de Diego RP, Jouanguy E, Zhang SY, Casanova JL. Inborn errors of anti-viral interferon immunity in humans. *Curr Opin Virol* . 2011;1:487-496.

524a. aa, under review

525. Hogquist KA, Baldwin TA, Jameson SC. Central tolerance: learning self-control in the thymus. *Nature reviews. Immunology*. 2005;5: 772-782.

526. Meffre E, Wardemann H. B-cell tolerance checkpoints in health and autoimmunity. *Curr Opin Immunol* . 2008;20:632-638.

527. von Boehmer H, Melchers F. Checkpoints in lymphocyte development and autoimmune disease. *Nat Immunol* . 2010;11:14-20.

528. Mackay F, Schneider P. Cracking the BAFF code. *Nat Rev Immunol* . 2009;9:491-502.

529. Neufeld M, Maclaren NK, Blizzard RM. Two types of autoimmune Addison's disease associated with different polyglandular autoimmune (PGA) syndromes. *Medicine*. 1981;60:355-362.

530. Michels AW, Gottlieb PA. Autoimmune polyglandular syndromes. *Nat Rev Endocrinol* . 2010;6:270-277.

531. Levin M, Newport M. Unravelling the genetic basis of susceptibility to mycobacterial infection. *J Pathol* . 1997;181:5-7.

532. Nagamine K, et al. Positional cloning of the APECED gene. *Nat Genet* . 1997;17:393-398.

533. Anderson MS, et al. Projection of an immunological self shadow within the thymus by the aire protein. *Science*. 2002;298:1395-1401.

534. Metzger TC, Anderson MS. Control of central and peripheral tolerance by Aire. *Immunol Rev* . 2011;241:89-103.

535. Kisand K, et al. Chronic mucocutaneous candidiasis in APECED or thymoma patients correlates with autoimmunity to Th17-associated cytokines. *J Exp Med* . 2010;207:299-308.

536. Puel A, et al. Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I. *J Exp Med*. 2010;207:291-297.

537. Russell WL, Russell LB, Gower JS. Exceptional inheritance of a sex-linked gene in the mouse explained on the basis that the X/O sex-chromosome constitution is female. *Proc Natl Acad Sci U S A* . 1959;45: 554-560.

538. Powell BR, Buist NR, Stenzel P. An X-linked syndrome of diarrhea, polyendocrinopathy, and fatal infection in infancy. *J Pediatr* . 1982;100:731-737.

539. Ferguson PJ, et al. Manifestations and linkage analysis in X-linked autoimmunity-immunodeficiency syndrome. *Am J Med Genetics* . 2000;90:390-397.

540. Means GD, Toy DY, Baum PR, Derry JM. A transcript map of a 2-Mb BAC contig in the proximal portion of the mouse X chromosome and regional mapping of the scurfy mutation. *Genomics*. 2000;65:213-223.

541. Brunkow ME, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* . 2001;27:68-73.

542. Bennett CL, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet*. 2001;27:20-21.

543. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*. 2003;4:330-336.

544. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003;299:1057-1061.

545. Hill JA, Benoist C, Mathis D. Treg cells: guardians for life. *Nat Immunol*. 2007;8:124-125).

546. Burroughs LM, et al. Stable hematopoietic cell engraftment after lowintensity nonmyeloablative conditioning in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. *J Allergy Clin Immunol*. 2010;126:1000-1005.

547. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol*. 2005;6:1142-1151.

548. Sharfe N, Dadi HK, Shahar M, Roifman CM. Human immune disorder arising from mutation of the alpha chain of the interleukin-2 receptor. *Proc Natl Acad Sci U S A*. 1997;94:3168-3171.

549. Caudy AA, Reddy ST, Chatila T, Atkinson JP, Verbsky JW. CD25 deficiency causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like syndrome, and defective IL-10 expression from CD4 lymphocytes. *J Allergy Clin Immunol*. 2007;119:482-487.

550. Yu A, Zhu L, Altman NH, Malek TR. A low interleukin-2 receptor signaling threshold supports the development and homeostasis of T regulatory cells. *Immunity*. 2009;30:204-217.

551. Davidson TS, DiPaolo RJ, Andersson J, Shevach EM. Cutting edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. *J Immunol*. 2007;178:4022-4026.

552. D'Cruz LM, Klein L. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. *Nat Immunol*. 2005;6:1152-1159.

553. Hennighausen L, Robinson GW. Interpretation of cytokine signaling through the transcription factors STAT5A and STAT5B. *Genes Develop*. 2008;22:711-721.

554. Kofoed EM, et al. Growth hormone insensitivity associated with a STAT5b mutation. *N Engl J Med*. 2003;349:1139-1147.

555. Nadeau K, Hwa V, Rosenfeld RG. STAT5b deficiency: an unsuspected cause of growth failure, immunodeficiency, and severe pulmonary disease. *J Pediatr*. 2011;158:701-708.

556. Canale VC, Smith CH. Chronic lymphadenopathy simulating malignant lymphoma. *J Pediatr*. 1967;70:891-899.

557. Sneller MC, et al. A novel lymphoproliferative/autoimmune syndrome resembling murine lpr/gld disease. *J Clin Invest*. 1992;90:334-341.

558. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature*. 1992;356:314-317.

559. Strasser A, Jost PJ, Nagata S. The many roles of FAS receptor signaling in the immune system. *Immunity*. 2009;30:180-192.

560. Rieux-Laucat F, et al. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science*. 1995;268:1347-1349.

561. Neven B, et al. A survey of 90 patients with autoimmune lymphoproliferative syndrome related to TNFRSF6 mutation. *Blood*. 2011;118:4798-4807.

562. Oliveira JB, et al. Revised diagnostic criteria and classification for the autoimmune lymphoproliferative syndrome (ALPS): report from the 2009 NIH International Workshop. *Blood*. 2010;116:e35-e40.

563. Teachey DT, Seif AE, Grupp SA. Advances in the management and understanding of autoimmune lymphoproliferative syndrome (ALPS). *Br J Haematol* . 2010;148:205-216.

564. Holzelova E, et al. Autoimmune lymphoproliferative syndrome with somatic Fas mutations. *N Engl J Med* . 2004;351:1409-1418.

565. Magerus-Chatinet A, et al. Onset of autoimmune lymphoproliferative syndrome (ALPS) in humans as a consequence of genetic defect accumulation. *J Clin Investigat*. 2011;121:106-112.

566. Bolze A, et al. Whole-exome-sequencing-based discovery of human FADD deficiency. *Am J Hum Genet*. 2010;87:873-881.

567. Wu J, et al. Fas ligand mutation in a patient with systemic lupus erythematosus and lymphoproliferative disease. *J Clin Invest*. 1996;98:1107-1113.

568. Del-Rey M, et al. A homozygous Fas ligand gene mutation in a patient causes a new type of autoimmune lymphoproliferative syndrome. *Blood*. 2006;108:1306-1312.

569. Chun HJ, et al. Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency. *Nature*. 2002;419:395-399.

570. Wang J, et al. Inherited human caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. *Cell*. 1999;98:47-58.

571. Brenner D, Mak TW. Mitochondrial cell death effectors. *Curr Opin Cell Biol* . 2009;21:871-877.

572. Oliveira JB, et al. NRAS mutation causes a human autoimmune lymphoproliferative syndrome. *Proc Natl Acad Sci U S A* . 2007;104:8953-8958.

573. Niemela JE, et al. Somatic KRAS mutations associated with a human nonmalignant syndrome of autoimmunity and abnormal leukocyte homeostasis. *Blood*. 2011;117:2883-2886.

574. Takagi M, et al. Autoimmune lymphoproliferative syndrome-like disease with somatic KRAS mutation. *Blood*. 2011;117:2887-2890.

575. Magerus-Chatinet A, et al. FAS-L, IL-10, and double-negative CD4- CD8- TCR alpha/beta+ T cells are reliable markers of autoimmune lymphoproliferative syndrome (ALPS) associated with FAS loss of function. *Blood*. 2009;113:3027-3030.

576. Farquhar JW, Claireaux AE. Familial haemophagocytic reticulosis. *Arch Dis Childhood*. 1952;27:519-525.

577. Cote M, et al. Munc18-2 deficiency causes familial hemophagocytic lymphohistiocytosis type 5 and impairs cytotoxic granule exocytosis in patient NK cells. *J Clin Invest*. 2009;119:3765-3773.

578. Stepp SE, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science*. 1999;286:1957-1959.

579. Ménasché G, et al. Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. *Nat Genetics*. 2000;25:173-176.

580. Feldmann J, et al. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). *Cell*. 2003;115:461-473.

581. zur Stadt U, et al. Linkage of familial hemophagocytic lymphohistiocytosis (FHL) type-4 to chromosome 6q24 and identification of mutations in syntaxin 11. *Hum Mol Gen*. 2005;14:827-834.

582. Pachlopnik Schmid J, et al. Inherited defects in lymphocyte cytotoxic activity. *Immunol Rev*. 2010;235:10-23.

583. de Saint Basile G, Menasche G, Fischer A. Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. *Nat Rev Immunol* . 2010;10:568-579.

584. Jenkins MR, Griffiths GM. The synapse and cytolytic machinery of cytotoxic T cells. *Curr Opin Immunol* . 2010;22:308-313.

585. Gholam C, Grigoriadou S, Gilmour KC, Gaspar HB. Familial haemophagocytic lymphohistiocytosis: advances in the genetic basis, diagnosis and management. *Clin Exp Immunol*. 2011;163:271-283.

586. Johnson TS, Villanueva J, Filipovich AH, Marsh RA, Bleesing JJ. Contemporary diagnostic methods for hemophagocytic lymphohistiocytic disorders. *J Immunol Methods*. 2011;364:1-13.

587. Marcenaro S, et al. Analysis of natural killer-cell function in familial hemophagocytic lymphohistiocytosis (FHL): defective CD107a surface expression heralds Munc13-4 defect and discriminates between genetic subtypes of the disease. *Blood*. 2006;108:2316-2323.

588. Jordan MB, Allen CE, Weitzman S, Filipovich AH, McClain KL. How I treat hemophagocytic lymphohistiocytosis. *Blood*. 2011;118:4041-4052.

589. Marsh RA, Jordan MB, Filipovich AH. Reduced-intensity conditioning haematopoietic cell transplantation for haemophagocytic lymphohistiocytosis: an important step forward. *Br J Haematol* . 2011;154:556-563.

590. Kaplan J, De Domenico I, Ward DM. Chediak-Higashi syndrome. *Curr Opin Hematol*. 2008;15:22-29.

591. Barbosa MD, et al. Identification of the homologous beige and Chediak-Higashi syndrome genes. *Nature*. 1996;382:262-265.

592. Nagle DL, et al. Identification and mutation analysis of the complete gene for Chediak-Higashi syndrome. *Nat Genet*. 1996;14:307-311.

593. Long HM, Taylor GS, Rickinson AB. Immune defence against EBV and EBV-associated disease. *Curr Opin Immunol* . 2011;23:258-264.

594. Rezaei N, Mahmoudi E, Aghamohammadi A, Das R, Nichols KE. X-linked lymphoproliferative syndrome: a genetic condition typified by the triad of infection, immunodeficiency and lymphoma. *Br J Haematol* . 2011;152:13-30.

595. Nichols KE, et al. Inactivating mutations in an SH2 domain-encoding gene in X-linked lymphoproliferative syndrome. *Proc Natl Acad Sci U S A* . 1998;95:13765-13770.

596. Sayos J, et al. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature*. 1998;395:462-469.

597. Cannons JL, Tangye SG, Schwartzberg PL. SLAM family receptors and SAP adaptors in immunity. *Ann Rev Immunol*. 2011;29:665-705.

598. Coffey AJ, et al. Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. *Nat Genet*. 1998;20:129-135.

599. Calpe S, et al. The SLAM and SAP gene families control innate and adaptive immune responses. *Adv Immunol*. 2008;97:177-250.

600. Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature*. 2008;455:764-769.

601. Nichols KE, et al. Regulation of NKT cell development by SAP, the protein defective in XLP. *Nat Med*. 2005;11:340-345.

602. Rigaud S, et al. XIAP deficiency in humans causes an X-linked lymphoproliferative syndrome. *Nature*. 2006;444:110-114.

603. Tabata Y, et al. Rapid detection of intracellular SH2D1A protein in cytotoxic lymphocytes from patients with X-linked lymphoproliferative disease and their family members. *Blood*. 2005;105: 3066-3071.

604. Marsh RA, Bleesing JJ, Filipovich AH. Using flow cytometry to screen patients for X-linked lymphoproliferative disease due to SAP deficiency and XIAP deficiency. *J Immunol Methods*. 2010;362:1-9.

605. Booth C, et al. X-linked lymphoproliferative disease due to SAP/SH2D1A deficiency: a multicenter study on the manifestations, management and outcome of the disease. *Blood*. 2011;117:53-62.

606. Wu C, et al. SAP controls T cell responses to virus and terminal differentiation of TH2 cells. *Nat Immunol*. 2001;2:410-414.

607. Botto M, et al. Complement in human diseases: Lessons from complement deficiencies. *Mol Immunol*. 2009;46:2774-2783.

608. Crow YJ. Type I interferonopathies: a novel set of inborn errors of immunity. *Ann N Y Acad Sci*. 2011;1238:91-98.

609. Polychronakos C, Li Q. Understanding type 1 diabetes through genetics: advances and prospects. *Nat Rev Genet*. 2011;12:781-792.

610. Picard C, Casanova JL, Abel L. Mendelian traits that confer predisposition or resistance to specific infections in humans. *Curr Opin Immunol*. 2006;18:383-390.

611. Barreiro LB, Quintana-Murci L. From evolutionary genetics to human immunology: how selection shapes host defence genes. *Nat Rev Genet*. 2010;11:17-30.

612. Metzger TC, Anderson MS. Control of central and peripheral tolerance *by* Aire. *Immunol Rev*. 2011;241:89-103.